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Stimulation of phosphorylation of lipocortin at threonine residues by epidermal growth factor (EGF) and the EGF receptor: Addition of protein kinase P with polylysine inhibits this effect

(protein threonine kinase/protein tyrosine kinase/cross talk/acetylcholine receptor/tubulin)

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ABSTRACT **In this paper we show that epidermal growth factor (EGF) stimulates the phosphorylation of lipocortin 1, at threonine as well as at tyrosine residues, by a highly purified preparation of the EGF receptor. The phosphorylation of threonine residues is catalyzed by an enzyme that contaminates the receptor preparations, since crude extracts of A431 plasma membranes contain larger amounts of the threonine kinase than does the receptor preparation. Protein kinase P (2.5 ng) inhibits both threonine and tyrosine phosphorylation of lipocortin 1 while greatly stimulating the autophosphorylation of the EGF receptor. Acetyllipocortin 1 is poorly phosphorylated at tyrosine residues by the EGF receptor kinase, but it becomes readily phosphorylated in the presence of polylysine. The most likely explanation for this observation is that there is an interaction between polylysine and acetyllipocortin that converts the latter into a suitable substrate for the EGF receptor. These and other experiments described in this paper point to a role of surface charges in the susceptibility of substrates to attack by protein kinases.**

There are numerous examples in the literature of "cross talk" at the enzyme level. The first example of stimulation of one protein kinase (PK) by another is phosphorylase kinase kinase (1). Cross talk between the catalytic subunit of cAMP-dependent PK (PK-A) and the Ca^{2+}- and phospholipid-dependent PK (PK-C) (2), between PK-C and the epidermal growth factor (EGF) receptor (3–5), between the polypeptide-dependent PK (PK-P) and the EGF receptor (6), and between insulin receptors and protein serine kinases (7–11) has been described. We observed several years ago (12) an EGF-stimulated phosphorylation of threonine residues with isolated membranes from A431 cells. Similar observations were made more recently with intact cells (13).

Cross talk at the substrate level takes on two forms. The first is the phosphorylation of a substrate by one kinase that influences the phosphorylation by another kinase, which was most strikingly demonstrated by experiments with glycogen synthase (14, 15). The second form is the interaction of substrates with other cellular components that influence susceptibility to protein kinase attack; this was first illustrated by the interaction of casein with spermine, which increases its phosphorylation by casein kinase 2 (16, 17).

In this paper, changes in function due to phosphorylation involving three interacting enzymes are described. A highly purified EGF receptor preparation contains a contaminating threonine kinase that is activated on addition of EGF. When 5 ng of pure PK-P, a serine kinase activated by basic polypeptides (18), was added to the EGF receptor, its autophosphorylation was stimulated, but the phosphorylation of lipocortin 1 at threonine residues was inhibited. A PK-P activator free of histone was isolated from human platelets or erythrocyte ghosts. The specificity of phosphorylation of tubulin and the acetylcholine receptor by PK-P in the presence of this activator differs from that in the presence of histone H1.

MATERIALS AND METHODS

Substrates, [γ-^{32}P]ATP, basic polypeptides, enzymes, cells, and plasma membrane preparations were obtained as described (6, 12). Phosvitin was purchased from Calbiochem. Cop 1, a basic random copolymer containing lysine, alanine, tyrosine, and glutamic acid, was donated by M. Sela (Weizmann Institute, Israel). Lipocortin and lipocortin-(10–346) were generous gifts of B. Pepinsky (Biogen). Affinity-purified EGF receptor was donated by S. Cohen (Vanderbilt University, Nashville, TN). Tubulin was given to us by J. Dinsmore and R. D. Sloboda (Dartmouth College), and the acetylcholine receptor was a gift of R. Huganir (Johns Hopkins University). PK activities were determined as described in the legends to figures and tables. Preparation and assay of PK-P (18), PAGE, gel alkalinization, autoradiography (6), and amino acid analysis (17) were performed as described.

