Novel cAMP targets in cell proliferation

Nieuwe cAMP doelmoleculen in celproliferatie

(met een samenvatting in het Nederlands)

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List of abbreviations

AC	Adenylate cyclase
AKAP	A-kinase associating protein
APP	Amyloid precursor protein
CAK	CDK-activating kinase
cAMP	Cyclic adenosine 3',5'-monophosphate
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
CIRC	Calcium-induced calcium-release
CKI	CDK inhibitor
CNG	Cyclic nucleotide-gated
CRE	cAMP responsive element
CREB	CRE binding protein
CREM	CRE modulator
DAG	Diacylglycerol
DBE	DAF-16 binding element
EGF	Epidermal growth factor
Epac	Exchange protein directly activated by cAMP
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GSK3	Glycogen synthase kinase 3
IBMX	Isobutylmethylxanthine
IGF-1	Insulin-like growth factor I
IR	Insulin receptor
IRS	Insulin receptor substrate
mantGDP	2',3'-bis(O)-N-methylantaranoloyl-guanosinediphosphate
MAPK	Mitogen activated protein kinase
MEF	Mouse embryo fibroblast
MEK	MAPK/ERK kinase
MnSOD	Manganese superoxide dismutase
NLS	Nuclear localization signal
p70S6	p70 ribosomal S6
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDK1	3-Phosphoinositide-dependent protein kinase 1
PDZ	PSD-95/DlgA/ZO-1
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PtdIns3P	3-Phosphorylated inositol lipid
RA	Ras-association
Rb	Retinoblastoma
RCBD	Related cyclic nucleotide binding domain
REM	Ras exchange motif
RI, RII	Regulatory subunit I and II
RTK	Receptor tyrosine kinase
SGK	Serum- and glucocorticoid-inducible kinase
SOS	Son of Sevenless
TSH	Thyroid-stimulating hormone
TSHR	TSH receptor

Chapter

General introduction

Cyclic AMP, a second messenger

Cyclic AMP (cAMP) is a second messenger that plays a crucial role in the intracellular signal transduction of various stimuli. It controls one of the most common and ubiquitous signalling pathways, the cAMP-dependent protein kinase (PKA) pathway, resulting in control of a wide variety of cellular events in almost all tissues of eukaryotes (reviewed in (1-3)).

Regulation of cAMP levels

cAMP was first identified as a small intracellular mediator in the 1950s (reviewed in (2)). It is generated from ATP by adenylate cyclases (ACs), and can be induced more than twentyfold upon activation of ACs by extracellular signals. Activity of ACs is regulated by G protein-coupled receptors (GPCRs) (figure 1), which are classified by the presence of seven transmembrane-spanning domains. Binding of extracellular ligands to GPCRs results in release of the Gα subunit from the trimeric Gαβγ complex and, depending on the Gα type, leads to stimulation or inhibition of ACs (reviewed in (3-5)). Inhibitory ($G_i\alpha$) subunits inhibit ACs by directly binding to the AC, and ACs are stimulated by stimulatory ($G_s\alpha$) subunits. ACs consist of two transmembrane clusters, a cytoplasmic N-terminus and two cytoplasmic loops, containing the catalytical domains. ACs are conserved in their catalytic domains, but vary significantly in other parts of the protein. $G_s\alpha$ stimulates ACs by facilitating the folding of the catalytic domain around its substrate, ATP. $G_i\alpha$, on the other hand, keeps the AC catalytic domain in an open conformation, not able to bind the substrate tightly. In mammals, nine membrane-bound isoforms (AC1-AC9) have been identified, which are differentially expressed and regulated. For instance, stimulatory action of Ca^{2+}/ calmodulin on ACs is seen with AC1 and AC8, which expression is limited to neuronal and secretory tissue. Apart from this, some AC subtypes are regulated by protein kinase C, and others can be desensitized by PKA through direct phosphorylation (reviewed in (6)).

Degradation of cAMP is mediated by cAMP phosphodiesterases (PDEs) (figure 1). PDEs hydrolyse cAMP into adenosine 5'-monophosphate and this is important for controlling cAMP resting state levels. From the 11 identified families of PDEs, PDE4, PDE7, and PDE8 are specific for cAMP, PDE4 accounting for most of the cAMP-hydrolyzing activity in the cell. PDE4 activity can be regulated by PKA, as phosphorylation of a conserved PKA consensus site, present in several PDE4 subtypes, leads to activation of the enzyme (reviewed in (7,8)). Inhibition of catalytic activity of PDE4 subtypes is achieved by mitogen activated protein kinase (MAPK)-dependent phosphorylation (9). This provides a feedback regulation on MAPK activity, as inhibition of PDE4 activity induces an increase in cAMP, which in turn suppresses the MAPK cascade via PKA in several cell lines (10). MAPK-induced phosphorylation of PDE4 exclusively results in inhibition of the PDE when no other sites are phosphorylated and is thus overruled by phosphorylation by PKA (reviewed in (7,8)). Furthermore, increased PDE4 activity has also been observed after activation of the phosphatidylinositol 3-kinase signalling pathway (11).

Important in cAMP signalling is that signalling occurs very locally and can have different outcomes, depending on the cAMP-inducing agent. In cardiac myocytes, both prostaglandin E1 and β-adrenergic receptor (β-AR) activation by epinephrine or isoproterenol result in accumulation of cAMP. Only β-AR agonists resulted in activation of PKA substrates involved in glycogen metabolism and contraction. Cell fractionation studies showed that, whereas both PGE1 and isoproterenol could activate soluble PKA, only isoproterenol

was able to activate non-soluble or membrane-bound PKA (reviewed in (12)). Furthermore, although cAMP has the potential to diffuse through water very quickly, use of a cAMP-biosensor has provided evidence that the diffusion rate of cAMP in a cell is rather low and a pool of cAMP below the plasma membrane does not equilibrate with the cytoplasm as rapidly as other molecules (reviewed in (8)). Localized cAMP-mediated activity is explained by localized induction and degradation of cAMP in specialized cellular compartments such as caveolae and lipid rafts. PDEs are important for regulating cAMP concentration in these microdomains as their unique intracellular targeting regulates the availability of cAMP to its effectors. Also ACs and GPCRs are not evenly distributed along the membrane. Furthermore, PKA can be targeted by several proteins, including Src family kinases, arrestins, receptor for activated C kinase 1 (RACK1) and A-kinase anchoring proteins (AKAPs). Especially AKAPs play a role in compartmentalization of PKA isoforms by anchoring to specific sites per AKAP isoform. This all contributes to a localized activation of cAMP effectors (reviewed in (3,5,7,8)).

Direct targets of cAMP

Although other cAMP-effectors have been identified, PKA is still the most common target (figure 1). PKA holoenzymes are inactive heterotetramers, composed of two regulatory (RIα and RIβ or RIIα and RIIβ) and two catalytic (Cα, Cβ, Cγ or PrKX) subunits. Binding of two cAMP molecules to each of the regulatory subunits, results in release and activation of the catalytic subunits. These catalytic subunits will phosphorylate various target molecules, both in the cytoplasm and in the nucleus (reviewed in (3,4,13)). The R-subunits exhibit different affinities for cAMP binding, resulting in different thresholds for activation of the holoenzymes. PKA type I holoenzymes typically have higher affinity for cAMP than type II holoenzymes. Besides cAMP affinity, localization of the holoenzymes is also different: PKA type I enzymes are generally cytoplasmic, and type II enzymes are specifically anchored to subcellular structures and compartments (3). Major nuclear targets of PKA are the transcription factors cAMP response element (CRE) binding protein (CREB) and CRE modulator (CREM). CREB and CREM bind optimally to palindromic CREs (sequence TGACGTCA) in promoters and upon phosphorylation by PKA, they recruit the coactivator, CREB binding protein (CBP) to the promoter (reviewed in (4)). Recently, a cAMP

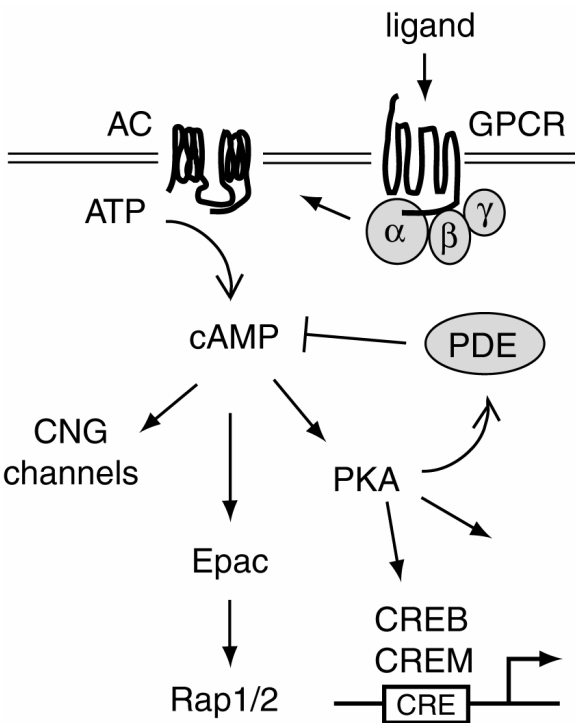

Figure 1. ***cAMP signalling.*** Ligand-stimulation of GPCRs results in release of the Gα-subunits, which will activate ACs to generate cAMP from ATP. This is in turn negatively regulated by degrading activity of PDEs. cAMP can bind to and activate its direct targets, PKA, Epac and CNG channels, resulting in activation of divergent signalling pathways. One role of PKA is regulation of transcription via activation of the transcription factors CREB and CREM, which bind to CREs in promoters. Furthermore, PKA provides a negative feedback loop by stimulating PDEs to degrade cAMP. Epac activation leads to activation of the small GTPases Rap1 and Rap2.

independent activation of PKA has been described, involving transforming growth factor β (TGFβ) which induced binding of smad3 and smad4 to the regulatory subunit of PKA. This resulted in dissociation of the holoenzyme and thus activation of PKA by TGFβ (14), although with a much lower potency than cAMP.

An additional effector system for cAMP signalling is achieved by the exchange proteins directly activated by cAMP 1 and 2 (Epac1 and -2; also named cAMP-GEFI and -II) (figure 1). These guanine nucleotide exchange factors (GEFs) are specific activators of the small GTPase Rap1 (15,16). The cAMP-binding domain of Epac1 can bind one molecule of cAMP, resulting in a conformational change of the protein, which will expose the active site of the catalytic domain, enabling the protein to bind to and activate Rap1 (17). It is well established that Epac via Rap1 is involved in integrin-mediated cell adhesion (18). Furthermore, Epac has also been described to mediate processes like insulin secretion and Ca^{2+}-induced Ca^{2+}-release in pancreatic β-cells (19,20).

In some tissue types, like kidney, testis, heart and the central nervous system, an alternative cAMP signalling pathway exist via the regulation of cyclic nucleotide-gated (CNG) channels (figure 1), such as pacemaker voltage-gated potassium channels or other channels involved in the transduction of sensory signals. These CNG channels contribute to the control of membrane potential and intracellular Ca^{2+} levels, and are opened more rapidly upon cAMP binding (reviewed in (5,13)).

Biological functions of cAMP

Dependent on the cell type and the subcellular localization of proteins involved in cAMP signalling, cAMP can exert different biological effects. This is schematically summarized in figure 2. Besides the regulation of cell proliferation, which will be described in a later section, cAMP is involved in the related processes of differentiation and apoptosis. cAMP has been reported to induce differentiation in, among others, neuronal cells, adipocytes and leukemic cells (21-25) and can inhibit differentiation in for instance myogenic cells (26,27). Apoptosis is induced by cAMP in B lymphocytes, thymocytes and myocytes (28-30) and is prevented by cAMP in other cell types, such as neutrophils, hepatocytes and pancreatic cells (31-33). The decision whether cells will proliferate or undergo differentiation might depend on the cAMP concentration, as in Schwann cells a low cAMP concentration induces proliferation, whereas a high cAMP concentration switches the cells to a differentiation program. This seems to be dependent on respectively transient or sustained activation of the mitogen activated protein kinase (MAPK) pathway (34), regulated by PKA.

The biological effect of cAMP has in some cases been shown to be dependent on the PKA subtype. Activity of PKA type Iα enzymes is necessary for the negative regulation of the T-cell and B-cell receptor, which has been shown using selective PKA agonists. Furthermore, RIα, and not RII subunits, were continuously associated with lipid rafts, where they co-localized with T-cell receptors. The importance of PKA-dependent regulation of immune function is shown in immune cell-related

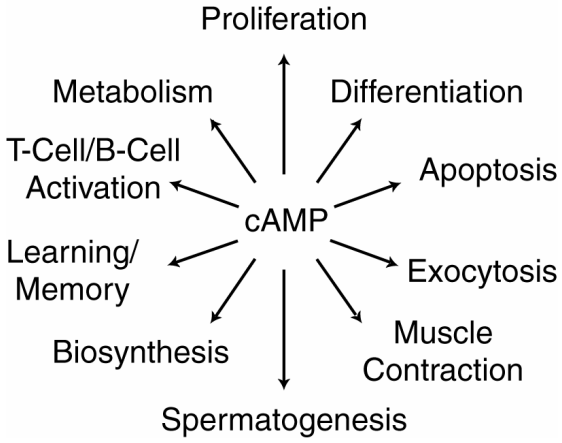

Figure 2. ***Schematic representation of biological functions of cAMP***

disease conditions. Sometimes, patients are immunodeficient due to elevated cAMP levels, which can be reversed using PKA antagonists. PKA RIIβ subunits have been shown to be important in adipocytes. Lypolysis is the major system for the release of stored energy from adipocytes and this process is increased by PKA activation. Mice lacking the PKA RIIβ subunit have markedly reduced deposits of white fat and are resistant to diet-induced obesity. This is due to compensation of the absence of PKA RIIβ levels by RIα subunits, which are more cAMP-responsive and as a consequence increase fat metabolism (reviewed in (3)).

Biological effects of cAMP can further be divided by PKA- or Epac-mediated processes. In neurons, cAMP-dependent signalling is very prominent and essential. cAMP is in these cells critical for learning and memory storage. Evidence is present for an important role for PKA signalling in long-term potentiation. cAMP levels can be increased in sensory neurons upon stimulation with neurotransmitters. Repetitive stimulation with neurotransmitters leads to translocation of PKA catalytic subunits to the nucleus, which will induce transcription in a CREB-dependent manner (reviewed in (35)). Epac, on the other hand, may increase neurotrophic activities and enhances memory via metabolism of amyloid precursor protein (APP) in neurons. Epac can, via activation of Rap1, regulate the small GTPase Rac to induce secretion of soluble amyloid β precursor protein α (sAPPα), which is beneficial for memory (36). Apoptosis-protection by cAMP in pancreatic β-cells is another example of a process which can involve either PKA or Epac, here dependent on the concentration of cAMP. Low cAMP levels, induced by glucagon-like peptide (GLP-1), prevent palmitate-induced apoptosis via a pathway involving PKA. High cAMP levels, induced by forskolin/IBMX or cAMP analogues, activate the Epac pathway, resulting in an anti-apoptotic effect (37). Thirdly, PKA and Epac can be involved in different aspects of cAMP-mediated exocytosis. This process plays a role in many secretory cell-types, like acid-secreting cells, renal principal cells and pancreatic β-cells. For the first two cell-types, PKA is involved in the translocation of proton pumps and water channels, which will induce respectively acid secretion and water reabsorption. In pancreatic β-cells, PKA can enhance insulin secretion (3), but cAMP can also affect exocytosis in these cells via Epac. It has been shown that an Epac specific cAMP-analogue can mobilize Ca^{2+} from intracellular stores in a process named Ca^{2+}-induced Ca^{2+}-release (CIRC), which is coupled to exocytosis (20).

cAMP and proliferation

The MAPK and PI3K pathway in proliferation

The MAPK pathway is an important pathway regulating growth and differentiation. In this pathway (figure 3A), the small GTPase Ras is activated by growth factors, leading to recruitment of the kinase Raf to the plasma membranes, where it becomes active. Activation of Raf results in sequential activation of the kinase MEK and MAPK. Raf related proteins comprise a family of three members, Raf1, A-Raf and B-Raf. Raf1 is ubiquitous expressed, whereas A-Raf seems more restricted to urogenital tissue and B-Raf to neuronal tissue, testis and haematopoietic cells. Studies of mice deficient for individual Raf members show that the family members can compensate for each other in MAPK activation, but also that B-Raf seems to be the main regulator of the MEK-MAPK pathway (reviewed in (38)). The MAPK pathway can be inhibited by cAMP in a PKA dependent manner (39-41) and this was reported to be important for reversal of the transformation phenotype of v-Raf-transformed cells (42,43). The inhibition of the pathway by PKA takes place at the level

of Raf1 (44,45). Phosphorylation of serine 43 of Raf1 by PKA has been shown to disrupt Raf1 binding to Ras-GTP (46). Furthermore, the phosphorylation of serine 621 has been reported to be involved in inhibition of catalytic activity by cAMP (42,47). However, both sites were found to be major sites of phosphorylation even in untreated cells (48), and an increase in phosphorylation on serine 621 in response to cAMP has not been demonstrated. In later studies then, it was found that phosphorylation of all three serines, 43, 233 and 259, had to be phosphorylated by PKA to completely block activation of the downstream kinase, MAPK (49). cAMP may induce inhibition of Raf1 via binding of 14-3-3 proteins to phosphorylated serines at position 233 and 259. Binding of 14-3-3 inhibits the membrane recruitment of Raf1 by Ras, which is a crucial event for Raf1 activation (50). Activation of the MAPK pathway by cAMP has been reported to be mediated by the Raf1 family member B-Raf, in a Rap1-dependent manner (51), but this may be cell type-dependent as different results on cAMP-mediated activation of the MAPK pathway have been reported (reviewed in (52)).

Although inhibition of MAPK often correlates with inhibition of proliferation, in many cases it appears not to affect growth. Even more striking, McKenzie et al. showed that treatment of CCL39 hamster fibroblasts with cAMP inhibited proliferation, without significantly affecting serum- or thrombin-stimulated MAPK activity (53). Recently, it has been reported that a Raf1 construct, containing the kinase domain of Raf1 fused to a modified estrogen receptor (ER), can stimulate DNA synthesis in quiescent NIH3T3, Rat1 and CCL39 fibroblasts. Although the fusion-construct itself is insensitive to cAMP, growth of these cells is still prohibited by cAMP, further proving that MAPK is not always

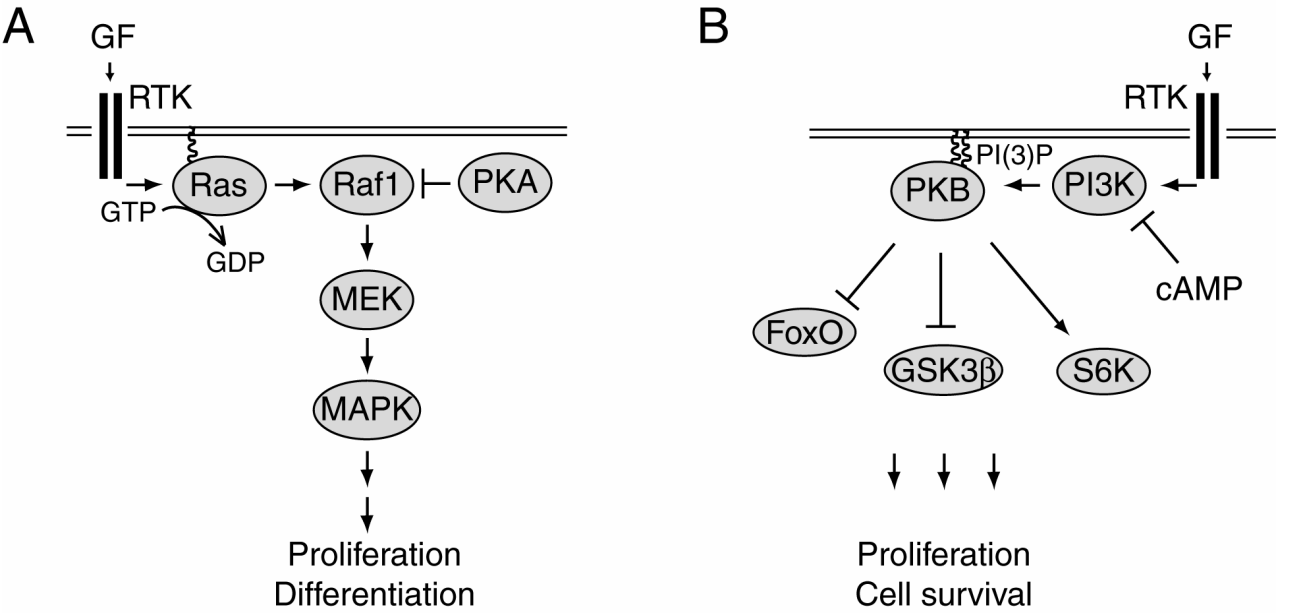

Figure 3. ***Schematic representation of the MAPK and PI3K pathway, controlling proliferation.*** Growth factors (GFs) can activate receptor tyrosine kinases (RTKs), resulting in activation of the small GTPase Ras (A) and the lipid kinase PI3K (B). Ras (A) is activated by exchange of GDP for GTP and recruits in activated state Raf1 to the plasma membrane. As a result, Raf1 becomes activated and activates a kinase cascade, consisting of MEK and MAPK. This leads to the induction of proliferation or differentiation. The pathway can be inhibited by cAMP via PKA at the level of Raf1. PI3K (B) is involved in the generation of PI(3)P lipids, leading to recruitment of PKB to the plasma membrane. As a result, PKB becomes activated and can affect several downstream targets. Inhibition of the S6K pathway and the direct targets GSK3 β and FoxO, results in proliferation and cell survival. Inhibition of the PI3K pathway by cAMP takes place at the level of PI3K.

involved in cAMP-induced inhibition of proliferation (54). Although the involvement of MAPK in a cAMP induced cell cycle arrest is not consistent, it can regulate some major cell cycle proteins. For instance, cyclin D1 is regulated by the MAPK pathway at different levels, as MAPK can transcriptionally upregulate cyclin D1 levels (55,56) but is also involved in assembly of cyclin D-CDK4/6 complexes and cyclin D1 turnover (57,58).

The phosphatidylinositol 3-kinase (PI3K) pathway is important in the control of cell survival and proliferation. Activation of the pathway (figure 3B) is initiated by growth factors, resulting in stimulation of PI3K to generate 3-phosphorylated inositol lipids (PtdIns3P). These lipids recruit protein kinase B (PKB) and also 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane. PDK1 phosphorylates PKB on threonine 308. Subsequently, serine 473 of PKB becomes phosphorylated by a so far not completely understood mechanism, leading to fully activated PKB. The PI3K pathway can be inhibited by cAMP, although the exact mechanism is not clear. It has been reported that cAMP can affect PDK1 membrane localization (59) and inhibit PtdIns3P levels (60), which can both be explained by inhibition of PI3K. Several downstream partners of PKB have been shown to play a role in proliferation. PKB can regulate cell cycle proteins involved in the G1 phase of the cell cycle via inactivation of FoxO transcription factors and GSK3β, which will be discussed later, and activation of p70S6 kinase (reviewed in (61,62)). p70S6 kinase is essential for control of the translational machinery of the cell (63) and, as a consequence, essential for induction of cellular proliferation by growth factors (reviewed in (64)). This has been shown in rat embryo fibroblasts, where injection of antibodies against p70S6 kinase could prevent serum-mediated cell cycle entry, due to almost complete inhibition of protein synthesis induction (65).

cAMP-dependent inhibition and stimulation of proliferation

Proliferation is stimulated by cAMP in a few cell types, like Swiss 3T3 fibroblasts (66) and thyrocytes (67). But in most cells, including smooth muscle cells (68,69), adipocytes (40), T-lymphocytes and macrophages (70,71), cultured astrocytes, glioma and myeloid cells (72,73), corneal endothelial cells (74), hepatocytes (75) and several types of fibroblasts (44,76-79), cAMP inhibits proliferation. Often, the effect on proliferation correlates with the ability of cAMP to activate or inhibit the MAPK pathway (80). In thyroid cells, though, the cAMP generating hormone TSH inhibits the MAPK pathway while stimulating proliferation. cAMP-induced proliferation in thyroid cells is Ras-dependent, (81) likely depending on activation of another Ras effector pathway, i.e. RalGDS (82) and on activation of p70S6 kinase. Necessity for activation of p70S6 kinase by cAMP is also seen in other cell types in which cAMP stimulates proliferation (83). It is not clear which factors determine whether signalling pathways involved in proliferation are inhibited or stimulated by cAMP. The type of receptor present on the cell to induce proliferation might play a role. Insulin-induced proliferation is inhibited by cAMP in insulin receptor containing cells (NIH3T3/IR cells), whereas NGF-induced proliferation is not affected by cAMP in NGF receptor containing cells (NIH3T3/trk cells) (84). Another aspect, that might play a role, is the subtype of the PKA regulatory subunits expressed in cells. Observations on this come from experiments with Chinese hamster ovary (CHO) cells, containing PKA mutations. Wild type CHO cells are inhibited in proliferation by cAMP, whereas several PKA mutant cells were found to be resistant to cAMP. This seemed to be

dependent on activity of PKA type II kinases but effects of type I holoenzymes can not be excluded (85). Contradictory results were obtained from experiments with mouse fibroblasts and thyroid cells. Thyroid cells contain predominantly RIIβ, whereas NIH3T3 mouse fibroblasts, in which proliferation is inhibited by cAMP, contain only low levels of RIIβ subunits. Co-expression of the TSH receptor (TSHR) and the PKA RIIβ subunit in NIH3T3 cells reverses the cAMP-inhibitory effect in these cells, indicating that stimulation of proliferation is mediated by type II enzymes. The growth stimulatory effect was accompanied by increased PKB and MAPK activity and inhibition of the PI3K, but not the MAPK pathway, could reverse the growth-stimulatory effect (86). Thus, compartmentalization, activation of additional signalling pathways, and the cellular context in which the cAMP signal develops may account for divergent biological effects (8).

cAMP and the control of cell cycle proteins

The main mechanism used by cAMP to inhibit proliferation is by arresting cells in the G1-phase of the cell cycle.

Progression through the G1-phase of the cell cycle

Proliferation of cells is a well controlled process, in which cells cycle through four different stages. These stages, G1, S, G2 and M-phase, are regulated by cyclins in complex with cyclin dependent kinases (CDKs) and by cyclin dependent kinase inhibitors (CKIs) (figure 4). Non-proliferating or quiescent cells reside in the G0-phase and can enter G1 upon stimulation with growth factors or cytokines. Mitogenic signals will induce D-type cyclins in early-G1, which will complex with CDK4 and CDK6 (58). The CDKs in these complexes have to be phosphorylated in the nucleus by CDK-activating kinase (CAK) to be active. Upon activation, the cyclin D-dependent kinases will phosphorylate their substrate retinoblastoma (Rb) in mid-G1, which results in initial induction of cyclin E and thereby activation of the cyclin E-CDK2 complexes. Active CDK2 phosphorylates Rb on additional sites, resulting in complete inhibition of Rb (57,87). Rb is an important growth suppressor, as it suppresses the E2F transcription factors, which are responsible for the induction of proteins, like cyclin A and E, necessary for DNA synthesis.

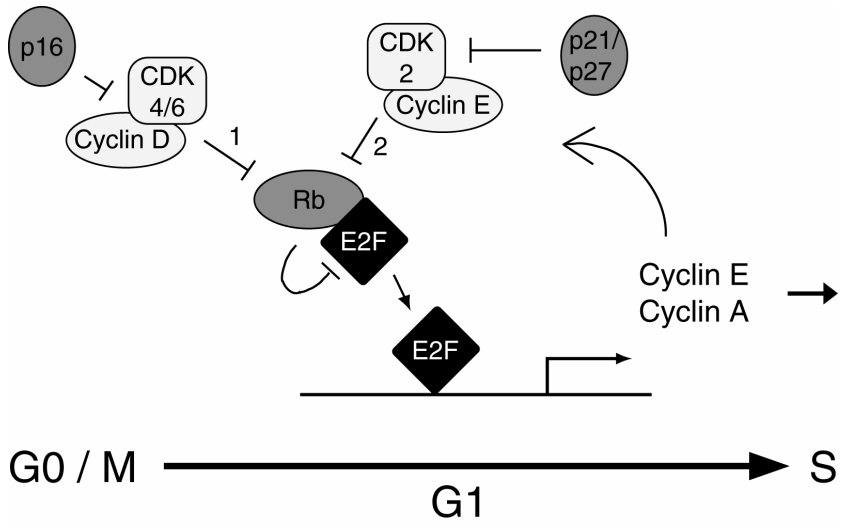

Figure 4. ***Control of G1-phase progression by cyclins, CDKs and CKIs.*** Cyclin D levels are induced when cells enter G1 from G0 or M-phase. Rb is completely inhibited in two steps. First, cyclin D in complex with CDK4 or CDK6 inhibits Rb, resulting in relieve of the inhibitory action of Rb on E2F. E2F transcriptionally induces cyclin E and A. Cyclin E in complex with CDK2 further inhibits Rb (step 2), resulting in increased production of cyclin E and A and irreversible progression to S-phase. Negative control on the cell cycle is provided by CKIs. INK4 CKIs, like p16, inhibit cyclin D-CDK4/6 complexes and Cip/Kip family CKIs, p21 and p27, inhibit cyclin E-CDK2 complexes.

Thus complete inhibition of Rb leads to a boost in cyclin E and A levels in late-G1, irreversibly committing the cell to enter S-phase. Physiological substrates of cyclin E and A are not well characterized, but cyclin E is active at the G1/S boundary and seems to be required for the initiation of DNA replication in early S-phase, whereas cyclin A is active during S-phase and may be important in controlling the switch from initiation to elongation of DNA replication (58,88). Later in cell cycle, cyclin A plays a role in G2, whereas cyclin B1 is induced later on, playing a role in mitosis in complex with CDK1. Cyclin A-CDK2 and cyclin B1-CDK1 complexes will keep Rb in its hyperphosphorylated form until cells exit mitosis, delivering Rb in hypo-phosphorylated form back in G1.

