Testis Specific Serine-Threonine Kinases
Regulation and their role in disease

Testis Specifieke Serine-Threonine Kinases
Regulatie en de rol in ziekten

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W. H. Gispen
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op donderdag 28 april 2005 des middags te 12.45 uur

door

Marta Bućko-Justyna

geboren op 5 october 1975 te Warszawa, Polen

Promotores:

Prof. Dr. Ir. Boudewijn M. Th. Burgering
University Medical Center Utrecht, The Netherlands

Prof. Dr. Maciej Żylicz
International Institute of Molecular and Cell Biology in Warsaw, and
University of Warsaw, Poland

ISBN: 90-393-3935-X

Cover: Puzzled scheme of RUSC2 assembly described in the thesis

The research described in this thesis was supported by research grant No 6 PO4B 006 19 from the State Committee for Scientific Research, Warsaw, Poland and by a grant from Utrecht University, The Netherlands as part of the collaborative research program, between the International Institute of Molecular and Cell Biology in Warsaw and the Academical Biomedical Center at Utrecht University. Research was performed at the Department of Molecular Biology, International Institute of Molecular and Cell Biology in Warsaw and Department of Physiological Chemistry, Center for Biomedical Genetics, University Medical Center Utrecht.

TABLE OF CONTENTS

Chapter

1

General introduction

TESTIS SPECIFIC PROTEIN KINASES

Protein kinases that function in testis

Spermatogenesis in mammals is a continuum of cellular differentiation comprising three principal phases. The first phase is spermacytogenesis, during which spermatogonia undergo mitotic cell division and generate a pool of spermatocytes. The second phase is the meiotic division of the spermatocytes, yielding four haploid round spermatids. The third phase, spermiogenesis, defines an elaborate process of spermatids cytodifferentiation, culminating in the release of characteristically shaped spermatozoa into the lumen of the seminiferous tubule. It is a complex process involving specific interactions between the developing germ cells, supporting Sertoli cells and hormone-producing Leydig cells.

An estimated two-thirds of mammalian genes is at some point expressed in adult or developing testis (1), with 5% to 10% of genes expressed exclusively there. Among the genes playing a role in testis function there is a large number of protein kinases. Kinases mediate the phosphorylation of substrate proteins and protein phosphorylation is the most common posttranslational protein modification in eukaryotes and a fundamental mechanism for the direct or indirect control of all cellular processes like control of cell division, cell adhesion and migration, cell to cell communication, and signal transduction. Protein kinases make up a large family of related enzymes (2) characterized by a homologous region of around 300 amino acids. Considering the importance of phosphorylation events in the regulation of cellular mechanisms, it comes as no surprise that several protein kinases have been shown to be involved in testis development and the process of spermatogenesis. For example, the kit receptor tyrosine kinase is critical for the migration of primordial germ cells (3). Another member of this group of receptor tyrosine kinases, Platelet-Derived Growth Factor Receptor a (Pdgfra), is involved in testis descent and development of Leydig cells (4). Disruption of the receptor serine-threonine kinase Bone Morphogenetic Protein Receptor 1 (Bmpr1) leads to the retention of female Mullerian ducts in males (5). Abl tyrosine kinase and Ataxia-Teleangiectasia Mutated (ATM) serine-threonine kinases participate in controlling meiosis during gametogenesis (6) (7). $C\alpha_2$ catalytic subunit of PKA is needed for the protein tyrosine phosphorylation that occurs late in the sequence of sperm maturation and for a negative feedback control of cAMP production (8).

All these kinases are, however, present in a variety of tissues and only a few of them are exclusively expressed in germ cells or in the testis. Testis-specific protein kinase 1 (TESK1) is one of the kinases expressed specifically in testis. TESK1 mRNA and protein expression has been shown to be limited to testicular germ cells at specific stages of spermatogenesis, particularly from the late pachytene spermatocytes to round spermatids (9,10). This suggests that TESK1 protein plays an important role in meiotic cell division and /or early spermiogenesis. Furthermore, TESK1 was demonstrated to convey dual specificity protein kinase activity, catalyzing autophosphorylation and phosphorylation of exogenous substrates on both serine/threonine and tyrosine residues (11). Another testis-specific protein kinase is Aie1/Aurora-C, first described by Tseng et al. (12). Northern blot and RNA *in situ* hybridization revealed that Aie1 mRNA was expressed in meiotically active germ cells (pachytene spermatocytes) in developing postnatal testis (13). It was also demonstrated in vitro that protein kinase A (PKA) phosphorylates Aie1 and the amino acids responsible for kinase activity were identified (14). The phosphoglycerate kinase-2 encoding gene *(PGK-2)*, an isomeric form of

ubiquitously expressed and X-linked *PGK-1* gene, is active specifically in testis and is likely to take over the function of PGK-1 in meiotic and postmeiotic spermatogenic cells (15,16). The microtubule-associated serine/threonine protein kinase (MAST205) colocalizes with the microtubular manchette of developing spermatids and may therefore function as a link between exogenous signals and microtubule organization possibly involved in sperm head shaping (17). Moreover it has been reported that MAST205 consists of a large heterologous protein complex and that kinase activity associated with MAST205 increases during the stages of mammalian spermatid maturation, coincident with the binding of this protein to manchette microtubules (18).

Although a testis specific expression pattern may suggest that these proteins are essential for testis development and/or spermatogenesis, not all testis specific proteins are indeed indispensable. For example, PASKIN (19) expression is strongly upregulated in postmeiotic germ cells during spermatogenesis but fertility, sperm production and sperm motility were not affected in PASKIN knockout male mice. Another example is the serine-threonine kinase, MAK (male germ cell-associated kinase) for which expression was shown to be highly restricted to testicular germ cells during and after meiosis (20). Therefore, it was suggested to play an important role in cellular processes of spermatogenesis. Surprisingly, later on it was shown that, in MAK-deficient (*Mak*$^{-/-}$) mice spermatogenesis and male fertility is normal, and thus the function of MAK appears not essential for this process (21) and it was suggested that it is rather involved in cellular function(s) of the mature sperm. However, other testis specific proteins are indeed essential for spermatogenesis. For example disruption of Casein Kinase 2a' (CK2a') (22), which is expressed in late stages of spermatogenesis, results in male mice in infertility, with oligospermia and globozoospermia ('round-headed' spermatozoa).

The last example of a family of testis specific proteins with a probable function in testis development and/or spermatogenesis, and the subject of this thesis, is a family of testis specific protein kinases, the TSSK family that is described below.

The family of Testis Specific Serine-threonine Kinases (TSSK)

Up to date the family of Testis Specific Serine-threonine Kinases (TSSK) comprises of four members (23). These kinases are expressed exclusively in testis during spermatogenesis. The first member of this family, TSSK1 was cloned by Bielke et al. (24) using degenerate oligonucleotides corresponding to two highly conserved motifs within the protein kinase catalytic domain and a PCR-based cloning strategy using RNA from a human neuroblastoma cell line. Interestingly, northern blot analysis of isolated TSSK1 expression detected RNA transcript only in RNA isolated from adult testes, reflecting the sensitivity of the PCR-based cloning strategy, allowing the detection of molecules at extremely low abundance. The sequence of TSSK1 harbors an open reading frame of 1092-bp encoding a putative protein of 364-aa. In the mouse, *Tssk1* is located on chromosome 16 A3 and its human homologue is located on chromosome 5. TSSK1 displays high homology to a group of yeast Ser/Thr kinases encoded by *SNF-1, nim-1, KIN-1* and *KIN-2* (24).

To search for additional family members, the same group (25) screened a mouse testis cDNA library under low stringency conditions using TSSK1 as a probe and isolated a clone containing a 1.3-kb cDNA insert, which was designated TSSK2. Sequence analysis revealed an open reading frame of 1071-bp encoding a 357-amino acid protein serine/threonine kinase, where the

kinase domain is located NH2-terminally and this is homologous to TSSK1 (84% identity). Mouse *Tssk2*, as well as mouse *Tssk1* is located on chromosome 16 A3 and is the mouse homologue of the human DiGeorge syndrome gene (DGS-G) which maps to chromosome 22. Northern blot analysis demonstrates an identical expression pattern for TSSK2 as that observed previously for TSSK1, namely limited to testis. Furthermore, it was demonstrated at the RNA and protein level that expression of TSSK1 and 2 was developmentally regulated, activated around the onset of spermatogenesis, persisting through adult life and absent in released sperm. Immunohistochemical analyses revealed that TSSK1 and TSSK2 expression is limited to meiotic and postmeiotic spermatogenic cells, respectively (26) (25) and that both proteins are absent in spermatocytes, Sertoli cells or in Leydig cells. Therefore the authors suggested that these kinases most probably participate in the reconstruction of the cytoplasm observed during sperm tail maturation.

Mouse Tssk3 has been originally described as the third member of the TSSK family (27). Characteristically, it was identified using low-stringency hybridization with a partial sequence obtained from cDNA amplification utilizing degenerate primers (28). The complete sequence of *hTSSK3* was published by Visconti et al. (29) shortly after it became available as a part of accessible Human Genomic Project sequences. The human *TSSK3* gene maps to chromosome 1 and is syntenic with the mouse *Tssk3* gene on chromosome 4 (29). Both the mouse and human sequence encode for a small protein of 29 kDa, consisting of a catalytic domain only that differs this protein from two previous members TSSK1 and 2 containing C-terminally extended sequence. Immunohistochemical studies in mice indicate that Tssk3 is present exclusively in testicular Leydig cells that synthesize androgens. The TSSK3 mRNA level is low at birth, increases substantially at puberty and remains high throughout adulthood. Thus TSSK3 seems to play an important role in adult testis. For TSSK3 there has been no specific substrate identified, unlike for mouse Tssk1 and 2 for which a putative substrate, TSKS (Testis-Specific Kinase Substrate) was shown to interact with these kinases using the yeast two-hybrid approach. It was also immunoprecipitated along with Tssk1 and Tssk2 from testicular extracts and phosphorylated in vitro by these kinases (25). The human homologue of TSKS was cloned and shown to interact with TSSK2 by Hao et al. (23). The expression pattern of TSKS is similar to TSSK1 and 2 and is limited to testicular spermatids as shown by northern blots, dot blots, western blots and immunohistochemical studies (23,25,30). The role of TSKS, but also of TSSK1 and 2, in testis development has yet to be established.

The sequence of the fourth member of the TSSK family, TSSK4, has been deduced using bioinformatics and it was found to be expressed only in the testis by real-time PCR (23), but the TSSK4 cDNA has not been cloned yet. Human *TSSK4* gene maps to chromosome 19 and has a mouse homologue in the syntenic region of mouse chromosome 8. Table 1 summarizes the chromosomal localization and expression pattern of proteins from TSSK family.

Although the mechanism of action of the TSSK family is not characterized, the involvement of phosphorylation events in signal transduction processes, the conservation of a kinase domain among members of TSSK family and the conserved pattern of expression limited to testis, suggest that TSSKs may play a role in mammalian germ cell differentiation and/or sperm development.

Table 1. The chromosomal localization and expression pattern of proteins from TSSK family

Name	Chromosome		Introns	Expression pattern
	mouse	human		
TSSK1	16	5	No	testes: late spermatids
TSSK2/DGS-G	16	22	No	testes: late spermatids
TSSK3	4	1	One	testes: Leydig cells
TSSK4	8	19	One	testes:?

Diseases and infertility

Disorders in spermatogenesis are the cause of most of the male infertility cases. Proteins involved in fertilization are likely to be expressed late in spermatogenesis, i.e. in meiosis or later. Candidate genes for male infertility have been identified by classical clinical genetic methods, animal model studies and through the use of cDNA microarrays to monitor changes in the gene expression profile in the developing testis. Classical methods involved karyotype analysis of families and/or individuals, who suffer from specific male infertility phenotypes. This led to the discovery of many chromosome abnormalities, including sex chromosome aneuploidies such as XXY and XYY syndromes (31-34) and autosomal chromosome translocations (35-37). Classical methods also identified a number of single gene defects involved in causing male infertility, such as mutations in genes encoding for hormones and for receptors, but also genes involved in more general clinical syndromes are often associated with causing infertility. For example myotonic dystrophy, cystic fibrosis and microdeletions on the long arm of the human Y chromosome (Yq) also involve severe defects of spermatogenesis (38-43). The disorders cystic fibrosis (44), Young syndrome (45) and congenital bilateral absence of the vas deference (CBAVD) (40) are caused by mutations in the same gene, *CFTR* (cystis fibrosis transmembrane regulator) and in all three cases a mutation in the CFTR gene is also likely to cause the male infertility that accompanies these syndromes. Another example is *ATM*, mutated in *ataxia-telangiectasia*, a DNA repair syndrome. One of the clinical symptoms of *ATM* mutations is male infertility due to abnormal meiotic progression and subsequent germ-cell degeneration (46). The substrates of this protein kinase include c-ABL, replication protein A, p53 and beta-adaptin. Since these targets are located both in the nucleus and the cytoplasm, the ATM protein is most likely involved in several distinct signaling pathways and one of these pathways, or even all, can be involved in causing male infertility.

Recently, knowledge concerning gene defects causing male infertility has also been derived from animal models such as transgenic mice and gene knockout mice. These include for example the casein kinase 2 alpha gene, of which the knockout mice

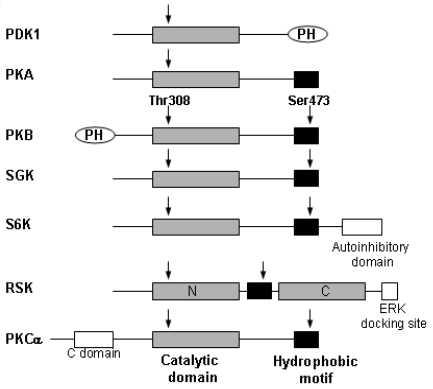
display defective development of the acrosome (22,47). Also to investigate the role of Camk4 (that in testis is expressed in spermatids) in spermatogenesis Wu et al. (48) generated mice with a targeted deletion of the *Camk4* gene. Male *Camk4* $^{(-/-)}$ mice were infertile with impairment of spermiogenesis in late elongating spermatids. The deposition of sperm basic nuclear proteins on chromatin was disrupted, with a specific loss of protamine-2 that is phosphorylated by Camk4. Defects in protamine-2 have been identified in sperm of infertile men (49), suggesting that the result of Wu at al. may have clinical implications. Also gene mutations in Drosophila can be informative such as mutations in the boule gene (related to human *DAZ* gene), which causes spermatogenic arrest at the primary spermatocyte stage in the fruit fly (50).

The search for candidate genes for male infertility using cDNA microarrays to monitor the expression profile of testis-specific genes and the use of the human genome databases, provided a vast number of male germ cells-specific genes expressed late in the development (during or after meiosis) (51,52). These genes include genes encoding proteases or proteins that interact with proteases, protein kinases, transcription factors, proteins associated with chromatin, channels or transporters, mitochondrial associated proteins, adhesion proteins, RNA polymerases, and genes involved in the interaction between spermatids and Sertoli cells (51). This extensive group provides potential targets for germ cell-targeted contraception and a large number of candidates that could be critical for fertilization and/or serve as cancer specific antigens.

REGULATION OF AGC KINASES ACTIVITY BY PDK1 PHOSPHORYLATION

3-Phosphoinositide-dependent protein kinase-1 (PDK1) is a serine/threonine kinase that acts downstream of PI 3-kinase in insulin and IGF-1 signaling pathways. It phosphorylates and activates a group of related protein enzymes termed AGC kinases

A.



B.

	Activation loop	Hydrophobic motif
PDK1	SFVGTAQYVSPE	
PKA	TLCGTPEYLAPE	FSEF
PKB	TFCGTPEYLAPE	FPQFSY
SGK	TFCGTPEYLAPE	FLGFSY
S6K	TFCGTIEYMAPE	FLGFTY
RSK	SFCGTVEYMAPE	FRGFSF
PKCα	TFCGTPDYIAPE	FEGFSY

Fig. 1. (A) Schematic representation of PDK1 and other AGC protein kinases. PDK1 and its substrates, members of AGC kinase family, contain a phosphorylation site at the activation loop (T-loop) of the catalytic domain (Thr^{308} in PKBα). Most PDK1 substrates also have a hydrophobic motif phosphorylation site. The figure is partially adapted from Biondi et al. (67). **(B)** Alignment of the amino acid sequences surrounding the T-loop and the hydrophobic motif of AGC kinases. The underlined residues correspond to those that become phosphorylated

(originally containing protein kinase A, cGMP-dependent protein kinase and protein kinase C), which mediate many of the physiological responses triggered by growth factors and hormones. Proteins in this family contain regions of high homology in their kinase domains and additionally they harbour a C-terminal non-catalytic region known as the hydrophobic motif (Figure 1). Upon growth factor stimulation of cells, the hydrophobic motif of AGC kinases becomes phosphorylated which enables PDK1 to interact with its kinase substrate through a docking site termed the 'PIF-pocket' (for PDK1-interacting fragment pocket), located on the small lobe of the PDK1 kinase domain (53-57). This interaction leads to increased catalytic activity of PDK1 that autophosphorylates (57) and phosphorylates the activation loop (T-loop) of AGC kinase (58). The structural information of the individual components of the PDK1-substrate interaction system provides a model showing that PDK1 acts as a sensor of protein conformation. PDK1 only interacts and phosphorylates AGC kinases when they are in their inactive conformation. The covalent modification (phosphorylation), catalyzed by PDK1 stabilizes the active conformation of the substrate kinase that becomes then 'invisible' for PDK1 by hiding its hydrophobic motif (59). Figure 2 illustrates the central features of this mechanism.

In contrast to other AGC kinases, PDK1 does not have an equivalent C-terminal extension to the catalytic core and lacks the hydrophobic motif (HM), but it is phosphorylated in the activation loop, and this phosphorylation has been found to be crucial for kinase activity. Recent evidence suggest that Ser-244 in PDK1 is an autophosphorylation site in the activation loop that is critical for PDK1 activity (60,61).

The protein kinases from the AGC family that are phosphorylated by PDK1 in the T-loop include protein kinase B (PKB/AKT), p70 ribosomal S6 kinase (p70S6K1/2), p90 ribosomal S6 kinase (p90RSK), serum- and glucocorticoid-induced protein kinase (SGK), protein kinase A (PKA), different isoforms of protein kinase C (PKC), PKC-related protein kinase 2 (PRK2) and p21-activated kinase (PAK1) (58,62). Actually, PDK1 was first identified by its ability to phosphorylate Thr-308 of PKBα in vitro (63). Insulin binding to its tyrosine kinase receptor induces the receptor to phosphorylate itself. This results in the recruitment of the lipid kinase, PI 3-kinase, to the plasma membrane, phosphorylation of its substrate phosphatydyloinositol (4,5) bisphosphate (PtdIns(4,5)P_2) and generation of PtdIns(3,4,5)P_3. A key effector of PtdIns(3,4,5)P_3 in insulin signaling is PKB that has a pleckstrin homology (PH) domain which binds to PtdIns(3,4,5)P_3. This results in PKB recruitment from the cytosol to the plasma membrane where it is phosphorylated by PDK1. PDK1 like PKB has a PtdIns(3,4,5)P_3 binding PH domain and it is likely that, upon growth factor or hormone stimulation, the colocalization of PKB and PDK1 induced through PtdIns(3,4,5)P_3, generated by PI 3-kinase, enables PDK1 to phosphorylate PKB at Thr-308 (64). Also phosphorylation of the T-loop of p70S6K1/2, SGK, PKA, PKCζ and PAK1 has been shown to be mediated by PDK1, in PI3K dependent manner (65) although these substrates of PDK1 do not harbor a PH domain and cannot interact directly with PtdIns(3,4,5)P_3. Recent evidence indicates that PtdIns(3,4,5)P_3 stimulates the phosphorylation of the hydrophobic motif by either activation of the hydrophobic motif kinase or by inhibiting the phosphatase dephosphorylating the hydrophobic motif (66). Other kinases like p90RSK, PKA and conventional isoforms of PKC are phosphorylated by PDK1 as well, despite that they are not dependent on PI3K for their activity. For example p90RSK requires prior docking to ERK in order to

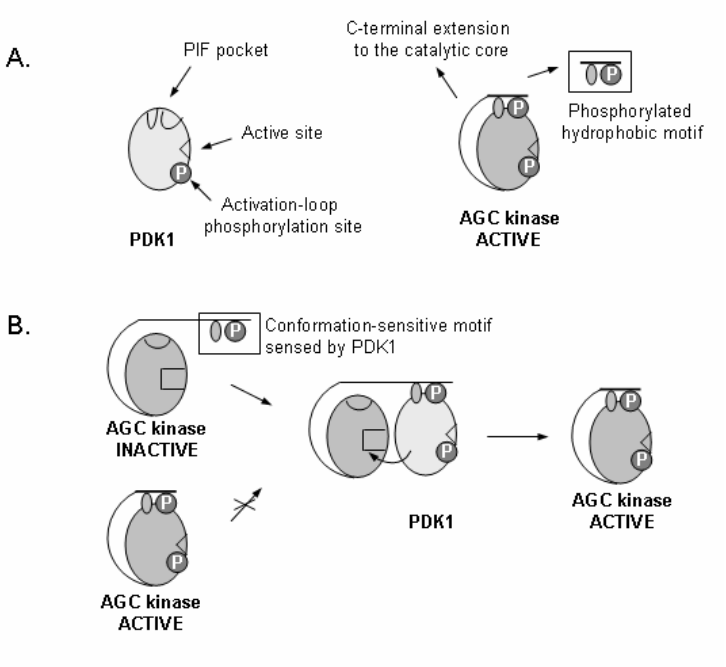


Fig. 2. Molecular mechanism of phosphoinositide-dependent protein kinase 1 (PDK1) conformational sensing. (A) Schemes of PDK1 and AGC kinases. Most AGC kinases are activated by the double phosphorylation of the hydrophobic motif (HM) phosphorylation site and the activation-loop phosphorylation site. These phosphorylations act together to stabilize the active conformation of AGC kinases. (B) PDK1 detects inactive conformations of AGC substrates. Interaction of phosphorylated HMs with PDK1 PIF pocket promotes activation of PDK1, enabling the phosphorylation of substrates at the activation loop. The figure is partially adapted from Biondi et al. (59).

phosphorylate and activate its C-terminal domain, which in turn results in the phosphorylation of the hydrophobic motif. This phosphorylation allows docking to PDK1, which phosphorylates the activation loop and enables full p90RSK activity (57,67). More insight into the role of PDK1 is provided by experiments with PDK1 deficient stem cells (*PDK1*$^{-/-}$) that were still viable but exhibited no activation of PKB, p70S6K or p90RSK in response to IGF-1 or the phorbol ester TPA, stimuli that induced their activation in PDK1$^{+/+}$ cells (68). In contrast, in PDK1-/- cells PKA was phosphorylated normally on Thr-197, shown to be phosphorylated in vitro by PDK1 (69), and PKA activity was still normal in these cells. This observation provides strong evidence that PDK1 mediates activation of PKB, p70S6K and p90RSK in vivo, but is not rate-limiting for activation of PKA. It was also demonstrated in vivo that PDK1 is required for PKC function: levels of PKC isoenzymes are dramatically decreased in stem cells deficient in PDK1, consistent with their instability in the non-phosphorylated form (70). The number of protein kinases regulated by PDK1 underlines the crucial role of this

protein in controlling multiple signaling pathways and cellular responses.

SIGNAL TRANSDUCTION BY THE C-JUN N-TERMINAL KINASE (JNK)

Oxidative stress in cellular signaling

Oxidative stress-induced cell damage is an important component of many diseases, including cardiovascular, autoimmune, neurodegenerative diseases, cancer, and the aging process. Oxidative stress is caused by the exposure of cells to reactive oxygen species (ROS). ROS include a variety of chemical species such as superoxide anion, hydroxyl radical, and hydrogen peroxide that are highly reactive with cellular components. The origin of these species can be either exogenous or endogenous. Exogenous sources of ROS include the exposure to ionizing radiation and sunlight, chemotherapeutic agents, or hypothermia. Endogenous ROS can be the result of physiological conditions (such as aerobic metabolism) or pathological situations (such as organ ischemia, Alzheimer's disease, and cancer) (71).

Cells have evolved protective mechanisms including antioxidants that detoxify ROS. When ROS levels exceed the antioxidant capacity of a cell the oxidative cell response is stimulated, which results in the increased production of antioxidants and repair proteins. Depending on its severity, oxidative stress can possibly lead to either cell necrosis or apoptosis. It has been proposed that ROS in these two types of cell death, function in two different ways. In necrosis, they serve as effectors, resulting in oxidative damage to lipids, nucleic acids, and proteins, while, in apoptosis, they may serve as signaling molecules via redox-sensitive pathways and cellular factors (72).

Among the main stress signaling pathways and/or central mediators activated in response to oxidant injury are the extracellular signal-regulated kinase (ERK), the phosphoinositide 3-kinase (PI3K) pathway, the nuclear factor (NF)-κB signaling system, and the heat shock response, generally considered as prosurvival responses while c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways, and p53 activation are associated with apoptosis (71). Figure 3 represents these major signaling pathways activated in response to oxidative stress. Activation of these pathways is not unique to oxidative stress and they are activated in response to other stresses as well as they play role in controlling normal growth and metabolism. Because of its potential involvement in the regulation of TSSK3 (Chapter 3), the subject of this thesis, we will concentrate below on the stress-activated pathways that include c-Jun N-terminal kinase. While signaling within this pathway is relatively well defined, the sensing

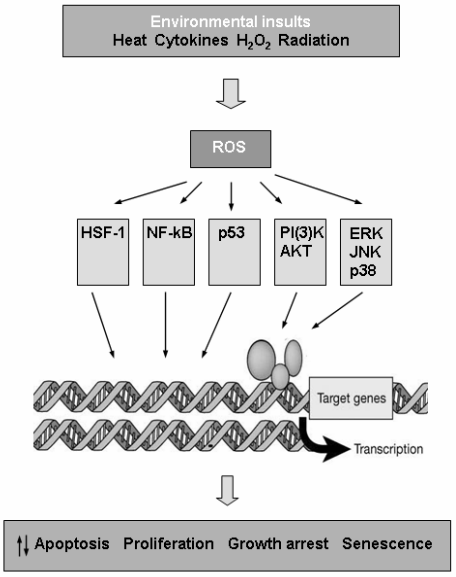


Fig. 3. Major signaling pathways activated in response to oxidative stress. The figure is adapted from Finkel, T and Hollbrook, N (71).

mechanism by which the JNK pathway is activated in response to oxidative stress is less understood. It has been suggested that ROS may have direct protein targets and the exposure of these proteins to an altered redox state alters the function of the target protein. For example, the redox regulatory protein thioredoxin (Trx) has been shown to bind to apoptosis signal-regulating kinase (ASK1), an upstream activator of JNK and p38, and under normal conditions this inhibits ASK1 activity. The rise in ROS levels results in the dissociation of Trx-ASK1 complex and subsequent activation of the downstream JNK and p38 (73). Similarly, glutathione S-transferase (GSTp) binds to JNK to inhibit its activation, but this interaction is also disrupted by oxidative stress (74). Recently, Veal et al. (75) presented in fission yeast a different regulatory mechanism where Tpx1 (a yeast homologue of mammalian 2-Cys peroxiredoxin) is a stimulator of peroxide-induced Sty1 (JNK) activation and has a direct role in peroxide-signaling through regulation of JNK oxidation.

Stress activated MAP kinase signaling

The stress Activated Protein Kinase (SAPK) group of mitogen-activated protein (MAP) kinases consist of the c-Jun N-terminal kinases (JNK) and the p38 kinases. JNKs are activated by treatment of cells with cytokines (like TNF and IL-1) and by exposure of cells to many forms of environmental stress (e.g. osmotic stress, redox stress and radiation) (76). JNK activation is also induced, in some cell types, in response to growth factors, heterotrimeric G-proteins (77), phorbol esters, or the activation of the T cell receptor in T lymphocytes (78). JNK was reported to play important roles in development, apoptosis, cell growth, and immune response (79),(80). Consequently, the JNK cascade has been implicated in the pathogenesis of many human diseases, including cancer (81), obesity and insulin resistance (82), muscular dystrophy (83), arthritis (84), aspects of heart disease (85), Parkinson's disease (86), and other neurological disorders characterized by abnormal cell death (87).

JNK kinases are encoded by three genes: *jnk1, jnk2* and *jnk3* (88,89). The *jnk1* and *jnk2* genes are ubiquitously expressed, while the *jnk3* gene is selectively expressed in brain, heart and testis. Transcripts derived from theses genes are alternatively spliced to create four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms (88).

Targets of the JNK signal transduction pathway include the transcription factors c-Jun, a component of AP-1 transcription complex and an important regulator of gene expression, other members of the AP-1 complex like JunB, JunD and activating transcription factor-2 (ATF2) (76), the Ets domain transcription factors Elk-1 and Sap-1 (89),(90-92) and the FOXO4 transcription factor (93). The phosphorylation of these transcription factors is thought to increase their transcriptional activity.

The JNK protein kinases are activated by dual phosphorylation on Tyr and Thr by the MAP kinase kinases, MKK4 and MKK7. These kinases are functionally distinct in several ways (79). First, although both kinases are dual-specificity kinases, in vitro experiments indicate that the Tyr site is phosphorylated selectively by MKK4, while Thr is preferred by MKK7 (94). Second, the MKK7 protein is primarily activated by cytokines and MKK4 is primarily activated by environmental stress. Finally, MKK4 (but not MKK7) can also activate p38. Target gene-disruption studies in mice showed that the simultaneous disruption of both *Mkk4* and *Mkk7* genes is required to block JNK activation in response to environmental stress but disruption of the *Mkk7* gene alone was sufficient to prevent JNK activation caused by proinflammatory cytokines (95). This demonstrated that MKK4 and MKK7 serve

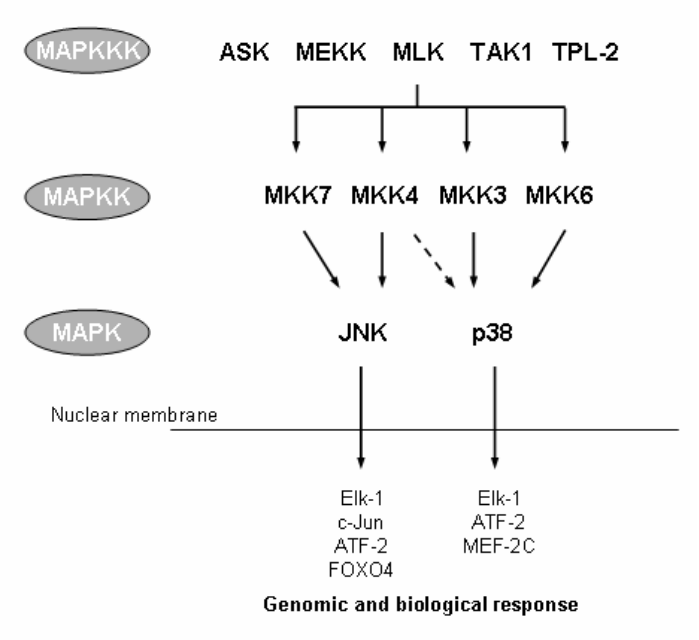


Fig. 4. Stress-activated MAP kinase signaling pathways. MAP kinase signaling pathways are structurally organized as a signaling cascade, whereby MAP kinases (MAPK) are activated by dual specificity MAP kinase kinases (MAPKK), which are themselves activated by a diverse group of MAP kinase kinase kinases (MAPKKK). The figure is adapted from Weston et al. (96)

different functions in the JNK signal transduction pathway.

Numerous proteins that function as MAP kinase kinase kinases (MAPKKK) in the JNK pathway have been described. These include the members of the MEKK group (MEKK1 to 4), the mixed linkage protein kinase group (MLK1, MLK2, MLK3, DLK and ZLK), the ASK group (ASK1 and ASK2), TAK1, and TPL-2 (79,96). Figure 4 represents a schematic structural organization of stress-activated MAP kinase signaling pathways. Until recently evidence to support this function was based mostly on transfection assays and in vitro kinase assays. Now, several studies on MAPKKK gene disruption in mice have recently been reported and these demonstrate in vivo the involvement of ASK1 (97), MEKK1, 2 and 3 (98-103), TPL-2 (104) and TAK1 (105) in the regulation of JNK pathway activation.