Assay of Purified EGF Receptor Kinase Activity with Lipocortin 1 or Acetyllipocortin as Substrate. The assay mixture contained, in a final volume of 50 μl, 20 mM Na Hepes (pH 7.4), 5 mM $MgCl_2$, 0.1 μg of purified EGF receptor, and 10 μM [γ-^{32}P]ATP (5000 cpm/pmol). The reaction mixture was incubated at 22°C for 30 min with and without 5 ng of EGF and with 6 μg of lipocortin 1 or 8 μg of acetylated lipocortin. In some experiments 200 μM $CaCl_2$, 250 ng of dicaproin, and 2.5 μg of freshly sonicated phosphatidylserine were added together with various amounts of polylysine and PK-P as indicated in the legends. The reaction was initiated by addition of [γ-^{32}P]ATP and, after 5 min at 22°C, was terminated by the addition of SDS sample buffer and analyzed by autoradiography.

Isoelectric Focusing. Isoelectric focusing was performed as described (19) with 10 μg each of lipocortin, acetyllipocortin, and standards. As standards, myoglobin, α-chymotrypsin, bovine hemoglobin, β-lactoglobulin, and ovalbumin obtained from Sigma were used.

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Abbreviations: PK, protein kinase; PK-A, catalytic subunit of cAMP-dependent PK; PK-C, Ca^{2+}- and phospholipid-dependent PK; PK-P, polypeptide-dependent PK; EGF, epidermal growth factor.

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Acetylation of Lipocortin. The reaction mixture contained, in a final volume of 1 ml, 0.25 M sucrose, 10 mM Tris SO_4 (pH 7.4), 1 mM EDTA, 600 μg of lipocortin, and 1 mg of acetic anhydride. After incubation for 20 min at 22°C with gentle shaking, the mixture was passed through a 10-ml Sephadex G-50 (coarse) column preequilibrated with 10 mM sodium bicarbonate/10 mM sodium acetate (pH 7.4) to remove unreacted acetic anhydride. The acetylated lipocortin was stored in aliquots at −70°C.

Purification of a PK-P Activator from Human Platelets. One unit (45 ml) of outdated human platelets was frozen and thawed six times and centrifuged for 20 min at 27,000 × *g*. The membrane pellet was washed with 10 mM Tris·HCl (pH 7.4). The supernatant and pellet wash were pooled and heated at 55°C for 5 min. The precipitated fibrinogen was removed by centrifugation for 30 min at 4200 × *g* at 5°C. After adjusting the supernatant to pH 7.4 by the addition of 1.0 M Tris base, 20 mM benzamidine, 1 mM EDTA, 0.1 mM diisopropyl fluorophosphate, 20 μg of leupeptin per ml, and 500 units of Trasylol per ml were added. Each unit of platelets yielded about 40 ml of extract containing about 100 mg of protein per ml. Five milliliters of the extract were loaded onto a 20-ml DEAE-Sephadex column equilibrated with distilled water. After collecting the flow through, the column was washed with 30 ml of H_2O. Proteins were eluted stepwise with 40 ml each of 0.3 M, 0.5 M, and 1 M NaCl. The eluted active fractions were dialyzed for 7 hr against 1 liter of 30% (wt/vol) polyethylene glycol and 20 mM Tris at pH 7.4 and concentrated to a volume of about 20 ml.

Ten milliliters of the concentrated fraction (*ca.* 1 mg/ml) were loaded onto a 2-ml hydroxyapatite column equilibrated with 20 mM Tris·HCl (pH 7.4), 10% (vol/vol) glycerol, and 10 mM thioglycerol. All activity was recovered in the flow through. After passing 1-ml samples through Sephadex G-25 column, aliquots of the preparation (0.4 mg/ml) were kept frozen at −70°C.