Negative control of G1 is achieved by activity of CKIs (57), leading to the inhibition of CDKs. There are two different classes of CKIs known, the INK4 family and the Cip/Kip family. The INK4 family specifically binds to and inhibits CDK4 and CDK6 and consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. These inhibitors are characterised by the presence of multiple ankyrin repeats. The second class of inhibitors consists of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and has a more broad function as it inhibits CDK2 by binding to the cyclin A/E-CDK2 complex and can stimulate cyclin D-dependent kinases (57,58). The Cip/Kip CKIs facilitate the assembly of cyclin D-CDK complexes by stimulating import and stability of cyclin D (89,90). The presence of these CKIs in cyclin D-complexes implicates furthermore that they are sequestered, and thus not available for inhibiting CDK2. In addition, CDK2 can inhibit $p27^{Kip1}$ directly, increasing its own activity. There is some debate on the function of Cip/Kip members for cyclin D1 complex activity. It has been reported that cyclin D-CDK4/6 complexes containing $p27^{Kip1}$ are inactive. Furthermore, overexpression of $p27^{Kip1}$ inactivates cyclin D-CDK complexes and immunodepletion of $p27^{Kip1}$ in macrophages restores the activation of CDK4 by CAK after inhibition by cAMP (91,92). Moreover, complex formation of cyclin D with CDK4 seems to be normal in p21/p27 double negative cells. The CKIs, though, do stabilize cyclin D, leading indirectly to enhanced complex formation (93). In contrast, it has been reported that p21/p27 double negative cells have severe reduction in CDK4 activity due to impaired complex formation with cyclin D1. This had, however no obvious effect on cell cycle progression. Apparently, these cells do well tolerate the absence of both CKIs (90).

CKIs can be induced by anti-proliferative agents, like TGFβ and cAMP and by DNA damaging agents. Common upregulated targets of TGFβ are $p15^{INK4b}$, $p21^{Cip1}$ and $p27^{Kip1}$ (94-96), transciptionally regulated via smads (97). Furthermore, $p27^{Kip1}$ can be transferred from cyclin D to E-type complexes due to competition of $p15^{INK4b}$, induced by TGFβ (98). cAMP can induce $p27^{Kip1}$ via various ways, which is described in a later section.

Regulation of cyclin D1 by cAMP

Cyclin D1 is one of the rate limiting proteins for G1 progression. Quiescent fibroblasts can not enter the cell cycle when they are unable to make cyclin D1 and the basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor c-Myc, which can be rescued by either of them (99). c-Myc does not likely directly upregulate cyclin D1, as in c-Myc-deficient cells cyclin D1 levels are only slightly reduced (100). Cyclin D1 levels do not fluctuate during cell cycle, like other cyclins, but are clearly induced upon growth factor stimulation of quiescent cells (101). The protein is highly unstable and can easily be downregulated by anti-proliferative agents like cAMP (figure 6). Although upregulation of cyclin D1 is a major requirement for cell cycle entry, its downregulation seems to play only a partial role in the inhibition of cell cycle entry (102).

cAMP-induced transcriptional downregulation of cyclin D1 is mainly regulated via the CRE in its promoter, which is regulated by CREB and ATF transcription factors (103), but also FoxO transcription factors have been described to regulate the cyclin D1 promoter (104,105). cAMP can affect cyclin D1 levels posttranscriptionally by inhibiting the PI3K pathway, resulting in GSK3β activation and p70S6 kinase inhibition. Cyclin D1 is phosphorylated by GSK3β on threonine 286, especially in complex with CDK4. This phosphorylation is essential for nuclear exclusion, which will lead to ubiquitin-dependent proteasomal degradation. GSK3β is negatively regulated by Ras and PI3K and its localization is regulated during cell cycle, entering the nucleus in S-phase where it can phosphorylate cyclin D1. Also in quiescent cells, GSK3β is active and mediates quick cyclin D1 turnover (106). p70S6 kinase is involved in translational control of cyclin D1 levels. This control can result in cyclin D1 induction prior to the induction of mRNA levels (107), although the importance of this is questionable as p70S6 kinase is not involved in mitogen-induced upregulation of cyclin D1, whereas PI3K is (63).

Regulation of p27^{Kip1} by cAMP

The cell cycle inhibitor p27^{Kip1} is important in regulating S-phase entry, by controlling CDK2 activity. Depletion of p27^{Kip1} by antisense oligonucleotides prevents mouse fibroblast cells from cell cycle arrest after serum withdrawal (108). The importance of p27^{Kip1} in controlling proliferation is also seen in p27^{Kip1} knockout mice, which have an overall enlarged size due to an increase in cell number (109-111). Furthermore, p27^{Kip1} is important for keeping cells in arrest in G0 (112). Its protein levels are therefore high in quiescent cells and decrease upon growth factor-induced cell cycle entry (113,114) and become even undetectable during S-phase (115). This is in contrast to its family member p21^{Cip1}, which is induced as cells enter cell cycle (116). p27^{Kip1} preferentially binds to cyclin-CDK complexes and is as a monomeric form only found in G0, where hardly any cyclin-CDK complexes are present. During G1-phase, p27^{Kip1} is associated with cyclin D-CDK complexes, and upon S-phase entry, p27^{Kip1} is redistributed to cyclin E/A-CDK2 complexes (114). The protein levels of p27^{Kip1} fluctuate extensively during the cell cycle, and this is achieved by rapid degradation via several mechanisms, e.g. ubiquitin-dependent (117) and -independent (118), caspase-mediated (119), jab1-dependent (120) and calpains-mediated (121) degradation.

The phosphorylation of p27^{Kip1} on threonine 187 (T187) by CDK2 (figure 5) plays an important role in the degradation upon growth factor stimulation. Phosphorylation on this site is followed by ubiquitination involving the E3 ubiquitin ligase Skp2 (122-124) and when this site is mutated to an alanine, the degradation in S and G2-phase is inhibited (125). p27^{Kip1} can also be degraded during G1, but this may not involve T187 phosphorylation or be mediated

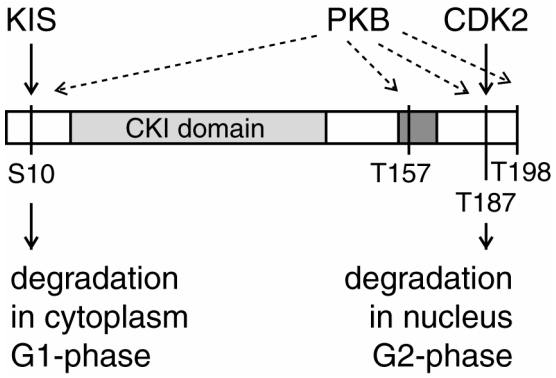

Figure 5. ***Phosphorylation-dependent regulation of p27^{Kip1}.*** p27^{Kip1} has been shown to be phosphorylated by KIS on serine 10, by PKB on serine 10 and threonine 157, 187 and 198 and by CDK2 on threonine 187. Threonine 157 is part of the NLS, which is shown as a dark-grey box. S10-phosphorylation results in export of p27^{Kip1} to the cytoplasm and degradation during the G1-phase of the cell cycle, whereas T187-phosphorylation by CDK2 is important for degradation of p27^{Kip1} during the G2-phase, taking place in the nucleus.

by Skp2. Instead, this may involve phosphorylation of serine 10 (S10), as phosphorylation on this site precedes CDK2 activation. S10 phosphorylation accelerates degradation, although it is not required for nuclear export and degradation of $p27^{Kip1}$ (126-128). Another signal is needed to initiate the degradation of $p27^{Kip1}$, mediated by S10-phosphorylation and it has been reported that this might be provided by the nuclear kinase KIS (figure 5). KIS can phosphorylate S10 of $p27^{Kip1}$ and can almost completely overcome a G1-arrest induced by wild type $p27^{Kip1}$, but not an arrest induced by the p27-S10A mutant. Furthermore, KIS RNAi increased G1-phase cells and reduced S10 phosphorylation, followed by $p27^{Kip1}$ accumulation in the nucleus (128). Other phosphorylation sites of $p27^{Kip1}$ that might play a role in its stability have also been reported. PKB can phosphorylate S10, T187 and T198 (figure 5), the latter creating a binding site for 14-3-3 proteins, likely resulting in nuclear exclusion of $p27^{Kip1}$ (129). Others reported that T157 is phosphorylated by PKB, which might lead to inhibition of import by shielding the nuclear localization signal (NLS), which is present in that region (130-132). In general, the picture emerges that $p27^{Kip1}$ degradation is executed in two steps. Upon mitogenic stimulation, S10 is phosphorylated, leading to nuclear export and the first step of degradation, taking place in the cytoplasm. This leads to a decline in $p27^{Kip1}$ levels under a certain threshold, ensuring CDK2 activation, which then starts by phosphorylating T187 the second and more pronounced step in $p27^{Kip1}$ degradation, taking place in the nucleus (127).

cAMP-dependent inhibition of proliferation often correlates with an upregulation of $p27^{Kip1}$ protein levels (74,92,133-135), likely via regulation of the MAPK and PI3K pathways (60,136-138). $p27^{Kip1}$ levels can be regulated by cAMP (figure 6) at the level of transcription, degradation and translation (115,139-141). Furthermore, it has been reported that cAMP can induce nuclear localization of $p27^{Kip1}$ and can inhibit the FGF-2 induced phosphorylation of $p27^{Kip1}$ on T187 in corneal endothelial cells (60,74).

Regulation of other cell cycle proteins by cAMP

The Myc oncogene plays a role in both the G1 phase and G2 phase of the cell cycle (100). It induces several cell cycle proteins, like cdk4, and cyclin D2, -E, and -A. Furthermore, Myc plays an important role in promoting proliferation by suppressing the CKIs $p15^{INK4b}$, $p21^{Cip1}$ and $p27^{Kip1}$. Importance of Myc regulation has been shown for TGFβ-mediated inhibition of proliferation: inhibition of Myc is essential for TGFβ to induce a cell cycle arrest. cAMP-dependent regulation of Myc (figure 6) often correlates with regulation of proliferation. In Bcr-Abl transformed myeloid cells, cAMP could inhibit Myc transcription via the E2F site of the Myc promoter. In these cells, cAMP inhibited proliferation by downregulating cyclin D1, leading to E2F sequestering and thus regulation of Myc (142). Furthermore, a decrease of Myc mRNA was dependent on cAMP in B-cells, fibroblasts and leukemic cells and correlated with inhibition of proliferation or differentiation (143-146), whereas a cAMP-dependent increase in Myc was seen in thyroid, Balb/c 3T3 and Swiss 3T3 cells, correlating with the induction of proliferation (147-149).

p53 is a transcription factor, induced in response to DNA damage, hypoxia and oncogene-activation and plays an important role in the decision to induce apoptosis or cell cycle arrest. Many pro-apoptotic proteins are induced by p53, to induce cell death upon activation of proteins that can drive cell proliferation, like Myc (150). A p53-induced cell cycle arrest is mediated by the cell cycle inhibitor $p21^{Cip1}$ and is transcriptionally upregulated by p53 under stress conditions (150,151). cAMP-dependent regulation of p53 (figure 6), and thereby $p21^{Cip1}$, has been reported and leads to inhibition of proliferation followed by apoptosis

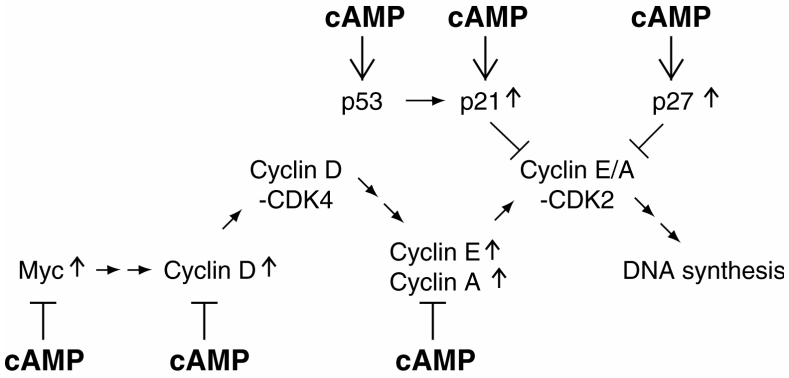

Figure 6. ***Cell cycle progression is sensitive to cAMP at multiple steps.*** Elevated levels of Myc can indirectly lead to upregulation of cyclin D levels, which in complex with CDK4 lead to upregulation of cyclin A and E levels. Cyclin A and E in complex with CDK2 will on their turn induce DNA synthesis. cAMP has been reported to inhibit the induction of Myc, cyclin D and cyclin A, all resulting in inhibition of DNA synthesis. Furthermore, negative regulation of cell cycle is enhanced by cAMP at different levels. cAMP stimulates p53 to upregulate $p21^{Cip1}$, and induces $p21^{Cip1}$ and $p27^{Kip1}$ directly.

in for instance vascular smooth muscle cells (152). In lung cells $p21^{Cip1}$ can be regulated in a PKA-dependent manner, but different mechanisms have been described. In a recent report, the PKA-dependent regulation of $p21^{Cip1}$ levels was induced by TGFβ, but independent of cAMP levels (14), whereas another report showed PKA and cAMP-dependent induction of $p21^{Cip1}$ levels, using sp1 sites in the $p21^{Cip1}$ promoter (153). Yet, another mechanism has been described for breast cancer cells, where $p21^{Cip1}$ can be induced by cAMP in a PKA-independent manner (154).

The cell cycle proteins cyclin D1, cyclin A, $p15^{Ink4b}$ and Rb do contain a CRE in their promoter and are therefore putatively transcriptionally regulated by cAMP (155). Besides cyclin D1 levels, cyclin A levels have been described to be regulated by cAMP (figure 6) in a cell cycle dependent manner. It has been shown that cAMP inhibits the induction of cyclin A in astrocytes in G1 and S-phase (156) and the cAMP-elevating hormone glucagon can upregulate cyclin A2 in late G1 in hepatocytes (157). In swiss-3T3 and human diploid fibroblasts, cAMP-dependent induction of cyclin A levels correlates with stimulation of proliferation (158,159). In contrast, in Rat1 fibroblasts cAMP-dependent reduction of cyclin A was not the important step to inhibit proliferation. Conditional E2F expression blocked in these cells, the cAMP-dependent decrease in cyclin A levels, but did not block cAMP-dependent inhibition of DNA synthesis (54).

Rap1 and its role in cAMP-dependent inhibition of proliferation

Rap1 is a small GTPase of the Ras family which can be activated by cAMP via the exchange factor Epac (15,16). Also a role of PKA in Rap1 activity has been proposed, as Rap1 can be phosphorylated by PKA on a single site (160,161). Since the finding that Rap1 could be activated by cAMP, a role for Rap1 in cAMP-dependent proliferation has been speculated for the cases where the effects on proliferation could not be explained by activity of PKA. Indeed, there are several reports, claiming a role for Rap1 in cAMP-dependent effects on proliferation and on important signalling pathways in proliferation. It has been reported that phosphorylation of Rap1 by PKA is essential for the cAMP induced inhibition of PKB (162) and the cAMP-induced stimulation of DNA synthesis of thyroid cells (163). Evidence for a capability of Rap1 to stimulate proliferation has also been provided by experiments in which cells overexpressing Rap1 were injected in nude mice and were found to form tumours (164). It has been proposed some time ago that the small

GTPase Rap1 can inhibit the MAPK pathway (165) and in this way is involved in cAMP-mediated inhibition of proliferation, in a PKA, C3G and Src dependent (166,167) or PKA independent manner (168). But in general, the data on this is conflicting and recently it has been shown with a cAMP-analogue, specific for Epac, that Rap1 is not involved in the regulation of the MAPK pathway (41).

Inhibition of proliferation by FoxO transcription factors

The family of FoxO transcription factors is comprised of FoxO1 (FKHR), FoxO3a (FKHR-L1) and FoxO4 (AFX). An important regulator of FoxO activity is PKB and FoxO1 has been shown to be phosphorylated at threonine 24, serine 256 and serine 319 by PKB, whereas FoxO3 and FoxO4 are phosphorylated by PKB at equivalent residues. PKB-dependent phosphorylation of FoxOs results in inhibition of their transcriptional activity due to cytoplasmic relocalization. Vice versa, inhibition of PKB signalling results in nuclear localization of FoxOs and activation of transcription. Overexpression of FoxOs does result in inhibition of proliferation of different cell types due to transcriptional upregulation of $p27^{Kip1}$ and/or reduction of cyclin D levels (figure 7). Upregulation of p27Kip1 is achieved by direct binding of FoxOs to Daf16 binding elements (DBEs) in the promoter, whereas regulation of the cyclin D1 promoter does not involve direct binding of FoxOs. Besides inhibition of proliferation, FoxOs can rescue cells from oxidative stress in a subset of cell lines, involving manganese superoxide dismutase (MnSOD), catalase and growth arrest and DNA damage protein 45 (GADD45), whereas FoxOs induce apoptosis in other cell types, mainly hematopoietic cells, involving Bim and Bcl-6 (reviewed in (169)).

FoxO transcription factors have been linked to cAMP signalling in cultured endometrial stromal cell. In these cells a biphasic pattern of cAMP-mediated induction of the prolactin promoter is seen during differentiation. A rapid, transient increase is mediated by a CRE-like sequence, and a delayed but strong and persistent stimulation of promoter activity was mediated by a cooperation of CCAAT/ enhancer-binding proteins (C/EBPs) and FoxO1a transcription factors. Both C/EBP and FoxO1a are induced by cAMP in these cells (reviewed in (4)).

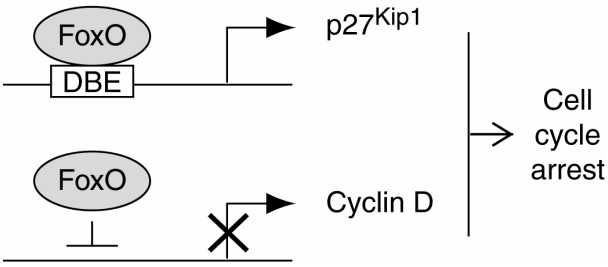

Figure 7. ***Schematic representation of a FoxO-mediated cell cycle arrest.*** FoxO transcription factors can transcriptionally induce $p27^{Kip1}$ levels by binding to DBE-sequences in the $p27^{Kip1}$ promoter. Transcription of cyclin D can be inhibited by FoxO transcription factors, although direct binding to the promoter has not been demonstrated. Both the induction of $p27^{Kip1}$ and the reduction of cyclin D levels contribute to a FoxO-mediated cell cycle arrest.

cAMP, cell cycle proteins and cancer

Deregulation of the cell cycle is an important aspect for the development of cancer. Several cell cycle proteins are subsequently found up- or downregulated in tumours, including some important cAMP targets, like cyclin D1 and $p27^{Kip1}$.

Cyclin D1 upregulation in cancer

In breast cancers, cyclins are often found upregulated, with cyclin D1 having the highest occurrence (170). The cyclin D1 locus on the genome contains in human cancers often amplifications and rearrangements. Furthermore, increased cyclin D1 mRNA stability has

been reported in breast cancers (171,172). Oncogenicity of cyclin D1 has also been shown using rat6 embryo fibroblasts overexpressing cyclin D1, which induced tumours when injected into athymic (nude) mice (172). In addition, it has been reported that cyclin D1-null mice are resistant to Ras and Neu induced breast cancers, although c-Myc and Wnt could still induce tumours in this tissue (173). While cyclin D1 abnormalities were more frequent in carcinomas of the breast, aberrant expression has also be seen in significant subsets of colorectal cancers, soft tissue sarcomas, uterine carcinomas and malignant melanomas (174). The upregulation of cyclin D1 in colon cancers suggests a link between the Wnt signalling pathway and cyclin D1, as inactivation of the adenomatous polyposis coli (APC) tumour suppressor, a component of the wnt pathway, is the single most common event in these tumour types. Indeed it has been found that the Wnt pathway can induce cyclin D1 transcriptionally via LEF1 and LEF1 binding sites in the cyclin D1 promoter (175).

p27^{Kip1} downregulation in cancer

p27^{Kip1} is a candidate tumour suppressor gene and abnormally low levels of the p27^{Kip1} protein are frequently found in human carcinomas. In general, reduced p27^{Kip1} levels correlate with a poor prognosis for cancer patients. Loss of both p27^{Kip1} alleles is a rare event in cancers, but loss of one allele is seen very often (176). It has been shown that knocking out p27^{Kip1} in mice results in multiple organ hyperplasia (109-111) and p27^{Kip1} null and heterozygous mice are predisposed to spontaneous and radiation- or chemical-induced tumours (177). The last means that the loss of one allele already might be enough to increase the susceptibility to carcinogenesis.

cAMP as anticancer drug

As cAMP is a potent inhibitor of proliferation in certain cell types, it is a potential anticancer drug. Experiments with animals have demonstrated that cAMP analogues can inhibit tumour growth in vivo and therefore, several cAMP analogues have been developed for the use as anticancer drugs. However, most of the first-generation analogues were not specific enough, were instable and poorly membrane permeable and had other side effects. Some of the most promising analogues have been used in clinical trials and, 8-Cl-cAMP, in particular, has proven to be beneficial for cancer patients (reviewed in (178)), although these effects of 8-Cl-cAMP turned out to be exerted mainly via its breakdown product 8-Cl-adenosine (179,180). New analogues have been developed, using the increased knowledge on signal transduction mechanism. These analogues exhibit higher membrane permeability, increased resistance against degradation, and improved target specificity and have led to new therapeutic strategies (reviewed in (178)).

Scope of this thesis

In this thesis the cAMP-dependent regulation of activity and expression levels of several putative cAMP-targets is investigated. Furthermore, the role of some new, genuine cAMP-targets in proliferation is examined.

In chapter 2 we address the question whether the guanine nucleotide exchange factors PDZ-GEF1 and -2 are regulated by cAMP to activate the small GTPase Rap1. We characterize both proteins and show that cAMP does hardly bind to PDZ-GEF in vitro or in vivo, nor changes its activity.

In chapter 3, the FoxO transcription factors are identified as mediators of a cAMP-induced cell cycle arrest in MEFs. FoxO transcription factors are known to be negatively regulated by the PI3K/PKB pathway and we show that cAMP can inhibit PKB-activity in MEFs.

Furthermore, we show that FoxO activity is regulated by cAMP and that a FoxO-mediated cAMP-induced cell cycle arrest likely involves $p27^{Kip1}$.

Screening for new cAMP-targets is described in chapter 4. We searched for genes that are regulated by cAMP at the (post-)transcriptional level by microarray analysis to find new targets involved in proliferation. These targets are discriminated for regulation by MAPK or not. A few of these new cAMP-targets are further investigated for their role in a cAMP-induced cell cycle arrest.

In the first addendum we describe that the PKB/FoxO-mediated mechanism, by which cAMP inhibits proliferation in MEFs, does not play a role in NIH3T3-A14 cells. In these cells $p27^{Kip1}$ is not regulated at the transcriptional level as in MEFs, but clearly translocated to the nucleus by cAMP. In the second addendum we try to identify new Rap1 targets, regulated by the Epac-specific cAMP analogue 8CPT-2'OMe-cAMP, by microarray analysis, whereas in the third addendum the insulin-regulated targets of NIH3T3-A14 cells are discussed.

The implications of the work described here and new models derived from it, are discussed in chapter 5.

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Chapter

2

Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2

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Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2

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PDZ-GEF1 (RA-GEF/nRapGEP/CNrasGEF) is a guanine nucleotide exchange factor (GEF) characterised by the presence of a PSD-95/DlgA/ZO-1 (PDZ) domain, a Ras-association (RA) domain and a region related to a cyclic nucleotide binding domain (RCBD). These domains are in addition to a Ras exchange motif (REM) and GEF domain characteristic for GEFs for Ras-like small GTPases. PDZ-GEF1 efficiently exchanges nucleotides of both Rap1 and Rap2, but has also been implicated in mediating cAMP-induced Ras activation through binding of cAMP to the RCBD. Here we describe a new family member, PDZ-GEF2, of which we isolated two splice variants (PDZ-GEF2A and 2B). PDZ-GEF2 contains in addition to the domains characteristic for PDZ-GEF1 a second, less conserved RCBD at the N-terminus. PDZ-GEF2 is also specific for both Rap1 and Rap2. We further investigated the possibility that PDZ-GEF2, like PDZ-GEF1, is a cAMP-responsive GEF for Ras. However, in contrast to previous results, we did not find any effect of both PDZ-GEF1 and PDZ-GEF2 on Ras in the absence or presence of cAMP. Moreover, affinity measurements by isothermic calorimetry showed that the RCBD of PDZ-GEF1 does not bind cAMP with a physiologically relevant affinity. We conclude that both PDZ-GEF1 and 2 are specific for Rap1 and Rap2 and unresponsive to cAMP and various other nucleotides.

INTRODUCTION

Rap1 is a Ras-like small GTPase that may function in a variety of different cellular processes, like integrin-mediated cell adhesion, cell proliferation and differentiation, and platelet activation (1,2). A variety of extra-cellular stimuli (e.g. growth hormones and cytokines) are able to activate Rap1 (3). Activation is mediated by guanine nucleotide exchange factors (GEFs) that substitute the bound GDP for GTP. Four types of Rap specific GEFs have been identified, some of which are regulated directly by second messengers. CD-GEF1 and 3 are presumably regulated by calcium and diacylglycerol (DAG), since both contain calcium and DAG binding domains (4-6). Epac1 and 2, are GEFs directly regulated by cAMP through binding of cAMP to a cAMP-binding domain very similar to those present in the regulatory domain of protein kinase A (PKA) (7-9). C3G is a GEF that is activated by receptor tyrosine kinases by binding to the activated receptor through the adapter protein Crk (10,11). The most recently identified GEF for Rap1 is PDZ-GEF1.

PDZ-GEF1 (also called RA-GEF-1, nRap GEP or CNrasGEF) (12-15) is characterised by the presence of a PSD-95/DlgA/ZO-1 (PDZ) domain, a Ras-association (RA) domain and a region related to a cyclic nucleotide binding domain (RCBD). In addition, it contains a Ras exchange motif (REM) and a GEF domain characteristic for GEFs for Ras-like small GTPases. At the C-terminus of PDZ-GEF1 a proline-rich regions and a PDZ binding motif are found. The GEF domain of PDZ-GEF1 efficiently exchanges nucleotides of both Rap1 and its close relative Rap2. The various other domains likely play a role in the regulation of activity or localisation of the protein.

The RA domain interacts with active Rap1 and also contributes to membrane localisation of PDZ-GEF1 (16). This domain may function in a positive feedback loop. The PDZ-binding motif was found to interact with the scaffolding protein S-SCAM (or MAGI-2) and MAGI-1 (14,17). These scaffolding proteins are localised to synaptic structures and cell adherens junctions respectively, implying that PDZ-GEF1 might be localised there as well. Indeed PDZ-GEF1 has been found to co-localise with β-catenin and ZO-1 at sites of cell-cell contact (18). Finally, also the PDZ domain plays a role in localisation, as deletion of a critical part of the PDZ domain affects plasma membrane localisation (15). PDZ domains are protein-protein interaction domains, usually interacting with the C-terminus of membrane proteins containing a PDZ-binding motif, but for PDZ-GEF1 the partner of the PDZ domain has not yet been identified. The stability of the PDZ-GEF protein is regulated by the E3 ligase Nedd4, which can bind to the proline-rich regions of PDZ-GEF1. Nedd4 ubiquitinates PDZ-GEF1, leading to degradation by the proteasome (19). The RCDB of PDZ-GEF1 is closely related to the cAMP-binding domain of Epac. However it lacks several critical residues involved in cAMP binding. The function of this domain in the regulation of PDZ-GEF1 with respect to GEF activity towards Rap1 and Rap2 is still unclear, although deletion of the RCBD results in vitro in more GEF activity (12). Intriguingly, Pham et al. reported that CNrasGEF, a protein identical to PDZ-GEF1, is a cAMP-responsive GEF for the small GTPase Ras (15).

We describe here the identification of PDZ-GEF2, a close relative of PDZ-GEF1, which is also a GEF specific for Rap1 and Rap2. No activity towards Ras was observed in vitro. PDZ-GEF2 contains in comparison to PDZ-GEF1 an extra, less conserved, RCBD at the N-terminus. We identified two splice variants of PDZ-GEF2 (2A and 2B). Recently, a third splice variant of PDZ-GEF2 (RA-GEF-2) was described (20). In addition we characterised both PDZ-GEF1 and 2 in further detail and found no evidence for cAMP binding by the RCBD, nor exchange activity towards Ras. We conclude that both PDZ-GEFs are specific for Rap1 and Rap2 and unresponsive to cAMP.

MATERIALS AND METHODS

Cloning of PDZ-GEF2A and 2B

Open reading frames (ORFs) of human PDZ-GEF2A and 2B were predicted on basis of ESTs, using a blast program (NCBI) (21), and on basis of intron/exon predictions for the genomic sequence by NIX analysis (http://www.hgmp. mrc.ac.uk/). Total RNA was isolated from umbilical cord tissue and Jurkat T-cells using RNAzol, according to the manufacturer's protocol (Campro Scientific). cDNA was generated from these total RNA extracts with poly-dT primers, using the Promega reverse transcription system. Three sets of primers were designed to amplify the whole coding sequence: N-for (gtcgacGAACTCACCCGTGGACC) and N-rev (TAAGTTGGATCCACGATGG) for the N-terminal part, M-for (gtcgacAGAGGGAGAAATTGTTA TGG) and M-rev (gcggccgcAAGGTACCATATGCAG G) for the middle part, C-for (TTGGGAAAAGTTACCA AGC) and CA-rev (gcggccgcAAATAGGTCATCCAAA GG) and CB-rev (gcggccgcTTCATCAGAGTGTCTTC C) respectively for the C-terminal part of PDZ-GEF2A and 2B. The PCR products were subcloned in pGEM-T vectors (Promega) and sequenced. Full-length PDZ-GEF2A, PDZ-GEF2A-*ΔRCBD* (amino acid 393-1601)

and PDZ-GEF2-*ΔC* (amino acid 1-1141) were cloned using the unique internal *Bam*HI and *Bgl*II sites and the by PCR created *Sal*I and *Not*I sites. The RCBD of PDZ-GEF2 (amino acid 1-417) was subcloned in the *Sal*I and *Bam*HI sites of pBluesript (Stratagene), using the by PCR introduced *Sal*I site and a PDZ-GEF2 internal *Bcl*I site. For expression in mammalian cells, these construct were subcloned in the *Sal*I and *Not*I sites of pMT2-HA, in frame with the HA-tag. For protein purification PDZ-GEF2-*ΔC*, PDZ-GEF2-*ΔRCBD-ΔC* (amino acid 393-1141) and the RCBD of PDZ-GEF2 were cloned in the *Xho*I and *Not*I sites of pGEX-4T3 (Pharmacia) in frame with the GST-tag, using the *Sal*I and *Not*I sites.

