Differential activation of p44^{mapk} (ERK1) by α -thrombin and thrombin-receptor peptide agonist

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α -Thrombin (thrombin), a potent mitogen for CCL39 hamster lung fibroblasts, stimulates phosphoinositide-specific phospholipase C (PI-PLC) and inhibits adenylate cyclase via cleavage of a specific G-protein-coupled receptor (TH-R), recently cloned from human and hamster cells. This action can be entirely mimicked by the synthetic peptide SFFLRNP, referred to here as TMP (thrombin-mimicking peptide). TMP corresponds to the first seven amino acids of the new N-terminus generated by thrombin cleavage of the hamster TH-R. Although thrombin and TMP apparently generate identical early transmembrane signals, only thrombin is mitogenic on its own. TMP needs to be associated with fibroblast growth factor (FGF), a tyrosine kinase-activating growth factor, to induce cell-cycle re-entry. Here, we have examined the early and late phase of p44 MAP kinase (p44^{mapk}) activation in G₀-arrested

CCL39 cells after stimulation by thrombin, TMP, FGF or TMP+FGF. We found that: (i) both thrombin and TMP rapidly activate p44^{mapk} in a dose-dependent manner with maximum activation at around 5 min, (ii) after the initial burst of activation, a second and long-lasting wave of activation is observed in response to thrombin (10–100 nM) but not to TMP (up to 300 μ M), (iii) FGF alone (25 ng/ml), like thrombin, rapidly and persistently activates p44^{mapk} (20-fold at 5 min and about 3-fold after 2 h), (iv) TMP added together with FGF strongly potentiates the second and sustained phase of p44^{mapk} activation. From these results we propose that: (1) thrombin-induced mitogenesis is mediated only in part by the TH-R recently cloned and (2) activation of p44^{mapk}, in particular the long-lasting phase that correlates with DNA synthesis, is an obligatory event for cell-cycle re-entry.

INTRODUCTION

α -Thrombin (thrombin) is a 'hormone' with pleiotypic effects as reflected by its remarkable ability to interact with a large variety of cell types. Depending on the cellular target, thrombin can induce secretion, vasodilatation, vasoconstriction or cell growth [for review see Fenton (1988)]. Its mitogenic action has been reported for various types of fibroblasts (Chen and Buchanan, 1975; Carney et al., 1978). For example, thrombin is the most potent growth factor for CCL39 cells, a line of Chinese hamster lung fibroblasts (Pouyssegur, 1990). Thrombin, added alone to arrested CCL39 cells, is capable of inducing cell-cycle re-entry as well as cell growth for several generations in chemically defined conditions (Pérez-Rodríguez et al., 1981; Van Obberghen-Schilling and Pouyssegur, 1983). The progression from G₀ to late G₁, leading to DNA replication, requires proteolytically active thrombin, and its presence is required for at least 6–8 h (Van Obberghen-Schilling et al., 1985). How this specific proteinase mediates these multiple cellular effects is still not completely resolved. In hamster fibroblasts, as in platelets, thrombin action is coupled to at least two classes of G-proteins, G_q and G_i, leading respectively to activation of phospholipase C and inhibition of adenylate cyclase (Paris and Pouyssegur, 1986; Magnaldo et al., 1988). Recently, the cloning of a functional thrombin receptor (TH-R) from human (Vu et al., 1991) and Chinese hamster cells (Rasmussen et al., 1991) has revealed unique features. TH-R, which belongs to the super family of G-protein-coupled receptors, is cleaved by thrombin, unmasking a 'tethered' ligand at the new extracellular N-terminus. A synthetic

heptamer, identical with the receptor sequence at the new N-terminus, inhibits adenylate cyclase and stimulates phospholipase C in growth-arrested CCL39 cells as potently as thrombin (Vouret-Craviari et al., 1992). This thrombin-mimicking peptide, referred to here as TMP, is, however, on its own unable to induce mitogenesis in CCL39 cells. TMP reveals its mitogenic potential only when associated with a tyrosine kinase-activating growth factor like platelet-derived growth factor, epidermal growth factor or fibroblast growth factor (FGF). This finding led us to propose that activation of TH-R is not sufficient to account for the full mitogenic response induced by thrombin.

