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Tyrosine phosphorylation and stimulation of protein kinase Cδ from porcine spleen by src in vitro

Dependence on the activated state of protein kinase Cδ

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Abstract

Native protein kinase Cδ from porcine spleen is phosphorylated in vitro by the tyrosine kinase src and to a much smaller extent by fyn. The tyrosine phosphorylation of PKCδ is restricted to the activated state of the enzyme, i.e. it occurs only in the presence of an activator, such as TPA or bryostatin. Upon phosphorylation at tyrosine, the apparent molecular weight of PKCδ increases by 6 kDa. Phosphorylation by src induces a stimulation of PKCδ activity apparently exhibiting some substrate selectivity. Other PKC isoenzymes, such as cPKC (α,β,γ), are not phosphorylated by src or only to a very small extent. This phosphorylation is not dependent on TPA and does not cause an increase in activity and molecular weight of the enzyme.

Key words: Protein kinase Cδ; Tyrosine phosphorylation; src; fyn; TPA; Bryostatin

1. Introduction

The PKC family, a group of 10 isoenzymes known so far with phospholipid-dependent serine/threonine kinase activity, plays a key role in signal transduction and is involved in the regulation of numerous cellular processes and probably in tumor promotion (for reviews see [1–4]). Over-expression of various PKCs in different cell lines has indicated that a certain isoenzyme might induce a specific effect, such as increased or reduced cell proliferation [5–10]. However, distinct functions of the different isoenzymes in signaling processes, especially with respect to selective phosphorylation of substrates, are unknown to date.

PKCδ was purified to homogeneity from porcine spleen [11]. It is a member of the Ca^{2+}-unresponsive, diacylglycerol (or TPA)-activatable subgroup of PKC isoenzymes, termed nPKC. Preliminary results indicate some substrate specificity of PKCδ as compared to Ca^{2+}-responsive isoenzymes (cPKC). In order to investigate whether PKC, and especially its substrate specificity, might possibly be affected by events taking place in other signaling pathways, we studied the effect of tyrosine kinases on PKCδ and cPKC. Interactions of tyrosine and serine/threonine kinases in signaling cascades are well known. The most intriguing example recently studied in great detail is provided by the tyrosine kinase → ras → raf → MAP-kinase cascade [12–14], in which PKC, possibly via GAP [15–17], might also be involved.

Here we show that purified PKCδ is phosphorylated and stimulated rather specifically by src in vitro. Tyrosine phosphorylation of PKCδ is dependent on the presence of a PKC activator (e.g. TPA or bryostatin), i.e. on the activated state of the enzyme. Tyrosine phosphorylation of PKCδ appears to modulate the substrate specificity of the kinase.

2. Materials and methods

2.1. Materials

TPA, bryostatin and Gö6983 were kindly provided by Dr. E. Hecker, German Cancer Research Center, Heidelberg, Dr. G.R. Pettit, State University of Arizona, Tempe, USA, and Gödecke AG, Freiburg, Germany, respectively. [γ^{32}P]ATP (spec. act. 3,000 Ci/mmol) was from DuPont-New England Nuclear (Waltham, MA, USA). Phosphatidyl serine (PS) and myelin basic protein were from Sigma, Munich, and protamine sulfate from Serva, Heidelberg, Germany. The anti-phosphotyrosine antibody was from Gibco BRL. Phosphotyrosine phosphatase-1B and src were from Upstate Biotechnology Inc., Lake Placid. In one experiment (see Fig. 1) preparations of src and fyn were used that were a generous gift of Dr. S.A. Courtneidge, EMBL, Heidelberg. The catalytic subunit of the EGF receptor was generously provided by Dr. D. Fabbro, Ciba Geigy, Basel. The pseudosubstrate-related peptides α and δ were synthesized by R. Pipkorn, German Cancer Research Center, Heidelberg, and had the following sequences: RFARKGSLRQKNV (α), MNRRGSIKQAKI (δ).

2.2. Purification of PKC

cPKC (a mixture of PKCα,β,γ) was purified from mouse brain [18] and PKCδ from porcine spleen [11] as described previously.

