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PHORBOL ESTER STIMULATES THE SYNTHESIS AND PHOSPHORYLATION OF A 48 kDA-INTRACELLULAR PROTEIN IN PLASMINOGEN ACTIVATOR SECRETING MELANOMA CELLS

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Phorbol ester (12-O-tetradecanoyl-phorbol 13-acetate) stimulates the secretion of tissue-type plasminogen activator by the melanoma cell line, Bowes. This effect is associated with increased levels of mRNAs for both tissue-type plasminogen activator and a 48 kDa-protein. Labelling of melanoma cells with L-[^{35}S]methionine allowed to identify an intracellular protein which, by 3 criteria, was identical with the in vitro translation product of the 48kDa-protein mRNA : (a) a Mr of 48,000 on electrophoresis in the presence of sodium dodecyl sulphate; (b) inducibility by phorbol ester and (c) failure of reducing agents to affect electrophoretic mobility. As detectable by L-[^{35}S]methionine labelling, the protein was mainly localized in the cytosol.

In vitro phosphorylation reactions, carried out on subcellular fractions revealed a membrane-associated protein which also had the three characteristics of the aforementioned 48 kDa-protein. Phosphorylation did not require Ca^{2+}-ions. Addition of phorbol ester to the reaction mixtures increased the phosphorylation.

Reconstitution experiments between membrane and cytosol fractions of phorbol ester-treated and untreated cells showed that the 48kDa protein occurs in a cytosolic, unphosphorylated and a membrane-bound, phosphorylated form and that the former is converted to the latter by a phorbol ester activated, membrane-associated protein kinase.

Phorbol esters, e.g. 12-O-tetradecanoyl-phorbol 13-acetate (TPA), stimulate the production of secreted as well as nonsecreted proteins, e.g. interferon-γ (1), ornithine decarboxylase (2) and tissue-type plasminogen activator (tPA) (3). Phorbol esters also cause increased phosphorylation of various intracellular phosphoproteins (4-12) and it has been suggested that some of these are regulators of secretion by cells (4,10,12). We have studied the stimulatory effect of phorbol ester on tPA production by a human melanoma cell line (Bowes). In this system enhanced production of tPA is accompanied by increased levels of mRNA for tPA (13,14), but also by the appearance of another mRNA which translates into a 48kDa protein in reticulocyte lysate (15).

The studies described here were directed at demonstrating a phorbol-ester-induced 48kDa protein in the cells and at testing whether this protein might be a substrate for phosphorylation reactions.

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In the present study, we describe a 48 kDa phosphoprotein, that is possibly involved in the phorbol-ester-induced cascade of intracellular events leading to the secretion of tissue-type plasminogen activator by a melanoma cell-line (Bowes).

MATERIALS AND METHODS

Materials. 12-O-Tetradecanoyl-phorbol 13-acetate (TPA) from Sigma Chemical Co. (St. Louis, MO, USA) was dissolved in dimethylsulfoxide (Sigma) in a concentration of 160 nM and stored by -20°C until use. γ-(^{32}P)-ATP (3000 Ci/mmol), L-[^{35}S]methionine (740 Ci/mmol), the [^{14}C]-methylated standardazation protein mixture were purchased from the Radiochemcial Centre Ltd. (Amersham, UK). The [^{14}C]-methylated standard mixture consisted of myosin (M_r : 200,000), phosphorylase b (M_r : 100,000 and 92,500), bovine serum albumin, carbonic anhydrase (M_r : 30,000) and lysozyme. Acrylamide, N,N'-bisacrylamide and N,N,-N',N'-tetramethylethylenediamine were from Biorad (Rockville Center, NY, USA). EDTA, sucrose, sodium dodecyl sulphate (SDS) and 4,2-(hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) were from Serva (Heidelberg, FRG). Trasylol was purchased from Bayer (Wupperthal, FRG).