To substantiate this proposal and to search for the missing link, we analysed the time course and magnitude of activation of a mitogen-activated protein kinase (MAP kinase) by either thrombin or TMP. MAP kinases, also referred to as extracellular regulated protein kinases (ERKs), are serine/threonine kinase rapidly activated in response to a variety of extracellular stimuli, including mitogens [for reviews see Cobb et al. (1991) and Thomas (1992)]. These kinases, also named 'switch kinases', have been hypothesized to play a key role in integrating various extracellular signals into a co-ordinated cellular response (Pouyssegur et al., 1990; Payne et al., 1991). In the present study we show that concentrations of thrombin and TMP, which activate inositol lipid breakdown to the same extent, also activate p44^{mapk} (ERK1). However, in contrast with thrombin, which induces a biphasic and second long-lasting phase of p44^{mapk} activation, TMP only stimulates the early phase of activation. TMP, however, is capable of potentiating the second phase of activation when associated with the co-mitogen FGF. These

Abbreviations used: TH-R, functional thrombin receptor from human; TMP, thrombin-mimicking peptide; MAP kinase, mitogen-activated protein kinase; ERK, extracellular regulated protein kinases; p44^{mapk}, p44 MAP kinase; FGF, fibroblast growth factor; MBP, myelin basic protein; DMEM, Dulbecco's modified Eagle's medium; PI-PLC, phosphoinositide-specific phospholipase C.

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findings support the idea that a persistent activation of MAP kinase, at least $p44^{mapk}$, is crucial to induce cell-cycle re-entry of resting cells.

MATERIALS AND METHODS

Materials

Highly purified human α -thrombin (3200 NIH units/mg) and human recombinant basic FGF were generous gifts of Dr. J. W. Fenton II (New York State Department of Health, Albany, NY, U.S.A.) and Dr. D. Gospodarowicz (University of California Medical Center, San Francisco, CA, U.S.A.) respectively. [32 P]P_i and [γ - 32 P]ATP were from Amersham (Les Ulis, France); bovine myelin basic protein (MBP) and BSA were purchased from Sigma (St. Louis, MO, U.S.A.); Triton X-100 was from Pierce, Rockford, IL, U.S.A. Antibody CA₁ is a rabbit polyclonal antibody raised against a C-terminal peptide of rat $p44^{mapk}$ corresponding to the sequence TARFQPGAPEAP (Scimeca et al., 1991). Antibodies α ICp⁴⁴ and f42 (see the Results section) were provided by Dr. Y. Wang and Dr. F. McKenzie respectively. TMP, the thrombin receptor peptide agonist 7-mer (SFFLRNP), was synthesized by Neosystem (Strasbourg, France).

Cell culture

The established Chinese hamster fibroblast line CCL39 (American Type Culture Collection) was cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL) containing 7.5% fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μ g/ml). Growth-arrested cells were obtained by 24 h of serum deprivation.

Immune-complex kinase assay

CCL39 cells in 12-well plates were incubated for 24 h in serum-free DMEM, then placed in a Hepes-buffered DMEM medium (20 mM Hepes, pH 7.4) just before the assay and processed as previously reported (Meloche et al., 1992a,b). Agonists were added for the indicated times at 37 °C and cells were then washed twice with ice-cold PBS, and lysed in 0.2 ml of Triton X-100 lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM pyrophosphate, 200 μ M orthonadate, 1 mM phenylmethanesulphonate fluoride, 1 mM phenanthroline, 1% Triton X-100) for 20 min at 0 °C. The lysates were incubated for 45 min at 4 °C with 2 μ l of non-immune rabbit serum coupled to Protein A-Sepharose and then clarified by centrifugation at 12000 *g* for 15 min. Precleared lysates were incubated for 2 h at 4 °C with 2 μ l of CA₁ antibody coupled to Protein A-Sepharose. After four washes in lysis buffer, the immune complexes were washed once with kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol, 10 mM *p*-nitrophenyl phosphate). MBP kinase assay was carried out in 40 μ l of kinase buffer containing 1 mg/ml MBP and 50 μ M [γ - 32 P]ATP (specific radioactivity 55 c.p.m./nmol). Reactions were initiated with ATP and incubated at 30 °C for 10 min. Assays were stopped by adding 40 μ l of 2 $\times$ Laemmli buffer, heated for 10 min at 95 °C and analysed by SDS/PAGE on 10% acrylamide gels. After migration, the gels were stained with Coomassie Blue, dried and exposed to film for autoradiography. Phosphate incorporation was measured by excising MBP from the gel and counting the radioactivity by liquid scintillation.