2.3. Tyrosine phosphorylation of PKC

cPKC or PKCδ was incubated with src or fyn and ATP (17 nM [^{32}P]ATP + 30 μM ATP) at 30°C for 15 min. The reaction mixture (total volume of 100 μl) contained: 50 μl of Tris-buffer (20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol), 4 mM $MgCl_2$, 0.1 mM

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PS, phospatidyl serine; MAP-kinase, mitogen activated protein kinase; GAP, GTPase activating protein.

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$MnSO_4$. Where indicated, 10 μg phosphatidyl serine (PS) and/or 10^{-7} M TPA were added. The reaction was stopped by the addition of 10% trichloroacetic acid, and the redissolved proteins were separated by SDS-PAGE. Tyrosin-phosphorylated proteins were visualized by immunoblotting with an anti-phosphotyrosine antibody (see [17]).

3. Results and discussion

PKCδ purified to homogeneity from porcine spleen [11] could be phosphorylated at tyrosine residue(s) by the tyrosine kinase src in vitro as shown by immunoblot analysis with an anti-phosphotyrosine antibody (Fig. 1a and b). Tyrosine phosphorylation of PKCδ was observed only in the presence of the phorbol ester TPA, i.e. in the activated state, but not in the absence of all cofactors and not in the presence of PS alone (Fig. 1a). Bryostatin, like TPA, was able to generate this activated form of the enzyme and to allow its tyrosine phosphorylation by src (data not shown). Tyrosine phosphorylation of PKCδ with fyn, another kinase of the src family, was much less effective than with src (Fig. 1b), even though both kinase preparations exhibited equal activities with respect to the phosphorylation of enolase. Preliminary results showed that the catalytic subunit of the EGF

