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Regulation of enzyme activity in vivo is determined by its cellular localization

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Introduction

Enzyme activity in vivo is regulated usually by conformational changes due to protein–protein interactions, interactions with other compounds as lipids and ions, and to the interaction between an enzyme and its substrate. In general, it can be stated that regulation of enzyme activity is dependent upon the interaction of the enzyme with another molecule, being activators, inhibitors and substrates. This means that the enzyme and its regulators have to be present at the same site in the cell in order to allow enzyme activity. The localization of these molecules is thus an important factor in the regulation of enzyme activity. During the last decades, enormous progress has been made in the methodology to localize proteins in cells, amongst them are the development of fluorescent probes in combination with confocal laser scanning microscopy (CLSM) and the development of immunogold labeling protocols and tomography on ultrastructural level.

In this contribution, we will describe the importance of the localization of proteins in their effect in the cells. These examples are related to growth factor induced signal transduction cascades which are essential in cell cycle progression. Polypeptide growth factors such as epidermal growth factor (EGF), platelet-derived growth factor or fibroblast growth factor exert their effects in the target cells by binding to specific plasma membrane bound receptors belonging to the class of receptor tyrosine kinases. Upon binding of growth factors, the receptors dimerize, which leads to activation and autophosphorylation of the receptor on tyrosine residues in the intracellular domain. This phosphorylation triggers the recruitment of a number of target proteins to the receptor; e.g., phosphoinositide-specific phospholipase Cγ (PLC-γ), the p85 kDa subunit of phosphatidylinositol 3-kinase, GTPase-activating protein, growth factor receptor binding protein 2 (Grb2) and members of the Src family of cytoplasmic tyrosine kinases. In some instances, binding of the receptor to a target molecule may result in phosphorylation and direct activation of this target

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(e.g., PLC-γ). In other cases, however, proteins without any enzymatic activity are bound, such as Grb2 and p85. These proteins serve as adaptor proteins to couple the activated receptor to other intermediates. All protein interactions consequently lead to modification (e.g., phosphorylation or dephosphorylation) and activation of other target proteins, thus creating signal transduction cascades that form a signal transduction network, which finally results in activation of nuclear transcription factors and induction of gene expression (amongst many others: Malarkey et al., 1995; Bokemeyer et al., 1996; Hulleman and Boonstra, 2001). One of the best-known signal transduction cascades activated by growth factors is the mitogen-activated protein kinase (MAPK) pathway. In general, the MAPK isoforms are activated by phosphorylation on regulatory threonine and tyrosine residues by dual specificity protein kinases, the MAP kinase kinases (MAPKK), which in turn are activated by phosphorylation by MAP kinase kinase kinases (MAPKKK). One of the most important MAPK pathways activated by growth factor receptors is the signal transduction pathway that leads to phosphorylation of $p44^{MAPK}$ and $p42^{MAPK}$ (also called ERK1 and ERK2, respectively). When growth factor receptors are activated, the adaptor protein Grb2 is bound to the receptor, together with the guanine-nucleotide exchange factor Sos. Binding of Sos leads to the activation of Ras, which subsequently recruits Raf-1 to the plasma membrane. Then, Raf-1 is activated and can, in turn, activate the MAP kinase kinase MEK (MAPK- or ERK kinase), which finally phosphorylates $p44/p42^{MAPK}$ (ERK1/2). Upon activation, ERK can phosphorylate targets in the cytoplasm, such as $p90^{RSK}$, cytoskeletal elements, $cPLA_2$ and others, or translocate to the nucleus where it may phosphorylate and activate several transcription factors, such as c-myc, c-jun, $p62^{TCF}$/Elk-1, c-Ets-1 and c-Ets-2 (amongst many others: Hulleman and Boonstra, 2001; Kolch, 2000; Bokemeyer et al., 1996; Hulleman and van Rossum, 2003; Jones and Kazlauskas, 2003).

