Protein Kinase C-dependent in Vivo Phosphorylation of Prourokinase Leads to the Formation of a Receptor Competitive Antagonist*

Paola Franco‡§, Ornella Massa‡§, Mar Garcia-Rocha‡, Ferdinando Chiardadonna‡, Ciro Iaccarino‡, Isabel Correas‡, Enrique Menedez‡, Jesus Avila‡, Francesco Blasi‡, and M. Patrizia Stoppelli‡**

From the ‡International Institute of Genetics and Biophysics, C.N.R., 80125 Naples, Italy, §Centro di Biologia Molecular, Facoltà di Scienze, Università Autonoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain, and ¶Dipartimento di Genetica, Universita di Milano and Department of Biology and Biotechnology, San Raffaele Scientific Institute, 20132 Milan, Italy

We recently reported that in vivo phosphorylation of urokinase-type-plasminogen activator on Ser138/303 prevents its catalytic-independent ability to promote myelomonocytic cell adherence and motility. We now show that Ca2⁺ activated, phospholipid-dependent protein kinase C from rat brain phosphorylates in vitro a peptide corresponding to prourokinase residues 133–143 (DGKKPSSPPEE) and the full-length molecule on Ser138/139. The in vivo involvement of the protein kinase C isoenzyme family is supported by the finding that inhibition of kinase C activity prevents prourokinase phosphorylation on Ser138/303 in A431 human carcinoma cells. Conversely, a short treatment of A431 cells with phorbol myristate acetate increases the extent of phosphorylated prourokinase and, concomitantly, affects its function; under these conditions, the capability of prourokinase to up-regulate U937 monocyte-like cell adherence is severely impaired, although receptor binding is unaltered. By the aid of a “phosphorylation-like” variant (Ser138 to Glu) we show that modification of Ser138 is sufficient to confer to prourokinase the antagonistic properties observed following in vivo stimulation of protein kinase C activity. These observations provide the first evidence that protein kinase C directs the formation of a receptor competitive antagonist by regulating the in vivo phosphorylation state of prourokinase.

Urokinase-type-plasminogen activator (uPA) catalyzes the conversion of plasminogen to active plasmin, a trypsin-like enzyme responsible for the lysis of fibrin and the degradation of many extracellular matrix components (1). This uPA-dependent proteolytic cascade is regulated by a complex network of interactions between specific domains of the protease and other macromolecules, such as plasminogen activator inhibitors (PAI-1, PAI-2 and others), a specific receptor (uPAR) and extracellular matrix components (vitronectin) (2–7).

Urokinase consists of an NH₂-terminal region sharing considerable homology with epidermal growth factor, a central kringle and a short proteolytically sensitive region which precedes a large carboxyl-terminal catalytic domain (8, 9). Whether soluble or receptor-bound, prourokinase (pro-uPA) is a zymogen that undergoes extracellular activation via the cleavage of the Lys158–Ile159 bond, thereby yielding a two-chain active urokinase capable of reacting with the inhibitors (10, 11). Recent evidence indicates that receptor-bound pro-uPA may fulfill additional functions besides those strictly dependent on its catalytic activity. Ligation of uPAR with uPA or with its noncatalytic amino-terminal fragment ATF (amino acids 1–135) stimulates intracellular biochemical pathways leading to a cellular response, which may involve changes in gene expression, protein phosphorylation, adhesion, migration, and metabolism. In particular, ligand-dependent uPAR activation leads to increased motility or adherence of myelomonocytic cell lines (12, 13).

Steadiy increasing evidence indicates that pro-uPA and uPAR syntheses are subjected to spatial and temporal regulation by oncogene activation, hormones, growth factors, and tumor promoters (14–16). In particular, protein kinase C (PKC), which belongs to an ubiquitous family of key regulatory isoenzymes in cell growth, differentiation, adhesion, carcinogenesis, and metastasis, regulates the uPA system at multiple levels (17, 18). It is known that PKC activation induces pro-uPA mRNA synthesis through a composite polyoma enhancer activator 3/activator protein 1 site located about 2 kilobase pairs upstream of the transcription initiation site in a variety of cell types, such as macrophages, keratinocytes, endothelial cells, and neurons, suggesting a highly conserved mechanism (19). Also, phorbol 12-myristate 13-acetate (PMA) stimulates uPAR synthesis in the U937 monocytic cell line and in migrating keratinocytes of wounded cultures (3, 20, 21). Taken together, these observations point to a complex network of interactions that link in vivo protein kinase C activation and regulation of urokinase function and localization.

