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α-Thrombin-induced tyrosine phosphorylation of 43000- and 41000-M_r proteins is independent of cytoplasmic alkalinization in quiescent fibroblasts

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Incubation of quiescent Chinese-hamster fibroblasts (CCL39) with α-thrombin, a potent mitogen for the cells, was found to stimulate the rapid phosphorylation of two 43000-M_r and two 41000-M_r proteins at tyrosine, threonine and/or serine, and two 63000-M_r proteins at serine. Insulin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and epidermal growth factor (EGF) are weak mitogens for cells; insulin and TPA did not stimulate the phosphorylation of those proteins significantly, whereas EGF stimulated their phosphorylation to the same extent as did α-thrombin. We analysed α-thrombin-induced protein phosphorylation at different external pH values in CCL39 and in the mutant derivative PS120, which lacks Na^+/H^+-antiport activity. We showed that cytoplasmic alkalinization, a common and early response to mitogens, is not required to trigger phosphorylation of 63000-, 43000- and 41000-M_r proteins, either at tyrosine or serine and threonine residues. This finding contrasts with the phosphorylation of ribosomal protein S6, which takes place only at permissive pH for reinitiation of DNA synthesis. These results, demonstrating that phosphorylation of 63000-, 43000- and 41000-M_r proteins and cytoplasmic alkalinization are not coupled, reinforce the idea that the site of action of intracellular pH controlling the commitment of G_0/G_1-phase-arrested cells to DNA synthesis might be restricted to mitogen-stimulated S6 phosphorylation.

INTRODUCTION

A variety of mitogens, such as PDGF, α-thrombin, EGF, FGF, interleukin 2 etc., have been shown to play a crucial role in the regulation of cell division; G_0/G_1-phase-arrested cells recommence DNA synthesis and enter mitosis when exposed to appropriate growth factors. The exact molecular mechanism by which these mitogens reinitiate DNA synthesis is, however, largely unknown. Some of the ubiquitous biochemical changes induced by mitogens are stimulation of ion fluxes across the plasma membrane (Pouysségur, 1985; Moolenaar, 1986) and protein phosphorylation (Hunter & Cooper, 1985). These biochemical changes occur rapidly after mitogen addition to quiescent cells and are thought to be implicated in the signalling pathways leading to reinitiation of DNA synthesis (Rozengurt, 1985).

Many growth-promoting agents have been shown to activate the amiloride-sensitive Na^+/H^+ antiporter, leading to a rapid rise in cytoplasmic pH (pH_{in}) of ~ 0.1–0.3 unit (Pouysségur, 1985). By using a line of Chinese-hamster fibroblasts (CCL39) and a mutant derivative that specifically lacks Na^+/H^+-antiport activity, pH_{in} has been demonstrated to exert a strict control on the rate of cell progression from G_0/G_1-phase into the S-phase (L'Allemain *et al.*, 1984*a*; Pouysségur *et al.*, 1984, 1985*a*,*b*).

Phosphorylation of cellular proteins has been commonly observed after activation of quiescent cells with a variety of growth factors (Cooper *et al.*, 1982, 1984; Chambard *et al.*, 1983; Gilmore & Martin, 1983; Nakamura *et al.*, 1983; Kohno, 1985; Kohno *et al.*, 1986). More particularly, the phosphorylation of two 43000-M_r and two 41000-M_r proteins, revealed by two-dimensional polyacrylamide-gel electrophoresis, appeared rapidly in response to diverse mitogenic agents (Cooper *et al.*, 1984; Kohno, 1985). Both sets of proteins contained phosphotyrosine, phosphothreonine and/or phosphoserine. The close correlation between mitogen action and the increased phosphorylation of these proteins is consistent with the notion that phosphorylation of such a common set of specific cellular proteins in limited number might be important for some early steps of the mitogenic-signalling pathway.

Because both an increase in pH_{in} and phosphorylation of 43000-M_r and 41000-M_r proteins are temporally associated in many systems, we addressed the question as to whether these two early events of the mitogenic response are coupled. By analysing phosphoproteins in G_0/G_1-phase-arrested Chinese-hamster fibroblasts (CCL39) and its mutant derivative lacking Na^+/H^+-antiport activity (PS120), we demonstrate here that the α-thrombin-induced phosphorylation of the 43000-M_r and 41000-M_r proteins is not critically regulated by pH_{in}. In contrast with ribosomal protein S6 (Chambard & Pouysségur, 1986), we found that the 43000- and

Abbreviations used: EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium.