One of the targets of the MAPK signal transduction pathway is $cPLA_2$. $cPLA_2$ is partially activated through phosphorylation by ERK1/2 and becomes fully activated when it is translocated to membranes by calcium (Börsch-Haubold et al., 1998). Several domains have been identified on the protein, such as a N-terminal Ca^{2+}-dependent lipid-binding (CaLB) domain (Clark et al., 1991; Nalefski et al., 1994). The CaLB domain preferentially binds to vesicles composed of phosphatidylcholine in response to physiological concentrations of Ca^{2+} (Nalefski et al., 1998). The CaLB domain did not exhibit any preference for phospholipid vesicles composed of saturated, unsaturated sn-2 fatty acyl chains or the carbonyl oxygens at the sn-1 or sn-2 linkage (Nalefski et al., 1998), and therefore it was concluded that the CaLB domain interacts primarily with the head group of phosphatidylcholine. Activation of $cPLA_2$ results in the release of arachidonic acid, which is the rate-limiting step in the biosynthesis of eicosanoids (i.e., prostaglandins, thromboxanes, leukotrienes, lipoxins, epoxyeicosatrienoic acid). These eicosanoids are formed via the cyclooxygenase, lipoxygenase or cytochrome p450 epoxygenase pathways, depending on the cell type, and are important regulators of many physiological responses in the cell, such as modulation and release of neurotransmitters, blood vessel tone and cell proliferation (Lynch et al., 1989; Capdevila et al., 2000; Korystov et al., 1998;

Piomelli, 1993). The important role of $cPLA_2$ in the control of cell proliferation is emphasized by the finding that most tumor cells produce elevated levels of eicosanoids, resulting in induced tumor growth, invasiveness and metastatic activity of the tumor cells (Reich and Martin, 1996). Accordingly, $cPLA_2$ was found to be over-expressed in oncogenic Ras-transformed non-small lung cancer cells and is thought to play a role in oncogenic Ras-transformation of rat-2 fibroblasts (van Putten et al., 2001; Yoo et al., 2001).

Of particular interest is the observation that the cytoskeleton plays an intermediate role in EGF-induced signal transduction. EGF and other growth factors cause a large change in the actin microfilament system (Rijken et al., 1991), and many components involved in signal transduction are associated with the cytoskeleton, including the receptor itself (van Bergen en Henegouwen et al., 1992; Payrastre et al., 1991; den Hartigh et al., 1992).

Effect of EGF on the localization of EGF-receptor substrates

A prominent substrate of the EGF receptor is PLC-γ (Meisenhelder et al., 1989). Activation of PLC-γ by the EGF receptor is accompanied by a translocation of PLC-γ from the cytoplasm to the plasma membrane (Diakonova et al., 1995). In addition, it was demonstrated that EGF caused an increase in PLC-γ activity associated with actin microfilaments. Using CLSM, it was demonstrated that EGF induced a transient co-localization between the EGF receptor and PLC-γ at specific areas on the plasma membrane, especially in newly formed membrane ruffles (Diakonova et al., 1995). In addition, a strong co-localization was observed between phosphotyrosine containing proteins and PLC-γ; also, this co-localization occurred in the newly formed membrane ruffles. These observations strongly suggested that the membrane ruffles, formed due to actin polymerization processes, constitute the site of action of EGF-induced signal transduction (Diakonova et al., 1995). These results clearly indicate that EGF-induced signal transduction occurs at specific sites at the cell surface and that proteins to be activated during this process are located at the right spot at the right time.

Expression and phosphorylation of MAP kinase during the ongoing cell cycle

MAP kinases play an important role in controlling G1 phase progression as deduced from studies in which serum-deprived G1 cells were activated by growth factors (Jones and Kazlauskas, 2003). However, regulation of MAP kinase activity occurring after the stimulation of growth-arrested cells to exponential growth is difficult to interpret, due to recovery processes and the timing of the G0 to G1 transition. Therefore, we have established MAP kinase expression and phosphorylation during the ongoing cell cycle using cells synchronized by mitotic shake-off (Boonstra et al., 1981). After synchronization, cell lysates of equal amounts of cells were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Western blots were probed with antibodies directed against $p42^{MAPK}$. As shown in Fig. 1, expression of $p42^{MAPK}$ was low in mitotic cells and increased in early G1