Regulation of JNK pathway by docking interactions

The assembly and localization of JNK signaling modules plays an important role in determining specificity and activity of JNK signaling. Assembly and localization is regulated by two types of protein-protein interactions i.e. by docking interactions between two proteins and coordinated simultaneous binding of multiple proteins to a scaffold proteins (106,107). The docking interaction is achieved through a specific conserved region present in a MAP kinase and its interacting molecule. Many studies

have highlighted the importance of docking interactions for enzymatic specificity and efficient signaling of MAPK pathways. Docking motifs are distinct from the phosphoacceptor sites and are present in the protein kinases of the MAPK cascade, downstream substrates, phosphatases, and scaffold proteins (108-110). The first MAPK docking motif identified was the delta domain (D domain) of the transcription factor c-Jun, which mediates binding to JNK and is essential for efficient JNK phosphorylation of c-Jun (111,112). Similar D domains have been identified in many other MAPK substrates, MAPKK and MAP phosphatases (108-110). Besides c-Jun also Elk-1 and ATF-2 contain a docking domain for JNK (113,114), as well as JunB that is in addition thought to recruit JNK kinases that can phosphorylate *in trans* other proteins like JunD (115). The N-terminal portion of MKK4 contains the MAPK-docking site and serves as a binding site for both the upstream activator, MEKK1, and the downstream target JNK (116). Some proteins, also in JNK pathway, contain docking domains whose primary function might not be to promote substrate phosphorylation by MAP kinases but to assemble the whole signaling module in response to a specific stimulation. This is facilitated by scaffold proteins that co-localize components of JNK pathway and modulate their signaling properties (106,107). Until now, four groups of potential scaffold proteins in the JNK signaling module have been reported: JIP, β-arrestin, CRK II and filamin. A fifth potential JNK scaffold is RUSC2 described in this thesis (Chapter 3), which will not be discussed here.

The JIP (JNK interacting proteins) family of scaffold proteins consists of three members JIP1, 2 and 3 that associate with MLKs, MKK7 and JNK and can potentiate JNK activation in a MLK-dependent manner (117-120). Recently, Whitmarsh et al. (121) showed in mice, with disruption of JIP1 gene, that JIP1 is necessary for stress-induced activation of JNK in hippocampal neurons. An alternatively spliced isoform of JIP3, JSAP1 interacts with JNK3, MKK4 and MEKK1 (122), thus functioning as a scaffold protein in the JNK3 cascade. Additionally, JIP3 was shown to be phosphorylated by ASK1 and the phosphorylation facilitated interactions of JIP3 with MKK4, MKK7 and JNK3. Furthermore, ASK1 dependent phosphorylation was necessary for recruitment and thereby activation of JNK3 in response to H_2O_2 (123). JIPs may integrate both positive and negative regulators of JNK, because apart from binding to protein kinases, JIP1 and JIP2 can associate also with the JNK phosphatase MKP-7 (via a region independent of JNK binding domain) leading to reduced JNK activity (124).

β-arrestins are versatile adapter proteins that form complexes with most G-protein-coupled receptors (GPCRs) following agonist binding and phosphorylation of receptors. In addition to their role in GPCR desensitization, they may function as GPCR signal transducers. Recent studies (125,126) demonstrate that β-arrestin-2, that is expressed in human heart, brain and testis, can recruit a JNK signaling module that consists of ASK1, MKK4 and JNK3 (but not JNK1 or 2). It binds directly to ASK1 and JNK3 and indirectly to MKK4. β-arrestin-2 appears to be required for JNK activation by the angiotensin II receptor.

CRK II was shown to interact with JNK1 and it was demonstrated that this interaction is a critical step, not only for the activation of JNK1 by CRK II, but also for Rac1-induced activation of JNK1 (127). The interaction of JNK with CRK II causes the recruitment of JNK to multi-protein complex that includes p130Cas and MKK4.

Another candidate protein to act as a scaffold in JNK signaling is filamin/ABP280 that is an actin-cross-linking protein, and therefore a determinant of cytoskeletal

architecture of the cell involved in cell adhesion and migration. Filamin binds to MKK4 (128) and TRAF2 (129), both of which are required for TNF-induced JNK activity. Moreover, TNF fails to activate JNK in melanoma cell line deficient in filamin and the reintroduction of filamin into these cells restores the TNF response (129).

To summarize, scaffold proteins appear to be essential components of the JNK signaling pathway that facilitate the flow of information from one kinase to another.

SCOPE OF THE THESIS

This thesis presents studies on the TSSK family of testis specific protein kinases. The aim was to further our understanding on this family of kinases in order to understand their potential role in testis development and/or spermatogenesis. At the time the study was started basically nothing was known on these kinases. Therefore we choose to first biochemically characterize one member of this family TSSK3. Chapter 2 presents evidence that TSSK3 is indeed a genuine protein kinase (which was formally not demonstrated yet) and characterizes in detail its biochemical properties (Chapter 2). In the same chapter we describe our initial efforts to characterize the signal transduction pathway in which TSSK3 may participate and it is shown that TSSK3 can be regulated in vitro by PDK1 through phosphorylation of a classical activation loop and that TSSK3 might be an in vivo a target of PDK1 signaling as well. Furthermore, analysis of a variety of peptide sequences defined the peptide sequence RRSSSY as an efficient and specific substrate for TSSK3.

To extend our knowledge concerning TSSK3 signaling we performed a yeast-two-hybrid analysis using TSSK3 as a bait. We identified, a novel, widely expressed protein, RUSC2 as an interacting partner but not a substrate for TSSK3 (Chapter 3). In addition we show that RUSC2 transiently interacts with JNK kinases after oxidative stress treatment and is phosphorylated by JNK in vitro and in vivo. These data thus led to the unexpected discovery that TSSK3 appears to function in oxidative stress-induced signaling involving RUSC2 and JNK. In the addendum to Chapter 3, we extend our studies on RUSC2 and show a possible link to the small GTPase Ras.

As discussed above the testis specific expression of a protein does not necessarily imply that there is also a testis-specific function. To obtain evidence for a testis specific function of the TSSK family, we reasoned that such a function could be reflected by a prevalence of mutations in TSSKs in male patients suffering from infertility. In Chapter 4 we describe a search for mutations in the genes coding for kinases TSSK1 and 2, in patients with infertility. In agreement with a testis specific function we identified several single nucleotide polymorphisms in TSSK2, a percentage of which results in a single amino acid change, with possible functional consequence. These data also suggest that in male patients suffering from infertility the TSSK2 locus is considerably more genetically unstable then the TSSK1 locus.

The possible consequences of these findings are discussed in Chapter 5.

REFERENCES

1. Shima, J. E., McLean, D. J., McCarrey, J. R., and Griswold, M. D. (2004) *Biol Reprod* 71, 319-330
2. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* 241, 42-52
3. Loveland, K. L., and Schlatt, S. (1997) *J Endocrinol* 153, 337-344
4. Brennan, J., Tilmann, C., and Capel, B. (2003) *Genes Dev* 17, 800-810
5. Jamin, S. P., Arango, N. A., Mishina, Y., Hanks, M. C., and Behringer, R. R. (2002) *Nat Genet* 32, 408-410

6. Kharbanda, S., Pandey, P., Morris, P. L., Whang, Y., Xu, Y., Sawant, S., Zhu, L. J., Kumar, N., Yuan, Z. M., Weichselbaum, R., Sawyers, C. L., Pandita, T. K., and Kufe, D. (1998) *Oncogene* 16, 1773-1777
7. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D., and Wynshaw Boris, A. (1996) *Cell* 86, 159-171
8. Nolan, M. A., Babcock, D. F., Wennemuth, G., Brown, W., Burton, K. A., and McKnight, G. S. (2004) *Proc Natl Acad Sci U S A* 101, 13483-13488
9. Toshima, J., Koji, T., and Mizuno, K. (1998) *Biochem Biophys Res Commun* 249, 107-112
10. Toshima, J., Nakagawara, K., Mori, M., Noda, T., and Mizuno, K. (1998) *Gene* 206, 237-245
11. Toshima, J., Tanaka, T., and Mizuno, K. (1999) *J Biol Chem* 274, 12171-12176
12. Tseng, T. C., Chen, S. H., Hsu, Y. P., and Tang, T. K. (1998) *DNA Cell Biol* 17, 823-833
13. Hu, H. M., Chuang, C. K., Lee, M. J., Tseng, T. C., and Tang, T. K. (2000) *DNA Cell Biol* 19, 679-688
14. Chen, S. H., and Tang, T. K. (2002) *DNA Cell Biol* 21, 41-46
15. McCarrey, J. R., Berg, W. M., Paragioudakis, S. J., Zhang, P. L., Dilworth, D. D., Arnold, B. L., and Rossi, J. J. (1992) *Dev Biol* 154, 160-168
16. McCarrey, J. R., Kumari, M., Aivaliotis, M. J., Wang, Z., Zhang, P., Marshall, F., and Vandeberg, J. L. (1996) *Dev Genet* 19, 321-332
17. Walden, P. D., and Cowan, N. J. (1993) *Mol Cell Biol* 13, 7625-7635
18. Walden, P. D., and Millette, C. F. (1996) *Biol Reprod* 55, 1039-1044
19. Katschinski, D. M., Marti, H. H., Wagner, K. F., Shibata, J., Eckhardt, K., Martin, F., Depping, R., Paasch, U., Gassmann, M., Ledermann, B., Desbaillets, I., and Wenger, R. H. (2003) *Mol Cell Biol* 23, 6780-6789
20. Jinno, A., Tanaka, K., Matsushime, H., Haneji, T., and Shibuya, M. (1993) *Mol Cell Biol* 13, 4146-4156
21. Shinkai, Y., Satoh, H., Takeda, N., Fukuda, M., Chiba, E., Kato, T., Kuramochi, T., and Araki, Y. (2002) *Mol Cell Biol* 22, 3276-3280
22. Xu, X., Toselli, P. A., Russell, L. D., and Seldin, D. C. (1999) *Nat Genet* 23, 118-121
23. Hao, Z., Jha, K. N., Kim, Y. H., Vemuganti, S., Westbrook, V. A., Chertihin, O., Markgraf, K., Flickinger, C. J., Coppola, M., Herr, J. C., and Visconti, P. E. (2004) *Mol Hum Reprod* 10, 433-444
24. Bielke, W., Blaschke, R. J., Miescher, G. C., Zurcher, G., Andres, A. C., and Ziemiecki, A. (1994) *Gene* 139, 235-239
25. Kueng, P., Nikolova, Z., Djonov, V., Hemphill, A., Rohrbach, V., Boehlen, D., Zuercher, G., Andres, A. C., and Ziemiecki, A. (1997) *J Cell Biol* 139, 1851-1859
26. Nayak, S., Galili, N., and Buck, C. A. (1998) *Mech Dev* 74, 171-174
27. Zuercher, G., Rohrbach, V., Andres, A. C., and Ziemiecki, A. (2000) *Mech Dev* 93, 175-177
28. Wilks, A. F. (1991) *Methods Enzymol* 200, 533-546
29. Visconti, P. E., Hao, Z., Purdon, M. A., Stein, P., Balsara, B. R., Testa, J. R., Herr, J. C., Moss, S. B., and Kopf, G. S. (2001) *Genomics* 77, 163-170
30. Scorilas, A., Yousef, G. M., Jung, K., Rajpert-De Meyts, E., Carsten, S., and Diamandis, E. P. (2001) *Biochem Biophys Res Commun* 285, 400-408
31. Eskenazi, B., Wyrobek, A. J., Kidd, S. A., Lowe, X., Moore, D., 2nd, Weisiger, K., and Aylstock, M. (2002) *Hum Reprod* 17, 576-583
32. McLachlan, R. I., Mallidis, C., Ma, K., Bhasin, S., and de Kretser, D. M. (1998) *Reprod Fertil Dev* 10, 97-104
33. Smyth, C. M., and Bremner, W. J. (1998) *Arch Intern Med* 158, 1309-1314
34. Wang, C., Baker, H. W., Burger, H. G., De Kretser, D. M., and Hudson, B. (1975) *Clin Endocrinol (Oxf)* 4, 399-411
35. Conn, C. M., Harper, J. C., Winston, R. M., and Delhanty, J. D. (1998) *Hum Genet* 102, 117-123
36. Frydman, N., Romana, S., Le Lorc'h, M., Vekemans, M., Frydman, R., and Tachdjian, G. (2001) *Hum Reprod* 16, 2274-2277
37. Veld, P. A., Weber, R. F., Los, F. J., den Hollander, N., Dhont, M., Pieters, M. H., and Van Hemel, J. O. (1997) *Hum Reprod* 12, 1642-1644
38. Augarten, A., Kerem, B. S., Kerem, E., Gazit, E., and Yahav, Y. (1994) *N Engl J Med* 330, 866
39. Augarten, A., Yahav, Y., Kerem, B. S., Halle, D., Laufer, J., Szeinberg, A., Dor, J., Mashiach, S., Gazit, E., and Madgar, I. (1994) *Lancet* 344, 1473-1474
40. Dumur, V., Gervais, R., Rigot, J. M., Delomel-Vinner, E., Decaestecker, B., Lafitte, J. J., and Roussel, P. (1996) *Hum Genet* 97, 7-10
41. Jou, S. B., Lin, H. M., Pan, H., Chiu, Y. L., Li, S. Y., Lee, C. C., and Hsiao, K. M. (2001) *Proc Natl Sci Counc Repub China B* 25, 40-44

42. Meng, M. V., Black, L. D., Cha, I., Ljung, B. M., Pera, R. A., and Turek, P. J. (2001) *Hum Reprod* 16, 529-533
43. Sokol, R. Z. (2001) *Curr Opin Pulm Med* 7, 421-426
44. Collins, A., and Morton, N. E. (1998) *Proc Natl Acad Sci U S A* 95, 1741-1745
45. Friedman, K. J., Teichtahl, H., De Kretser, D. M., Temple-Smith, P., Southwick, G. J., Silverman, L. M., Highsmith, W. E., Jr., Boucher, R. C., and Knowles, M. R. (1995) *Am J Respir Crit Care Med* 152, 1353-1357
46. Boder, E. (1975) *Birth Defects Orig Artic Ser* 11, 255-270
47. Xu, E. Y., Moore, F. L., and Pera, R. A. (2001) *Proc Natl Acad Sci U S A* 98, 7414-7419
48. Wu, J. Y., Ribar, T. J., Cummings, D. E., Burton, K. A., McKnight, G. S., and Means, A. R. (2000) *Nat Genet* 25, 448-452
49. Kramer, J. A., Zhang, S., Yaron, Y., Zhao, Y., and Krawetz, S. A. (1997) *Genet Test* 1, 125-129
50. Eberhart, C. G., Maines, J. Z., and Wasserman, S. A. (1996) *Nature* 381, 783-785
51. Schultz, N., Hamra, F. K., and Garbers, D. L. (2003) *Proc Natl Acad Sci U S A* 100, 12201-12206
52. Truong, B. N., Moses, E. K., Armes, J. E., Venter, D. J., and Baker, H. W. (2003) *Asian J Androl* 5, 137-147
53. Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M., Currie, R. A., and Alessi, D. R. (2000) *Embo J* 19, 979-988
54. Biondi, R. M., Kieloch, A., Currie, R. A., Deak, M., and Alessi, D. R. (2001) *Embo J* 20, 4380-4390
55. Biondi, R. M., Komander, D., Thomas, C. C., Lizcano, J. M., Deak, M., Alessi, D. R., and van Aalten, D. M. (2002) *Embo J* 21, 4219-4228
56. Frodin, M., Antal, T. L., Dummler, B. A., Jensen, C. J., Deak, M., Gammeltoft, S., and Biondi, R. M. (2002) *Embo J* 21, 5396-5407
57. Frodin, M., Jensen, C. J., Merienne, K., and Gammeltoft, S. (2000) *Embo J* 19, 2924-2934
58. Vanhaesebroeck, B., and Alessi, D. R. (2000) *Biochem J* 346 Pt 3, 561-576
59. Biondi, R. M. (2004) *Trends Biochem Sci* 29, 136-142
60. Chen, H., Nystrom, F. H., Dong, L. Q., Li, Y., Song, S., Liu, F., and Quon, M. J. (2001) *Biochemistry* 40, 11851-11859
61. Wick, M. J., Ramos, F. J., Chen, H., Quon, M. J., Dong, L. Q., and Liu, F. (2003) *J Biol Chem* 278, 42913-42919
62. King, C. C., Gardiner, E. M., Zenke, F. T., Bohl, B. P., Newton, A. C., Hemmings, B. A., and Bokoch, G. M. (2000) *J Biol Chem* 275, 41201-41209
63. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr Biol* 7, 261-269
64. Anderson, K. E., Coadwell, J., Stephens, L. R., and Hawkins, P. T. (1998) *Curr Biol* 8, 684-691
65. Leslie, N. R., Biondi, R. M., and Alessi, D. R. (2001) *Chem Rev* 101, 2365-2380
66. Lizcano, J. M., and Alessi, D. R. (2002) *Curr Biol* 12, R236-238
67. Biondi, R. M., and Nebreda, A. R. (2003) *Biochem J* 372, 1-13
68. Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D. R. (2000) *Curr Biol* 10, 439-448
69. Moore, M. J., Kanter, J. R., Jones, K. C., and Taylor, S. S. (2002) *J Biol Chem* 277, 47878-47884
70. Balendran, A., Hare, G. R., Kieloch, A., Williams, M. R., and Alessi, D. R. (2000) *FEBS Lett* 484, 217-223
71. Finkel, T., and Holbrook, N. J. (2000) *Nature* 408, 239-247
72. Sarafian, T. A., and Bredesen, D. E. (1994) *Free Radic Res* 21, 1-8
73. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *Embo J* 17, 2596-2606
74. Adler, V., Yin, Z., Fuchs, S. Y., Benezra, M., Rosario, L., Tew, K. D., Pincus, M. R., Sardana, M., Henderson, C. J., Wolf, C. R., Davis, R. J., and Ronai, Z. (1999) *Embo J* 18, 1321-1334
75. Veal, E. A., Findlay, V. J., Day, A. M., Bozonet, S. M., Evans, J. M., Quinn, J., and Morgan, B. A. (2004) *Mol Cell* 15, 129-139
76. Ip, Y. T., and Davis, R. J. (1998) *Curr Opin Cell Biol* 10, 205-219
77. Coso, O. A., Teramoto, H., Simonds, W. F., and Gutkind, J. S. (1996) *J Biol Chem* 271, 3963-3966
78. Cuenda, A. (2000) *Int J Biochem Cell Biol* 32, 581-587
79. Davis, R. J. (2000) *Cell* 103, 239-252
80. Kyriakis, J. M., and Avruch, J. (2001) *Physiol Rev* 81, 807-869
81. Kennedy, N. J., Sluss, H. K., Jones, S. N., Bar-Sagi, D., Flavell, R. A., and Davis, R. J. (2003) *Genes Dev* 17, 629-637

82. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M., and Hotamisligil, G. S. (2002) *Nature* 420, 333-336
83. Kolodziejczyk, S. M., Walsh, G. S., Balazsi, K., Seale, P., Sandoz, J., Hierlihy, A. M., Rudnicki, M. A., Chamberlain, J. S., Miller, F. D., and Megeney, L. A. (2001) *Curr Biol* 11, 1278-1282
84. Han, Z., Boyle, D. L., Chang, L., Bennett, B., Karin, M., Yang, L., Manning, A. M., and Firestein, G. S. (2001) *J Clin Invest* 108, 73-81
85. Bishopric, N. H., Andreka, P., Slepak, T., and Webster, K. A. (2001) *Curr Opin Pharmacol* 1, 141-150
86. Xia, X. G., Harding, T., Weller, M., Bieneman, A., Uney, J. B., and Schulz, J. B. (2001) *Proc Natl Acad Sci U S A* 98, 10433-10438
87. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) *Nature* 389, 865-870
88. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) *Embo J* 15, 2760-2770
89. Whitmarsh, A. J., and Davis, R. J. (1996) *J Mol Med* 74, 589-607
90. Strahl, T., Gille, H., and Shaw, P. E. (1996) *Proc Natl Acad Sci U S A* 93, 11563-11568
91. Janknecht, R., and Hunter, T. (1997) *Embo J* 16, 1620-1627
92. Janknecht, R., and Hunter, T. (1997) *J Biol Chem* 272, 4219-4224
93. Essers, M. A., Weijzen, S., de Vries-Smits, A. M., Saarloos, I., de Ruiter, N. D., Bos, J. L., and Burgering, B. M. (2004) *Embo J* 23, 4802-4812
94. Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998) *Curr Biol* 8, 1387-1390
95. Tournier, C., Dong, C., Turner, T. K., Jones, S. N., Flavell, R. A., and Davis, R. J. (2001) *Genes Dev* 15, 1419-1426
96. Weston, C. R., and Davis, R. J. (2002) *Curr Opin Genet Dev* 12, 14-21
97. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) *EMBO Rep* 2, 222-228
98. Garrington, T. P., Ishizuka, T., Papst, P. J., Chayama, K., Webb, S., Yujiri, T., Sun, W., Sather, S., Russell, D. M., Gibson, S. B., Keller, G., Gelfand, E. W., and Johnson, G. L. (2000) *Embo J* 19, 5387-5395
99. Schaefer, B. C., Ware, M. F., Marrack, P., Fanger, G. R., Kappler, J. W., Johnson, G. L., and Monks, C. R. (1999) *Immunity* 11, 411-421
100. Xia, Y., Makris, C., Su, B., Li, E., Yang, J., Nemerow, G. R., and Karin, M. (2000) *Proc Natl Acad Sci U S A* 97, 5243-5248
101. Yang, J., Boerm, M., McCarty, M., Bucana, C., Fidler, I. J., Zhuang, Y., and Su, B. (2000) *Nat Genet* 24, 309-313
102. Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., and Su, B. (2001) *Nat Immunol* 2, 620-624
103. Yujiri, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsu, Y., Clarke, P., Tyler, K., Oka, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) *Proc Natl Acad Sci U S A* 97, 7272-7277
104. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) *Cell* 103, 1071-1083
105. Vidal, S., Khush, R. S., Leulier, F., Tzou, P., Nakamura, M., and Lemaitre, B. (2001) *Genes Dev* 15, 1900-1912
106. Morrison, D. K., and Davis, R. J. (2003) *Annu Rev Cell Dev Biol* 19, 91-118
107. Whitmarsh, A. J., and Davis, R. J. (1998) *Trends Biochem Sci* 23, 481-485
108. Enslen, H., and Davis, R. J. (2001) *Biol Cell* 93, 5-14
109. Sharrocks, A. D., Yang, S. H., and Galanis, A. (2000) *Trends Biochem Sci* 25, 448-453
110. Tanoue, T., and Nishida, E. (2003) *Cell Signal* 15, 455-462
111. Dai, T., Rubie, E., Franklin, C. C., Kraft, A., Gillespie, D. A., Avruch, J., Kyriakis, J. M., and Woodgett, J. R. (1995) *Oncogene* 10, 849-855
112. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev* 7, 2135-2148
113. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) *Science* 267, 389-393
114. Livingstone, C., Patel, G., and Jones, N. (1995) *Embo J* 14, 1785-1797
115. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) *Cell* 87, 929-939
116. Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. (1998) *Genes Dev* 12, 3369-3381
117. Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997) *Science* 277, 693-696
118. Kelkar, N., Gupta, S., Dickens, M., and Davis, R. J. (2000) *Mol Cell Biol* 20, 1030-1043
119. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998) *Science* 281, 1671-1674

120. Yasuda, J., Whitmarsh, A. J., Cavanagh, J., Sharma, M., and Davis, R. J. (1999) *Mol Cell Biol* 19, 7245-7254
121. Whitmarsh, A. J., Kuan, C. Y., Kennedy, N. J., Kelkar, N., Haydar, T. F., Mordes, J. P., Appel, M., Rossini, A. A., Jones, S. N., Flavell, R. A., Rakic, P., and Davis, R. J. (2001) *Genes Dev* 15, 2421-2432
122. Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K. I. (1999) *Mol Cell Biol* 19, 7539-7548
123. Matsuura, H., Nishitoh, H., Takeda, K., Matsuzawa, A., Amagasa, T., Ito, M., Yoshioka, K., and Ichijo, H. (2002) *J Biol Chem* 277, 40703-40709
124. Willoughby, E. A., Perkins, G. R., Collins, M. K., and Whitmarsh, A. J. (2003) *J Biol Chem* 278, 10731-10736
125. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) *Science* 290, 1574-1577
126. Miller, W. E., and Lefkowitz, R. J. (2001) *Curr Opin Cell Biol* 13, 139-145
127. Girardin, S. E., and Yaniv, M. (2001) *Embo J* 20, 3437-3446
128. Marti, A., Luo, Z., Cunningham, C., Ohta, Y., Hartwig, J., Stossel, T. P., Kyriakis, J. M., and Avruch, J. (1997) *J Biol Chem* 272, 2620-2628
129. Leonardi, A., Ellinger-Ziegelbauer, H., Franzoso, G., Brown, K., and Siebenlist, U. (2000) *J Biol Chem* 275, 271-278

Chapter

2

Characterization of Testis Specific Serine-threonine Kinase 3 and its activation by Phosphoinositide-Dependent Kinase -1-dependent signaling

Submitted

Characterization of Testis Specific Serine-threonine Kinase 3 and its activation by Phosphoinositide-Dependent Kinase -1-dependent signaling

Marta BUCKO-JUSTYNA[1]*, Leszek LIPINSKI[1,2]*, Boudewijn M. T. BURGERING[3], Lech TRZECIAK[1]

[1]Department of Molecular Biology, International Institute of Molecular and Cell Biology in Warsaw, Ks. Trojdena 4, 02-109 Warsaw, Poland, [2]Labolatory of Molecular Medicine, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland, [3]Department of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands

* These authors contributed equally to this work

SUMMARY

The family of testis specific serine-threonine kinases (TSSKs) consists of four members whose expression is confined almost exclusively to testis. Very little is known about their physiological role and mechanisms of action. We cloned human and mouse TSSK3 and analyzed its biochemical properties, substrate specificity and *in vitro* activation. *In vitro* TSSK3 exhibited the ability to autophosphorylate and to phosphorylate test substrates such as histones, myelin basic protein and casein. Interestingly, in keeping with it being testis specific, TSSK3 exhibited maximal *in vitro* kinase activity at a temperature of 32^{0}C. Sequence comparison indicated the existence within the TSSK3 catalytic domain of a so-called 'T-loop' a structure present in the AGC-family of protein kinases. To test if this T-loop is engaged in TSSK3 regulation, we mutated the critical threonine within the T-loop to alanine (T168A) and this resulted in an inactive TSSK3 kinase. Furthermore *in vitro* Thr-168 is phosphorylated by the T-loop kinase phosphoinositide-dependent protein kinase-1 (PDK1) and phosphorylation by PDK1 increased *in vitro* TSSK3 kinase activity, suggesting that TSSK3 can be regulated in the same way as AGC-kinase family members. Furthermore, analysis of peptide sequences defines the peptide sequence RRSSSY, in which the second serine serves as phospho-acceptor, as an efficient and specific substrate for TSSK3.

INTRODUCTION

Phosphorylation of proteins by protein kinases constitutes a major regulatory mechanism in *Eukarya*, affecting virtually every cellular process. The human genome contains genes encoding for over 500 protein kinases [1] and a substantial number of these are relatively well known as their mode of regulation, targets and functional roles have been studied in multiple tissues. Meanwhile, a substantial number of kinases was cloned using molecular screening methods based on sequence conservation only, and a further 70 kinases were not identified until the assembled genome sequence was scanned [1]. Not surprisingly, many of these kinases have remained poorly characterized, thus leaving a

substantial gap in our understanding of cellular regulatory networks. Here we describe a study on one of such uncharacterized kinase, the Testis-Specific Protein Kinase 3 (TSSK3).

The mouse TSSK3 has been originally described as a third member of the subfamily of protein kinases expressed in testis [2]. Characteristically, it was identified using low-stringency hybridization with a partial sequence obtained from cDNA amplification utilizing degenerate primers [3]. Our group has independently obtained a fragment of human *TSSK3* sequence, employing the same degenerate primers method to study kinases expressed in human AGS cell line (L.T. unpublished). The complete sequence of *hTSSK3* was published by Visconti et al. [4] shortly after it became available as a part of accessible Human Genomic Project sequences. Both the mouse and human sequence encode for a small protein of 29 kDa, consisting of a catalytic domain only. Interestingly, TSSK3 has no orthologues in non-mammals. Immunohistochemical studies in mice indicate that TSSK3 is present exclusively in testicular Leydig cells [2], unlike the two other members of TSSK subfamily, TSSK1 and TSSK2, whose expression is limited to meiotic and postmeiotic spermatogenic cells, respectively [5] [6]. The TSSK3 mRNA level is low at birth, increases substantially at puberty and remains high throughout adulthood. Thus TSSK3 seems to play an important role in adult testis.

Testis comprises of an interstitial compartment with Leydig cells and seminiferous tubules containing Sertoli cells, spermatogenic cells and peritubular myoid cells. Despite this apparently simple structure, the development of testis is rather complicated, involving migration of germ cells and regression of developing female reproductive tract [7] followed by a descent of the formed testis to the scrotal sac [8], where the temperature is about 5°C lower than in the abdomen.

Testis in adults continuously performs two main functions: Leydig cells synthesize androgens, while seminiferous tubules produce sperm [9]. The latter is a large scale process, comprising of an intense proliferation coupled to meiotic divisions [10], and requires a very precise control. An estimated two-thirds of mammalian genes is at some point expressed in adult or developing testis [11], with 5% to 10% of genes expressed exclusively there; moreover, testis makes an extensive use of alternative splicing [12] and translational control [13].

Among genes playing role in testis function there is a large number of protein kinases. Several ones have been already shown to be indispensable. For example, kit receptor tyrosine kinase is critical for migration of primordial germ cells [14]. Another member of this group, Platelet-Derived Growth Factor Receptor a (Pdgfra), is involved in testis descent and development of Leydig cells [15]. Disruption of the receptor serine-threonine kinase Bone Morphogenetic Protein Receptor 1 (Bmpr1) leads to the retention of female Mullerian ducts in males [16]. Abl tyrosine kinase and Ataxia-Teleangiectasia Mutated (ATM) serine-threonine kinases participate in controlling meiosis during gametogenesis [17] [18]. All these kinases are, however, present in a variety of tissues. Thus it is important to elucidate also the role of kinases expressed exclusively in testis in order to better understand the regulation of its function, especially in light of an increasing rate of male infertility on one hand and possible applications to the development of male contraceptives on the other, given the recent therapeutic success of small inhibitors of protein kinases such as imatinib. Among testis-specific kinases, some appeared indispensable, such as Casein Kinase 2a' (CK2a') [19]; whereas others, surprisingly,

were not (for example, PASK [20]).

In this paper we present evidence that TSSK3 is a genuine kinase that can be regulated *in vitro* by PDK1 through phosphorylation of a classical activation loop and that it might be *in vivo* a target of PDK1 signaling as well. We also show that the peptide RRSSSY is specifically phosphorylated by TSSK3, what should direct future searches for TSSK3 substrates and help define its function in testis.

EXPERIMENTAL PROCEDURES

TSSK3 constructs

PCR, restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit on Applied Biosystems automated DNA sequencers.

Total RNA from mouse and human testis was isolated by homogenization in TRI REAGENT (Sigma) as described by the manufacturer. First strand cDNA synthesis was performed from 5µg of total RNA using the Fermentas RevertAid kit with oligo-dT primers according to manufacturers suggestions.

The full-length TSSK3 coding sequence was PCR amplified from a human or mouse testis cDNA, respectively using oligonucleotide primers GGTGGTCATATGGAGGACTTTCTRCTCT / CAC TTGCCATTGCTTTTATCA and ligated into *Sma*I site of pUC 18 vector.

The *E.coli* pGEX-mTSSK3 or pGEX-hTSSK3 plasmids were constructed using pGEX-4T-2, which expresses the target protein as a fusion protein with GST. The full-length human and mouse TSSK3 were subcloned from pUC18mTSSK3 or pUC18hTSSK3, respectively into multicloning site of pGEX-4T-2 using *BamH*I, *Eco*RI restriction sites. The sequence was put in frame by cutting of *Bam*HI, *Nde*I fragment, filling in protruding ends and religation.

In order to generate mammalian expression constructs encoding the full-length human or mouse HA-tagged TSSK3 (HA-hTSSK3 or HA-mTSSK3 respectively), the following primer pair was used: primer 1/primer 2 (GCGCTGTCGACCATGGAGGACTTTCTGCTCT/ CATTGAATTCCTCAAGTGCTTGCTAGCCATG). The forward (5') primer contained a *Sal*I site, whereas the reverse (3') primer contained an *Eco*RI site. The amplified products were digested with the corresponding enzymes and subcloned into *Sal*I/*Eco*RI cut pMT2-HA vector.

To generate point mutants of GST-TSSK3, site-directed mutagenesis was engaged [21]. Six point mutants were created in pGEX-mTSSK3: K39R, the Lys-39-to Arg mutation; T168A, the Thr-168-to Ala mutation; T168D, substitution of Thr-168 to Asp; S166A, the Ser-166-to-Ala mutation; S166G, the Ser-166-to-Gly mutation and S166D, with substitution of Ser-166-to Asp.

GST-TSSK3 protein purification from *E.coli*

GST-hTSSK3 or GST-mTSSK3 was over-expressed in *E.coli* BL21 RIL [DE3] strain. 1 litre culture was grown at 30^{O}C (OD_{600nm}=0.6). Induction was carried out 4h with 1 mM isopropyl β-D-thiogalactosidase (IPTG) at 20^{0}C, and the cells were harvested by centrifugation. Bacterial pellet was incubated (0.5h, 4^{O}C) in 20 ml lysis buffer (50 mM Tris/HCl (pH 7.5); 1 mM EDTA; 5% (v/v) glycerol; 0,1% 2-mercaptoethanol; 1 mM PMSF;) containing 0.5mg/ml lizozyme and cells were disrupted by adding 5 ml 5M NaCl at 42^{O}C for 5 min. The protein was purified by one-step affinity chromatography using GSH-agarose. After washing the column (50 mM Tris/HCl (pH 7.5); 200 mM NaCl; 1 mM EDTA; 5% (v/v) glycerol; 0,1% 2-mercaptoethanol; 1 mM PMSF) the protein was eluted in the washing buffer containing 10 mM glutathione and it was analyzed by SDS-PAGE. In the purified fraction there was a major band at approximately 55 kDa (Fig. 1A) what is consistent with the predicted molecular mass of the fusion protein.