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41000-M_r proteins can be fully phosphorylated by growth factors at an acidic pH that does not allow reinitiation of DNA synthesis.

MATERIALS AND METHODS

Cell culture

The Chinese-hamster lung fibroblast cell line CCL39 (American Type Culture Collection) and mutant derivative PS120, lacking the amiloride-sensitive Na^+/H^+-antiport activity (Pouysségur *et al.*, 1984), were maintained in DMEM supplemented with 5% fetal-calf serum and 0.025 M-$NaHCO_3$ at 37 °C in CO_2/air (1:19). Cells were arrested in G_0/G_1-phase by incubation in serum-free medium for 24 h as previously described (Pouysségur *et al.*, 1980). Experiments at different pH_{out} values were performed in $NaHCO_3$-free medium buffered with 0.025 M-Mops in the absence of CO_2.

Radiolabelling of cells and cell lysis

Cells incubated in serum-free medium for 17 h were labelled for a further 7 h at 37 °C in serum-free Mops-buffered DMEM, pH 7.4, containing only 10 μM-sodium phosphate and 1 mCi of [^{32}P]orthophosphate/ml. For the experiments at different pH_{out} values, cells were labelled for 5 h as described above, then the labelling medium was replaced with the same serum-free labelling medium, except that the pH was respectively adjusted to 6.9, 7.3 or 7.7, and incubation was continued for further 2 h. Growth factors were then added and the cells incubated for 15 min. [^{32}P]Orthophosphate-labelled cultures were chilled, washed twice with ice-cold phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/1.5 mM-KH_2PO_4/8.1 mM-Na_2HPO_4, pH 7.4), then lysed and treated with nuclease as described previously (Kohno, 1985).

Two-dimensional gel electrophoresis

Cell lysates were subjected to isoelectric focusing in 9.2 M-urea for 7000 V·h with pH 5–8 ampholytes, followed by SDS/10%-(w/v)-polyacrylamide-slab-gel electrophoresis (O'Farrell, 1975). After electrophoresis, the gels were fixed with trichloroacetic acid/acetic acid/methanol/water (1:1:3:5, by vol.) and dried. ^{32}P-labelled polypeptides were located by autoradiography, a fluorescent screen being used. For the detection of alkali-resistant phosphoester bonds in phosphoproteins, gels were incubated, after fixation, in 1 M-NaOH at 45 °C for 1.5 h, then neutralized and dried (Cheng & Chen, 1981). For the measurement of the pH profile, a pI calibration kit (BDH, Poole, Dorset, U.K.) was used; marker proteins were detected by Coomassie Blue staining after fixing the first-dimension isoelectric-focusing tubular gel run in parallel. M_r markers were located on slab gels by staining. The gels are shown with the basic end at the left.

Phospho-amino acid analysis

Portions from non-alkali-treated or alkali-treated gels were incubated in 5.7 M-HCl for 1 h at 110 °C, and released phospho-amino acids were recovered from the hydrolysates as described by Cooper & Hunter (1983). Recovered phospho-amino acids were separated by electrophoresis at pH 3.5 (pyridine/acetic acid/water, 1:10:189 by vol.) on thin-layer cellulose plates. Thin-layer plates were stained with ninhydrin to locate the phospho-amino acid markers. The ^{32}P-labelled phospho-amino acids were located by autoradiography.

Table 1. Response of CCL39 and PS120 cells to mitogen stimulation

Culture of growth-arrested CCL39 and its derivative PS120 (lacking Na^+/H^+-antiport activity) were exposed to mitogens, and [^{3}H]thymidine incorporation into the acid-insoluble fraction or nuclei over 24 h after mitogen addition was determined in duplicate cultures as described in the Materials and methods section. The experiments below were performed on sister cultures corresponding to those shown in the following Figures: expt. 1 and Fig. 1; expt. 2 and Fig. 3; expt. 3 and Fig. 4. Expt. 1 was repeated four times and expts. 2 and 3 twice. Results were always essentially the same, and those shown are the representative. Abbreviation used: ND, not determined.

