Journal of Cellular Biochemistry 86:394–402 (2002)

Identification of PSF as a Protein Kinase Cα-Binding Protein in the Cell Nucleus

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Abstract Protein kinase C (PKC) isoforms are present in the cell nucleus in diverse cell lines and tissues. Since little is known about proteins interacting with PKC inside the cell nucleus, we used Neuro-2a neuroblastoma cells, in which PKCα is present in the nucleus, to screen for nuclear binding partners for PKC. Applying overlay assays, we detected several nuclear proteins which bind to PKCα. Specificity of binding was shown by its dependence on PKC activation by phorbol ester, calcium, and phosphatidylserine. The PKC-binding proteins were partially purified and analyzed by microsequencing and mass spectrometry. Four proteins could be identified: PTB-associated splicing factor (PSF), p68 RNA helicase, and the heterogeneous nuclear ribonucleoprotein (hnRNP) proteins A3 and L. In the case of PSF, binding to PKC could also be demonstrated in a GST-pull-down assay using GST-PKCα, expressed in insect cells. Phosphorylation experiments revealed that PSF is a weak in vitro substrate for PKCα. J. Cell. Biochem. 86: 394–402, 2002. © 2002 Wiley-Liss, Inc.

Key words: protein kinase C; PSF; nucleus; anchoring; PKC binding protein

Protein kinase C (PKC) is involved in the regulation of a wide variety of cellular processes [Parker, 1994; Newton, 1995; Nishizuka, 1995]. Many of these, such as the induction of proliferation or differentiation, involve nuclear events. Accordingly, there are several examples for PKC isozymes present in the cell nucleus, either constitutively or dependent on their activation [reviewed in, Martelli et al., 1999; Buchner, 2000]. Additionally, various substrates of PKC have been identified in the cell nucleus, for example, lamin B, B23, and DNA topoisomerases [Buchner, 1995; Martelli et al., 1999]. Although for several proteins, for example, DNA topoisomerase II, the influence of phosphorylation by PKC on its activity has been demonstrated, much of the function of PKC in the cell nucleus remains to be clarified.

Furthermore, it is not known how PKC enters the nucleus and how it is retained there. PKC has none of the known nuclear localization signals (NLS) which mediate the import of proteins into the nucleus, i.e., the "classical" from SV40 large T antigen, a bipartite NLS, or the M9 region from hnRNP A1 [Kalderon et al., 1984; Robbins et al., 1991; Siomi and Dreyfuss, 1995]. Earlier observations strongly indicate that the nuclear transport of PKCα is different from that of NLS-containing proteins [Schmalz et al., 1996, 1998].

Nevertheless, nuclear import alone cannot explain the differential compartmentalization in the nucleus of PKC isozymes depending on cell type and state [Buchner, 1995; Martelli et al., 1999]. To explain the complicated compartmentalization of PKC isozymes, a set of

Abbreviations used: ESI, electrospray ionization; hnRNP, heterogeneous nuclear ribonucleoprotein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PSF, PTB-associated splicing factor; PTB, polypyrimidine tract-binding protein; RACK, receptor for activated C kinase; TBS, tris-buffered saline; TFA, trifluoroacetic acid.

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: SFB 515; Grant sponsor: Fonds der Chemischen Industrie; Grant sponsor: Graduiertenkolleg "Signalketten in lebenden Systemen".

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Received 2 November 2001; Accepted 3 April 2002

DOI 10.1002/jcb.10233

binding partners for PKC has been proposed and a variety of binding proteins for PKC have been identified [for recent reviews see Ron and Kazanietz, 1999; Jaken and Parker, 2000]. These proteins can be classified as "receptors for activated C-kinase" (RACKs) [Mochly-Rosen et al., 1991] or "substrates interacting with C-kinase" (STICKs) [Chapline et al., 1998], scaffolding proteins like AKAP79, and other proteins which do not fit into either of these groups [Ron and Kazanietz, 1999]. AKAP79 originally was defined as a binding protein for the RII subunit of protein kinase A [Klauck et al., 1996]. It has separate binding sites for PKC, PKA, and calcineurin and is a good example for the similar anchoring of both kinases, PKA and PKC. AKAP79 as well as the STICKs are substrates for PKC whereas the RACKs are not [Ron and Kazanietz, 1999].