RESULTS

Cross Talk at the Enzyme Level: Stimulation of Phosphorylation of Lipocortin at Threonine Residues by EGF and the EGF Receptor and Inhibition by Polylysine-Stimulated PK-P. Lipocortin 1, a 35-kDa protein, was shown to be an excellent substrate for the EGF receptor kinase in the presence of Ca^{2+} (20, 21). Solubilized membranes of A431 cells or purified EGF receptor preparations were incubated in the presence of lipocortin 1 and Ca^{2+} for 30 min at 4°C with and without EGF. After PAGE the sharp bands of lipocortin were cut out and analyzed for phosphoamino acids as described previously (12). It can be seen from Table 1 that with crude membrane extracts from A431 cells most of the EGF-stimulated counts were found in phosphothreonine, less in phosphotyrosine, and the least in phosphoserine. It appears that the A431 membranes also contain a threonine (serine) protein kinase that is stimulated by EGF and the EGF receptor tyrosine kinase. Surprisingly, the purified EGF receptor preparation also catalyzed EGF-stimulated phosphorylation of threonine residues in lipocortin 1 (Table 1). As shown in Fig. 1*A*, the stimulation of lipocortin phosphorylation by EGF with the purified EGF receptor preparation (Fig. 1*A*, lanes 1 and 2) was increased by addition of Ca^{2+} (Fig. 1*A*, lanes 3 and 4). Addition of 0.5 μg of polylysine inhibited phosphorylation of lipocortin, both in the absence and presence of Ca^{2+} (Fig. 1*A*, lanes 5 and 6 and lanes 7 and 8). Lipids markedly stimulated lipocortin phosphorylation (Fig. 1*A*, lanes 9 and 10) but did not eliminate the inhibition by polylysine (Fig. 1*A*, lanes 11 and 12). Fig. 1*B* shows that a large fraction of the radioactivity in the lipocortin band was removed by alkaline treatment of the gel, which preferentially cleaves serine and threonine phosphates. However, the general pattern of inhibitions and stimulations was similar to that observed when total phosphorylations (tyrosine plus threonine and serine) were measured as shown in Fig. 1*A*. In the same experiment the EGF-dependent autophosphorylation of the EGF receptor at tyrosine residues was markedly stimulated by polylysine (Fig. 1*B*, lanes 6–8) as was shown previously (6). We conclude that receptor preparations purified by either EGF affinity chromatography (used for these experiments) or by tyrosine agarose chromatography (data not shown) are contaminated with a threonine (serine) kinase that is stimulated

Table 1. Phosphoamino acid analysis of lipocortin phosphorylated by membrane extracts of A431 cells or by purified EGF receptor

	Crude extract		EGF receptor	
Amino acid	− EGF	+ EGF	− EGF	+ EGF
Tyrosine	1700	2900	5700	13,000
Threonine	6900	9100	2600	8,200
Serine	1500	1900	1700	2,800

Triton extracts of A431 membranes (8 μg of protein) or purified EGF receptor (0.1 μg of protein) with and without EGF were labeled with [γ-^{32}P]ATP (5000 cpm/pmol) in the presence of 200 μM $CaCl_2$ and 6 μg of lipocortin 1 as described in *Materials and Methods*. The lipocortin band was excised from the gel and analyzed for phosphoamino acids as described (12). The radioactivity (cpm) was measured in a Beckman scintillation counter.