Cell	Expt.	Treatment	[^{3}H]Thymidine incorporation (c.p.m./culture)	Nuclei labelled (%)
CCL39	1	Control	780	0.5
		Insulin (10 μg/ml)	1630	2.0
		TPA (100 ng/ml)	2300	2.5
		EGF (100 ng/ml)	3480	4.0
		α-Thrombin (1 unit/ml)	54400	43.5
	2	pH_{out} 6.9		
		Control	400	ND
		α-Thrombin (1 unit/ml)	1200	3.5
		pH_{out} 7.3		
		Control	550	ND
		α-Thrombin (1 unit/ml)	13360	28.0
		pH_{out} 7.7		
		Control	500	1.0
		α-Thrombin (1 unit/ml)	15400	35.5
PS120	3	pH_{out} 6.9		
		Control	440	1.0
		α-Thrombin (1 unit/ml)	520	1.0
		pH_{out} 7.3		
		Control	880	1.0
		α-Thrombin (1 unit/ml)	2080	3.0
		pH_{out} 7.7		
		Control	1100	1.5
		α-Thrombin (1 unit/ml)	19700	30.5

Measurement of growth stimulation

Measurements of thymidine incorporation were performed with sister cultures treated as described above for radiolabelling, except that [^{32}P]orthophosphate was omitted and all the media contained 1 mM-sodium phosphate. DNA synthesis was quantified by measuring the amount of [^{3}H]thymidine incorporated into acid-insoluble materials or by counting the labelled nuclei after 24 h of incubation with mitogens and [^{3}H]thymidine (Van Obberghen-Schilling *et al.*, 1983).

Materials

Highly purified human α-thrombin [2660 NIH (National Institutes of Health) units/mg was generously