Urokinase function is also subjected to post-translational control; we found that the human proenzyme undergoes intra-
cellular serine phosphorylation in A431 human carcinoma cells, resulting in a reduction of its sensitivity to the inhibitor PAI-1 (22–24). According to other reports, phosphorylated urinary urokinase activates plasminogen with a greater catalytic efficiency and is neither inhibited by PAI-1 nor by PAI-2 (25). Receptor-bound urokinase is phosphorylated on tyrosine and serine residues in a human metastatic carcinomatous cell line (26). Others have shown that urokinase from human urine and from HT1080 fibrosarcoma cells contains phosphothreonine residues, although no functional effects have been reported as yet (27, 28). Recent data from this laboratory indicate that two phosphorylation sites are located within the A and B chains of pro-uPA(Se138/303) from A431 human carcinoma cells and that pro-uPA phosphorylation renders the protease unable to activate uPAR-dependent signaling in myeloid cells (29). Interestingly, the nonsignaling serine phosphorylated pro-uPA binds to uPAR with unaltered affinity, such as a naturally occurring competitive antagonist (29). In this work, we attempted to shed light on the generation of such a molecule by analyzing the kinase pathway that directs pro-uPA phosphorylation on Ser138/303 in vivo. First, we report that protein kinase C from rat brain is able to directly modify Ser138 and/or Ser139 in vitro. Second, we show that in vivo modulation of protein kinase C activity regulates the extent of pro-uPA phosphorylation on Ser138/303 in the A431-P1 cell line. Finally, we present evidence that PKC-dependent in vivo phosphorylation of pro-uPA, as well as the replacement of Ser138 with a glutamic acid residue, markedly inhibit pro-uPA signaling ability, yet do not alter receptor binding.

EXPERIMENTAL PROCEDURES

Materials—Purified, recombinant human pro-uPA from Escherichia coli and mutant A125 (recombinant pro-uPA missing residues 11–135 of pro-uPA) were kindly provided by Dr. P. Sarmentos, Farmitalia Carlo Erba, Milan, Italy. ATF was a gift of Dr. Wang, Abbott Laboratories (Abbott Park, IL). uPA enzyme-linked immunosorbent assay kit was from American Diagnostica (Greenwich, CT). Phorbol 12-myristate 14-acetate (PMA), H-7, bisindolylmaleimide, the myristoylated PKC (19–27) peptide, 2-amino-3'-methoxyflavone (PD-98059), 4-(4-fluorophenyl)-2',4'-methylenedioxyphenyl)-5(6)-3H-methylene-cytosine, (SB-203580), TGF-β, and vitamin D3 were from Calbiochem-Novabiochem (La Jolla, CA). [32P]orthophosphate and [35S]methionine and methionine-free medium of HeLa-stable transfectants, as described previously (29). In this work, we attempted to shed light on the generation of such a molecule by analyzing the kinase pathway that directs pro-uPA phosphorylation on Ser138/303 in vivo. First, we report that protein kinase C from rat brain is able to directly modify Ser138 and/or Ser139 in vitro. Second, we show that in vivo modulation of protein kinase C activity regulates the extent of pro-uPA phosphorylation on Ser138/303 in the A431-P1 cell line. Finally, we present evidence that PKC-dependent in vivo phosphorylation of pro-uPA, as well as the replacement of Ser138 with a glutamic acid residue, markedly inhibit pro-uPA signaling ability, yet do not alter receptor binding.