Cells and Culture Media. The human melanoma cell line (Bowes) was grown to confluency in stoppered culture flasks (80 or 175 cm^2) or in semi-microtiterplates (Nunc Plastics, Roskile, Denmark; cat. no. 43982). Modified EMEM, supplemented with sodium bicarbonate (1.35 mg/ml), heat-inactivated calf serum (10 v/v %) and non-essential amino acids were used.

Stimulation of Melanoma Cells with 12-O-Tetradecanoyl-Phorbol 13-Acetate. Confluent cultures of melanoma cells were treated with 160 nM of TPA for indicated time intervals. Three different groups were compared : a) control cell cultures received new culture medium at -24 h and 0 h; b) cells that were "primed plus induced" received a primary phorbol ester treatment at -24 h (= priming) and a secondary at 0 h (= induction); c) the cultures that were "induced only" received new culture medium at -24 h and phorbol ester-induction at 0 h. The time interval of the induction itself depended on the purpose of the experiment.

Analysis of Cell Lysates for Newly Synthesized Proteins. At time zero in the above schedule the cell cultures were washed with Dulbecco's phosphate-buffered saline and labelled by addition of 10 pmol L-[^{35}S]methionine in 250 µl methionine-depleted EMEM. After 30 min labelling, the supernatant fluids were discarded, the cells washed and lysed with 200 µl buffer (10 mM Tris-HCl, pH 7.4; 4 w/v % SDS). Equal aliquots of the lysates were analyzed by electrophoresis.

Cell-fractionation. Melanoma cell cultures from group b and c were induced for 5 and 7 h, respectively and harvested after resuspension (13). Control cells from group a were harvested after 7 h culture in fresh medium. They were labelled in the presence of 1 nmol L-[^{35}S]methionine in 10 ml medium as above. Resuspended cells were centrifuged (5 min, 120 x g, 4°C) and frozen overnight (-20°C). All further fractionation steps were performed at 4°C. The cells were homogenized in 10 mM Tris-HCl, pH 7.2; 1 mM EDTA; 100 U/ml aprotinine; 250 mM sucrose and centrifuged (10 min, 1085 x g). The resulting "pellet 1" was washed two times with 200 µl of the homogenization buffer and resuspended in 300 µl 10 mM Tris-HCl, pH 7.2, 100 U/ml aprotinine. The supernatant fluid was centrifuged again (1 h, 100,000 x g) resulting in "pellet 2" and "cytosol". Pellet 2 was washed in 100 µl of the homogenization buffer, centrifuged again for 1 h at 100,000 x g and resuspended in 150 µl 10 mM Tris-HCl, pH 7.2, 100 U/ml aprotinine. The supernatant fluid was added to the cytosol and buffered with Tris-HCl, aprotinine to 1 ml. The incorporation of L-[^{35}]methionine was monitored by scintillation counting of trichloro acetic acid precipitable material. For electrophoretic analysis the gel slabs were loaded with samples containing equal amounts of radioactivity.

Phosphorylation Reactions.

a) Phosphorylation of the crude membrane fraction ("pellet 1"). Protein contents of the membrane fractions were determined with the method of Brad-

ford (16). Aliquots (150 µg) from group a (control) and c (only induced) were mixed with HEPES buffer pH 7.4 (final concentration 40 mM) and reacted in the presence of 2 x 10^{-6} M γ-(^{32}P)-ATP as a label. Protein phosphorylation was effected by the following (final concentrations) : EDTA (5 mM); bivalent ions (5 mM Ca^{2+}, 10 mM Mg^{2+}, 0.5 mM Mn^{2+}); dibutyryl-cAMP (0.3 mM) and TPA (160 nM). The phosphorylation rections were terminated by addition of an equal volume of buffer (10 mM Tris-HCl; pH 6.8; 2 % w/v SDS). The samples were stored at -20°C until electrophoretic analysis. The nature of the phosphorylation was investigated by incubation of the gel slabs for 2 h at 55°C in 1 M KOH (17).

b) Phosphorylation of pellet 2 and cytosol. Pellet 2 and cytosol were phosphorylated as the crude membrane fraction, but only in the presence of EDTA and TPA.