Cloning, purification and phosphorylation of GST-peptides

In order to clone peptides for phosphorylation reactions with TSSK3, pairs of oligonulcleotides were ordered coding for: peptide 1, KQSPSSSPT; peptide 2, KLRRSSSVG; peptide 3, LRRSSSVGY; peptide 4, KRRSSSYHV; peptide NEG, PRPASVPPS; peptide PKA (kemptide), LRRASLG, and mutant peptides: peptide 2(V8Y) KLRRSSSYG, with substitution of Val-8 to Tyr, peptide 4(S4,5C), KRRCCSYHV, with substitution of Ser-4 and Ser-5 to Cys, peptide 4(S5,6C), KRRSCCYHV with substitution of Ser-5 and Ser-6 to Cys and peptide 4(S4,6C), KRRCSCYHV with substitution of Ser-4 and Ser-6 to Cys. Oligonucleotides contained *Eco*RI overhang on 5' site and *Not*I overhang on 3' site to ligate annealed oligonucleotides into pGEX-6P-1 vector cut with *Eco*RI, *Not*I. Additionally, oligonucleotides contained *Kpn*I restriction site to select for correct clones. The pGEX-6P-1 constructs encoding GST-peptides were

transformed into BL21 E.*coli* cells and 0.5-liter culture was grown at 37^0C in Luria broth containing 100 μg/ml ampicilin until the absorbance at 600 nm was 0.6 and 0.1 mM IPTG was then added. The cells were cultured for further 5h at 25^0C, resuspended in 10 ml of ice-cold lysis buffer containing 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 5% glycerol, 0.03% (by vol.) 2-mercaptoethanol. The suspension was sonicated and the lysates centrifuged at 4^0C for 45 min at 50 000 ***g*** and incubated with 0.25 ml of GSH-agarose (Sigma) for 2h. The resin was washed in wash buffer containg 50 mM Tris/HCl pH 7.5, 400 mM NaCl, 5% glycerol, 0.03% (by vol.) 2-mercaptoethanol and resuspended in wash buffer plus 10 mM glutathion to elute GST-peptides from the resin. The kinase assays with purified GST-peptides and TSSK3 were carried out as described in *Substrate phosphorylation assays*. Additionally, after autoradiography, bands of phosphorylated peptides were excised from the gel and [^{32}P]Pi incorporation was determined by liquid scintillation counting. Results were normalized to 1μg of GST-TSSK3.

Substrate phosphorylation assays

Substrate phosphorylation assays were performed in 20 μl of kinase reaction buffer containing 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 5 mM $MnCl_2$ or 5 mM $MnCl_2$ only, 15 μM ATP, 3 μCi of [γ-^{32}P] ATP (3000 Ci/mmol), 0.17 mg/ml casein or 0.33 mg/ml MBP (myelin basic protein) or 0.33 mg/ml histone HI with 1 μg of GST-TSSK3 at 30^0C for 30 min.

For temperature dependence assay, the reaction was performed in the range of temperatures 28-42^0C, for pH optimum 50 mM Hepes was used with pH set from 6.8 to 8.0 changing by 0.2 per sample. In experiment testing cation requirements the concentrations of $MgCl_2$ or $MnCl_2$ varied from 0 to 9 mM while the concentration of other reaction components remained constant. In ATP dependence assay the concentration of ATP was increasing from 5 to 300 μM with maintaining constant 3 μCi of [γ-^{32}P] ATP. For triphosphonucleotides competition experiment reaction was carried out with 15μM ATP, 3 μCi of [γ-^{32}P] ATP and the increasing concentrations of CTP or GTP were added (0-375 μM).

All of the above reactions were terminated by adding Laemmli sample buffer and heating samples at 100^0C for 10 min. Aliquots were separated by SDS/PAGE, and after staining with 0.1% Coomassie Brilliant Blue the gels were vacuum-dried and exposed to X-ray film at –80^0C.

TSSK3 *in vitro* phosphorylation and activation assays

GST-TSSK3 attached to glutathione–agarose was subjected to *in vitro* phosphorylation by catalytic subunit of PDK1 (kindly provided by D.Alessi, MRC Dundee [22, 23]) in buffer A containing 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 2.5 μM PKI peptide, 10 mM $Mg(Ac)_2$, with 0.9 nM PDK1. Incubation was carried out for 30 min at 30^0C. In parallel samples with GST-TSSK3 were phosphorylated by catalytic subunit of PKA (Promega) in buffer B: 40 mM Tris/HCl pH 7.4, 20 mM $Mg(Ac)_2$ with 0.3 μM PKA followed by incubation at 30^0C for 30 min or by PKB [24] in buffer C: 50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT with 0.3 μM PKB followed by incubation at 37^0C for 30 min. All buffers were supplemented with 100 μM unlabelled ATP and 5 μCi of [γ-^{32}P] ATP per reaction. Subsequent phosphorylation of TSSK3 with PDK1 and PKA was also performed using cold ATP, then the protein kinase was washed away and PKA or PDK1, respectively was used for phosphorylation of GST-TSSK3 with [γ-^{32}P]ATP.

The phosphorylation reactions of GST-TSSK3^{K39R} by Myc-PDK1 [23] immunoprecipitated from 293T cells and of HA-TSSK3^{K39R} immunoprecipitated from 293T cells, by PDK1 catalytic subunit were carried out in buffer A.

For assaying GST-TSSK3 activation, a coupled kinase assay was performed. GST-TSSK3 attached to glutathione-agarose beads was pre-phosphorylated using cold-ATP by either PDK1 or PKA. After washing away PDK1 or PKA, GST-TSSK3 activity was assayed using [γ-^{32}P]ATP and Histone f2a as a substrate. Subsequent phosphorylation with PDK1 and PKA were also performed as in phosphorylation assay described above. All of the above reactions were terminated by addition of 5x Laemmli sample buffer, proteins were separated by SDS/PAGE and after staining with 0.1% Coomassie Brilliant Blue the gels were vacuum-dried and exposed to X-ray film at –80^0C.

Cells and transfections

Insulin receptor-overexpressing mouse NIH 3T3 cells (A14) and HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Sigma) and 1% antibiotic suspension (penicillin and streptomycin; Sigma) and 2mM L-glutamine. Prior to stimulation, cells were deprived of serum for 18h. Insulin or LY294002 were added at a final concentration of 1μg/ml or 10μM, respectively. Transfections were

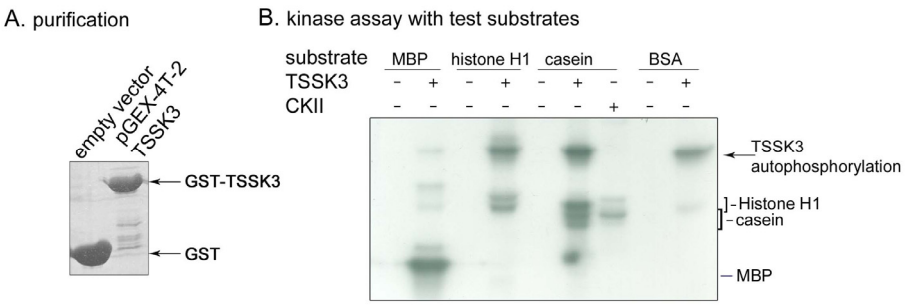


Fig. 1. Purification of GST-TSSK3 (A) and kinase assay with test substrates
(A) Comassie Brilliant Blue stained protein gel of purified mouse GST-TSSK3 kinase. TSSK3 was expressed in *E.coli* BL21 as a fusion with GST that allows for one-step affinity purification on glutathione beads. **(B)** Autoradiogram of TSSK3 kinase assay (using [γ-^{32}P]ATP) with test substrates: MBP; histone H1; casein; BSA, negative control. CKII, casein kinase II was used as positive control for casein phosphorylation. The reaction was carried out in 30^{0}C in the kinase buffer supplemented with 5 mM $MgCl_2$ and 5 mM $MnCl_2$, 15μM ATP, 3 μCi of [γ-^{32}P] ATP.

carried out using the $CaPO_4$ method for A14 cells, PEI reagent (Polyethylenimine, *Polysciences, Inc.*) was used to transfect HEK 293T cells.

Antibodies

The following antibodies were used: 12CA5 for HA-tagged proteins and 9E10 for Myc-tagged proteins, anti-phospho AKT (T308) (Transduction Laboratories)

Immunoprecipitation and in vitro kinase assays.

A14 or HEK 293T cells were lysed in ice-cold kinase lysis buffer containing: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% piridinium betain, 5 mM EDTA, 10 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and lysates were cleared for 10 min at 20 000 ***g*** at 4^{0}C. HA-TSSK3 or HA-PKB was immunoprecipitated by protein A-Sepharose beads coupled to the 12CA5 monoclonal antibody; Myc-PDK1 by 9E10 monoclonal antibody and rotation at 4^{0}C for 2h. Beads were washed twice with kinase lysis buffer and once with kinase reaction buffer. For kinase reactions, the beads were incubated in kinase buffer (containing 3 μCi of [γ-^{32}P] ATP per reaction) with histone 2B (for HA-TSSK3 or HA-PKB) or with GST or GST-TSSK3 (for Myc-PDK1) at 30^{0}C for 30 min, taken up in 5x Laemmli sample buffer, and analysed by SDS/PAGE followed by autoradiography.

Phosphoamino acid analysis

GST-TSSK3 fusion protein after autophosphorylation or PDK1 phosphorylation was separated by SDS/PAGE and immobilized on polyvinylidene difluoride membrane (Pall Cor.). The region of the membrane containing the ^{32}P-labeled was excised and incubated with 6N HCl for 1h at 110^{O}C. The hydrolysates were separated by thin-layer chromatography [25], ^{32}P-labeled phosphoamino acids were detected by autoradiography and compared with phosphoamino acid standards (Sigma) stained with ninhydrin.

RESULTS

Cloning, expression and substrates phosphorylation of TSSK3

To analyze the function of the family of testis specific kinases we chose to clone full-length human and mouse TSSK3. To biochemically characterize TSSK3 kinase *in vitro* we expressed human and mouse TSSK3 as GST-fusion protein and purified GST-TSSK3 (Fig. 1A) was then assayed for possible kinase activity. Since specific substrates for TSSK3 are unknown, we used artificial substrates like myelin basic protein (MBP), histone HI and casein to detect kinase activity of purified GST-TSSK3 in the presence of [γ-^{32}P]ATP and 5 mM $MgCl_2$ and 5mM $MnCl_2$. The phosphorylated proteins were separated by SDS-PAGE and analyzed by autoradiography (Fig. 1B). All three tested

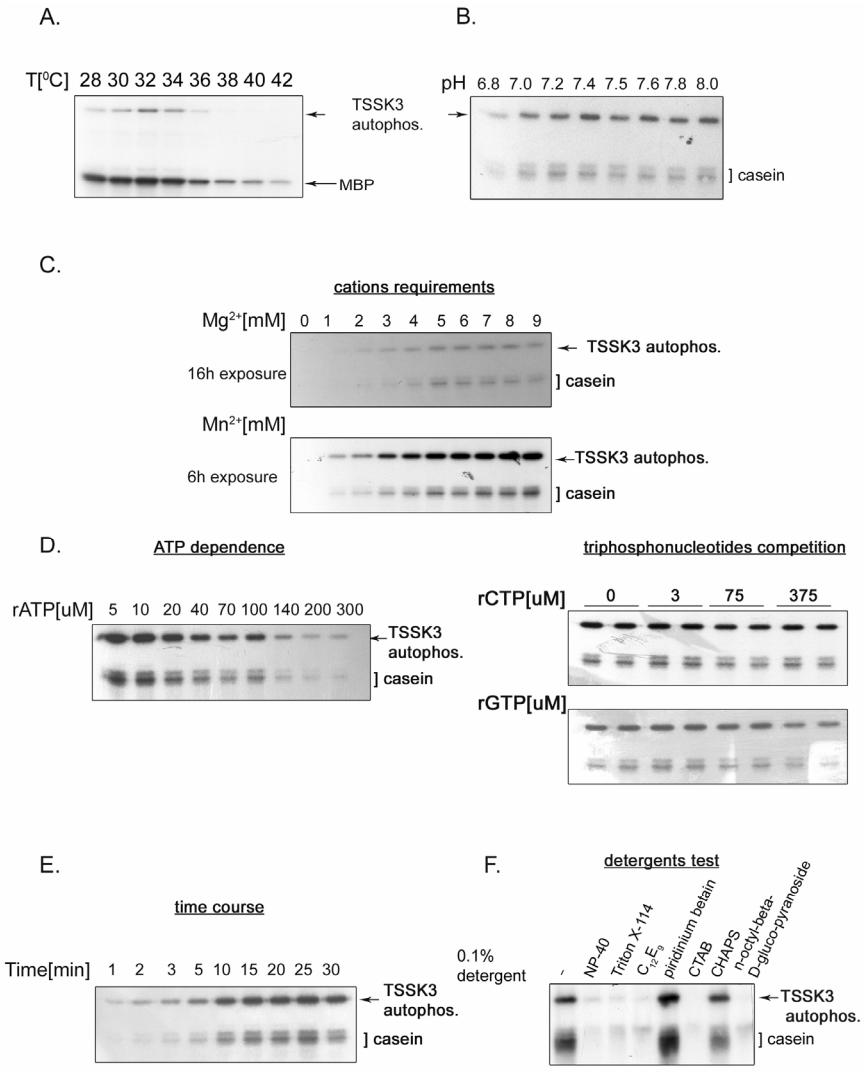


Fig. 2. Determination of requirements of TSSK3 for its activity

Mouse GST-TSSK3 protein was subjected to several *in vitro* phosphorylation reactions with test substrates to determine **(A)** temperature; **(B)** pH; **(C)** divalent metal cation concentrations (Mn^{2+}, Mg^{2+}), **(D)** ATP dependence (the concentration of ATP was increasing from 5 to 300 µM with maintaining constant 3 µCi of [γ-^{32}P] ATP) and triphosphonucleotide competition (reaction was carried out with 15µM ATP, 3 µCi of [γ-^{32}P] ATP and the increasing concentrations of CTP or GTP were added), **(E)** Time course of TSSK3 autophosphorylation and phosphorylation of casein carried out in standard experimental conditions described in *Experimental Procedures* **(F)** Detergents test. Buffer used for TSSK3 kinase assays was supplemented with 0.1% of various detergents, and TSSK3 activity was tested in the temperature of 32^{0}C. Proteins were fractionated by SDS-PAGE and visualized by autoradiography. In all experiment the concentration of ATP was 15 µM (besides D, left panel), the kinase buffer was supplemented with 5 mM $MgCl_2$ and 5 mM $MnCl_2$ (A, B) or 5 mM $MnCl_2$ only (D, E, F) and the kinase reaction was carried out in 32^{0}C (besides A).

substrates are phosporylated by recombinant TSSK3, although with different efficiency. This demonstrated that TSSK3 is a genuine protein kinase. We also observed a significant level of autophosphorylation of TSSK3.

Characterization of the optimal conditions required for maximal kinase activity of the purified recombinant TSSK3

To carry out biochemical characterization of purified recombinant TSSK3 protein kinase, we determined the temperature requirements (Fig. 2A), the pH optimum (Fig. 2B) and divalent metal cation requirements (Fig. 2C) of TSSK3 to optimize *in vitro* kinase assay conditions. The enzyme has a broad optimal pH range with the maximal activity at pH 7.4, therefore all subsequent assays were conducted at this pH. TSSK3 exhibits its highest activity at lower temperatures, for substrate phosphorylation in the range 28-34^{0}C and with highest autophosphorylation level at 32^{0}C. These temperature requirements support the previous reports about TSSK3 as a protein kinase expressed exclusively in testis [2, 26]. Since temperature is an important factor in sperm production the position of testes provides a lower temperature (at least 4-5^{0}C, in human and 4-7^{0}C, in mouse) than within the rest of the body [27].

Triphosphonucleotide binding to the catalytic domain of protein kinases is mediated by divalent cations, mainly Mn^{2+} or Mg^{2+}. The divalent cation preference of TSSK3 was determined by measuring kinase activity in the presence of various concentrations of Mg^{2+} or Mn^{2+} with casein as the phosphate-accepting substrate (Fig. 2C). It was found that TSSK3 prefers Mn^{2+} to Mg^{2+} for the maximal activity with 5mM concentration of $MnCl_2$ as sufficient for efficient phosphorylation of the test substrate casein.

The kinase reaction of TSSK3 is ATP dependent (Fig. 2D). Increasing the concentration of the non-radioactive γ-phosphate group (rATP) and maintaining the same concentration of [γ-^{32}P]ATP, decreased the ability of TSSK3 to transfer radioactive ATP on the substrate whereas, increasing concentrations of rCTP or rGTP, did not compete with ATP.

Furthermore, we determined the *in vitro* kinetics of TSSK3 activity towards a test substrate (casein) (Fig. 2E). The total incorporation of radioactive phosphate group seems to reach the maximum level after 10 minutes of the reaction and it does not change after this time point. In our search for the best conditions to study TSSK3 kinase we performed an additional experiment testing the detergent resistance of TSSK3 by conducting a phosphorylation reaction with test substrate (casein) in the presence (in kinase buffer) of 0.1% of various detergents (Fig. 2F). TSSK3 is very sensitive to most of the commonly used detergents and only piridinium betain and CHAPS do not abolish its activity. Taken together these results established the conditions for the *in vitro* kinase reactions with TSSK3 in further experiments.

TSSK3 kinase can be activated *in vitro* by autophosphorylation or PDK1-mediated phosphorylation within activation/T-loop motif

Analysis of the TSSK3 primary sequence revealed the presence of a structure reminiscent of the activation loop of protein kinases belonging to the AGC kinase family [28] (Fig. 3A). Within this family of kinases the threonine or serine residue within the T-loop needs to be phosphorylated in order to obtain maximal kinase activity. As TSSK3 purified from bacteria displays already kinase activity, we reasoned that T-loop phosphorylation may occur in part through autophosphorylation. To study the potential involvement of the T-loop in regulating TSSK3 kinase activity we mutated the T-loop

residue threonine 168 to alanine (T168A) to prevent phosphorylation, or to aspartate (T168D) to mimic T-loop phosphorylation. We also mutated serine 166 to alanine (S166A), glycine (S166G) or to aspartate (S166D) as this may either be part of the recognition motif for T168 phosphorylation or potentially phosphorylated by autophosphorylation and thereby replacing in part the requirement for T168 phosphorylation. Kinase activity of these mutants was compared to a classical kinase-dead mutation in which the critical lysine of the ATP binding packet was mutated to arginine (K39R) (Fig. 3B). As expected, the kinase dead mutant (K39R) and T-loop mutant (T168A) lost completely their kinase activity. Mutating Ser-166 (S166A, S166G) abolished autophosphorylation capability of recombinant TSSK3 and decreased its kinase activity towards a substrate but the substitution of Ser-166 with negatively charged Asp (mimicking the negatively charged phosphate group) almost rescued kinase activity to the wild type level. At the same time substitution of Thr-168 with Asp resulted in significant activation of TSSK3, compared to wild type TSSK3. Importantly, the T168D mutant retained autophosphorylation activity, while S166D mutant was not able to autophosphorylate. Based on these results we propose that *in vitro* Ser-166 is phosphorylated by autophosphorylation within activation loop while Thr-168 is supposedly the site involved in the regulation of TSSK-3 activity by other kinases. Thin layer chromatography of hydrolysates of ^{32}P-labeled GST-TSSK3 wild type (Fig. 3C) shows that it is indeed serine that is autophosphorylated on TSSK3. Thus these data showed that Ser-166 and Thr-168 located within a T-loop play a significant role in the regulation of TSSK3 activity and suggests that the mechanism of its activation is similar to that of the AGC kinase family.

For a number of AGC kinases the 3-phosphoinositide-dependent protein kinase-1 (PDK1) has been shown to be responsible for T-loop phosphorylation (e.g. PKB [29], p70S6K [30], PKC [31]). In all cases described thus far, T-loop phosphorylation results in kinase activation. On the other hand, the sequence within the T-loop is also highly conserved in the Ca^{2+}-and calmodulin-dependent protein kinase family (CaMK) to which TSSK3 is classified [1] and yet it was shown that PDK1 does not phosphorylate CaMK kinases [30]. Interestingly, recently MEK1/2, was reported to be phosphorylated by PDK1 [32] and they also possess the PDK1-mediated phosphorylation sites in their T-loop. So the classification of a protein kinase to a certain family does not help, in this case, in the prediction whether a kinase will be a PDK1 substrate.

We therefore set out to investigate whether PDK1 can phosphorylate *in vitro* Thr-168 of TSSK3, homologous to threonines phosphorylated by PDK1 in other kinases (see Fig. 3A). Purified active PDK1 (catalytic subunit) could efficiently phosphorylate wild type GST-TSSK3^{WT} but not GST-TSSK3^{T168A} (Fig. 3D). Furthermore, full length Myc-PDK1 immunoprecipitated from 293T cells efficiently phosphorylated GST-TSSK3^{K39R} (Fig. 3E, upper panel) and HA-TSSK3^{K39R} immunoprecipitated from 293T cells as well (Fig. 3E, middle panel). To further support that PDK1 phosphorylates threonine on TSSK3 we performed phosphoamino acid mapping of GST-TSSK3 WT or kinase dead mutant phosphorylated by PDK1 under conditions that prevent TSSK3 autophosphorylation. As only threonine phosphorylation was observed, this confirmed that indeed Thr-168 is located within a T-loop that can be phosphorylated by PDK1 and that PDK1 can act as an upstream kinase in the regulation of TSSK3 (Fig. 3E, lower panel).

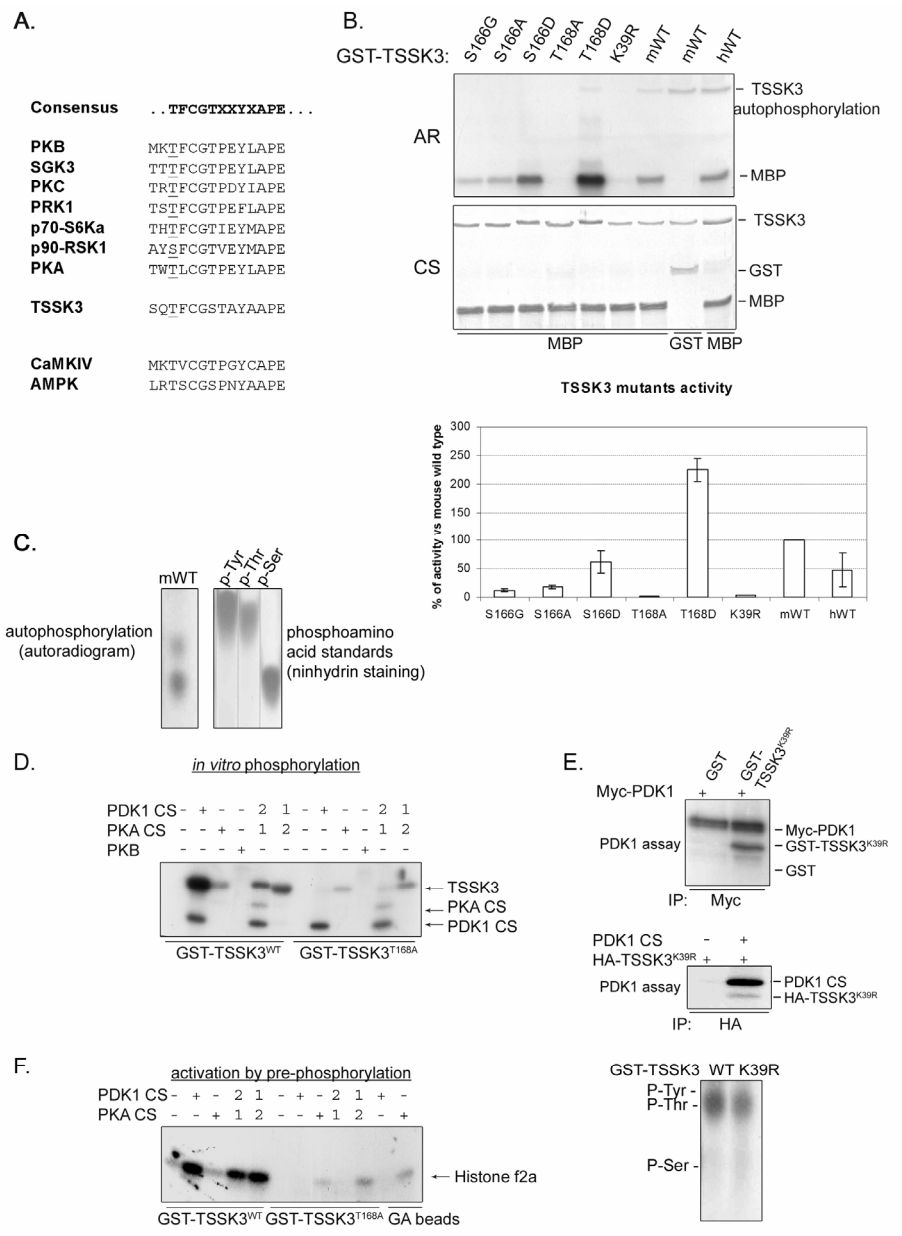


Fig. 3. TSSK3 kinase can be activated by autophosphorylation or PDK1-mediated phosphorylation within activation/T-loop motif.
(A) Alignment of the amino acid sequences surrounding the T-loop motif of AGC kinases and CaMK kinases in comparison with (mouse and human) TSSK3 T-loop sequence. The underlined residues correspond to those that become phosphorylated. Substrates data taken from [30], [28]. **(B)** Upper panel: Test of kinase activity of different mouse GST-TSSK3 mutants in *in vitro* kinase assay using MBP as test substrate; K39R, kinase dead mutant; T168A, T-loop mutant; T168D, kinase active mutant; S166A, S166G, S166D, T-loop mutants; hWT,

In these experiments, addressing the ability of PDK1 to phosphorylate TSSK3, we used active PKB and PKA (catalytic subunit) as controls. Indeed, as expected, since TSSK3 lacks a PKB consensus phosphorylation sequence, we did not observe PKB mediated phosphorylation, yet surprisingly we observed significant phosphorylation by PKA *in vitro*. To determine the consequence of *in vitro* TSSK3 phosphorylation on TSSK3 activity we performed a coupled kinase assay. GST-TSSK3 attached to glutathione-agarose beads was pre-phosphorylated using cold-ATP by either PDK1 or PKA. After washing away PDK1 or PKA, GST-TSSK3 activity was assayed using [γ-^{32}P]ATP and Histone f2a as a substrate (Fig. 3F). This experiment showed that phosphorylation of TSSK3 at Thr-168 results in a significant increase in TSSK3 activity. In parallel, although PKA can phosphorylate TSSK3, pre-phosphorylation did not resulted in increased TSSK3 activation in this assay.

TSSK3 can be activated in the mammalian cells by insulin

Having established *in vitro* that PDK1 can indeed function as an upstream kinase in TSSK3 regulation we turned to an *in vivo* model system in which PDK1 is active. Insulin treatment of A14 cells (NIH3T3 cells over-expressing the human insulin receptor) results in a rapid and strong activation of PKB (also known as c-Akt) [33] and this is mediated by PI3K and PDK1. Thus A14 cells were transfected with HA-tagged TSSK3, and treated with insulin for several time periods. Following cell lysis HA-tagged TSSK3 was isolated by immunoprecipitation and TSSK3 activity was measured *in vitro* using [γ-^{32}P]ATP (Fig. 4A). We were able to observe an increase in TSSK3 activity towards test substrate following insulin or EGF treatment (data not shown). This result suggests that PDK1 might be involved in TSSK3 activation *in vivo* in cells. However, when A14 cells were pretreated before insulin stimulation with the PI(3)K inhibitor LY294002 this inhibited insulin-induced PKB activation but

---

human wild type; mWT, mouse wild type, AR, autoradiography; CS, Coomassie staining; purified GST (glutathione-S-transpherase) was used as negative control of phosphorylation. Lower panel: bands of phosphorylated MBP by TSSK3 mutants were excited from gel and their radioactivity was measured by scintillation counting. Data are representative of three independent experiments and compared to mouse wild type TSSK3 activity taken as 100%. **(C)** One-dimensional thin layer chromatography of hydrolysates of ^{32}P-labeled mouse GST-TSSK3 wild type (mWT). The positions of standard phosphoamino acids are indicated, p-Ser, phosphoserine; p-Thr, phosphothreonine; p-Tyr, phosphotyrosine. **(D)** *In vitro* phosphorylation of mouse GST-TSSK3 wild type (GST-TSSK3^{WT}) or T168A mutant (GST-TSSK3^{T168A}) by PDK1 CS (catalytic subunit, 0.9nM), PKA CS (catalytic subunit, 0.3 μM) or PKB (0.3 μM) kinases. **(E)** 293T cells were transfected with expression vectors encoding Myc-PDK1 or HA-TSSK3^{K39R}, as indicated. Ectopic Myc-PDK1 or HA-TSSK3^{K39R} were isolated from the cell lysates by immunoprecipitation by anti-Myc or anti-HA antibody, respectively and assayed for PDK1 kinase activity with GST-TSSK3^{K39R} as a substrate (upper panel) or PDK1 catalytic subunit was added to immunoprecipitated protein (middle panel) and kinase reaction was carried out. Lowest panel: One-dimensional thin layer chromatography of hydrolysates of ^{32}P-labeled GST-TSSK3 mutants phosphorylated by Myc-PDK1 in conditions preventing TSSK3-WT autophosphorylation (absence of Mn^{2+}ions and addition of PKI peptide to PDK1 kinase buffer). **(F)** TSSK3 activation after *in vitro* pre-phosphorylation with PDK1 CS or PKA CS; Histone f2a was used as a test substrate for assaying activity of GST-TSSK3^{WT} or GST-TSSK3^{T168A} attached to glutathione-agarose beads (GA beads); TSSK3 was pre-phosphorylated with PDK1 (0.9nM) or PKA (0.3 μM), using cold ATP, washed twice (to remove PDK1 and PKA kinases), subjected to kinase assay with [γ-^{32}P]ATP. Proteins were fractionated by SDS-PAGE and visualized by autoradiography. Numbers 1 and 2 (C, E) indicate the order of the kinases used, in the samples where the subsequent phosophorylation with PKA and PDK1 was performed

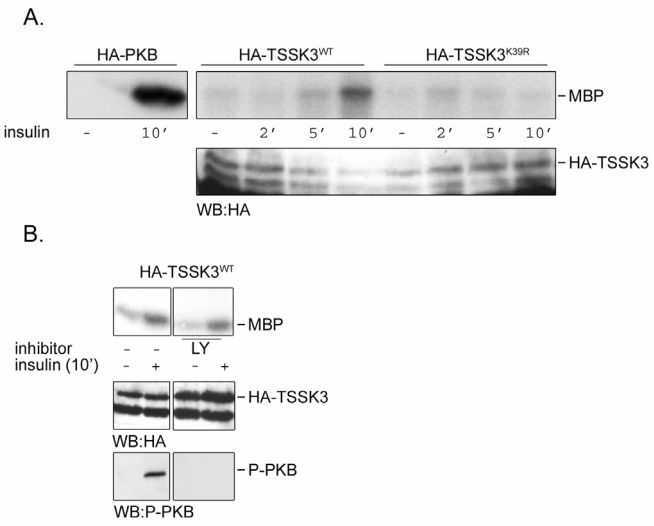


Fig. 4. TSSK3 can be activated in the cells by insulin
A14 cells were transfected with HA-tagged TSSK3 WT (wiled type) or K39R (kinase dead mutant) or HA-tagged PKB, and treated with insulin (1µg/ml final concentration) for indicated time periods (A) or 10 µM LY294002 (LY) followed by insulin (B). Following cell lysis, HA-tagged TSSK3 was isolated by immunoprecipitation and TSSK3 activity was measured *in vitro* using MBP as the test substrate. Blots were probed for expression of HA-TSSK3 (A, B). Total lysates were probed for phosphorylated PKBpT308 (B).

did not cause a decrease in TSSK3 activation by insulin (Fig. 4B). Thus the involvement of PDK1 in TSSK3 activation would be different from its involvement in the activation of PKB.

TSSK3 specifically phosphorylates *in vitro* the amino acid sequence motif RRSSSY.