Protein expression and purification

GST-tagged PDZ-GEF proteins were expressed in *E.coli* BL21 by induction with 0.1 mM IPTG for 20 h at room temperature. The bacteria were collected and lysed in ice-cold phosphate-buffered saline containing 0.5% Triton X100 and protease inhibitors and sonicated 6 times for 20 seconds. Lysates were cleared by centrifugation at 10,000 x g and glycerol was added at 10% final concentration. The GST fusion proteins were purified by incubation of the lysate with glutathione-agarose beads (Sigma) and eluted with 10mM glutathione in buffer containing 50 mM Tris, pH 7.5; 100 mM NaCl; 10% glycerol and 2mM $MgCl_2$. Purification of small GTPases and the proteins used for the experiment shown in figure 5 were described elsewhere (22,23).

Cell culture, transfection and stable cell lines

NIH-3T3-A14 cells and Rat1 fibroblasts were cultured in DMEM, supplemented with 10% fetal bovine serum and 0.05% glutamine. Cells were transfected, using the calcium-phosphate precipitation method (24). Stable cell lines were made by transfecting NIH-3T3-A14 cells and Rat1 cells with the vector pBabe, which contains the puromycin resistance gene, or with pBabe together with a HA-tagged PDZ-GEF construct (ratio 1:10). Two days after transfection, medium containing 2 μg/ml puromycin (Sigma) was added. Single colonies were picked and tested for expression. Monoclonal cell lines were made from clones expressing full-length PDZ-GEF1, by limiting dilution.

Northern and Western blotting

A multiple tissue northern with poly(A) RNA from various human tissues (Clontech) was probed, according to the protocol of the manufacturer, with a ^{32}P-radioactive labelled PDZ-GEF2 probe, coding for the sequence spanning the GEF domain. Western blotting of total lysates and protein samples, isolated with an activation specific probe, was performed using polyvinylidene difluoride membranes (NEN^{TM}). Antibodies used to detect the proteins were anti HA (12CA5; sc-805, Santa Cruz), Rap-1/Krev-1 (sc-65, Santa Cruz), Ras (Transduction Laboratories) and phospho-CREB (Ser133, Cell Signaling).

In vitro activation of small GTPases

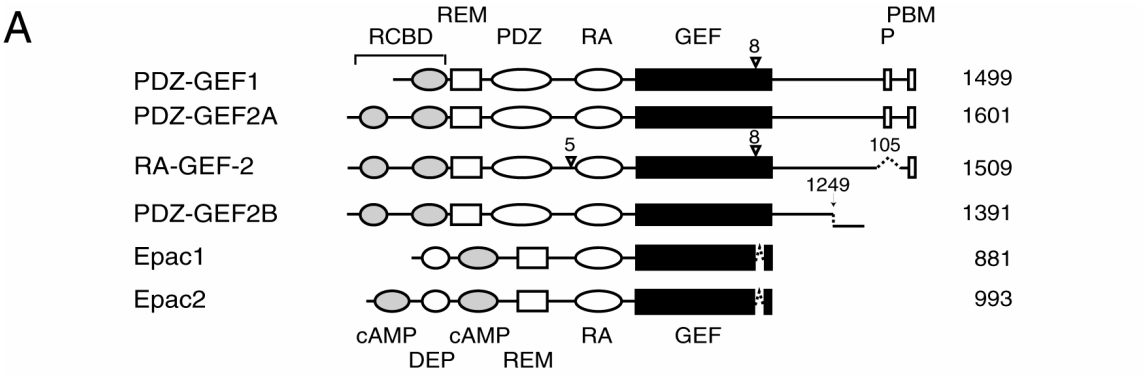
GEF activity was measured in vitro as described (22). Briefly, 100 to 400 nM of the purified small GTPase loaded with fluorescent labelled 2',3'-bis(O)-N-methyl-antharanoloyl-guanosinediphosphate (mantGDP), was incubated with 20 to 200 nM of purified GEF in the presence of excess unlabeled GDP. Nucleotides were added at 100 μM final concentrations. Release of mantGDP was measured in real time as a decrease in fluorescence. Reaction rates were calculated from fitted, single exponential curves. Cell extracts were made as follows. Cells were sheared 20 times through a 23G needle in buffer containing 50 mM Tris, pH 7.5; 100 mM NaCl; 10% glycerol; 2mM $MgCl_2$. Lysates were cleared by centrifugation at 10,000 x g and molecules smaller than 3 kDa were collected by spinning the lysates through a 3 kDa size column (Centricon).

In vivo activation of small GTPases

NIH-3T3-A14 cells were transiently transfected with HA-tagged PDZ-GEF constructs in combination with HA-tagged Rap1 (cloned in pMT2-HA vector). Alternatively, cell lines stably expressing HA-tagged PDZ-GEF1 or PDZ-GEF2-*ΔC* were used. For the experiment shown in figure 3E cells were serum starved for 20 h prior to lysis. Cells were stimulated with forskolin (20 μM) in combination with IBMX (1 mM) for 5 or 15 minutes or with EGF (25 ng/ml) for 2 or 5 minutes. GTP-bound Rap was isolated using purified GST-RalGDS-RBD protein as an activation specific probe as described (25) and for GTP-bound Ras purified GST-Raf-RBD was used (26).

Isothermal titration calorimetry (ITC) measurements

ITC measurements were done for cAMP binding to the RCBD domain of PDZ-GEF1 (amino acid 1-268) and to the cAMP-binding domain of Epac1. The isolated domains (cleaved from the GST-tag) were thermostatted in the cell of the apparatus to 25°C and cAMP was injected from a syringe, in 40 steps, up to a 2-4 fold molar excess. The cell contains 1.36 ml protein solution (0.63 mM PDZ-GEF1-RCBD or 66.7 μM Epac1 cAMP-binding domain) in buffer containing 50 mM Tris-HCl pH 7.6; 50 mM NaCl; 5% glycerol and 5 mM DTE. Typically cAMP was added in steps of 6 μl every four minutes. The data were analysed using the manufacturers software.


A
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RCBD
PDZ
RA
GEF
PBM
P
8
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PDZ-GEF2A
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RA-GEF-2
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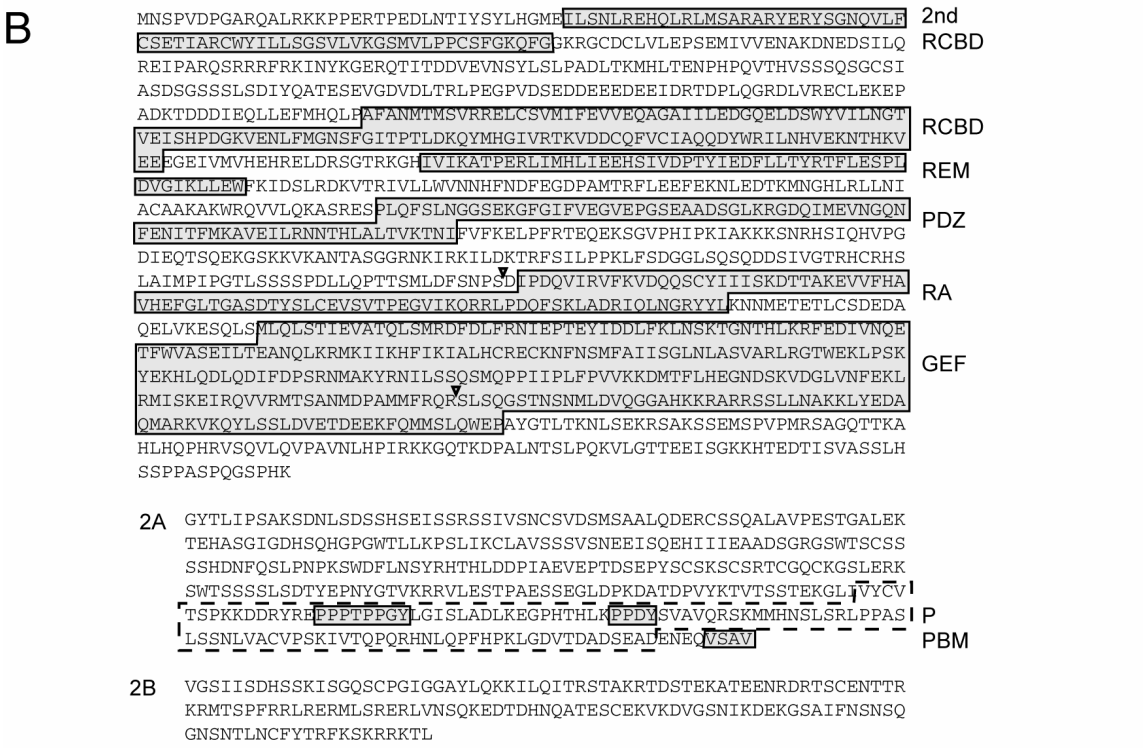
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2nd
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RCBD
REIPARQSRRRFRKINYKGERQTITDDVEVNSYLSLPADLTKMHLTENPHPQVTHVSSSQSGCSI
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ADKTDDDIEQLLEFMHQLPAFANMTMSVRRELCSVMIFEVVEQAGAIILEDGQELDSWYVILNGT
RCBD
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EEEGEIVMVHEHRELDRSGTRKGHIVIKATPERLIMHLIEEHSIVDPTYIEDFLLTYRTFLESPL
REM
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SSPPASPQGSPHK
2A
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P
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PBM
2B
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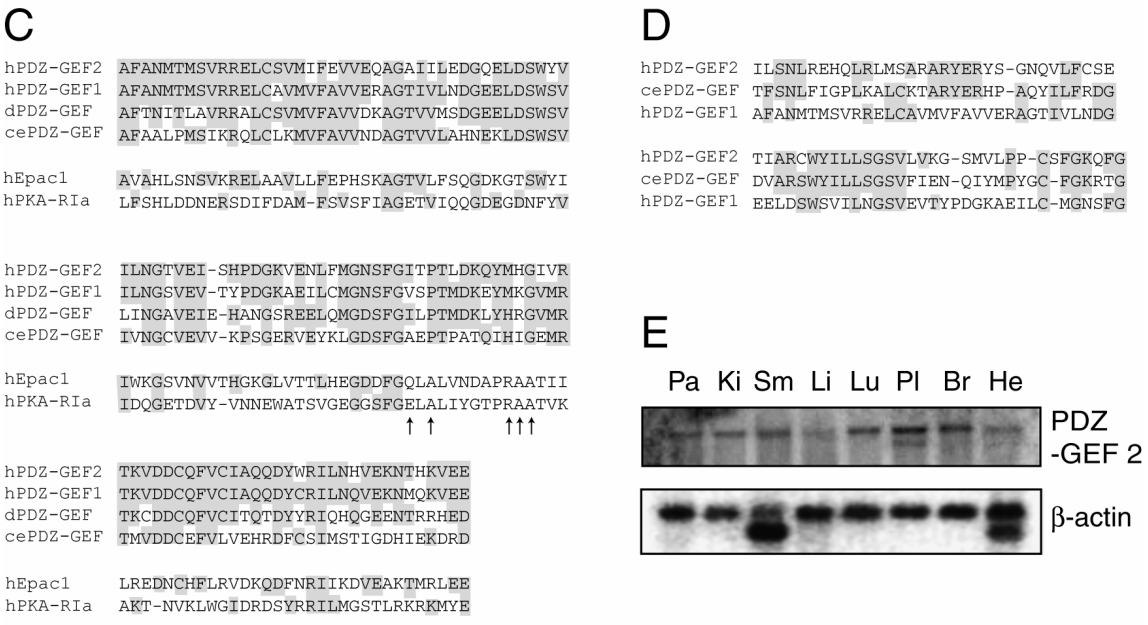
C
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dPDZ-GEF AFTNITLAVRRALCSVMVFAVVDKAGTVVMSDGEELDSWSV
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hPKA-RIa AKT-NVKLWGIDRDSYRRILMGSTLRKRKMYE
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cePDZ-GEF TFSNLFIGPLKALCKTARYERHP-AQYILFRDG
hPDZ-GEF1 AFANMTMSVRRELCAVMVFAVVERAGTIVLNDG
hPDZ-GEF2 TIARCWYILLSGSVLVKG-SMVLPP-CSFGKQFG
cePDZ-GEF DVARSWYILLSGSVFIEN-QIYMPYGC-FGKRTG
hPDZ-GEF1 EELDSWSVILNGSVEVTYPDGKAEILC-MGNSFG
E
Pa Ki Sm Li Lu Pl Br He
PDZ
-GEF 2
β-actin

RESULTS

Cloning of PDZ-GEF2A and 2B

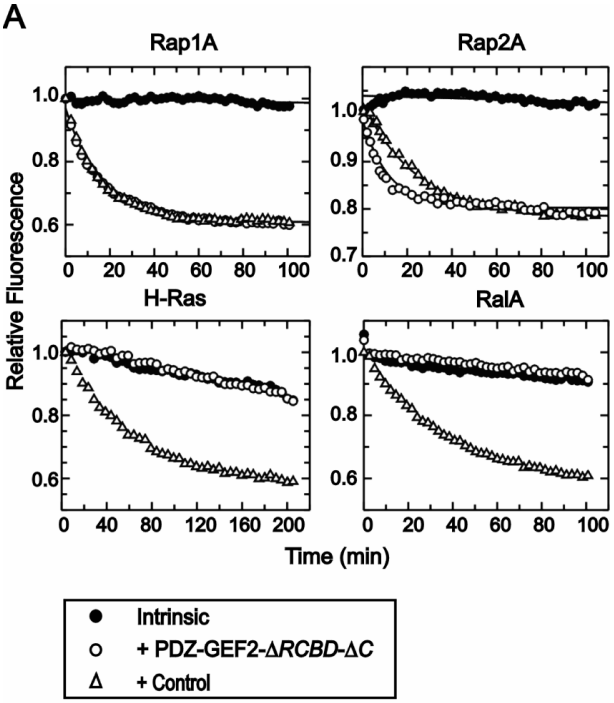
The human PDZ-GEF2 gene was identified in a genomic sequence of chromosome 5 by homology searches with PDZ-GEF1 (12). The intron/exon structure was predicted and primers were designed to amplify cDNA from human umbilical cord cDNA and from Jurkat T-cell cDNA. Two different mRNAs were identified, which differ in their C-terminus due to alternative splicing. Like PDZ-GEF1 (12,17), PDZ-GEF2 is rather ubiquitously expressed (figure 1E). The transcript is about 9 kb and slightly larger than the PDZ-GEF1 transcript. Using a probe specific for PDZ-GEF2B mRNA we did not obtain a clear signal, indicating that the expression of this splice isoform is low or restricted. PDZ-GEF2A encodes a protein of 1601 amino acids, whereas PDZ-GEF2B encodes a protein of 1391 amino acids. The two proteins differ after amino acid residue 1249. PDZ-GEF2A is most homologous to PDZ-GEF1, with all the domains conserved. PDZ-GEF2B lacks the C-terminal proline-rich sequences and the PDZ binding motif, which are replaced by a sequence with no apparent homology (figure 1A and B). Unlike PDZ-GEF1, PDZ-GEF2 contains an additional, N-terminal domain, which is distantly related to the RCBD (figure 1D). This domain is also present in the single PDZ-GEF from *C. elegans*. This situation is reminiscent to that for Epac, in that Epac1 contains a single and Epac2 a double cAMP-binding motif (figure 1A).

Figure 1. ***PDZ-GEF organisation.*** (A) Domain organisation of PDZ-GEFs in comparison to Epac. The arrow with amino acid number 1249 indicates the site from which PDZ-GEF2A and 2B differ. Extra or deleted exons, compared to PDZ-GEF2A are indicated with amino acid counts in PDZ-GEF1 and RA-GEF-2, a sequence recently published by Gao et al. (20). P stands for proline-rich region and PBM stands for PDZ binding motif. (B) Complete amino acid sequence of PDZ-GEF2A and 2B. The C-terminal ends where they differ are given separately, indicated by '2A' and '2B'. Domains are lifted out with grey boxes. Sites where RA-GEF-2 has small extra exons are indicated with arrowheads, and the exon that is not present in RA-GEF-2 is in the PDZ-GEF2A C-terminal part shown by a box with dashed sites. (C) Alignment of the RCBD of PDZ-GEFs from different species and the cAMP-binding domain of Epac1 and PKA. Identical amino acids are in a grey background. Amino acids in PKA that are involved in cAMP binding are indicated with arrows. (D) Alignment of the less conserved second RCBD domain of human PDZ-GEF2 and *C. elegans* PDZ-GEF with part of the RCBD of human PDZ-GEF1. (E) Tissue distribution of PDZ-GEF2. A Northern blot containing poly(A) mRNA from tissue of the pancreas (Pa), kidney (Ki), smooth muscles (Sm), liver (Li), lung (Lu), placenta (Pl), brain (Br) and heart (He) was probed with a PDZ-GEF2 sequence, spanning the catalytic domain.

PDZ-GEF2 specifically activates both Rap1 and Rap2 in vitro

To investigate the specificity of PDZ-GEF2 in vitro, a GST fusion protein named PDZ-GEF2-*ΔRCBD-ΔC* (figure 3A) was purified. This PDZ-GEF2 fusion protein lacks both the N-terminus, including the RCBD domains (1-391) and the C-terminus (1142-1601). For comparison we used a similar construct for PDZ-GEF1 (12). The proteins were incubated with different small GTPases loaded with mantGDP, in the presence of an excess of unlabeled GDP. Exchange activity was measured in real time as a decrease in fluorescence. PDZ-GEF2 clearly showed exchange activity towards both Rap1 and Rap2, but not to H-Ras or RalA (figure 2A). Also no effect of PDZ-GEF1 and 2 on N-Ras was observed (data not shown). The catalytic activities were quantified as reaction rates calculated from fitted single exponential curves and compared to the intrinsic GTPase activities. PDZ-GEF2, like PDZ-GEF1, increased nucleotide exchange several hundred-folds and was slightly more active towards Rap2 than to Rap1 (figure 2B) (12). From these results we conclude that in vitro both PDZ-GEF1 and 2 are GEFs for Rap1 and Rap 2, but not for Ras.

A



B

	Rate (per min)	Fold induction
Rap1A intrinsic	0.0002	
+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0623	310
Rap2A intrinsic	0.0002	
+ PDZ-GEF1-Δ*RCBD*-Δ*C*	0.0413	210
+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0849	420

Figure 2. ***In vitro activation of Rap1 and Rap2 by PDZ-GEF2.*** (A) Release of mantGDP from the loaded small GTPases, measured in vitro in real time in the presence or absence of exchange factors. The intrinsic activity of the relevant small GTPases (black dots) and the activity of PDZ-GEF2-*ΔRCBD-ΔC* towards the GTPase (open circles) are shown. As a control, Rap1 was incubated with Epac1-*ΔDEP* in the presence of cAMP, Rap2 with PDZ-GEF1-*ΔRCBD-ΔC*, H-Ras with CalDAGGEF3 and RalA with EDTA (open triangles). (B) Reaction rates for figure 2A are given for fitted curves for Rap1 and Rap2 together with PDZ-GEF, and the fold induction was calculated.

PDZ-GEF2 does activate Rap1 in vivo, but not Ras

NIH-3T3-A14 cells were transiently transfected with increasing amounts of HA-tagged PDZ-GEF1, PDZ-GEF2A or PDZ-GEF2A-*ΔRCBD* (figure 3A) together with HA-tagged Rap1A. Activation of HA-Rap1 was measured using GST-RalGDS-RBD as an activation specific probe (25). All the PDZ-GEF constructs used were able to activate Rap1 in vivo in a concentration dependent manner (figure 3B). To further study the regulation of Rap1 and possibly Ras by PDZ-GEF1 and 2, we constructed Rat1 and NIH-3T3-A14 cell lines stably expressing different PDZ-GEF constructs, i.e. full-length PDZ-GEF1, PDZ-GEF2-*ΔC*, PDZ-GEF1-*ΔRCBD-ΔC* and PDZ-GEF2-*ΔRCBD* (figure 3A). Strikingly, the cell lines expressing a PDZ-GEF construct lacking the RCBD domain (PDZ-GEF1-*ΔRCBD-ΔC* and PDZ-GEF2-*ΔRCBD*) were unstable. Analysis of a number of Rat1 cells expressing full-length PDZ-GEF1 did not show enhanced Rap1 activity (figure 3C). Similar results were obtained for PDZ-GEF1 expressing NIH-3T3-A14 cells (data not shown). These results suggest that full-length PDZ-GEF1, when stably expressed, is normally inactive.
Interestingly, cells expressing PDZ-GEF2-*ΔC* did show elevated levels of Rap1-GTP (figure 3D), raising the possibility that the C-terminus might play an inhibitory role in the regulation of catalytic activity.

The cell lines stably expressing PDZ-GEF1 were used to investigate whether extra-cellular stimuli or second messengers could enhance PDZ-GEF activity. However, using EGF or endothelin, two known activators of Rap1 (3), we did not detect more Rap1-GTP in cells expressing full-length PDZ-GEF1 compared to the parental cell line (data not shown). Since previously it was reported that PDZ-GEF1 is responsive to cAMP with respect to Ras activation, we measured this effect using forskolin to activate adenylate cyclase. However, forskolin did not cause activation of Ras, but as expected did induce CREB phosphorylation (figure 3E). Also in transient transfection experiments, using full length PDZ-GEF1, Rap1 was not further activated upon stimulation with cAMP (data not shown).

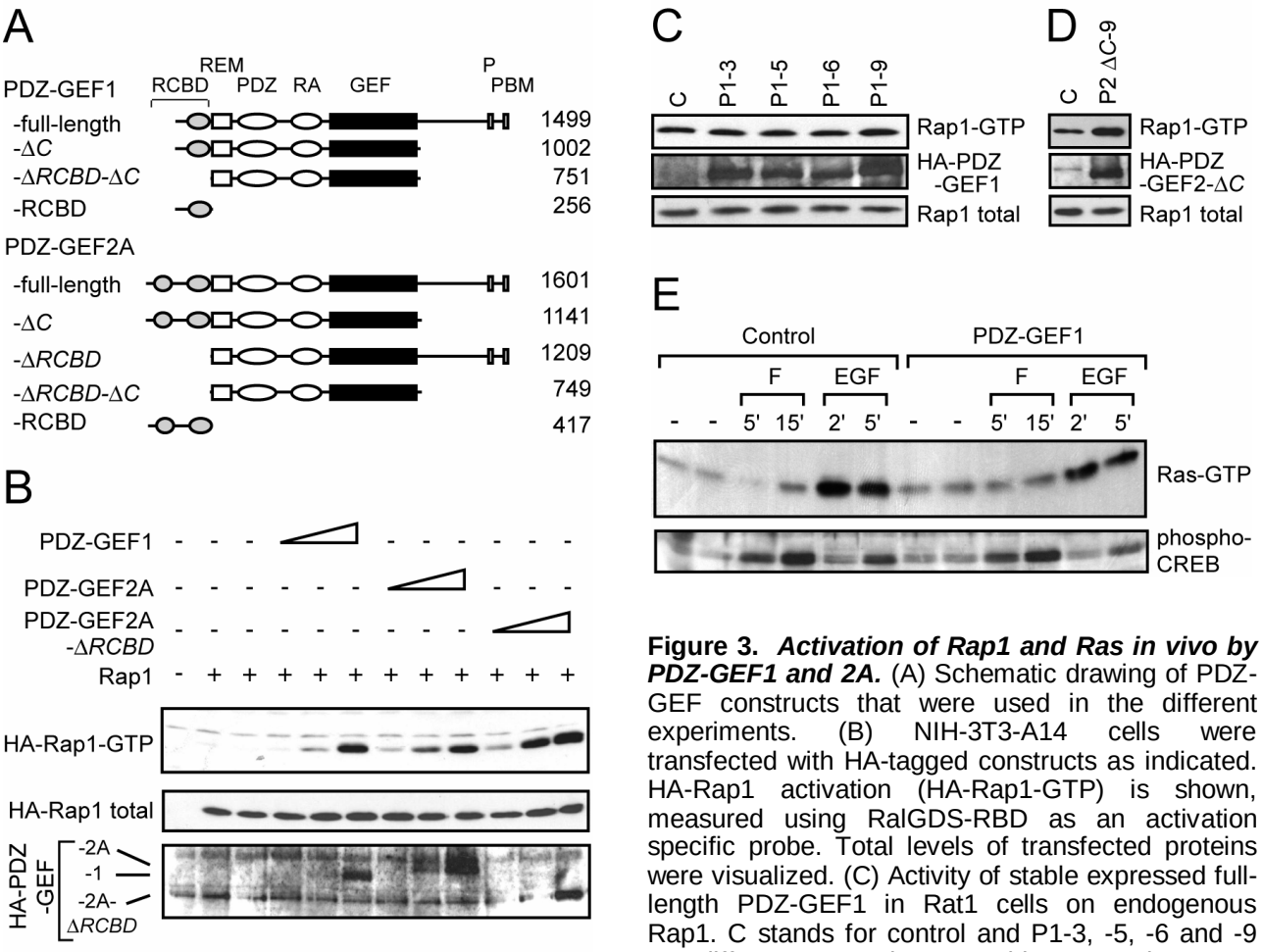


Figure 3. ***Activation of Rap1 and Ras in vivo by PDZ-GEF1 and 2A.*** (A) Schematic drawing of PDZ-GEF constructs that were used in the different experiments. (B) NIH-3T3-A14 cells were transfected with HA-tagged constructs as indicated. HA-Rap1 activation (HA-Rap1-GTP) is shown, measured using RalGDS-RBD as an activation specific probe. Total levels of transfected proteins were visualized. (C) Activity of stable expressed full-length PDZ-GEF1 in Rat1 cells on endogenous Rap1. C stands for control and P1-3, -5, -6 and -9 are different Rat1 clones, stably expressing PDZ-GEF1. (D) PDZ-GEF2-*ΔC*, stably expressed in NIH-3T3-A14 cells (clone P2-ΔC-9), activity towards Rap1, compared to the parental cell line (C). (E) A Rat1 cell line, stably expressing PDZ-GEF1, and a control cell line were stimulated with forskolin (F) and EGF for the indicated time scales. Ras activation (Ras-GTP) is shown, measured using Raf-RBD as an activation specific probe. The amount of phosphorylated CREB upon stimulation in the same protein samples was visualized as a control.

RCBD of PDZ-GEF1 and 2 did not respond to cAMP or other small molecules

To further investigate whether PDZ-GEF is responsive to cAMP, we measured the effect of cyclic nucleotides on PDZ-GEF activity in vitro. However, neither cAMP nor 8-Br-cGMP was able to stimulate PDZ-GEF2 catalytic activity towards Rap2 or H-Ras (figure 4A and B). Both Rap2 and H-Ras could be activated in this type of assay, as shown in figure 2A. In contrast, Epac1-*ΔDEP*, which shows hardly any activity towards Rap2 by itselves, was strongly stimulated by cAMP (figure 4C). From these and previous results with PDZ-GEF1 (12) we conclude that in vitro both PDZ-GEFs are unresponsive to cAMP. To further corroborate these findings, we determined the affinity of the RCBD of PDZ-GEF1 for cAMP in comparison to the cAMP-binding domain of Epac1 (figure 5). For PDZ-GEF1 the affinity was is in the mM range, whereas for Epac the affinity was in the μM range. Since the 1 mM affinity is very unlikely to be physiologically relevant we conclude that cAMP is not a binding partner for the RCBD of PDZ-GEFs.

A large part of the RCBD of PDZ-GEF is very homologues to the Epac cAMP-binding domain, with the notable exception of cAMP interacting amino acids (figure 1C). It may be

that other, perhaps cAMP-like second messengers can interact with the RCBD and activate PDZ-GEF. We therefore tested several different nucleotides in the in vitro assay using GST-PDZ-GEF1-*ΔC* (figure 3A) and Rap2A-mantGDP. However, AMP, ADP, GDP, ATP, GTP, ADP-ribose, cyclic ADP-ribose, β-NAADP, adenine, adenosine and guanosine did not affect PDZ-GEF1 activity (figure 4D, data not shown and (12)). To test more generally second messengers for their ability to activate PDZ-GEFs, Jurkat T-cells were stimulated with Lipopolysaccharide (LPS) or the phorbolester TPA, both known to activate Rap1. Extracts containing molecules smaller than 3 kDa were used in the assay in vitro. No effect was observed on PDZ-GEF1 activity (figure 4E). Also lysates from fMLP-stimulated neutrophils did not affect PDZ-GEF activity (data not shown). In contrast, a similar extract from forskolin-stimulated Jurkat cells did stimulate Epac1-*ΔDEP*, most likely due to the presence of cAMP (figure 4F), while lysates of cells stimulated by for instance serum did not stimulate Epac1 activity (data not shown).