EXPERIMENTAL PROCEDURES

Materials—Purified, recombinant human pro-uPA from Escherichia coli and mutant A125 (recombinant pro-uPA missing residues 11–135 of pro-uPA) were kindly provided by Dr. P. Sarmentos, Farmitalia Carlo Erba, Milan, Italy. ATF was a gift of Dr. Wang, Abbott Laboratories (Abbott Park, IL). uPA enzyme-linked immunosorbent assay kit was from American Diagnostica (Greenwich, CT). Phorbol 12-myristate 14-acetate (PMA), H-7, bisindolylmaleimide, the myristoylated PKC (19–27) peptide, 2-amino-3'-methoxyflavone (PD-98059), 4-(4-fluorophenyl)-2',4'-methylenedioxyphenyl)-5(6)-3H-methylene-cytosine, (SB-203580), TGF-β, and vitamin D3 were from Calbiochem-Novabiochem (La Jolla, CA). [32P]orthophosphate and [35S]methionine and methionine-free medium of HeLa-stable transfectants, as described previously (29). In this work, we attempted to shed light on the generation of such a molecule by analyzing the kinase pathway that directs pro-uPA phosphorylation on Ser138/303 in vivo. First, we report that protein kinase C from rat brain is able to directly modify Ser138 and/or Ser139 in vitro. Second, we show that in vivo modulation of protein kinase C activity regulates the extent of pro-uPA phosphorylation on Ser138/303 in the A431-P1 cell line. Finally, we present evidence that PKC-dependent in vivo phosphorylation of pro-uPA, as well as the replacement of Ser138 with a glutamic acid residue, markedly inhibit pro-uPA signaling ability, yet do not alter receptor binding.

EXPERIMENTAL PROCEDURES

Materials—Purified, recombinant human pro-uPA from Escherichia coli and mutant A125 (recombinant pro-uPA missing residues 11–135 of pro-uPA) were kindly provided by Dr. P. Sarmentos, Farmitalia Carlo Erba, Milan, Italy. ATF was a gift of Dr. Wang, Abbott Laboratories (Abbott Park, IL). uPA enzyme-linked immunosorbent assay kit was from American Diagnostica (Greenwich, CT). Phorbol 12-myristate 14-acetate (PMA), H-7, bisindolylmaleimide, the myristoylated PKC (19–27) peptide, 2-amino-3'-methoxyflavone (PD-98059), 4-(4-fluorophenyl)-2',4'-methylenedioxyphenyl)-5(6)-3H-methylene-cytosine, (SB-203580), TGF-β, and vitamin D3 were from Calbiochem-Novabiochem (La Jolla, CA). [32P]orthophosphate and [35S]methionine and methionine-free medium of HeLa-stable transfectants, as described previously (29). In this work, we attempted to shed light on the generation of such a molecule by analyzing the kinase pathway that directs pro-uPA phosphorylation on Ser138/303 in vivo. First, we report that protein kinase C from rat brain is able to directly modify Ser138 and/or Ser139 in vitro. Second, we show that in vivo modulation of protein kinase C activity regulates the extent of pro-uPA phosphorylation on Ser138/303 in the A431-P1 cell line. Finally, we present evidence that PKC-dependent in vivo phosphorylation of pro-uPA, as well as the replacement of Ser138 with a glutamic acid residue, markedly inhibit pro-uPA signaling ability, yet do not alter receptor binding.
kinase II (34). However, purified preparations of these kinases failed to phosphorylate in vitro pro-uPA from E. coli, under standard reaction conditions (not shown). On the contrary, when recombinant pro-uPA was incubated with rat brain PKC in the presence of 1 mM ATP, 10 μCi of [γ-32P]ATP, 150 mg/ml phosphatidylserine, and 200 ng/ml PMA, a specific phosphorylation reaction was observed. As shown in Fig. 1A, prourokinase incorporates [32P]phosphate in the presence of PKC, whereas no phosphorylation occurs in its absence, and a dramatic reduction of the resulting specific activity was observed in the presence of H-7 or bisindolylmaleimide or a myristoylated PKC (19–27) peptide. As expected, the mitogen-activated protein kinase kinase inhibitor PD-98059 did not inhibit pro-uPA phosphorylation by PKC. The Coomassie staining of the gel revealed equal amounts of recombinant pro-uPA in all samples (Fig. 1B). In control samples, phosphorylation of histone H1 yielded a strongly labeled band of about 35 kDa, whereas bovine serum albumin was not phosphorylated (not shown). Under the same conditions, prourokinase purified from the conditioned medium of the A431-P1 cell line overexpressing human pro-uPA (23) is also modified by PKC (not shown). The susceptibility of A431-P1 pro-uPA to in vitro phosphorylation is not surprising, as we have previously shown that about half of the secreted molecules are not phosphorylated, neither on A nor on B chain (24).