Western-blot analysis

CCL39 cells were washed twice with ice-cold PBS and scraped into 10 ml of the same medium. Cells were then spun for 10 min at 1000 *g* and the pellet was frozen for at least 2 h. Then 5 ml of 10 mM Tris (pH 7.5)/0.5 mM EDTA was added and cells were kept on ice for 5 min. Homogenized cells were centrifuged at 4 °C for 10 min at 500 *g* and the supernatant, which contained the cytosolic fraction, was centrifuged for 1 h at 100000 *g*. Proteins were quantified and analysed by SDS/PAGE on a 10% gel. At the end of the migration, proteins were transferred to Hybond-C Extra membranes (Amersham) in 25 mM Tris/0.19 M glycine. Membranes were blotted in a TN buffer (50 mM Tris/150 mM NaCl) containing 5% non-fat dry milk. The blot was then incubated with the indicated antibodies (1:1000 dilution) in blotting solution for 2 h at room temperature. After six washes of 5 min in TN buffer containing 0.05% Triton X-100, the second antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000, Sigma), was added for 1 h. After six additional washes the blot was developed by adding horseradish peroxidase substrate diaminobenzidine in the presence of NiCl₂.

Labelling and immunoprecipitation *in vivo*

Quiescent cells were washed once in phosphate-free DMEM and incubated for 15 min in the same medium. Cells were then labelled with 200 μ Ci/ml [32 P]orthophosphate for 5 h; agonists were added during the period of labelling. Stimulated cells were then washed and lysed in 1 ml of Triton X-100 lysis buffer. Lysates were processed as described above and incubated with 15 μ l of CA₁ antibody preadsorbed on protein A-Sepharose. Immune complexes were washed six times in Triton X-100 lysis buffer. The final pellet was resuspended in 80 μ l of Laemmli buffer, heated for 10 min at 95 °C and the proteins were separated by SDS/PAGE on 10% gels. For detection of phosphotyrosine-containing proteins, the gels were incubated for 2 h in 1 M KOH at 55 °C. The alkali-treated gels were neutralized in 10% acetic acid/10% methanol and dried before autoradiography.

Phosphoamino acid analysis

The $p44^{mapk}$ phosphorylation was performed as described above, except that 1.1 mCi/ml [32 P]orthophosphate instead of 200 μ Ci/ml was used to label the cells. Samples were submitted to SDS/PAGE. After electrophoresis, the gel was blotted on a nitrocellulose membrane and 32 P-labelled $p44^{mapk}$ was localized by autoradiography. Nitrocellulose pieces corresponding to the labelled $p44^{mapk}$ were excised and incubated for 90 min at 110 °C in 6 M HCl to hydrolyse the amino acids. Phosphoamino acids were separated on cellulose thin-layer plates by electrophoresis at pH 3.5 for 2 h at 1000 V and were analysed by autoradiography.

Reinitiation of DNA synthesis

Quiescent cells in 24-well plates were stimulated for 24 h by different agonists in a serum-free DMEM/Ham's F12 medium (1:1, v/v) containing [3 H]thymidine (1 μ Ci/ml; 3 μ M) and 10 μ g/ml fatty acid-free BSA. [3 H]Thymidine incorporated into trichloroacetic acid-precipitable material was recovered with 0.1 M NaOH and quantified by liquid-scintillation counting.

Other methods

Protein concentration was measured using the BCA protein assay kit (Pierce) with BSA as standard. SDS/PAGE was performed using the buffer system of Laemmli (1970). Prestained molecular-mass markers were from Bio-Rad (Paris, France).