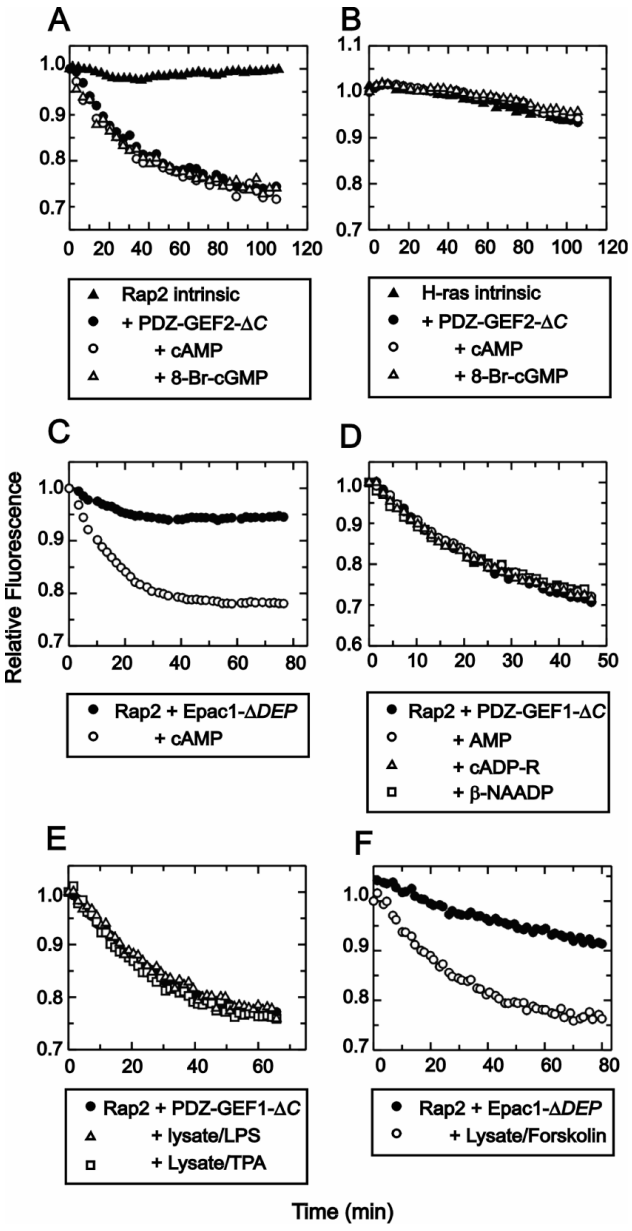
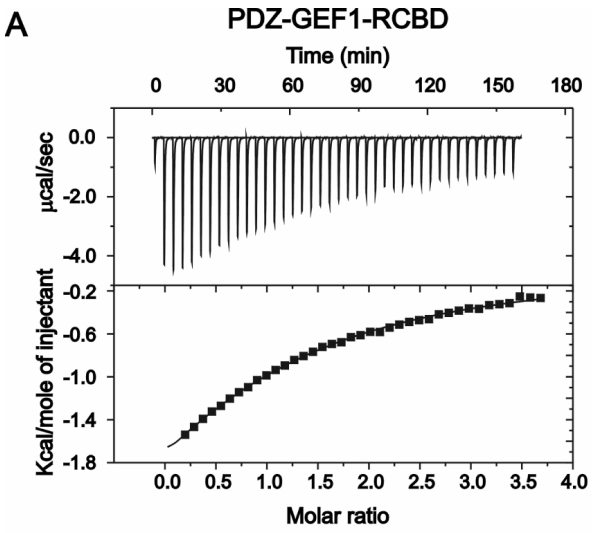
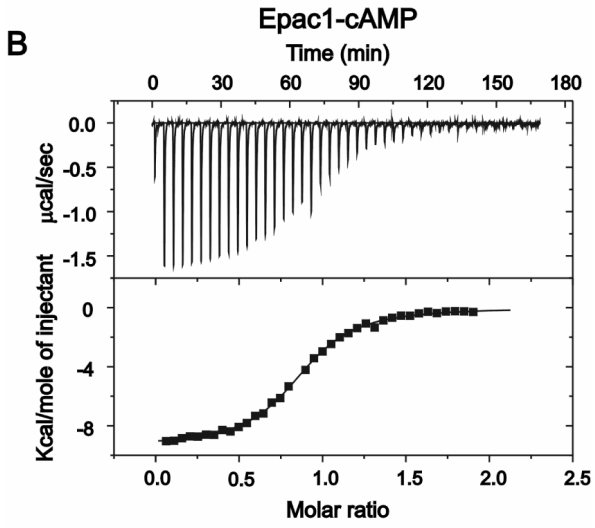


Figure 4. ***Role of second messengers in PDZ-GEF activation.*** MantGDP loaded small GTPases were in vitro incubated with PDZ-GEF1-*ΔC*, 2-*ΔC* or Epac1-*ΔDEP* in the presence or absence of second messengers. Intrinsic GTPase activity is shown by black triangles (A,B). Black dots indicate the activity of the exchange factor alone towards the GTPase. Open circles, triangles and squares show the activity of the exchange factor in the presence of the second messengers as indicated below. (A,B) Respectively Rap2A and H-Ras activation by PDZ-GEF2-*ΔC* in the presence of cAMP (open circles) or 8Br-cGMP (open triangles). (C) Rap2A activation by Epac1-*ΔDEP* in the presence of cAMP. (D) Rap2A activation by PDZ-GEF1-*ΔC* in the presence of AMP (open circles), cADP-Ribose (open triangles) or β-NAADP (open squares). (E,F) Rap2A activation by respectively PDZ-GEF1-*ΔC* and Epac1-*ΔDEP* in the presence of molecules smaller than 3 kDa from Jurkat T-cell lysates. Lysates from none stimulated cells (black dots) were added in both cases and lysates from cells stimulated for 5 minutes with LPS (open triangles) or TPA (open squares) were added (E) and lysates from cells stimulated for 10 minutes with forskolin (open circles) were added (F).

The RCBD of PDZ-GEF2 is in vitro not auto-inhibitory

Previously it was shown that the cAMP-binding domain of Epac clearly plays an inhibitory role, which is released in the presence of cAMP (7,9). Besides it was shown that PDZ-GEF1-*ΔC* in vitro is less active than PDZ-GEF1-*ΔRCBD-ΔC* and that the PDZ-GEF1-*ΔRCBD-ΔC* protein could be inhibited by the cAMP-binding domain of Epac1 (9,12). Based on these





C

	K_d (µM)
PDZ-GEF1-RCBD	1.0×10^3
Epac1-cAMP	2.8

Figure 5. ***Affinity of the PDZ-GEF1-RCBD for cAMP.*** The affinities of the isolated RCBD domain of PDZ-GEF1 (A) and the cAMP-binding domain of Epac1 (B) were determined by ITC. The upper parts of the graphics show the time dependent heating power detected after each injection of cAMP. In the lower parts, the integrated heating power is normalised to the concentration of injected cAMP and plotted against the molar ratio of the nucleotide and the protein. (C) Calculated Kds from the graphics above are plotted in a table.

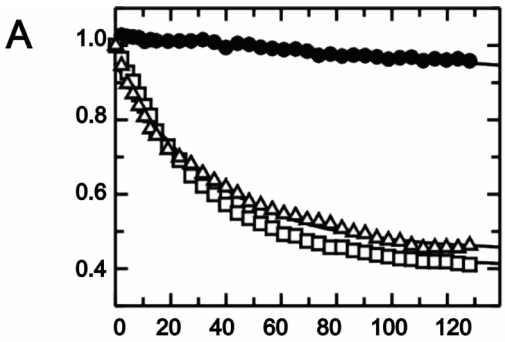
previous experiments we predicted an auto-inhibitory role for the RCBD of PDZ-GEF2. As shown by the calculated reaction rates, deletion of the RCBD had only a minor effect on the activity of the protein (figure 6A). In agreement with this, the GST-PDZ-GEF2-*ΔRCBD-ΔC* protein (figure 3A) could not be inhibited by the cAMP-binding domain of Epac1, or by the PDZ-GEF2-RCBD (figure 6B). We conclude from this that the influence of the RCBD on the activity of PDZ-GEF1 and 2 is different.

DISCUSSION

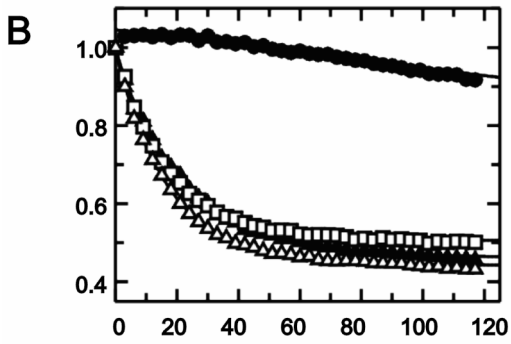
Here we describe the identification of two splice variants (2A and 2B) of a new PDZ-GEF family member, PDZ-GEF2. Like PDZ-GEF1 (12), PDZ-GEF2 is a guanine nucleotide exchange factor specific for Rap1 and Rap2. PDZ-GEF2 differs from PDZGEF1 in that it contains an N-terminal extension that has a distant similarity to the RCBD of PDZ-GEF1. This region is conserved in the single orthologue of PDZ-GEF in *C. elegans* (Ce-RA-GEF (13)) (figure 1D). Another difference resides in the RA domains, since the RA domain of PDZ-GEF1 interacts with GTP-bound Rap1, whereas the RA domain of PDZ-GEF2 interacts with GTP-bound M-Ras (16,20). The main differences between the PDZ-GEF2 isoforms are found at the C-terminus. PDZ-GEF2B lacks the proline-rich region and the PDZ binding motif, which are present in PDZ-GEF2A and PDZ-GEF1. Recently a third splice variant of PDZ-GEF2, RA-GEF-2, was identified (20). This splice variant has small additional sequences just upstream of the RA domain and at the end of the GEF region, but it lacks the proline-rich region of PDZ-GEF2A (figure 1A and 1B). These differences between the splice variants might be important for subcellular localisation and stability. For example, both PY motifs in the proline-rich region of PDZ-GEF2A may function as Nedd4 binding sites. For PDZ-

GEF1 it has been reported that the E3 ligase Nedd4 regulates its degradation, by binding to these sites (19). Second, the PDZ binding motif at the C-terminus, which is absent in PDZ-GEF2B, can bind to a PDZ domain of S-SCAM (or MAGI-2) and MAGI-1, resulting in a location at sites of cell-cell contact (14,17). This localisation may have an inhibitory effect on the activity of PDZ-GEFs since in general PDZ-GEFs lacking the C-terminus have a higher activity than full-length PDZ-GEFs (figure 3D). The RA domain is another part of PDZ-GEF1 that was found to influence the activity of PDZ-GEF1 (16). Surprisingly, we did not find elevated PDZ-GEF activity upon EGF and endothelin stimulation in cell lines expressing PDZ-GEF1. Since it is known that both stimuli activate Rap1, which can bind to the RA domain of PDZ-GEF1, it appears that GTP loaded Rap1 is not sufficient to activate this exchange factor. Indeed, in vitro Rap1 could also not activate PDZ-GEF1, although the RA domain in vivo was required for optimal activity (16). This suggests that either another small GTPase may activate PDZ-GEF1 via the RA domain, or that an additional signal is required.

One intriguing but controversial issue is the function of the RCDB in PDZ-GEFs. This domain is closely related to the cAMP-binding domain of Epac, PKA and cyclic nucleotide gated ion channels. However, residues that are critically involved in cAMP binding in PKA, like the 'PRAAT' sequence, are lacking (figure 1C). This suggests that PDZ-GEFs do not bind cAMP (or cGMP). Indeed we and others did not find any evidence for the regulation of PDZ-GEFs by cAMP in vitro, or in vivo (12-14), (this study). Here we demonstrate by direct affinity measurements using isothermic calorimetry that the affinity of the RCDB of PDZ-GEF1 for cAMP is too low to be physiologically relevant (figure 5). This result is at variance with observations by Pham et al. who suggested that PDZ-GEF1 (CNrasGEF)



		Rate (per min)	Fold induction
●	Rap1A intrinsic	0.0011	
□	+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0319	29
△	+ PDZ-GEF2-Δ*C*	0.0280	25



		Rate (per min)	Fold induction
●	Rap1A intrinsic	0.0016	
▲	+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0457	29
□	+ 15x Epac1-cAMP	0.0578	36
△	+ 15x PDZ-GEF2-RCBD	0.0584	37

Figure 6. ***Effect of the RCBD on PDZ-GEF2 activity in vitro.*** (A) Catalytic activity of PDZ-GEF2-*ΔC* (open triangles) and PDZ-GEF2-*ΔRCBD-ΔC* (open squares) measured in vitro as release of mantGDP from Rap1A. Intrinsic Rap1 activity is shown by black dots. Fold inductions were calculated from reaction rates of the fitted curves and plotted in a table. (B**)** Catalytic activity of PDZ-GEF2-*ΔRCBD-ΔC* (black triangles) measured as in figure 6A in the presence of 15-fold excess in molar concentrations of the PDZ-GEF2-RCBD (open triangles) or the Epac1 cAMP-binding domain (open squares). Fold inductions were calculated from reaction rates of the fitted curves and plotted in a table.

mediates cAMP-induced Ras activation through direct binding of cAMP to PDZ-GEF1. Activation of Rap1 however was not induced by cAMP in their experiments. Strikingly, in the same study no effect of cAMP was observed in vitro on the activation of Ras by immuno-precipitated PDZ-GEF1. This and our results indicate that indeed PDZ-GEFs are not responsive to cAMP and that the reported effect on Ras is indirect.

We previously hypothesised that the RCBD of PDZ-GEF1 has an auto-inhibitory function, which would be relieved by an unidentified second messenger (12). This was based on analogy to Epac, in which the cAMP-binding domain is clearly an auto-inhibitory domain that completely blocks Epac activity in vitro. Only in the presence of cAMP or after deletion of the cAMP-binding domain, Epac is active (7,9). For PDZ-GEF1 it was found that the RCBD has some inhibitory effect (12). However, we have not been able to identify a second messenger that could enhance the activity of the RCBD-containing protein (figure 4). Furthermore, deletion of the RCBD from our PDZ-GEF2 protein did not significantly affect its in vitro activity (figure 6). Together, these data indicate that the RCBD does not function as an inhibitory domain. On the other hand it should be realised that our studies in tissue culture cells still support our initial hypothesis. Of note, we completely failed to isolate cell lines stably expressing PDZ-GEF-*ΔRCBDs*, although we could easily establish cell lines expressing full-length PDZ-GEF1. Moreover, full-length PDZ-GEF1 expressing cell lines did not show any enhanced Rap1 activity. To our knowledge no other stable cell lines expressing activated RapGEFs have been reported. This suggests that cells cannot support highly active PDZ-GEFs and select against it. This may imply that the RCBD requires an additional protein for its regulation.

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Chapter

3

Activation of FoxO transcription factors contributes to the anti-proliferative effect of cAMP

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Submitted

Activation of FoxO transcription factors contributes to the anti-proliferative effect of cAMP

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cAMP is a potent inhibitor of cell proliferation in a variety of cell lines. Downregulation of cyclin D1 and upregulation of the cell cycle inhibitor $p27^{Kip1}$ are two mechanisms by which cAMP may induce a G1-arrest. Here we show that cAMP inhibits proliferation of cells that constitutively express cyclin D1 or are deficient for Rb, demonstrating that changes in these cell cycle regulators does not account for the cAMP-induced growth effects in MEFs. Interestingly, the anti-proliferative effect of cAMP mimics the effect previously observed for FoxO transcription factors. These transcription factors are under negative control of PKB. We show that in mouse embryo fibroblasts (MEFs) cAMP strongly induces transcriptional activation of FoxO4 through the inhibition of protein kinase B. Accordingly not only $p27^{Kip1}$, but also the FoxO target MnSOD is upregulated by cAMP. Importantly, introduction of dominant negative FoxO partially rescues cAMP-induced inhibition of proliferation. From these results we conclude that inhibition of PKB and subsequent activation of FoxO transcription factors mediates an anti-proliferative effect of cAMP.

INTRODUCTION

cAMP is an important regulator of proliferation, through modulation of cAMP-dependent protein kinase (PKA). Depending on the cell type, cAMP can either stimulate or inhibit proliferation. Inhibition of proliferation by cAMP takes place in the G1-phase of the cell cycle, and involves the cyclin-dependent kinase (CDK) complexes cyclin D1-CDK4/6 and cyclin E/A-CDK2 as well as the CDK inhibitors (CKIs) $p21^{Cip1}$ and $p27^{Kip1}$. Indeed, inhibition of cell proliferation by cAMP has most often been reported to correlate with and partially depend on a decrease in cyclin D1 or an increase in $p27^{Kip1}$ (1-6). The main pathways involved in the regulation of $p27^{Kip1}$ and cyclin D1 are the MAPK and PI3K pathways (7-10). Inhibition of the MAPK pathway by cAMP is well documented and takes place at the level of Raf kinase (11,12), which is phosphorylated by PKA (13-16). Inhibition of the MAPK pathway alone however, can not account for the G1-arrest induced by cAMP (16-18). Also the PI3K pathway may be inhibited by cAMP, but this has only been shown in a restricted number of cell types and the mechanism is elusive. It has been suggested that cAMP inhibits the pathway at the level of PI3K itself, resulting in disturbed PIP3 formation and PDK1 membrane localization (19,20).

Cyclin D1 in complex with CDK4 or CDK6 promotes proliferation by inhibiting the retinoblastoma (Rb) protein, which on its turn

blocks E2F transcription factors and thus the induction of genes involved in DNA replication (21). MAPK regulates cyclin D1 levels transcriptionally, via the ATF/CREB family of transcription factors and the CREB response element (CRE) in the cyclin D1 promoter (22). The PI3K/PKB pathway regulates cyclin D1 levels transcriptionally via regulation of FoxO transcription factors (23,24), at the translational level involving p70S6 kinase (10,25), and posttranscriptional by targeting it for degradation using GSK3β (26).

The cell cycle inhibitor $p27^{Kip1}$ acts during late G1-phase by binding and inhibiting CDK2-cyclin E/A complexes (21). Cells can only progress through the cell cycle when $p27^{Kip1}$ is dissociated from the CDK2-cyclin E/A complexes and this is in general achieved by degradation of $p27^{Kip1}$ (27,28). Degradation of $p27^{Kip1}$ is usually initiated through phosphorylation by CDK2 (29,30), but may also involve calpains (31), caspases (32) or jab1 (33). Furthermore, $p27^{Kip1}$ protein can be regulated at the transcriptional level by FoxO transcription factors (9) or by translational regulation (34,35). Finally, $p27^{Kip1}$ activity can also be diminished by sequestering it in cyclin D-CDK complexes (21) or by nuclear exclusion, which is preceded by phosphorylation of $p27^{Kip1}$ by PKB or KIS (36-40).

The picture that emerges is one in which cAMP affects different cell cycle components by multiple mechanisms. This, together with cell-type differences has obstructed identification of a uniform mechanism of cAMP-induced cell cycle arrest. For example, it has been hypothesized that the combined downregulation of cyclin D1 and upregulation of $p27^{Kip1}$ would be sufficient (41), but actual proof for this is lacking. Interestingly, upregulation of $p27^{Kip1}$ and downregulation of cyclin D1 is part of the program by which FoxO transcription factors induce a G1 arrest in non-hematopoietic cells (9,23,24). These FoxO transcription factors are negatively regulated by the PI3K/PKB pathway, and direct phosphorylation of FoxOs by PKB leads to nuclear exclusion (42,43). Therefore, we have tested here the involvement of FoxO in a cAMP-induced growth arrest. We show that in mouse embryo fibroblasts apart from the MAPK pathway, the PKB/FoxO pathway plays a prominent role in the anti-proliferative effect of cAMP.

MATERIALS AND METHODS

Cell culture, plasmids, antibodies and reagents

Culturing of Rb-deficient 3T3 cells, MEFs constitutively expressing cyclin D1, $p27^{Kip1}$-deficient MEFs and wild type MEFs has been described before (9,23). All cells used were cultured in DMEM, supplemented with 10% fetal bovine serum and 0.05% glutamine.

pMT2-HA-FoxO4, -DB, -A3, pSG5-gagPKB, pGL3-6xDBE-luc and pGL2-$p27^{Kip1}$-1609-luc have been described (9,44-47).

Antibodies directed against the following proteins were used: phospho-Thr202/Tyr204-p44/42 MAPK, phospho-Ser473 and -Thr308-PKB (Cell Signaling), PKB (44), cyclin D1 (Immunotech), GAPDH (Chemicon), HA (12CA5; (44)), ERK2 (48), p27 (Transduction Laboratories) and MnSOD (Stressgen).

The following stimuli and inhibitors were used at the following concentrations: forskolin (10 μM; ICN), IBMX (0.5 mM; Sigma), insulin (1 μg/ml; Sigma), U0126 (10 μM; Biomol Research Laboratories) and LY294002 (10 μM; Sigma).

Transfections and immunoblotting

Cells were transfected, using the calcium phosphate precipitation method, αPEI (49) or using the transfection agent fugene (Roche). In the case of pBabe-puro co-transfections, the cells were selected for at least 24 hours with 1 μg/ml puromycin. Following stimulation, cells were lysed by scraping in Ripa lysisbuffer (20 mM Tris-HCl pH 7.5, 1 % triton X-100, 0.5 % Na-DOC, 0.1 % SDS, 10 mM EDTA, 150 mM NaCl), supplemented with aprotinin, leupeptin, trypsin inhibitor, Na_3VO_4 and NaF. Ripa lysates were cleared by centrifugation and protein amounts were equalized, using standard Bradford protein quantification methods. Proteins were separated on SDS-polyacrylamide gels and blotted to polyvinylidene difluoride membranes (NEN™), following incubations

with primary and secondary antibodies. Proteins were visualized using standard enhanced chemiluminescence and auto-radiography.

Proliferation assay

Cells were plated in multi-well plates in triplicates, following stimulation the next day. Medium and stimuli were refreshed every two days and the cells were stained at 3 different days, 0 to 6 days after stimulation. To stain, the cells were fixed for 10 minutes in 10 % acetic acid at RT, prior to 10 minutes staining at RT with 0.4 % crystal violet, dissolved in 10 % acetic acid. The plates were washed twice with water and dried overnight. Proliferation was quantified by measuring the optical density at wavelength 560 nm of the in 10 % acetic acid re-dissolved crystal violet stain in the plates.

Flow cytometry

For determination of cell cycle distributions, untransfected cells or cells transfected with the indicated constructs together with pBabe-puro were used. Cells were stimulated for 24 or 48 hours as indicated and transfected cells were selected with 1 µg/ml puromycin for at least 24 hours. Cells were detached using EDTA, containing trypsin and washed twice, following fixation in 70 % ethanol for at least 16 hours at 4 ºC. Subsequently, cells were washed and resuspended in PBS, containing 0.1 % BSA, 0.25 mg/ml RNase and propidium iodide and were incubated for 30 minutes at 37 ºC prior to DNA profile measurements. Flow cytometry analysis was performed using a FACScalibur instrument (Becton Dickinson).

Luciferase assay

Cells were transfected in triplicates with renilla-luc and either 6xDBE-luc or $p27^{Kip1}$-promoter-luc, together with either pMT2-HA empty vector or pMT2-HA-FoxO4 and cells were stimulated as indicated. Two days after transfection, cells were lysed in passive lysis buffer (Promega) for 30 minutes at RT, where after lysates were cleared. Luciferase counts were measured using the dual-luciferase reporter assay system (Promega).

Immune fluorescence

Cells were plated on cover slides and stimulated for the indicated time points, followed by fixation in 4 % paraformaldehyde solution in PBS. Cells were blocked with blocking buffer (0.1 % saponin and 0.5 % BSA in PBS), followed by incubation with an anti-$p27^{Kip1}$ antibody. Subsequently, the cells were incubated with a donkey-anti-mouse-Cy3 conjugated antibody and DAPI. Cells were visualized using a Zeiss fluorescence microscope.

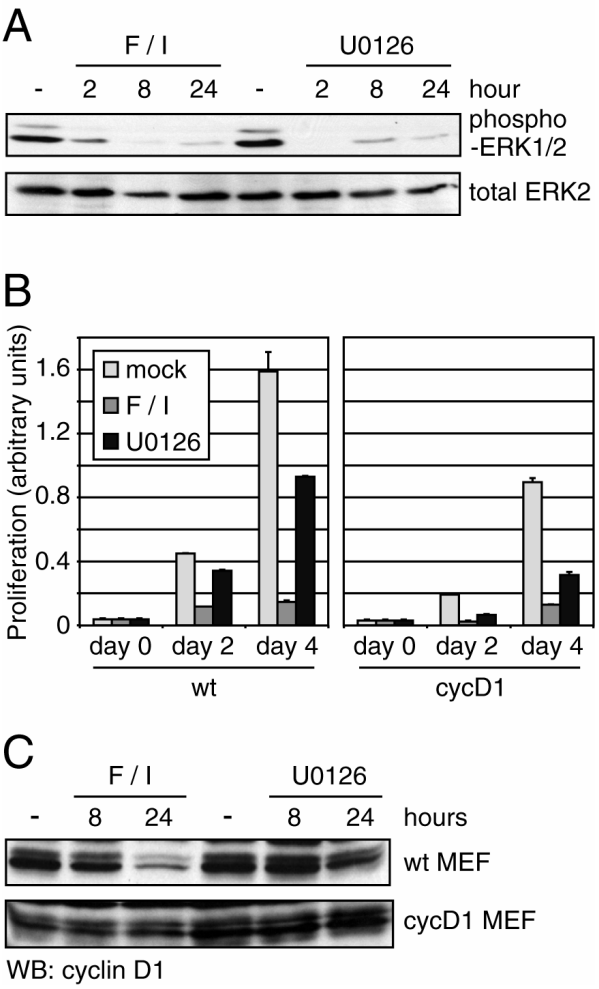

Figure 1. ***Inhibition of the MAPK pathway and cyclin D1 by cAMP is not sufficient for induction of a G1-arrest.*** (A) ERK-phosphorylation is inhibited to the same extent by cAMP and U0126. MEFs were stimulated for the indicated time points with forskolin/IBMX (F/I), U0126 or were left untreated. The amount of phosphorylated ERK1 and -2 and total ERK2 were visualized on a western blot. (B) Wild type (wt) and cycD1 MEFs are sensitive to cAMP-induced growth inhibition. Proliferation of wt and cycD1 MEFs was measured upon mock, forskolin/IBMX or U0126 treatment. The amount of cells was measured using crystal violet staining as read-out at 0, 2 and 4 days after stimulation. (C) Cyclin D1 protein levels are downregulated by cAMP in wt, but not in cycD1 MEFs. Wt and cycD1 MEFs were stimulated with forskolin/IBMX or U0126 for the indicated time points. Total amounts of cyclin D1 were visualized by immunoblotting.

RESULTS

Inhibition of the MAPK pathway and cyclin D1 by cAMP is not sufficient for the induction of a G1-arrest in cycling mouse embryo fibroblasts

We choose to study the growth inhibitory effects of cAMP in mouse embryo fibroblasts (MEFs) based on the fact that these cells are known to be responsive to cAMP and the existence of MEFs, derived from mice in which various cell cycle regulators have been deleted by targeted gene disruption. First we compared the effects of the cAMP-elevating compound forskolin (in combination with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX)) and that of the MEK-inhibitor U0126 on wild type MEFs grown in the presence of serum. Whereas both compounds inhibited the ERK pathway equally well (Figure 1A), forskolin/IBMX was clearly more effective in inhibiting proliferation (Figure 1B left panel). We then investigated the level of cyclin D1 under both conditions and found that cyclin D1 was clearly down-regulated by forskolin/IBMX, but only weakly and transiently down regulated by U0126 (Figure 1C upper panel). To see if this difference in cyclin D1 expression could explain the difference in growth rate, we made use of cycD1 MEFs, in which cyclin D1 is expressed under the control of a constitutive promoter at levels comparable to those seen in proliferating cells (23). As expected, forskolin/IBMX did not change cyclin D1 expression in these cells (Figure 1C lower panel). Despite this, the inhibition of growth by forskolin/IBMX was only slightly less as compared to that of wild type cells. Furthermore, forskolin/IBMX was still more potent in inhibiting proliferation than U0126 (Figure 1B right panel and Table 1). These findings are consistent with other data showing a partial involvement of the MAPK pathway and/or cyclin D1 in a cAMP-induced cell cycle arrest (16-18).

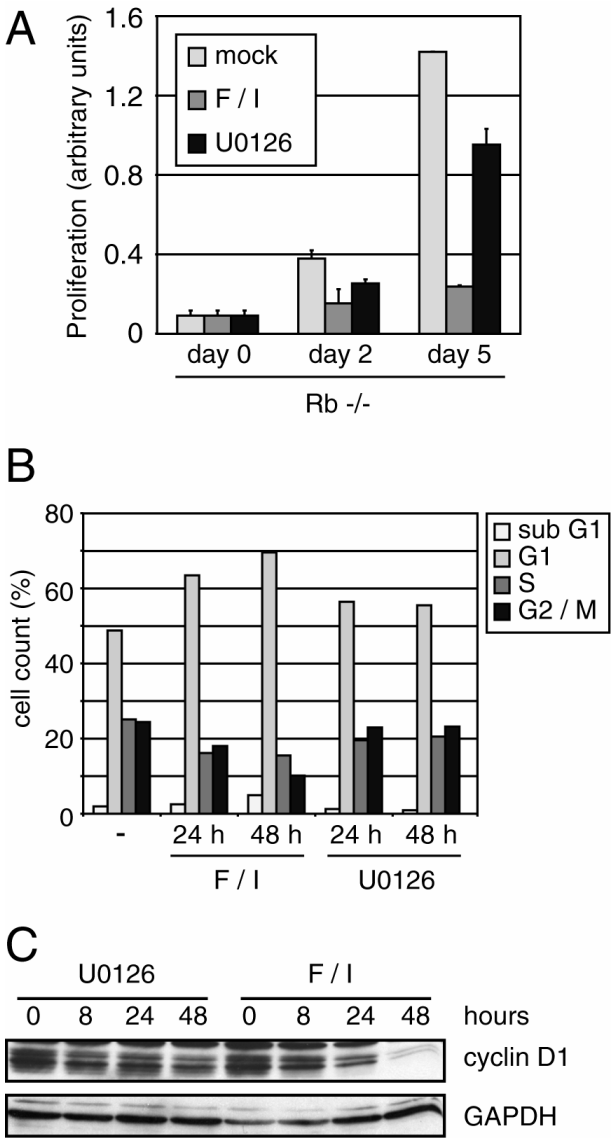

Figure 2. ***Effect of Rb-deficiency on cAMP-induced G1 arrest.*** (A) Rb-deficient cells are inhibited in proliferation by cAMP. Proliferation of $Rb^{-/-}$ cells was assayed as in Figure 1A. (B) cAMP induces a G1-arrest in Rb-deficient cells. $Rb^{-/-}$ cells were assayed for their cell cycle profile. Cells were treated for 24 or 48 hours with forskolin/IBMX (F/I) or U0126 or were left untreated. The cell cycle profile was determined by FACS analysis, using propidium iodide as staining. The percentage of cells per phase of the cell cycle was calculated and plotted. (C) Cyclin D1 is downregulated by cAMP in Rb-deficient cells. $Rb^{-/-}$ cells were stimulated with U0126 and forskolin/IBMX for the indicated time points. Cyclin D1 protein levels and GAPDH as a control were visualized by immunoblotting.