Analysis of Proteins on Sodium Dodecylsulphate/Polyacrylamide Slab Gel Electrophoresis. Cell protein fractions were analyzed on vertical slab gels, containing 0.1 % SDS (18). Stacking and separating gels of 5 % w/v and 15 % w/v acrylamide were used respectively. Protein samples were buffered in 125 mM Tris-HCl, pH 6.8, 4 % w/v SDS, heated for 2 min at 95°C and loaded. Reduction of the samples was done by addition of 2-mercaptoethanol to a final concentration of 1 % (v/v). For autoradiography the gels were prepared as described (13). Scanning of the autoradiographs was as in reference 15.

RESULTS

12-O-Tetradecanoyl-phorbol 13-acetate induces a 48 kDa protein in Bowes melanoma cells. Subcellular fractions of L-[^{35}S]methionine labelled melanoma cells were examined for the presence of a phorbol ester-inducible 48 kDa protein. For these experiments we resorted to two phase phorbol ester-treatments (i.e. "priming" and "induction") a procedure which in several other systems (19-21) has been shown to be several-fold more effective than simple induction. Sets of cell cultures were incubated for 24 h in serum-free medium with 100 ng/ml phorbol ester (= priming); the medium was then (= time point 0 h) renewed and a second phorbol ester-treatment was instated for 3.5, 5 or 7 h (= induction) and cells as well as supernatants were harvested. Parallel sets of cultures were incubated in plain serum-free medium (unprimed cells) and after 24 hrs the inducing phorbol ester-stimulus was given. The results of this experiment are shown in Fig. 1. Comparison of lanes $I_{3.5}$, I_5 and I_7 with lane Co illustrates that induction only did not cause significant alterations in protein synthesis patterns. Parallel lanes (I and P+I) in this figure compare protein synthesis patterns in cultures which had received induction only with those having received priming and induction (P+I). These comparisons show that the intensity of a 48 kDa-band was enhanced by the phorbol ester-priming procedure. This enhancement was observed with all induction times used, but was most pronounced with an induction time of 3.5 hrs.

In order to determine the subcellular localisation of the phorbol ester-induced 48 kDa-protein subsequent experiments were similarly designed, but the cells were homogenized instead of lysed. The homogenates were fractionated by ultracentrifugation and fractions were analyzed separately. The results of these experiments, as shown in Fig. 2, confirm that synthesis of a 48 kDa protein is greatly enhanced in cultures which are both primed and induced with