RESULTS

CA₁ antiserum specifically recognizes p44^{mapk}

Chinese hamster lung fibroblasts, like the majority of cells, express both isoforms of MAP kinase, p42^{mapk} and p44^{mapk} (Boulton and Cobb, 1991; Meloche et al., 1992a,b). This can easily be visualized when a total cellular extract of growing CCL39 fibroblasts is immunoblotted with the antiserum α ICp44 raised against the last 16 amino acids of rat p44^{mapk} which recognizes both isoforms (Figure 1). Although these kinases are very similar, we have been able to generate antisera that discriminate between them. Figure 1 shows that antiserum f42 raised against the last 14 residues of rat p42^{mapk} specifically recognizes p42^{mapk} whereas antiserum CA₁ directed against the last 12 residues of p44^{mapk} reacts only with the more slowly migrating isoform. In addition, CA₁ antiserum is capable of specifically immunoprecipitating p44^{mapk} (Scimeca et al., 1991). Therefore we used an immunoprecipitation assay to analyse the activation of p44^{mapk} in response to thrombin and TMP.

Thrombin, in contrast with TMP, induces a persistent activation of p44^{mapk}

Thrombin at 10 units/ml and TMP at 100 μ M, concentrations that are equipotent for stimulation of phosphoinositide-

Figure 1 Specificity of anti-p44^{mapk} CA₁ antiserum

Cytosolic proteins from exponentially growing CCL39 cells were prepared as described in the Materials and methods section and loaded on a 10% polyacrylamide gel. After separation by electrophoresis, proteins were transferred to Hybond C-Extra membrane and the membrane was probed with different antibodies. Lane 1, preimmune serum; lane 2, α Cp44 serum which recognizes p42^{mapk} and p44^{mapk}; lane 3, f42 serum which recognizes p42^{mapk}; lane 4, CA₁ antibody which recognizes only p44^{mapk}. Arrows indicate the positions of p42^{mapk} and p44^{mapk}. Molecular-mass markers, BSA (80 kDa) and ovalbumin (49.5 kDa), are indicated on the right.

Figure 2 Time course of stimulation of p44^{mapk} activity

Quiescent CCL39 cells were stimulated with 10 units/ml thrombin (■) or 100 μ M TMP (△) for the indicated times. After immunoprecipitation of p44^{mapk} from the cell lysates, MBP kinase activity was measured as described in the Materials and methods section. Similar results were obtained in three independent experiments.

stimulated phospholipase C (PI-PLC) in quiescent CCL39 cells (Vouret-Craviari et al., 1992), were compared for their capacity to stimulate p44^{mapk}. The time course of p44^{mapk} activation is illustrated in Figure 2. As we previously reported (Meloche et al., 1992b), thrombin rapidly and persistently activates p44^{mapk}. This activation (3–8-fold above basal) peaks around 5 min and persists for at least 4 h at a lower level (2–4-fold) in the presence of the agonist. TMP, however, although it is competent to induce to similar levels in the initial peak, fails to activate p44^{mapk} persistently. In response to TMP the kinase activity rapidly declines to basal levels after 15–20 min. The experiment depicted in Figure 2 was repeated three times and yielded the same results with variations only in the magnitude of stimulation. Figure 3 shows the dose-dependence of p44^{mapk} activation at an early (5 min) or late time (120 min) after stimulation. If we consider the early peak, maximum activation is obtained with 0.1 unit/ml thrombin and with 30 μ M TMP. Note that at higher agonist concentrations, the capacity to activate p44^{mapk} decreases. This is only an apparent decrease since we observe that at higher concentrations of agonist the peak is reached sooner; thus, with 10 units/ml thrombin, the 5 min time point is already in the declining phase (results not shown). This would indicate that in parallel to the stimulatory process, there is an efficient mechanism that inactivates p44^{mapk}. Activation of p44^{mapk} at the late time (120 min) is also dose-dependent and increases steadily with increasing thrombin concentrations to reach maximal levels at the highest dose of thrombin tested. In contrast, TMP, even at the highest concentration examined (300 μ M), fails to elevate the basal level of p44^{mapk} significantly.