Localization of the in Vitro Phosphorylation Site(s)—To identify the protease domain(s) modified by PKC, in vitro phosphorylated 32P-pro-recombinant pro-uPA was subjected to limited proteolytic degradation with plasmin, which cleaves the Lys 158–Ile159 bond, thereby generating two fragments. Under these conditions, all the radioactivity previously incorporated in the intact 45-kDa protein was retained by the 18-kDa amino-terminal fragment (Fig. 2A). Accordingly, incubation of preactivated recombinant pro-uPA with PKC results in the exclusive phosphorylation of the 18-kDa fragment (Fig. 2A). In both cases, the stronger intensity of the 18-kDa fragments compared with the single-chain 45-kDa pro-uPA suggests that the PKC target region may undergo a conformational change following activation. To further restrict the analysis, Δ125 (35) and a proteolytic fragment comprising the amino acids 1–135 (ATF) were employed as substrates (depicted in Fig. 2C). Δ125 was highly susceptible to PKC-dependent phosphorylation, whereas ATF was slightly modified either in the presence of Δ125 or in its absence (Fig. 2B). These results, taken together, strongly suggest that the major phosphorylation site is located between amino acids 135 and 158, as this region is included in Δ125 and in the A chain of plasmin-cleaved pro-uPA but is not in the ATF. Analysis of the 135–158 region reveals three potential phosphate acceptors, two serine residues at 138 and 139 and a threonine residue at 152 (Fig. 2A). However, amino acid analysis of in vitro phosphorylated recombinant 32P-pro-uPA exclusively showed the occurrence of 32P-phosphoserine, suggesting that PKC indeed modifies Ser138 and/or Ser139 (not shown).

The latter possibility is in agreement with the results of an experiment in which recombinant pro-uPA was first phosphorylated with PKC in the presence of [γ-32P]ATP, then extensively digested with plasmin, reduced, and carboxymethylated (see “Experimental Procedures”). The resulting peptides were fractionated by RP-HPLC, and the fractions containing radioactivity were subjected to sequencing. The most abundant radiolabeled peptide was eluted in fraction 114 (Fig. 3) and showed the amino-terminal sequence PSSPPEEL . . . , in agreement with Ser138/139 being the predominant phosphate acceptor. Confirmatory data were obtained with a peptide corresponding to pro-uPA residues 133–143 (DGKKPSSPPEE), which was included as a substrate in an in vitro phosphorylation reaction with PKC (see “Experimental Procedures”). In Fig. 4A, the
position of the substrate peptide within the pro-uPA molecule is depicted. In this case, the \(^{32}\)P-phosphorylated products were separated onto a HPLC column, and two radioactive fractions were obtained, the first eluted in the void volume of the column, corresponding to non incorporated \([\gamma-^{32}\)P]ATP and the second containing the \(^{32}\)P-phosphorylated peptide (Fig. 4B). Under the same conditions, peptide KENSTDYPEWQLK, which is a substrate for casein kinase II, did not exhibit a second peak, indicating that it was not appreciably modified by PKC (not shown).

**In Vivo Phosphorylation of Pro-uPA and Dependence on PKC Activity**—The activity of protein kinase C can be modulated in vivo by different effectors, which may consequently affect pro-uPA phosphorylation state. This possibility was tested in a set of experiments in which PKC activity was stimulated with 100 ng/ml PMA or down-regulated either by a prolonged treatment with 1 \(\mu\)g/ml PMA or with the kinase inhibitor H-7. A431-P1 cells were metabolically labeled either with \([35\)S]methionine to ensure an internal control of pro-uPA synthesis and secretion (23).