Since the natural substrates for TSSK3 have not been identified yet and the amino acid sequences recognized by TSSK3 are not characterized, we set out to determine a specific substrate sequence for TSSK3. To this end, we used PepChip Kinase slides (Pepscan Systems) to scan 200 peptides of nine amino acids length, for the ability of TSSK3 to phosphorylate them. This resulted in choosing four of the best-phosphorylated peptides for further analysis (Fig. 5A). We cloned them into pGEX-6P-1 vector in frame with GST, expressed the fusions in *E.coli* and purified these by one-step affinity chromatography. Two control peptides were cloned and purified in parallel, a peptide phosphorylated neither by TSSK3 nor by PKA (peptideNEG) and peptide known as an artificial test substrate for PKA (kemptide). All purified GST-peptides were tested in an *in vitro* kinase assay as potential substrate for PKA and TSSK3. This showed that TSSK3 displays highest activity towards peptide 4: KRRSSSYHV (Fig. 5B). Next we set out to investigate which serine(s) within this sequence is phosphorylated by TSSK3. Therefore we made subsequent mutations of the neighboring three serines, by substituting them (two at once, leaving one serine) with also polar cysteine. Furthermore, peptides 2, 3 and 4 obviously share a common core -RRSSS- what prompted us to test which amino acids in surrounding sequence are responsible for TSSK3 specific phosphoryla-

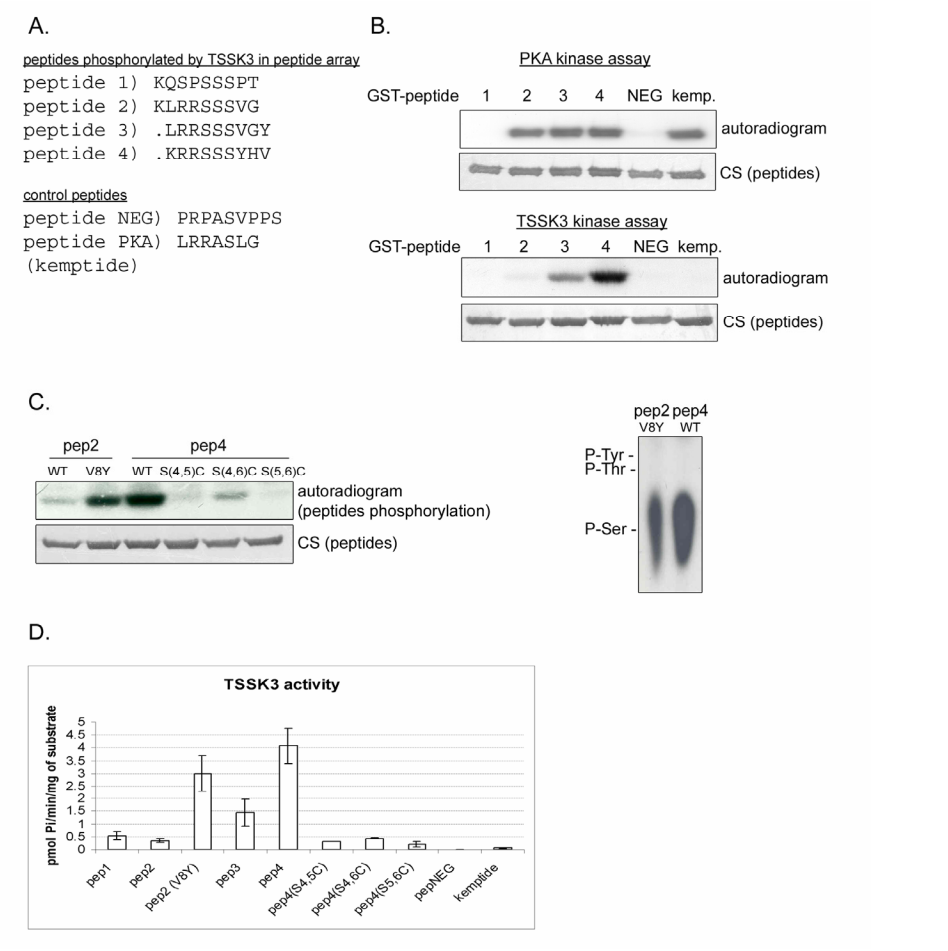


Fig. 5. TSSK3 specifically phosphorylates *in vitro* selected peptides sequences
(A) alignment of the amino acid sequences of four peptides phosphorylated by GST-TSSK3 in peptide array; sequences of control peptides: peptide NEG (not phosphorylated by GST-TSSK3 nor PKA in peptide array) and peptide PKA (kemptide, a positive control for PKA phosporylation) are also indicated. **(B)** purified GST-TSSK3 was subjected to *in vitro* phosphorylation reaction using peptides 1, 2, 3, 4, NEG and kemptide (pep1, 2, 3, 4, NEG and kemp, respectively) as substrates; all peptides were cloned in fusion with GST on pGEX-6P-1 vector, expressed in *E.coli* BL21 and purified by one-step affinity chromatography on glutathione beads. After phosphorylation reaction, proteins were subjected to SDS-PAGE, stained with Coomassie Brilliant Blue (CS) and analysed by autoradiography. **(C)** Left panel: kinase reaction was carried out as in (B) with mutant peptides, pep2(V8Y) with substitution of Val-8 to Ala and pep4 mutants with substitutions of Ser to Cys as indicated. Right panel: One-dimensional thin layer chromatography of hydrolysates of ^{32}P-labeled GST-peptides phosphorylated by TSSK3. The positions of standard phosphoamino acids are indicated **(D)** bands of peptides used in (B, C) in TSSK3 kinase assay, were excited from gel and their radioactivity was measured by scintillation counting. Results were normalized to 1µg of GST-TSSK3.

-tion. We substituted Val in peptide 2 (barely phosphorylated by TSSK3) for Tyr to create the sequence more resembling the best-phosphorylated peptide 4. We tested all newly created peptides for ability to be phosphorylated by TSSK3 (Fig. 5C). We observed that mutating any serines in peptide 4 significantly decreased its phosphorylation by TSSK3 showing that within the consensus sequence a preference for serine exists. Nevertheless, when Ser-5 was left not mutated we could observe some phosphorylation of peptide 4, suggesting that this is the site phosphorylated by TSSK3. Our next finding was that the substitution of Val to Tyr in peptide 2 reconstituted the phosphorylation of this peptide by TSSK3 almost to level of peptide 4 phosphorylation (Fig. 5C, D). This suggests two possible explanations: 1) Tyr on +2 position from phosphorylated Ser (like in peptide 4 and mutated peptide 2) is necessary for recognition of the target amino acid by TSSK3, therefore creating a kind of recognition motif for TSSK3 or 2) Tyr present in peptide 3, 4 and mutated peptide 2 is the amino acid that is also phosphorylated by TSSK3, thus TSSK3 is a dual specificity kinase. To test this, we performed phosphoamino acid mapping of mutated peptide 2 and wild type peptide 4 and we identified only serine to be phosphorylated by TSSK3 (fig. 5C right panel). Therefore we suggest that we identified the specifically recognized and phosphorylated by TSSK3 amino acid sequence consisting of the core -RRSSSY-.

DISCUSSION

In the present work, we provided experimental evidence that TSSK3 protein is indeed a protein kinase and thus complementing the protein sequence analysis of TSSK family of kinases [26] that classifies TSSK3 as a member of testis specific serine/threonine kinases family, containing a short sequence motif in the kinase subdomain VIB (DKCEN) diagnostic for ser/thr kinases and expressed exclusively in testis [2, 26]. We showed the mechanism of regulation of TSSK3 activity by autophosphorylation and PDK1 phosphorylation in the "activation loop". The latter is of special interest in the view of a recent publication on the identification of a testis and brain specific isoform of mouse PDK1, mPDK-1β [34] where the authors suggest that this isoform may play an important role in regulating spermatogenesis. Thus an attractive possibility is that mPDK-1β may function in the regulation of TSSK3 activity.

At present a number of examples have been described of protein kinases phosphorylated on residues located within the activation loop [35], but among them there are few testis specific kinases. One of these is the dual specificity kinase TESK1 (Testis-Specific Protein Kinase 1) [36] with, similarly to TSSK3, an expression pattern limited to testis. For TESK1, as well as shown here for TSSK3, the autophosphorylation of a serine residue located in the activation loop seems to be an important regulatory mechanism for the protein kinase activity. However, unlike TESK1, TSSK3 contains in the activation loop also a threonine residue, which is equivalent to the Ser/Thr residue of the members of the AGC family protein kinases that is phosphorylated by PDK1 [28]. We show *in vitro* that indeed TSSK3 activity can be regulated by PDK1 phosphorylation in the T-loop. This provides the first example of a testis specific kinase regulated in this way and apparently differs from the mechanism of regulation of dual specificity kinase TESK1. It has been suggested that *in vivo* in cells PDK1 is a constitutively active kinase [23], although some reports claim that insulin treatment of cells may also slightly (2-fold) enhance PDK1 kinase activity [37]. Therefore

it is thought that the role of PDK1 in the activation of other kinases is governed by its cellular location. For example in insulin-induced activation of PKB/Akt the insulin-induced transient increase in 3'-phosphorylated inositide lipids, is thought to act as a recruitment signal for PDK1 to the plasma membrane, where it may co-localize and phosphorylate/activate PKB/Akt [38]. Consistent with this insulin treatment of cells resulted in activation of TSSK3, albeit weakly. However, pretreatment with LY294002 to inhibit insulin-induced PI-3K activation did not inhibit TSSK3 activation. Thus TSSK3 activation apparently does not require membrane localization of PDK1. As TSSK3 consists essentially of a kinase domain [2], it is well conceivable that in cells other adaptors/effector(s) may be necessary for maximum activation and/or activation by PDK1 of TSSK3, like it was suggested for PKCξ phosphorylation and activation by PDK1 [39]. Thus we hypothesize that, in order to efficiently recruit PDK1 to TSSK3, co-factors or additional modifications of TSSK3 are required. This is further supported by our observation that bacterially produced TSSK3 is very sensitive to detergents (Fig. 2F) suggesting that it is rapidly unfolding in the absence of a co-factor. As TSSK3 is a testis specific kinase such a co-factor is not necessarily expressed in the A14 cells that we have employed here to analyze *in vivo* activation of TSSK3 by insulin and this may explain why the activation is rather small. Therefore we are currently investigating the possible existence of regulatory, protecting and/or scaffolding factors for TSSK3 and we have already obtained potential interaction partners by yeast-two-hybrid screening (data not shown) that may be key proteins in the *in vivo* regulation of TSSK3.

Thus far most of described protein kinases phosphorylated by PDK1 are members of AGC-family protein kinases [28] but there are also PDK1 substrates outside this family like PAK1 [40] or MEK1/2 [32] both from STE group. According to the human kinome [1] TSSK3 is classified as a member of CaMK kinase family and it is shown that PDK1 does not phosphorylate CaMK kinases [30]. The cases of PAK1, MEK1/2 and described here TSSK3 phosphorylated and activated by PDK1 show that in classifying protein kinases into a separate families apart from DNA and amino acid sequence analysis, also other features should be taken into consideration like mechanism of protein regulation.

We identified in this study the specific consensus motif -RRSSSY- phosphorylated by TSSK3. The natural substrate for TSSK3 has not been found yet unlike for the two other testis specific kinase family members TSSK1 and TSSK2. TSKS, testis specific kinase substrate, has been reported as a putative substrate for TSSK1 [6] and TSSK2 [6, 26]. Its amino acid sequence does not contain –RRSSSY- motif what is consistent with the finding that TSSK3 does not phosphorylate TSKS [2]. Besides, peptide 4 with -RRSSSY- sequence is very weakly phosphorylated by TSSK2 (data not shown). This shows the differences in substrate specificity of TSSK1, 2 and 3 what is in agreement with reports about different localization of these kinases in mature testis. TSSK3 is localized in the androgen-producing Leydig cells [2] whereas TSSK1 and 2 are expressed exclusively during the cytodifferentiation of late spermatids to sperms [6] what suggests that TSSK3 represents a more distantly related TSSK family member. Moreover, despite the very high homology at the amino acid level between human TSSK members (TSSK3 protein has 47.5 and 49% identity with TSSK1 and TSSK2 respectively), the TSSK3 protein lacks the approximately 100 amino acid C-terminal extension located outside the kinase domain that is present in TSSK1 and 2. To conclude, we showed substrate specificity of TSSK3 and we propose the

peptide sequence for TSSK3 phosphorylation experiments that can be used in further study on TSSK3 regulation and can give a hint of possible natural substrates for TSSK3 and its function in spermatogenesis.

ACKNOWLEDGMENTS

This work was supported by KBN research grant No 6 PO4B 006 19 (1114/PO4/2000/19) for LT, and Centre of Excellence in Molecular Bio-Medicine, contract No QLK6-CT-2002-90363. We thank prof. D.R. Alessi for providing PDK1 CS protein and Myc-PDK1 expression construct, Dr W. van Workum and Dr J. Joore from ServiceXS for a gift of test PepChip Kinase slides and help with the identification of phosphorylared peptides. Mouse and human testis tissues were the kind gifts from prof. J. Ostrowski and Dr R. Nowak, respectively. We thank prof. A. Zylicz, prof. M. Zylicz, A. Helwak, M. Gutkowska, G. Kudla for experimental support and many helpful discussions.

REFERENCES

1 Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) Science **298**, 1912-34

2 Zuercher, G., Rohrbach, V., Andres, A. C. and Ziemiecki, A. (2000) Mech Dev **93**, 175-7

3 Wilks, A. F. (1991) Methods Enzymol **200**, 533-46

4 Visconti, P. E., Hao, Z., Purdon, M. A., Stein, P., Balsara, B. R., Testa, J. R., Herr, J. C., Moss, S. B. and Kopf, G. S. (2001) Genomics **77**, 163-70

5 Nayak, S., Galili, N. and Buck, C. A. (1998) Mech Dev **74**, 171-4

6 Kueng, P., Nikolova, Z., Djonov, V., Hemphill, A., Rohrbach, V., Boehlen, D., Zuercher, G., Andres, A. C. and Ziemiecki, A. (1997) J Cell Biol **139**, 1851-9

7 Brennan, J. and Capel, B. (2004) Nat Rev Genet **5**, 509-21

8 Klonisch, T., Fowler, P. A. and Hombach-Klonisch, S. (2004) Dev Biol **270**, 1-18

9 Plant, T. M. and Marshall, G. R. (2001) Endocr Rev **22**, 764-86

10 Sassone-Corsi, P. (2002) Science **296**, 2176-8

11 Shima, J. E., McLean, D. J., McCarrey, J. R. and Griswold, M. D. (2004) Biol Reprod **71**, 319-30

12 Venables, J. P. (2002) Curr Opin Genet Dev **12**, 615-19

13 Wolgemuth, D. J., Laurion, E. and Lele, K. M. (2002) Recent Prog Horm Res **57**, 75-101

14 Loveland, K. L. and Schlatt, S. (1997) J Endocrinol **153**, 337-44

15 Brennan, J., Tilmann, C. and Capel, B. (2003) Genes Dev **17**, 800-10

16 Jamin, S. P., Arango, N. A., Mishina, Y., Hanks, M. C. and Behringer, R. R. (2002) Nat Genet **32**, 408-10

17 Kharbanda, S., Pandey, P., Morris, P. L., Whang, Y., Xu, Y., Sawant, S., Zhu, L. J., Kumar, N., Yuan, Z. M., Weichselbaum, R., Sawyers, C. L., Pandita, T. K. and Kufe, D. (1998) Oncogene **16**, 1773-7

18 Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D. and Wynshaw Boris, A. (1996) Cell **86**, 159-71

19 Xu, X., Toselli, P. A., Russell, L. D. and Seldin, D. C. (1999) Nat Genet **23**, 118-21

20 Katschinski, D. M., Marti, H. H., Wagner, K. F., Shibata, J., Eckhardt, K., Martin, F., Depping, R., Paasch, U., Gassmann, M., Ledermann, B., Desbaillets, I. and Wenger, R. H. (2003) Mol Cell Biol **23**, 6780-9

21 Costa, G. L., Bauer, J. C., McGowan, B., Angert, M. and Weiner, M. P. (1996) Methods Mol Biol **57**, 239-48

22 Deprez, J., Bertrand, L., Alessi, D. R., Krause, U., Hue, L. and Rider, M. H. (2000) Biochem J **347 Pt 1**, 305-12

23 Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A. and Bownes, M. (1997) Curr Biol **7**, 776-89

24 van Weeren, P. C., de Bruyn, K. M., de Vries-Smits, A. M., van Lint, J. and Burgering, B. M. (1998) J Biol Chem **273**, 13150-6
25 Neufeld, E., Goren, H. J. and Boland, D. (1989) Anal Biochem **177**, 138-43
26 Hao, Z., Jha, K. N., Kim, Y. H., Vemuganti, S., Westbrook, V. A., Chertihin, O., Markgraf, K., Flickinger, C. J., Coppola, M., Herr, J. C. and Visconti, P. E. (2004) Mol Hum Reprod **10**, 433-44
27 Sarge, K. D. (1995) J Biol Chem **270**, 18745-8
28 Vanhaesebroeck, B. and Alessi, D. R. (2000) Biochem J **346 Pt 3**, 561-76
29 Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997) Curr Biol **7**, 261-9
30 Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A. and Thomas, G. (1998) Science **279**, 707-10
31 Dutil, E. M., Toker, A. and Newton, A. C. (1998) Curr Biol **8**, 1366-75
32 Sato, S., Fujita, N. and Tsuruo, T. (2004) J Biol Chem **279**, 33759-67
33 Burgering, B. M. and Coffer, P. J. (1995) Nature **376**, 599-602
34 Dong, L. Q., Ramos, F. J., Wick, M. J., Lim, M. A., Guo, Z., Strong, R., Richardson, A. and Liu, F. (2002) Biochem Biophys Res Commun **294**, 136-44
35 Johnson, L. N., Noble, M. E. and Owen, D. J. (1996) Cell **85**, 149-58
36 Toshima, J., Tanaka, T. and Mizuno, K. (1999) J Biol Chem **274**, 12171-6
37 Chen, H., Nystrom, F. H., Dong, L. Q., Li, Y., Song, S., Liu, F. and Quon, M. J. (2001) Biochemistry **40**, 11851-9
38 Anderson, K. E., Coadwell, J., Stephens, L. R. and Hawkins, P. T. (1998) Curr Biol **8**, 684-91
39 Dong, L. Q., Zhang, R. B., Langlais, P., He, H., Clark, M., Zhu, L. and Liu, F. (1999) J Biol Chem **274**, 8117-22
40 King, C. C., Gardiner, E. M., Zenke, F. T., Bohl, B. P., Newton, A. C., Hemmings, B. A. and Bokoch, G. M. (2000) J Biol Chem **275**, 41201-9

Chapter

3

A novel TSSK3-interacting protein, RUSC2 is a scaffolding protein and a substrate for c- Jun N-terminal kinase (JNK)

Submitted

A novel TSSK3-interacting protein, RUSC2 is a scaffolding protein and a substrate for c- Jun N-terminal kinase (JNK)

Marta BUCKO-JUSTYNA[1], Lech TRZECIAK[1], Leszek LIPINSKI[1,2], Boudewijn M. T. BURGERING[3#]

[1]Department of Molecular Biology, International Institute of Molecular and Cell Biology in Warsaw, Ks. Trojdena 4, 02-109 Warsaw, Poland, [2]Labolatory of Molecular Medicine, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland, [3]Department of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands

SUMMARY

We have recently characterized the biochemical properties and *in vitro* regulation of Testis Specific Serine-threonine Kinase 3 (TSSK3). Since TSSK3 consists solely of a kinase domain and harbors no regulatory domains itself, we searched for potential regulatory interacting proteins by a yeast two-hybrid approach. We identified KIAA0375, recently annotated as RUN and SH3 domain containing protein 2 (RUSC2 (1)), an uncharacterized and ubiquitously expressed protein, as a TSSK3 binding partner. Although RUSC2 binds to TSSK3, it is not a substrate for TSSK3. Binding of TSSK3 to RUSC2 is enhanced by increased cellular oxidative stress and we therefore analyzed the involvement of stress activated kinases. We observed binding of Jun NH_2-terminal kinase (JNK) and phosphorylation by JNK of RUSC2. Binding of JNK to RUSC2 requires activation of JNK, but does not appear to critically require so-called kinase interaction motifs (KIMs) (2-4). Binding and phosphorylation of RUSC2 by JNK however, is required for binding of TSSK3 to RUSC2. In addition JNK and TSSK3 binding are not mutually exclusive as both can be found simultaneously in complex with RUSC2. Taken together our results suggest that JNK acts upstream in the assembly of a complex between JNK, RUSC2 and TSSK3, following cellular oxidative stress. This identifies RUSC2 as a novel type of scaffold protein in JNK signaling. As TSSK3 is exclusively expressed within the testis this also suggests that this scaffold complex is involved in mediating a testis-specific stress response.

INTRODUCTION

The mouse testis specific kinase 3 (TSSK3) belongs to a small subfamily of protein kinases expressed exclusively in the testis (5). Characteristically, it was identified using low stringency hybridization with a partial sequence obtained from cDNA amplification utilizing degenerate primers (6). Our group has independently obtained a fragment of the human TSSK3 sequence, employing the same degenerate primers method to study kinases expressed in the

human AGS cell line (L.T. unpublished). The complete sequence of *hTSSK3* was published by Visconti et al. (7) shortly after it became available as a part of accessible Human Genomic Project sequences. Both the mouse and human sequence encode for a small protein of 29kDa, consisting of a catalytic domain only. Interestingly, TSSK3 has no orthologues in non-mammals.

Immunohistochemical studies in mice indicate that TSSK3 is present exclusively in testicular Leydig cells (5), unlike the two other members of TSSK subfamily, TSSK1 and TSSK2, whose expression is limited to meiotic and postmeiotic spermatogenic cells, respectively (8,9). The TSSK3 mRNA level is low at birth, increases substantially at puberty and remains high throughout adulthood. Thus TSSK3 seems to play an important role in adult testis.

To further understand a possible role in testis development for the TSSK family, and TSSK3 in particular, we have recently started to characterize biochemically TSSK3 (M. Bucko-Justyna et al. submitted). TSSK3 is indeed a genuine serine-threonine kinase, which in agreement with its testis-specific expression displays optimal kinase activity at approximately 32^{o}C. Interestingly, TSSK3 is regulated by phosphorylation of two residues within the T-loop. *In vitro* one residue (Ser168) is phosphorylated by autophosphorylation whereas the other residue (Thr168) can be phosphorylated by PDK1, the T-loop kinase of the ACG-kinase family. *In vitro* PDK1 phosphorylation enhances TSSK3 kinase activity and *in vivo*, treatment of cells with ligands (e.g. insulin) that activate PDK1 stimulates kinase activity of ectopically expressed TSSK3. Although this study revealed some aspects of TSSK3 regulation it was clear that because TSSK3 is expressed in testis only, essential co-factors for TSSK3 regulation might be lacking in a system employing ectopic expression of TSSK3 in fibroblast cell lines. Furthermore, whereas TSSK1 and TSSK2 contain, in addition to the kinase domain, potential regulatory domains, the TSSK3 protein consists solely of a kinase domain.

Therefore, we investigated the possible existence of regulatory, protecting and/or scaffolding factors for TSSK3. Here we present evidence that KIAA0375 acts as scaffold for TSSK3 and that binding of TSSK3 to KIAA0375 tethers TSSK3 to JNK signaling. Recently, the cDNA sequence of human and mouse KIAA0375 was analyzed *in silico* by Strausberg et al. (10) and named RUSC2 for RUN and SH3 domain containing protein 2, so hereafter KIAA0375 is referred to as RUSC2. Tethering of TSSK3 to JNK signaling is achieved by binding of JNK to RUSC2 and thus in addition RUSC2 is identified as a novel JNK binding partner. JNK interaction with RUSC2 requires activation of JNK and JNK phosphorylates RUSC2. JNK-mediated phosphorylation apparently regulates the binding of TSSK3, as inhibition of JNK activation inhibits TSSK3 recruitment to RUSC2. Finally, we show that treatment of cells with hydrogen peroxide, to increased cellular oxidative stress, stimulates the formation of a complex between RUSC2, JNK and TSSK3. Thus our data suggest that, in contrast to the previously described JNK scaffolds of the JIP family, RUSC2 also recruits a potential JNK target.

EXPERIMENTAL PROCEDURES

cDNA constructs

PCR, restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit on Applied Biosystems automated DNA sequencers.

Total RNA from mouse and human testis was isolated by homogenization in TRI REAGENT (Sigma) as described by the manufacturer. First strand cDNA synthesis was performed from 5µg of total RNA using the Fermentas RevertAid kit with oligo-dT primers according to manufacturers' suggestions.

TSSK3 constructs. The full-length TSSK3 coding sequence was PCR amplified from a human testis cDNA, using oligonucleotide primers GGTGGTCATATGGAGGACTTTCTRCTCT / CACTTGCCATTGCTTTTATCA and ligated into *Sma*I site of pUC 18 vector. The *E.coli* pGEX-hTSSK3 plasmid was constructed using pGEX-4T-2, which expresses the target protein as a fusion protein with GST. The full-length human TSSK3 was subcloned from pUC18hTSSK3 into multicloning site of pGEX-4T-2 using *BamH*I, *Eco*RI restriction sites. The sequence was put in frame by cutting of *Bam*HI, *Nde*I fragment, filling in protruding ends and religation. The same PCR fragment of TSSK3 was used to generate pAS2-1hTSSK3 construct for yeast two-hybrid screen. TSSK3 PCR fragment was ligated into pUC57 vector (InsT/AClone System, Fermentas), and subcloned into pAS2-1 using *Nde*I, *Bam*HI restriction sites. In order to generate mammalian expression constructs encoding the full-length human or mouse HA-tagged TSSK3 (HA-hTSSK3 or HA-mTSSK3 respectively), the following primer pair was used: primer1/primer2 (GCGCTGTCGACCATGGA GGACTTTCTGCTCT/CATTGAATTCCTCAAGTG CTTGCTAGCCATG). The forward (5') primer contained a *Sal*I site, whereas the reverse (3') primer contained an *Eco*RI site. The amplified products were digested with the corresponding enzymes and subcloned into *Sal*I/*Eco*RI cut pMT2-HA vector. To generate point mutants of HA-TSSK3, site-directed mutagenesis was used (11). Two kinase-dead point mutants were created by site-directed mutagenesis in HA-hTSSK3: K39R, a Lys-39-to Arg mutation and T168A, a Thr-168-to Ala mutation. Flag-TSSK3 was generated by PCR amplification of human testis cDNA using the following primer pair: primer 1(GCGCTAAAGCTTATGGAGGACTTTCTGCTCT C) and primer 2 (CATTGAATTCCTCAAGTGCTTG CTAGCCATG) containing *Hind*III/*EcoR*I sites, respectively, and ligation into pFLAG-CMV-4 vector (Sigma) cut with *Hind*III/*EcoR*I enzymes.

RUSC2 constructs. GST-RUSC2_26 for expression in *E.coli* was generated by subcloning *EcoR*I/*Xho*I fragment from human brain cDNA library (on pACT2) construct of RUSC2_26 into pGEX-6P-2 vector cut with *EcoR*I/*Xho*I; mammalian expression constructs, HA-RUSC2_26 and HA-RUSC2_31 were created by subcloning *Bgl*II/*Xho*I fragments from pACT2 RUSC2_26 or pACT2 RUSC2_31, respectively, into pCDNA 3.1(+) vector (Invitrogen) digested with *BamH*I/*Xho*I; Flag-RUSC2 (full length) was generated by PCR reaction on pBluescript II SK(+) RUSC2 (Kazusa DNA Research Institute) using following primer pairs: primer 1 (CGAAGCGGCCGCAATGGATAGT) and primer 2 (CCAGCATGCACAGGAATTCAGTTT) containing *Not*I/*EcoR*I sites, respectively, and ligation of the resulting PCR product digested with *Not*I/*EcoR*I with pFLAG-CMV-4 vector cut with the same enzymes; Flag-RUSC2 RUN was made by cutting off *Kpn*I fragment from Flag-RUSC2 construct and religation. To generate Flag-RUSC2 SH3, Flag-RUSC2 was digested with *Kpn*I and a fragment containing SH3 domain was isolated and ligated with modified pFLAG-CMV-4 cut with *Kpn*I. To modify pFLAG-CMV-4 for this purpose, *Bgl*II/*EcoR*V small fragment was cut off, the remaining protruding DNA ends were filled in and relegated. To generate deletion mutants Flag-RUSC2 RUNΔKIM1, Flag-RUSC2 SH3 ΔKIM2, Flag-RUSC2 ΔKIM2 or Flag-RUSC2 ΔKIM1ΔKIM2 site-directed mutagenesis was engaged (11). In order to clone KIM sequences present in RUSC2 (GST-KIM1, GST-KIM2) or in c-Jun (GST-KIM c-Jun) in fusion with GST, pairs of oligonucleotides were obtained coding for: KIM1, RVSQDLLL; KIM2, RLPSDWLSL or KIM c-Jun, KQSMTLNL. Oligonucleotides contained *Eco*RI overhang on 5' site and *Not*I overhang on 3' site to ligate annealed oligonucleotides into pGEX-6P-1 vector cut with *Eco*RI, *Not*I. Additionally, oligonucleotides contained *Kpn*I restriction site to select for correct clones.

Other constructs. pMT2-HA-JNK1 (12) , HA-JNK3 was a kind gift from prof. J. Ostrowski, Flag-MKK4 DN was a gift from H. Nishina and is described elsewhere (13), GST-Jun (14)

Yeast two-hybrid screen

To identify novel TSSK3-interacting proteins, a two-hybrid screen was carried out using MATCHMAKER GAL4 Two-hybrid system (Clontech). A human brain cDNA library was screened using human TSSK3. Of 1 x 10^6 independent clones screened, 8 were found to show a reproducible interaction with TSSK3. The cDNAs contained within these clones were purified and sequenced.

Protein purification from *E.coli*

GST-TSSK3 or GST-RUSC2_26 was purified as follows: protein constructs were over-expressed in *E.coli* BL21 RIL [DE3] strain. 1 litre culture was grown at 30^OC (OD_{600nm}=0.6). Induction was carried out for 4 hours with 1 mM isopropyl β-D-thiogalactosidase (IPTG) at 20^0C, and the cells were harvested by centrifugation. Bacterial pellet was incubated (0.5h, 4^OC) in 20 ml lysis buffer (50 mM Tris/HCl (pH 7.5); 1 mM EDTA; 5% (v/v) glycerol; 0,1% 2-mercaptoethanol; 1 mM PMSF) containing 0.5mg/ml lysozyme and cells were disrupted by

adding 5 ml 5 M NaCl at 42^{O}C for 5 min. The protein was purified by one-step affinity chromatography using GSH-agarose (Sigma). After washing the column (50 mM Tris/HCl (pH 7.5); 200 mM NaCl; 1 mM EDTA; 5% (v/v) glycerol; 0,1% 2-mercaptoethanol; 1 mM PMSF) the protein was eluted (or was left on the GSH agarose beads if the pull-down assay was performed) in the washing buffer containing 10 mM glutathione and it was analyzed by SDS-PAGE.

GST-KIMs or GST-c-Jun were transformed into BL21 E.*coli* cells and 0.5-liter culture was grown at 30^{0}C in Luria broth containing 100 µg/ml ampicilin until the absorbance at 600 nm was 0.6 and 0.1 mM IPTG was then added. The cells were cultured for further 4h at 20^{0}C, resuspended in 10 ml of ice-cold lysis buffer containing 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 5% glycerol, 0.03% (by vol.) 2-mercaptoethanol. The suspension was sonicated and the lysates centrifuged at 4^{0}C for 60 min at 100 000 ***g*** and incubated with 0.25 ml of GSH-agarose (Sigma) for 1h. The resin was washed 4 times with lysis buffer: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DOC, 5 mM EDTA, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and subjected to pull down assays.

GST pull-down assays

For HA-TSSK or HA-RUSC2_26 pull-down assays, glutathione-Agarose beads were loaded with GST-RUSC2_26 or GST-TSSK3 wild type, respectively. 293T cells were transfected with HA-TSSK3 (wild type, K39R or T168A mutants) or with HA-RUSC2_26, followed by cell lysis in lysis buffer containing: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% piridinium betain, 5 mM EDTA, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin. For HA-JNK3 pull down assays glutathione-Agarose beads were loaded with GST-KIM motifs or GST c-Jun. 293T cells were transfected with HA-JNK3, stimulated with 100 µM H_2O_2 for indicated time periods and lysed in the lysis buffer: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DOC, 5 mM EDTA, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin. Cell extracts were incubated with GSH-immobilized proteins for 1h at 4^{0}C. After 4 times washing in lysis buffer, bound proteins were resuspended in Laemmli sample buffer, resolved by SDS-PAGE, and HA-TSSK3, HA-RUSC2_26 or HA-JNK3 were detected by Western blotting using anti-HA antibody.

Cells culture and transfections

HEK-293T cells, HeLa cells and JNK9 MEFs (JNK1-/-, JNK2-/- mouse embryo fibroblasts) (15) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Sigma) and 1% antibiotic suspension (penicillin and streptomycin; Sigma) and 2mM L-glutamine. Prior to stimulation, cells were deprived of serum for 18h, but not when H_2O_2 was used. EGF or H_2O_2 were added at a final concentration of 40ng/ml or 200µM, respectively. Transfections were carried out using PEI reagent (Polyethylenimine, *Polysciences, Inc.*) to transfect HEK 293T cells, Fugene 6 transfection reagent (Roche) was used to transfect JNK9 cells or Lipofectamine (Invitrogen) for transfection of HeLa cells.