TMP potentiates FGF-stimulated p44^{mapk} activity

On the basis of the fact that suppression of thrombin-induced second and persistent phase of p44^{mapk} activation abolishes its mitogenic potential (Meloche et al., 1992b), we hypothesized that long-lasting p44^{mapk} activation is essential for cell-cycle entry. This notion is consistent with the fact that TMP alone, which activates p44^{mapk} only transiently, fails to induce mitogenicity in CCL39 cells (Vouret-Craviari et al., 1992). TMP, however, is mitogenic when associated with FGF or other tyrosine kinase-activating growth factors. We therefore analysed whether TMP could potentiate the long-lasting phase of p44^{mapk} activation when associated with the action of FGF (Figures 4 and 5). Figure 4(a) shows that FGF alone induces two phases of p44^{mapk} activation: the first phase, which reaches 15–20-fold above basal, declines sharply between 30 and 50 min and is followed by a second phase (3–4-fold above basal) which persists for at least 3 h. In addition, Figure 4(a) shows that 100 μ M TMP strongly synergizes with FGF to activate p44^{mapk} at later times (2–3 h). It is interesting to note the parallelism between the late phase of p44^{mapk} activation and the index of cell-cycle re-entry measured by the percentage of maximal thymidine incorporation obtained with 10% fetal calf serum (compare Figures 4a and 4b).

It has now been well documented that activation of p42^{mapk} and p44^{mapk} is the result of dual phosphorylation at tyrosine and threonine residues in the critically conserved T*E Y*V A T cluster (Anderson et al., 1990; Rossomando et al., 1991; G. Pages, Ph. Lenormand, S. Meloche and J. Pouyssegur, unpublished work). It was therefore important to see whether the Tyr/Thr phosphorylation with a 1:1 stoichiometry exists throughout the course of p44^{mapk} activation and whether an imbalance in phosphorylation ratio might be the basis of the failure of TMP to activate p44^{mapk} persistently. ³²P-labelled quiescent CCL39 cells were stimulated with each agonist, lysed and p44^{mapk} was immunoprecipitated with CA₁ antibodies.

Figure 3 Dose-response of p44^{mapk} kinase activation by thrombin and TMP

Quiescent CCL39 cells were treated for 5 min (■, ▲) or 120 min (□, △) with increasing concentrations of thrombin (a) or TMP (b). After immunoprecipitation of the p44^{mapk}, MBP kinase activity was measured as described in the Materials and methods section. The same results were obtained in two separate experiments.

Figure 4 Synergistic effect of FGF and TMP on p44^{mapk} activity and on thymidine incorporation

(a) MBP kinase activity. Quiescent CCL39 cells were stimulated with 25 ng/ml FGF (○) or FGF + 100 μM TMP (●) for the indicated times, then lysed in a Triton X-100 lysis buffer. Cell lysates were immunoprecipitated with CA₁ antiserum, and MBP kinase activity in the immune complex was measured as described in the Materials and methods section. The same results were obtained twice. (b) Reinitiation of DNA synthesis in CCL39 cells. Cells were stimulated with 25 ng/ml FGF (FGF), 10 units/ml thrombin (Thr), 100 μM TMP (7-mer) or FGF + TMP (FGF + 7-mer). [³H]TdR incorporation was expressed as the percentage of maximal stimulation obtained with 10% fetal calf serum. The mean values from five determinations (± S.E.M.) are plotted.