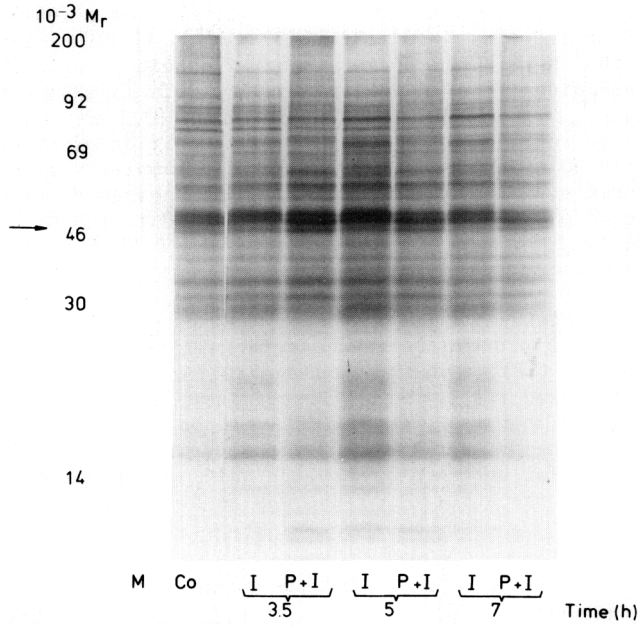

Fig. 1. Autoradiogram showing the electrophoetic analysis of melanoma cell lysates: kinetic study of the 48,000-Da protein induction.
12-O-Tetradecanoyl-phorbol 13-acetate-induced (lanes I) and primed plus induced (lanes P + I) cells were labelled for 0.5 h with L-[^{35}S]methionine and lysed at parallel induction times (3.5, 5 or 7 h). A lysate of L-[^{35}S]methionine-labelled untreated cells (control : lane Co) was taken as reference. Equal aliquots of lysates were applied to gel slab. The arrow indicates the 48,000-Da, 12-O-tetradecanoyl-phorbol 13-acetate inducible band. M = molecular mass standard.

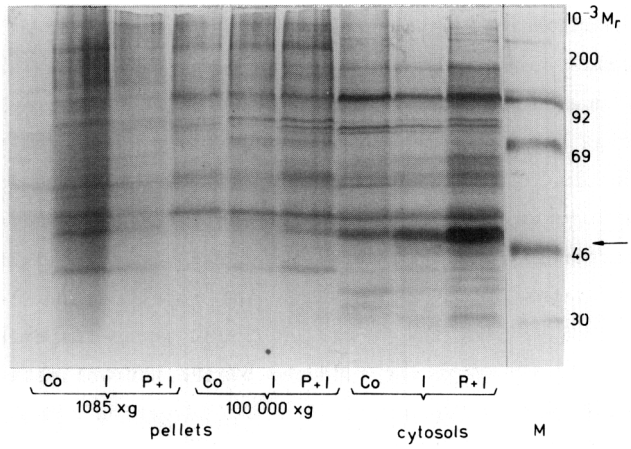

Fig. 2. Autoradiogram showing the electrophoretic analysis of melanoma cell fractions.
Untreated (control : lanes Co), tetradecanoyl-phorbol acetate-induced (lanes I) and primed plus induced (lanes P + I) cells were labelled with L-[^{35}S]methionine for 0.5 h before cell harvesting. Cells were homogenized and fractionated, resulting in a 1085 x g, 100,000 x g pellet and cytosol. L-[^{35}S]methionine incorporation was measured by scintillation counting after protein precipitation with trichloroacetic acid and samples, containing 30,000 counts per minute, were loaded on gel slab. The arrow indicates the 48,000-Da band. M = molecular mass standard.

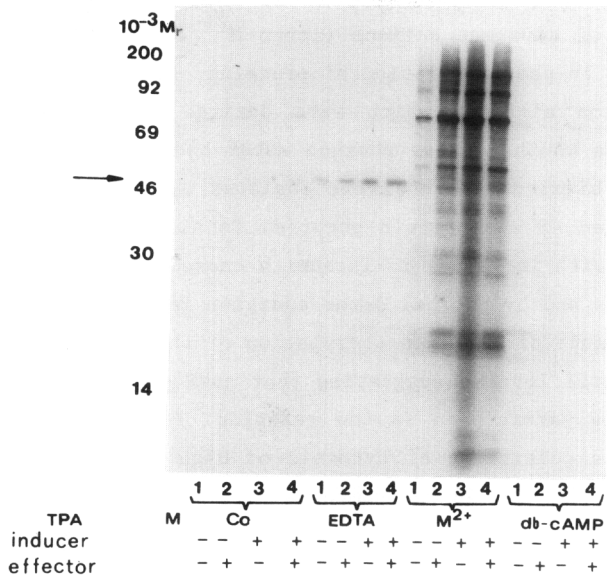

Fig. 3. Autoradiogram showing the in vitro phosphorylated proteins in the 1085 x g-pellet-cell fraction : influence of supplementary added agents upon the phosphorylated protein pattern.
1085 x g pellets from 12-O-tetradecanoyl-phorbol 13-acetate-induced and non-induced melanoma cells were incubated in the presence of γ-[^{32}P]ATP as a label. Phosphorylation was done without supplementary addition of influencing factors (= control : Co) or with addition of EDTA, bivalent ions (Ca^{2+}, Mg^{2+}, Mn^{2+}: M^{2+}) or dibutyryl-cAMP (db-cAMP). 12-O-Tetradecanoyl-phorbol 13-acetate (TPA) was added (+) or not (-) as inducer to the cells in culture or as phosphorylation effector to the 1085 x g-pellet fraction. Samples, reduced by addition of 2-mercaptoethanol, were applied to the gel slab. The arrow indicates the 48,000-Da protein. M = molecular mass standard.