Table 1. ***Comparison of growth inhibition by cAMP and U0126 in different cell lines***

	Treatment			
	Forskolin / IBMX		U0126	
Cell type	Inhibition (%)	P value	Inhibition (%)	P value
wt MEF (n=3)	90 ± 1.7	-	43 ± 4.2	-
cycD1 MEF (n=2)	86 ± 0.3	0.0452*	65 ± 1.2	0.0062*
$p27^{-/-}$ MEF (n=4)	64 ± 6.6	0.0012*	51 ± 7.1	0.1712
$Rb^{-/-}$ 3T3 (n=2)	82 ± 1.8	0.0178*	34 ± 3.8	0.0983

Cells were treated with forskolin/IBMX, U0126 or left untreated and were grown until untreated cells reached confluency. At this time point, the amount of cells was measured and the percentage of growth inhibition in comparison to untreated cells was calculated of 2 to 4 independent experiments. The percentage of growth inhibition plus standard deviations and the statistical significance (P value) in comparison to wt MEFs are shown. P values with higher confidence then 95% are indicated with an asterix (*)

Consecutively, we tested the role of Rb, which acts downstream of cyclin D1, in the anti-proliferative effect of cAMP. However, proliferation of Rb-deficient 3T3 cells was still inhibited by forskolin/IBMX, to a comparable level as seen in cycD1 MEFs (Figure 2A and Table 1). Interference of apoptosis in the measurement of proliferation could be excluded, as there was no significant increase in apoptotic cells (Figure 2B, sub G1 fraction). Furthermore, forskolin/IBMX clearly induced a G1-arrest in the $Rb^{-/-}$ cells, whereas U0126 led to a minor increase (Figure 2B) and the change in cyclin D1 levels seen after forskolin/IBMX treatment was identical to that seen in the wild type MEFs (Figure 2C). We conclude that promoting the inhibitory action of Rb on E2F is not the only mechanism for a cAMP-induced G1 arrest in MEFs. This is in agreement with our observation that constitutively expressing cyclin D1 hardly affects the anti-proliferative effect of cAMP.

$p27^{Kip1}$ plays an important role in inhibition of proliferation by cAMP

To see if in addition to MAPK, $p27^{Kip1}$ is involved in a cAMP-induced G1-arrest of MEFs we first measured the protein levels of this cell cycle inhibitor. A clear increase in $p27^{Kip1}$ levels up to at least 48 hours after stimulation with forskolin/IBMX was seen, whereas U0126 gave a weaker and more transient increase (Figure 3A). Furthermore, stimulation with forskolin/IBMX induced translocation of $p27^{Kip1}$ to the nucleus (Figure 3B), where it is known to exert its inhibitory action. In contrast, hardly any $p27^{Kip1}$ translocated after stimulation with U0126 (data not shown). To investigate the importance of $p27^{Kip1}$ for inhibition of proliferation by cAMP, we made use of $p27^{Kip1}$-deficient MEFs. Although these cells grow slower than wild type cells, proliferation of the $p27^{-/-}$ MEFs was significantly less inhibited by treatment with forskolin/IBMX as compared to wild type cells. Furthermore, the residual inhibition of proliferation by cAMP was almost comparable to the effect of U0126 on proliferation (Figure 3C and Table 1). Importantly, the inhibition of proliferation was not due to an increase in apoptosis in these cells, as the amount of cells in the sub G1 fraction of the cell cycle profile was comparable to wild type MEFs (data not shown). This demonstrates that upregulation of $p27^{Kip1}$ in MEFs contributes to cAMP-induced growth arrest but is not solely responsible for this effect.

cAMP regulates p27^{Kip1} levels via transcriptional activity of FoxO transcription factors

To test if cyclin D1 in the absence of p27^{Kip1} upregulation would overcome a cAMP-induced G1-arrest, we tried to generate p27^{Kip1} knock-out MEFs, constitutively expressing cyclin D1. However, whereas we could express cyclin D1 in wild type MEFs, p27^{Kip1} knock-out MEFs expressing cyclin D1 appeared unstable. We then reasoned that FoxO transcription factors have been shown to inhibit proliferation by regulating both cyclin D1 and p27^{Kip1} levels (9,23,24). To investigate whether these transcription factors play a role in a cAMP-induced arrest, the effect of forskolin/IBMX on FoxO activity was tested. A reporter construct containing six DAF-16 family protein-binding elements (DBEs), which is known to be activated by FoxO family members (46), was used for this purpose. Luciferase activity was increased by co-transfection of FoxO4 and clearly elevated upon stimulation with cAMP (Figure 4A). cAMP was not able to increase luciferase activity in the absence of co-transfected FoxO4 or in the presence of constitutive active FoxO4 (data not shown), indicating that cAMP indeed regulates 6xDBE activity via FoxO transcription factors. The next step was to investigate whether regulation of the p27^{Kip1} promoter by cAMP was FoxO-dependent. To this end, a reporter construct containing the p27^{Kip1} promoter (-1609 to +178) (47) was used. Treatment of cells with forskolin/IBMX elevated FoxO4-induced luciferase activity (Figure 4B), whereas in the absence of co-transfected FoxO4, cAMP was not able to stimulate the p27^{Kip1} promoter (data not shown). Furthermore, we investigated whether protein levels of p27^{Kip1} are increased by cAMP via FoxOs. We transiently transfected MEFs with active PKB (gagPKB), the DNA binding domain of FoxO4 (FoxO4-DB), which acts as dominant negative FoxO, or active FoxO4 (FoxO4-A3) and examined the effect of cAMP on the total levels of p27^{Kip1}. Whereas

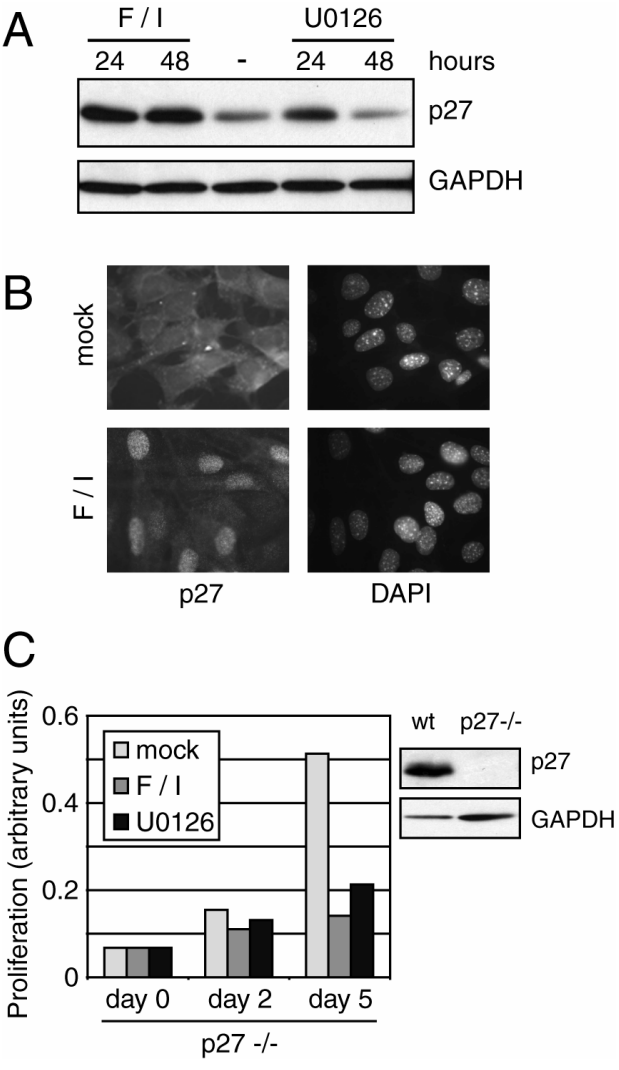

Figure 3. ***p27^{Kip1} plays an important role in inhibition of proliferation by cAMP.*** (A) p27^{Kip1} proteins levels are elevated by cAMP. Wild type MEFs were stimulated for 24 or 48 hours with forskolin/IBMX (F/I) or U0126, or were left untreated. Total p27^{Kip1} levels were visualized by immunoblotting with GAPDH levels as a control. (B) cAMP induces nuclear localization of p27^{Kip1}. Cells were stimulated as indicated and fixed to slides. p27^{Kip1} protein was stained by immunofluorescence and nuclei were stained with DAPI. (C) Inhibition of proliferation of p27-deficient cells is partially reversed by cAMP. p27$^{-/-}$ MEFs were subjected to a proliferation assay as in 1A. The presence of p27^{Kip1} protein was checked on a western blot with GAPDH as a control.

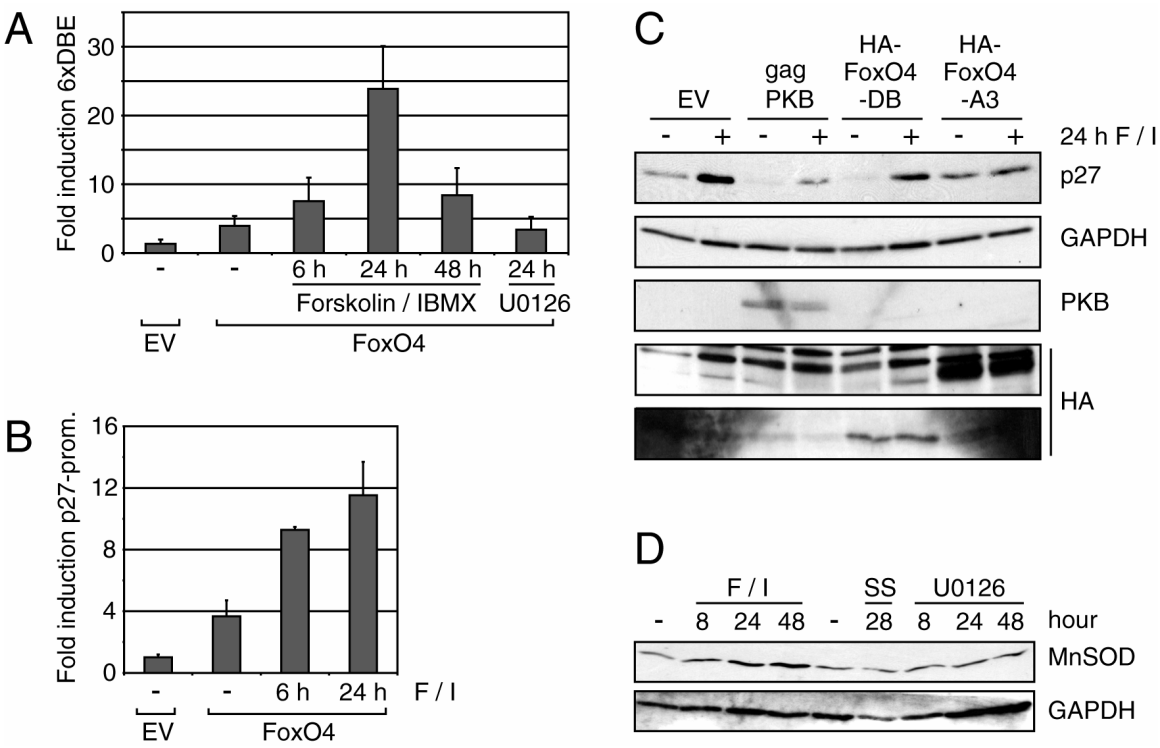

Figure 4. ***cAMP regulates FoxO transcriptional activity.*** (A) MEFs were transfected with the FoxO binding sites containing luciferase construct and either co-transfected with empty vector (EV) or a FoxO4 construct. Cells were stimulated for the indicated time points with forskolin/IBMX (F/I) or U0126. Fold induction of normalized luciferase counts in relation to unstimulated, empty vector transfected cells was calculated and plotted. (B) cAMP increases $p27^{Kip1}$-promoter activity in a FoxO-dependent manner. The experiment was performed as in A, except that the $p27^{Kip1}$-promoter luciferase construct was used. (C) cAMP regulates $p27^{Kip1}$ protein levels partially via PKB and FoxOs. MEFs were transfected either with empty vector, gagPKB, HA-tagged FoxO4-A3 or HA-tagged FoxO4-DB and cells were stimulated for 24 hours with forskolin/IBMX or were left untreated. Proteins were separated and blotted and membranes were probed for total $p27^{Kip1}$ levels. Membranes were probed for PKB or HA to visualize the transfected proteins. As a control for equal loading, the blot was probed for GAPDH. (D) cAMP upregulates MnSOD protein levels. MEFs were stimulated with forskolin/IBMX or with U0126 for 8, 24 or 48 hours or the cells were serum starved (SS) for 28 hours. MnSOD levels were visualized by immunoblotting and GAPDH was used as a control.

active FoxO4 in the absence of cAMP increased $p27^{Kip1}$ levels, the basal level of $p27^{Kip1}$ was lower in the presence of active PKB or dominant negative FoxO4. Furthermore, the increase of $p27^{Kip1}$ levels caused by cAMP was clearly reduced by overexpression of active PKB and to a lesser extent by dominant negative FoxO4. No complete inhibition of the cAMP-induced $p27^{Kip1}$ levels was seen, which may be explained by the fact that not all cells were transfected or by additional inputs on the $p27^{Kip1}$ promoter. Active FoxO4 could, as reported before, induce $p27^{Kip1}$ protein levels, but interestingly, forskolin/IBMX did not increase this (Figure 4C). Together, these data show that cAMP can regulate FoxO transcriptional activity and that PKB and FoxO mediate cAMP-induced expression of $p27^{Kip1}$.

If cAMP is able to regulate FoxO transcription factors, FoxO targets other than $p27^{Kip1}$ are predicted to be regulated as well. We were particularly interested in the FoxO target manganese superoxide dismutase (MnSOD), as we previously showed that MnSOD by scavenging reactive oxygen species (ROS) plays a role in protecting cells from going into apoptosis (50). Thus, MnSOD might protect cAMP-arrested cells, which are not apoptotic

(data not shown). Indeed, MnSOD protein levels were increased in MEFs after stimulation with forskolin/IBMX for 24 or 48 hours, whereas U0126 did not have an effect (Figure 4D).

FoxO transcription factors are involved in cAMP-induced G1 arrest

We next addressed the question of how cAMP regulates FoxO transcription factors. Given that the PI3K/PKB pathway directly affects FoxO activity (45), we measured PKB phosphorylation as a read-out for PI3K activity. Phosphorylation of both serine 473 and threonine 308 of PKB was decreased rapidly upon stimulation with forskolin/IBMX and this inhibition was sustained (Figure 5A). Inhibition of PKB phosphorylation is observed from at least 6 hours until 48 hours after stimulation, thus correlating with $p27^{Kip1}$ upregulation. In contrast, U0126 led to a reduced activity of PKB only at later time points. Furthermore, inhibition of the PI3K pathway using the inhibitor LY294002 resulted in an increase in G1 in Rb-deficient cells, almost comparable to the effect of forskolin/IBMX (data not shown). Thus, cAMP-mediated growth arrest might be largely dependent on activation of FoxOs due to inhibition of PI3K/PKB by cAMP.

To prove that FoxO transcription factors are indeed important in a cAMP-induced cell cycle arrest, the DNA binding domain of FoxO4 (FoxO4-DB) was overexpressed in MEFs. This protein is expected to act as a dominant negative for all FoxO family members. We assessed the inhibition of proliferation by determining the increase in the G1-phase of the cell cycle by cAMP. A clear, albeit incomplete, reversion of cAMP-induced inhibition of proliferation was seen both at 24 and 48 hours by overexpression of FoxO4-DB (Figure 5B). Together these data demonstrate a clear involvement of FoxO transcription factors in cAMP-induced cell cycle arrest.

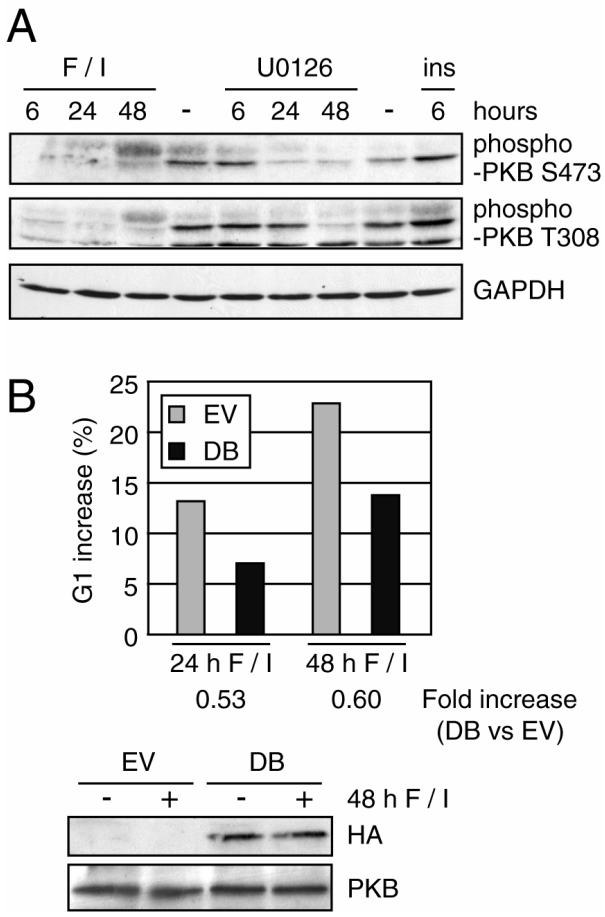

Figure 5. ***FoxOs are involved in a cAMP-induced cell cycle arrest.*** (A) PKB-phosphorylation is inhibited by cAMP. Cells were stimulated for 6, 24 or 48 hours with forskolin/IBMX (F/I) or U0126, or 6 hours with insulin (ins), or were left untreated. Equal protein amounts were blotted and blots were probed for both phosphorylated PKB on serine 473 and on threonine 308 and as a control blots were probed for GAPDH. (B) MEFs were transfected with either an empty vector (EV) or HA-tagged FoxO4-DB (DB). In two separate experiments, the cells were treated with forskolin/IBMX for either 24 or 48 hours. The cell cycle profile was determined as in Figure 2B. The percentage increase in G1-phase cells of forskolin/IBMX stimulated cells, compared to unstimulated cells was calculated and plotted. Furthermore, the fold increase in G1 of DB-transfected cells versus EV-transfected cells is shown in numbers. Part of the cells used for FACS analysis was lysed before fixation to check for the presence of transfected constructs. Lysates were separated and blotted and blots were stained for HA to visualize HA-FoxO4-DB and total PKB levels were visualized as a control.

DISCUSSION

The mechanism of inhibition of proliferation by cAMP is a complex process, analysis of which is further complicated by cell type-dependent differences. Consistent with previous reports, we show here that inhibition of the MAPK pathway and cyclin D1 levels can not fully account for the cAMP-induced cell cycle arrest in MEFs, and that $p27^{Kip1}$ upregulation is clearly important as well. This is evident from the finding that cAMP-induced inhibition of proliferation is partly reversed in $p27^{Kip1}$-deficient cells. Apparently, the anti-proliferative effect of cAMP in MEFs can not be explained by upregulation of $p27^{Kip1}$ alone. In other cell types, the most likely partner in the process of inhibition of proliferation is cyclin D1. For example, in hamster fibroblasts (41) and T cells (5) overexpression of cyclin D at least partially overcomes the anti-proliferative effect of cAMP. However, constitutive expression of cyclin D1 in MEFs has hardly any effect on cAMP-induced growth arrest. Moreover, Rb-deficient cells are also clearly inhibited in proliferation by cAMP, indicating that changes in cyclin D1 and Rb activity are not sufficient to explain the cAMP effect. It could be argued that in the Rb-deficient cells proliferation is still inhibited due to redundancy between Rb family members. We could exclude this, as MEFs deficient for all three Rb family members, Rb, p130 and p170 (51), are still arrested upon cAMP treatment, which is only partly due to the induction of apoptosis (H.B.K, unpublished observation).

Here we report that FoxO transcription factors, which are under the negative control of the PI3K/PKB pathway, are involved in the anti-proliferative effect of cAMP in MEFs. First, FoxO transcription factors can downregulate cyclinD1 and upregulate $p27^{Kip1}$ (this report and (9,24,52)). Furthermore, at least one other direct target of FoxO transcription factors, namely MnSOD, is upregulated following cAMP treatment. Regulation of FoxO transcription factors by cAMP is clearly observed in co-transfection experiments in which transcription from a 6xDBE luciferase construct was increased by forskolin/IBMX. In contrast, a constitutively active version of FoxO4, FoxO4-A3 did not respond to cAMP. More direct evidence for a role of FoxO transcription factors in the anti-proliferative effect of cAMP comes from ectopic expression of dominant negative FoxO4. FoxO4-DB clearly interferes in growth inhibition by cAMP, although this effect is not complete. This may be due to the fact that not all cells were transfected or that FoxO4-DB cannot fully block the action of endogenous FoxO transcription factors. An equally likely possibility however is that not all growth inhibitory effects of cAMP are mediated via activation of FoxOs. In line with this is the observation that ectopic expression of cyclin D1 does partially protect cells from a FoxO4-induced cell cycle arrest (23), whereas ectopic expression of cyclin D1 does hardly affect a cAMP-induced cell cycle arrest (Table 1). The most likely way by which cAMP activates FoxOs is via inhibition of PI3K. Although we did not directly measure decreases in PIP3 levels, the PI3K-regulated protein kinase B was clearly less phosphorylated at S437 and T308. Furthermore, the induction of $p27^{Kip1}$ protein levels by cAMP could clearly be suppressed by active PKB.

Besides its anti-proliferative effect, cAMP is known to protect certain cell types from apoptosis (53-55). We now show that this might also be the case in mouse fibroblasts via a mechanism using MnSOD. MnSOD is a known target of FoxOs (50) and is clearly upregulated by cAMP, which is thus a likely consequence of stimulation of FoxOs on FoxO binding sites in the MnSOD promoter. MnSOD upregulation might provide the cells with a mechanism in which cells can be protected when PKB is inhibited.

Apart from cyclin D1 or Rb, $p21^{Cip1}$ might be involved next to $p27^{Kip1}$ in a cAMP-induced cell cycle arrest. Although we were not able to show an increase in $p21^{Cip1}$ levels upon cAMP treatment in wild type MEFs (data not shown), this has been shown by others in other cell types (56,57). Furthermore, we recently obtained preliminary evidence that FoxO transcription factors can regulate $p21^{Cip1}$ levels (B.M.B., unpublished observation). It would therefore be interesting to see if cAMP can still inhibit proliferation of p21/p27 double knockout cells.

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Chapter

4

Expression profiling of cAMP-regulated genes via MAPK-dependent and -independent pathways

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To be submitted

Expression profiling of cAMP-regulated genes via MAPK-dependent and -independent pathways

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In many cell types cAMP inhibits cell proliferation by interfering in among others the MAPK pathway and in expression of cell cycle regulators like cyclin D1 and $p27^{Kip1}$. To identify genes whose expression may contribute to cAMP-induced inhibition of proliferation we counterselected for genes controlled by MAPK, since inhibition of the MAPK pathway is not crucial for cAMP-dependent growth arrest. We investigated the effect of cAMP on global gene expression by performing microarray analysis on cAMP-treated NIH3T3-A14 cells. To discriminate between cAMP-effects that are mediated or not by the MAPK pathway, we have compared cAMP-induced changes in expression profiles with profiles induced by the MEK-inhibitor U0126. We found several genes which were reported before to be involved in proliferation and/or regulated by cAMP or MAPK, e.g. cyclin D1. Furthermore, we identified and verified the growth factors HDGF and Gas6, transcriptional repressor TSC-22, putative E3-ligase KIAA1593 and phosphoprotein stathmin as new cAMP-regulated genes.

INTRODUCTION

cAMP is an important regulator of proliferation in various cell lines via stimulation of the cAMP-dependent protein kinase (PKA). It is known that cAMP can regulate proliferation by regulating the MAPK pathway (1), but inhibition of MAPK alone can not explain the cAMP-induced growth arrest (2,3). Other mechanisms have been described as well, including inhibition of the PI3K/PKB pathway, which is also important for proliferation (4). Often, regulation of the cell cycle regulator cyclin D1 and the cell cycle inhibitor $p27^{Kip1}$ by the PI3K or MAPK pathway correlates with the anti-proliferative effect of cAMP (5-10). In part this is due to transcriptional contributions, as the promoter of cyclin D1 is regulated by the MAPK pathway via the ATF/CREB family of transcription factors (11), and the promoter of both cyclin D1 and $p27^{Kip1}$ can be regulated by the PI3K pathway, involving FoxO transcription factors (12-14). Recently, we have shown that FoxO transcription factors, under negative control of the PI3K/PKB pathway, play an important role in the anti-proliferative effect of cAMP in mouse embryo fibroblasts (chapter 3). Besides transcriptional control via regulation of the PI3K and MAPK pathway, cAMP can directly influence transcription of many targets via the CREB family of transcription factors (15). These proteins bind to cAMP response elements (CREs) in promoters and are activated upon phosphorylation by PKA, resulting in recruitment of the coactivator CREB binding protein (CBP) to the promoter. Given the fact that the anti-proliferative effect of cAMP can not be fully explained by inhibition of the above mentioned signalling pathways, it is likely that CREB mediated transcription contributes to the cAMP-induced growth arrest.

We used microarray analysis to study global changes in mRNA expression linked to the process of inhibition of NIH3T3-A14 cell proliferation by cAMP. In these cells cAMP inhibits the Raf-MEK-ERK pathway, but importantly, this inhibition is not sufficient to inhibit proliferation. We therefore discriminated between MAPK-dependent and -independent cAMP targets using the MEK-inhibitor U0126. Several new cAMP-regulated genes were found, which may either be involved in blocking cell proliferation or help the cell to adjust to a G1 arrest.

MATERIALS AND METHODS

Constructs, antibodies and stimuli

pKH3-HA-HDGF and pSG5-HA-TSC-22 constructs were a gift of Allen Everett and Paul van de Saag respectively (16,17). The open reading frame of TSC-22 plus HA-tag was subcloned in pcDNA4/TO (Invitrogen) for inducible expression of TSC-22. Full length TSC-22 was amplified by PCR using TSC-22for (gaattcACCAT GTACCCATATGATGTTCCTGATTATGC) and TSC-22rev (gcggccgcAGCTATGCGGTTGGTC) primers and pSG5-HA-TSC-22 as a template and the product was cloned in pGEM-T (Promega). Using the introduced *Eco*RI and *Not*I site, TSC-22 was subcloned in the corresponding sites of pcDNA4/TO.

The following antibodies were used: phosphorylated ERK1/2, stathmin (Cell Signaling), ERK2 (18), cyclin D1 (Immunotech), TSC-22 (19), 12CA5 (20) and GAPDH (Chemicon).

The following stimuli were used at the indicated concentrations: forskolin (10 µM; ICN), IBMX (0.5 mM; Sigma), U0126 (10 µM; Biomol Research Laboratories), insulin (1 µg/ml; Sigma) and camptothecin (4 µg/ml; ICN).

Cell culture and generation of stable cell lines

NIH3T3-A14 cell line has been described before (21). TSC-22 expressing lines were generated by transfecting NIH3T3-A14 and LS174T-TR cells with pcDNA4/TO-HA-TSC-22 and selected with respectively 200 and 500 µg/ml zeocin (Invitrogen). NIH3T3-A14 clones with different TSC-22 expression levels and LS174T clones with inducible TSC-22 expression were selected. NIH3T3-derived cell lines were cultured in DMEM and LS174T-derived cell lines were cultured in RPMI, all supplemented with 10% fetal bovine serum and 0.05% glutamine. In addition, LS174T-TR cells were grown in the presence of 10 µg/ml blasticidin (Invitrogen).

RNA-isolation

NIH3T3-A14 cells were stimulated for 2, 8 or 24 hours with either forskolin/IBMX, U0126 or insulin and were mock stimulated for every time point and stimulation type. After stimulation, cells were washed twice with cold PBS, followed by lysis in Trizol reagent (Invitrogen). Trizol-lysates were stored at -20 ºC until further use. To isolate RNA, 1/5 volume chloroform was thoroughly mixed through the lysates and incubated for a few minutes at RT. The sample was centrifuged for 15 minutes, 3000 rpm at 4 ºC in a table-centrifuge. The RNA-containing phase was transferred and RNA was precipitated with 0.7 volumes of 2-propanol and dissolved in RNase free water. In parallel to RNA-isolations, protein of untreated or forskolin/IBMX, U0126 or insulin treated NIH3T3-A14 cells was isolated to determine ERK-phosphorylation. Furthermore, NIH3T3-A14 cells were in parallel to RNA-isolation subjected to a proliferation assay.

Preparation of labelled cDNA

Total RNA was cleared for residual DNA by treatment with DNaseI (Ambion) for 30 minutes at 37 ºC. DNase was subsequently removed using DNase inactivation reagent (Ambion). cDNA was generated from 10 to 20 µg total RNA per sample using oligo $dT_{12\text{-}18}$-primers and SuperScript™ II Reverse Transcriptase (Gibco/Life Sciences). During this reaction 2-aminoallyl-dUTP (Sigma) was incorporated and RNA templates were removed afterwards by alkaline lysis using NaOH. cDNA was purified using Microcon-30 columns (Millipore) and Cy 3- or Cy 5-fluorophores (Amersham) were coupled to the incorporated 2-aminoallyl-dUTP, during incubation for at least 60 minutes at RT in the dark. The coupling reaction was quenched with hydroxylamine (Sigma) and free dyes were removed using Chromaspin-30 columns (Clontech). Efficiency of cDNA synthesis and dye incorporation was measured using a spectrophotometer (UV1240 mini, Shimadzu). 150 to 250 ng of Cy 3-labelled cDNA, prepared from unstimulated cells, was combined with an equal amount of Cy 5-labelled cDNA, prepared from stimulated cells, or the other way around in a dye-swap experiment with cDNA from separately isolated RNA.

Hybridization of mouse cDNA microarrays

15-K mouse cDNA microarrays (manufactured at the Netherlands Cancer Institute) were used. Microarrays were incubated for at least 45 minutes at 42 ºC in pre-hybridization buffer (5x SSC, 25 % formamide, 2.5 % SDS, 1 % BSA). Hybridization was performed using 300 to 500 ng of combined Cy 3- and Cy 5-labeled samples (with a specific activity of 2-4% labelled nucleotides) and diluted in an equal volume of 2x hybridization buffer (50 % formamide, 10x SSC, 0.2 % SDS, 0.2 mg/ml sheared Herring sperm DNA). Samples were loaded on microarrays using LifterSlips (Erie Scientific), followed by an incubation for 16 to 20 hours at 42 ºC in hybridization chambers (Corning). LifterSlips were carefully removed during the first wash, at low-stringency (1x SSC, 0.2 % SDS) and microarrays were then subjected to a high-stringency wash (0.1x SSC, 0.2 % SDS) and a wash with SDS-free buffer (0.1x SSC). Slides were dried quickly by a 1 minute spin at 500 rpm in a table-centrifuge, or with pressured-air.

Scanning and data analysis

Slides were scanned in a Scanarray 4000 XL (Perkin Elmer Biosystems). Image analysis was carried out using Imagene 4.0 (Biodiscovery). Data was normalized on genes using intensity dependent (Lowess) normalization (22) and analyzed using Genespring 5.0.3. (Silicon Genetics).