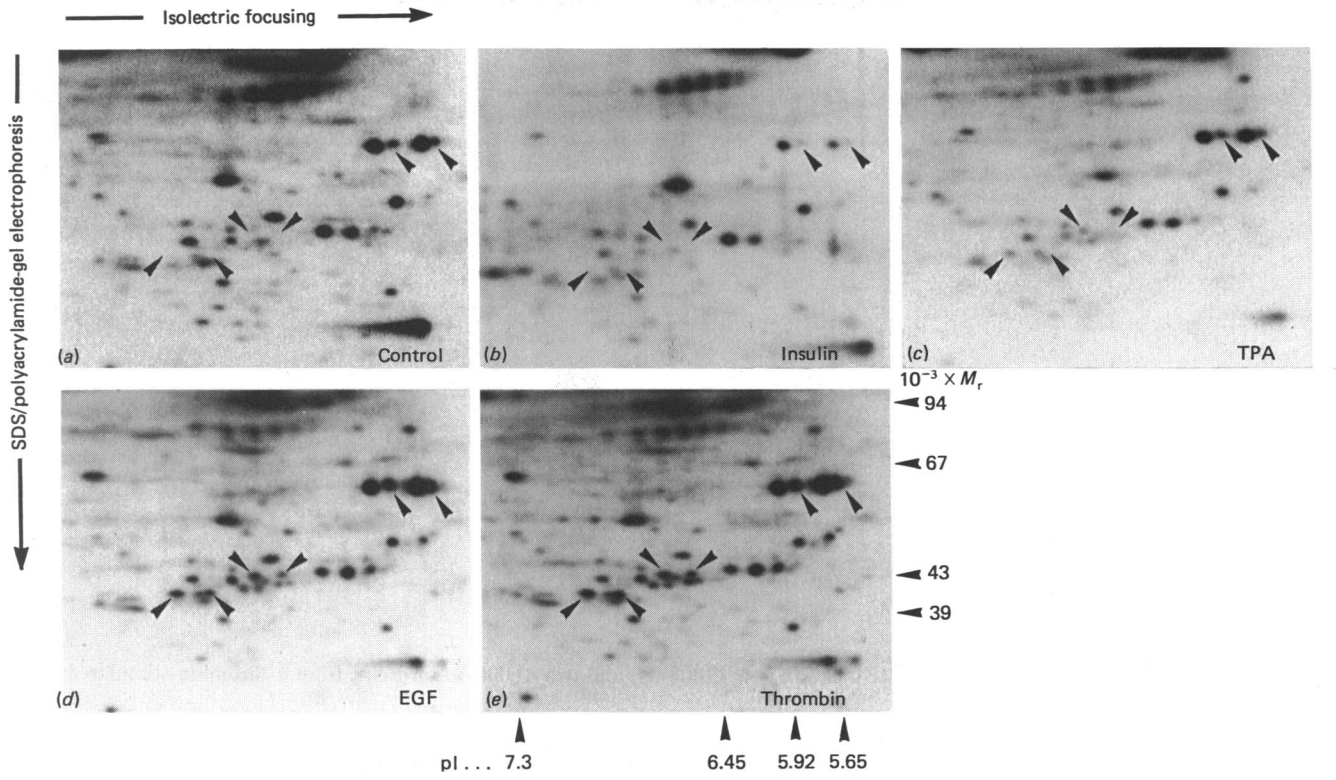

Fig. 1. Alkali-resistant phosphoproteins of CCL39 cells

Growth-arrested CCL39 cells were labelled for 7 h at 37 °C in serum-free DMEM containing 25 mM-Mops, pH 7.4, 10 μM-sodium phosphate and 1 mCi of [^{32}P]orthophosphate/ml. Cells were treated for 15 min with 10 μg of insulin/ml (*b*), 100 ng of TPA/ml (*c*), 100 ng of EGF/ml (*d*) and 1 unit of α-thrombin/ml (*e*), then cells were lysed and analysed by two-dimensional gel electrophoresis as described in the Materials and methods section. The gels were fixed, stained with Coomassie Blue, incubated in 1 M-NaOH at 45 °C for 1.5 h, then neutralized, dried, and exposed to film. Each gel contained the lysate of about 3×10^4 cells. Arrowheads indicate positions of two 63000-M_r, two 43000-M_r and two 41000-M_r proteins. Mobilities of M_r markers [phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000) and *Escherichia coli* RNA polymerase σ-subunit (39000)] and mobilities of isoelectric-point (pI) markers [metmyoglobin (equine) (7.3), metmyoglobin (porcine) (6.45), trifluoroacetylated metmyoglobin (porcine) (5.92) and azurin (*Pseudomonas aeruginosa*) (5.65)] are indicated on (*e*).

provided by Dr J. W. Fenton II (New York State Department of Health, Albany, NY, U.S.A.). Purified EGF from mouse submaxillary glands was purchased from Toyobo Co. (Osaka, Japan). Insulin and TPA were from Sigma (St. Louis, MO, U.S.A.), and [^{32}P]orthophosphate (carrier-free) and [*methyl*-^{3}H]thymidine were from New England Nuclear (Paris, France).

RESULTS AND DISCUSSION

α-Thrombin stimulates alkali-resistant phosphorylation of 63000-, 43000- and 41000-M_r proteins in G_0/G_1-phase-arrested CCL39 cells

Addition of 1 unit of highly purified α-thrombin/ml to G_0/G_1-phase-arrested culture of CCL39 cells stimulated DNA synthesis more than 80-fold when determined by the increased number of labelled nuclei (Table 1). Under such conditions, quiescent CCL39 cells, labelled for 7 h with [^{32}P]orthophosphate, were exposed to 1 unit of α-thrombin/ml for 15 min, lysed, and the proteins were analysed by two-dimensional gel electrophoresis. Before autoradiography, the gels were incubated in alkali. This procedure, known to remove phosphate preferentially from phosphoserine-containing proteins, facilitates the detection of proteins phosphorylated at tyrosine (Cheng & Chen, 1981). As shown in Figs. 1(*a*) and 1(*e*), at least six proteins whose alkali-resistant phosphorylation increased reproducibly after thrombin stimulation were found. These proteins have apparent M_r values and apparent isoelectric points of about 63000/5.93 (pp63–2), 63000/5.60 (pp63–4), 43000/6.68 (pp43-B), 43000/6.55 (pp43-A), 41000/7.00 (pp41-B) and 41000/6.85 (pp41-A).

The phosphoproteins from thrombin-treated CCL39 cells were excised from the non-alkali-treated gels (for pp63–2, pp63–4, pp43-B and pp43-A) or alkali-treated gels (for pp41-B and pp41-A, because separation of these phosphoproteins from other ones was incomplete in non-alkali-treated gels) and subjected to partial acid hydrolysis. As shown in Fig. 2, pp63–2 and pp63–4, although both are totally alkali-resistant, contain only phosphoserine, whereas pp43-B and pp41-B contain phosphotyrosine and phosphoserine, and pp43-A and pp41-A contain phosphotyrosine, phosphoserine and phosphothreonine.