phorbol ester. The intensity of the 48 kDa band, relative to other labelled proteins, was highest in cytosol, with only minor signals in the low- and high-speed pellets.

12-O-Tetradecanoyl-phorbol 13-acetate induces a 48 kDa-phosphoprotein. Fractions of control and phorbol ester-treated cells were run in phosphorylation reactions in vitro. γ-[^{32}P]-ATP was used as a label and the phosphoproteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and autoradiography. The effect of bivalent cations was evaluated by extra addition of Ca^{2+}, Mg^{2+} and Mn^{2+} to the reaction mixtures or by chelation of endogenous bivalent cations with added EDTA. The possible participation of cAMP-dependent protein kinase was studied by additions of its effector, dibutyryl-cAMP. Furthermore, all reactions were done in parallel with and without addition of phorbol ester as phosphorylation effector. Fig. 3 shows the autoradiogram from reaction mixtures containing the low-speed pellets. In the absence of phosphorylation effectors (lanes Co: 1 to 4), a faint 48 kDa-phosphorylated band was detectable only in reaction mixtures containing homogenates from phorbol ester-treated cells with extra phorbol ester added in

in vitro. Addition of divalent cations (lanes M^{2+} : 1 to 4) caused an expected overall increase in phosphorylation of proteins. The 48 kDa-band was only seen in those reaction mixtures which were derived from phorbol ester-treated cells, regardless whether extra phorbol ester had been added *in vitro* or not. When endogenous bivalent cations were chelated by addition of EDTA, a clear-cut phosphorylated 48 kDa-protein occurred in all 4 reaction mixtures (lanes EDTA : 1 to 4), with increases in intensity caused both by phorbol ester-treatment of the cells and by phorbol ester addition *in vitro*.

In the presence of cAMP phosphorylation of the 48 kDa-protein was not enhanced over control levels; suggesting that cAMP-dependent protein kinase did not significantly participate in the reaction. It should be noted here that reaction mixtures consisting of cytosols or high speed pellets did not yield detectable phosphorylated 48 kDa-proteins.

The electrophoretic relative mobility of the [^{32}P]-labelled 48 kDa-protein was unaffected by the addition of reducing agents. Alkali treatment of the SDS-PAGE gels caused disappearance of the [^{32}P]-labelled 48 kDa-protein band, which indicates that phosphorylation occurred at serine and/or threonine residues.

Reconstitution experiments. In the previous experiments it was shown that a phorbol ester-induced 48 kDa-protein, detectable by [^{35}S]methionine incorporation into nascent protein, mainly occurred in the cytosol and much less in the membrane fractions of cells. On the other hand a phorbol ester-induced phosphoprotein with the same molecular weight, was found exclusively in the 1085 x g membrane fraction. The possibility was therefore considered that these two proteins are related in that the former is converted to the latter by a membrane-associated kinase. In order to test this possibility reconstitution experiments were done. Membranes (1085x g pellets) and cytosols obtained from phorbol ester-treated and control cells were recombined in 3 different combinations as indicated in Table 1 and Fig. 4. These mixtures were run in phosphorylation reactions in presence of EDTA and phorbol ester. After 90 min the reaction mixtures were analyzed by liquid scintillation counting of incorporated [^{32}P]. Table 1 shows that *in vitro* addition of TPA to the reaction mixture increased significantly the incorporation of [^{32}P] into protein when the pellet component was derived from TPA-treated cells. Parallel reaction mixtures were centrifuged and [^{32}P]-labelled proteins in the resulting pellets and supernatants were analyzed by SDS-PAGE and semi-quantitative analysis of scanning plots. As illustrated by Fig. 4, phosphorylated proteins occurred both in the cytosol and the pellet, but the phosphorylated 48 kDa-protein was found exclusively in the pellet of all reconstituted reaction mixtures (Fig. 4, tracks a, c, e), thereby confirming our previous finding that the 48 kDa-phosphoprotein was tightly membrane-bound.