Real time-PCR

cDNA was generated from total RNA samples with the use of a reverse transcription system (Promega), according to the manufacturers protocol. Primers were designed such that the amplicon length was 50-150 bp and contained intron/exon boundaries. The following sets of primers were used for respectively mouse Gas6, HDGF, KIAA1593, stathmin and TSC-22: mGas6-for (CCGTGGGCGGCATTC) and -rev (TCCAGGCGAGG GTTAATCG), mHDGF-for (ACCAGCCAAGGAGAAG AACG) and -rev (TGGTCTCCTGACTCCTTGGG), mKIAA1593-for (GCCGTCCCTGGATGATCTG) and -rev (CTTCTGGATAGTCCAGCAATTGC), mStathmin -for (GATGGCGGCCAAGCTG) and -rev (TTCTTCCG CACCTCTTCCAC) and mTSC-22-for (CTTTGCTGGG AACCGAAAACG) and -rev (TTGTCGATAGCTACC ACACTTGC). The real time-PCR was performed using the Sybr green kit (Applied Biosystems), according to the manufacturers protocol, and PBGD primers were used as a control for equal cDNA amounts. Real time-PCRs were monitored and analyzed using Applied Biosystems Sequence Detection System. The in- and decrease of PCR-products upon stimulations was determined in relation to PBGD product formation in the same samples and the absolute in- and decrease was calculated by converting the exact, corrected cycle difference of unstimulated versus stimulated samples to fold inductions.

Western blotting

Following stimulation, cells were lysed by scraping in Ripa lysisbuffer (20 mM Tris-HCl pH 7.5, 1 % triton X-100, 0.5 % Na-DOC, 0.1 % SDS, 10 mM EDTA, 150 mM NaCl), supplemented with aprotinin, leupeptin, trypsin inhibitor, Na_3VO_4 and NaF. Ripa lysates were cleared by centrifugation and protein amounts were equalized, using standard Bradford protein quantification. Proteins were separated on SDS-polyacrylamide gels and blotted to polyvinylidene difluoride membranes (NEN^{TM}), following incubations with primary and secondary antibodies. Proteins were visualized using standard enhanced chemiluminescence and autoradiography.

Proliferation assay

Cells were plated in 12-wells or 6-wells plates in triplicates, followed by stimulations the next day. Induction of LS174T-derived cell lines was done with 10 µg/ml tetracycline. Medium, tetracyclin and stimuli were refreshed every two days and the cells were stained at 3 to 4 different days, 0 to 6 days after stimulation. To stain, the cells were fixed for 10 minutes in 10 % acetic acid at RT, prior to 10 minutes staining at RT with 0.4 % crystal violet, dissolved in 10 % acetic acid. The plates were washed twice with water and dried overnight. Proliferation was quantified by measuring the optical density at wavelength 560 nm of the in 10 % acetic acid re-dissolved crystal violet stain in the plates.

Flow cytometry

For determination of cell cycle distributions, untransfected cells or cells transfected with the indicated constructs together with pBabe-puro were used. Cells were stimulated for 24 or 48 hours as indicated and transfected cells were selected with 1 µg/ml puromycin for at least 24 hours. Cells were detached using EDTA, containing trypsin and washed twice, following fixation in 70 % ethanol for at least 16 hours at 4 ºC. Subsequently, cells were washed and resuspended in PBS, containing 0.1 % BSA, 0.25 mg/ml RNase and propidium iodide and were incubated for 30 minutes at 37 ºC prior to DNA profile measurements. Flow cytometry analysis was performed using a FACScalibur instrument (Becton Dickinson).

RESULTS

Proliferation of NIH3T3-A14 cells is inhibited by cAMP in a mainly MAPK-independent manner

We used the insulin receptor-overexpressing NIH3T3-A14 cell line (21) as a model system for cAMP-induced inhibition of cell proliferation, since previous results have indicated that this cell line is highly sensitive to cAMP-induced inhibition of proliferation. We examined the cAMP-dependent effects on proliferation of NIH3T3-A14 cells, for the purpose to use these cells for the profiling of genes involved in cAMP-induced inhibition of proliferation. In the presence of serum we observed that proliferation of these cells was clearly inhibited after stimulation with the cAMP-generating compound forskolin in combination with the phosphodiesterase inhibitor, IBMX, whereas the MEK-inhibitor U0126 only slightly inhibited proliferation (figure 1A). In addition, cell cycle profile analysis demonstrated that cells are arrested in G1 upon forskolin/IBMX treatment for 24 and 48 hours. U0126 treatment gave a transient increase in G1 at 24 hours, but this effect was diminished after 48 hours (figure 1B). As expected, both forskolin/IBMX and U0126 inhibited ERK activity to the same extent up to at least 24 hours after stimulation, whereas insulin stimulated ERK activity (figure 1C). Thus, as reported before (3), MAPK is only marginally involved in the anti-proliferative effect of cAMP in NIH3T3-A14 cells, which thus provides a good model system to investigate cAMP-induced inhibition of proliferation.

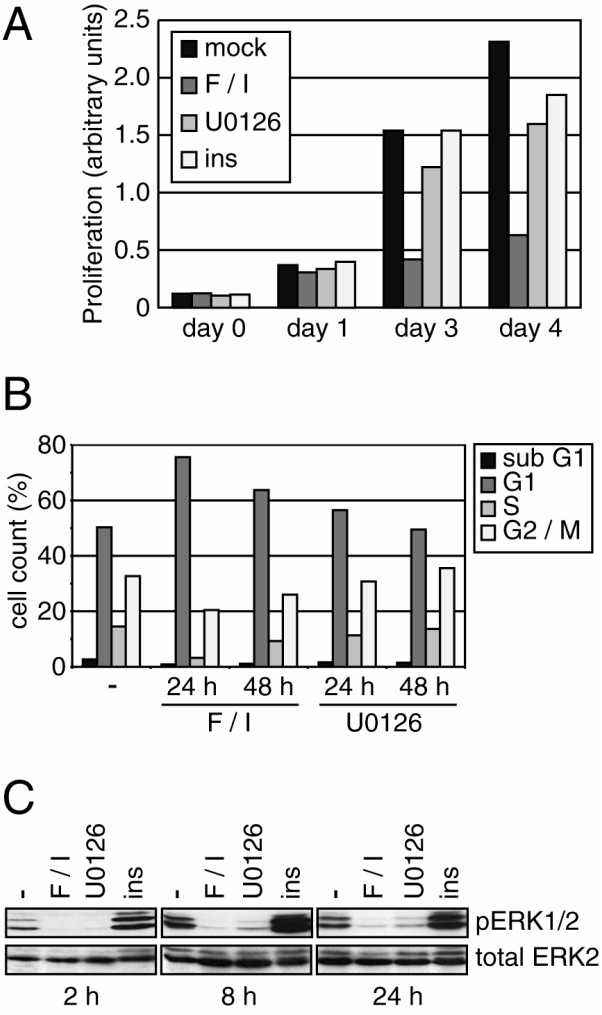

Figure 1. ***Effect of cAMP and MAPK on proliferation of NIH3T3-A14 cells.*** (A) Proliferation of NIH3T3-A14 cells is less sensitive to U0126 than cAMP. Proliferation of NIH3T3-A14 cells was measured upon mock, forskolin/IBMX (F/I), U0126 or insulin (ins) treatment. The amount of cells was measured using crystal violet staining as read-out at the indicated days after stimulation. (B) cAMP induces a G1-arrest in NIH3T3-A14 cells. Cells were treated for 24 and 48 hours with forskolin/IBMX or U0126 or were left untreated. The cell cycle profile of NIH3T3-A14 cells was determined by FACS analysis, using propidium iodide as staining. The percentage of cells per phase of the cell cycle was calculated and plotted. (C) ERK-phosphorylation is inhibited to the same extent by cAMP and U0126. NIH3T3-A14 cells were stimulated with forskolin/IBMX, U0126 or insulin for 2, 8 or 24 hours. The amount of phosphorylated ERK1 and -2 and total ERK2 was visualized by immunoblotting.

Expression profiling of cAMP versus U0126 effects in NIH3T3-A14 cells

We performed microarray experiments to study forskolin/IBMX and U0126 effects on transcription and proliferation. U0126 was used to be able to exclude MAPK-regulated genes, as MAPK can have pronounced effects on transcription by regulating multiple

Table 1. ***Sustained forskolin/IBMX regulated transcriptional targets***

		F / I		
Acc. No.	**Gene description**	**2 h**	**8 h**	**24 h**
Growth factors		**mRNA fold induction**		
BG086784	Hepatoma derived growth factor (HDGF)	-1.1	-1.9	-2.7
BG088548	Growth arrest specific 6 (Gas6)	1.2	-1.4	-1.4
BG077749	Macrophage migration inhibitory factor (MIF)	1.2	-1.4	-2.5
Transcriptional and translational proteins				
BG080816	Transcriptional repressor, TSC-22	2.8	1.9	2.0
BG085396		1.8	1.7	1.8
BG084814	Zinc finger protein, KIAA0714	1.1	2.0	1.5
BG080414		1.1	2.4	1.5
AU042135	Zinc finger protein, KIAA1577	2.1	1.5	1.7
BG067430	Histon H3 3B	2.3	2.9	-1.4
AW551507		1.9	2.4	-1.3
Ion channel and transport proteins				
BG063584	K^+ channel, KCNQ1	2.1	1.6	1.1
BG067025	Ion co-transporter, Slc16a1	1.7	1.3	-1.0
BG069505	Na-K-Cl co-transporter, Slc21a2	-1.2	-1.3	-1.8
Ubiquitination related proteins				
BG087165	HECT E3 ligase, KIAA1593	1.6	2.2	2.1
BG076898	E2 ubiquitin-conjugating enzyme	-1.0	-1.4	-1.5
Metabolic enzymes				
BG077839	Aspartyl aminopeptidase, Dnpep	-1.2	-1.5	-1.7
BG088828	Ornithine antizyme inhibitor (Oazi)	2.1	2.1	1.1
BG070710		1.8	2.3	1.2
Cytoskeletal proteins				
BG073184	Vimentin	-1.1	-1.6	-1.9
-	Beta-actin	1.0	-1.5	-2.6
Divers signalling proteins				
BG073186	Tyrosine phosphatase, PTP4a1	1.7	1.4	-1.2
BG068173		2.2	1.8	-1.2
BG073409	Phosphoprotein, stathmin	1.0	-1.6	-3.7
BG074892	Phospholipid binding protein, Annexin A6	1.0	-1.5	-2.6
BG069792	Unknown, ARPF domain containing protein	2.9	1.2	-1.2
BG084513	RNA binding protein, hnRNP	-1.0	-1.5	-1.4

transcription factors (23). Genes with an intermediate to high expression level, whose expression was in- or decreased significantly by forskolin/IBMX for two consecutive time points and were not affected by U0126, were selected. These sustained cAMP-regulated genes, which were not regulated via MAPK, were listed in table 1. Early cAMP-regulated genes were listed as well (table 2) and they had to pass the criteria that forskolin/IBMX increased expression significantly at 2 hours after treatment, but not at other time points, while U0126 did not increase expression levels at any time point. Furthermore, putative MAPK regulated genes were listed (table 3). They had to pass the criteria that, both U0126 and forskolin/IBMX changed expression levels in the same direction significantly at two consecutive time points of treatment.

Validation of gene expression of sustained-regulated cAMP-targets

To verify and validate the regulation of the selected genes, we performed a real time-PCR for the most promising putative sustained-regulated cAMP-targets, being TSC-22, HDGF, KIAA1593, Gas6 and stathmin. Real time-PCR was performed with cDNA generated from independently/newly isolated RNA. We tested TSC-22 and stathmin mRNA expression at 2, 8 and 24 hours after forskolin/IBMX or U0126 treatment. mRNA levels changed according to the profiles found in the microarray, with TSC-22 being upregulated at all time points by forskolin/IBMX and stathmin levels being downregulated in time. U0126 did hardly change mRNA levels of either genes (figure 2A and B and figure 2D and E). As expected, the changes in expression were more pronounced when measured by real time-PCR than by

Table 2. ***Early forskolin/IBMX upregulated transcriptional targets***

Acc. No.	Gene description	Known links to cAMP or proliferation
Transcriptional and translational proteins		
BG079103	WD40 domain containing transcriptional corepressor, Hira	
BG069866	Translation initiation factor, Eif4a2	Linked to proliferation (32), contains CRE
BG082196	Zinc finger protein, RM1	
BG072227	T-Box transcription factor, TBX1	Induces differentiation (33)
BG080285		
BG084290	Inhibitor of differentiation, ID2	Regulated by cAMP (62), TGFβ (63)
AW557873	Inhibitor of differentiation, ID3	Correlates with differentiation, regulated by cAMP, insulin and TPA (64)
BG074838	Zinc finger protein, Nolz-1	Differentiation related (34)
BG067918	Cell cycle regulator protein, Btg2/TIS21/PC3	Inhibits proliferation, transcriptionally induced by cAMP and growth factors, contains CRE (31)
BG080666	Immediate early response gene, Gly96/IEX-1/IER3	Cell type dependent effects on proliferation, induced by cAMP, protection from apoptosis (35,36)
Receptor and channel proteins		
BG077994	G-protein coupled receptor, RAI3	Induced by retinoic acid, differentiation linked (65)
BG085927	Arginine vasopressin receptor, Avr1A	Induces proliferation (66)
BG080898	Potential cation channel, TRPC7	
Metabolic proteins		
BG078582	Dihydrofolate reductase, Dhfr	Linked to proliferation (67), inhibited by cAMP (68)
BG076728	Thioredoxin reductase, Txnrd1	Induces proliferation, upregulated in cancer (69)
BG088005	S-adenosylmethionine decarboxylase, Amd1	Induces proliferation (70), upregulated by cAMP (71), contains CRE
Diverse signalling proteins		
BG070255	Phosphodiesterase, PDE7A	Induced by cAMP (72), contains CRE (73)
BG070961	Deubiquitinating enzyme, USP2	Likely involved in proliferation based on domains
BG086271	p53-binding protein, 53BP2/ASPP2	Anti-proliferative (74)
BG073504	Glycogen phosphorylase, pyg1	Upregulated by cAMP (75)
BG067594	Polycomb-group protein, Sex comb on midleg homolog 1	
AU044106	Chaperone protein, DNAJ/HSP40	
AW547238	Unknown ribosomal protein	
BG063600	Putative actin cytoskeleton controlling protein	
AW552763	Chemokine-like factor superfamily 3	

Table 3. ***Genes regulated both by forskolin/IBMX and U0126***

Acc. No.	Gene description	Known links to MAPK, cAMP or proliferation	mRNA levels
Transcriptional and translational proteins			
BG085740	Ets-related transcription factor, Etv5/ERM	Upregulated by Ras and in cancer, linked to proliferation (54,76)	down
BG064464	Transcriptional repressor, Plfap/Ebp1	Inhibits proliferation (77)	down
BG069836	Transcriptional repressor, TARDBP/TDP-43		down
BG067001	Transcriptional co-activator, Rbpsuh/RBP-J	Regulated by Notch signalling (78)	down
BG086749	Eukaryotic translation initiation factor 4E (Eif4E)	Regulated by mTor/S6K, malignant (59)	down
BG076791	Eukaryotic translation elongation factor 1-delta (TEF-1δ)	Oncogenic potential (58)	down
Cytoskelet related proteins			
BG064838	Alpha-tubulin gene, M-alpha-2		down
BG069637	Tubulin alpha 1 (Tuba1)		down
BG064830	Tubulin beta-2 protein		down
Diverse signalling proteins			
BG072439	Serum and glucocorticoid-dependent protein kinase (Sgk)	MAPK- and cAMP-regulated, involved in proliferation (55,60,79)	down
BG070254	E1 ubiquitin activating protein		down
BG083088	Cyclin D1	MAPK- and cAMP-regulated cell cycle protein (6,25)	down
BG087671	Growth arrest specific 1 (Gas1)	Involved in proliferation (80)	up
BG085460	Ras-like small GTPase, TC21	Involved in transformation (81)	down
BG072299	Calcitonin-receptor activity modifying protein 2 (Ramp2)		up
BG064796	Iron-storage protein, Ferritin light chain	Likely involved in proliferation (82)	
BG064795			
BG064794			up
BG086954	Beta-amyloid precursor protein	Induced by MAPK, induces proliferation (56,57)	up
BG064823	Phosphoglycerate mutase, Pgam1		down
BG075139	Methyladenosine phosphorylase		down
BG086754	Ribonucleoprotein, hnRNP A3	Downregulated by MEK-inhibitor PD98059 (83)	down
BG087654	Helicase, hells/lsh	Induces proliferation in lymphoid tissue (84)	down
BG073147	Chloride intracellular channel, Clic1	Upregulated in hepatocarcinoma (85)	down
BG088178	Unknown endocytosis associated protein		down

microarray analysis. Also at the protein level, TSC-22 and stathmin showed the expected changes. TSC-22 protein levels were clearly increased endogenously by forskolin/IBMX treatment in NIH3T3-A14 cells and mouse embryo fibroblasts (figure 2C), whereas stathmin levels were found downregulated by this treatment (figure 2F). In a second real time-PCR experiment we tested all five selected genes at one time point of forskolin/IBMX stimulation, and again changes in mRNA levels did confirm the microarray data: TSC-22 and KIAA1593 were upregulated by cAMP, whereas stathmin, Gas6 and HDGF were down regulated (figure 2A, 2D, 3A-C and 3E). As a control for the microarray experiment, the protein levels of cyclin D1 and the cell cycle inhibitor $p27^{Kip1}$ were examined. Cyclin D1 is listed in table 4 as a putative MAPK regulated gene, as it has been described before as MAPK-

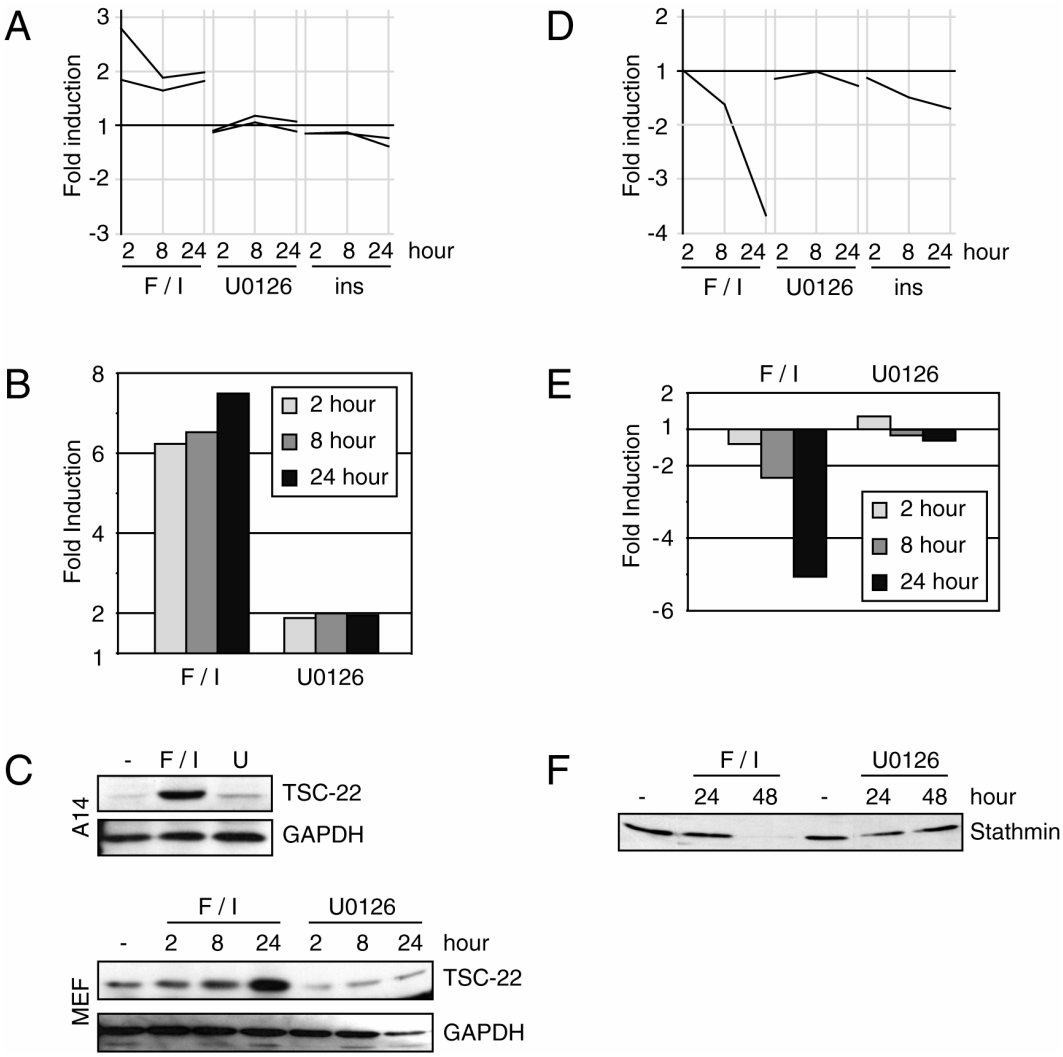

Figure 2. ***Verification of TSC-22 and stathmin as cAMP-regulated genes.*** (A) and (D) Average fold induction of TSC-22 (A) and stathmin (D) mRNA levels are shown, as retrieved from the microarray data. NIH3T3-A14 cells were stimulated for 2, 8 and 24 hours with either, forskolin/IBMX (F/I), U0126 or insulin (ins) and microarrays were hybridized and analysed as described in materials and methods. (B) and (E) TSC-22 and stathmin RNA levels were determined by real time-PCR. NIH3T3-A14 cells stimulated with forskolin/IBMX or U0126 for 2, 8 and 24 hours and a real time-PCR was performed as described in materials and methods. Average fold inductions of TSC-22 (B) and stathmin (E) mRNA levels are shown. (C) and (F) TSC-22 and stathmin protein levels are regulated by cAMP. NIH3T3-A14 cells and mouse embryo fibroblasts (MEFs) were treated for the indicated time points with forskolin/IBMX or U0126 (U) or were left untreated. TSC-22 protein levels in NIH3T3-A14 cells and MEFs were visualized on a western blot with GAPDH levels as a control for equal loading (C) and stathmin protein levels were visualized in NIH3T3-A14 cells (F).

regulated gene (24,25). The profile of cyclin D1 mRNA expression, retrieved from the microarray data, showed a clear decrease in cyclin D1 mRNA levels by forskolin/IBMX treatment, whereas U0126 changed the levels with different kinetics (figure 3D). We observed that cyclin D1 protein levels were clearly downregulated, 24 hours after forskolin/IBMX treatment, whereas U0126 had hardly any effect (figure 3F). $p27^{Kip1}$ mRNA levels were not changed by any stimulus in the microarray experiment, which correlated with the finding that protein levels were also not changed upon forskolin/IBMX or U0126 treatment (data not shown).

Examination of cAMP regulated genes

Genes involved in a cAMP-induced cell cycle arrest are likely continuously regulated by cAMP, as long as cAMP is present, or are shortly induced by cAMP to initiate prolonged regulation of other proteins. We found 21 different genes that were long term regulated and 24 genes that were regulated only at early time points by forskolin/IBMX (table 1 and 2). Of these cAMP-regulated genes, 9 have been reported before to be regulated at the transcriptional level by cAMP and 18, mostly growth factors and transcriptional and translational proteins, have been described to be linked to or involved in proliferation.

Furthermore, 23 genes regulated both by U0126 and forskolin/IBMX were identified in this screen (table 3). As both stimuli result in inhibition of the MAPK pathway, these genes are putative MAPK regulated genes. Most of the 23 genes were downregulated when MAPK was inhibited, thus MAPK mostly stimulates transcription, rather than inhibiting it. These putative MAPK-regulated genes included 5 genes (Etv5, SGK1, cyclin D1, hnRNP A3 and Beta-amyloid precursor protein), which were reported before to be regulated by MAPK, including 2 genes reported to be regulated by cAMP (cyclin D1 and SGK1). Ten of the putative MAPK-regulated genes were reported

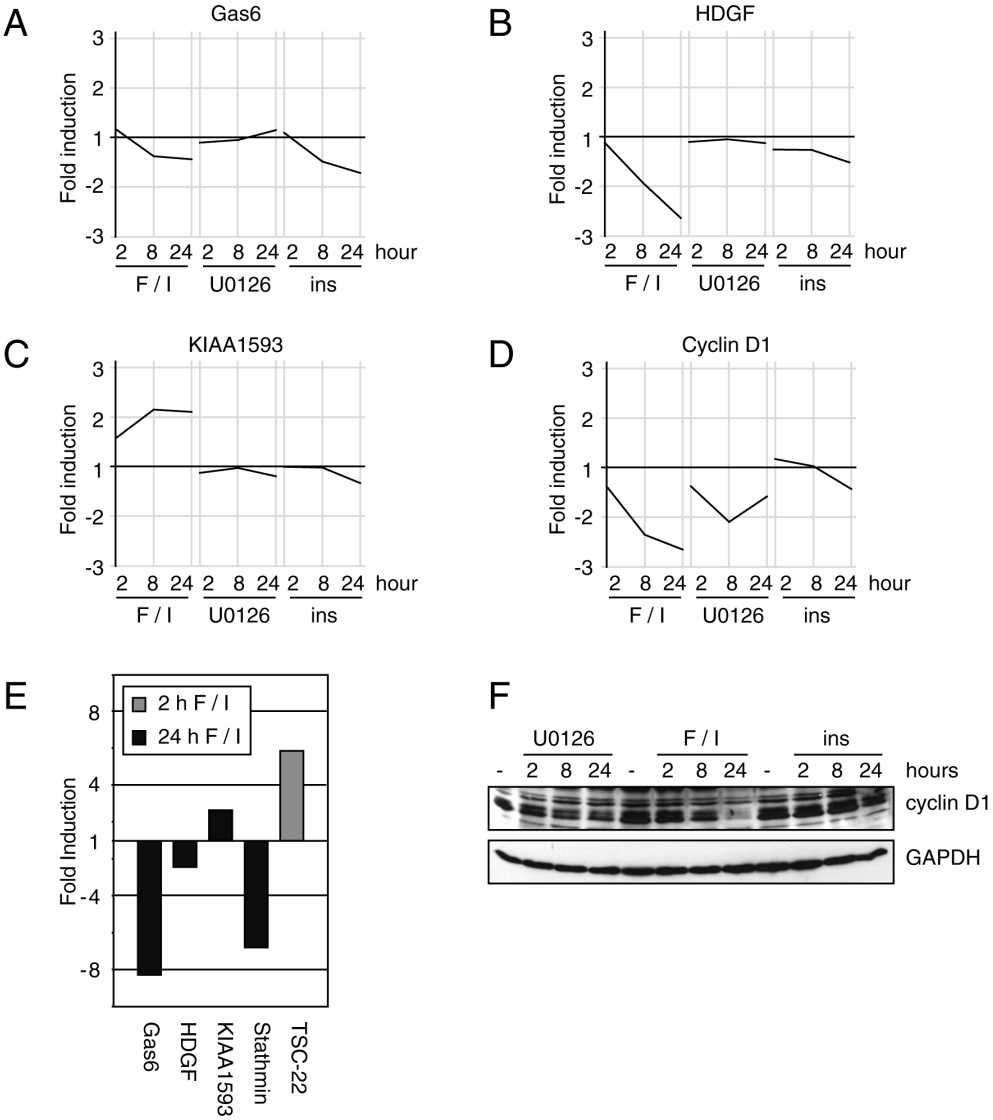

Figure 3. ***Verification of the regulation of Gas6, HDGF, KIAA 1593 and cyclin D1 by cAMP.*** (A-D) Average fold inductions of mRNA levels in the microarray are determined for Gas6 (A), HDGF (B), KIAA1593 (C) and cyclin D1 (D) as in figure 2A. (E) mRNA fold inductions upon forskolin/ IBMX treatment for the indicated time point was measured by real time-PCR. mRNA levels were measured as in figure 2B. (F) Cyclin D1 protein levels are reduced by cAMP. NIH-3T3-A14 cells were stimulated with forskolin/IBMX, U0126 or insulin for 2, 8 and 24 hours or were left untreated. Cyclin D1 protein levels were visua-lized by immunoblotting and the blot was probed for GAPDH as a control.

to be involved in proliferation or to have oncogenic potential. The kinetics in the regulation of most genes by forskolin/IBMX and U0126 were not identical, suggesting that either cAMP may regulate these targets via other signalling pathways than by inhibition of the MAPK pathway, or that U0126 has additional effects.