These PKC binding proteins are thought to direct PKC isoforms to various cytoplasmic compartments, for example, to focal contacts [Hyatt et al., 1994b], the Golgi apparatus [Csukai et al., 1997], caveolae [Oka et al., 1997], and elements of the cytoskeleton [Blobe et al., 1996; Prekeris et al., 1996]. In contrast to that, very little is known about interaction partners of PKC in the cell nucleus.

We therefore started a screen for PKC binding proteins present in the nucleus of Neuro-2a neuroblastoma cells. In these cells, PKCα is present in the cytoplasm and to a smaller extent also in the cell nucleus [Buchner et al., 1997]. The nuclear PKCα in Neuro-2a cells shows a reduced extractability from the nucleus after stimulation, suggesting a tighter association with nuclear structures [Buchner et al., 1997]. Using overlay assays, we could demonstrate the presence of PKC-binding proteins in a nuclear fraction. Four of these proteins were identified as PTB-associated splicing factor (PSF), p68 RNA helicase, hnRNP A3, and hnRNP L. Focussing on PSF, we found that this protein also interacts with PKCα in a GST-pull-down assay and that it is a weak in vitro substrate for PKCα.

MATERIALS AND METHODS

Cell Culture

Neuro-2a cells, kindly supplied by G. Schultz (Freie Universität Berlin), were grown in Dulbecco's modified Eagle's medium, containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37°C.

Sf9 insect cells were grown in serum-free Sf 900 II medium (GIBCO) containing penicillin (50 U/ml) and streptomycin (50 μg/ml) at 27°C either in monolayer or in suspension cultures. The cells of the suspension cultures were passaged when the density increased beyond 2.5×10^6 cells/ml. To overexpress GST-PKCα, cultures with a density of $>1.5 \times 10^6$ cells/ml were used for infection with the recombinant baculovirus at a multiplicity of infection (MOI) of 10. The recombinant baculovirus was obtained from S. Wagner from our group.

Cell Fractionation

Cell fractionation of Neuro-2a cells was performed as described [Emig et al., 1995; Buchner et al., 1997]. The nuclei isolated in this way show a very small degree of contamination with other cellular compartments as demonstrated by electron microscopy and marker protein analysis [Emig et al., 1995; Buchner et al., 1997]. The particulate fraction was obtained by centrifugation of the homogenate for 1 h at 100,000*g*.

Preparation of Nuclear Extract

Nuclei of Neuro-2a cells were resuspended in binding buffer and then homogenized on ice using a Branson sonifier 250 (30 s at setting 3). The homogenized nuclei were shortly spun down in an Eppendorf centrifuge to remove debris and the supernatant was collected and used for the in-solution binding assays.

Overlay Assay

The overlay assays were performed as described [Liao et al., 1994] with slight modifications. Proteins were separated by gel electrophoresis and blotted on nitrocellulose membranes. After staining with Ponceau red, membranes were blocked by a solution of 5% skimmed milk powder in tris-buffered saline (TBS), containing 20 mM Tris/HCl, pH 7.4, and 0.5 M NaCl. After blocking, blots were incubated for 1 h with a solution of 3 μg/ml PKC purified from bovine brain (mixture of conventional PKC isoforms, prepared according to Krüger et al. [1990] in TBS containing 1% BSA, 10 μg/ml leupeptin, 10 μg/ml aprotinin. For testing the co-factor dependence of PKC binding, an overlay solution containing 6 μg/ml PKC was used. Under activating conditions, the assay additionally

contained phosphatidylserine (20 μg/ml), $CaCl_2$ (1 mM), and PMA (160 nM). After rinsing with PBS (phosphate buffered saline; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4), blots were fixed with 0.5% formaldehyde and quenched with 2% glycine, both in PBS. Then the membranes were probed with a monoclonal antibody against PKCα (UBI, Lake Placid) and secondary antibodies coupled to enzymes (SIGMA, Deisenhofen). Alkaline phosphatase was used for detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt), horseradish peroxidase for enhanced chemiluminescence detection.

Ion-Exchange Chromatography

For partial purification of nucleoplasmic proteins, 5 ml of the nucleoplasmic fraction (supernatant of the nuclear envelope preparation) were applied onto a MonoQ-FPLC column (Pharmacia). Proteins were eluted with a gradient from 0–1 M NaCl in a buffer containing 20-mM triethanolamine (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 10 mM β-mercaptoethanol. The gradient was run with a flow rate of 1 ml/min with an increase in buffer B of 2% per minute.