In the first experiment, cells prelabeled for 4 h were further incubated with fresh medium containing either \([35\)S]methionine or \([^{32}\)P]orthophosphate with or without 100 ng/ml PMA. Aliquots of the resulting conditioned media were subjected to quantitative immunoprecipitation with anti-uPA antibody and separated by SDS-PAGE under reducing conditions. This analysis reveals single-chain pro-uPA and two-chain uPA deriving from partial serum-dependent proenzyme activation occurring in culture. As shown in Fig. 5A, after 30 min of incubation with PMA, the level of \(^{35}\)S-pro-uPA/uPA is unaltered, whereas \(^{32}\)P-labeled pro-uPA/uPA exhibit a marked increase. Quantitation of the resulting bands by autoradiogram scanning revealed a PKC-dependent 3-fold increase of overall phosphorylation; furthermore, the increased phosphorylation of both A and B chain suggests that modification of both Ser\(^{138}\) and Ser\(^{303}\) is dependent on PKC activation. In parallel experiments, we found that the addition of PMA does not stimulate the in vivo phosphorylation of His-pro-uPA\(^{138E/303E}\) (histidine-tagged pro-uPA variant in which Ser\(^{138/303}\) are no longer available), confirming that PKC exclusively modulates phosphorylation of Ser\(^{138/303}\) (not shown).

Down-regulation of PKC can be achieved by treating the cells with 1 \(\mu\)g/ml PMA for 24 h. Therefore, A431 cells were either subjected to a 16-h treatment with 100 ng/ml PMA or to a 24-h treatment with 1 \(\mu\)g/ml PMA. Samples of conditioned media from equal cell numbers were subjected to quantitative immunoprecipitation with 5B4 monoclonal antibody (Fig. 5B). Following PMA induction of PKC activity, there is again about a 3-fold increase in pro-uPA phosphorylation (Fig. 5B). On the contrary, pro-uPA phosphorylation is abolished if PKC activity is down-regulated, indicating that phosphorylation of Ser\(^{138/303}\) is totally PKC-dependent (Fig. 5B). In the third experiment, the cells were incubated with 100 \(\mu\)M H-7 or with diluents for 17 h, in the presence of each radiolabeled isotope (Fig. 5C). Furthermore, incubation of A431-P1 cells with bisindolylmaleimide or myristoylated PKC (19–27) peptide, both specific inhibitors of PKC, results in a strong reduction of pro-uPA phosphorylation level. On the contrary, inhibition of p38 mitogen-activated protein kinase by SB-203580 (not shown) or inhibition of mitogen-activated protein kinase by PD-98059 (Fig. 5D) did not alter pro-uPA phosphorylation.

In keeping with our previous results, PKC inhibitors do not alter pro-uPA secretion but they cause a strong reduction of the overall phosphorylation level, confirming that protein kinase C activity is responsible for most of pro-urokinase phosphorylation.
tion in uninduced A431 cells.