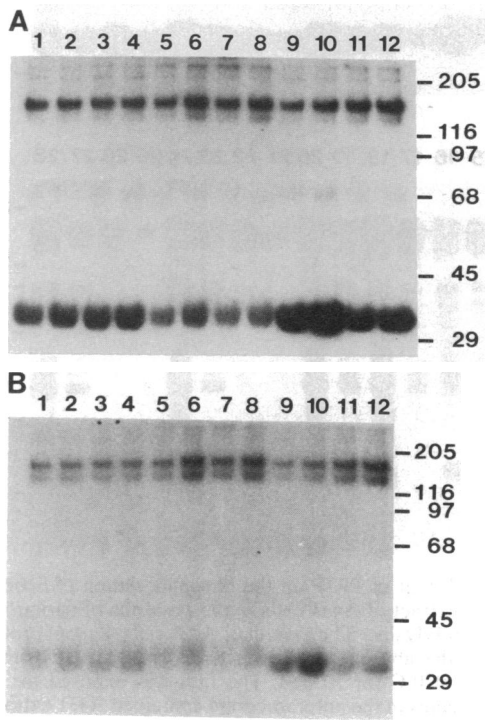

FIG. 1. Effect of calcium, polylysine, and lipids on the phosphorylation of lipocortin by the EGF receptor. Purified EGF receptor (0.1 μg) was incubated with 6 μg of lipocortin and 10 μM [γ-^{32}P]ATP at 22°C for 5 min as described in *Materials and Methods* in the presence or absence of 0.2 μg of polylysine. (*A*) Lane 1, EGF receptor + lipocortin; lane 2, receptor + lipocortin + EGF; lane 3, receptor + lipocortin + $CaCl_2$; lane 4, receptor + lipocortin + $CaCl_2$ + EGF; lane 5, receptor + lipocortin + polylysine; lane 6, receptor + lipocortin + polylysine + EGF; lane 7, receptor + lipocortin + polylysine + $CaCl_2$; lane 8, receptor + lipocortin + polylysine + $CaCl_2$ + EGF; lane 9, receptor + lipocortin + $CaCl_2$ + lipid; lane 10, receptor + lipocortin + $CaCl_2$ + lipid + EGF; lane 11, receptor + lipocortin + $CaCl_2$ + lipid + polylysine; lane 12, receptor + lipocortin + $CaCl_2$ + lipid + polylysine + EGF. The film was exposed for 17 hr at −70°C. (*B*) Same gel as in *A* after alkaline treatment. The film was exposed for 85 hr at −70°C.

by the EGF receptor tyrosine kinase. The possibility that the EGF receptor itself catalyzed EGF-stimulated phosphorylation of threonine residues in lipocortin seems less likely in view of the relatively lower capacity for threonine phosphorylation by the purified receptor compared to that by the crude membrane extract (Table 1). The possibility that the threonine phosphorylation was catalyzed by contaminating PK-C was ruled out, since the purified receptor preparation did not catalyze a Ca^{2+}- and lipid-dependent phosphorylation of histone. Thus, an unidentified protein kinase that preferentially phosphorylates threonine residues is present in purified preparations of the EGF receptor.

However, since the purified EGF receptor is less responsive to EGF than crude membrane extracts (ref. 22; compare Fig. 1 with Fig. 2), in the next experiment membrane extracts were used. As shown in Fig. 2, autophosphorylation of the

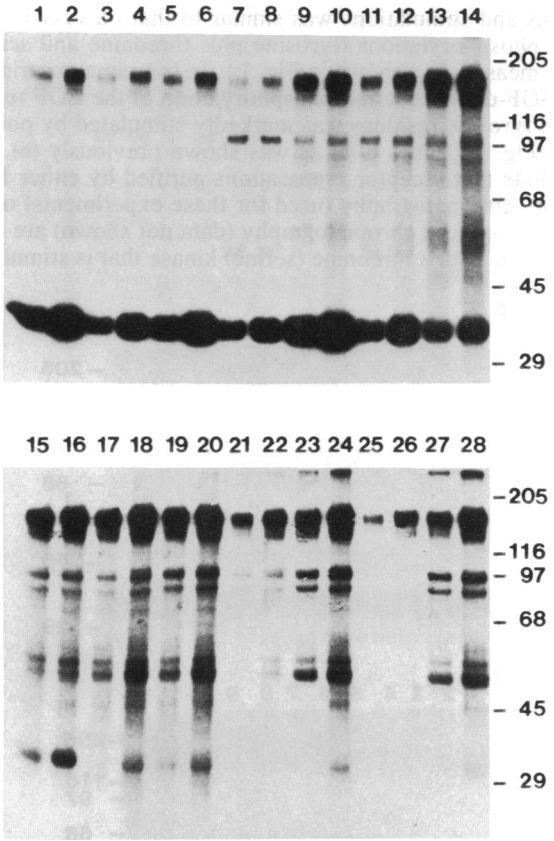

FIG. 2. Effect of PK-P on the phosphorylation of lipocortin by membrane extract of A431 cells in the presence of various concentrations of polylysine. The extract of A431 cells (8 μg of protein) was assayed in the absence or presence of 5 ng of EGF with 10 μM [γ-^{32}P]ATP at 0°C for 30 min. The film was exposed for 17 hr at −70°C. All lanes in the autoradiogram contained A431 extract + 200 μM $CaCl_2$ + 6 μg of lipocortin (control). Lane 1, control; lane 2, control + EGF; lane 3, control + 2.5 ng of PK-P; lane 4, same as lane 3 + EGF; lane 5, control + 0.05 μg of polylysine; lane 6, same as lane 5 + EGF; lane 7, control + 0.05 μg of polylysine + 2.5 ng of PK-P; lane 8, same as lane 7 + EGF; lane 9, control + 0.2 μg of polylysine; lane 10, same as lane 9 + EGF; lane 11, control + 0.2 μg of polylysine + 2.5 ng of PK-P; lane 12, same as lane 11 + EGF; lane 13, control + 0.5 μg of polylysine; lane 14, same as lane 13 + EGF; lane 15, control + 0.5 μg of polylysine + 2.5 ng of PK-P; lane 16, same as lane 15 + EGF; lane 17, control + 1 μg of polylysine; lane 18, same as lane 17 + EGF; lane 19, control + 1 μg of polylysine + 2.5 ng of PK-P; lane 20, same as lane 19 + EGF; lane 21, control + 2 μg of polylysine; lane 22, same as lane 21 + EGF; lane 23, control + 2 μg of polylysine + 2.5 ng of PK-P; lane 24, same as lane 23 + EGF; lane 25, control + 5 μg of polylysine; lane 26, same as lane 25 + EGF; lane 27, control + 5 μg of polylysine + 2.5 ng of PK-P; lane 28, same as lane 27 + EGF.