Protein Identification

FPLC fractions from the ion-exchange chromatography were further separated by SDS–PAGE. Proteins were stained with Coomassie Blue. Bands of interest (PKC-binding in the overlay assay) were cut out from the gel, and the proteins were digested in the gel matrix with trypsin according to Eckerskorn and Lottspeich [1989]. The resulting peptides were eluted from the gel matrix, separated by reverse-phase HPLC, and analyzed using electrospray ionization (ESI) mass spectrometry (TSQ 700, Finnigan), and micro-sequencing with an automated gas-phase sequencer (473 A, Applied Biosystems). Proteins were finally identified by sequence comparison with databases (SWISSPROT, using PCGENE).

Isolation of GST-Fusion Proteins

Sf9 cells were harvested 72 h after infection by a 15-min centrifugation (8000*g*). The pellet was resuspended in homogenization buffer (140 mM NaCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4, pH 7.5) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Five to ten milliliters of buffer were used for 10^8 cells. The cells were homogenized on ice using a Branson sonifier 250 (3 min at setting 4).

After centrifugation (100,000*g*, 25 min, 4°C), the clear supernatant was applied to a 1 ml-column of glutathion agarose (Clontech) at 4°C, equilibrated with 10-bed volumes of homogenization buffer. The column was washed with 10-bed volumes of homogenization buffer and then the enzyme was eluted with 50 mM Tris/HCl (pH 8.0) supplemented with 10-mM glutathion. The eluate was dialyzed against binding buffer (20 mM Tris/HCl (pH 7.4), 100 mM NaCl; 2 mM Mg acetate; 2 mM DTE; 0.1% Tween-20; 0.1% casaminoacids; 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF).

In-Solution Binding Assay

In each sample, 1.0-μg GST-PKCα was incubated with 100 μg nuclear extract, 50 μl packed glutathion agarose beads, and the effectors in a final volume of 400 μl binding buffer for 90 min at 4°C with constant shaking. At the end of the incubation, the beads were collected by centrifugation at 2000*g* for 20 s. Thirty microliters of the supernatant containing the unbound proteins were kept for further analysis. After removal of the rest of the supernatant, the beads were then washed four times by resuspension in 0.5 ml binding buffer and centrifugation as before. Finally, 50 μl sample buffer was added to the pellets and heated at 95°C for 5 min. The proteins were then analyzed by SDS–PAGE and Western blotting.

The antibody against PSF, raised in rabbit against the peptide CQREMEEQMRRQREES (amino acids 582–596 of the PSF sequence), was supplied by Pineda Antikörper Service (Berlin). For Western blotting, the serum was used at a 1:2,500 to 1:5,000 dilution and detected one single band at about 100 kDa. In some experiments we used an antibody against PSF which was kindly provided by J. G. Patton (Vanderbildt University, Nashville, TN).

Detection of PSF Phosphorylation With PKCα

The phosphorylation reaction was performed in a total volume of 50 μl of phosphorylation buffer (20 mM Tris/HCl (pH 7.4); 4 mM Mg acetate; 1 mM DTE; 0.1 mM $CaCl_2$; 50 μg/ml phosphatidylserine; 5 μg/ml diolein (1,2 dioleoyl-glycerol) 20 μM ATP supplemented with [γ^{32}P]-ATP to give approximately 1 μCi/assay). About 25 μg nuclear extract protein and 1.5 μg PKC were used in each assay.

The reaction was started by the addition of ATP. The samples were incubated for 10 min at 30°C, then put on ice and 50 μl 2 × NP40 buffer (200 mM NaCl; 1% NP40; 100 mM Tris/HCl (pH 8.0); 20 mM NaF; 2 mM Na-vanadate; 2 μM okadaic acid) were added.

After addition of anti-PSF-antibody (1:500), the samples were incubated for 60 min on ice, before 20 μl protein-G-sepharose (Pharmacia Biotech) were added. Then the samples were incubated for 1 h at 4°C. Finally, the beads were collected by centrifugation at 2000*g* for 20 s. The beads were then washed four times by resuspension in 100 μl 1 × NP40 buffer and centrifugation as before. The pelleted beads were processed by adding 50 μl of sample buffer and heating at 95°C for 5 min. The eluted proteins were then analyzed by SDS–PAGE and Western blotting. For autoradiography, the blotting membrane was exposed to a Biomax MS film (Kodak) at −80°C before Western blot analysis.