TSC-22 is not involved in a cAMP-induced cell cycle arrest

TSC-22 has been described to be a transcriptional repressor (17). We now show that TSC-22 is upregulated by cAMP in MEFs and NIH3T3-A14 cells (figure 2C), in which cAMP has an anti-proliferative effect (figure 1A and data not shown). It has not been proven that induction of TSC-22 actually results in inhibition of proliferation, and therefore we constructed NIH3T3-A14 cells overexpressing TSC-22 and LS174T cells, inducible overexpressing TSC-22. We performed proliferation assays with several clones of both stable cell-types. Overexpression of TSC-22 in NIH3T3-A14 cells resulted in a small increase in proliferation, compared to empty vector cell lines, instead of a reduction in proliferation. On the other hand, forskolin/IBMX did inhibit proliferation of TSC-22 expressing cells slightly better than proliferation of empty vector cell lines (figure 4A), indicating some synergy of TSC-22 with other factors to inhibit proliferation. TSC-22 expression in LS174T stable cell lines was induced with tetracycline. Proliferation of these cells treated with tetracycline may be slightly reduced, compared to uninduced cells, but this was not conclusive due to an effect of tetracycline itself on proliferation of empty vector lines (data not shown). We concluded that induced expression of TSC-22 does not play a significant role in inhibition of proliferation. It has also been reported that induction of TSC-22 can induce apoptosis (26-28). As in NIH3T3-A14 cells no apoptosis is induced upon cAMP treatment (sub

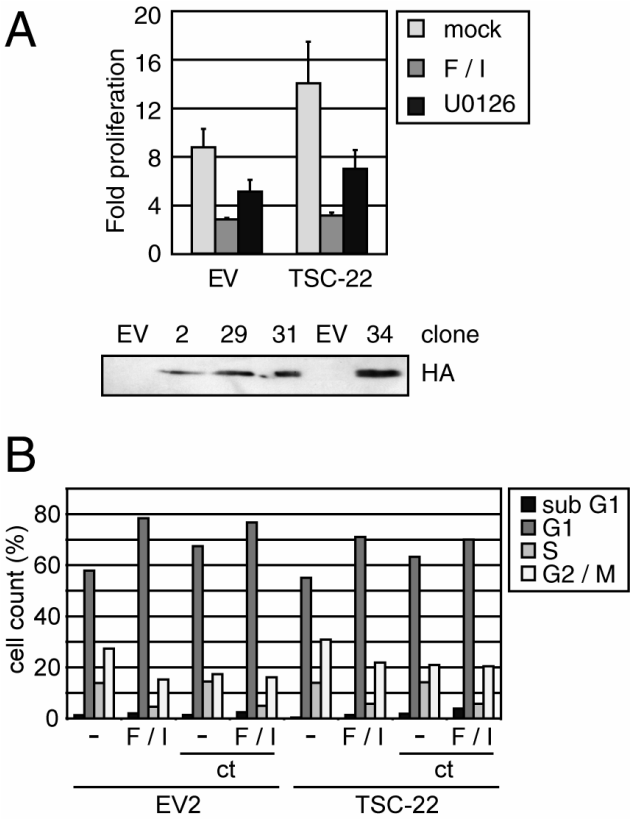

Figure 4. ***TSC-22 is not involved in cAMP-dependent inhibition of proliferation.*** (A) Different clones of TSC-22 or empty vector (EV) expressing stable NIH3T3-A14 cell lines were assayed for their proliferation upon treatment with forskolin/IBMX (F/I) or U0126. The average increase in cells of different clones compared to day 0, and the expression of HA-tagged TSC-22 in the different cell lines is shown. (B) TSC-22 does not induce apoptosis or a G1-arrest in NIH3T3-A14 cells. NIH3T3-A14 stably expressing TSC-22 or an empty vector were assayed for their DNA profile as in figure 1B. Cells were treated mock or with forskolin/IBMX, in the absence or presence of camptothecin (ct).

G1 fraction in figure 1B), TSC-22 is not likely involved in induction of apoptosis in these cells. To test this further, we challenged NIH3T3-A14 cells with the DNA-damaging agent camptothecin in empty vector and TSC-22 expressing cells, in the absence or presence of forskolin/IBMX. Although camptothecin streatment did not lead to an increase in the amount of cells in the sub-G1 fraction, it did lead to an increase in G1 in the empty vector

cell line. Apparently, the cells are not apoptotic, but instead arrested. This increase in G1 was not changed in the TSC-22 expressing line (figure 4B), suggesting that TSC-22 does not play a role in inducing a G1-arrest.

HDGF may be part of the mechanism by which cAMP inhibits proliferation

HDGF is a growth factor which has been reported to induce proliferation of different cell types and to be oncogenic (16,29,30), but cAMP-dependent regulation has not been described before. Therefore induction of a cAMP-dependent cell cycle arrest was examined in the presence or absence of overexpressed HDGF. The cAMP-mediated increase of G1-phase cells was clearly inhibited for a part by introduction of HDGF (figure 5), and also HDGF alone slightly reduced the amount of G1-phase cells. Thus, increased HDGF levels may promote proliferation of NIH3T3-A14 cells, thereby overruling cAMP-effects.

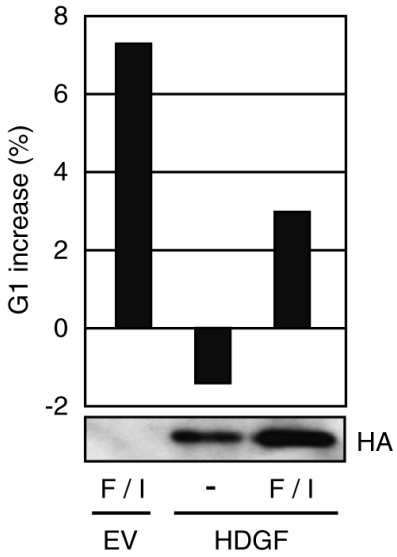

Figure 5. ***HDGF is involved in a cAMP-induced cell cycle arrest.*** NIH3T3-A14 cells were transfected with either an empty vector (EV) or with HDGF. Cells were treated with forskolin/IBMX (F/I) for 48 hours, or were left untreated and the cell cycle profile was determined as in figure 1B. The percentage increase in G1-phase cells, compared to EV-transfected, unstimulated cells was plotted and the expression of HA-tagged HDGF is shown.

DISCUSSION

We searched for genes that are transcriptionally regulated by cAMP, and involved in inhibition of proliferation. To this end we performed a microarray experiment to find new cAMP-regulated targets, discriminated by regulation by MAPK or not regulated by MAPK. We found 68 genes regulated by cAMP of which 11 known cAMP-regulated genes and 29 genes known to be related to proliferation. 23 Genes of the cAMP-regulated genes were found as putative MAPK targets, of which 5 were reported before to be regulated by MAPK. We verified 5 new and 2 known cAMP-regulated genes by real time-PCR and/or protein levels and all these genes turned-out to confirm the microarray data (figure 2 and 3 and data not shown), showing that the microarray data is reliable.

MAPK-independent cAMP targets

Early cAMP-targets are putatively involved in regulation of other proteins involved in a cAMP-induced cell cycle arrest. Therefore, especially proteins involved in transcription or translation and modifying proteins, like kinases and ligases, are interesting. Several transcription and translation related proteins were found to be upregulated by forskolin/IBMX, but not by U0126 at 2 hours after stimulation. Among these genes, Btg2 and Eif4a2 are known to contain a cAMP response element (CRE) in their promoter, which makes them likely cAMP-responsive genes via PKA and CREB. Indeed Btg2 has been reported to be induced transcriptionally by cAMP. Btg2 is a transcriptional co-activator, also named TIS21 or PC3, which has an anti-proliferative effect by impairing G1-S progression by a so far unknown mechanism (reviewed in (31)), which would fit in a cAMP-induced arrest. Translation initiation factor Eif4a has not been reported to be regulated by cAMP, but is linked to proliferation as *Drosophila* Eif4a could

suppress the PTEN mutant proliferative advantage (32). Furthermore, the function of two other early cAMP-regulated transcription factors, TBX1 and the recently identified Nolz-1, are not well known, but interesting as they have been described to be involved in induction of differentiation (33,34), a proliferation-related process. Finally, another interesting transcription factor, Gly96, was found to be regulated at early timepoints by cAMP. Gly96, also named IEX-1 or IER3, has emerged before in a screen for cAMP-regulated genes (35) and has cell-type dependent effects on cell cycle progression and apoptosis and is highly expressed in many tumour cell lines (reviewed in (36)). It would be interesting to examine if the cell-type dependency of Gly96 corresponds with the cell type-dependent growth regulation by cAMP.

cAMP-induced, prolonged-regulated genes comprise for a large part transcription factors, but also some growth factors. The latter were all found downregulated upon cAMP treatment. Among these growth factors, HDGF regulation by cAMP could be verified by us. HDGF is a NLS-containing, secreted growth factor with mitogenic properties. Nuclear translocation is essential for its proliferation-inducing activity, which is seen in different cell types, including fibroblasts (37,38). HDGF is also an oncogenic growth factor, as NIH3T3 cells overexpressing HDGF form tumours, when injected in nude mice (29). We now show that HDGF can participate in a cAMP-induced cell cycle arrest, as overexpression of HDGF could compensate partly for a cAMP-induced G1-increase. Besides HDGF, we verified the downregulation of Gas6, a ligand for the transforming receptor tyrosine kinase Axl. Gas6 was reported to induce cell survival of NIH3T3 cells, which involved Src and PI3K (39). In general, gas6 seems to function as growth promoting, anti-apoptotic cell survival protein in several cell types (39-41). The third growth factor, macrophage migration inhibitory factor (MIF), has been shown before to be induced by cAMP, via its CRE in the promoter (42). MIF is a regulator of inflammatory and immune responses, but also involved in proliferation. Recombinant MIF can induce for instance the proliferation of quiescent NIH3T3 cells, which is suggested to be mediated by the MAPK pathway, whereas inhibition of serum-induced secretion of MIF with anti-MIF antibodies could reduce DNA synthesis. Furthermore, MIF has pronounced effects on tumour-growth by mediating angiogenesis and antagonizing p53 function (reviewed in (43)). Besides HDGF and Gas6, we verified some other interesting targets, which might be involved in proliferation. For instance, the transcriptional repressor TSC-22 (17) upregulates the cell cycle inhibitor $p21^{Cip1}$ in intestinal epithelial cells, resulting in inhibition of proliferation (44). It has been reported that cAMP also can regulate $p21^{Cip1}$ levels (45), but this could not be repeated by us in our cell system (data not shown). TSC-22 has been found in several screens upregulated by different agents, like TGFβ, progestins, vesnarinone, and PPARγ, which all can inhibit proliferation (44,46-48), like cAMP. A putative role for TSC-22 in proliferation is further suggested as TSC-22 levels are downregulated in human brain tumours (49) and antisense constructs against TSC-22 induce proliferation in a salivary gland cancer cell line. In the same cell-line the anti-cancer drug vesnarinone induced expression of TSC-22, although overexpression of TSC-22 did not affect proliferation (50). The latter is in agreement with our results, as NIH3T3 and LS174T cells stably overexpressing TSC-22 were not significantly inhibited in proliferation. Furthermore, it has been reported that overexpression of TSC-22 induces (sensitivity to) apoptosis in tumour cell lines (26-28,51), but as in NIH3T3-A14 cells no apoptosis is induced upon cAMP treatment or after challenge with camptothecin in NIH3T3-A14 cells overexpressing TSC-22, TSC-22 is not

likely involved in induction of apoptosis in these cells.

Another target that we verified and found upregulated by cAMP is the RCC1-like and HECT E3 ligase-domain containing protein, KIAA1593. KIAA1593 protein and function is unknown, but is conserved in among others *C. Elegans* and *Drosophila* and has homology in sequence and domain organization to Ceb1, which has been found in a screen for cyclin E-$p21^{Cip1}$-complex binding proteins. Ceb1 binds to several cyclins in HEK293T cells and is upregulated in a background of p53- or Rb-inactivation (52), which makes the involvement of KIAA1593 in proliferation also plausible. Finally, stathmin was verified, of which it has been reported that both overexpression and antisense disruption results in inhibition of proliferation of K562 leukemic cells and accumulation of cells in the G2/M phase of the cell cycle. This is due to a role of stathmin in the generation of the mitotic spindle, by promoting microtubule depolymerization. Stathmin is regulated by phosphorylation in a cell cycle dependent manner and phosphorylation is mainly increased in M-phase, leading to inhibition of the protein and built-up of the mitotic spindle. Both phosphorylation of stathmin to form the mitotic spindle, as dephosphorylation of stathmin to disassemble the spindle, are essential to progress cell cycle. Stathmin is phosphorylated by MAPK, PKA, cdc2 and calmodulin-dependent kinase, and is highly expressed in many types of cancer and correlates with prognostic factors for breast cancer. Although stathmin is not likely involved in the transformation process itself, it has been suggested to maintain the transformed phenotype of cells and is indeed needed to maintain the high proliferation rate of cancer cells (reviewed in (53)). In our cell system, stathmin mRNA levels were clearly downregulated by cAMP in time, but protein levels were downregulated only after 48 hours of cAMP-treatment. Therefore, stathmin is likely downregulated as a consequence of a cAMP-induced cell cycle arrest. Further, a G2/M cell cycle arrest induced by stathmin does not fit in the mechanism of a cAMP-cell cycle arrest in the G1 -phase of the cell cycle.

Further experiments should be performed to establish the role of other targets in cAMP-dependent inhibition of proliferation.

MAPK-dependent cAMP targets

Interestingly, although MAPK does not play a major role in proliferation of NIH3T3-A14 cells, MAPK-dependent regulation of several targets often correlates with a role in proliferation in various cell lines. This has for instance been reported for our MAPK targets Etv5, SGK1, cyclin D1 and Beta-amyloid precursor protein (table 3) (25,54-57). Furthermore, several MAPK targets are oncogenic or upregulated in cancer, like Etv5, TEF-1δ and Eif4E (54,58,59). cAMP-dependent regulation of MAPK targets though is less common described and only SGK1 and cyclin D1 have been reported before in this (6,60). Downregulation of Cyclin D1 correlates with a cAMP-induced cell cycle arrest in several cell types and its regulation at the mRNA level in the microarray does correlate with its protein levels in NIH3T3-A14 cells. Therefore this is a good validation of the screen. Serum and glucocorticoid-inducible serine/threonine kinase (SGK) stimulates several ion-channels, including ENaC and KCNQ1 (reviewed in (61)). Interestingly, the latter was found upregulated by cAMP in the microarray as well (table 1), supporting a model of cAMP-dependent regulation at different levels.

In general, inhibition of these genes by U0126 is not sufficient to inhibit cell proliferation, showing the robustness of the regulation of cell proliferation.

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ADDENDUM 1

cAMP-induced inhibition of proliferation of NIH3T3-A14 may involve nuclear translocation rather than transcriptional regulation of $p27^{Kip1}$

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In chapter 3 we show that in MEFs, apart from inhibition of the MAPK pathway, inhibition of PKB activity and consequent induction of $p27^{Kip1}$ levels play a role in the anti-proliferative effect of cAMP. Furthermore, a reduction in cyclin D1 levels was observed. Also for NIH3T3-A14 cells, cAMP is a potent inhibitor of proliferation, which was investigated in chapter 4. This inhibition of proliferation is accompanied by a decrease in MAPK activity (chapter 4, figure 1C) and a decrease in cyclin D1 expression levels (chapter 4, figure 3F). The question is raised whether in NIH3T3-A14 cells the PI3K/PKB pathway is also an important target of cAMP to inhibit proliferation.

Therefore, the regulation of PKB and several components of the PKB signalling pathway by cAMP in NIH3T3-A14 cells was investigated (figure 1A). In contrast to MEFs (chapter 3), PKB was not inhibited by cAMP at early time points. We only observed inhibition of PKB 24 to 48 hours after cAMP treatment, indicating an indirect effect of cAMP. In addition, phosphorylation of the PKB target GSK3β (1) and the indirect target p70S6 kinase (reviewed in (2)) were not affected by cAMP treatment. In contrast, cAMP did inhibit ERK-phosphorylation and cell proliferation. Importantly, cAMP was not able to induce $p27^{Kip1}$ protein levels (figure 1A), which correlates with the inability of cAMP to induce $p27^{Kip1}$ promoter activity, nor in the presence or in the absence of FoxO4 in NIH3T3-A14 cells (data not shown). Apparently, regulation of the PI3K/PKB pathway and expression levels of $p27^{Kip1}$ is not part of the program via which cAMP inhibits proliferation of NIH3T3-A14 cells.

Interestingly, apart from increasing expression levels, treatment of MEFs with cAMP resulted in nuclear localization of $p27^{Kip1}$. We hypothesized that translocation of $p27^{Kip1}$ might be an alternative mechanism to regulate the protein in NIH3T3-A14 cells, and therefore the subcellular localization of $p27^{Kip1}$ was investigated. $p27^{Kip1}$ resided in the cytoplasm of cycling NIH3T3-A14 cells and translocated to the nucleus upon serum starvation. More importantly, $p27^{Kip1}$ translocated to the nucleus upon forskolin/IBMX treatment (figure 1B). Apparently, increased cAMP can shift the balance of import and export of $p27^{Kip1}$ by a mechanism that does not rely on increase in protein levels. As a control, cells were stimulated with U0126, but this did not result in nuclear translocation, showing that the cAMP-effect on import is MAPK-independent. In addition, we tested the effect of the PI3K-inhibitor LY294002 on $p27^{Kip1}$ import. LY294002 treatment alone led to only partial nuclear staining and furthermore, it enhanced cAMP-induced $p27^{Kip1}$ nuclear staining (figure

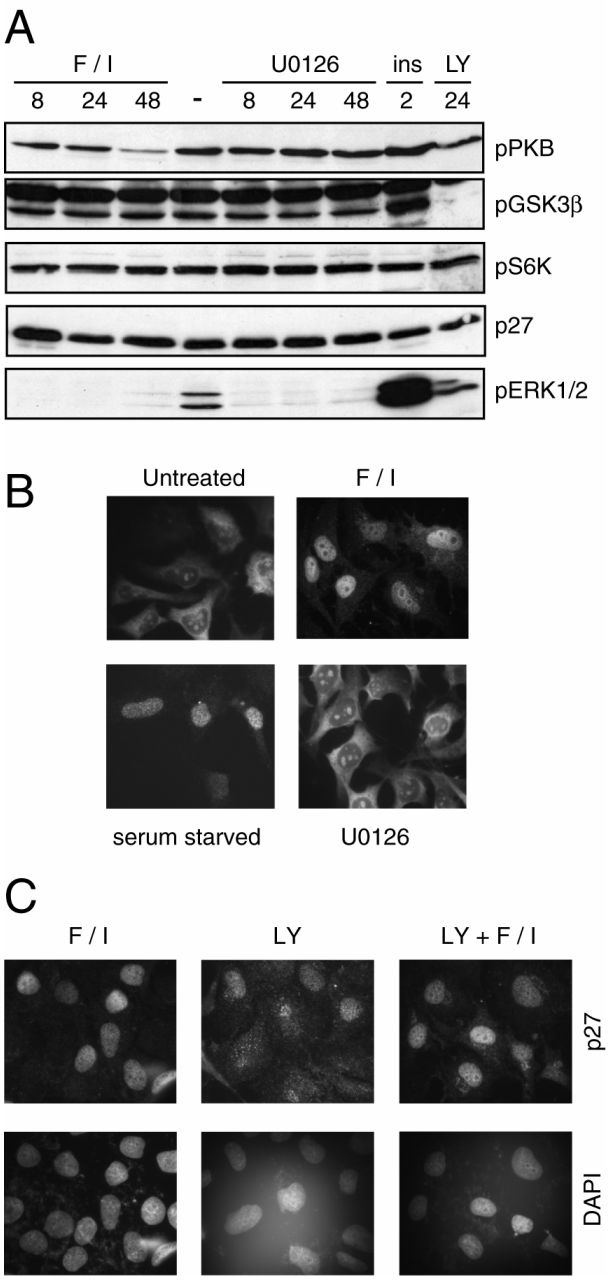

Figure 1. ***cAMP-dependent regulation of different components of the PI3K/PKB signalling pathway.*** (A) Regulation of the PKB pathways by cAMP. NIH3T3-A14 cells were treated for the indicated time points with forskolin (10 μM; ICN)/IBMX (0.5 mM; Sigma) (F/I), U0126 (10 μM; Biomol Research Laboratories), insulin (ins; 1 μg/ml; Sigma), or LY294002 (LY; 10 μM; Sigma) or were left untreated. Blots containing total lysates were probed for PKB phosphorylated at serine 473, GSK3β phospho-rylated at serine 21/9, p70S6 kinase phosphorylated at threonine 389, ERK 1/2 phosphorylated at threonine 202 and tyrosine 204 (Cell Signaling), and $p27^{Kip1}$ (Transduction Laboratories). (B) cAMP induces nuclear localization of $p27^{Kip1}$. NIH3T3-A14 cells were treated for 24 hours with forskolin/IBMX, U0126, were left untreated, or were serum starved for 36 hours. Cells were fixed to slides and $p27^{Kip1}$ protein was stained by immunofluorescence. (C) cAMP and LY294002 induce nuclear localization of $p27^{Kip1}$ via separate pathways. NIH3T3-A14 cells were stimulated for 24 hours with forskolin/IBMX, LY294002 or a combination of both stimuli and $p27^{Kip1}$ protein was visualized as described in B. Nuclei were stained with DAPI.

1C). This indicates that the cAMP pathway and PI3K pathway might act synergistically to import $p27^{Kip1}$.

In conclusion, in NIH3T3-A14 cells PKB activity is only indirectly inhibited by cAMP at later time points and PKB targets are clearly not regulated at all by cAMP. The indirect PKB target, $p27^{Kip1}$, can nevertheless be regulated by cAMP, at the level of localization in a PKB-independent manner. Thus, induction of nuclear localization of $p27^{Kip1}$ can be part of the mechanism via which cAMP induces a cell cycle arrest. This means that cAMP-dependent growth inhibition of both NIH3T3-A14 cells and MEFs may involve $p27^{Kip1}$, but that the mechanism is different.

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ADDENDUM 2

Gene expression profiling of 8CPT-2'OMe-cAMP-treated NIH3T3-A14-Epac1 cells

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The role of Rap1, a member of the Ras-family of small GTPases, in integrin-mediated cell adhesion is well established (1-3). Although Rap1 is regulated by various growth factor and second messengers (4), other functions of Rap1 are still mainly undefined. Several other members of the Ras superfamily of small GTPases have been reported to play a role in different cellular processes. For instance, the best characterized function of Rho family GTPases is regulation of the actin cytoskeleton (reviewed in (5)), but in addition they control processes like cell polarity, vesicular trafficking and gene expression. Rho-dependent gene expression plays a role in the control of cell cycle progression in different cell types, using different mechanisms. For instance, Rac can induce interleukin-2 expression via the c-Jun N-terminal kinase pathway in T-cells, and Rac regulates the expression of cyclin D1 and D2 respectively in endothelial cells and B-cells. Furthermore, Rho has been described to inhibit expression of the CDK-inhibitor $p21^{Cip1}$ and to induce cyclin D1 via regulation of the MAPK pathway (reviewed in: (6,7)). We investigated whether Rap1, in analogy to Rac and Rho GTPases, also regulates gene expression, using microarray analysis. To this end, we made use of the fact that 8CPT-2'OMe-cAMP is a very specific activator of the Rap1-specific guanine nucleotide exchange factor Epac (8). RNA was isolated from NIH3T3-A14-Epac1 cells, which are NIH3T3-A14 cells stably expressing Epac1, and used for microarray analysis. We used adherent cells to exclude genes which are induced as a result of the adhesion process.

NIH3T3-A14-Epac1 cells were stimulated with 50 μM 8CPT-2'OMe-cAMP (Biolog) for 1, 3, 8 and 24 hours or were left untreated. Microarrays (15-K mouse cDNA microarrays; manufactured at the Netherlands Cancer Institute) were hybridized with labeled cDNA of unstimulated versus stimulated cells and at the same time a dye swap experiment was performed. Genes whose mRNA level was changed by at least 1.2 fold for at least two consecutive time points are presented in table 1. We found 22 genes fitting the criteria, which are putative Rap1-regulated genes. Although they need to be verified, real time-PCRs in chapter 4 showed that the actual fold induction in a microarray experiment normally should exceed this 1.2 fold, and this has also been observed by others (9). We mostly found genes of which the function is unknown or not investigated to a great extent. The most significant changes were found at 8 hours of 8CPT-2'OMe-cAMP treatment for corticosteroid binding globulin (Cbg) and septin 7/CDC10. Interestingly, Cbg has been reported before to be regulated by cAMP (10). The cAMP-effector involved in Cbg regulation has not been investigated so far, but our results indicate that this is Epac. Cbg binds with high

affinity to corticosteroids, thereby regulating the effect of a stress-response to cells. Cbg generally regulates the availability of steroids to tissues and directs the delivery of hormones to specific sites (reviewed in (11)). For the other 8CPT-2'OMe-cAMP-regulated target, septin 7, not much is known about the processes in which it is involved. Septins are a family of guanine nucleotide binding proteins, which seem to be structural components of filaments. They were identified in a genetic screen for yeast mutants defective in cytokinesis. Besides cytokinesis, septins are involved in bud-site selection and cell polarity in budding yeast (reviewed in (12-14)). This shows an intriguing resemblance with the function of the Rap1 orthologue in yeast, bud1, which is also involved in bud-site selection (reviewed in (15)). In mammalian cells, septins are also involved in cytokinesis. Interestingly, septins were found to bind to some components of the exocyst complex and to SNARE proteins, assuming a role in exocytosis and vesicle fusion (reviewed in (12-14)).

The deubiquitinating enzyme Fam, which is the *Drosophila* fat facets homologue is another interesting 8CPT-2'OMe-cAMP target. In *Drosophila* eye development, fat facets is involved in inhibition of neural development of specific facet precursor cells of the compound eye, limiting the number of photoreceptor cells per facet to eight. Interestingly, fat facets genetically interacts with Rap1 in Drosophila, as in addition to the mentioned essential

Table 1. ***8CPT-2'OMe-cAMP transcriptionally regulated genes***

		8CPT-2'OMe-cAMP			
Acc. No.	**Gene description**	**1 h**	**3 h**	**8 h**	**24 h**
Transcriptional and translational protein		**mRNA fold induction**			
BG081324	KRAB zinc finger protein	1.0	1.3	1.2	1.4
BG063901	Eukaryotic release factor (Hbs1-like)	1.1	1.3	1.3	1.6
BG078832	Wilms' tumour associating protein (WTAP)	1.3	2.5	1.3	1.2
BG077900	bHLH transcription factor HAND1	-1.1	-1.5	-1.7	-1.5
AW557129	Poly(A) polymerase III	1.2	-1.3	-1.8	-1.2
DNA / RNA related proteins					
BG065520	Small nuclear Ribonucleoprotein (U2-snRNP B)	1.8	1.5	2.2	1.5
BG073458	Topoisomerase II beta	1.0	1.2	1.3	1.2
BG063903	RNA metabolic protein, DEAD-box protein	1.6	1.4	1.9	1.9
BG069712	45S pre rRNA	-1.1	-1.3	-2.3	-1.4
Divers signalling proteins					
BG087442	Deubiquitinating enzyme Fam	1.0	1.7	1.3	1.7
AW539669	Ubiquitin binding protein (p62/SQSTM1)	2.3	2.2	1.9	1.9
BG088372	Tau-tubulin kinase	-1.2	1.2	1.3	1.3
BG075647	Cyclin I	1.4	1.5	-1.3	1.1
BG078850	Corticosteroid binding globulin (Cbg)	2.1	1.5	4.2	1.2
BG077286	Septin 7/CDC10	1.3	1.8	4.3	-1.2
BG064589	Vacuolar ATPase (V-ATPase)	1.5	1.2	1.7	1.5
BG077301	Syntaxin 4A (Stx4a)	1.7	1.6	1.5	-1.2
BG075033	BC-box family protein Muf1	-1.2	1.3	1.3	1.5
BG088292	JNK-1 interacting protein (JIP3)	-1.2	-1.1	-1.4	-1.3
BG068258	Hermansky-Pudlak Syndrome 1 homolog (HPS1)	-1.1	-1.1	-1.2	-1.4
BG076893	RhoB	-1.1	-1.3	-1.7	-1.2
BG075095	TPR repeat containing protein	-1.4	-1.2	-1.4	-1.4

function in early eye development, fat facets functions later in eye development as well, which involves Ras1 and Rap1 (16). In mammalian cells, Fam has been shown to interact and co-localize with the putative Rap1 effector AF-6 and Fam can prevent AF-6 ubiquitination, thereby stabilizing the protein (17). Fam dependent stabilization was further shown for the wnt-signalling component β-catenin (18) and recently it has been reported that Fam interacts with β-catenin and E-cadherin in subconfluent cell cultures. This interaction was suggested to take place during traffic of the proteins to the plasma membrane (19). Although *Drosophila* fat facets and mouse Fam functionally are conserved, shown by substitution of fat facets by Fam in *Drosophila*, *Drosophila* AF-6 (canoe) and β-catenin (armadillo) are not genetically linked to fat facets functions in *Drosophila* eye development (20). Still, it would be interesting to see if Rap1, via induction of Fam and stabilization of AF-6 can induce cell adhesion in mammalian cells.

Overall, we did find a few genes regulated by 8CPT-2'OMe-cAMP, which suggests that the main role of Rap1 is to regulate cell adhesion, but in addition plays a role in transcriptional regulation of some genes. These genes might give clues for other processes that are regulated by Rap1 activation. It would be interesting to see if Rap1 activation would induce other expression profiles in non-adherent cells or in cells that are in the process of adhesion. This would contribute to the identification of Rap1 functions.

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ADDENDUM 3

Microarray analysis of insulin-regulated genes in NIH3T3-A14 cells

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Insulin is a hormone that regulates many cellular processes like glucose uptake, glycogen, lipid and protein synthesis, protein translation, transcription and modulation of cellular growth and differentiation. Decreased secretion of insulin and decreased sensitivity to insulin (insulin resistance) results in type II diabetes. Furthermore, insulin resistance is often associated with disease conditions like obesity, hypertension and atherosclerosis. Insulin is secreted by pancreatic beta-cells. Important is also the autocrine-positive feedback loop of insulin on its own secretion, involving insulin receptor signalling. The insulin receptor is a tyrosine kinase receptor and its direct targets include c-Cbl, Shc and the docking proteins Gab1, $p60^{DOK}$ and insulin receptor substrate (IRS) proteins. These substrates couple to different signalling pathways and are differently expressed and distributed in the cell. IRS proteins are the main mediators of insulin signals at the receptor. Mice deficient for IRS-1 are insulin resistant and mice lacking IRS-2 exhibit impaired glucose tolerance and diabetes due to insulin resistance and a defect in insulin secretion. The most important signalling molecule coupled to IRS proteins is phosphatidylinositol 3-kinase (PI3K), which signals to PKB, atypical PKCs (PKCζ and PKCλ) and p70S6 kinase pathways. These pathways can also be activated upon stimulation with other growth factors, but this does not mimic insulin action, likely due to activation of PI3K at different locations in the cell. The insulin receptor can also couple independently of IRS proteins to the MAPK pathway, via Grb2-Sos-Ras. The PI3K pathways are though the major pathways responsible for the metabolic processes of insulin (reviewed in (1-3)).

We searched for genes that are regulated by insulin in NIH3T3-A14 cells by microarray analysis. NIH3T3-A14 cells are well characterized by our group, but notwithstanding, the panel of genes transcriptionally regulated by insulin is largely unidentified. Furthermore, investigating insulin-regulated transcription can be used as a control for cAMP- and U0126-regulated transcription (chapter 4). NIH3T3-A14 cells overexpress the insulin receptor and both the Ras-MAPK and the PI3K-PKB pathway are clearly activated upon insulin treatment. The MAPK pathway is activated by insulin up to at least 24 hours after treatment (chapter 4, figure 1C). We used for our screen NIH3T3-A14 cells growing in the presence of serum, to get more insights in insulin-induced expression patterns independently of cell cycle entry. The cells were treated for 2, 8 and 24 hours with insulin (1 μg/ml Sigma) and a microarray experiment was performed as described in chapter 4. The most significant differentially regulated genes whose expression was changed for at least 2 consecutive time points were selected and are presented in table 1. Interestingly, they include

the transcription factors Egr-1, Gly96, BTG1 and Fra-1, regulated by insulin in a similar pattern. They were induced to high levels after 2 hours, with intermediate expression at 8 hours and back to basal after 24 hours of stimulation. Since insulin signalling remains high for at least 24 hours, there has to be a negative feedback loop on the regulation of these transcription factors.