Table 1. Effect of TPA on protein phosphorylation in reaction mixtures, reconstituted from cytosolic and pelleted cell fractions

Reaction mixture		TCA-precipitable [^{32}P]cpm	
Composed of cytosol from cells	Containing membrane pellet from cells treated with	Without added TPA	With added TPA
-	-	8585	6462
-	TPA	7344	19746
TPA	TPA	6724	22050

Cytosolic and 1085 x g pelleted fractions of untreated and TPA-treated melanoma cells were mixed and incubated in the presence of γ-[^{32}P]-ATP and EDTA (5 mM). To one set of mixtures TPA (160 nM) was added; the other set remained without added TPA. After 90 min the [^{32}P]-incorporation into TCA precipitable material was assayed.

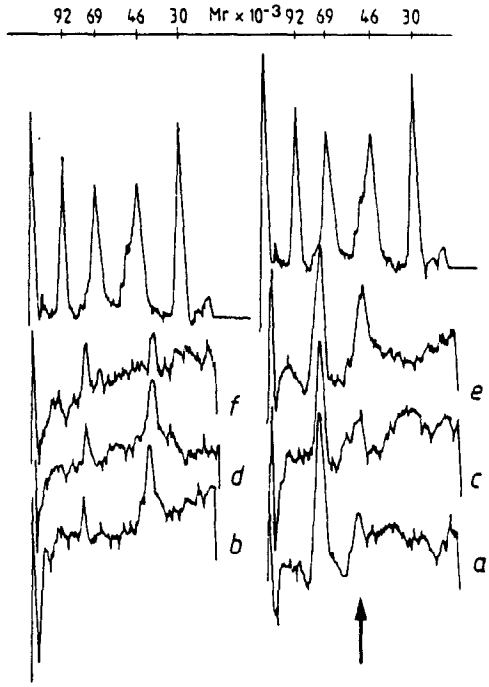

Fig. 4. Semiquantitative scanning analysis of the phosphorylated proteins from a reconstitution experiment.
The 1085 x g-pellet and cytosol from 12-O-tetradecanoyl-phorbol 13-acetate-induced or non-induced melanoma cells were mixed and in vitro phosphorylated in presence of γ-[^{32}P]ATP, EDTA and 12-O-tetradecanoyl-phorbol 13-acetate as in Fig. 3.
The phosphorylation reaction mixture was centrifuged and samples of the resulting pellets and supernatants were applied to the gel slab. Scanning plots of the autoradiography are indicated as follows :
- Pellet (track a) and supernatant (track b) of the reaction mixture, reconstituted from the 1085 x g pellet and cytosol of non-induced melanoma cells.
- Pellet (track c) and supernatant (track d) of the reaction mixture, reconstituted from the 1085 x g-pellet of 12-O-tetradecanoyl-phorbol 13-acetate-induced and the cytosol of non-induced cells.
- Pellet (track e) and supernatant (track f) of the reaction mixture, consisting of the 1085 x g pellet and cytosol of 12-O-tetradecanoyl-phorbol 13-acetate-induced cells.
Molecular mass standards are indicated on top. Arrow indicates 48kDa phosphoprotein.