Antibodies

The following antibodies were used: 12CA5 for HA-tagged proteins, anti-Flag M2 monoclonal antibody (Sigma) for Flag-tagged proteins, monoclonal anti-phospho-JNK(G-7) (Santa Cruz).

Immunoprecipitation and *in vitro* kinase assays

For immunoprecipitation of HA-TSSK3 the cells were lysed in ice-cold lysis buffer containing: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% piridinium betain, 5 mM EDTA, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin. For other immunoprecipitations cells were lysed in ice-cold lysis buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DOC, 5 mM EDTA, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin. Lysates were cleared for 10 min at 20 000 ***g*** at 4^{0}C. HA-tagged (HA-RUSC2_26, HA-RUSC2_31, HA-TSSK3, HA-JNK1, HA-JNK3) or Flag-tagged proteins (Flag-BAP (Sigma), Flag-TSSK3, Flag-RUSC2, Flag-RUSC2 SH3, Flag-RUSC2 RUN) were immunoprecipitated by protein A-Sepharose beads coupled to the 12CA5 monoclonal antibody or anti-Flag monoclonal antibody, respectively and rotation at 4^{0}C for 2h. For co-immunoprecipitation assays beads were washed three times with lysis buffer, for *in vitro* kinase reactions the immunoprecipitate was washed twice with lysis buffer and once with kinase reaction buffer lacking rATP. Kinase reaction was carried out at 30^{0}C for 30 min with the purified kinases: GST-p38 (Upstate), HIS-JNK1 (Upstate), GST-TSSK3 (M. Bucko-Justyna et al. submitted). The kinase buffer for GST-p38 and HIS-JNK1 contained: 25mM Tris/HCl pH 7.5, 2mM $MgCl_2$, 2 mM DTT, 100 µM ATP, 3 µCi of [γ-^{32}P] ATP (3000 Ci/mmol); for GST-TSSK3: 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 5 mM $MnCl_2$, 15 µM ATP, 3 µCi of [γ-^{32}P] ATP (3000 Ci/mmol). Kinase reaction was stopped by adding 5x Laemmli sample buffer followed by SDS-

Name of the protein	Number of clones picked up in the screen
RanBPM/RanBP9 (Ran-binding protein)	3
KIAA0375/RUSC2 (RUN and SH3 domain containing protein 2)	2 (two different length clones)
Jab1/CSN5 (Jun-activation-domain-binding-protein)	1
DOK-5 (Docking protein 5)	1
LAMR1 (Laminin receptor 1)	1
KIAA0317	1
ZNF 76 (Zinc finger protein 76)	1
QDPR (Quinoid dihydropteridine reductase)	1

Table 1. Proteins picked up in yeast two-hybrid screen with TSSK3

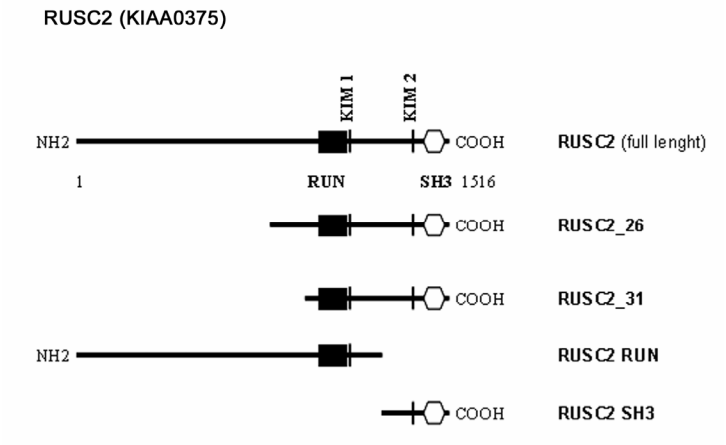


Fig. 1. RUSC2 domain structure and constructs used in this study
Illustration of RUSC2 and the constructs used in the present work. RUN, SH3 domains and potential JNK binding motifs (KIMs- kinase interaction motifs) are indicated.

PAGE and analysis by immunoblot or autoradiography (kinase assays).

RESULTS

TSSK3 Interacts with RUSC2

To identify potential interacting proteins for TSSK3 we performed a yeast-two-hybrid screen, using human TSSK3 as a bait and a human brain cDNA library as a prey. Out of 1 x 10^6 transformants, we initially obtained 70 positive colonies, which after further selection resulted in the identification of 8 candidate proteins for interaction with TSSK3 (Table 1).

Next we verified the yeast-two-hybrid interactions by co-immunoprecipitation in mammalian cells. The cDNA sequences from the positive yeast-two-hybrid clones were cloned into a mammalian expression vector, in frame with the HA-tag. All HA-tagged proteins were co-expressed with Flag-tagged

TSSK3 in HeLa cells. In the absence of any specific cellular treatment, we observed only co-immunoprecipitation of TSSK3 with RUSC2. The estimated molecular weight of the RUSC2 protein is 180 kDa, and thus far two domains have been allocated: a RUN domain and a SH3 domain, both located within the C-terminal part (Fig. 1). From the yeast-two-hybrid screen we obtained two partial RUSC2 cDNAs of different length (RUSC2_26 and RUSC2_31) (Fig. 1). They both lack most of the N-terminal part of protein but do contain the RUN and SH3 domain and they are both able to interact with TSSK3 (Fig. 2A). The interaction with the longer version, RUSC2_26, however appeared consistently stronger. Therefore, RUSC_26 cDNA was used for the subsequent experiments. In agreement with our initial result, co-immunoprecipitation between HA-RUSC2_26 and FLAG-TSSK3 was observed in both directions (Fig. 2B). To further confirm this interaction we constructed Glutathione-S-Transferase fusion proteins (GST-RUSC2_26 and GST-TSSK3) and purified GST fusion proteins attached to glutathione-agarose beads were used to pull down HA-TSSK3 or HA-RUSC2_26, respectively, from 293T lysates ectopically expressing these constructs (Fig. 2C). Indeed HA-RUSC2_26 and Flag-TSSK3 could be affinity purified using GST-TSSK3 and GST-RUSC2_26, respectively. Interestingly, GST-RUSC2_26 (upper panel) was able to associate with wild type TSSK3 as well as with two different TSSK3 mutants (K39R, kinase-dead and the T-loop mutant T168A, M. Bucko-Justyna et al. submitted), suggesting that TSSK3 kinase activity is not required for the interaction with RUSC2_26. Taken together our data provide evidence that RUSC2, through its C-terminal part interacts directly with TSSK3.

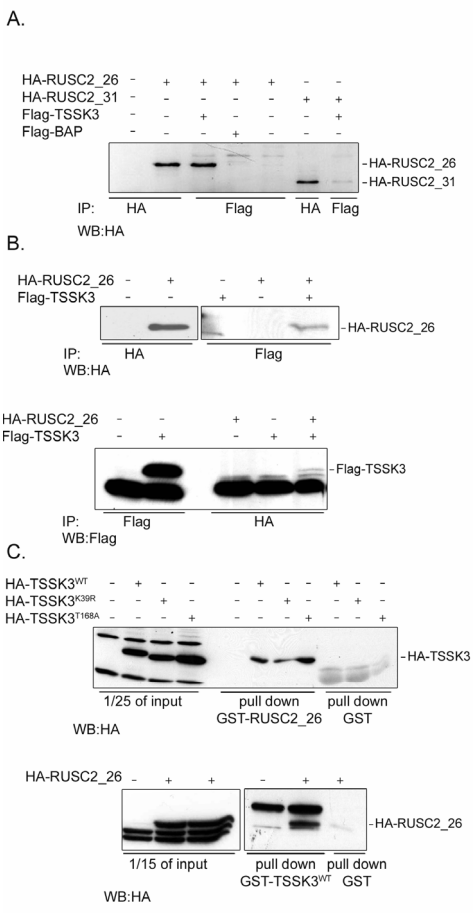


Fig. 2. TSSK3 Interacts with RUSC2

(A) (B) HeLa cells (A) or 293T cells (B) were transfected with the indicated combinations of plasmids. Two different HA-tagged clones of RUSC2 (RUSC2_26 and RUSC2_31) were used in co-transfections with Flag-tagged TSSK3. Co-immunoprecipitations were performed using anti-FLAG or anti-HA antibody. Flag-BAP (bacterial alkaline phosphatase) was used as negative control. Proteins were resolved by SDS-PAGE and analyzed using anti-HA or anti-FLAG Ab. **(C)** 293T cells were transfected with wild type (WT), kinase-dead (K39R), T-loop mutant (T168A) HA-tagged TSSK3 (upper panel) or HA-RUSC2_26 (lower panel). HA-TSSK3 was pulled down using RUSC2-GST bound to GA beads (upper panel) or HA-RUSC2_26 was pulled down by GST-TSSK3^{WT} (lower panel). GST only was utilized as a negative control for pull down assay specificity. Proteins were resolved by SDS-PAGE and analyzed with anti-HA antibody.

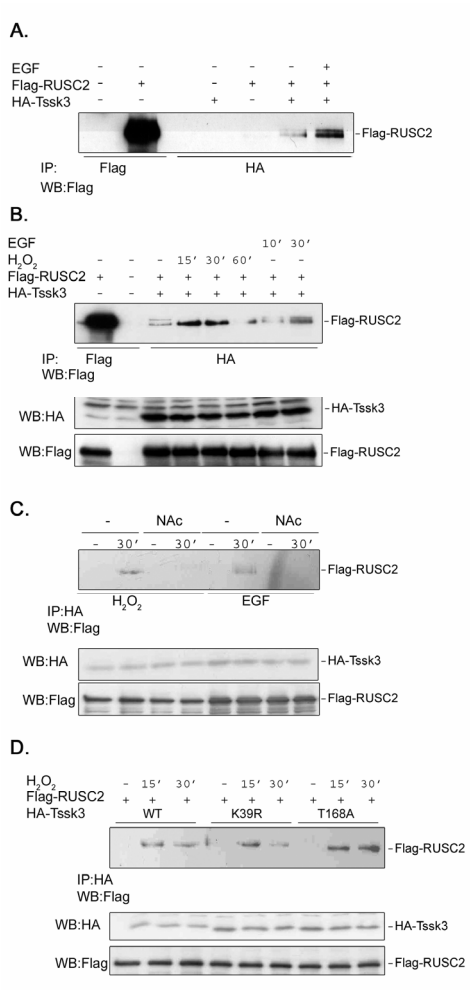


Fig. 3. EGF or H_2O_2 promotes the interaction of TSSK3 with RUSC2 in intact cells

293T cells were transiently transfected with expression vectors encoding Flag-RUSC2 (full length) and HA-TSSK3, as indicated. At 36 h of transfection, the cells were untreated or treated with EGF for 20 min (A) or EGF or H_2O_2 (B) for indicated time periods. Cell lysates were subjected to immunoprecipitation with anti-HA or anti-Flag (positive control samples) antibody, and the resulting immunoprecipitates were subjected to immunoblot analysis with anti-Flag antibody. Corresponding cell lysates were immunoblotted with the indicated antibodies to evaluate the expression of HA-TSSK3 and Flag-RUSC2 (B). **(C)** 293T cells co- transfected with Flag-RUSC2 and HA-TSSK3, were treated with 10 mM NAc and the next day treated with EGF or H_2O_2 for 30 min. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. SDS-PAGE gels were developed by anti-Flag antibody. **(D)** 293T cells were transfected with wild type (WT), kinase-dead (K39R) or T-loop mutant (T168A) HA-tagged TSSK3 together with Flag-RUSC2 and treated with H_2O_2 for the indicated time periods. HA-TSSK3 was immunoprecipitated from the cell lysates with anti-HA antibody and the binding of Flag-RUSC2 was monitored using anti-Flag antibody.

EGF or H_2O_2 Promotes the Interaction of TSSK3 with RUSC2 in Intact Cells

Having established the interaction of RUSC2 clones 26 or 31 with TSSK3, we next cloned the full length RUSC2 cDNA into pFLAG-CMV-4 vector (Flag-RUSC2). 293T cells were co-transfected with vectors encoding HA-tagged wild type TSSK3 and Flag-RUSC2, and were again subjected to co-immunoprecipitation analysis (Fig. 3A). This demonstrated that also full length RUSC2 associated with HA-TSSK3 in cells. Furthermore, exposure of the cells to EGF resulted in an increased interaction between Flag-RUSC2 and HA-TSSK3. In addition to EGF, we also examined whether increased cellular oxidative stress applied to the cells has any effect on the interaction between TSSK3 and RUSC2 (Fig. 3B). Already after 15 min of H_2O_2 treatment of the cells, we observed a significant increase in interaction of both proteins. Importantly, the EGF-induced RUSC2-TSSK3 interaction apparently also involved an increase in cellular oxidative stress, as pretreatment with N-acetyl-L-cysteine (NAC), which enhances scavenging of oxygen radicals, reduced EGF-induced RUSC2-TSSK3 interaction (Fig. 3C).

Furthermore, treatment of cells with H_2O_2 also induced the binding of RUSC2 to different kinase-inactive TSSK3 mutants (Fig. 3D), confirming our observation from the GST-pull down experiments that binding of TSSK3 to RUSC2 does not require TSSK3 kinase activity (Fig. 2C). However, this also

suggests that following H_2O_2 treatment either TSSK3 or RUSC2 is modified, for example through phosphorylation, to enhance complex formation.

RUSC2 is phosphorylated by JNK1 and not by TSSK3

Given that RUSC2 was isolated in Y2H screen as a co-partner of TSSK3 protein kinase we first tested whether RUSC2 might be a substrate for TSSK3. Therefore, Flag-tagged RUSC2 was transiently expressed in 293T cells, purified by immunoprecipitation and subjected to an *in vitro* kinase assay with purified active TSSK3 (M.Bucko-Justyna et al. submitted). As controls for specificity we included the stress kinases p38α and JNK1 (Fig. 4A). The two latter stress-responsive kinases were included in our analysis, since we observed increased binding of RUSC2 to TSSK3 following H_2O_2 treatment of cells, which also induced p38α and JNK activity (data not shown). TSSK3 was not able to phosphorylate RUSC2. This is in agreement with the observation that RUSC2 lacks the RRSSSY sequence which we have previously defined as a consensus sequence for TSSK3 phosphorylation (M.Bucko-Justyna et al. submitted). Interestingly JNK1, but not p38 α very efficiently phosphorylated RUSC2. As a result of this phosphorylation event, we observed a clear shift in migration on SDS-PAGE gel of Flag-RUSC2 (Fig. 4A lower panel). This prompted us to investigate whether JNK phosphorylates RUSC2 in the cells. To this end we used JNK9 cells (JNK1, 2 -/- cells that also do not express JNK3 (15)) transfected with Flag-RUSC2 alone or a combination of Flag-RUSC2 with HA-JNK1 (Fig. 4B). As indicated by the lack of a mobility shift of Flag-RUSC2 following H_2O_2 treatment of JNK9 cells, JNK phosphorylates RUSC2 also *in vivo*. This is further corroborated by the observation that the reconstitution of JNK expression in JNK9 cells results in the recovery of a mobility shift following H_2O_2 treatment. Taken together these data indicate that JNK phosphorylates RUSC2 both *in vitro* and *in vivo*.

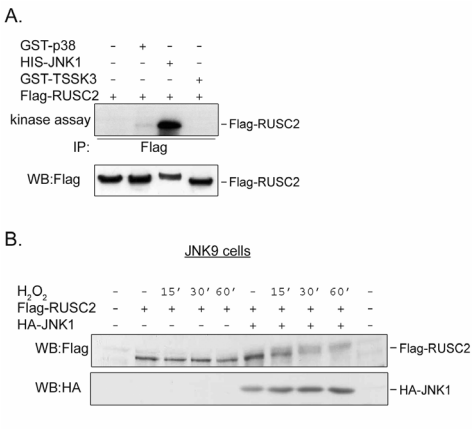


Fig. 4. RUSC2 can serve as a substrate for JNK1 and not for TSSK3

(A) 293T cells were transfected with expression vector encoding Flag-RUSC2. Ectopic Flag-RUSC2 was isolated from the cell lysates by immunoprecipitation with anti-Flag antibody followed by a kinase reaction using GST-p38, HIS-JNK1 or GST-TSSK3 recombinant protein kinases, as indicated, and in the presence of [γ-^{32}P]ATP. Proteins were fractionated by SDS-PAGE and visualized by autoradiography. The expression of Flag-RUSC2 was evaluated by re-probing the same membrane with anti-HA antibody. **(B)** JNK1, 2-/- cells (JNK9) were transfected with Flag-RUSC2 only or in co-transfection with HA-JNK1. At 36 h of transfection, the cells were untreated or treated with H_2O_2 for indicated time periods. Cell lysates were immunoblotted with anti-Flag antibody to visualize Flag-RUSC2 migration or with anti-HA antibody to evaluate HA-JNK1 expression

JNK kinases transiently bind to RUSC2 after hydrogen peroxide treatment

Since our results demonstrate that RUSC2 is a possible substrate for JNK, we next investigated whether these two proteins

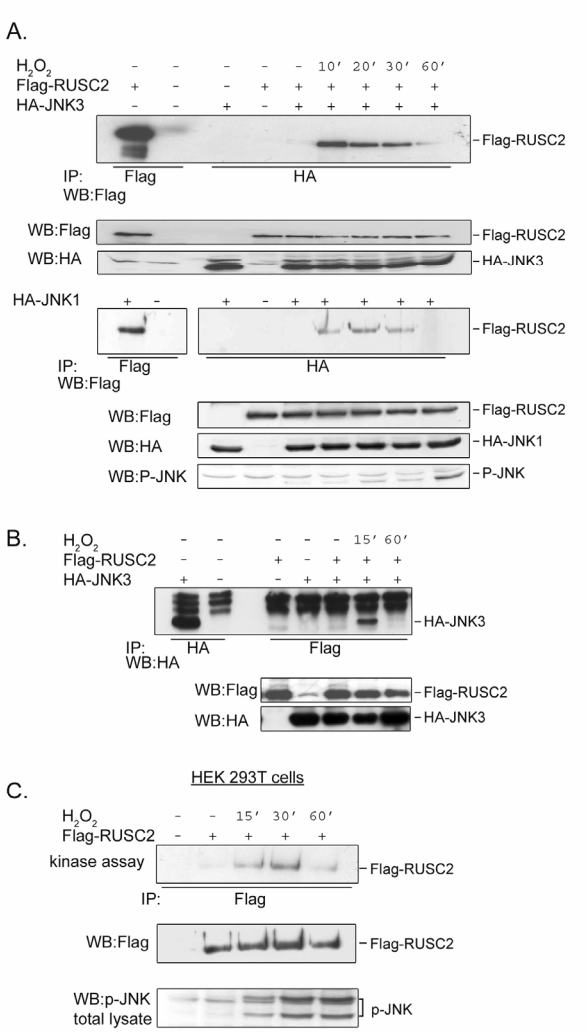


Fig. 5. JNK kinases transiently bind to RUSC2 after hydrogen peroxide treatment
(A, B) 293T cells were transiently transfected with expression vectors encoding Flag-RUSC2, HA-JNK3 or HA-JNK1 in the indicated combinations. At 36 h of transfection, the cells were untreated or treated with H_2O_2 for indicated time periods and cell lysates were subjected to immunoprecipitation assays with anti-Flag or anti-HA antibody followed by protein separation by SDS-PAGE and immunoblotting with anti-Flag antibody to test for Flag-RUSC2 binding to co-expressed JNK proteins (A) or with anti-HA to test for HA-JNK co-immunoprecipitation (B). Total lysates were probed with anti-Flag or anti-HA antibody to test for protein expression (A, B) or with anti-p-JNK antibody (A). **(C)** 293T cells were transfected with expression vector encoding Flag-RUSC2. Prior cell lysis, cells were treated with H_2O_2 for indicated time periods and ectopic Flag-RUSC2 was isolated from the cell lysates by immunoprecipitation with anti-Flag antibody. Next the kinase assay was performed in the immunoprecipitated samples with [γ-^{32}P]ATP without addition of any external kinase. Proteins were fractionated by SDS-PAGE and visualized by autoradiography. Expression of Flag-RUSC2 was tested by re-probing the same blot with anti-Flag antibody.

physically interact in intact cells. 293T cells were co-transfected with Flag-RUSC2 and HA-JNK1 or HA-JNK3 and were then subjected to co-immunoprecipitation analysis. RUSC2 was found to bind to JNK1 as well as to JNK3, however, only after activation of JNK induced by H_2O_2 treatment. This suggests that in contrast to TSSK3 binding to RUSC2 which does not require TSSK3 activity, JNK binding to RUSC2 required JNK kinase activity (Fig. 5A, B). Interestingly, JNK binding to RUSC2 is transient since prolonged treatment of the cells with H_2O_2 resulting in full and prolonged activation (phosphorylation) of JNK (Fig. 5A, bottom panel), resulted in dissociation of RUSC2 from JNK. This suggests the subsequent order of events: after H_2O_2 treatment of cells the initial phase of JNK activation is sufficient for efficient binding to RUSC2, then JNK phosphorylates RUSC2 and dissociates from the complex. This model is further supported by a coupled experiment (Fig. 5C) where transiently transfected Flag-RUSC2 was immuno-precipitated from H_2O_2 treated 293T cells followed by a kinase reaction with [γ-^{32}P]ATP. Autoradiography analysis revealed the same kinetics of RUSC2 phosphorylation as its binding to JNK (compare Fig. 5A and C). Furthermore, this provides also evidence that endogenous JNK associates with and phosphorylates RUSC2.

Inhibition of MKK4 activation abolishes RUSC2 binding to JNK and to TSSK3

As shown above the kinetics of binding of JNK to RUSC2 and the phosphorylation of RUSC2 by JNK, following H_2O_2 treatment of cells suggested that JNK binding requires JNK activity. JNK activation following H_2O_2 treatment of cells most likely involves the activation of the JNK upstream kinase MKK4 (16). Thus we determined whether MKK4 binds to RUSC2 and we also determined

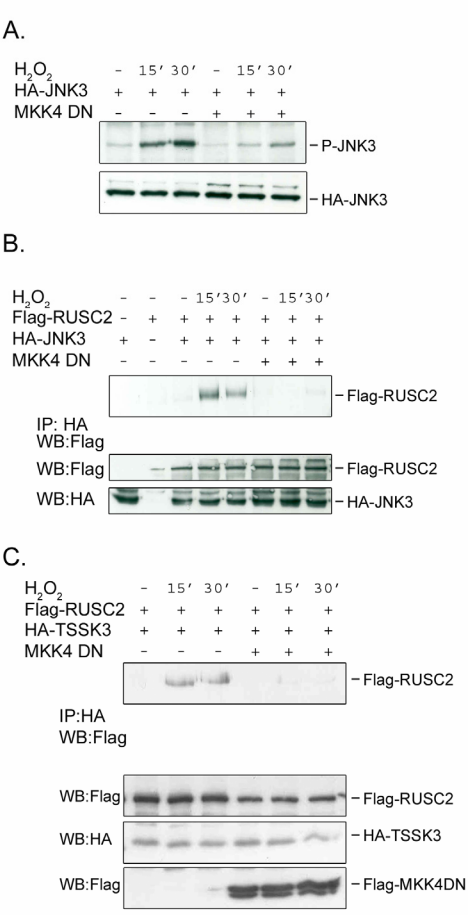


Fig. 6. Inhibition of JNK activation abolishes binding of JNK and TSSK3 to RUSC2

(A) HA-JNK3 was transfected alone or together with Flag-MKK4-DN into 293T cells treated afterwards with 200 µM H_2O_2 for increasing periods of time. The phosphorylation status of HA-JNK3 was tested with anti p-JNK antibody. The protein expression was visualized by immunoblotting total cell lysates. 293T cells were transiently co-transfected with expression vectors encoding Flag-RUSC2 and HA-JNK3 **(B)** or Flag-RUSC2 and HA-TSSK3 **(C)** in combination with Flag-MKK4-DN (dominant negative) as indicated. Prior cell lysis, cells were treated with H_2O_2 for indicated time. Cell lysates were subjected to immunoprecipitation assays with anti-HA antibody followed by protein separation by SDS-PAGE and immunoblotting with anti-Flag antibody.

whether MKK4 activity is essential for activation of JNK in our cells, and the consequent binding to RUSC2. We did not observe binding between RUSC2 and MKK4 (data not shown). However, H_2O_2-induced JNK activation was efficiently inhibited by dominant-negative MKK4 (Fig. 6A) indicating that in our cells indeed H_2O_2-induced JNK activation predominantly involves MKK4. Inhibition of JNK activation by dominant-negative MKK4 blocked the interaction between JNK and RUSC2 (Fig. 6B). This is consistent with our previous data showing that JNK activation is required for binding to RUSC2. Interestingly, co-expression of the MKK4 dominant negative mutant also severely reduced H_2O_2-induced interaction between RUSC2 and TSSK3 (Fig. 6C). In keeping with JNK acting upstream of TSSK3 in binding to RUSC2, we did not observe an effect of dominant negative kinase-dead TSSK3 expression on JNK binding to RUSC2 (Fig. 7). Also we did not obtain evidence that JNK could be phosphorylated by TSSK3 (data not shown). Taken together these results suggest that activated JNK binds to RUSC2 and this in turn results in the binding of TSSK3 to RUSC2. As we do not observe binding of MKK4 to RUSC2 (data not shown) this excludes RUSC2 as a scaffold similar to proteins of the JIP family, which bind in addition to JNK also the upstream activators of JNK. More likely RUSC2 may act as a JNK signaling scaffold in which JNK is the most upstream component of a signaling cascade and acts upstream from TSSK3.

Oxidative stress induces complex formation between RUSC2, JNK3 and TSSK3

If indeed RUSC2 would function as a scaffold to tether JNK and TSSK3, we would expect that binding of JNK and TSSK3 to RUSC2 is not mutually exclusive. Thus RUSC2 was immunoprecipitated from cells

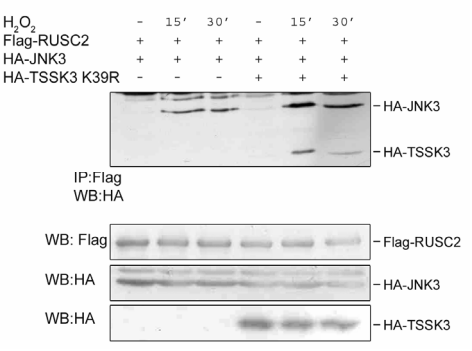


Fig. 7. Cellular oxidative stress induces complex formation between RUSC2, JNK3 and TSSK3. 293T cells were transiently co-transfected with expression vectors encoding Flag-RUSC2 and HA-JNK3 with or without HA-TSSK3 K39R (kinase dead) as indicated. After treatment of cells with 200 μM H_2O_2 for the indicated time points, Flag-RUSC2 was immunoprecipitated with anti Flag antibody and bound proteins were visualized by anti-HA antibody. Total lysates were probed with anti-Flag or anti-HA antibody to test for protein expression.

and simultaneous binding of JNK and TSSK3 to RUSC2 was analyzed by immunoblotting (Fig. 7). After H_2O_2 treatment of the cells, a complex of TSSK3, RUSC2 and JNK3 can be immunoprecipitated. This is consistent with the idea that RUSC2 acts as a scaffold to tether JNK and TSSK3.

KIM motifs are not responsible for binding of RUSC2 to JNK

As indicated above (Fig. 1) two separate domains can be identified in RUSC2, a RUN domain and a SH3 domain (1,17). To analyze which part of RUSC2 binds to JNK, we created two complementary constructs (Flag tagged) containing either of these two domains, named RUSC2 RUN and RUSC2 SH3 (Fig. 1) and we tested them in co-immunoprecipitation experiments with HA-JNK3. Figure 8A shows that both parts of RUSC2 are able to bind JNK under oxidative stress conditions (H_2O_2) although JNK

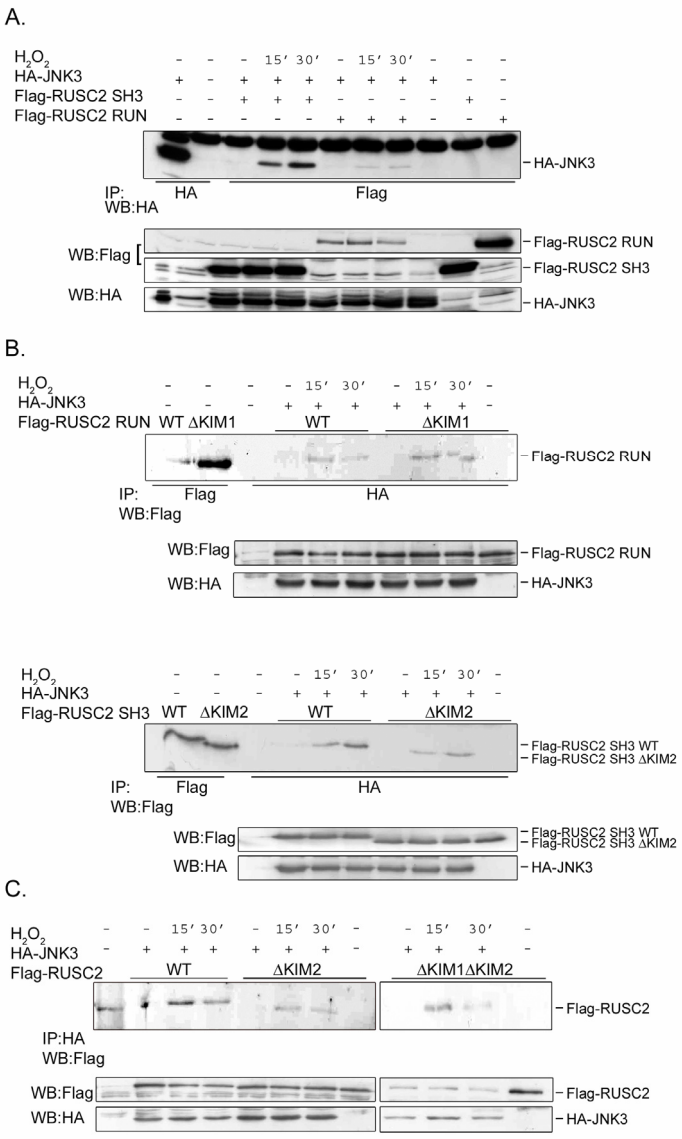


Fig. 8. KIM motifs are not solely responsible for binding of RUSC2 to JNK

(A) 293T cells were transfected with N-terminally or C-terminally deleted constructs of RUSC2, Flag-RUSC2 SH3 or Flag-RUSC2 RUN, respectively (see Fig. 1) together with HA-JNK3. Cells were stimulated with H_2O_2 for indicated time and cell lysates were subjected to immunoprecipitation assays with anti-Flag antibody, followed by protein separation by SDS-PAGE and immunoblotting with anti-HA antibody. **(B)** The KIM deletion constructs of Flag-RUSC2 RUN (ΔKIM1) (upper panel) or Falg-RUSC2 SH3 (ΔKIM2) (lower panel) were tested for binding to HA-JNK3 after oxidative stress (200 μM H_2O_2) in co-immunoprecipitation assay. HA-JNK3 was immunoprecipitated with anti HA antibody and bound proteins were visualized by anti-Flag antibody. **(C)** Full length Flag-RUSC2 constructs with deletion of either KIM2 (ΔKIM2) or both KIM1 and KIM2 (ΔKIM1 ΔKIM2) were tested as in (B). All immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-HA (A) or anti-Flag (B, C) antibody. The total lysates were tested for protein expression

interaction with RUSC2 SH3 construct appeared stronger compared to interaction with RUSC2 RUN construct. This result implicates that there are likely multiple motifs within RUSC2 to which JNK can bind. A previously defined JNK interaction motif is the so-called kinase interaction motif (KIM). Such a motif with the consensus $K/R(X)_{3-5}LXL$, where X stands for any amino acid, is found for example in the c-Jun δ domain. In RUSC2 KIM-like sequences (Fig. 1) are located between amino acids 1193-1200 (RVSQDLLL) just after RUN domain and 1404-1412 (RLPSDWLSL) in front of SH3 domain. To determine whether these KIM sequences are required for JNK binding we prepared several deletion constructs Flag-RUSC2 RUN ΔKIM1, Flag-RUSC2 SH3 ΔKIM2 with deletion of KIM1 or KIM2, respectively and full length RUSC2, Flag-RUSC2 ΔKIM2 or Flag-RUSC2 ΔKIM1 ΔKIM2 with deletion of either KIM2 or both KIMs. Next we tested these deletion constructs in co-immunoprecipitation assays for binding to HA-tagged JNK3. This showed that neither KIM appears essential for oxidative stress-induced binding of JNK3 to RUSC2 (Fig 8B, C). Nevertheless, we could observe some reduced binding of constructs in which the KIM2 motif was deleted, suggesting that KIM2 may contribute to binding of JNK to RUSC2. The KIMs within RUSC2 have been identified on the basis of sequence homology. Because we did not obtain evidence that either KIM is sufficient for JNK binding we wished to validate this identification. Therefore we tested whether these KIM sequences from RUSC2 could bind JNK at all. Thus the KIM motifs from RUSC2 were cloned in fusion with GST and used to perform a pull down assay with HA-JNK3 transiently expressed in 293T cells (Fig. 9). As a control we used the KIM

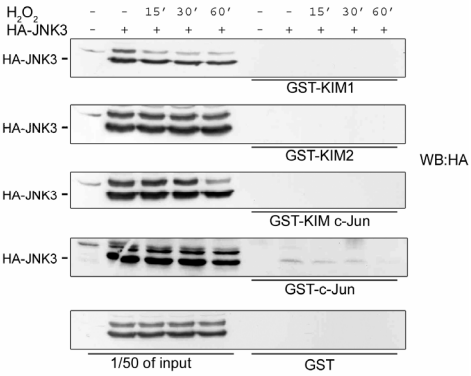


Fig. 9. The KIM sequence is not sufficient for binding JNK3

293T cells were transfected with HA-JNK3 and treated with 200 μM H_2O_2. Cell lysates were prepared and incubated with GST-KIM1, GST-KIM2, GST-KIM c-Jun or GST-c-Jun (1-115) bound to GA beads. GST only was utilized as a negative control for pull down assay specificity. Proteins bound to the GST fusion proteins were resolved by SDS-PAGE and analyzed for the presence of HA-JNK3, by immunoblotting with anti-HA antibody.

sequence present within the δ-domain of c-Jun fused in frame to GST. The GST-KIM sequences from RUSC2 did not bind JNK in this experimental setting and interestingly also the KIM sequence from c-Jun did not bind, whereas a larger fragment, GST c-Jun N-terminal part (1-115) encompassing the KIM-domain, was capable of binding to ectopically expressed HA-JNK3. As far as known to us, the involvement of the KIM motif in JNK binding was only established by mutating certain amino acids of KIM motif, resulting in loss of JNK binding (18,19), whereas it has not been shown that KIM motif by itself can bind JNK. Thus our results suggests that a KIM motif can be necessary but is not sufficient for binding to JNK and apparently in JNK binding to RUSC2, other sequences are likely to be involved, but identification clearly needs more detailed studies.