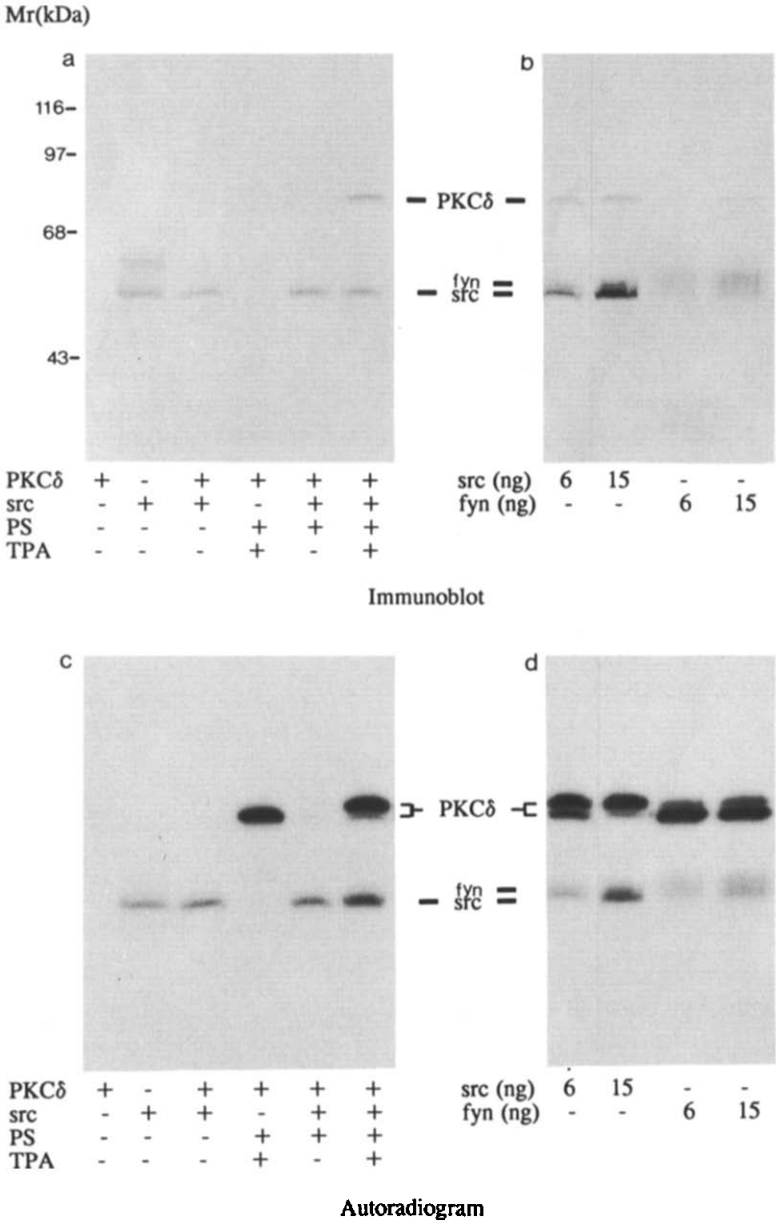

Fig. 1. Tyrosine phosphorylation of PKCδ. 0.26 μg of PKCδ from porcine spleen [11] were incubated with 15 ng of src from Upstate Biotechnology Inc. (a,c) or 6 and 15 ng of src or fyn provided by Dr. S.A. Courtneidge (b,d), as described in Section 2. The latter preparations of src and fyn exhibited equal activities with respect to the phosphorylation of enolase. Tyrosine-phosphorylated proteins were visualized by immunoblotting with an anti-phosphotyrosine antibody (a,b), and total phosphorylated proteins by autoradiography (c,d).

receptor was barely, if at all, able to phosphorylate PKCδ. This indicated some selectivity towards the tyrosine kinase. The tyrosine phosphorylation of PKCδ caused an increase in its apparent molecular weight of around 6 kDa. In the absence of src PKCδ was just autophosphorylated and appeared as a 76 kDa protein (see also [11]) in the autoradiogram, whereas in the presence of src PKCδ became tyrosine-phosphorylated in addition to its autophosphorylation, exhibiting an apparent molecular weight of 82 kDa (Figs. 1c and 2a). Incorporation of phosphate in addition to that incorporated by autophosphorylation of PKCδ became evident also by the increase in radioactivity of PKCδ phosphorylated in the presence of src over that in the absence of src (4,615 cpm vs. 2,517 cpm; see Fig. 2a). Tyrosine-phosphorylated PKCδ appeared in the immunoblot (Figs. 1a and 2b) at the same location (82 kDa) as in the autoradiogram (Figs. 1c and 2a). The molecular weight shift could be reversed by treatment of the tyrosine-phosphorylated PKCδ with phosphotyrosine phosphatase-1B (data not shown). The portion of PKCδ that became tyrosine-phosphorylated (see the band shift) was dependent on the amount of src in the assay. With the larger amount of src (15 ng) almost all PKCδ molecules appeared to be tyrosine-phosphorylated since just a faint band was visible at 76 kDa (Fig. 1d). In contrast, the same amount of fyn caused just a small portion of the 76 kDa band to shift to 82 kDa (Fig. 1d). This is in accordance with the poor tyrosine phosphorylation of PKCδ by fyn that we could demonstrate by immunoblotting with an anti-phosphotyrosine antibody (see above, Fig. 1b). Preliminary results with PKCδ fragments obtained after limited digestion with trypsin indicated that the phosphorylated tyrosine residue(s) might be located downstream of the pseudosubstrate sequence RRGAIK. In this region only the tyrosine residues 155, 311, 332, 372, 446 and 628 (amino acid sequence of murine PKCδ, see [18]) are PKCδ-specific (not present in amino acid sequences of other PKC isoenzymes) and conserved in murine [19], rat [20] and human [21] PKCδ. Inhibition of PKC activity, and thus also of autophosphorylation, by means of a highly specific PKC inhibitor (the staurosporine-related compound Gö6983, see [22]) did not affect tyrosine phosphorylation of PKCδ (Fig. 2c). This finding indicates that it was not the TPA-induced stimulation of the activity and autophosphorylation of the enzyme but rather a conformational change of the kinase occurring concomitantly with its activation that might be an essential prerequisite of tyrosine phosphorylation. This special feature points to some physiological significance of the tyrosine phosphorylation of PKCδ.