EGF receptor as well as lipocortin phosphorylation were greatly enhanced by EGF (Fig. 2, lanes 1 and 2). Added PK-P at low concentrations (2.5 ng) in the absence of PK-P activator inhibited these phosphorylations significantly (Fig. 2, lanes 3 and 4) as described previously for EGF receptor autophosphorylation (6). Addition of PK-P with increasing amounts of polylysine induced the expected stimulation of autophosphorylation of EGF receptor and of endogenous substrates (Fig. 2, lanes 13–28), but it had the opposite effect on lipocortin phosphorylation, particularly when more than 1 μg of polylysine was added (Fig. 2, lanes 15–28). Since the inhibition of lipocortin phosphorylation was influenced by both PK-P and polylysine (compare lanes 13 and 14 with lanes 15 and 16 in Fig. 2) and tyrosine phosphorylation of the EGF receptor was still stimulated even at the highest levels of polylysine, it was concluded that we are dealing with a double cross talk by PK-P, a stimulation of EGF receptor autophosphorylation and inhibition of lipocortin phosphorylation. It seems likely that the inhibition of lipocortin phosphorylation was caused by an interaction of lipocortin with polylysine, resulting in a change in conformation and charge distribution. To obtain further evidence for this concept the following experiments were performed.

Cross Talk at the Substrate Level. *(i) EGF receptor PK.* Lipocortin was acetylated with acetic anhydride until its isoelectric point was shifted from pH 6.8 to pH 4.9. In contrast to lipocortin, which was phosphorylated at tyrosine residues by the EGF receptor and inhibited by increasing amounts of polylysine (Fig. 3*A*), acetyllipocortin was a very poor substrate for EGF receptor but became an increasingly better substrate in the presence of increasing amounts of polylysine (Fig. 3*B*). It thus appears that acetylation of lipocortin, which results in a more negative surface charge, reduced its susceptibility to phosphorylation by the EGF receptor. Various possible explanations for these observations will be discussed below.

(ii) PK-P from yeast plasma membrane. Many examples of cross talk at the substrate level between polylysine, histones, or cop 1 as cosubstrates and casein, phosvitin, tubulin, and the acetylcholine receptor as substrates for PK-P were observed. Since neither histones nor polylysine are physiological activators of PK-P in the plasma membrane, a natural activator was isolated from human platelets or erythrocyte ghosts (containing no histones) and was tested with a variety of substrates that are of physiological interest. As shown in Fig. 4, the phosphorylation of tubulin by PK-P was greatly enhanced by histone 1 (Fig. 4, lane 4), whereas the platelet activator (Fig. 4, lane 6) was much less effective. The reverse was true for some contaminants in the tubulin preparation, which responded better to the platelet activator. In the case of acetylcholine receptor, the platelet activator (Fig. 4, lane 12) was also superior to histone, which was most clearly seen at the α subunit at 40 kDa.

DISCUSSION

This paper is focused on two major themes. The first is a demonstration of cross talk between the EGF-dependent EGF receptor and a threonine (serine) kinase that phosphorylates lipocortin 1 and is present in the plasma membrane of A431 cells as well as in a purified preparation of EGF receptor (Table 1). We have previously shown EGF-stimulated threonine phosphorylation with crude membrane extracts (12). Activated PK-P inhibits this threonine kinase while it greatly stimulates the autophosphorylation of the EGF receptor at tyrosine residues, representing an interesting example of double cross talk. These observations may be of considerable physiological significance since it is well-known that in intact cells EGF (13) as well as insulin (7) stimulate the phosphorylation of serine (threonine) residues of their receptors,