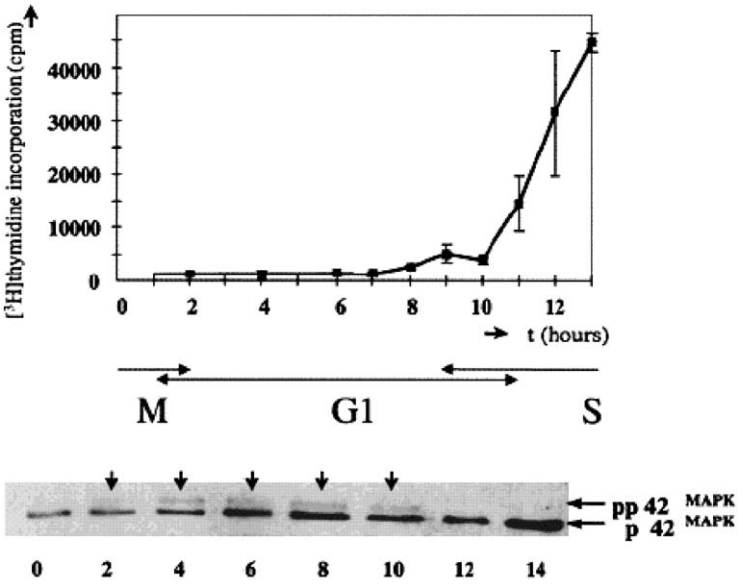

Fig. 1. [^{3}H]thymidine incorporation in CHO cells synchronized by mitotic shake-off in the upper part. [^{3}H]thymidine was added in a concentration of 5 μCi/ml to medium containing synchronized cells. Cells were replated and grown at 37°C. At the indicated times after replating, the radioactivity of the samples was measured using scintillation counting as described previously. Data are presented as mean + SD ($N = 3$). In the lower part is shown the expression of $p42^{MAPK}$ during the ongoing cell cycle as detected on Western blot. Synchronized cells were lysed in RIPA buffer at the indicated times after replating. Cell lysates of equal amounts of cells were put on gel; a representative experiment is shown. During G1 phase a gel-mobolity shift is observed as indicated by black arrows. Taken from Hulleman et al. (1999).

phase. During the remainder of G1 and S phase, the MAPK levels remained constant. A second increase was observed later in S phase (Hulleman et al., 1999).

MAP kinase activation is dependent upon phosphorylation by MEK, and can be visualized by a gel-mobility shift. As shown in Fig. 1, a gel-mobility shift was observed during G1 phase, indicating that MAP kinase is active during the G1 phase of the cell cycle. No mobility-shift was observed in mitotic cells and in cells in early S phase (Hulleman et al., 1999). Interestingly, the level of phosphorylated MAP kinase was rather low in comparison with the levels observed when serum-starved cells were activated by growth factors. These observations indicate that either a small but constant fraction of the MAP kinase population is active during the G1 phase, while the majority of the population remains inactive, or that a small but changing population is active during the various phases of the G1 phase. Since MAP kinase is able to phosphorylate a large number of both cytoplasmic and nuclear proteins, it is tempting to suggest that the latter possibility reflects the real situation. Since the various substrates reside in different sites in the cell, it also seems likely that the MAP kinase localization varies during the G1 phase. Therefore, immunofluorescence studies were performed in synchronized cells to localize MAP kinase and phosphorylated MAP kinase using confocal scanning laser microscopy (CSLM).

Localization of MAP kinase during the ongoing cell cycle

Optical sections obtained by CSLM showed that MAP kinase was present in both the cytoplasm and nucleus and at the plasma membrane in a ruffle-like appearance in cells early in G1 phase (Fig. 2). In mid-G1 phase, a strong accumulation of MAP kinase was observed in the nucleus. This nuclear localization was observed during a relatively short period of time; cells in late G1 phase exhibited a perinuclear localization. In these latter cells, virtually no plasma membrane labeling was seen (Fig. 2) (Hulleman et al., 1999). Labeling the cells with an antibody directed against the phosphorylated MAP kinase showed that the distribution of the phosphorylated MAP kinase was similar to that of the total pool of MAP kinase, except for the plasma membrane. These observations indeed demonstrate that the localization of the MAP kinase is dependent upon the phase of the cell cycle, and furthermore that MAP kinase present at the plasma membrane is not phosphorylated. It is tempting to suggest that the presence of active MAP kinase in discrete sites in the cell will result in the phosphorylation of specific substrates. Since $cPLA_2$ is a well-known substrate of MAP kinase it was of interest to establish the activation of this enzyme during the ongoing cell cycle.