DISCUSSION

Here we present a previously uncharacterized protein, RUSC2, as a novel interacting partner for two kinases: testis specific serine-threonine kinase 3 (TSSK3) and c- Jun N-terminal kinase (JNK).

We have recently demonstrated (M. Bucko-Justyna et al. submitted) that TSSK3 activity is regulated by phosphorylation of two residues within the T-loop, Ser-166 and Thr-168. *In vitro* Ser-166 phosphorylation is due to autophosphorylation, whereas Thr-168 can be phosphorylated by PDK1. However, insulin treatment of cells which enhances PDK1 activity (20) resulted only in a weak activation of TSSK3. As we performed these experiments in fibroblast cell lines which do not express endogenous TSSK3, and TSSK3 consists essentially of a kinase domain (5), we reasoned that in cells other adaptors/co-activator(s) may be necessary for maximum activation of TSSK3 by PDK1. We employed a yeast-two-hybrid strategy to find possible interactors of TSSK3. We isolated RUSC2 as an interaction partner of TSSK3 and show that *in vivo* RUSC2 and TSSK3 indeed interact, and that EGF and H_2O_2 treatment of cells enhanced this interaction. EGF-induced binding of TSSK3 is sensitive to scavenging of oxygen radicals, suggesting that cellular oxidative stress induces binding of TSSK3 to RUSC2. Unfortunately, co-expression of RUSC2 did not enhance insulin-induced activation of TSSK3 and we could not observe an interaction between PDK1 and RUSC2 (data not shown). Therefore RUSC2 is not a missing link between PDK1 and TSSK3. Alternatively, although PDK1 can act as T-loop kinase for TSSK3 *in vitro*, it is well conceivable that *in vivo* another type of T-loop kinase (e.g. LKB (21), MSTs (22)) not regulated by insulin is involved in TSSK3 regulation and this T-loop kinase may bind to RUSC2. This possibility is currently being studied.

In the present study we provide evidence that RUSC2 acts to tether TSSK3 towards JNK signaling. Both TSSK3 and JNK binding is induced by H_2O_2 treatment of cells and JNK, but not TSSK3, can phosphorylate RUSC2 *in vitro* and *in vivo*. Furthermore, inhibition of JNK activation prevents binding of TSSK3 following H_2O_2 treatment of cells. In addition TSSK3 activity is not required for binding to RUSC2 and kinase-dead TSSK3 does not prevent binding of JNK to RUSC2 induced by H_2O_2. Binding of JNK and TSSK3 to RUSC2 is not mutually exclusive indicating that JNK and TSSK3 bind to separate domains of RUSC2. Taken together our data suggest the following model. Following increased cellular oxidative stress, MKK4 activation results in JNK activation. Active JNK binds to RUSC2 and phosphorylates RUSC2. Phosphorylated RUSC2 in turn binds TSSK3 and JNK is subsequently released from RUSC2.

It is obvious that there are still several outstanding questions. For example, TSSK3 expression is limited to testis and the role of TSSK3 in testis biology is unknown. Furthering our understanding of TSSK3 regulation may provide insight into the role of TSSK3 in testis and in this respect it is noteworthy that we observed that H_2O_2 stimulation of the cells leads also to the transient interaction of RUSC2 with JNK1 and JNK3. Compared to JNK1 and JNK2, JNK3 has a limited pattern of expression, but importantly is expressed in testis (23). Thus in testis JNK3 is likely to be involved in the regulation of TSSK3. Although JNK3-/- mice have been generated a testis phenotype has not been described and it thus may be of interest to analyze these mice in this respect in more detail.

Recently it has been demonstrated that β-arrestin2 can bind JNK3 (24). Furthermore, also ASK1, which can activate JNK3 and MKK4 can form a protein complex with β-arrestin2 (24,25). Thus, besides members of

the JIP family (26-28), other scaffolds such as β-arrestin2 can be involved in regulating the specificity of JNK signaling, and RUSC2 provides yet another example. In contrast however to JIPs and β-arrestin, we did not observe binding of MKK4 to RUSC2. Although we did not test binding of MKK7 because MKK7 may not be involved in H_2O_2-induced JNK activation (16,29), this suggests that RUSC2 functions in providing specificity in downstream JNK signaling rather then in JNK activation itself. We did not obtain evidence for TSSK3 phosphorylation and/or activation by JNK. This is in agreement with our previous biochemical characterization of TSSK3 kinase activity that demonstrates the involvement of Ser166 and Thr168 phosphorylation in regulating kinase activity and both residues cannot be phosphorylated by JNK (data not shown). One likely possibility is that RUSC2 binds in addition to JNK and TSSK3, a specific TSSK3 substrate. Thus rather then regulating TSSK3 kinase activity, JNK may provide specificity to TSSK3 signaling. Recently we have identified the consensus sequence for TSSK3 phosphorylation (M. Bucko-Justyna et al. submitted) and combined with yeast-two-hybrid analysis using RUSC2 as a prey this may result in the identification of such a TSSK3 specific substrate and in addition shed light on the role of TSSK3 in testis.

On the basis of sequence homology we identified so-called KIM sequences shown to be involved in binding of JNK to c-Jun and other transcription factors that can bind JNK or other MAP kinases (3). A KIM sequence was recently also found in the JNK-interacting protein Sab that associates with mitochondria suggesting that this motif is not specific to transcription factors (19). Our results show that binding of JNK to RUSC2 may involve these putative KIM motifs present in RUSC2, especially KIM2, but the KIM motifs do not appear to be essential. Besides the KIM motif a multitude of other JNK docking sites have been described (for a review see (3,4)) some of these (e.g. the FxFP docking site) can be identified within RUSC2 and thus may serve as additional JNK binding site(s). Further work will be required to define the JNK interacting domain(s) within RUSC2.

In conclusion, we have identified RUSC2 (KIAA0375) a previously uncharacterized protein lacking any obvious enzymatic activity as a novel interacting protein for JNK and TSSK3. The sequence of interaction events described in this study indicates TSSK3 as a novel downstream component of JNK signaling and suggests that the interaction between TSSK3 and RUSC2 is likely involved in conveying specificity to TSSK3 downstream signaling during a cellular stress response.

ACKNOWLEDGMENTS

This work was supported by KBN research grant No 6 PO4B 006 19 (1114/PO4/2000/19) for LT, and Centre of Excellence in Molecular Bio-Medicine, contract No QLK6-CT-2002-90363. We thank prof. M. Zylicz for many helpful discussions.

REFERENCES

1. Katoh, M. (2004) *Oncol Rep* 12, 933-938
2. Enslen, H., and Davis, R. J. (2001) *Biol Cell* 93, 5-14
3. Sharrocks, A. D., Yang, S. H., and Galanis, A. (2000) *Trends Biochem Sci* 25, 448-453
4. Tanoue, T., and Nishida, E. (2003) *Cell Signal* 15, 455-462
5. Zuercher, G., Rohrbach, V., Andres, A. C., and Ziemiecki, A. (2000) *Mech Dev* 93, 175-177
6. Wilks, A. F. (1991) *Methods Enzymol* 200, 533-546
7. Visconti, P. E., Hao, Z., Purdon, M. A., Stein, P., Balsara, B. R., Testa, J. R., Herr, J. C., Moss, S. B., and Kopf, G. S. (2001) *Genomics* 77, 163-170
8. Kueng, P., Nikolova, Z., Djonov, V., Hemphill,

A., Rohrbach, V., Boehlen, D., Zuercher, G., Andres, A. C., and Ziemiecki, A. (1997) *J Cell Biol* 139, 1851-1859

9. Nayak, S., Galili, N., and Buck, C. A. (1998) *Mech Dev* 74, 171-174
10. Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., Zeeberg, B., Buetow, K. H., Schaefer, C. F., Bhat, N. K., Hopkins, R. F., Jordan, H., Moore, T., Max, S. I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A. A., Rubin, G. M., Hong, L., Stapleton, M., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Brownstein, M. J., Usdin, T. B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S. S., Loquellano, N. A., Peters, G. J., Abramson, R. D., Mullahy, S. J., Bosak, S. A., McEwan, P. J., McKernan, K. J., Malek, J. A., Gunaratne, P. H., Richards, S., Worley, K. C., Hale, S., Garcia, A. M., Gay, L. J., Hulyk, S. W., Villalon, D. K., Muzny, D. M., Sodergren, E. J., Lu, X., Gibbs, R. A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Young, A. C., Shevchenko, Y., Bouffard, G. G., Blakesley, R. W., Touchman, J. W., Green, E. D., Dickson, M. C., Rodriguez, A. C., Grimwood, J., Schmutz, J., Myers, R. M., Butterfield, Y. S., Krzywinski, M. I., Skalska, U., Smailus, D. E., Schnerch, A., Schein, J. E., Jones, S. J., and Marra, M. A. (2002) *Proc Natl Acad Sci U S A* 99, 16899-16903
11. Costa, G. L., Bauer, J. C., McGowan, B., Angert, M., and Weiner, M. P. (1996) *Methods Mol Biol* 57, 239-248
12. de Groot, R. P., van Dijk, T. B., Caldenhoven, E., Coffer, P. J., Raaijmakers, J. A., Lammers, J. W., and Koenderman, L. (1997) *J Biol Chem* 272, 2319-2325
13. Kishimoto, H., Nakagawa, K., Watanabe, T., Kitagawa, D., Momose, H., Seo, J., Nishitai, G., Shimizu, N., Ohata, S., Tanemura, S., Asaka, S., Goto, T., Fukushi, H., Yoshida, H., Suzuki, A., Sasaki, T., Wada, T., Penninger, J. M., Nishina, H., and Katada, T. (2003) *J Biol Chem* 278, 16595-16601
14. de Ruiter, N. D., Wolthuis, R. M., van Dam, H., Burgering, B. M., and Bos, J. L. (2000) *Mol Cell Biol* 20, 8480-8488
15. Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M., and Wagner, E. F. (1999) *Mech Dev* 89, 115-124
16. Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998) *Curr Biol* 8, 1387-1390
17. Callebaut, I., de Gunzburg, J., Goud, B., and Mornon, J. P. (2001) *Trends Biochem Sci* 26, 79-83
18. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) *Cell* 87, 929-939
19. Wiltshire, C., Matsushita, M., Tsukada, S., Gillespie, D. A., and May, G. H. (2002) *Biochem J* 367, 577-585
20. Chen, H., Nystrom, F. H., Dong, L. Q., Li, Y., Song, S., Liu, F., and Quon, M. J. (2001) *Biochemistry* 40, 11851-11859
21. Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G., and Alessi, D. R. (2004) *Embo J* 23, 833-843
22. Praskova, M., Khoklatchev, A., Ortiz-Vega, S., and Avruch, J. (2004) *Biochem J* 381, 453-462
23. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) *Embo J* 15, 2760-2770
24. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) *Science* 290, 1574-1577
25. Miller, W. E., and Lefkowitz, R. J. (2001) *Curr Opin Cell Biol* 13, 139-145
26. Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K. I. (1999) *Mol Cell Biol* 19, 7539-7548
27. Yasuda, J., Whitmarsh, A. J., Cavanagh, J., Sharma, M., and Davis, R. J. (1999) *Mol Cell Biol* 19, 7245-7254
28. Whitmarsh, A. J., Kuan, C. Y., Kennedy, N. J., Kelkar, N., Haydar, T. F., Mordes, J. P., Appel, M., Rossini, A. A., Jones, S. N., Flavell, R. A., Rakic, P., and Davis, R. J. (2001) *Genes Dev* 15, 2421-2432

29. Davis, R. J. (2000) *Cell* 103, 239-252

Addendum

Chapter 3

Interaction of RUSC2 with the active form of the small GTPase H-Ras

Interaction of RUSC2 with the active form of the small GTPase H-Ras

Marta BUCKO-JUSTYNA[1], Alida M.M. de VRIES-SMITS[2], Boudewijn M. T. BURGERING[2]

[1]Department of Molecular Biology, International Institute of Molecular and Cell Biology in Warsaw, Ks. Trojdena 4, 02-109 Warsaw, Poland, [2]Department of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands

The Ras GTPases operate as molecular switches that link extracellular stimuli with a diverse range of biological outcomes. These proteins cycle between GDP-bound inactive and GTP bound active forms. The switch is activated by guanine-nucleotide exchange factors (GEFs) and it is turned off through hydrolysis of GTP by the intrinsic GTPase activity, which is stimulated by GTPase activating proteins (GAPs) (1-4). In their activated forms, Ras GTPases interact with effector molecules that activate downstream pathways. These effector proteins contain characteristic domains that mediate their direct protein interactions with Ras GTPases. Examples of defined Ras-interaction domains are the RA (Ras-associating) domain such as present in RalGDS (5), the RafRBD (Ras-binding domain) that mediates the interaction between Ras and Raf (6,7) or the PBD (p21-Rho-binding domain), that is responsible for the interaction of Rac with PAK (8). Callebaut, I. et al. (9) recently described a new prototype of Ras interacting domain named the RUN domain [after RPIP8 (Rap2 interacting protein 8), UNC-14 and NESCA (new molecule containing SH3 at the carboxyl-terminus)] and proposed that the RUN domain containing proteins could function as specific effectors for some proteins of the Ras superfamily.

One of the proteins containing such a RUN domain is RUSC2 (10) that we studied in Chapter 3. To analyze whether RUSC2 could be an effector of any GTPase from Ras superfamily, we tested a series of GTP-bound active mutants of small GTPases for a possible interaction with RUSC2 in co-immunoprecipitation assays and we observed binding of H-RasV12 to RUSC2 (Fig. 1A). Interestingly, RUSC2 did not bind to the inactive (dominant-negative) H-Ras mutant H-RasN17 (Fig.1B). Treatment of A14 cells, NIH3T3 cells that overexpress the human insulin receptor, with insulin activates many signaling cascades, including the activation of Ras and Ras-mediated downstream signaling, resulted in a small but reproducible induction of RUSC2 binding to wild type H-Ras transiently overexpressed in these cells (Fig. 1C).

The rationale for testing the binding of a small GTPase to RUSC2 was the presence of a RUN domain, a potential Ras small GTPase binding domain. Thus we tested whether the RUN domain is involved in mediating the interaction between H-Ras and RUSC2. A partial RUSC2 clone encompassing either the RUN domain (Flag-RUSC2 RUN) or the SH3 domain (Flag-RUSC2 SH3) were tested for binding to active H-Ras in a co-immunoprecipitation assay (Fig. 1D).

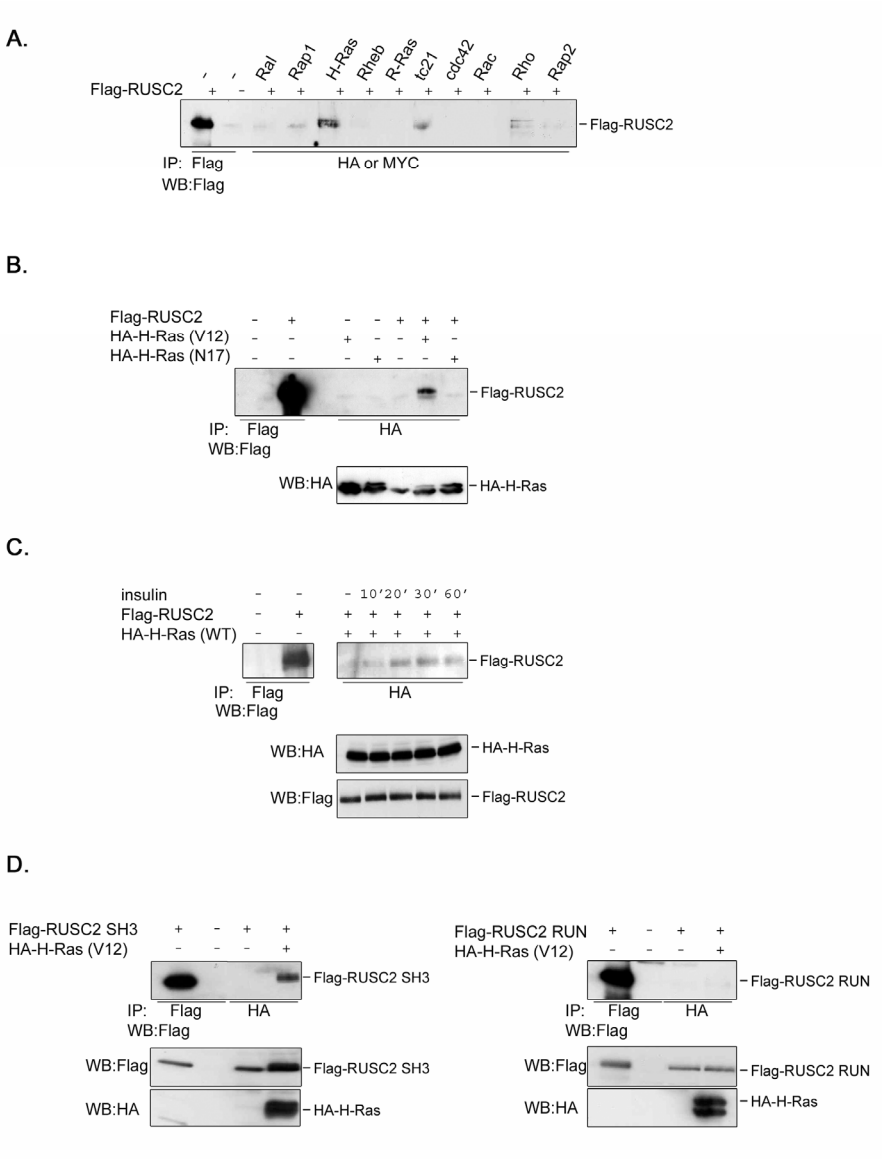
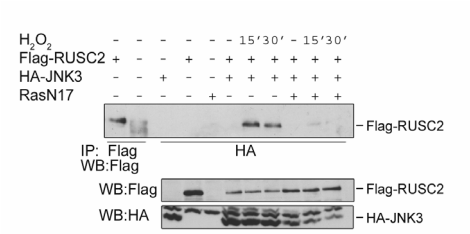


Fig. 1. RUSC2 interacts with active form of H-Ras

(A) 293T cells were transfected with Flag-RUSC2 together with different Myc or HA tagged active forms of small GTPases, as indicated. Small GTPases were immunoprecipitated using anti-HA or anti-Myc antibody, and binding of RUSC2 to small GTPases was analyzed on blot. **(B)** 293T cells were transfected with Flag-RUSC2 together with HA-H-RasV12, active mutant or HA-H-RasN17, dominant-negative mutant. H-Ras was immunoprecipitated using 12CA5 monoclonal antibody, and binding of RUSC2 was analyzed on blot using anti-Flag antibody. **(C)** A14 cells were transfected with Flag-RUSC2 together with HA-H-RasWT, wild type. Cells were treated with 1μg/ml insulin for indicated time. H-Ras was immunoprecipitated with anti-HA antibody and binding of RUSC2 was analyzed on blot. **(D)** 293T cells were transfected with HA-H-RasV12 together with different domains of RUSC2, Flag-RUSC2 SH3 or Flag-RUSC2 RUN. H-Ras was immunoprecipitated using 12CA5 monoclonal antibody, and binding of RUSC2 domains was analyzed on blot using anti-Flag antibody.



Fig, 2. Inhibition of Ras by RasN17 abolishes RUSC2 binding to JNK
293T cells were transiently co-transfected with expression vectors encoding Flag-RUSC2 and HA-JNK3 in combination with RasN17 (dominant negative). Prior cell lysis, cells were treated with H_2O_2 for indicated time. Cell lysates were subjected to immunoprecipitation assays with anti-HA antibody followed by protein separation by SDS-PAGE and immunoblotting with anti-Flag antibody.

Surprisingly, Flag-RUSC2 SH3 and not Flag-RUSC2 RUN interacted with active HA-H-Ras. Within the part of RUSC2 containing the SH3 domain no apparent other domains can be identified that possibly could mediate binding to H-Ras. SH3 domains interact with proline-rich domains, which are not present in H-Ras and indeed we are not aware of any example in literature demonstrating a direct interaction between Ras and a SH3 domain. Thus, we conclude that the interaction between H-Ras and RUSC2 is likely to be indirect.

In Chapter 3 we described the identification of RUSC2 as a protein interacting with both TSSK3 and JNK and demonstrated the regulation of this interaction by cellular oxidative stress. Inhibition of JNK activation following increased cellular oxidative stress resulted in the inhibition of both JNK and TSSK3 binding. Several Ras family GTPases have been implicated in the regulation of JNK activation, including Rac, Ras and Ral (11). We did not observe binding of the small GTPases Ral or Rac to RUSC2 (Fig.1A) but we did observe binding of active H-Ras to RUSC2. Furthermore we observed a mobility shift of Flag-RUSC2 SH3 when co-transfected with H-Ras V12 and not H-Ras N17 (compare Fig. 1D right lanes) and previously we have shown (Chapter 3) that JNK phosphorylation of RUSC2 induces a mobility shift of RUSC2. Thus, it is conceivable that in this experimental setting active H-Ras, through the activation of JNK would regulate the interaction between RUSC2 and JNK. To test this we transfected 293T cells with Flag-RUSC2, HA-JNK3 and dominant-negative RasN17 (Fig. 2). H_2O_2 induced interaction between RUSC2 and JNK3 was severely reduced in the presence of RasN17 suggesting that indeed Ras acts upstream of JNK in the regulation of JNK activity and consequently in the interaction between JNK and RUSC2. To further corroborate this possibility we tested whether inhibition of JNK activation by expression of dominant negative MKK4 would not affect binding of Ras to RUSC2. To our surprise we observed a strong induction of wild type H-Ras binding to RUSC2 after treatment of cells with H_2O_2 and a clear reduction in H-Ras

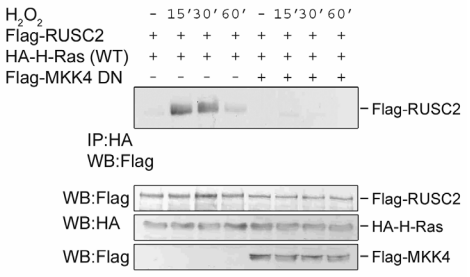


Fig. 3. Cellular oxidative stress induces H-Ras-RUSC2 interaction and inhibition of JNK activation abolishes it
293T cells were transiently co-transfected with expression vectors encoding Flag-RUSC2 and HA-H-Ras (WT), wild type in combination with MKK4-DN (dominant negative). Prior cell lysis, cells were treated with H_2O_2 for indicated time. Cell lysates were subjected to immunoprecipitation assays with anti-HA antibody followed by protein separation by SDS-PAGE and immunoblotting with anti-Flag antibody. Total lysates were probed with anti-Flag or anti-HA antibody to test for protein expression.

binding in the presence of dominant-negative MKK4 (Fig. 3). This result suggests that Ras acts upstream of JNK in the signaling cascade leading to the activation of JNK, but is downstream of JNK with respect to the binding to RUSC2. Insulin induces a robust activation of Ras in A14 cells, yet we observed only a small increase in binding of H-Ras to RUSC2. Although at present not tested H_2O_2 treatment of 293T cells will at the most induce Ras activation similar to insulin treatment of A14 cells, yet we observed an unexpected strong induced binding of Ras to RUSC2. This could therefore indicate that RUSC2 may play an important role in regulating Ras function independent of its GTP loading for example in providing a spatial component.

MATERIALS AND METHODS

cDNA constructs

Flag-RUSC2 (full length) was generated by PCR reaction on pBluescript II SK(+) RUSC2 (Kazusa DNA Research Institute) using following primer pairs: primer 1/primer 2 (CGAAGCGGCCGCAATGGATA GT / CCAGCATGCACAGGAATTCAGTTT) containing *Not*I/*Eco*RI sites, respectively, and ligation of the resulting PCR product digested with *Not*I/*Eco*RI with pFLAG-CMV-4 vector cut with the same enzymes; Flag-RUSC2 RUN was made by cutting off *Kpn*I fragment from Flag-RUSC2 construct and religation. To generate Flag-RUSC2 SH3, Flag-RUSC2 was digested with *Kpn*I and a fragment containing SH3 domain was isolated and ligated with modified pFLAG-CMV-4 cut with *Kpn*I. To modify pFLAG-CMV-4 for this purpose, *Bgl*II/*Eco*RV small fragment was cut off, the remaining protruding DNA ends were filled in and relegated.

pMT2HA-H-RasWT, pMT2HA-H-RasV12, pMT2HA-H-RasN17 have been described previously (12), HA-JNK3 was a kind gift from prof. J. Ostrowski, Flag-MKK4 DN was a gift from H. Nishina and is described elsewhere (13).

Cells and transfections

HEK-293T cells and A14 cells (mouse NIH3T3 cells overexpressing the human insulin receptor (14)) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Sigma) and 1% antibiotic suspension (penicillin and streptomycin; Sigma) and 2mM L-glutamine. Insulin was added at a final concentration of 1µg/ml. Transfections were carried out using the $CaPO_4$ method for A14 cells and FuGENE6 reagent (Roche) was used to transfect HEK 293T cells.

Antibodies

The following antibodies were used: 12CA5 for HA-tagged proteins, 9E10 for Myc-tagged proteins (both produced using hybridoma cell lines) and anti-Flag M2 monoclonal antibody (Sigma) for Flag-tagged proteins.

Immunoprecipitations and Western blots

Cells were lysed in ice-cold lysis buffer containing: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DOC, 5 mM EDTA, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin. Lysates were cleared for 10 min at 20 000 *g* at 4^0C, and incubated for 2 hours at 4^0C with either 12CA5 (HA), 9E10 (myc) or Flag antibody and 50 µl pre-washed protein A beads. Immunoprecipitations were washed three times with lysis buffer, cleared of all supernatant and suspended in Laemmli sample buffer. Samples were subjected to SDS/PAGE and transferred to PVDF. Western blot analysis was performed under standard conditions, using indicated antibodies.

REFERENCES

1. Ahmadian, M. R., Stege, P., Scheffzek, K., and Wittinghofer, A. (1997) *Nat Struct Biol* 4, 686-689
2. Bos, J. L. (1989) *Cancer Res* 49, 4682-4689
3. Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., De Rooij, J., Bos, J. L., and Wittinghofer, A. (2003) *Nat Struct Biol* 10, 26-32
4. Scheffzek, K., Ahmadian, M. R., and Wittinghofer, A. (1998) *Trends Biochem Sci* 23, 257-262
5. Ponting, C. P., and Benjamin, D. R. (1996) *Trends Biochem Sci* 21, 422-425
6. Huang, L., Weng, X., Hofer, F., Martin, G. S., and Kim, S. H. (1997) *Nat Struct Biol* 4, 609-615
7. Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) *Nature* 375, 554-560
8. Thompson, G., Owen, D., Chalk, P. A., and Lowe, P. N. (1998) *Biochemistry* 37, 7885-7891

9. Callebaut, I., de Gunzburg, J., Goud, B., and Mornon, J. P. (2001) *Trends Biochem Sci* 26, 79-83
10. Katoh, M. (2004) *Oncol Rep* 12, 933-938
11. Coso, O. A., Teramoto, H., Simonds, W. F., and Gutkind, J. S. (1996) *J Biol Chem* 271, 3963-3966
12. Zwartkruis, F. J., Wolthuis, R. M., Nabben, N. M., Franke, B., and Bos, J. L. (1998) *Embo J* 17, 5905-5912
13. Kishimoto, H., Nakagawa, K., Watanabe, T., Kitagawa, D., Momose, H., Seo, J., Nishitai, G., Shimizu, N., Ohata, S., Tanemura, S., Asaka, S., Goto, T., Fukushi, H., Yoshida, H., Suzuki, A., Sasaki, T., Wada, T., Penninger, J. M., Nishina, H., and Katada, T. (2003) *J Biol Chem* 278, 16595-16601
14. Burgering, B. M., and Coffer, P. J. (1995) *Nature* 376, 599-602

Chapter

4

Search for mutations in genes coding for TSSK1 and TSSK2 in patients with infertility

To be Submitted

Search for mutations in genes coding for TSSK1 and TSSK2 in patients with infertility

Marta BUCKO-JUSTYNA[1], Paulien POLDERMAN[2], Lech TRZECIAK[1], Boudewijn M. T. BURGERING[2]

[1] Department of Molecular Biology, International Institute of Molecular and Cell Biology in Warsaw, Ks. Trojdena 4, 02-109 Warsaw, Poland, [2]Department of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center Utrecht, 3584 CG Utrecht, The Netherlands

Although various genetic factors have been implicated in human male infertility, the causative genes for the different types of male infertility have not been elucidated. Testis specific serine-threonine kinase 1 and 2 (TSSK1 and 2) whose expression is limited to meiotic and postmeiotic spermatogenic cells, respectively, are potential candidates to play a role in the processes of fertilization. The sequencing of the entire coding regions of human *TSSK1* and *TSSK2* genes, in 120 infertile male patients revealed three single nucleotide polymorphisms (SNPs) in *TSSK2* coding region, which did not cause any amino acid substitutions, and one SNP in the *TSSK2* gene, which changed threonine residue, located in the C-terminal regulatory domain of *TSSK2*, to methionine (T280M). We did not observe any SNPs in *TSSK1* gene. This suggests that T280M mutation in *TSSK2* gene may result in spermatogenic failure and that *TSSK2* might be a candidate gene for molecular marker for genetic diagnosis of male infertility.

INTRODUCTION

Spermatogenesis is a complex process of mitotic and meiotic divisions of germ cells finally resulting in the formation of haploid spermatozoa. The general organization of spermatogenesis is essentially the same in all mammals and can be divided into three distinct phases: (i) spermacytogenesis, during which spermatogonia undergo mitotic cell division and generate a pool of spermatocytes; (ii) the meiotic phase, which yields the haploid spermatids; and (iii) spermiogenesis whereby each spermatid undergoes an elaborate process of cytodifferantiation before being released as viable sperm into the lumen of seminiferous tubules. Meiosis is critical for sexual reproduction. Male infertility affects ~ 10% of the couples with a desire to have children (1). Environmental factors or infections contribute to infertility to some extent, but genetic factors also play a pivotal role in causing male infertility. Some genetic causes of male infertility are known (2), but the causes of most types of male infertility are unclear. Examples of gene defects that cause only infertility without causing other abnormalities, are for example: men with Yq microdeletions, XXY karotype and androgen insensitivity who usually display testicular atrophy and possible signs of androgen

deficiency (3). Other gene defects, such as those causing mytonic dystrophy and cystic fibrosis are not primarily characterized by a infertility but this is often one of a range of characteristic symptoms affecting general health (4,5). Therefore the discovery of new genetic causes of male infertility, specifically involved in testis development and spermatogenesis may have therapeutic implications. The most likely candidates for this are of course genes that are specifically expressed in the testis and not in other tissues.