These results are essentially identical with those previously obtained with human, mouse and rat fibroblasts stimulated with a variety of mitogens, including EGF, FGF, PDGF and TPA. The two 41000-M_r phosphoproteins and four 63000-M_r phospho-

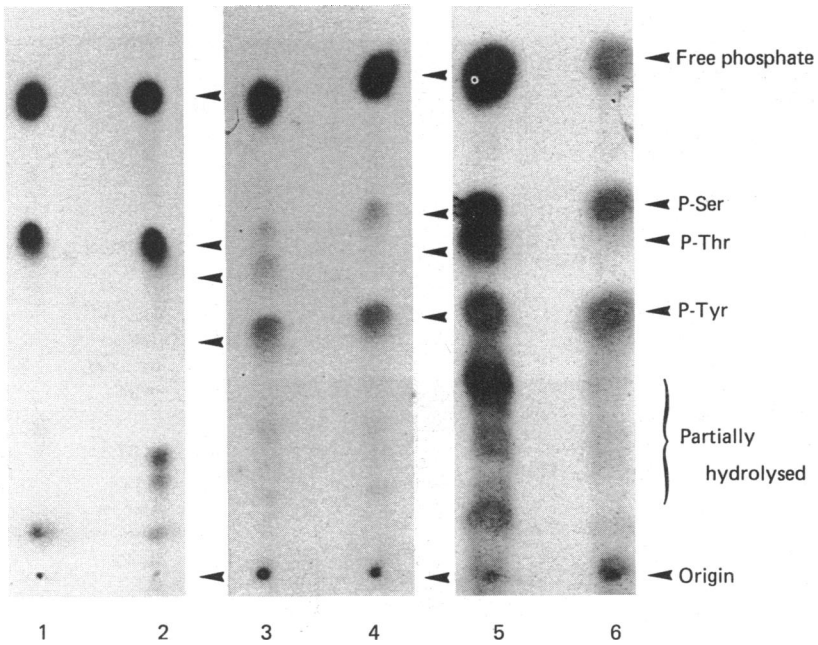

Fig. 2. Phospho-amino acid analysis of two 63000-M_r, two 43000-M_r and two 41000-M_r proteins from α-thrombin-stimulated CCL39 cells

Proteins containing the two 63000-M_r proteins and two 43000-M_r proteins were cut from non-alkali-treated gels, or two 41000-M_r proteins were cut from alkali-treated gels, of CCL39 cells labelled with [^{32}P]orthophosphate and treated for 15 min with 1 unit of α-thrombin/ml. Partial acid hydrolysis was performed, and phospho-amino acids were separated on a cellulose plate by electrophoresis (1000 V, 60 min) at pH 3.5 (pyridine/acetic acid/water, 1:10:189, by vol.) as described in the Materials and methods section. Marker phospho-amino acids are phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), identified by ninydrin staining. 1, pp63–2; 2, pp63–4; 3, pp41-A; pp41-B; 5, pp43-A; 6, pp43-B.

proteins are structurally related to each other as judged by analysis of phosphopeptide maps (Kohno, 1985).

Effect of other agents on the protein phosphorylation

Insulin or TPA was not, by itself, mitogenic for CCL39 cells (Table 1), and did not stimulate significantly the alkali-resistant phosphorylation of the proteins described above (Figs. 1*b* and 1*c*). EGF was also a weak mitogen for the cells; in contrast with insulin and TPA, it stimulated the phosphorylation of 63000-, 43000- and 41000-M_r proteins to the same extent as did α-thrombin (Fig. 1*d*). This is the first example of a discrepancy between the extent of mitogen-induced DNA synthesis and phosphorylation of those proteins. We had previously showed a good correlation between the extent of mitogen-stimulated DNA synthesis and the increased level of mitogen-induced phosphorylation of 63000-, 43000- and 41000-M_r protein in mouse 3T3 cells (Kohno, 1985).

CCL39 cells do possess receptors for insulin and EGF; both contain an intrinsic tyrosine kinase activity similar to that characterized in PDGF receptor (Cohen *et al.*, 1980; Ushiro & Cohen, 1980; Ek *et al.*, 1982; Kasuga *et al.*, 1982; Nishimura *et al.*, 1982). The fact that EGF, but not insulin, stimulated the tyrosine phosphorylation of 43000- and 41000-M_r proteins implies the direct involvement of EGF-receptor kinase activity, but not of insulin-receptor kinase activity, in the phosphorylation, at least in part, of those cellular proteins. These results and, in particular, the weak mitogenic activity of EGF for CCL39 cells suggest that increased phosphorylation of such a common set of cellular proteins is not sufficient to set in motion the progression of cells into the S-phase.