$cPLA_2$ activity during the ongoing cell cycle

In CHO cells, synchronized by mitotic shake-off, the activity of $cPLA_2$ remained constant during M and early G1 phase, followed by a small decrease in mid-G1 (Fig. 3). During G1 phase a transient increase was measured and a strong increase in early S-phase (Fig. 3). A similar pattern of $cPLA_2$ activity was measured during the ongoing cell cycle of neuroblastoma N2A cells (van Rossum et al., 2001a). The changes in $cPLA_2$ activity were not due to changes in expression level of the enzyme. These observations suggest that the cell cycle related changes in $cPLA_2$ activity are due to other factors as phosphorylation and/or a rise in cytosolic Ca^{2+} concentration. In order to establish the role of ERK1/2 in this cell cycle dependent $cPLA_2$ activation, cells were treated with the MEK inhibitor U0126. Under these conditions, a complete inhibition of ERK1/2 phosphorylation and hence activation was observed (van Rossum et al., 2001a). The activity of $cPLA_2$ in control cells again showed a decrease after mitosis, followed by a peak in G1 and a peak after the G1/S transition. In cells treated with the MEK inhibitor, $cPLA_2$ activity was lower as compared to control cells, but the pattern of activity was similar, indicating that ERK1/2 activity is not the sole source of activation during the various cell cycle phases. To further evaluate the role of phosphorylation in cell cycle dependent $cPLA_2$ activity, the cell lysates were treated with phosphatase and it was shown that phosphatase caused also a significant reduction in $cPLA_2$ activity in all cell cycle phases (van Rossum et al., 2001a).

In summary, these observations demonstrated that the activation of MAP kinase immediately after mitosis does not result in $cPLA_2$ activation. During the remainder of the G1 phase, the MAP kinase phosphorylation level, and consequently activity, remains constant, but during this phase a transient peak in $cPLA_2$ activity was