Effects of PKC Activity Modulation on Receptor Binding and Signaling Ability of A431 Pro-uPA—Previous functional analysis of pro-uPA phosphorylated on Ser\(^{138} / 303\) revealed a severe impairment of its catalytic-independent ability to promote myelomonocytic motility and adherence (29). The above demonstrated PKC-dependence of such a modification leads to the prediction that \(\text{in vivo}\) direct stimulation of PKC activity should result in a reduction of pro-uPA signaling ability. Considering a PKC-dependent 3-fold increase in the overall phosphorylation level, we expect that all secreted pro-uPA will at least bear one phosphate group/molecule. To test the effect of PKC activation on pro-uPA function, subconfluent A431-P1 cells were either left unstimulated or subjected to a 16-h treatment with 100 ng/ml PMA. The protease was then purified from the cell-conditioned medium, quantitated, and tested in a receptor competition assay for binding of \(\text{\textsuperscript{125}I}-\text{ATF}\) to U937 monocyte-like cells. As shown in Fig. 6A, both pro-uPA preparations exhibit comparable \(K_t\) values for uPAR. This finding is in agreement with our previous results obtained with phosphorylated and unphosphorylated pro-uPA purified from A431-P1-conditioned medium by \(\text{Fe}^{3+}\)-chelated chromatography (29). The effect of PKC-dependent pro-uPA phosphorylation on uPAR-mediated signaling was tested in a U937 monocyte-like cell adhesion assay. These cells were “primed” with TGF-β/ vitamin D\(_3\) for 20 h and then subjected to an adhesion assay, in the presence of 10 nM pro-uPA purified from A431-P1 cells, either stimulated with 100 ng/ml PMA for 16 h or left unstimulated. In this experiment, prourokinase from untreated A431-P1 cells causes about 40% of the total cells to adhere to the culture dish. On the contrary, pro-uPA from PMA-treated A431-P1 cells is markedly hampered in its proadhesive ability, over a wide concentration range (Fig. 6B). Interestingly, a “phosphorylation-like” histidine-tagged pro-uPA variant, carrying a Ser to Glu substitution, which is expected to mimic the functional consequences of phosphorylation on Ser\(^{138}\), is similarly impaired at inducing cell adherence, up to 10 nM, although it binds to uPAR (Fig. 6, A and B). This result suggests that, although PKC regulates the overall phosphorylation level, modification of Ser\(^{138}\) is mainly responsible for the dramatic inhibition observed. Confirmatory results were obtained in a U937 cell migration assay, employing pro-uPA as a chemotactic agent; we found that pro-uPA from PMA-treated cells retains only 20–30% of the chemotactic ability of pro-uPA from uninduced A431-P1 cells (not shown). These data indicate that protein kinase C activity down-regulates pro-uPA chemotactic and proadhesive ability without affecting uPAR binding. In addition, they show that a single amino acid substitution in pro-uPA mimics the functional effects observed following \(\text{in vivo}\) stimulation of PKC activity, therefore emphasizing the central regulatory role of PKC-dependent phosphorylation on Ser\(^{138}\).

DISCUSSION

The results presented in this study assign a role to PKC isoenzyme family in the \(\text{in vivo}\) regulation of prourokinase phosphorylation on Ser\(^{138}/303\). The modification of Ser\(^{138}\), in particular, results in a severe impairment of receptor-depend-
Naturally Occurring Inhibitor of Urokinase Receptor Signaling

Fig. 6. Effect of in vivo PKC activity on pro-uPA receptor binding and proadhesive ability. Panel A, the result of a competition between **ATF** (10^5 cpm/sample) and the indicated nanomolar concentrations of A431 pro-uPA from untreated cells (C), from cells treated with 100 ng/ml PMA for 16 h (A), or purified His-pro-uPA 138E (■) to U937 cell uPARs is shown. Cell-bound radioactivity is reported as a function of unlabeled competitor concentration. Maximal binding in the absence of competitor was 1168 cpm (S.D. = 48). Data are shown as the mean of three independent experiments performed in duplicate; standard deviations are indicated by error bars. Panel B, U937 cells were grown to 0.8 x 10^6 cells/ml, then diluted 1:2 and treated with TGF-β vitamin D3 for 20 h. Then they were incubated with A431-P1 pro-uPA from untreated cells (C), from cells treated with 100 ng/ml PMA for 16 h (A), or with His-pro-uPA 138E (■). The number of adherent and nonadherent cells was counted 30 min later and reported as a percentage of the total cell number. The background adherence due to the TGF-β vitamin D3 addition was 10.5% (S.D. = 1.13). The data represent the average of three experiments performed in duplicate with standard deviations indicated by error bars.

ent pro-uPA ability to promote myeloid cell adherence, although it does not alter receptor binding. The latter site, which lies outside of the receptor binding domain, is a target of in vitro phosphorylation by rat brain protein kinase C. Analysis of the sequence surrounding Ser 138 suggests that accessibility and secondary structure of the region surrounding Ser 138 may be relevant to PKC recognition. The lack of signaling by the pro-uPA variants carrying Glu 138 indicates that the aforementioned region, designated “connecting peptide” and including the zymogen activation site, although not required for binding, is a potential inhibitor of uPA signaling function by a PKC-mediated mechanism. Interestingly, secondary structure prediction, according to the Garnier et al. (36) method, generates a four-turns model for the 132–158 region, which is completely disrupted by the introduction of a negatively charged amino acid at 138.2 The relevance of this region is also suggested by the finding that removal of Lys 135 or Lys 158 with carboxypeptidase A impairs the proadhesive effect, yet does not affect receptor binding (37). Therefore, the possibility exists that the uPA-dependent signal can be delivered by a transient association of activated uPAR with a transmembrane receptor, which is modulated by a balance between negatively and positively charged amino acids at specific positions in the 135–158 region.