Egr-1, BTG1 and Fra-1 are reported previously to be insulin-, MAPK- and/or PI3K-regulated (4-6). Egr-1 is an insulin-responsive gene in for instance vascular endothelial cells, hepatoma cells and adipocytes. In vascular endothelial cells, insulin induces egr-1 expression via the MAPK and PI3K pathway and egr-1 antisense oligos block insulin-induced DNA synthesis. Also in hepatoma cells, egr-1 expression was ERK/MAPK-dependent, whereas the p38/MAPK and PI3K pathway could influence ERK/MAPK-induced egr-1 expression (4,7,8). Egr-1 has also been identified in similar types of microarray screens using human and NIH3T3 mouse fibroblast cell lines, as serum and IGF-1-responsive gene respectively (9,10). The second transcription factor, B-cell translocation gene 1 (BTG1), is a protein that can inhibit proliferation of NIH3T3 cells (11) and was recently identified in a microarray screen as direct target of FoxO3a in an erythroid cell line (5). However, FoxO transcription factors are negatively regulated by the PI3K/PKB pathway (12,13), thus FoxO3a-induced BTG1 expression is the opposite of what we find, as BTG1 is induced by insulin in our screen. This means that FoxO transcription factors are not involved in the regulation of BTG1 levels in NIH3T3-A14 cells. The third transcription factor, Fra-1, belongs to the Fos family of transcription factors and this family, together with the Jun family, forms the activator protein-1 (AP-1) transcriptional complex. It has been shown that insulin induces Fra-1 transcription in a MAPK-dependent manner (6).

Most of the genes identified in the screen are downregulated by insulin, while several of them are reported to be regulated by MAPK or PI3K signalling, e.g. ID1, dab2, TIMP-2, clusterin, $GABA_A$-receptor and TC21 (14-21). If insulin mediates inhibition of these genes via MAPK or PI3K in NIH3T3-A14 cells awaits further experimentation. As insulin stimulates the MAPK and PI3K pathways in NIH3T3-A14 cells, activation of the pathways has likely an inhibitory role on transcription of these genes or does involve other signalling pathways.

Several of the insulin-regulated genes that we identified are involved in proliferation, either in a stimulating or inhibiting manner. These include, apart from the genes described above, dab2, TIMP-2, gly96, ID1, TC21, ERK3 and the growth factors. An example is Drosophila disabled homolog 2 (dab2), also named p96 or differentially expressed in ovarian carcinoma-2 (DOC-2), which is a potential tumour suppressor gene with a growth inhibitory effect on several cancer types. This occurs likely by suppression of the MAPK pathway as dab2 can interrupt the binding between Grb2 and SOS, necessary for ras-MAPK activation (22). Dab2 is downregulated by insulin in our screen, likely enhancing a growth stimulatory effect. A second example is tissue inhibitor of metalloproteinase-2 (TIMP-2), which has been shown to have variable effects on proliferation. TIMP-2 plays a role in the homeostasis of the extracellular matrix (ECM) by inhibiting activity of matrix-degrading matrix metalloproteinases (MMPs). TIMP-2 can inhibit the growth of among others basic FGF-stimulated endothelial cells and of human melanoma cells grown in the presence of collagen. However, TIMP-2 has been shown to stimulate the growth of a large variety of normal and malignant cells in vitro (reviewed in (23)). In NIH3T3 cells, proliferation can be promoted by decreasing TIMP-2 expression levels, resulting in increased MMP-2 activity

Table 1. ***Insulin regulated genes***

		Insulin		
Acc. No.	**Gene description**	**2 h**	**8 h**	**24 h**
Transcription regulating and DNA binding proteins		**mRNA fold induction**		
BG070825	Egr-1	3.5	1.5	1.1
BG080666	Gly96/IEX-1/IER3	3.5	2.0	1.1
BG067594	Polycomb-group protein, sex comb on midleg homolog 1	3.0	1.6	1.1
BG072743	B-Cell translocation gene 1 (BTG1)	2.1	1.5	1.1
BG063519	Fos-related antigen 1 (Fra-1)	1.5	1.3	1.0
BG076619	Helix-loop-helix DNA binding protein regulator, ID1	-1.5	-2.0	-1.7
BG083139	Musculus pituitary tumor-transforming, Pttg1/securin	-1.1	-1.4	-1.6
BG069168		-1.0	-1.4	-1.5
Mitogen/cytokine regulated proteins				
BG080700	Tumor necrosis factor family member receptor, TWEAKR/Fn14	1.8	1.5	1.4
BG072253	Mitogen-activated protein kinase, ERK3/p97MAPK	1.2	1.5	1.4
BG072156	Interferon-induced transmembrane protein, fragilis/Ifitm3/Mil-1	-1.2	-1.7	-2.4
BG085400	Dab2/Doc-2/p96	-1.3	-1.8	-2.2
Cytoskeletal/membrane proteins				
BG080898	Ion channel, TRPM7	2.1	1.5	1.0
BG063159	Cell adhesion molecule, nectin3	1.3	1.8	1.5
BG088523	Integral transmembrane protein 2B, Itm2b	-1.2	-1.6	-2.3
BG063081	Thymosin beta-10	-1.1	-1.3	-2.1
BG063173	Alpha-actinin-4 (Actn4)	-1.1	-1.3	-2.0
BG078578	Actin binding LIM protein, abLIM/UNC-115	-1.3	-1.8	-1.5
Enzymes				
BG080845	TC21/R-Ras2	-1.5	-1.6	-2.6
BG088451	Tissue inhibitor of metalloproteinase, TIMP-2	-1.2	-1.5	-2.6
BG073415	HECT E3 ubiquitin ligase, Nedd4	-1.2	-1.4	-1.6
BG083522	Cdc kinase subunit 2 (CKS2)	-1.3	-1.3	-1.6
Growth factors				
BG088548	Growth arrest specific 6 (Gas6)	1.1	-1.5	-1.7
BG080688	Colony stimulating factor 1 (Csf1)	-1.2	-1.4	-1.5
BG083515	Lens epithelium derived growth factor (LEDGF)	-1.2	-1.3	-1.5
Diverse signalling proteins				
BG074158	Steroid sensitive gene-1 (SSG1)	-1.2	-1.5	-3.2
BG064795	Ferritin L-subunit gene	1.1	-1.8	-2.3
BG064796		1.0	-1.9	-2.1
BG079620	γ-synuclein	-1.1	-1.4	-1.9
BG073409	Stathmin	-1.1	-1.5	-1.7
BG072209	Clusterin/ApoJ/SGP-2	-1.0	-1.3	-1.7
BG085378	Myristoylated alanine rich C kinase substrate (Marcks)	-1.2	-1.4	-1.5
BG076653	Peripheral benzodiazepine/$GABA_A$ receptor	-1.1	-1.4	-1.5

(17). It is therefore likely that in NIH3T3-A14 cells insulin-mediated reduction of TIMP-2 levels contributes to increased proliferation.

In conclusion, some known and several novel insulin-responsive genes were identified by microarray analysis using NIH3T3-A14 cells. The finding of known insulin-, MAPK- and PI3K-regulated genes, verifies the microarray screen. In general, a part of the insulin-regulated genes are known regulators of cell proliferation. Insulin-mediated regulation of proteins like BTG1, ERK3, Gas6 and LEDGF, would predict an inhibitory effect on proliferation (11,24-26), whereas regulation of a few other genes like egr-1 and dab2, would result in stimulation of proliferation (4,22). This is in accordance with the finding that insulin by itself does not have a significant growth stimulatory effect on NIH3T3-A14 cells (chapter 4 figure 1A). It may be that insulin regulates a subset of growth regulatory genes in NIH3T3-A14 cells, resulting in an equilibrium of proliferative and anti-proliferative genes, not resulting in a net growth effect. The finding of proliferation-associated genes is in contrast to what Dupont et al. has reported (10). They performed a microarray screen using NIH3T3 cells, overexpressing either the insulin or IGF-1 receptor. They did find proliferation-associated genes after IGF-1 treatment, but not after insulin treatment. Only a few insulin-regulated genes were identified by them, which may be explained by the use of a higher cut-off for differentially regulated genes. In our screen, the most significant differentially regulated novel insulin-responsive genes are gly96, sex comb on midleg homolog 1 and steroid sensitive gene-1 (SSG1). The finding of these and other novel insulin-responsive genes may accelerate the identification of functions of insulin-signalling in fibroblasts.

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Chapter

General discussion

cAMP functions as a second messenger in a broad range of cellular effects. At the start of this thesis, the lab had discovered that cAMP not only activates PKA, but also the Rap1GEF Epac. In addition, another Rap1GEF, PDZ-GEF, was identified, which possesses a putative cAMP binding domain. The aim of my work was first to investigate whether PDZ-GEF is a real cAMP target and if not, whether other ligands for this GEF could be found. Secondly, the effect of cAMP-activated Rap1 on gene expression was investigated to find novel functions of Rap1. This was followed by the analysis of effects of cAMP on gene expression in general, specifically to find genes involved in cell proliferation. Several novel cAMP-regulated genes were identified, of which some were analysed for their role in the anti-proliferative effect of cAMP. Furthermore, the question whether FoxO transcription factors are mediators of the cAMP-induced cell cycle arrest was addressed.

Regulation of PDZ-GEF

In addition to Epac, also the Rap1GEF PDZ-GEF has a domain homologous to the cAMP-binding domain in PKA. However, critical residues required for cAMP binding are lacking. Indeed, extensive binding studies, including ITC measurements, show that cAMP does not bind to the related cAMP-binding domain (RCBD) of PDZ-GEF. In addition, cAMP does not activate PDZ-GEF in vitro as well as in vivo. Also PDZ-GEF2 did not respond to cAMP (**chapter 2**). We were therefore surprised that Pham et al. reported that cAMP did bind to and regulate PDZ-GEF1. However, surprisingly the activity towards Rap1 was not affected, and in contrast, PDZ-GEF1 now activated Ras (1). Moreover, this group reported that PDZ-GEF can bind to β1-adrenergic receptors and that the cAMP-generating hormone, isoproterenol, is also able to activate Ras via PDZ-GEF1 (2). We have however, never observed any activity of PDZ-GEF towards Ras and do not have a simple explanation for this discrepancy. Furthermore, it has been shown that PDZ-GEF1 can interact with the synaptic scaffolding molecule S-SCAM (3), which can bind to β1-adrenergic receptors (4), showing that the interaction of PDZ-GEF1 and β1-adrenergic receptors might be indirect.

PDZ-GEF contains multiple domains, of which several interact with proteins that function at cell junctions, but regulation of this protein is still largely unknown. The question is what then the function is of the RCBD if cAMP does not bind to it. Importantly the domain is highly conserved during evolution, suggesting a conserved function as well. We searched for a ligand that can activate PDZ-GEF, but were so far unsuccessful in spite of the use of different approaches (**chapter 2**). One putative ligand, which was tested in vitro in a candidate approach, was cyclic ADP-ribose (cADP-R). This molecule is interesting since the target protein is still not known. It is involved in calcium signalling via activation of the ryanodine receptor (reviewed in (5)). It may be that proteins in vivo synergize with other factors to activate PDZ-GEF and therefore cADP-R is still a candidate for involvement in PDZ-GEF regulation in vivo. Recently a membrane permeable analogue of cADP-R has been synthesized (6), which may be a useful tool for testing cADP-R effects on PDZ-GEF and Rap1 activity in cell lines.

cAMP-mediated Rap1 effects

The small GTPase Rap1 can be activated by cAMP and has been implicated in proliferation, although involvement in integrin-mediated cell adhesion is the main established role. We searched for genes regulated by cAMP-activated Rap1 to find new functions of Rap1

(**addendum 2**). Rap1 has been described to play a role in regulation of both the MAPK and PKB pathway, which indicates that Rap1 may be involved in transcriptional regulation. Although we did find some Rap1-regulated genes, this is not easily explained by Rap1-mediated regulation of the MAPK or PI3K pathway. Our lab has shown using 8CPT-2'OMe-cAMP that Rap1 is not involved in regulation of the MAPK pathway (7). The role of Rap1 in PKB signalling is less clear as Rap1 has both been shown to activate and inhibit PKB activity, either in an Epac- or PKA-dependent manner (8-10). We were not able to show a connection between Rap1 and PKB (data not shown) and this is confirmed by the microarray data, as no established PKB targets were found. Concerning Epac versus PKA effects on Rap1 activity, it is clear that Epac is involved in activation of Rap1 (11,12). In contrast, PKA may have an inhibitory or stimulatory role on Rap1 activity (8,13). Interestingly, the PKA inhibitor H89 induces Rap1 activity in several cell lines (data not shown) which may imply that PKA has an inhibitory effect on Rap1. It should however, be taken in account that this inhibitor is not specific for PKA (14). It has been reported that Rap1 does contain a PKA-phosphorylation site, which is phosphorylated in vitro and in vivo by PKA (15). Furthermore, PKA has been shown to phosphorylate certain sites of the Rap1 GTPase activating protein (Rap1GAP) in vitro and these sites are also phosphorylated in vivo upon cAMP treatment (16,17). The question is if PKA-mediated phosphorylation of Rap1 or Rap1GAP changes Rap1 activity or its ability to become activated. It has been reported that in platelets thrombin can stimulate Rap1 activity independent of its phosphorylation status (18), ruling out direct effects of PKA on the ability of Rap1 to become activated. Recently, it has been shown that in thyroid cells induction of proliferation by TSH/cAMP induces a TSH/cAMP-dependent negative feedback loop. TSH/cAMP could prevent degradation of Rap1GAP, likely via a mechanism involving GSK3β, and overexpression of Rap1GAP was sufficient to reverse the growth-promoting effect of TSH/cAMP (19). This indicates that PKA may inhibit Rap1 activity by a mechanism involving an increase in Rap1GAP levels, either due to direct phosphorylation or indirect effects.

The picture emerges that PKA and Epac can regulate the same proteins or processes at different steps. A new example comes from the finding of β-amyloid precursor protein (APP) as a putative cAMP/MAPK regulated gene by microarray analysis (**chapter 4**). Amyloid β protein is the major component of senile plaques in Alzheimer's disease. Epac-activated Rap1 has been reported to play a role in secretion of the non-amyloidogenic sAPPα, which is the large product of splitting APP by α-secretases (20). A model can be proposed in which cAMP induces APP transcriptionally via activation of PKA and inhibition of the MAPK pathway. Consequently, cAMP-activated Rap1 plays a role in the processing and secretion of APP.

Regulation of cell cycle proteins by cAMP at different levels

cAMP can regulate cyclin D1 and $p27^{Kip1}$ and for both proteins it has been shown that they are important for a cAMP-induced cell cycle arrest (**chapter 3** and (21,22)). Investigation of the importance of certain cell cycle proteins in the anti-proliferative effect of cAMP is hindered by the complicated regulation of these proteins. cAMP often regulates proteins at different levels, which may be cell type dependent as well. It is generally believed that regulation of $p27^{Kip1}$ expression levels is the main mechanism to control this protein. Results from **chapter 3** and **addendum 1** show that $p27^{Kip1}$ may be regulated in NIH3T3-A14 cells solely by translocation; whereas in MEFs $p27^{Kip1}$ is regulated at the level of transcription, although regulation via translocation, degradation or

translational pathways can not be excluded. In MEFs, regulation of the localization of $p27^{Kip1}$ can be assumed from experiments using active FoxO4. Active FoxO4 is able to increase $p27^{Kip1}$ levels (chapter 3, figure 4C), but unable to give nuclear localization (data not shown). On the other hand, cAMP both increases $p27^{Kip1}$ levels and induces nuclear localization (chapter 3, figure 4A-B). The mechanism via which cAMP regulates $p27^{Kip1}$ localization is not known, but phosphorylation seems to play a key-role in the regulation of import and export (**chapter 1**). PKB has been reported to phosphorylate $p27^{Kip1}$ at different sites, including threonine 157, which resides in the nuclear localization signal (NLS) of human $p27^{Kip1}$ (23-25). Regulation of export by phosphorylation of the NLS is also described for other proteins, e.g. C/EBPβ phosphorylation in the NLS induces binding of Crm1 and nuclear export (26). However, this is not likely a general mechanism to inhibit $p27^{Kip1}$ import, as this site is not conserved in murine $p27^{Kip1}$. Another site that is important for export of $p27^{Kip1}$ is serine 10 (S10). This site is conserved among species and phosphorylation of this site was shown to participate in export of the protein and to overcome a G1-arrest (27). Preventing or inhibiting the phosphorylation of S10 by cAMP is thus far still an option for regulation of $p27^{Kip1}$ export, but cAMP may equally well use a mechanism of general PKA-mediated import. The latter is a possibility since import of unrelated NLS-conjugated proteins could be blocked by inhibition of PKA in human fibroblasts, independent of direct phosphorylation of the transported protein (28).

Also during cAMP-dependent stimulation of proliferation in thyroid cells, $p27^{Kip1}$ can be regulated in different manners. In these cells, TSH/cAMP plus serum firstly decreases nuclear expression of $p27^{Kip1}$ in a p70S6 kinase-dependent manner, leading to induced CDK2 activity. Subsequently, TSH synergizes with serum to decrease $p27^{Kip1}$ protein levels in a CDK2-dependent manner (29). It may therefore be that localization, rather than expression levels, are important for regulation of $p27^{Kip1}$. Interestingly, the extent of $p27^{Kip1}$ mislocalization in breast cancer sections correlates with disease prognosis (reviewed in (30)).

General mechanism for cAMP anti-proliferative effect

In **chapter 3** we show that proliferation of MEFs is inhibited by cAMP by preventing PKB to inhibit FoxO transcription factors. This mechanism was predicted based on the similarities in inhibition of proliferation by FoxOs and cAMP. Another interesting link between FoxOs and cAMP is that under certain conditions the induction of a G2-M arrest has been reported. FoxOs can induce a G1 and G2-M arrest in mouse myoblasts. The G2-M arrest involved FoxO-mediated induction of the stress-inducible gene GADD45 (31). cAMP is also able to induce a G2 arrest next to G1 arrest in several fibroblasts (32). This might provide cells with a back-up mechanism to inhibit proliferation when cells can not be arrested in G1. Interestingly, the microtubule regulating protein stathmin was found downregulated by cAMP in the microarray experiment (**chapter 4**). Stathmin has been reported to play a role in a G2-M arrest, either due to upregulation or downregulation of the protein. Stathmin is regulated quite late by cAMP and is likely regulated after a G1 arrest is induced and might take part in such a 'back-up' system.

As in MEFs the PI3K/PKB pathway is an important target of cAMP to inhibit proliferation (**chapter 3**), the most relevant question is if cAMP can affect proliferation via a general mechanism, using this pathway. Results obtained from experiments with NIH3T3-A14 cells show that in these cells the PI3K/PKB pathway may not mediate the cAMP-induced inhibition of proliferation (**addendum 1**). The main difference between

these cells is overexpression of the insulin receptor in NIH3T3-A14 cells and not in MEFs. One could speculate that the moderate cAMP effects on the PI3K pathway in NIH3T3-A14 cells (addendum 1, figure 1A) as compared to MEFs (chapter 3, figure 5A) are caused by increased basal activity of this pathway due to the presence of the insulin receptor. In contrast, the MAPK is equally well inhibited by cAMP in both cell lines and has in line with this, partial effects on proliferation in both cases. Thus, whereas in MEFs inhibition of proliferation by cAMP can be explained by inhibition of both the MAPK and PI3K pathway, this has to involve other signalling pathways, apart from the MAPK pathway, in NIH3T3-A14 cells. Results from the microarray experiment described in **chapter 4** might give hints for these other pathways. For instance, HDGF seems to reverse partly the cAMP-induced growth arrest in NIH3T3-A14 cells (chapter 4, figure 5). Apparently, cAMP can use some alternative pathways to inhibit proliferation when other paths are overruled due to overexpression of growth factor receptors. This questions the usefulness of targets like FoxOs for anti-cancer treatment as many cancers do overexpress tyrosine kinase growth receptors, like the EGF- or HER2-receptor (reviewed in (33)). It is therefore important to find the alternative pathways regulated by cAMP to inhibit proliferation. It has been shown that the TGFβ pathway directly activates PKA (34). Also crosstalk between the PI3K/PKB pathway and the TGFβ pathway has been reported, by direct interaction of both PKB and FoxOs to the TGFβ pathway mediator Smad3 (35-37). It would be interesting to investigate whether PKA can activate the TGFβ pathway, especially because TGFβ is a potent inhibitor of proliferation.

Future perspectives

For the continuation of research on the mechanism of cAMP-induced inhibition of proliferation by microarray analysis, it should be taken in account that cell cycle proteins often are regulated at different levels. More importantly, transcriptional regulation may not be the most important mechanism, as cyclins and CKIs are highly unstable proteins, showing a limitation of the use of microarray analysis. However, the microarray experiment performed with NIH3T3-A14 cells (**chapter 4**) might for the previous discussed reasons still be useful for finding new targets involved in proliferation or cAMP-induced profiles and may be a suitable system for cancer research. More importantly, comparison of our microarray data with other data sets and using different cell types, including cancer and non-cancer cells may help to find a general mechanism used by cAMP to inhibit proliferation. This would contribute to determination of targets useful for cancer therapy.

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Summary

cAMP is a second messenger that plays a role in a wide variety of biological processes, one of which is the regulation of cell proliferation. Adenylate cyclases generate cAMP in the cell upon activation, followed by binding to and activation of its direct targets, PKA and Epac. PKA is a protein kinase that can regulate the activity of key signalling pathways like the MAPK and PI3K pathway. Inhibition of these pathways plays a role in the inhibition of proliferation of several cell types. This is often achieved by regulation of expression levels of important cell cycle regulators, like cyclin D1 and the CDK inhibitor $p27^{Kip1}$. The importance of tight regulation of these cell cycle proteins is that aberrant expression can result in deregulated cell cycle and cancer. The second important cAMP target is Epac, which is a guanine nucleotide exchange factor for the small GTPase Rap1. Activation of Epac by cAMP leads to activation of Rap1, which has a well established role in cell adhesion.

In this thesis it has been investigated whether certain proteins or genes are regulated by cAMP and whether this contributes to the regulation of cell proliferation.

Chapter 2 addresses the question whether the Rap1GEF PDZ-GEF can be controlled by cAMP. It has been reported that this exchange factor can directly bind cAMP, resulting in activation of the small GTPase Ras. We show that PDZ-GEFs are specific for the Rap GTPases and that PDZ-GEFs are not activated by cAMP, do not bind directly cAMP, nor can activate Ras. In addition we searched for ligands that can activate PDZ-GEF, but so far this has not been successful.

In **chapter 3** the regulation of FoxO transcription factors by cAMP and their role in the anti-proliferative effect of cAMP is described. FoxOs can inhibit proliferation of various cell lines via regulation of cyclin D and $p27^{Kip1}$. We show that cAMP can regulate the activity of these transcription factors in MEFs via inhibition of the PI3K/PKB pathway and that this is part of the mechanism by which cAMP upregulates $p27^{Kip1}$ levels. Moreover, we show that activation of the FoxO transcription factors is an important aspect of the anti-proliferative effect of cAMP in MEFs.

To identify novel genes involved in the inhibition of proliferation by cAMP, cAMP-induced expression profiles were determined. This is described in **chapter 4**. Several novel cAMP-regulated genes were identified, discriminated for their regulation by the MAPK pathway or not. HDGF was identified as one of these novel cAMP-regulated genes, and we show that HDGF is a candidate that may be involved in cAMP-dependent inhibition of proliferation.

Addendum 1 describes that proliferation of NIH3T3-A14 cells is inhibited by cAMP via a mechanism that does not involve the PKB/FoxO pathway. In line with this, $p27^{Kip1}$ protein levels are not increased upon cAMP treatment. Interestingly, we observed that in these cells cAMP regulates instead the subcellular localization of $p27^{Kip1}$. In **addendum 2** we describe the effect of cAMP-activated Rap1 on gene expression. The purpose was to identify novel functions of Rap1, apart from the induction of cell adhesion. We identified some genes which expression was changed upon activation of Rap1, indicating that Rap1 can be involved in signalling pathways that control transcription or mRNA stability. In **addendum 3** we determine insulin-induced expression profiles as a control for cAMP-regulated genes in NIH3T3-A14 cells.

In **chapter 5** the achieved results are discussed. A main point is that regulation of $p27^{Kip1}$ localization or regulation of novel cAMP-regulated genes, which were identified by microarray analysis, may be part of a general mechanism of cAMP-induced inhibition of cell proliferation.

Samenvatting

cAMP is een boodschappermolecuul dat een rol speelt in een grote variëteit aan biologische processen, waaronder de regulatie van celproliferatie. Het enzym adenylaat cyclase kan aangezet worden tot het genereren van cAMP, dat vervolgens zijn doeleiwitten, PKA en Epac, kan activeren. PKA is een kinase die belangrijke signaaltransductieroutes, zoals de MAPK en PI3K route, kan reguleren. Remming van deze routes kan een rol spelen in de remming van proliferatie van verscheidene celtypes. Dit wordt vaak bereikt door de expressie van belangrijke celcyclus regulerende eiwitten, zoals cycline D1 en $p27^{Kip1}$, te veranderen. Dit is belangrijk, want abnormale expressie kan leiden tot een ontregelde celcyclus en kanker. Het andere cAMP doeleiwit, Epac, is een guanine nucleotide uitwisselingsfactor voor het kleine GTPase Rap1. Dit speelt een rol in de hechting van cellen aan de extracellulaire matrix.

In dit proefschrift is vooral onderzocht of bepaalde eiwitten en genen gereguleerd worden door cAMP en zo ja, of dit een rol speelt in de proliferatie van cellen.

De vraagstelling in **hoofdstuk 2** is of de Rap1GEF PDZ-GEF gereguleerd kan worden door cAMP. Voor deze GEF is eerder beschreven dat het cAMP kan binden, resulterend in activatie van de kleine GTPase Ras. Wij laten echter zien dat PDZ-GEF alleen de Rap GTPasen kan activeren en niet gereguleerd wordt door cAMP. Daarnaast hebben wij gezocht naar kleine moleculen die wel betrokken zijn bij de regulatie van PDZ-GEF, maar dit heeft tot nu toe nog geen resultaat opgeleverd.

In **hoofdstuk 3** is de regulatie van FoxO transcriptiefactoren door cAMP en de essentie hiervan voor de remming van celproliferatie beschreven. FoxO's kunnen groei remmen door transcriptionele regulatie van cycline D en $p27^{Kip1}$. Wij laten zien dat in MEFs cAMP FoxO's kan activeren door remming van de PI3K/PKB route en dat dit deel uitmaakt van het mechanisme dat cAMP gebruikt om $p27^{Kip1}$ eiwit expressie te verhogen. Verder laten we zien dat regulatie van FoxO's belangrijk is voor het remmen van groei door cAMP in deze cellen.

Om nieuwe genen te ontdekken die betrokken zijn bij de remming van celproliferatie door cAMP, hebben we in **hoofdstuk 4** cAMP geïnduceerde genexpressie profielen bepaald. Verscheidene nieuwe cAMP gereguleerde genen werden ontdekt en onderverdeeld in MAPK gereguleerd of niet MAPK gereguleerd. HDGF werd hierbij geïdentificeerd als een interessant gen dat mogelijk een rol speelt in de remming van celproliferatie door cAMP.

Addendum 1 beschrijft dat de PKB/FoxO route niet betrokken is bij groeiremming van NIH3T3-A14 cellen door cAMP en dat in deze cellen de eiwitexpressie van $p27^{Kip1}$ niet verhoogd wordt na behandeling met cAMP. Een interessante vinding was echter dat de lokalisatie van $p27^{Kip1}$ in de cel veranderd door stimulatie met cAMP. In **addendum 2** beschrijven we het effect van actief Rap1 op genexpressie met als doel nieuwe functies van Rap1 te ontdekken. Rap1 werd hiertoe geactiveerd met cAMP en enkele Rap1 gereguleerde genen werden geïdentificeerd. Dit duidt erop dat Rap1 een rol kan spelen in signaleringsroutes die leiden tot regulatie van transcriptie of stabiliteit van mRNA. In **addendum 3** is de bepaling van insuline-geïnduceerde genexpressieprofielen beschreven, als een controle voor cAMP gereguleerde genen in NIH3T3-A14 cellen.

Hoofdstuk 5 is een discussie van de behaalde resultaten. Een belangrijk punt is dat de cellulaire lokalisatie van $p27^{Kip1}$ en de regulatie van nieuwe door cAMP gereguleerde genen onderdeel kunnen zijn van een algemeen mechanisme via welke cAMP celproliferatie kan remmen.

Curriculum vitae

Bea Kuiperij werd geboren op 23 november 1974 te Almelo. In 1993 behaalde zij haar VWO diploma aan de CSG Jan van Arkel te Hardenberg. Vervolgens begon zij haar studie aan het HLO van de Hogeschool van Drenthe te Emmen, waar zij haar propedeuse in 1994 behaalde. Zij vervolgde deze studie met als richting biotechnologie aan het HLO van de Hanzehogeschool, Hogeschool van Groningen. Tijdens deze studie werd onderzoekservaring opgedaan tijdens een stage bij de vakgroep Medische Microbiologie aan de Universitei t van Groningen onder begeleiding van Dr. S. Welling-Wester en Ir. D.F. Westra. In 1997 werd het diploma voor deze studie behaald. Van september 1997 tot mei 2000 heeft zij gewerkt als research analiste bij de vakgroep Moleculaire Cel Biologie van het Leids Universitair Medisch Centrum, onder begeleiding van Prof. Dr. A.J. van der Eb en Dr. A. Zantema. Vervolgens werd het promotie-onderzoek, zoals beschreven in dit proefschrift, gedaan in de periode van mei 2000 tot augustus 2004 onder begeleiding van Prof. Dr. J.L. Bos en Dr. Ir. G.J.T. Zwartkruis bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht. Vanaf september 2004 is zij werkzaam als post-doctoraal onderzoeker bij de afdeling Neurologie van het Universitair Medisch Centrum Utrecht onder begeleiding van Dr. F.L. van Muiswinkel.

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Bea