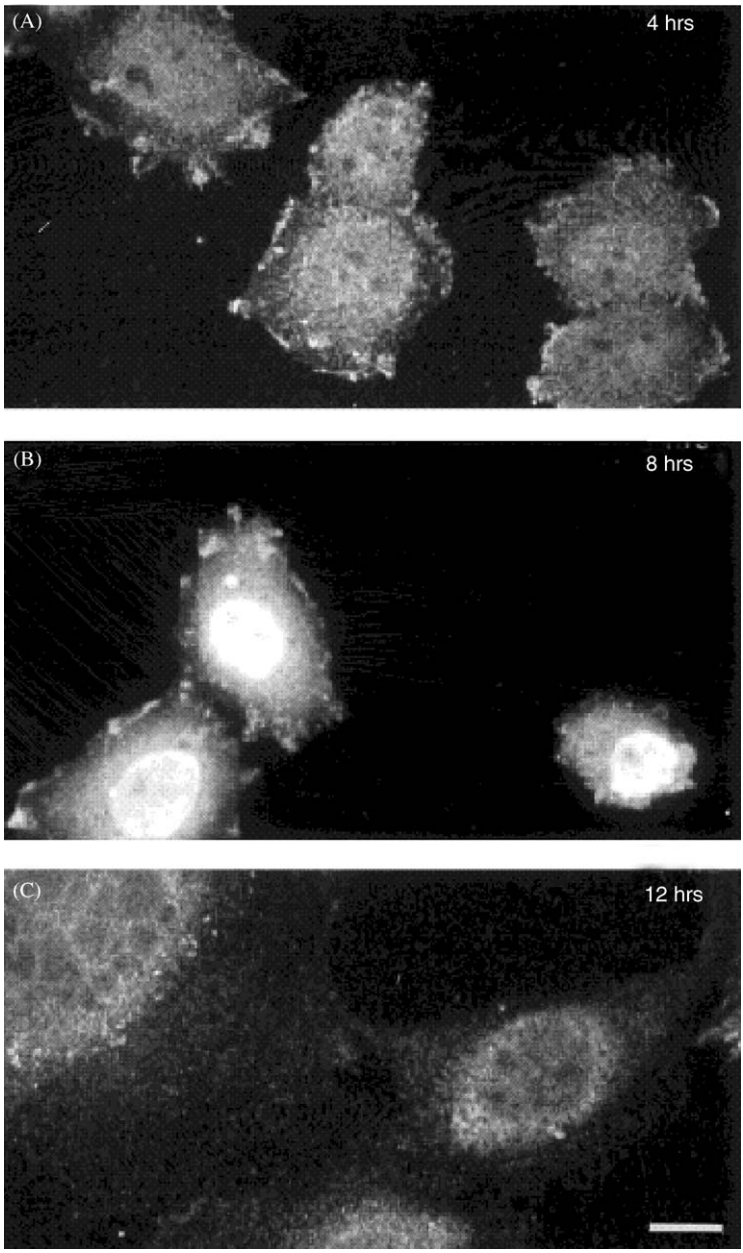

Fig. 2. Localization of $p42^{MAPK}$ in CHO cells during the ongoing cell cycle. Cells were synchronized via mitotic shake-off, replated and grown at 37°C. At 4 h (A), 8 h (B), and 12 h (C) after mitosis, cells were labeled with fluorescent probe directed against $p42^{MAPK}$as described previously (Hulleman et al., 1999). A CSLM was used to photograph optical sections at 0.6 μm from the basal side of the cells. Scale bar = 10 μm. Taken from Hulleman et al. (1999).

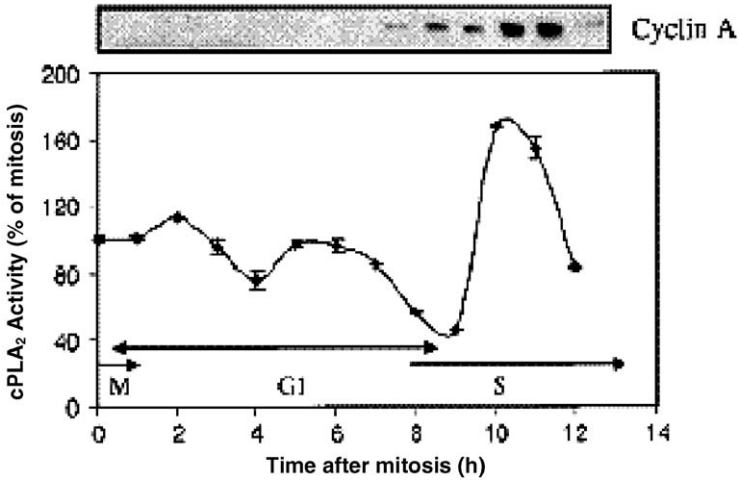

Fig. 3. Activity of $cPLA_2$ in CHO cells during the ongoing cell cycle. Cells were synchronized by mitotic shake-off and were harvested after different hours after mitosis. $cPLA_2$ activity was measured of 2×10^5 cells using the in vitro assay as described in Materials and Methods. G1/S phase transition was detected on Western blot by cyclin A expression. Taken from van Rossum et al. (2001a, b).

determined. Finally, in early S phase the MAP kinase phosphorylation was decreased, but in contrast the $cPLA_2$ activity was strongly increased. So, no clear relationship is seen between MAP kinase activity and $cPLA_2$ activity during the G1 phase of the cell cycle, but the two are related because inhibition of MAP kinase activity resulted in a strong inhibition of $cPLA_2$ activity. This means that, in addition, also other components are involved in regulation of $cPLA_2$ activity during the cell cycle. In order to understand the molecular mechanisms responsible for the regulation of $cPLA_2$ activity during the cell cycle, all components playing a role in $cPLA_2$ activity have to be identified and a detailed analysis on the $cPLA_2$ and MAP kinase localization has to be performed.

$cPLA_2$ localization

$cPLA_2$ was visualized by conventional immunofluorescence microscopy in randomly growing cells as described in detail previously (Bunt et al., 1997). These studies demonstrated that $cPLA_2$ was present in the cytoplasm in small clusters (Fig. 4). No preference was observed for a particular cellular membrane, such as the plasma membrane and the nuclear membrane. This random nature of the distribution was supported by the fact that confocal optical sections revealed a punctate labeling on all depths of the cell. Addition of EGF resulted in a clear activation of $cPLA_2$ but no effect was observed on the localization of the enzyme (Bunt et al., 1997). Since the resolution of light microscopy is too low to identify specific organelles or membranes in cells, the precise localization of $cPLA_2$ has to be analyzed using electron microscopy. $cPLA_2$ was immunogold labeled on cryosections.