Tyrosine phosphorylation of 43000- and 41000-M_r proteins was also induced by α-thrombin; however, so far, the nature of the mitogenic α-thrombin receptor is unknown (Van Obberghen-Schilling & Pouysségur, 1985). Tumour promoters such as TPA also stimulated tyrosine phosphorylation of the proteins under the conditions where they are found to be mitogenic for cells (Cooper *et al.*, 1984; Kohno, 1985); the receptor of tumour promoters is widely believed to be a serine/threonine-specific protein kinase, C-kinase (Castagna *et al.*, 1982; Niedel *et al.*, 1983; Nishizuka, 1984). Thus mitogens such as α-thrombin and TPA could stimulate phosphorylation of 43000- and 41000-M_r proteins at tyrosine by an indirect mechanism, for instance, by activating the EGF- or PDGF-receptor kinase, c-*src*, or some other uncharacterized cellular tyrosine kinase. Other possibilities to consider are (i) that mitogens inhibit a phosphotyrosine phosphatase or (ii) induce a conformational change of the phosphorylated protein modifying its susceptibility to phosphorylation/dephosphorylation on tyrosine.

pH-dependence for reinitiation of DNA synthesis

α-Thrombin stimulation of DNA synthesis reinitiation was assayed in G_0/G_1-phase-arrested cultures of CCL39 and its derivative, PS120, at different external pH values (pH_{out}) under the same conditions as those used for analysis of protein phosphorylation (Table 1). In both cell types, stimulation of [^{3}H]thymidine incorporation

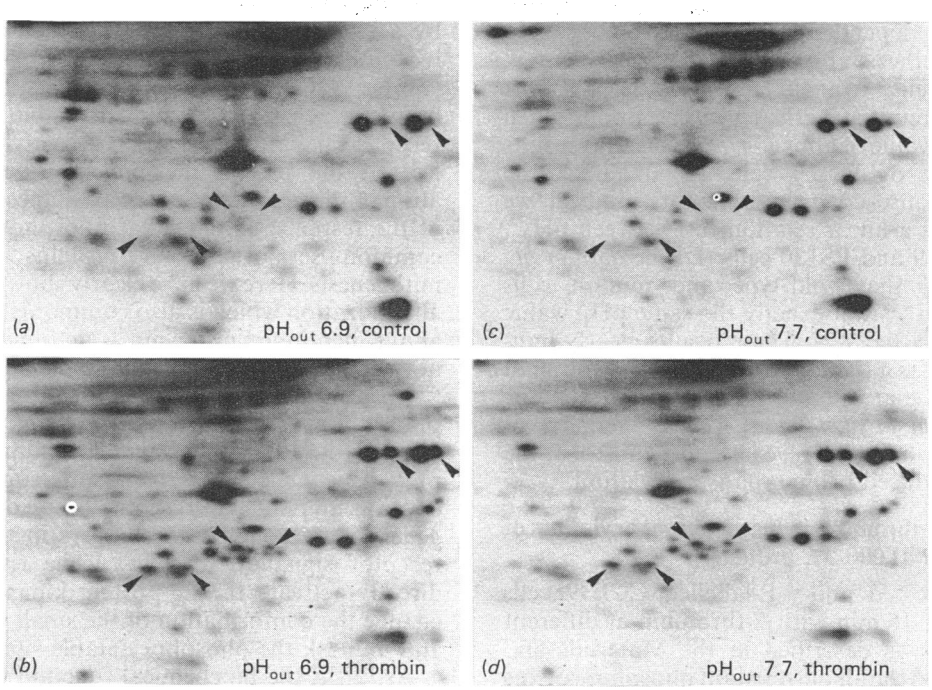

Fig. 3. pH-dependence of α-thrombin-induced alkali-resistant phosphoproteins of CCL39 cells

Growth-arrested CCL39 cells were labelled with [^{32}P]orthophosphate for 7 h; during the last 2 h of labelling, medium was replaced with that of different pH$_{out}$ values: 6.9 (*a*, *b*) or 7.7 (*c*, *d*). Cells were treated with 1 unit of α-thrombin/ml for 15 min (*b*, *d*). Labelled phosphoproteins were analysed by two-dimensional gel electrophoresis and the gels were treated with 1 M-NaOH as described in the legend of Fig. 1. Each gel contained the cell lysates of about 4×10^4 cells. The acidic end of the first dimension is to the right, as in Fig. 1. Arrowheads indicate the positions of the two 63000-M_r, two 43000-M_r and two 41000-M_r proteins.