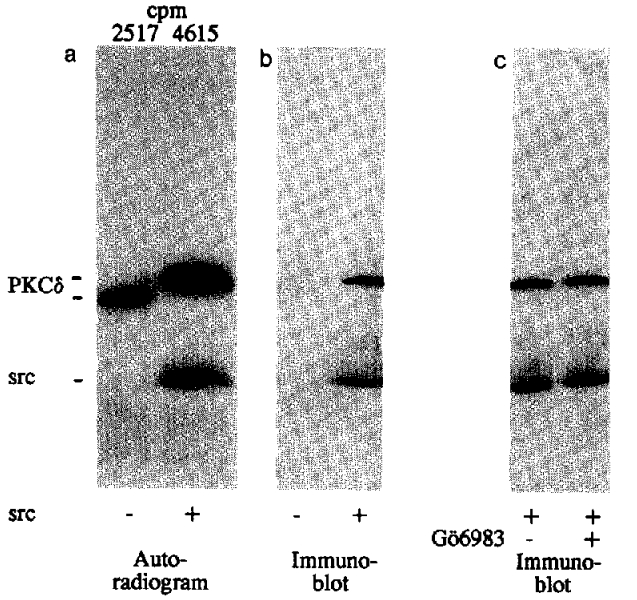

Fig. 2. Tyrosine phosphorylation of PKCδ in addition to its autophosphorylation: independence of PKC activity. Phosphorylation in the presence of PS/TPA was performed and proteins were visualized by autoradiography (a) and immunoblotting (b,c) as in Fig. 1. (a) The radioactivity in each PKCδ band cut out of the blot was determined by scintillation counting and is given on top of the figure. (c) Phosphorylation of PKCδ with src was performed in the absence (−) or presence (+) of the PKC inhibitor Gö6983 (1 μM).

A specific physiological role of this phosphorylation was further indicated by our finding that incubation with src caused an increase in the activity of PKCδ (Table 1). but not of cPKC (PKCα,β,γ purified from mouse brain, see [18], data not shown). Again, this effect was observed only in the presence of TPA. Intriguingly, the increase in kinase activity proved to be dependent on the PKC

Table 1
Kinase activity of PKCδ and tyrosine-phosphorylated PKCδ (PKCδ(Tyr-P)) as determined with various substrates

Substrate	PKCδ activity (cpm)	PKCδ(Tyr-P) activity (cpm)	Increase (%)
Myelin basic protein	71,608 ± 2867	147,975 ± 8880	107
Histone-IIIS	14,936 ± 750	26,832 ± 2055	80
Pseudosubstrate-related peptide δ	230,080 ± 11504	320,326 ± 14713	39
Pseudosubstrate-related peptide α	104,027 ± 7807	84,033 ± 6954	0 (−19)
Protamine sulfate	213,917 ± 17253	203,478 ± 11274	0 (−5)

PKCδ was phosphorylated with src in the presence of PS/TPA as described in the legend to Fig. 1. The activity of PKCδ and PKCδ(Tyr-P) was determined with various substrates in the presence of 10 μg PS and 10^{-7} M TPA as described previously [27]. The values are the mean of two experiments.