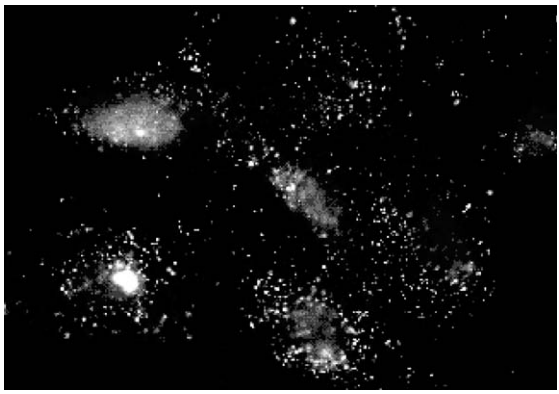

Fig. 4. Immunofluorescence detection of endogenous $cPLA_2$ in CHO cells. Randomly growing cells were labeled as described previously in detail (Bunt et al., 1997).

Under these conditions, small clusters of gold particles were observed throughout the cytosol of the cell. Labeling was not found in a specific organellar structure and no membrane was found to be associated to the clusters. The clusters were however not randomly distributed in the cytosol but were located in the proximity of organellar membranes without preference for a particular membrane (Fig. 5). In fact, labeling was found near all organellar membranes, except for the Golgi system. Minor amounts of labeling were found at the plasma membrane. Also using this methodology, no effect was observed upon activation of the cells with EGF (Bunt et al., 1997). It was proposed that the presence of $cPLA_2$ in clusters reflected a localized inactive pool from which active monomers were recruited (Bunt et al., 2000). Using an electron microscopic in vitro approach, it was demonstrated that $cPLA_2$ monomers, but not the clusters, bind to membranes in a Ca^{2+}-dependent manner. This binding was accompanied with hydrolytic activity. These observations suggested that regulation of $cPLA_2$ activity is achieved by the release of small amounts of monomers from the clusters. The majority of the $cPLA_2$ molecules in the cell are present in clusters, and this suggests that, as was seen for MAP kinase (see above), also the fraction of active $cPLA_2$ molecules in the cell is a small fraction from the population.

However, in order to exhibit enzymatic activity, $cPLA_2$ has to associate to MAP kinase to become phosphorylated and activated, but also to membranes to be able to hydrolyze phospholipids. Since the membrane association was not visible using electron microscopy, the association was demonstrated using biochemical extraction of the cells. Thus fibroblasts were stimulated with EGF under conditions known to result in $cPLA_2$ activation. Subsequently, the cells were homogenized and a particulate fraction was prepared. It was clearly demonstrated by Western blotting that EGF resulted in an increase in the association of $cPLA_2$ to the particulate fraction with a maximum effect in 5 min. This translocation was demonstrated to be dependent upon Ca^{2+} (Schalkwijk et al., 1995). In conclusion: the activation of $cPLA_2$ was related to a translocation to membranes as demonstrated only by