Two members of the human testis specific serine-threonine kinase family, TSSK1 and TSSK2 were chosen in this study to screen for mutations or single nucleotide polymorphisms (SNPs) in patients with infertility. The human TSSK1 and TSSK2 genes encode proteins of 367 and 358 amino acids respectively, and are mapped to chromosomes 5 and 22 respectively (6). Interestingly, human TSSK2 (also called DGSG) has been characterized as one of 11 putative transcription units encoded in the minimal DiGeorge critical region (MDGCR) of 250 kb, located in the proximal arm of human chromosome 22 (7-9). Deletion of this area is suspected to be involved in the pathogenesis of the DiGeorge and velo-cardio-facial syndromes (8). Both syndromes represent developmental disorders associated with a spectrum of malformations including hypoplasia of the thymus and parathyroid glands, cardiovascular anomalies, and mild craniofacial dysmorphia. Up to date only for the transcription factor tbx1, located within this chromosome region, evidence has been obtained for its involvement in (some) of the developmental disorders associated with DiGeorge's syndrome (10-12). Thus, it is presently unclear if the *TSSK2/DGSG* gene may be involved in this syndrome. It is likely that the development of the disorder is sensitive to the dosage of a product encoded by a combination of genes within MDGCR.

Both *TSSK1* and *TSSK2* genes are intronless and relatively short (1104 and 1077 nucleotides, respectively) what makes them easy to analyze by sequencing. Additionally, because proteins involved specifically in fertilization are likely expressed late in spermatogenesis, TSSK1 and 2 with expression limited to meiotic and postmeiotic spermatogenic cells, respectively (9,13) represent potential participants in the process of fertilization.

In the current study, we analyzed the coding sequences of human *TSSK1* and *TSSK2* genes, using genomic DNA from infertile men, in search for mutations or SNPs that could potentially alter TSSK1 and/or 2 protein kinase function/activity and therefore result in male fertility dysfunction.

MATERIAL AND METHODS

120 men with different spermatogenic defects resulting in infertility like azoospermia (lack of sperm cells in semen), severe oligospermia (less than $5x10^6$ sperm cells/ml semen) and with oligospermia (5-$10x10^6$ sperm cells/ml semen), all of them of Polish origin, were included in the study.

DNA samples isolated from peripheral blood leukocytes of the patients were a kind gift from prof. J. Jaruzelska from Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland (14). For mutation screening, genomic DNA was amplified by PCR using primers specific for human *TSSK1* sequence: primer1 / primer2 (TCCAGGATGTAAAT GAGCACACTG / TCTGGCTCCACCCTTGACTTC TTC) or for human *TSSK2*: primer1 / primer2 (CTCCGGTAGTGTAAATGAGGACAA / TTA GTTTACGTGAAGGCCGACTGC). Amplification products of *TSSK1* and *TSSK2* genes were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on ABI PRISM fluorescent automated DNA sequencers (Applied Biosystems).

RESULTS AND DISCUSSION

Direct sequencing of *TSSK1* and *TSSK 2* genes performed in 120 infertile men revealed three synonymous (no change in amino acid)

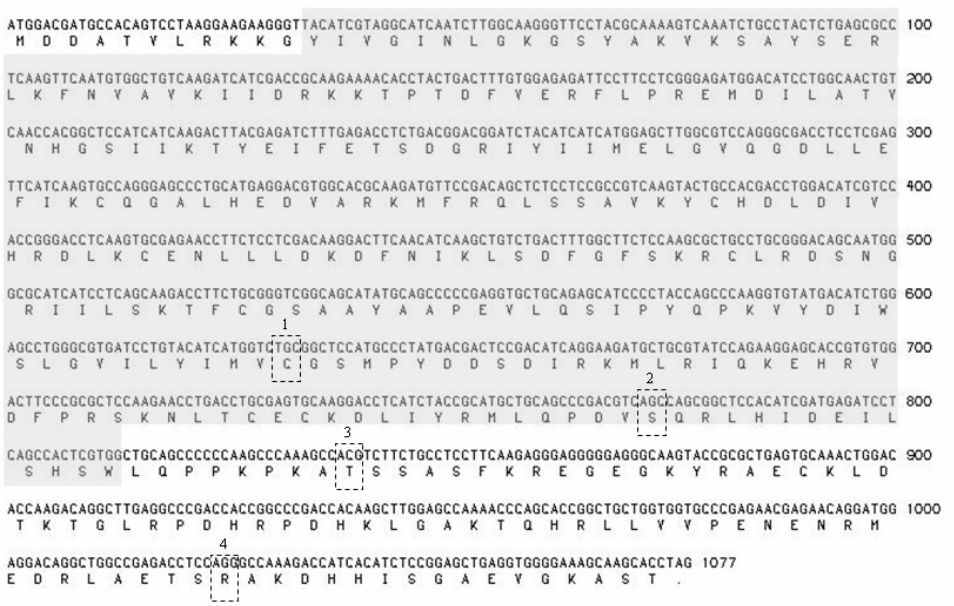


Fig. 1. Nucleotide and amino acid sequence of human TSSK2. The kinase domain is shaded. The polymorphism positions found in this study are boxed and numbered: 1) Cys211 (TGC to TGT), 2) Ser258 (AGC to AGT), 3) Thr280 to Met280 (ACG to ATG), 4) Arg342 (AGG to AGA)

SNPs present in *TSSK2*. They include changes in one patient in codon 211 (TGC (Cys) to TGT (Cys)), four patients in codon 258 (AGC (Ser) to AGT (Ser)) and two patients in codon 342 (AGG (Arg) to AGA (Arg)). The residues 211 and 258 are located within the kinase domain of TSSK2 while residue 342 locates on C-terminal regulatory part of the kinase (Fig. 1). Additionally, sequencing of *TSSK2* revealed the existence of a missense mutation in 7 cases at codon 280 (ACG to ATG) resulting in the threonine to methionine change. Two patients appeared heterozygous for this mutation and the remaining 5 patients were homozygous for this mutation (Fig. 2). Thr280 is located outside the kinase domain of TSSK2, within the C-terminal extension. TSSK2 is a putative protein kinase that contains a potential T-loop necessary for activation within the kinase domain (L. Lipinski personal communication). Thus TSSK2 is likely to be regulated by for example an upstream acting kinase(s) and the C-terminal extension outside the kinase domain may for example function as a lid on the kinase domain that opens up after phosphorylation to allow activation of TSSK2. This would be reminiscent to the regulation of p70S6 kinase which is extensively phosphorylated in its C-terminal non-catalytic domain and this is essential to make p70S6 kinase accessible for PDK-1 to phosphorylate the T-loop and induce activation of p70S6 kinase (15,16). In such a model the change of potential phosphorylation site within this (regulatory) domain, Thr280 to Met, might result in improper regulation or even loss of function of TSSK2 and therefore a potential cause of defects in spermatogenesis, the process in which TSSK2 is suspected to be involved (6,9). Further *in vitro* studies using cells

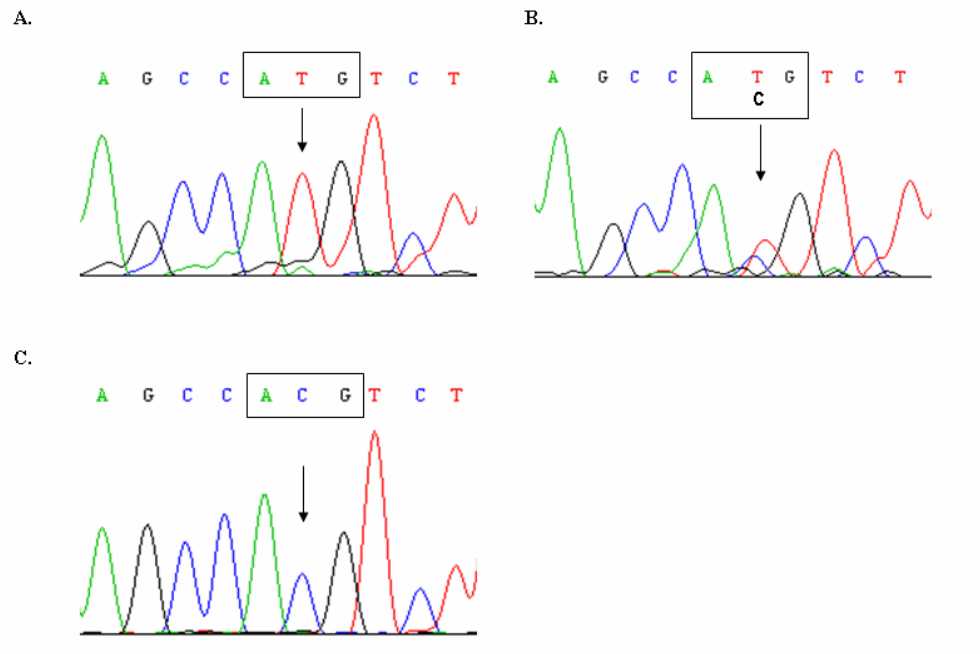


Fig. 2. Chromatograms from direct sequencing of *TSSK2* gene.
A. Arrow indicates C to T change at codon 280 (ACG to ATG) coding for threonine, found in five patients. **B.** Arrow indicates the T/C heterozygosity at codon 280, found in two patients. **C.** Wild type sequence in a healthy individual. The codon 280 is boxed.

transfected with T280M mutation in *TSSK2* gene, or transgenic animals are required to analyze the consequences of this mutation on TSSK2 activity / regulation.

In contrast to *TSSK2*, we were not able to find any SNPs in the sequence of *TSSK1* analyzed on genomic DNA of the same pool of infertile men patients. The human TSSK1 and TSSK2 show very high homology at the amino acid level. The identity between TSSK1 and TSSK2 is 83% in the kinase region. This identity, however, decreases to 72% across the entire ORF, reflecting the divergence of the C-terminal amino acid sequence. The mutation that we found in the C-terminus of TSSK2 (T280M) seems therefore to be specific for TSSK2. Since we did not have a statistically important number of genomic DNAs obtained from proven-fertile male, a good comparison of the occurrence of SNPs in TSSK2 between infertile males and the healthy males, we cannot draw at present a definitive conclusion on the importance of SNPs that we found in *TSSK2*, for male infertility. Nevertheless, it is clear that compared to TSSK1, at least TSSK2 lies within a chromosomal region that is genetically less stable in patients suffering from infertility. This at least suggests a link between infertility and genetic defects in TSSK2. Alternatively, the novel concept is evolving that SNPs, potentially modifying gene functions, might be tolerated in reproduction when their effects are subtle and the frequency among the population is high. Indeed, a number of such SNPs have been reported recently, and for some of them the association with reproductive function, such

as sperm production (SNPs in FSH receptor gene (17)) or different hormone sensitivities (18), has been shown. For others, like for *DAZL* (deleted in azoospermia-like) SNPs were demonstrated not to be associated with spermatogenic failure and therefore do not represent a molecular marker for genetic diagnosis of male infertility (19). If SNPs of TSSK2 can indeed be such a molecular marker needs to be studied further, in *in vitro* studies as well as by analyzing additional pools of DNA of infertile patients in parallel with healthy individuals. This would enable more robust statistical analysis of the prevalence of certain TSSK2 SNPs in the population.

ACKNOWLEDGEMENTS

This work was supported by KBN research grant No 6 PO4B 006 19 (1114/PO4/2000/19) for LT and a VICI-NWO grant to BMTB**.** We thank prof. J. Jaruzelska (Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland) for a kind gift of genomic DNA samples from 120 infertile men.

REFERENCES

1. Nieschlang, E., Behre, H.M. (2000) *Springer, Heidelberg* **2nd edn.**
2. Baker, H. W. (2000) *Baillieres Best Pract Res Clin Endocrinol Metab* **14**, 409-422
3. McLachlan, R. I., Mallidis, C., Ma, K., Bhasin, S., and de Kretser, D. M. (1998) *Reprod Fertil Dev* **10**, 97-104
4. Jou, S. B., Lin, H. M., Pan, H., Chiu, Y. L., Li, S. Y., Lee, C. C., and Hsiao, K. M. (2001) *Proc Natl Sci Counc Repub China B* **25**, 40-44
5. Sokol, R. Z. (2001) *Curr Opin Pulm Med* **7**, 421-426
6. Hao, Z., Jha, K. N., Kim, Y. H., Vemuganti, S., Westbrook, V. A., Chertihin, O., Markgraf, K., Flickinger, C. J., Coppola, M., Herr, J. C., and Visconti, P. E. (2004) *Mol Hum Reprod* **10**, 433-444
7. Galili, N., Baldwin, H. S., Lund, J., Reeves, R., Gong, W., Wang, Z., Roe, B. A., Emanuel, B. S., Nayak, S., Mickanin, C., Budarf, M. I., and Buck, C. A. (1997) *Genome Res* **7**, 399
8. Gong, W., Emanuel, B. S., Collins, J., Kim, D. H., Wang, Z., Chen, F., Zhang, G., Roe, B., and Budarf, M. L. (1996) *Hum Mol Genet* **5**, 789-800
9. Kueng, P., Nikolova, Z., Djonov, V., Hemphill, A., Rohrbach, V., Boehlen, D., Zuercher, G., Andres, A. C., and Ziemiecki, A. (1997) *J Cell Biol* **139**, 1851-1859
10. Epstein, J. A. (2001) *Trends Genet* **17**, S13-17
11. Lindsay, E. A., Vitelli, F., Su, H., Morishima, M., Huynh, T., Pramparo, T., Jurecic, V., Ogunrinu, G., Sutherland, H. F., Scambler, P. J., Bradley, A., and Baldini, A. (2001) *Nature* **410**, 97-101
12. Merscher, S., Funke, B., Epstein, J. A., Heyer, J., Puech, A., Lu, M. M., Xavier, R. J., Demay, M. B., Russell, R. G., Factor, S., Tokooya, K., Jore, B. S., Lopez, M., Pandita, R. K., Lia, M., Carrion, D., Xu, H., Schorle, H., Kobler, J. B., Scambler, P., Wynshaw-Boris, A., Skoultchi, A. I., Morrow, B. E., and Kucherlapati, R. (2001) *Cell* **104**, 619-629
13. Nayak, S., Galili, N., and Buck, C. A. (1998) *Mech Dev* **74**, 171-174
14. Jaruzelska, J., Korcz, A., Wojda, A., Jedrzejczak, P., Bierla, J., Surmacz, T., Pawelczyk, L., Page, D. C., and Kotecki, M. (2001) *J Med Genet* **38**, 798-802
15. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) *Curr Biol* **8**, 69-81
16. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998) *Science* **279**, 707-710
17. Simoni, M., Nieschlag, E., and Gromoll, J. (2002) *Hum Reprod Update* **8**, 413-421
18. Eckardstein, S. V., Schmidt, A., Kamischke, A., Simoni, M., Gromoll, J., and Nieschlag, E. (2002) *Clin Endocrinol (Oxf)* **57**, 647-655
19. Tschanter, P., Kostova, E., Luetjens, C. M., Cooper, T. G., Nieschlag, E., and Gromoll, J. (2004) *Hum Reprod* **19**, 2771-2776

Chapter

5

Discussion

Spermatogenesis is a complex process of mitotic and meiotic divisions of germ cells finally resulting in the formation of haploid spermatozoa and involves specific interactions between the developing germ cells, supporting Sertoli cells and hormone-producing Leydig cells. An estimated two-thirds of mammalian genes is at some point expressed in adult or developing testis (1), with 5% to 10% of genes expressed exclusively there. Among genes playing role in testis function there is a large number of protein kinases but only a few of them are exclusively expressed in germ cells or in the testis. The family of Testis Specific Serine-threonine Kinases (TSSK) comprises four members up to date (2). These kinases are expressed exclusively in testis during spermatogenesis but very little is known about their physiological role and mechanisms of action. The research presented in this thesis is mainly focused on TSSK3 characterization, regulation and interaction properties (Chapter 2 and 3). The other two members of the TSSK family, TSSK1 and 2 are also studied, but only with respect to the possible presence or absence of genetic modifications within these genes, in human male infertility (Chapter 4).

TSSK3 regulation by autophosphorylation and PDK1 phosphorylation

One of the best studied signaling events controlled by PtdIns(3,4,5)P3, comprises the activation of the AGC family of protein kinases, including protein kinase B (PKB/c-Akt) (3), p70 ribosomal S6 kinase (p70S6K) (4), serum- and glucocorticoid-induced protein kinase (SGK) (5) and protein kinase C (PKC) (6), all of which play crucial roles in regulating physiological processes relevant to metabolism, growth, proliferation and survival. Much research has focused on understanding the mechanism by which AGC kinases are activated following insulin and growth factor stimulation and it was shown that phosphorylation at two highly conserved Ser/Thr residues is critical (7-11). One is located within the so-called T-loop (also known as activation-loop) of the catalytic domain, whilst the other is C-terminal to the catalytic domain in a region termed the hydrophobic motif. Phosphorylation of both residues is required for the maximal activation of these enzymes. The 3-phosphoinositide-dependent protein kinase-1 (PDK1) has been shown in several cases to be responsible for the T-loop phosphorylation of these kinases. In all cases described thus far, T-loop phosphorylation results in kinase activation. Analysis of the TSSK3 primary sequence revealed the presence of a structure reminiscent of the activation loop of protein kinases belonging to the AGC kinase family. In Chapter 2, we show the mechanism of regulation of TSSK3 activity by autophosphorylation and PDK1 phospho-rylation in this activation loop. We show *in vitro* that TSSK3 activity is regulated by phosphorylation of two residues within the T-loop, Ser-166 and Thr-168. *In vitro* Ser-166 phosphorylation is due to autophospho-rylation. In this respect TSSK3 is not exceptional as several cases of T-loop autophosphorylation have been described already. For example, PKA isolated from *E. coli* autophosphorylates *in vitro* on Thr-197 located in the T-loop. However in cells Thr-197 is phosphorylated not through autophosphorylation but by a heterologous kinase, which is likely to be PDK1 (12). Another example comprises the dual specificity kinase TESK1 (Testis-specific Protein Kinase 1) (13) with, similarly to TSSK3, an expression pattern limited to testis. For TESK1, as well as shown in Chapter 2 for TSSK3, the autopho-sphorylation of a serine residue located in the activation loop seems to be an important regulatory mechanism for the protein kinase

activity. Again this is only shown *in vitro* and *in vivo* autophosphorylation may not be involved. Interestingly Ser-166 of TSSK3 lies within a consensus for phosphorylation by ATM (LSQ (14)). As mentioned in the introduction loss of ATM results, amongst others abnormalities, in male infertility. Thus it would be interesting to test whether ATM functions *in vivo* as an upstream kinase for Ser-166 phosphorylation of TSSK3. In addition, ATM is responsive to genotoxic stress and we have shown that TSSK3 is at least in part also regulated by cellular stress. Albeit that we tested oxidative stress and thus far not genotoxic stress, it should be noted that genotoxic stress is often accompanied by a change in cellular redox. Taken together it seems worthwhile to test the aforementioned possibility that ATM is involved in TSSK3 regulation. Contrary to Ser-166, Thr-168 in TSSK3 activation loop can be phosphorylated *in vitro* by PDK1 and this results in TSSK3 activation. Phosphorylation by PDK1 is especially interesting given the recent identification of a testis and brain specific isoform of mouse PDK1, mPDK-1β (15). The authors suggest that this isoform may play an important role in regulating spermatogenesis. Thus an attractive possibility emerges that mPDK-1β may function in the regulation of TSSK3 activity. However, insulin treatment of cells which enhances PDK1 activity (16) resulted only in a weak activation of TSSK3. Several possible explanations for this observation can be put forward. First of all, it has been suggested that *in vivo* in cells PDK1 is a constitutively active kinase (17). PDK1 immunoprecipitated from either unstimulated or growth factor/insulin-stimulated cells, possesses similar -high- catalytic activity, suggesting that PDK1 activity is not directly altered by agonist stimulation (17). PDK1 is also a member of the AGC family of protein kinases and like all other AGC kinases, requires phosphorylation at its own T-loop residue (Ser-241) in order to be activated (18). The finding that bacterially expressed PDK1 is stoichiometrically phosphorylated at Ser-241 and fully active, indicates that PDK1 possesses the intrinsic ability to phosphorylate its own T-loop residue, perhaps explaining why it is constitutively active in mammalian cells. Thus, the ability of PDK1 to phosphorylate its substrates is most likely regulated through the regulation of PDK1 localization and thus by the ability of PDK1 to interact with its substrate in a spatially specific manner. In most cases this involves prior modification of the substrates (e.g. phosphorylation of the hydrophobic motif) in order to convert the substrate into forms that can readily interact and become phosphorylated by PDK1. The paradigm for this model is the activation of PKB by PDK1. Insulin induces the activation of PKB/Akt by inducing a transient increase in 3'-phosphorylated inositide lipids, thought to act as a recruitment signal for PDK1 and PKB to the plasma membrane, where they may co-localize and it results in phosphorylation /activation of PKB/Akt (19). Consistent with this insulin treatment of cells resulted in weak activation of TSSK3 and pretreatment with LY294002 to inhibit insulin-induced PI-3K activation did not inhibit TSSK3 activation. Thus TSSK3 activation apparently does not require membrane localization of PDK1. In addition to this TSSK3 consists essentially of a kinase domain (20) and it does not contain the C-terminal hydrophobic motif present in most AGC kinases. Thus TSSK3 likely lacks a motif to enable efficient binding of PDK1. Thus if we assume that PDK1 indeed acts as an upstream kinase in cells it is well conceivable that other adaptors/effector(s) are necessary for maximum activation of TSSK3 by PDK1. This is similar to what has been suggested for PKCξ phosphorylation and activation by PDK1 (21). Thus we hypothesized that, in order to efficiently recruit PDK1 to TSSK3, co-factors or additional modifications of TSSK3 are

required. As we performed our experiments in fibroblast cell lines which do not express endogenous TSSK3, the above argument are likely to be even more relevant. We employed a yeast-two-hybrid strategy to find possible interacting proteins for TSSK3. We isolated RUSC2 as an interaction partner of TSSK3 and show that *in vivo* RUSC2 and TSSK3 indeed interact (Chapter 3). Unfortunately, co-expression of RUSC2 did not enhance insulin-induced activation of TSSK3 and we could not observe an interaction between PDK1 and RUSC2. Therefore, we have to conclude that RUSC2 is not the missing link between PDK1 and TSSK3. Of course the possibility remains open that although PDK1 can act as T-loop kinase for TSSK3 *in vitro*, *in vivo* another type of T-loop kinase is involved in TSSK3 regulation. An example of such an alternative T-loop kinase is LKB1 (21) which functions in the regulation of AMP-Kinase and AMPK-related kinases. To achieve maximal activation of AMPK by LKB1 the accessory proteins STRAD and MO25 subunits are essential. Bearing this example in mind it is again conceivable that TSSK3 is regulated by such a T-loop kinase that binds RUSC2 that in turn serves as a docking platform for these two kinases.

Clearly, further work needs to be carried out to characterize the mechanism of regulation and function of TSSK3. In future studies it will be important to define one or more physiological substrates for TSSK3. In Chapter 2, we identified the specific consensus motif -RRSSSY- phosphorylated by TSSK3. A natural substrate for TSSK3 has not been found yet. For the two other testis specific kinase family members TSSK1 and TSSK2 a substrate has been found and named TSKS, testis specific kinase substrate (2,22). Its amino acid sequence does not contain -RRSSSY- motif and this is consistent with the finding that TSSK3 does not phosphorylate TSKS (20). A database search revealed the existence of two human proteins that contain the exact –RRSSSY- motif: HBSC II (Sodium channel protein, brain II alpha subunit) (23) and CrkRS (Cell division cycle 2-related protein kinase 7) (24). Since HBSC II is reported to be expressed only in brain, CrkRS that is ubiquitously expressed may be a more likely candidate TSSK3 substrate. In tissues two primary CrkRS transcripts were recognized by northern blots using a probe covering domains I-V from CrkRS (24). Interestingly, an additional transcript of CrkRS was detected specifically in testis. We are currently investigating if in our model system CrkRS could be specifically phosphorylated by TSSK3 and therefore could be physiological TSSK3 substrate.

RUSC2 assembly model

Oxidative stress is caused by the exposure of cells to reactive oxygen species (ROS). Cells have evolved protective mechanisms including antioxidants that detoxify ROS. When ROS levels exceed the antioxidant capacity of a cell the oxidative cell response is stimulated, which results in the increased production of antioxidants and repair proteins. In Chapter 3 and addendum to Chapter 3 we investigated the oxidative stress activated signaling events that result in the activation of the JNK pathway and the assembly of several proteins: RUSC2, JNK, TSSK3 and H-Ras. Our study results in a hypothetical model of assembly of these proteins in response to increase in cellular oxidative stress (Fig.1).

The increase in cellular oxidative stress, in this case due to hydrogen peroxide treatment of cells, causes the activation of stress-responsive JNK pathway. Our results show that activation of JNK through phosphorylation, results in binding of JNK

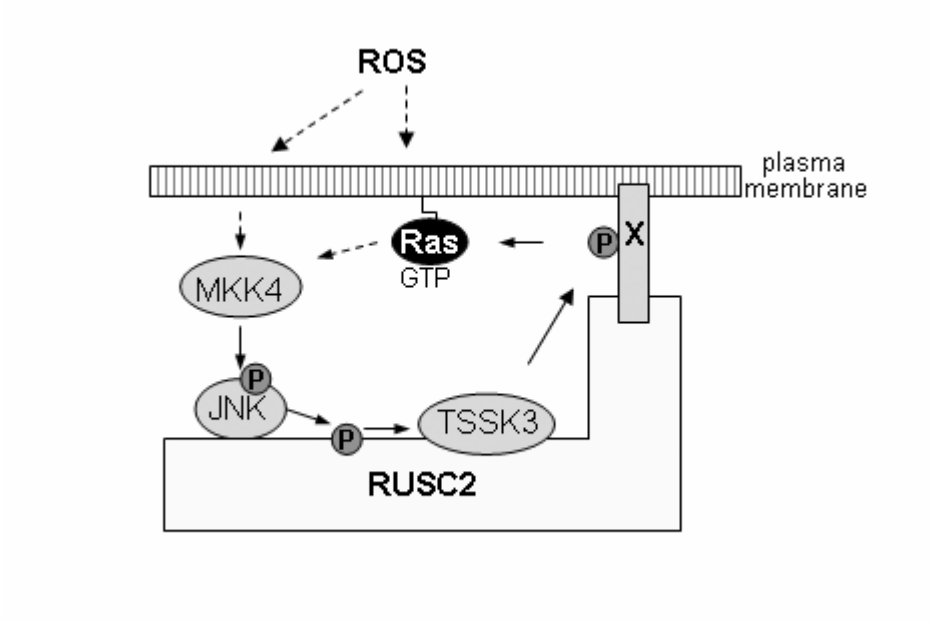


Fig. 1. RUSC assembly model in response to increased cellular oxidative stress.
In Chapter 3 and addendum to Chapter 3 we show the stress-dependent interactions of JNK, TSSK3 and H-Ras with RUSC2. This figure summarizes the above findings in a hypothetical model of subsequent signaling events that occur as a result of increase in cellular oxidative stress. Following increased cellular oxidative stress, MKK4 activation, mediated by Ras or other upstream proteins, results in JNK activation. Active JNK binds to RUSC2 and phosphorylates RUSC2. Phosphorylated RUSC2 in turn binds TSSK3 and JNK is subsequently released from RUSC2. RUSC2 (in its SH3 domain) binds also a specific TSSK3 substrate (X) that when phosphorylated by TSSK3 is recruited to the plasma membrane where it serves as a regulator of Ras activity. Based on our experiments thus far we consider it the most likely possibility, that GTP-bound Ras forms a complex with X-RUSC2 and that this interaction either stabilizes RasGTP and thus increases the duration of Ras signaling or that it serves to downregulate Ras activity, whereby RasGDP is then released from the complex.
The locations of proteins on RUSC2 are strictly schematic, since the interaction domains for JNK and TSSK3 has not been specified yet.

to RUSC2 and its consequent phosphorylation by JNK. The JNK protein kinases are activated by dual phosphorylation on Tyr and Thr by MAPKK, MKK4 and MKK7 (25). Since the MKK7 protein is primarily activated by cytokines and MKK4 is primarily activated by environmental stress, we tested the inhibition of JNK pathway with MKK4 dominant negative mutant and observed the severe reduction in binding of JNK to RUSC2 after treatment of cells with H_2O_2. Thus MKK4 is likely to be involved in the activation of JNK-RUSC2 interaction. Several Ras family GTPases have been implicated in the regulation of JNK activation, including Rac, Ras and Ral (26). We demonstrated that H_2O_2 induced interaction between RUSC2 and JNK3 was severely reduced in the presence of RasN17 (GDP-bound inactive mutant) suggesting that Ras acts upstream of JNK in the regulation of JNK activity and consequently in the interaction between JNK and RUSC2. It cannot be ruled out that stress activated MKK4-JNK-RUSC2 pathway is also regulated by other upstream proteins. A good candidate is for example apoptosis signal-regulating kinase (ASK1), an upstream

activator of JNK and p38 that is activated due to increase in oxidative stress (27,28). ASK1 dependent phosphorylation was shown to be necessary for recruitment and thereby activation of JNK3 in response to H_2O_2 (29).

Our results also indicate what the possible events are downstream of JNK-RUSC2 assembly. We observed H_2O_2 induced binding of TSSK3 to RUSC2, independent of TSSK3 activity and inhibition of JNK activation prevents binding of TSSK3 following H_2O_2 treatment of cells. In contrast, kinase-dead TSSK3 does not prevent binding of JNK to RUSC2 induced by H_2O_2. Moreover, TSSK3 appears in the complex with RUSC2 and JNK showing that binding of JNK and TSSK3 to RUSC2 is not mutually exclusive and indicating that JNK and TSSK3 bind to separate domains of RUSC2. Therefore, we propose that active JNK binds to RUSC2 and phosphorylates RUSC2. Phosphorylated RUSC2 in turn binds TSSK3 and JNK is subsequently released from RUSC2. An interesting finding contributing to this model is that active GTP-bound H-Ras is able to bind to RUSC2, while its inactive GDP-bound form is not capable of this interaction. Furthermore, this interaction seems to be indirect since we demonstrate that only SH3 domain containing part of RUSC2 binds to H-Ras, and SH3 domains interact with proline-rich domains that are absent in Ras. There is likely possibility that RUSC2 binds in addition to JNK and TSSK3, a specific TSSK3 substrate (X protein) probably containing a proline-rich region. Phosphorylation by TSSK3 may serve two functions. First, it may stabilize binding of X to RUSC2. Second, it may serve to promote plasma membrane docking and thereby indirectly promote binding to Ras. Third, it may act as a direct signal for Ras binding. Although we observe binding of RasV12 and not RasN17 to RUSC2, we cannot conclude that RUSC2 only binds the activated form of Ras since RasV12 and not RasN17 activates JNK. Thus at present it remains possible that binding of RUSC2 to Ras may function in a positive feed-forward mechanism, or a negative feed-back mechanism. To discriminate between these possibilities Ras activation should be measured in the presence and absence of RUSC2. Alternatively, our observation of a strong induction of wild type H-Ras binding to RUSC2 after treatment of cells with H_2O_2 and a clear reduction in H-Ras binding in the presence of dominant-negative MKK4 may also suggest that RUSC2 binding solely stabilizes RasGTP rather then to participate in a Ras activation/inhibition mechanism. There are still several important questions to be answered. First of all, what is the X protein that fits our model. It should be a physiological substrate for TSSK3 that contains proline-rich region able to bind to SH3 domain of RUSC2, and supposedly also contain a Ras-binding domain. In Chapter 2 we described the identification of the specific consensus motif -RRSSSY- phosphorylated by TSSK3. As we mentioned above the protein containing such a motif is CrkRS that interestingly also harbors extensive proline-rich regions, consensus binding sites for SH3 and WW domain binding (24). Thus apart from testing if CrkRS can be phosphorylated by TSSK3 it would be very attractive to test the possible interaction of CrkRS with RUSC2 in response to oxidative stress. Unfortunately, subcellular fractionation and immunofluorescence studies show that CrkRS is a nuclear protein (24). In that case one would predict that assembly of the RUSC2 would occur at the nuclear membrane rather then the plasma membrane. Although this possibility can not be ruled out at present we do not consider this a very likely possibility. Alternatively, another protein can serve as an X protein, for example a NORE-1 family member which contains a proline-rich region and a Ras-binding domain, shown to directly and specifically interact with Ras in a GTP-

dependent manner (30,31). An exact consensus sequence for TSSK3 phosphorylation is not present in this protein family, but similar sequences are present so the possibility that a NORE-1 family member is a TSSK3 substrate remains open.

We have to bear in mind that TSSK3 is expressed exclusively in testis, therefore it is of interest to understand how the above complex may function in testis. It is noteworthy that we observed that H_2O_2 stimulation of the cells leads to the transient interaction of RUSC2 with JNK1 and JNK3. Compared to JNK1 and JNK2, JNK3 has a limited pattern of expression, but importantly is expressed in testis (32). Thus in testis JNK3 rather then JNK1 and JNK2, is likely to be involved in the regulation of TSSK3 and RUSC2 serves a scaffold for these proteins providing specificity in downstream JNK signaling rather then in JNK activation itself. It may therefore be of interest to analyze JNK3-/- mice in this respect in more detail and the creation of TSSK3-/- mice seems desirable to analyze stress response events in our model. Finally, it should be noted that the temperature of the testis is on the average 5^{o}C lower compared to body temperature. So it will be of interest to test whether heat- or cold-shock will impact TSSK3-RUSC2 assembly in a manner similar to oxidative stress. This because heat- or cold-shock will likely to be the most physiological type of stress encountered within the testis. Many of the above mentioned possibilities are worthwhile testing but the exact role of this interaction in the context of testis function remains to be elucidated.