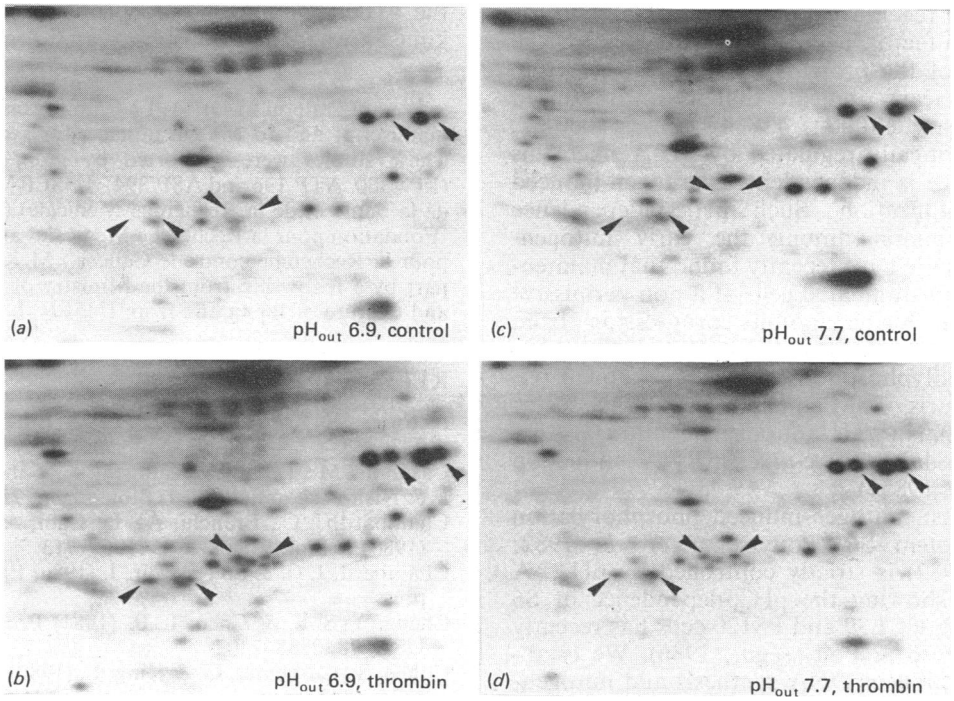

Fig. 4. pH-dependence of α-thrombin-induced alkali-resistant phosphoproteins of PS120 cells

Growth-arrested PS120 cells were labelled with [^{32}P]orthophosphate at pH$_{out}$ 6.9 (*a*, *b*) or 7.7 (*c*, *d*), treated with 1 unit of α-thrombin/ml for 15 min, and alkali-resistant phosphoproteins were analysed as described in the legend of Fig. 3. Each gel contained the cell lysates of about 4×10^4 cells. The acidic end of the first dimension is to the right, as in Fig. 1. Arrowheads indicate the positions of the two 63000-M_r, two 43000-M_r and 41000-M_r proteins.

was clearly pH_{out}-dependent. CCL39 cells begin to reinitiate DNA synthesis at a pH_{out} value of about 6.9, whereas PS120 cells, which lack Na^+/H^+-antiport activity, begin to reinitiate DNA synthesis only when exposed to a pH_{out} value of above ~ 7.3. This shift results from the inability of PS120 cells to alkalinize the cytoplasm in response to growth factors. Indeed we recently established a linear relationship between pH_{out} and pH_{in} in CCL39 and PS120 cells (L'Allemain *et al.*, 1984*b*) and found that wild-type and mutant cells reinitiate DNA synthesis at exactly the same pH_{in} value (lower-threshold $pH_{in} = 7.2$). These results clearly indicated that pH_{in} exerts a tight control on the commitment of the G_0/G_1-phase-arrested cells for entry into the S-phase (Pouysségur *et al.*, 1985*a*). It was therefore of interest to analyse whether pH_{in} exerted its control at the level of mitogen-induced protein phosphorylation.

pH-dependence for thrombin-induced phosphorylation of 63000-, 43000- and 41000-M_r proteins