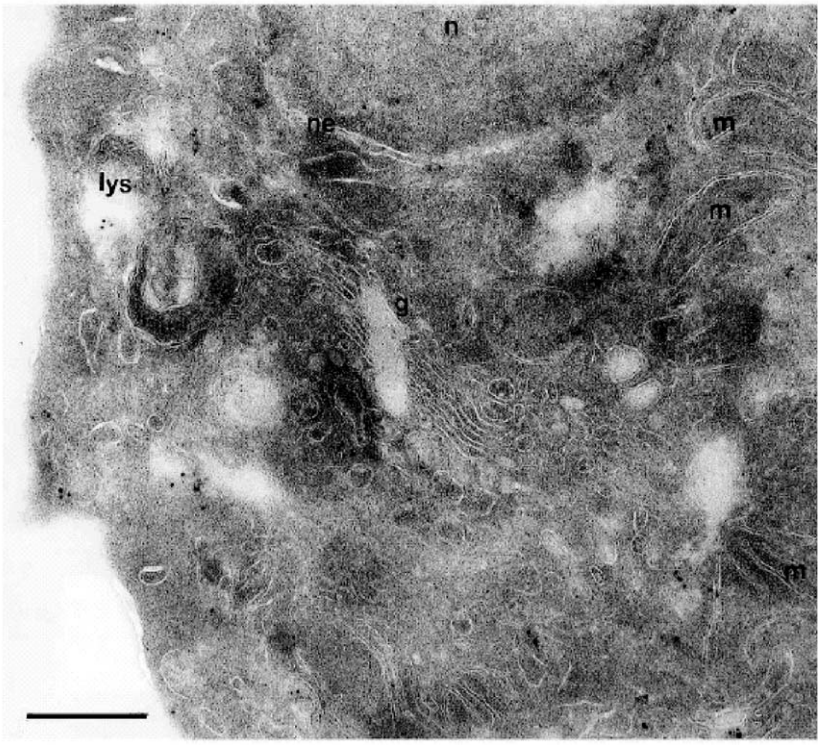

Fig. 5. Electron microscopic localization of $cPLA_2$ in ultrathin cryosections of HER14 fibroblasts. Immunogold labeling was performed as described previously (Bunt et al., 1997). g: Golgi; n: nucleus; ne: nuclear envelope; lys: lysosome; m: mitochondria. Bar: 300 nm. Taken from Bunt et al. (1997).

biochemical fractionation. The membrane-associated fraction of $cPLA_2$ consisted of monomers, as shown by electron microscopy (Bunt et al., 2000). Cellular homogenates were coated to a grid and $cPLA_2$ monomers were added to these homogenates. By electron microscopy, it was shown that the $cPLA_2$ monomers were present exclusively at membrane structures (Bunt et al., 2000).

$cPLA_2$ activation has been studied most intensively in cells starved for serum and subsequently activated by a growth factor such as EGF (van Rossum et al., 2001b; Schalkwijk et al., 1995; Spaargaren et al., 1992). Growth factors usually activate several signal transduction cascades including the MAP kinase pathways and the activation of PLC, which result ultimately in the release of Ca^{2+} from intracellular sources. These two properties are required for $cPLA_2$ activation. In studies aiming to characterize the role of MAP kinase activity and of Ca^{2+} in $cPLA_2$ activation, two different fibroblast cell lines were used, HERc13 and HER14, respectively (Schalkwijk et al., 1996). Of specific interest was the observation that EGF did not cause a release of arachidonic acid in HERc13 cells, in contrast to HER14 cells. Both cell lines were able to bind EGF with similar binding characteristics and in both cell lines EGF caused activation of MAP kinase. But EGF did cause an increase in

intracellular Ca^{2+} in HER14 cells, but not in HERc13 cells (Schalkwijk et al., 1996). It is tempting to suggest that the absence of a Ca^{2+} signal is related to the absence of the EGF-induced $cPLA_2$ activation in HERc13 cells. Therefore, the Ca^{2+} ionophore A23187 was added to the cells in order to realize an EGF-independent rise in intracellular Ca^{2+} concentrations. It was demonstrated that A23187 on its own caused an increase in $cPLA_2$ activity in both HERc13 and HER14 cells. Pretreatment of the cells with EGF and subsequently with A23187 caused a further increase in $cPLA_2$ activity in both cell lines. Thus the inability of HERc13 cells to respond to EGF by activation of $cPLA_2$ is most likely due to the inability of the cells to provoke a rise in intracellular Ca^{2+} on EGF stimulation.

Next, the temporal relationship between $cPLA_2$ phosphorylation and the Ca^{2+}-induced translocation was studied. A23187 was demonstrated to be unable to phosphorylate and activate MAP kinase in HERc13 cells, while EGF caused a strong MAP kinase phosphorylation (Schalkwijk et al., 1996). The presence of A23187 did not influence the EGF-induced MAP kinase phosphorylation, demonstrating that this process is Ca^{2+}-independent. Subsequently, cells were stimulated with EGF for different time periods, followed by incubation for 5 min with A23187. Under these conditions, it was demonstrated that EGF caused a 2-fold stimulation of $cPLA_2$ activity in vivo in HERc13 cells with a maximum in 2.5 min (Fig. 6). Alternatively, cells were incubated for 5 min with A23187, followed for several periods of time with EGF. Interestingly, under these conditions no stimulation of $cPLA_2$ activity above background was detected. These results clearly demonstrate that the phosphorylation of $cPLA_2$ has to precede the Ca^{2+}-induced membrane translocation for full activation (Schalkwijk et al., 1996). In conclusion,