TSSK2, a candidate gene for molecular marker for genetic diagnosis of male infertility?

Recent studies demonstrate that there is a vast number of genes expressed specifically by male germ cells late in development (33). This group provides the potential targets for germ-cell directed contraception and the candidate proteins that could be critical for fertilization. Therefore an attractive idea is to search among these genes for single nucleotide polymorphisms (SNP) or mutations that could serve as molecular markers for genetic diagnosis of male infertility. Because proteins involved specifically in fertilization are likely to be expressed late in spermatogenesis, TSSK1 and TSSK2 protein kinases whose expression is limited to meiotic and postmeiotic spermatogenic cells, respectively (34) (22) appeared good candidates to search for possible genetic changes that could result in spermatogenic failure. In addition these genes do not contain introns so can be sequenced relatively easily. In Chapter 4 we analyzed (by direct sequencing) the sequences of human genes coding for TSSK1 and TSSK 2 protein kinases. The genomic DNA from 120 patients with different spermatogenic defects resulting in infertility served as matrix. Beside synonymous SNPs, that cause no change in amino acids, we identified a SNP in *TSSK2* sequence, located in the C-terminal part of the protein, outside the kinase domain that changes the threonine residue to methionine (T280M). Interestingly, we did not find, in the tested group, any SNPs in *TSSK1* sequence what may reflect the differences in the amino acid sequence of the C-terminal part of both kinases (2) and suggests that T280M change is TSSK2 specific.

In general, studying SNP profiles may reveal relevant genes associated with a disease. Association study can detect and indicate which pattern is most likely associated with

the disease-causing genes. Thus, in our future study it would be important to analyze the SNP pattern for *TSSK2* in statistically important group of proven-fertile men to be able to compare this pattern with a group of infertile men analyzed in this study and draw conclusions about possible implication of T280M mutation for spermatogenic failure causing male infertility. Moreover, it would be interesting to analyze the consequences of T280M mutation for biochemical properties of TSSK2 kinase. Since TSSK2 is a putative protein kinase that contains a potential T-loop necessary for activation within the kinase domain, thus it is likely to be regulated by for example an upstream acting kinase(s) in the C-terminal regulatory domain. *In vitro* kinase assay studies of T280M mutated recombinant TSSK2 with its physiological substrate TSKS (2,22) would reveal if this mutation changes the activity (or even causes a loss of function) of TSSK2 towards its substrate. Also, studies in the cell systems with TSSK2 T280M mammalian expression constructs could disclose if the proper regulation of TSSK2 is impaired due to this change. Finally, the creation of transgenic animals could be a solution to validate (or contradict) the involvement of TSSK2 in spermatogenesis.

In conclusion, the data described in this thesis contribute to the better characterization of protein kinases from TSSK family. Novel information is presented on several important aspects. The mode of regulation of TSSK3 by autophosphorylation and PDK1-like phosphorylation is provided. Secondly, the assembly model of a novel protein complex: TSSK3, RUSC2, JNK, and H-Ras under the conditions of cellular oxidative stress is described. This part in addition discloses a novel scaffold protein, RUSC2 involved in JNK stress signaling and also suggests a possible involvement of testis specific kinase, TSSK3. Finally, a candidate gene (*TSSK2*) is proposed as a novel molecular marker for genetic diagnosis of male infertility.

REFERENCES

1. Shima, J. E., McLean, D. J., McCarrey, J. R., and Griswold, M. D. (2004) *Biol Reprod* **71**, 319-330
2. Hao, Z., Jha, K. N., Kim, Y. H., Vemuganti, S., Westbrook, V. A., Chertihin, O., Markgraf, K., Flickinger, C. J., Coppola, M., Herr, J. C., and Visconti, P. E. (2004) *Mol Hum Reprod* **10**, 433-444
3. Brazil, D. P., and Hemmings, B. A. (2001) *Trends Biochem Sci* **26**, 657-664
4. Avruch, J., Belham, C., Weng, Q., Hara, K., and Yonezawa, K. (2001) *Prog Mol Subcell Biol* **26**, 115-154
5. Lang, F., and Cohen, P. (2001) *Sci STKE* **2001**, RE17
6. Dutil, E. M., Toker, A., and Newton, A. C. (1998) *Curr Biol* **8**, 1366-1375
7. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *Embo J* **15**, 6541-6551
8. Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E., and Thomas, G. (1995) *Embo J* **14**, 5279-5287
9. Kobayashi, T., and Cohen, P. (1999) *Biochem J* **339 (Pt 2)**, 319-328
10. Kobayashi, T., Deak, M., Morrice, N., and Cohen, P. (1999) *Biochem J* **344 Pt 1**, 189-197
11. Park, J., Leong, M. L., Buse, P., Maiyar, A. C., Firestone, G. L., and Hemmings, B. A. (1999) *Embo J* **18**, 3024-3033
12. Moore, M. J., Kanter, J. R., Jones, K. C., and Taylor, S. S. (2002) *J Biol Chem* **277**, 47878-47884
13. Toshima, J., Tanaka, T., and Mizuno, K. (1999) *J Biol Chem* **274**, 12171-12176

14. Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999) *J Biol Chem* **274**, 37538-37543
15. Dong, L. Q., Ramos, F. J., Wick, M. J., Lim, M. A., Guo, Z., Strong, R., Richardson, A., and Liu, F. (2002) *Biochem Biophys Res Commun* **294**, 136-144
16. Chen, H., Nystrom, F. H., Dong, L. Q., Li, Y., Song, S., Liu, F., and Quon, M. J. (2001) *Biochemistry* **40**, 11851-11859
17. Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A., and Bownes, M. (1997) *Curr Biol* **7**, 776-789
18. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) *Biochem J* **342 (Pt 2)**, 287-292
19. Anderson, K. E., Coadwell, J., Stephens, L. R., and Hawkins, P. T. (1998) *Curr Biol* **8**, 684-691
20. Zuercher, G., Rohrbach, V., Andres, A. C., and Ziemiecki, A. (2000) *Mech Dev* **93**, 175-177
21. Dong, L. Q., Zhang, R. B., Langlais, P., He, H., Clark, M., Zhu, L., and Liu, F. (1999) *J Biol Chem* **274**, 8117-8122
22. Kueng, P., Nikolova, Z., Djonov, V., Hemphill, A., Rohrbach, V., Boehlen, D., Zuercher, G., Andres, A. C., and Ziemiecki, A. (1997) *J Cell Biol* **139**, 1851-1859
23. Lu, C. M., Han, J., Rado, T. A., and Brown, G. B. (1992) *FEBS Lett* **303**, 53-58
24. Ko, T. K., Kelly, E., and Pines, J. (2001) *J Cell Sci* **114**, 2591-2603
25. Davis, R. J. (2000) *Cell* **103**, 239-252
26. Coso, O. A., Teramoto, H., Simonds, W. F., and Gutkind, J. S. (1996) *J Biol Chem* **271**, 3963-3966
27. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) *Science* **275**, 90-94
28. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *Embo J* **17**, 2596-2606
29. Matsuura, H., Nishitoh, H., Takeda, K., Matsuzawa, A., Amagasa, T., Ito, M., Yoshioka, K., and Ichijo, H. (2002) *J Biol Chem* **277**, 40703-40709
30. Praskova, M., Khoklatchev, A., Ortiz-Vega, S., and Avruch, J. (2004) *Biochem J* **381**, 453-462
31. Vavvas, D., Li, X., Avruch, J., and Zhang, X. F. (1998) *J Biol Chem* **273**, 5439-5442
32. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) *Embo J* **15**, 2760-2770
33. Schultz, N., Hamra, F. K., and Garbers, D. L. (2003) *Proc Natl Acad Sci U S A* **100**, 12201-12206
34. Nayak, S., Galili, N., and Buck, C. A. (1998) *Mech Dev* **74**, 171-174

SUMMARY

The phosphorylation of proteins is one of the most important post-translational modifications found in the living cells and is involved in the control, either directly or indirectly, of all processes occurring in the cell. The enzymes responsible for protein phosphorylation are the protein kinases that catalyze phosphoryl transfer from ATP to proteins. A major challenge in the signal transduction field is to define sequence, structural and mechanistic features responsible for the substrate selectivity, regulation and cellular function of individual protein kinases. We took part in this challenge and studied the family of Testis Specific Serine-threonine Kinases (TSSK) that comprises four members up to date. These kinases are expressed exclusively in testis during spermatogenesis and therefore they are the potential candidates that could be critical for fertilization. The main aim of this thesis was to give more insight into the biochemical properties and possible function(s) of TSSK3. **Chapter 2** describes the analysis of biochemical properties, substrate specificity and *in vitro* activation of one of TSSK family members, TSSK3. *In vitro* TSSK3 exhibited the ability to autophosphorylate and to phosphorylate test substrates providing the evidence that TSSK3 is a genuine kinase. Sequence comparison indicated the existence within the TSSK3 catalytic domain of a so-called 'T-loop' a structure present in the AGC-family of protein kinases. To test if this T-loop is engaged in TSSK3 regulation, we mutated the critical threonine within the T-loop to alanine (T168A) and this resulted in an inactive TSSK3 kinase. Furthermore *in vitro* Thr-168 is phosphorylated by the T-loop kinase phosphoinositide-dependent protein kinase-1 (PDK1) and phosphorylation by PDK1 increased *in vitro* TSSK3 kinase activity, suggesting that TSSK3 can be regulated in the same way as AGC-kinase family members. Furthermore, analysis of a range of peptide sequences defines the peptide sequence, RRSSSY, as an efficient and specific substrate for TSSK3. In **Chapter 3**, we searched for TSSK3 potential regulatory interacting proteins by a yeast two-hybrid approach. We identified, a novel, widely expressed protein, RUSC2 and we showed that it binds to but is not a substrate for TSSK3. We also observed it to be phosphorylated by Jun NH_2-terminal kinase (JNK). Next, we demonstrated that RUSC2 transiently interacts with JNK kinases after oxidative stress treatment and that TSSK3 appears in this stress-induced complex as well. We illustrated that the interaction between JNK and RUSC2 is not dependent on the kinase interaction motifs (KIMs) present in RUSC2 protein sequence. Furthermore we showed that this interaction is abolished by inhibitory mutant of MKK4. In this chapter we pointed out the possible connection between RUSC2, TSSK3 and oxidative stress induced JNK signaling pathway. In the **Addendum** to Chapter 3, the experiments aiming to reveal function of RUSC2 are continued and they provide another RUSC2 interacting protein, the small GTPase H-Ras, that when it is in active GTP-bound conformation binds to RUSC2. **Chapter 4** describes our search for mutations in two other genes of the TSSK family, TSSK1 and 2, in patients with infertility. Since these proteins are expressed exclusively in testis, in meiotic and postmeiotic spermatogenic cells, and they have simple DNA structure (intronless), and thus easy to analyze, they are good candidates to study the potential occurrence of mutations in DNA

from infertile patients. We found, single nucleotide polymorphism (SNP) in the *TSSK2* gene, which changed threonine residue, located in the C-terminal regulatory domain of *TSSK2*, to methionine (T280M) that may be correlated with spermatogenic failure. Thus, *TSSK2* might be a candidate gene for molecular marker for genetic diagnosis of male infertility. In **Chapter 5**, a model for RUSC2 assembly with TSSK3, JNK and H-Ras is proposed and the possible directions of future studies on TSSK family and their role in signal transduction are discussed.

SAMENVATTING

Fosforylering van eiwitten is een van de meest belangrijke post-translationele modificaties die in levende cellen wordt gevonden en is betrokken bij de controle van zowel direkte als indirekte processen die in de cel plaatsvinden. De verantwoordelijke enzymen voor deze fosforyleringen zijn eiwit kinasen die de fosforyl transfer van ATP naar eiwitten katalyzeert. Een belangrijke uitdaging in het signaal transductie onderzoek is om de structurele en mechanistische kenmerken die verantwoordelijk zijn voor substraat selectiviteit, regulatie en cellulaire funktie van individuele eiwit kinasen te ontrafelen. Om deze vraagstellingen te beantwoorden hebben we een familie van Testis Specifieke Serine-threonine Kinasen (TSSK), die bestaan uit 4 leden, bestudeerd. Deze kinasen worden exclusief in de testis tot expressie gebracht gedurende spermatogenese en zijn daardoor potentieel belangrijk voor de vruchtbaarheid. Het voorname doel van dit proefschrift is om meer inzicht te krijgen in de biochemische eigenschappen en de mogelijke functies van TSSK3. **Hoofdstuk 2** beschrijft de biochemische eigenschappen, substraat specificiteit en *in vitro* aktivatie van een van de TSSK kinasen, TSSK3. In *in vitro* experimenten hebben we laten zien dat TSSK3 kon autofosforyleren en testsubstraten kon fosforyleren en daarmee een echte kinase is. Sequentie vergelijkingen wijzen erop dat in het katalytische domein van TSSK3 een zogeheten “T-loop”, een structuur specifiek voor de familie van AGC kinasen, aanwezig is. Om te testen of deze loop daadwerkelijk van belang is voor de regulatie van TSSK3, hebben we een alanine mutatie in deze T-loop aangebracht op positie 168 en dit resulteerde in een inactief kinase. Verder laten we zien dat *in vitro*, deze positie wordt gefosforyleerd door de T-loop kinase PDK1 (phosphoinositide-dependent protein kinase) en dat deze fosforylering ertoe leidde dat de *in vitro* kinase aktiviteit verhoogd wordt. Dit suggereert dat TSSK3 op dezelfde manier gereguleerd kan worden als andere AGC-kinase familie leden. Daarnaast bleek uit analyse van een reeks peptide sequenties dat de peptide sequentie RRSSSY een efficiënt en specifiek substraat is voor TSSK3.

In **hoofdstuk 3** hebben we gezocht naar potentiële interacterende eiwitten door middel van een Yeast Two-Hybrid techniek. We vonden een nieuw eiwit, genaamd RUSC2, dat breed tot expressie komt. We laten zien dat RUSC2 bindt aan TSSK3, maar dat het geen substraat is. We zagen tevens dat RUSC2 een transiënte interactie heeft met JNK kinasen na behandeling met oxidatieve stress en dat TSSK3 in dit stress-geïnduceerde komplex zit. We laten zien dat de interactie tussen JNK en RUSC niet afhankelijk is van kinase interactie motieven (KIMs) die aanwezig zijn in de eiwitsequentie van RUSC. We laten tevens zien dat de interactie verstoord wordt door de inhibitoire MKK4 mutant. Samenvattend laten we in dit hoofdstuk de mogelijke connectie tussen RUSK, TSSK3 en oxidatieve stress-geïnduceerde JNK activatie zien. In het **addendum van hoofdstuk 3** hebben we op zoek naar de funktie van dit eiwit gekeken naar een ander interacterend eiwit, de kleine GTPase H-Ras, die in zijn actieve GTP-gebonden conformatie aan RUSC2 bindt.

Hoofdstuk 4 beschrijft ons onderzoek naar mutaties in twee andere genen van de TSSK familie, TSSK1 en TSSK2, in onvruchtbare patiënten. Aangezien deze eiwitten uitsluitend geproduceerd worden in de testis in meiotische en post-meiotische spermatogene cellen, een eenvoudige intronloze DNA struktuur hebben, en daardoor makkelijk te

analyzeren zijn, zijn ze goede kandidaten om de mogelijke aanwezigheid van mutaties in onvruchtbare patienten te bestuderen. We hebben een single nucleotide polymorphism (SNP) in het *TSSK2* gen met een verandering van een treonine naar een metionine in het C-terminale regulatoire domain van *TSSK2* gevonden, die mogelijk gecorreleerd is met onvruchtbaarheid in mannen.

In **hoofdstuk 5** beschrijven we een model voor de binding van RUSC2 met TSSK3, JNK en Ha-Ras en geven we een aantal richtingen voor verder onderzoek aan.

Kindly translated by Arjan Brenkman

STRESZCZENIE W JĘZYKU POLSKIM
(DLA LAIKÓW)

Podstawowym budulcem, z jakiego składają się komórki są białka. Fosforylacja białek jest jedną z najważniejszych modyfikacji, jakie zachodzą w komórkach istot żywych i jest zaangażowana, pośrednio bądź bezpośrednio we wszystkie procesy w komórkach. Enzymy odpowiedzialne za proces fosforylacji białek to kinazy białkowe, które katalizują przeniesienie reszt fosforanowych z ATP na białko. Tylko trzy aminokwasy (elementy składowe każdego białka) mogą podlegać tej modyfikacji i są to seryna, treonina i tyrozyna. Szacuje się, że aż 25% wszystkich białek w komórce jest fosforylowanych w czasie procesów metabolicznych. Uszkodzenia (mutacje) w genach kodujących kinazy w wielu przypadkach prowadzą do chorób m. in.: nowotworów, miażdżycy, niedoborów immunologicznych, chorób zapalnych, a także zaburzeń płodności. Podstawowym wyzwaniem na polu przekaźnictwa sygnału w komórce jest poznanie tych enzymów, ich właściwości, które decydują o wyborze substratu, regulacji aktywności i funkcji poszczególnych kinaz białkowych. Głównym tematem tej pracy jest rodzina kinaz białkowych nazwanych Jądrowo-Specyficzne Kinazy Serynowe w której skład wchodzą 4 białka. Kinazy te funkcjonują tylko w jądrach męskich podczas procesu powstawania plemników i dlatego są potencjalnymi kandydatami mogącymi mieć znaczenie dla płodności mężczyzn. Głównym celem tej pracy jest charakterystyka właściwości biochemicznych i poznanie ewentualnych funkcji jednego z przedstawicieli tych kinaz: TSSK3 (od angielskiego zwrotu Tesits Specific Serine-Threonine Kinase 3).

W **Rozdziale 2** analizowane są właściwości biochemiczne oraz specyficzność substratowa enzymu TSSK3. W modelu *in vitro* (w probówce, poza organizmem żywym czy też kulturą komórkową), TSSK3 wykazuje zdolność do autofosforylacji (fosforylacji samej siebie) oraz do fosforylacji tak zwanych substratów testowych, co dowodzi, że rzeczywiście TSSK3 posiada właściwości kinazy białkowej. Analizy sekwencji aminokwasowej wykazały, iż TSSK3 w swojej sekwencji posiada tak zwaną pętlę aktywacyjną charakterystyczną dla kinaz z rodziny AGC odpowiedzialną za regulację aktywności tych kinaz. Aby zbadać czy pętla ta jest powiązana również z regulacją aktywności TSSK3, kluczowy

aminokwas w pętli aktywacyjnej, treonina nr 168 (Thr-168), został podmieniony na inny aminokwas, alaninę co spowodowało całkowitą utratę aktywności enzymatycznej TSSK3. Dodatkowo, wykazaliśmy, iż ta sama treonina 168 jest fosforylowana przez inną kinazę białkową, o nazwie PDK-1, pełniącą ważną funkcję w przekaźnictwie sygnału w komórce. Fosforylacja ta powoduje znaczny wzrost aktywności enzymatycznej TSSK3. Nasze wyniki sugerują, że aktywność enzymatyczna TSSK3 może być regulowana w ten sam sposób, co aktywność przedstawicieli rodziny kinaz białkowych AGC znacznie lepiej poznanych i opisanych. Ponieważ naturalny substrat dla TSSK3 nie został jeszcze zidentyfikowany podjęliśmy próby identyfikacji przynajmniej krótkich sekwencji aminokwasowych, które mogą być specyficznie fosforylowane przez TSSK3 i znaleźliśmy sekwencję, RRSSSY, która może służyć za wydajny i specyficzny substrat dla TSSK3.

W **Rozdziale 3** badania zostały rozszerzone o poszukiwanie potencjalnych białek oddziałujących z TSSK3 i przez to pełniących funkcję naturalnych regulatorów dla TSSK3. W tym celu wykorzystaliśmy system oparty na drożdżach piekarskich i z kilu milionów białek wyizolowaliśmy, specyficznie oddziałujące z TSSK3 białko nazwane RUSC2. Zademonstrowaliśmy, że RUSC2 oddziałuje z TSSK3, ale nie jest przez nie fosforylowane (nie jest substratem dla TSSK3). Co ciekawe RUSC2 okazało się substratem i białkiem oddziałującym również z inną kinazą białkową JNK. Kinaza JNK jest bardzo ważnym białkiem związanym z odpowiedzią organizmu na stres oksydatywny, czyli na zwiększenie obecności szkodliwych pochodnych tlenu (wolnych rodników) w komórce. Wolne rodniki są związkami tlenu obecnie uznawanymi za jedną z przyczyn procesu starzenia się. Po wywołaniu stresu oksydatywnego w komórkach, znacznie wzmacnia się wiązanie RUSC2 do JNK, a także doprowadza to do pojawienia się TSSK3 w tym kompleksie białek. Natomiast, gdy w naszym modelu sztucznie zablokujemy aktywację kinazy JNK, (która w naturze jest aktywowana między innymi przez stres oksydatywny) to powyższy kompleks nie może powstać. Zatem aktywność enzymatyczna JNK jest niezbędna, aby pozostałe białka, TSSK3 i RUSC2 mogły ze sobą oddziaływać, co zapewne dzieje się w określonym celu, dla nas jeszcze niejasnym. Ponieważ zaangażowana jest w to kinaza białkowa obecna tylko w jądrach męskich (TSSK3), przypuszczamy, że tworzenie

kompleksu pomiędzy RUSC2, TSSK3 i JNK może mieć znaczenie w procesach odpowiedzialnych za płodność.

Rozdział 4 opisuje poszukiwanie mutacji w dwóch pozostałych genach z rodziny TSSK, TSSK1 i TSSK2 u bezpłodnych mężczyzn. Ponieważ geny te kodują białka występujące tylko w jądrach męskich, w czasie procesu podziału komórek z których powstają plemniki, a także mają prostą, łatwą do analizy strukturę DNA, są bardzo dobrymi kandydatami do poszukiwania mutacji (zmian w DNA) prowadzących do bezpłodności u mężczyzn. W genie kodującym TSSK2 znaleźliśmy jedną, powtarzającą się u kilku pacjentów mutację, która zmienia aminokwas treoninę (która znajduje się w części regulacyjnej białka i może być potencjalnie fosforylowana) na inny aminokwas, metioninę. Sądzimy, iż ta mutacja może mieć związek z defektami w produkcji plemników u badanych pacjentów i w związku z tym TSSK2 mogłoby służyć jako genetyczny marker w diagnostyce bezpłodności u mężczyzn.

W ostatnim, podsumowującym **Rozdziale 5** został zaproponowany model oddziaływań wspomnianych powyżej białek RUSC2, TSSK3, JNK oraz H-Ras który powstał na podstawie badań prezentowanych w tej pracy. Dyskutowane są również kierunki dalszych badań nad rodziną TSSK i jej rolą w przekaźnictwie sygnału w komórkach organizmów żywych.

Curriculum vitae

Marta Bućko-Justyna was born on the 5th of October 1975 in Warsaw in Poland. She obtained her secondary school diploma in June 1994. In October the same year she entered the Warsaw University to study chemistry, but after one year of study she changed the faculty within the same University to biology. In February 1999 she got an Erasmus-Socrates scholarship and for six months she joined the group of Prof. Leslie A. Grivell in the Institute of Molecular Cell Biology (BioCentrum) at the University of Amsterdam. The research done in this period was the basis for her Master Thesis entitled "The role of the Leigh Disease associated gene, *SURF-1*, in the biosynthesis of cytochrome *c* oxidase, using yeast homologue *SHY1* as a model" written under the supervision of Prof. Ewa Bartnik from the Department of Genetics at the Warsaw University. On 1st of March 2000 she obtained her MSc degree in Molecular Biology. From May 2000 till April 2005 she worked as graduate student under the supervision of Prof. Maciej Żylicz in the Department of Molecular Biology in the International Institute of Molecular and Cell Biology in Warsaw and Prof. Boudewijn M. Th. Burgering in Department of Physiological Chemistry, Center for Biomedical Genetics, University Medical Center Utrecht. Research performed during that period was focused on Testis Specific Serine-Threonine Kinases and is described in this PhD Thesis.

List of publications

Nijtmans LG, Artal Sanz M, **Bucko M**, Farhoud MH, Feenstra M, Hakkaart GA, Zeviani M, Grivell LA.
Shy1p occurs in a high molecular weight complex and is required for efficient assembly of cytochrome c oxidase in yeast. FEBS Lett. 2001 Jun 1;498(1):46-51

Bucko-Justyna M, Lipinski L, Burgering BMT, Trzeciak L
Characterization of Testis Specific Serine-threonine Kinase 3 and its activation by Phosphoinositide-Dependent Kinase -1-dependent signaling. (2005) *Submitted*

Bucko-Justyna M, Trzeciak L, Lipinski L, Burgering BMT
A novel TSSK3-interacting protein, RUSC2 is a scaffolding protein and a substrate for c- Jun N-terminal kinase (JNK). (2005) *Submitted*

ACKNOWLEDGEMENTS, PODZIĘKOWANIA

And finally we come to the nicest and the most interesting part of this book. The acknowledgements. There are many people who contributed to my thesis and many events that influenced my work during the last few years. I would like at least to mention them here.

The Dutch Story

First of all, the person without whom this thesis would never come to life, prof. Boudewijn Burgering. I am so grateful that you appeared on my scientific way, Boudewijn. You are the person who knew how to make me believe in myself. Every time I was working in your lab I was getting so much energy and good spirit that I almost could move the mountains. Thank you for showing me how much fun one can have while "doing" science and for appreciating my (sometimes desperate) efforts to be a good scientist. Prof. Bos, Hans thank you for taking me originally under your wings, thanks to you I could start the cooperation with your lab, learn so much and meet all these extraordinary people. The atmosphere in the lab in Utrecht was so great thanks to people who work there and who made me laugh every day. Everyone was so helpful that very fast I could integrate with the lab and I was feeling like at second home. I couldn't wait till my returns to Utrecht. Marta R. my paranimf, thank you for your help with all the stuff before the defense and of course for "crazy dancing" and very nice company at Spetses and in Utrecht. Jurgen, your sense of humor always makes me fall on my knees and remember, "I am still waiting for the stimulation". Armando, you were always so helpful that I still feel guilty that I was using you but at least I know that. Thank you very much for taking care for me whenever I needed it and a nice word every rainy and sunny day. I will never forget your "stamppot". Pieter there is probably no way for you to learn how to pronounce my name but I enjoyed playing tennis with you and your company in the lab. Marike it was a pleasure to meet you and to talk to you every now and then, thanks for introducing me to the lab on my first, difficult days. Lidia, thank you for being there and for help with experiments, and Miranda thank you for bringing me good

news on Fridays after developing Lydia's blots. Arjan, you always believed in me, it kept me alive even when you locked my jacket in the office overnight in the wintertime. Bea, you had a hard life with me on Spetses! but you stayed friendly and always helpful, thank you for that. Paulien, thank you for help with the sequencing and very fruitful tennis meetings. Holger, you always could find time to talk to me and serve with your advice, I am grateful for that. Ingrid, the small lab chats with you in the morning made my day to start nicely. Oh, I would forget "prettig avond". Sanne, thanks for the constructs, good advice and interest in my work. Jorrit, Joost and Roland, you were my first office mates. Thanks to your company I learned that if I survive your jokes guys, I can survive everything and everyone!!! Jorrit, I really don't want to use your towel. Taisa, szkoda że w pracy nigdy nie miałam zbyt dużo czasu żeby spokojnie pogadać. Mimo to, sama Twoja obecność, pięknej, polskiej dziewczyny dodawała mi otuchy. Aniu, mam nadzieję, że Twoje życie na obczyźnie potoczy się tak jak sobie zamarzyłaś i że będziesz szczęśliwa, pomimo, że czasem klonowanie nie wychodzi. Lars, thanks to Anna I got to know you better and I am happy for that. Good luck with your thesis and future scientific life. Don't forget to visit us in Warsaw. Mike and June, the Chinese ones in jungle of Dutch. I keep fingers for both of you and "dziękuję" and "proszę". I wreszcie moi ukochani koledzy, z polskiej części Holandii, Monika i Marcin. Nie znajduję nawet słów jak Wam podziękować za nieustanną opiekę nade mną, niezliczone spotkania, nocne Polaków rozmowy i pomoc w każdej, najdrobniejszej sprawie. Dzięki Wam nie musiałam przebijać głową muru, który Wy juz wielokrotnie przebiliście. Bardzo sie cieszę, że teraz mieszkacie w naszej stolicy i spotykamy sie tak często. Edyto, Twoja obecność w Utrechcie dodała kolorytu wszystkim wydarzeniom, które razem przeszliśmy, dziękuję Ci bardzo za to i życzę poukładania wszystkiego jak tylko sobie zapragniesz. Wojtku, Twój spokój i optymizm działal jak balsam w każdej sytuacji. Bardzo się ciesze, że Cię poznałam. My Italian friends, Francesca and Francesco, my life in Utrecht wouldn't be the same without you. Thank you for living together, creating a specific, warm and crazy atmosphere and teaching me to cook lots of tasty dishes. Manuel, I hope we will never loose contact, meeting you was a very special experience in my life. I would also

like to thank all those who I accidentally forgot to mention and who for sure had an influence on my life and work during last few years.

The Polish Story/Część Polska

Na początku chciałabym podziękować mojemu szefowi Prof. Maciejowi Żyliczowi, który się mną opiekował przez te wszystkie lata i na którego mogłam liczyć w każdej sprawie. Dziękuję również prof. Alicji Żylicz za cenne rady w kwestiach technicznych i nie tylko....
Wszyscy z którymi pracowałam w zespole w Warszawie mieli swój udział w powstaniu mojego doktoratu, począwszy od Lecha T., który był moim doradcą i swego rodzaju mentorem, poprzez Leszka, który pracował ze mną w projekcie i poświęcił swoje zdrowie psychiczne abym ja mogła napisać doktorat :) a skończywszy na pani Wandzie, która zawsze dbała aby wszystko było przygotowane do pracy i na swoim miejscu tak jak lubię ☺. Grzesiu, nasz mistrzu, życzę ci powodzenia w Hameryce i nie zapominaj o prawdziwym życiu, które się toczy poza pracą. Olu, dziękuję Ci za pogodny uśmiech na początek dnia i za to że zawsze znajdowałaś czas żeby porozmawiać. Gosiu, dziękuję Ci za zainteresowanie moją pracą i cenne sugestie. Asiu, nigdy nie byłaś „nowa" i to w Tobie cenię. Fajnie się z Tobą pracowało. Dawid, nie spotkałam jeszcze większego optymisty i cynika w jednym, praca z Tobą i słowne utarczki to bardzo ciekawe doświadczenie. Bartosz, życzę powodzenia w wielkim świecie nauki. Grażynko, bez Ciebie ten lab by nie funkcjonował. Dziękuję Ci bardzo za pomoc w potrzebie zarówno laboratoryjnej jak i mentalnej. Doktorku nasz, Marcinie przeniosłeś swoje udziały z Utrechtu do Warszawy, życzę powodzenia we wspaniałej karierze naukowej.
Jest też wiele osób niezwiązanych ze środowiskiem naukowym, którym również chciałabym podziękować. Kochani moi przyjaciele, Marenko i Pawle, Paulinko i Rafale, Agnieszko i Wojtku, Renatko i wielu innych, dziękuję Wam bardzo za to że jesteście, za wasze zainteresowanie, że zawsze mogłam wam ponarzekać jak to „strasznie" jest w tej pracy i jaka to ja jestem „biedna". Między innymi dzięki Wam miałam po co wracać do Polski z wyjazdów naukowych do Utrechtu.
I na koniec chiałabym podziękować moim najbliższym, mojej Babci Halusi, która zawsze we mnie wierzyła i chętnie wysłuchiwała o moich

kłopotach i radościach, o moich sukcesach i porażkach i zawsze wkładała „kawałek serca" do mojego ciasta. Rodzice kochani, śledziliście z zapałem moją karierę od pierwszego mojego kroku aż po dziś, to dzięki Wam jestem teraz tu gdzie jestem. Dziękuję wam bardzo za nieustające wsparcie, za dodawanie mi otuchy, krytykę, gdy było trzeba i miłość, która dodawała mi sił w trudnych chwilach. Pawełku, kochanie moje, mężu mój. W czasie powstawania tego doktoratu, wiele się wydarzyło w naszym życiu, nasze małżeństwo, kupno mieszkania, moje wyjazdy do Utrechtu, moje ciągłe zmagania z doświadczeniami, które nie „działają". Dziękuję Ci, że zawsze potrafisz mnie rozweselić, pomóc spojrzeć na sprawy z zupełnie innej perspektywy i pokazać co jest naprawdę ważne. Przy Tobie nawet perspektywa opublikowania wyników mojej ciężkiej pracy w 'Acta Biochemika CHOMIKA' jest zabawna i atrakcyjna. Dziekuję Ci za to.