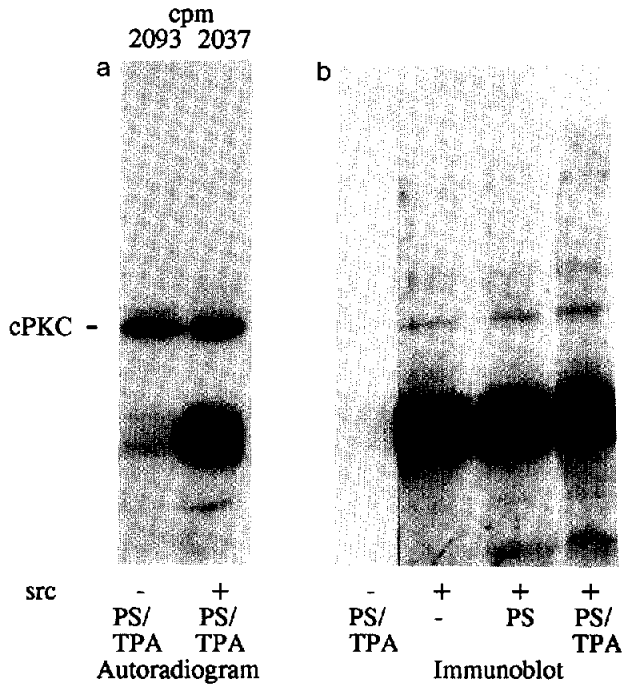

Fig. 3. Phosphorylation of cPKC with src. cPKC (PKCα,β,γ purified from murine brain [18]) was incubated with src and [^{32}P]ATP as described for PKCδ in the legend to Fig. 1. Phosphorylated proteins were visualized either by autoradiography (a) or immunoblotting (b) and the radioactivity in each cPKC band (see top of the figure) was determined as described in Fig. 2.

substrate used for the assay (Table 1). Incorporation of phosphate into myelin basic protein was stimulated by 107%, into histone by 80%, and into the pseudosubstrate-related pepdie δ by 39%. No stimulation of kinase activity was observed with protamine sulfate and the pseudosubstrate-related peptide α as substrates. Phosphorylation of the substrate proteins was entirely due to PKCδ, since src was not able to phosphorylate any of these substrates (data not shown). It is conceivable that specific substrates exist in cells which are phosphorylated exclusively or at least much more effectively by the tyrosine-phosphorylated PKCδ. Very recently, when this paper was in preparation, tyrosine phosphorylation of PKCδ in response to treatment of PKCδ-over-expressing cells with TPA [23] or to transfection of cells with ras^{Ha} [24] was reported. Our finding of a stimulatory effect of tyrosine phosphorylation on the activity of PKCδ is in agreement with the first, but in contrast to the latter report, which describes an inhibition of kinase activity upon tyrosine phosphorylation.

Tyrosine phosphorylation appeared to be rather specific for the δ-isoenzyme, since incubation with src did not cause any incorporation of phosphate into cPKC exceeding that caused by autophosphorylation (see Fig. 3a and compare with Fig. 2a). Nevertheless, weak tyrosine phosphorylation of cPKC might have occurred as indicated by the immunoblot (Fig. 3b). However, it did not cause an increase in either molecular weight or enzyme activity (see above) and was not dependent on TPA. Preliminary results with recombinant PKC isoenzymes from baculovirus-infected insect cells indicate that some isoenzymes other than PKCδ, such as PKCζ, might also become tyrosine-phosphorylated by src to a very small extent. Again, however, phosphorylation of these isoenzymes was not dependent on TPA and no increase in molecular weight was observed. Thus, the tyrosine phosphorylation of PKCδ differs clearly from that of other PKC isoenzymes and may have a specific quality. In intact cells, besides PKCδ no other PKC isoenzyme was found to be tyrosine-phosphorylated [23,24], indicating that the weak tyrosine phosphorylation of other PKC isoenzymes seen in vitro does not occur in vivo and thus might be physiologically irrelevant.

According to our results, tyrosine phosphorylation of PKCδ specifically causing an increase in its molecular weight is restricted to the activated form of the enzyme. Thus, tyrosine phosphorylation of PKCδ in vitro and upon treatment of cells with TPA appears to be due to the exposure of some tyrosine residue(s) as a consequence of TPA activation of PKCδ. However, we cannot exclude the possibility that TPA treatment of intact cells additionally causes activation of tyrosine kinases, such as src. It is tempting to speculate that, by means of the specific tyrosine phosphorylation of PKCδ and its activation for selective substrate phosphorylation, an interaction between PKC-mediated and tyrosine kinase-dependent signaling pathways is achieved. Tyrosine kinases of the src family are known to bind to growth factor and cytokine receptors and to be part of so-called signal transduction complexes together with other signaling molecules, e.g. for instance ras, raf and rasGAP (for reviews see [25,26]). Recently we have shown that rasGAP forms a complex with and is phosphorylated by PKC [17]. Thus, via such signal transduction complexes PKC might get in contact with tyrosine kinase-dependent signaling pathways.

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