RESULTS

We performed overlay assays according to Liao et al. [1994] to detect proteins binding to activated PKCα in nuclear fractions of Neuro-2a cells. Using this method, PKC-binding proteins could indeed be observed in nuclear fractions (Fig. 1). Binding of PKCα to nuclear proteins depends, in many cases, on the presence of PKC activators, as most PKC-binding proteins were detected in the presence of phorbol ester, calcium, and phosphatidylserine (Fig. 1). Calcium ions or phosphatidylserine alone caused only a small increase in the PKC binding of blotted proteins when compared to the case where PKC without activators was applied (Fig. 1). In the following analysis, we focussed on those proteins that showed preferential or strongly enhanced binding in the presence of activators.

Since in Neuro-2a cells PKCα is present in the nucleoplasm [Buchner et al., 1997], we focussed on the identification of PKC-binding proteins in a nucleoplasmic fraction. After partial purification by FPLC ion-exchange chromatography (data not shown), five proteins were digested with trypsin, and the resulting peptides were analyzed by micro-sequencing and mass spectrometry. Figure 2 shows an example of this procedure. The eluted peptides of a 102-kDa protein were separated by reverse-phase HPLC. The indicated peak was further analyzed by mass spectrometry and Edman sequencing. The

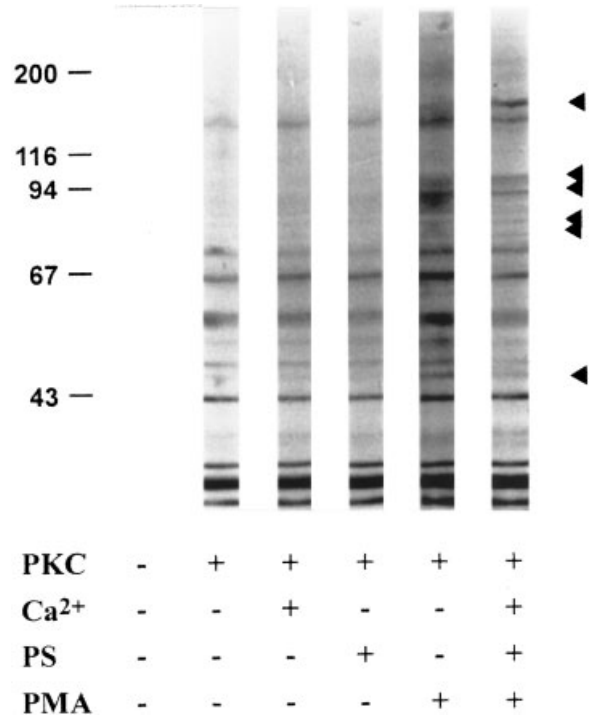

Fig. 1. PKC binding proteins in the nuclear fraction. The nuclear fraction of neuro-2a cells was subjected to overlay assays with PKC in the presence or absence of the PKC activators calcium-ions, phosphatidylserine (PS), and phorbol ester (PMA). The arrowheads mark proteins which show full binding of PKC only in the presence of all PKC activators, especially PMA. The time for development of the color reaction was the same for all lanes and adjusted in a way to achieve an optimal visualization of the bands of the binding proteins. Therefore, the band reflecting endogenous PKCα, which is apparent at other incubation times, especially in lanes without PKC in the overlay solution, is not visible in this case.

determination of a sequence of 11 amino acids led to the identification of the peptide as a 23 amino acid tryptic peptide from the splicing factor PSF with a theoretical mass of 2428.64 Da (measured mass: 2428.0 Da).

For all proteins, the sequence of at least one peptide could be completely determined. The following proteins were identified (Table I): PSF, p68 RNA helicase, hnRNP L, hnRNP A3, and hnRNP A1. Only one protein was found in the database for each of the peptide sequences.

Since hnRNP A1 is one of the most abundant proteins of the nucleus [Dreyfuss et al., 1993], and because the alignment of the protein band to the band in the overlay assay was less clear than for the other proteins, we performed an immuno-precipitation with a monoclonal antibody against hnRNP A1. Immuno-purified hnRNP A1 did not bind PKC in the overlay assay (data not shown). Therefore the originally