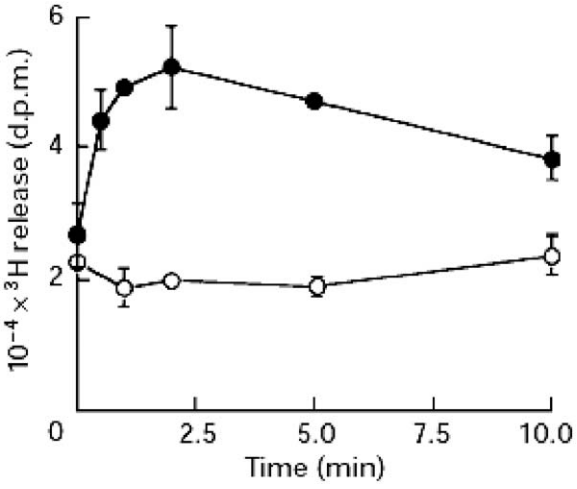

Fig. 6. $cPLA_2$ activity induced by EGF and A23187 in HERc13 cells. HERc13 cells were labeled with [^{3}H]arachidonoc acid as described previously (Schalkwijk et al., 1996). Subsequently, the cells were stimulated with 1 μM A23187 for 5 min followed by 200 ng/ml EGF for the indicated time (○), or with EGF for the indicated time, followed by A23187 for 5 min (●).

$cPLA_2$ is present as an inactive molecular complex in clusters or aggregates of which the composition is not yet known. Upon activation of MAP kinase, $cPLA_2$ is released from the clusters as a monomer to only a small extent, and translocates to membranes where the rise in Ca^{2+} is sufficient to induce the binding to the CaLB domain. Then $cPLA_2$ monomers bind to phosphatidylcholine and possibly phosphatidylinositol 4,5 P_2; both lipids are present in the inner leaflet of the membrane, followed by release of arachidonic acid.

In conclusion, these studies clearly demonstrate that localization of proteins is an essential feature in the regulation of enzyme activity. A full understanding of the role of proteins in cells requires also a detailed knowledge of this cellular localization.

Summary

Regulation of enzyme activity in vivo depends not only upon the active state of the enzyme, but in addition also on the cellular localization, as will be shown for two enzymes which play an important role in cell cycle regulation, MAPK and cytosolic phospholipase A_2 ($cPLA_2$), respectively.

MAPK plays an essential role in cell cycle regulation. Mitogenic stimulation of quiescent cells by growth factors results in a strong phosphorylation of MAPK, followed by a translocation to the nucleus, where it phosphorylates several transcription factors required for cell cycle progression. Inhibition of MAPK phosphorylation and hence activation resulted in an arrest of cell cycle progression. We have studied the MAPK phosphorylation and localization during the G1 phase of synchronized Chinese Hamster Ovary (CHO) cells. It was demonstrated that MAPK was phosphorylated within 1 h after mitosis in a growth factor dependent manner. In this cell cycle phase, the MAPK was localized throughout the cytoplasm and nucleus, with a preference for the cell periphery. During the remainder of the G1 phase, MAPK was phosphorylated, but interestingly translocated to the nucleus only in mid-G1 phase. Inhibition of the phosphorylation caused inhibition of the nuclear translocation and S phase entry. After the transient nuclear localization, MAPK was found in the cytoplasm and not in the nucleus. These observations demonstrate that the localization of the enzyme is tightly regulated during the G1 phase of the cell cycle, and apparently determines progression through this phase.

$cPLA_2$ is a key enzyme in the release of arachidonic acid and subsequent production of eicosanoids, which play an important role in a variety of biological processes, including mitogenic signalling by EGF. It has been demonstrated that EGF treatment caused a transient Ca^{2+}-dependent translocation of $cPLA_2$ from the cytosol to the membrane fraction, due to an EGF-induced rise in Ca^{2+} concentration. In addition, it was demonstrated that EGF treatment of the cells resulted in phosphorylation of $cPLA_2$ on serine residues, due to MAPK activity. The EGF-induced activation of $cPLA_2$ was reversed upon phosphatase treatment, demonstrating that $cPLA_2$ activation is determined by its phosphorylation state. Full activation of $cPLA_2$ requires both the MAPK-induced phosphorylation and the Ca^{2+}-dependent membrane translocation. Studies in two fibroblast cell lines

revealed that in vivo the phosphorylation of $cPLA_2$ by MAPK has to precede the Ca^{2+}-dependent translocation for full activation of the enzyme. Translocation followed by MAPK activation did not result in $cPLA_2$ activation, clearly demonstrating the importance of the localization of the enzyme for its activity regulation.

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