Figure 5(a) shows that 5 min of treatment with 25 ng/ml FGF (lane 2), 10 units/ml thrombin (lane 3) or 30 μM TMP (lane 4) induces a large increase in phosphorylation in comparison with the non-stimulated cells (lane 1). Phosphorylation of p44^{mapk} is, however, less intense after 2 h of stimulation with the same agonists. The level of total phosphorylation is in accordance with the activation of p44^{mapk}. Note the lack of phosphorylation in response to TMP (lane 5) and the synergistic phosphorylation when TMP and FGF are added together (lane 6). Treatment with KOH to highlight phosphotyrosine residues indicates a

Figure 5 Phosphorylation of p44^{mapk} in CCL39 cells

Quiescent CCL39 cells were labelled with 200 μCi/ml [³²P]P_i for 5 h. Agonists were present during the last 5 min (lanes 1–4) or 120 min (lanes 5–8) of labelling. Agonists include: no addition (lane 1), FGF 25 ng/ml (lanes 2 and 7), thrombin 10 units/ml (lanes 3 and 8), TMP 30 μM (lanes 4 and 5). Lane 6, cells were stimulated by FGF + TMP for 120 min. The cells were lysed with Triton X-100 lysis buffer, and immunoprecipitated with CA₁ antibody coupled to Protein A-Sepharose. Immune complexes were washed and proteins were analysed by SDS/PAGE. (a) Autoradiogram of labelled immunoprecipitated p44^{mapk} after 1 day of exposure. (b) Autoradiogram of the same gel treated for 2 h with 1 M KOH after 7 days of exposure. Molecular-mass standards (kDa) are indicated on the right. (c) Phosphoamino acid analysis. Quiescent CCL39 cells were labelled with 1.1 mCi/ml [³²P] for 5 h. Agonists were present during the last 2 h of labelling. Phosphoamino acid analysis was performed as described in the Materials and methods section. Agonists include: no addition (lane 1); FGF 25 ng/ml (lane 2); FGF + TMP 300 μM (lane 3); TMP 300 μM (lane 4) and thrombin 10 units/ml (lane 5). The autoradiogram was exposed for 5 days. Arrows on the right indicate the position of P-Ser, P-Thr and P-Tyr. The positions of phosphoamino acid standards are outlined in lane 1.

strict parallelism between the levels of P-Tyr and the respective p44^{mapk} activities (Figure 5b). We substantiated this result by performing phosphoamino acid analysis at a late time of stimulation (2 h). Figure 5(c) shows that, in all situations analysed (thrombin, TMP, FGF and TMP + FGF), the ratio P-Tyr/P-Thr remains constant and only the total phosphoamino acids vary in proportion to the level of p44^{mapk} activation. Specific dephosphorylation of either tyrosine or threonine residue does not seem to account for the rapid decrease in p44^{mapk} activity after TMP stimulation. In agreement with our previous report (Kohn and Pouyssegur, 1986), activation of p44^{mapk} is also associated with phosphorylation on serine residues (Figure 5c). The significance of this phosphorylation for MAP kinase activity has not yet been determined.

DISCUSSION

Reinitiation of DNA synthesis is a very complex process. G₀-arrested cells require the presence of the growth factor(s) for several hours to pass the G₁ restriction point that controls cell cycle entry (Van Obberghen-Schilling et al., 1985; Pardee, 1989). To be efficient in normal and growth-arrested cells, a mitogen must not only be continuously present but also capable of activating multiple transmembrane signalling pathways. Accordingly, an optimal mitogenic action is often obtained by

combining different classes of co-mitogens (Rozengurt, 1986). Consequently, potent growth factors, capable of acting alone, are known to trigger multiple signalling pathways, a situation that prevails, for example, for thrombin and platelet-derived growth factor (Pouyssegur and Seuwen, 1992).