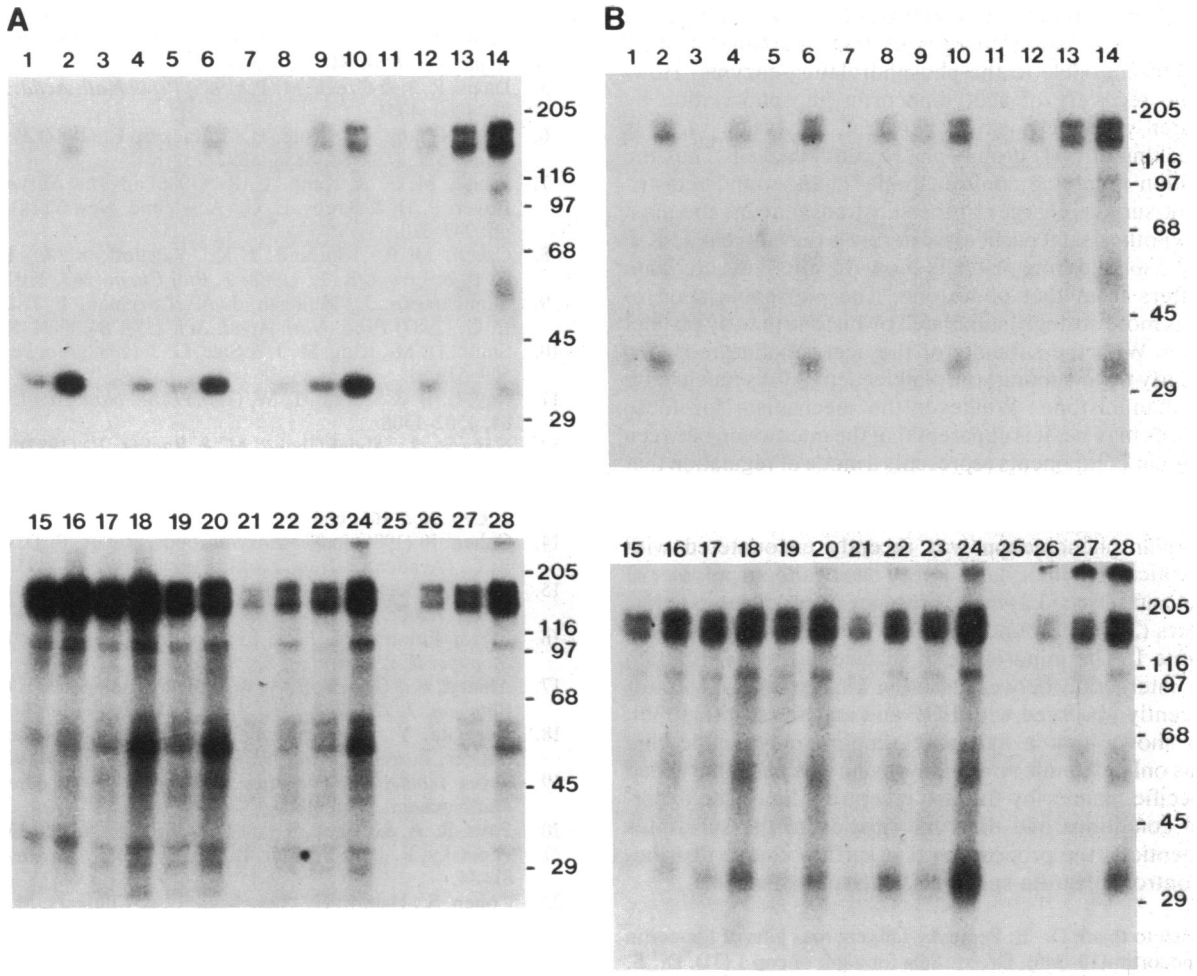

FIG. 3. Effect of various concentrations of polylysine on the phosphorylation of tyrosine residues in lipocortin (*A*) and acetylated lipocortin (*B*) in the presence of PK-P. (*A*) The experiments were performed as described in the legend of Fig. 2 for lipocortin. (*B*) The conditions were the same as in *A*, except that 8 μg of acetylated lipocortin was used instead of 6 μg of lipocortin. The autoradiograms were taken after treatment of the gels with 1 M NaOH.