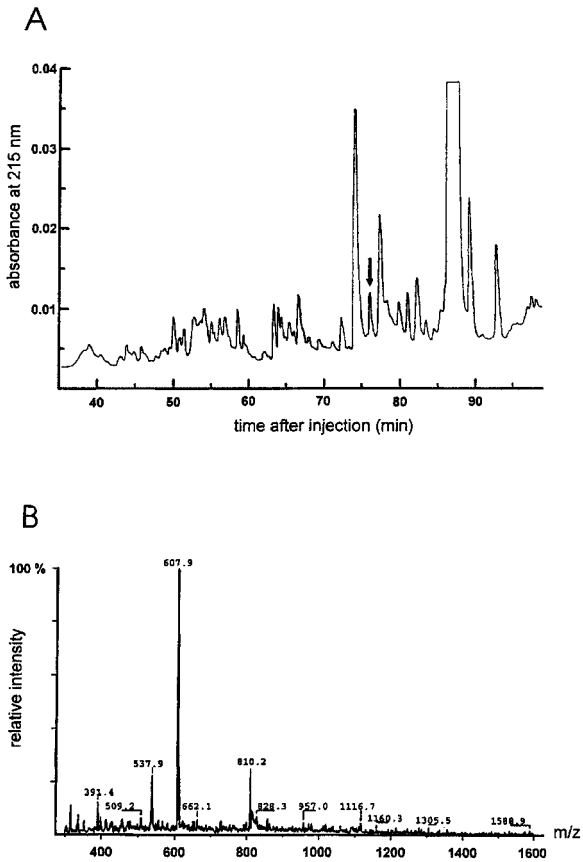

Fig. 2. Protein identification by HPLC and ESI mass spectrometry. Tryptic peptides of a 102-kDa protein, shown to bind PKCα in the overlay assay, were separated and analyzed. (**A**) Separation of eluted peptides with reverse-phase HPLC; (**B**) electrospray mass spectrometry of the indicated peak. The main component has a mass of 2,428.0 Da and appears mainly three- and fourfold protonated (m/z = 607.9 and 810.2). Edman sequencing led to the identification of the peptide as a 23 amino acid peptide from the splicing factor PTB-associated splicing factor (PSF).

observed binding of PKC was probably caused by another (yet unidentified) protein comigrating with hnRNP A1.

PSF has been shown to bind protein phosphatase 1 [Hirano et al., 1996] and is a developmentally regulated neuronal protein [Chanas-Sacre et al., 1999], making it a particularly interesting candidate PKC-binding protein, so that we focussed further experiments on PSF.

To investigate whether PSF indeed binds to PKC, we used a second approach: nuclear extracts were incubated together with a GST-PKCα fusion protein and the fusion protein was captured together with bound proteins by glutathion beads. Binding of PSF was then analyzed by Western blotting. Using this procedure, we found that GST-PKCα alone already can bind some PSF, whereas binding is clearly enhanced in the presence of Ca^{2+}, staurosporine, or phorbol ester (Fig. 3). Incubation of glutathion beads with nuclear extract alone (without GST-PKCα) did not recover detectable amounts of PSF (not shown).

Since several (but not all) PKC-binding proteins identified by overlay assays are substrates of PKC [Mochly-Rosen, 1995], and because it is known that, for example, the prevalent PKC substrate MARCKS binds to PKC in overlay assays [Hyatt et al., 1994a], we tested whether PSF is a substrate for PKC. Proteins in nuclear extracts were phosphorylated in vitro with GST-PKCα under stimulating conditions, followed by immunoprecipitation of PSF. Autoradiography revealed that PSF is only a very weak substrate for PKCα (Fig. 4). Without addition of PKCα no phosphorylation of PSF could be detected. Analysis of the same blots with antibodies against PSF revealed that PSF was precipitated in equal amounts in both cases.

TABLE I. Identified Nuclear PKC Binding Proteins

1	2	3	4	5
Apparent molecular weight (kDa)	Peptide sequence	Peptide mass; measured	Peptide mass; predicted	Identified protein
102	DXLESEDXY-HEXQ...	2428.0	2428.64 (after identification)	PSF = PTB-associated splicing factor
68	GLDVEDVK; (GG)FNTF(R)	874.2; ND	873.96; 797.87	P68 RNA helicase
67	FSTPEQA(AK)	978.0	978.49	hnRNP L
37	IETIEVMEDR	1233.9	1234.38	hnRNP A3

Proteins with the apparent molecular weights listed in column (1) were analyzed by Edman-sequencing and mass spectrometry as described. The measured masses of the peptides (3) were in good agreement with the theoretical masses of peptides (4) having the obtained sequences (2). These data allowed the identification of the proteins listed in (5). Amino acids which were not unambiguously identified by micro-sequencing are in brackets; X, unidentified amino acids.