G_0/G_1-phase-arrested and ^{32}P-labelled CCL39 cells were stimulated for 15 min with α-thrombin at different external pH values as described in the Materials and methods section, and the alkali-resistant phosphoproteins were analysed. As shown in Fig. 3, phosphorylation of 63000-, 43000- and 41000-M_r proteins was induced to the same extent at both pH_{out} values, 6.9 and 7.7, although the level of α-thrombin-stimulated DNA synthesis was 10 times higher at pH_{out} 7.7 than at pH_{out} 6.9. This pH-independence of the phosphorylation of such a common set of cellular proteins was even more evident in PS120 cells (Fig. 4). Indeed α-thrombin, which failed to reinitiate DNA synthesis below pH_{out} 7.3, stimulated the phosphorylation of those proteins to the same extent at pH_{out} 6.9 and 7.7. Increasing pH_{out} to 7.7, which is a way to reach a pH_{in} permissive value in the absence of growth factor, had, by itself, no effect on the phosphorylation of the proteins described above (Fig. 3*c*). Our results clearly indicate that mitogen-induced phosphorylation of 63000-, 43000- and 41000-M_r proteins is not critically regulated by pH_{in}, since this biochemical change is independent of mitogen-induced cytoplasmic alkalinization. Such pH_{in}-independence seems rather common among the early mitogen-stimulated events. We have recently found that maintenance of α-thrombin-stimulated cells at a non-permissive pH ($pH_{out} = 6.6$), which inhibits progression from G_0/G_1- to S-phase, does not prevent α-thrombin stimulation of polyphosphoinositide breakdown (G. L'Allemain, S. Paris, I. Magnaldo & J. Pouysségur, unpublished work), Na^+/H^+-antiport activation (L'Allemain *et al.*, 1984*b*) and c-*myc* mRNA induction (Pouysségur *et al.*, 1985*b*).

In sharp contrast, mitogen-induced phosphorylation of ribosomal protein S6 (Pouysségur *et al.*, 1982; Thomas *et al.*, 1982) is strictly controlled by pH_{in}. A detailed account showing the pH_{in}-dependence of S6 phosphorylation in CCL39 and PS120 cells has recently appeared (Chambard & Pouysségur, 1986). We established that reinitiation of DNA synthesis and mitogen-induced S6-protein phosphorylation display the same pH_{in}-dependence, suggesting that S6 phosphorylation is one of the pH_{in}-sensitive limiting steps in growth control.

In conclusion, rapid phosphorylation of two 43000- and two 41000-M_r proteins at tyrosine, threonine and/or serine, and of two 63000-M_r proteins at serine is induced by a variety of mitogens. This includes factors whose receptors are known to possess tyrosine kinase activity (EGF, PDGF), factors whose receptors have not yet been characterized (FGF, α-thrombin) or factors whose receptors have no tyrosine kinase activity (TPA). Regardless of the precise mechanism inducing phosphorylation of those proteins in each instance, the commonality of the response implies that phosphorylation of such a common set of cellular proteins plays a role in mitogenesis. Here we have clearly shown that cytoplasmic alkalinization which is also commonly induced by all the above-mentioned mitogens, is an ionic event apparently not needed to trigger protein phosphorylation.

It is interesting that the mitogen-induced phosphorylation at serine residues of the 63000-M_r protein and of the ribosomal protein S6 have very distinct pH_{in}-dependencies. This result suggests that pH_{in} controls S6 phosphorylation at a site distal from the step generating secondary messengers in the growth-factor-receptor signalling pathway. The rise in pH_{in} could directly activate the S6-protein kinase or more simply change the conformation of the small ribosomal subunit and unmask the phosphorylatable sites in S6.

As far as the biochemical function of the 41000- and 43000-M_r proteins is concerned, a possible role, although speculative, is that they represent the phosphorylated forms of GTP-binding proteins. Recently it has been shown that the free α-subunit of G_i (M_r 41000) is a good substrate for protein kinase C *in vitro* (Katada *et al.*, 1985). This result is of potential interest, since it is known that α-thrombin in platelets (Aktories & Jakobs, 1984) or in CCL39 cells (S. Paris & J. Pouysségur, unpublished work) is a potent inhibitor of activated adenylate cyclase, presumably through G_i activation. Therefore the phosphorylation at serine and threonine in the 41000- and 43000-M_r protein could represent the kinase C-mediated phosphorylated forms of G_i.

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