sometimes to an even greater extent than tyrosine residues. It is of great interest that phosphorylation of threonine residues of lipocortin occurs even with a highly purified EGF receptor. This suggests a strong physical association of the

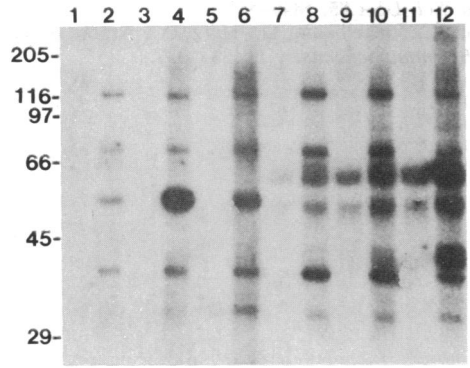

FIG. 4. Phosphorylation of acetylcholine receptor and tubulin by PK-P in the presence of histone or platelet activators. The assay conditions were as described (6) with 1 μg of acetylcholine receptor or tubulin as substrate and 2.5 ng of PK-P in the absence or presence of 5 μg of activator. The film was exposed for 9 hr at −70°C. Lane 1, tubulin; lane 2, tubulin + PK-P; lane 3, tubulin + histone; lane 4, tubulin + histone + PK-P; lane 5, tubulin + activator; lane 6, tubulin + platelet activator + PK-P; lanes 7–12, same as lanes 1–6 except that the acetylcholine receptor was used instead of tubulin.

threonine kinase with the EGF receptor. There is ample evidence (cf. ref. 8) that signal transduction from protein-tyrosine kinase takes place by means of protein-serine kinases. Ribosomal S_6 phosphorylation at serine (threonine) residues is stimulated by EGF (23), insulin (24), nerve growth factor (25), and *src* transformation (26). Phosphorylation of the protein kinases present in low amounts are not readily detectable by ^{32}P labeling or by precipitation with antibodies. More sensitive tests are required to detect the protein kinases involved in such a cascade (27).

De *et al.* (28) have demonstrated that only tyrosine residues of lipocortin-(13–346) are phosphorylated by EGF receptor, whereas it was shown in this paper that full-length lipocortin 1 is phosphorylated at threonine residues as well. Indeed, we observed that lipocortin missing 9 amino acids at the NH_2 terminus is no longer a substrate for the threonine kinase, thus explaining the apparent discrepancy. Lipocortin 1 does not contain threonine among the first 12 amino acids, suggesting that the NH_2 terminus influences the susceptibility of lipocortin 1 to phosphorylation by the protein-threonine kinase.

The second theme involves the proposition that surface charge distribution in a substrate has a profound influence on susceptibility to phosphorylation. The experiments on the effect of polylysine on phosphorylation of lipocortin and acetyllipocortin recorded here are consistent with this formulation. Acetylation of a protein does not only change

surface charges. Blockage of NH_2 groups could well give rise to major conformation changes so that threonine residues become inaccessible to the phosphorylating enzyme. However, the recovery of acetyllipocortin phosphorylation by interaction with the positively charged polylysine suggests that blocking of NH_2 groups *per se* is not critical. Thus the proposition involving conformational changes and redistribution of surface charges in the substrate remains the most likely hypothesis. Of particular interest is the fact that a PK-P activator isolated from platelets shows a pattern of activation that differs from that of histone. The phosphorylation of tubulin is more strongly stimulated by histone than by platelet activator. With the subunits of the acetylcholine receptor, particularly the α subunit, the platelet activator is much more potent than histone. Whatever the mechanism for these differences may be, it is apparent that the interaction between intracellular components represents a mode of regulation that could profoundly change the overall pattern of phosphorylation and function.

A similar phenomenon was recently encountered with PK-C, which phosphorylates a very basic random polymer of arginine and serine (3:1) in the presence of endogenous acidic activators (29) that are not required for the phosphorylation of histone 1. The importance of surface charge distribution for the interaction between protein kinases and substrates was recently observed with EGF and insulin receptors (30). It was shown that a synthetic random polypeptide that contains only glutamic acid and tyrosine was phosphorylated in a specific manner by the two receptors. Since the experimental conditions and the conformation of the substrates were identical, the proposition that surface charge distributions control substrate specificity appears reasonable.

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