In the in vitro experiments, no appreciable phosphorylation of Ser 103 by PKC takes place, suggesting several interpretations. It is possible that the conformation attained by the mature protein, either recombinant or secreted from A431 cells may not totally resemble that of intracellular pro-uPA (22). Alternatively, an unknown kinase may be responsible for the modification of Ser 103 in vivo, provided its activity is regulated by PKC. We cannot distinguish between these two possibilities, at the moment. Conversely, phosphorylation of Ser 138, which plays a central role in the modulation of pro-uPA signaling ability, is totally PKC-dependent both in vitro and in vivo. These data support the hypothesis that PKC may directly modify Ser 138 in vivo. In agreement with this possibility, a direct role of pp60Src and protein kinase C in pro-uPA phosphorylation by metastatic and tumor cells has been suggested by other authors (26). On the other hand, we have previously shown that pro-uPA serine phosphorylation does not occur in cell culture medium, but inside A431 cells (22). The analysis of these data poses an interesting topological dilemma, as most of the known PKC isozymes are cytosolic and pro-uPA is a secreted zymogen. However, extensive studies indicate localization of PKC in a variety of intracellular compartments different from the plasma membrane, including the Golgi complex, the perinucleus, the cytoskeleton, and the focal contacts of rat embryo fibroblasts (38). Among the atypical isozymes of PKC is the 1 form, belonging to the ζ subgroup, which has been detected over the luminal surfaces of acinar cells in pancreatic cells (39). Interestingly, an ecto-protein kinase with a catalytic specificity similar to PKC has been recently discovered on the cell surface of brain neurons (40). Given the growing information about the structure and physiology of PKC-related and PKC-like enzymes, we cannot exclude that a particular type will phosphorylate secreted proteins. Interestingly, pro-uPA susceptibility to in vitro phosphorylation by PKC is shared by other secreted proteins, such as the platelet coagulation factor Va and matrix vitronectin (41, 42).

In any event, our results provide an important step toward understanding the formation and the occurrence of natural receptor antagonists, which may be shared by other ligand-receptor systems. In this study, we have shown that protein

2 C. Iaccarino and M. P. Stoppelli, unpublished results.
kinase C in vivo dictates the conversion of an agonist into a receptor-competitive antagonist, which blocks the signaling by nonphosphorylated pro-uPA. This functionally resembles the case of IL-1α and IL-1β, the unique cytokines for which a naturally occurring antagonist is known (IL-1RA); crystal structure of the IL-1RA/receptor complex has shown that, unlike the agonists, the “receptor trigger site” of the antagonist is not in direct contact with the receptor (43). In our system, the di-substituted variant is unable to mobilize the receptor, which may be due to a peculiar binding mode of phosphorylated pro-uPA (29). The possibility exists that negatively charged domains D2 and/or D3 or with a negative regulator of cell adhesion. Alternatively, the negative effect may be due to a peculiar binding mode of phosphorylated pro-uPA forms. In addition, we have shown that phosphorylation of Ser138 can be functionally mimicked by a Ser to Glu substitution in the protease, offering the naturally occurring antagonist is known (IL-1RA); crystal structure of the IL-1RA/receptor complex has shown that, unlike the agonists, the “receptor trigger site” of the antagonist is not in direct contact with the receptor (43).

REFERENCES

Protein Kinase C-dependent in Vivo Phosphorylation of Prourokinase Leads to the Formation of a Receptor Competitive Antagonist
Paola Franco, Ornella Massa, Mar Garcia-Rocha, Ferdinando Chiaradonna, Ciro Iaccarino, Isabel Correas, Enrique Mendez, Jesus Avila, Francesco Blasi and M. Patrizia Stoppelli


Access the most updated version of this article at http://www.jbc.org/content/273/42/27734

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 16 of which can be accessed free at http://www.jbc.org/content/273/42/27734.full.html#ref-list-1