Here we have shown that the potent mitogen, thrombin, induces a sharp and persistent activation of $p44^{mapk}$, whereas TMP, a co-mitogen, induces only a transient phase of activation. What is surprising with this result is that thrombin and TMP are both acting on the same receptor generating apparently the same intracellular second messengers (Vouret-Craviari et al., 1992). Although they act through the same G-protein-coupled receptor, TMP acts as a classical hormone whereas thrombin cleaves the receptor generating a tethered ligand (Vu et al., 1991). This difference in receptor activation cannot, however, account for the different biological actions observed. We previously demonstrated that TMP is as potent as thrombin for stimulating PI-PLC, phosphocholine-stimulated PLC, phospholipase 2A and inhibiting adenylate cyclase (Vouret-Craviari et al., 1992; F. McKenzie, K. Seuwen, E. Van Obberghen-Schilling and J. Pouyssegur, unpublished work). This action can be demonstrated not only at early times of stimulation but also after several hours as judged by the continuous production of diacylglycerol consequent to phosphocholine-stimulated PLC activation (F. McKenzie, K. Seuwen and J. Pouyssegur, unpublished work). Moreover we checked that TMP is stable during the course of the experiment and that the rate of desensitization of the PI-PLC effect is identical with equally potent concentrations of either TMP or thrombin (Vouret-Craviari et al., 1992). Therefore, the present finding, which highlights a differential intracellular signalling between TMP and thrombin, has at least two major implications. The first one is that the thrombin-specific G-protein-coupled receptor cloned (Rasmussen et al., 1991; Vu et al., 1991) cannot by itself account for the full mitogenic potential of thrombin. We have to postulate that thrombin activates an additional surface receptor, the signalling of which co-operates with the G-protein-coupled receptor identified. The nature of this receptor is unknown at the present time.

The second implication is that the persistent phase of $p44^{mapk}$ activation plays a crucial role in the G_0 - to S-phase progression. This result is in accordance with the effects of carbachol observed in CCL39 cells expressing muscarinic M1 receptors. In these cells, carbachol elicits a strong but transient stimulation of $p44^{mapk}$. Like TMP, carbachol is not mitogenic, although it is able to elicit a strong and persistent activation of PI- and PC-PLCs as robust as the one generated by thrombin (Kahan et al., 1992; Seuwen et al., 1991; F. McKenzie, K. Seuwen and J. Pouyssegur, unpublished work). In addition, it was recently demonstrated that not only the second phase of $p44^{mapk}$ activation was missing in response to carbachol but also the second activation phase of S6 kinase (Kahan et al., 1992) and the long-lasting induction of *c-jun* transcription (J. Trejo, J. C. Chambard, M. Karin and J. H. Brown, unpublished work). These results emphasize the notion that persistent and sustained signalling is obligatory to engage the cells irreversibly into the S-phase. This notion was reinforced with the capacity of TMP to enhance the FGF-induced second phase of $p44^{mapk}$ activation.

What therefore appears crucial, to understand the mitogenic action of thrombin fully, is the elucidation of the various signals that participate in the activation of the second and long-lasting phase of $p44^{mapk}$. Recent work from our laboratory has shown that in contrast with the first phase which can be activated simply by phorbol esters, carbachol or TMP, implying a PKC action, the second phase initiated by thrombin is much more complex to

dissect. It appears to result from a combined action involving PKC, G_i -activated signals and some unidentified additional pathway(s). Indeed, the second phase of $p44^{mapk}$ activation initiated by thrombin is strongly attenuated by down-regulation of PKC and completely abolished by pertussis toxin (Kahan et al., 1992). Yet activation of both PKC and G_i -coupled effector systems by TMP is insufficient to activate $p44^{mapk}$ persistently, a finding that emphasizes the need for additional signals. Even though PI-PLC is rapidly desensitized, the production of diacylglycerol steadily increases in response to carbachol, TMP or thrombin, most probably via PC-PLC activation (F. McKenzie, K. Seuwen and J. Pouyssegur, unpublished work). So it is puzzling to see that PKC is able to activate $p44^{mapk}$ at early but not at late times. One possible explanation is that this activating process (PKC/ Ca^{2+} signals) also concomitantly turns on phosphatases that dominate at later times, inactivating $p44^{mapk}$ (Anderson et al., 1990). To counteract the inhibitory action, another signal, mandatory for cell-cycle progression, is needed such as tyrosine kinase activation (Pouyssegur and Seuwen, 1992). In conclusion, the persistent activation of $p44^{mapk}$ as well as $p42^{mapk}$ (our unpublished results) could be considered as an excellent read out system for monitoring cell-cycle re-entry. With the molecular advances on the MAP kinases (Boulton et al., 1990; Meloche et al., 1992a) and the activator, MAP kinase kinase (Gomez and Cohen, 1991; Matsuda et al., 1992) it will be possible to sort out the positive and negative signals that set in motion persistent activation of these kinases, a prerequisite for S-phase entry.

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