Furthermore, the intensity of the 48 kDa-band, relative to that of the 73 kDa-band in the pellet (which was only little influenced by the constitutive parts of the reaction mixture), was highest when both constituents of the reaction mixture were derived from phorbol ester-treated cells (Fig. 4, track e). The 48 kDa-band was virtually absent when both constituents were derived from untreated cells. The hybrid reaction mixture, composed of membranes from phorbol ester-treated and cytosol from untreated cells, yielded of intermediary intensity of the 48 kDa-band, suggesting that membranes and cytosol both contribute to the generation of the membrane-bound 48 kDa-phosphoprotein. One possibility is that a membrane-associated protein kinase phosphorylates a cytosol 48 kDa-protein, whereafter this 48 kDa-protein possesses a high membrane affinity.

DISCUSSION

We have described several physicochemical characteristics as well as the subcellular distribution of (a) 48 kDa-protein(s), which is (are) coinduced with tissue-type plasminogen activator in melanoma cells treated with phorbol ester. Our findings are three-fold. First, we showed that cells treated with phorbol ester accumulate an mRNA translatable by reticulocyte lysate to a non-secreted, unglycosylated 48 kDa-protein : 48 kDa-r (15). Secondly, we demonstrated the phorbol ester-induced *de novo* synthesis of a 48 kDa-protein in the cytosol : 48 kDa-c. Thirdly, although this protein was not clearly detectable as such in the membrane fraction, exposure of the cell homogenates to phosphorylation reactions in the presence of labelled ATP, allowed to demonstrate the phorbol ester-induced appearance of a membrane-associated, phosphorylated 48 kDa-protein : 48 kDa-p. The postulate that these three products (48 kDa-r, 48 kDa-c and 48 kDa-p) are forms of the same protein is not proven but is supported by the observation that all three are phorbol ester-induced and migrate at near identical positions in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate under reducing as well as non-reducing conditions. Further evidence comes from the reconstitution experiments which indicated that a cytosolic protein contributes to the generation of the membrane-associated 48 kDa-phosphoprotein. Definitive proof for the identity of the 48 kDa protein species would require a monospecific antiserum which has until now been unavailable.

The study of the *in vitro* phosphorylation of the 48 kDa-protein was of particular interest, since it provided possible clues to the function of the protein. First, the amounts of the phosphorylated 48 kDa-phosphoprotein were enhanced by phorbol ester, whether added to cell culture or to the phosphorylation reaction mixture *in vitro*. Secondly, the phosphorylation of the 48 kDa-protein was not influenced by the addition of c-AMP, indicating that cAMP-dependent protein kinase is not involved. Third, phosphorylation of the

48 kDa-protein in cell homogenates was independent of divalent cations. This raises the question whether the reaction is indeed catalyzed by protein kinase C, which has been defined as Ca^{2+}-dependent. In reaction mixtures containing 5 mM EDTA, the free Ca^{2+}-concentration is lowered to a value of approximately 0.1 nM. Nevertheless, under these conditions the 48 kDa-protein was found to be efficiently phosphorylated. A plausible explanation for this observation may be that chelation of divalent cations blocks most of the kinases, thereby making sufficient ATP for a kinase whose action is Ca^{2+}-independent. This implies that, either there exists an as yet undefined calcium-independent, membrane-bound, phorbol ester-activated protein kinase, or that, in the presence of phorbol ester, the minimum free Ca^{2+}-concentration required to activate protein kinase C is at least 1000 or 100 times less than that derived from studies on blood platelets (22-24) or neutrophils (25), respectively.

Since the 48 kDa-protein is coinduced with tissue-type plasminogen activator, we have suggested (13) that it may be an intracellular signal molecule controlling secretory processes. This hypothesis is further supported by the data presented here. In particular, the observation that the 48 kDa-protein can be phosphorylated and dislocated from cytosol to the membrane is concordant with a possible role of messenger molecule. This as well as the parallelism in phorbol ester-induced phosphorylation and enhanced tissue-type plasminogen activator secretion support the idea that the 48 kDa-protein is part of the signal cascade leading from phorbol ester receptor activation to enhanced secretion of tissue-type plasminogen activator.

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