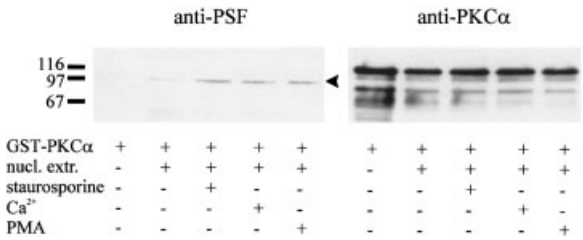

Fig. 3. PSF binding to GST-PKCα. One microgram of GST-PKCα was incubated with nuclear extract (100-μg protein) and glutathione-agarose beads in the absence or presence of staurosporine, Ca^{++}, and PMA as described. After collection of the beads by centrifugation and washing, bound proteins were analyzed by Western blot analysis using antibodies against PSF. Presence of either of the activators lead to an increase in the amount of PSF (arrowhead) associated with the pelleted beads. Reprobing of the same blot with antibodies against PKCα showed that the amount of GST-PKCα was about the same in all cases. Similar results were obtained in three independent experiments.

Since all PKC-binding proteins which we identified, interact with RNA, we tested PKC's possible involvement in splicing. However, in our hands PKC did not influence either the overall splicing efficiency of nuclear extracts or the splice-site selection in the alternative splicing of the tyrosine kinase src (data not shown).

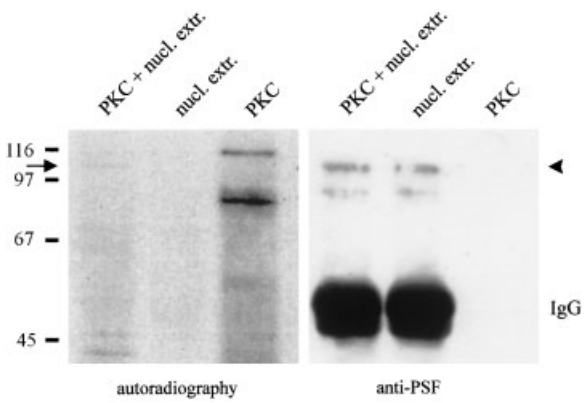

Fig. 4. In vitro phosphorylation of PSF by PKC. Nuclear extracts were phosphorylated in vitro with or without GST-PKCα. After immunoprecipitation with an antibody against PSF, the precipitated proteins were separated by SDS–PAGE, blotted onto nitrocellulose and analyzed by autoradiography (**left panel**) and by probing with the PSF antibody (**right panel**). Phosphorylation of nuclear extract proteins with GST-PKCα (**first lane**) leads to the detection of only a very weak radioactive band at about 100 kDa in the autoradiography (arrow; left panel), which comigrates with PSF (arrowhead; right panel). Incubation without PKC (nuclear extracts; **second lane**) did not lead to a detectable phosphorylation of PSF, indicating that PSF is not phosphorylated to a detectable degree by other kinases present in the nuclear extracts. In the **third lane** (PKC), a sample of GST-PKCα incubated without nuclear extract was applied to the gel to demonstrate autophosphorylation-activity.

DISCUSSION

The presence of PKC isoforms in the cell nucleus under various conditions [Buchner, 1995; Martelli et al., 1999] and the existence of a variety of cytoplasmic PKC-binding proteins [Ron and Kazanietz, 1999; Jaken and Parker, 2000] made it probable that PKC-binding proteins are also present in the nucleus. Using blot overlay assays, we were able to detect several PKCα-binding proteins in a nuclear fraction. Since the yeast two-hybrid system does not allow to search specifically for proteins belonging to a defined subcellular compartment (in our case the cell nucleus), we could not use this method, which in other cases has successfully been applied for the identification of protein PKC-binding proteins [Staudinger et al., 1995]. On the other hand, the overlay assay, as we and others have performed it, includes a denaturing gel electrophoresis followed by partial renaturation of the proteins on the blot membrane. Since not all proteins show this behavior, certainly not all PKC-binding proteins can be found in this way.

Overlay assays in combination with partial purification and peptide sequencing were further used to identify some nuclear PKC-binding proteins as hnRNP A3, hnRNP L, p68 RNA helicase, and PSF. These proteins are all involved in the processing of RNA but differ markedly in their function and exact nuclear location [Pinol-Roma et al., 1989; Iggo et al., 1991; Good et al., 1993; Hahm et al., 1998].

PSF is an essential splicing factor, which although interacting with the polypyrimidine tract-binding protein PTB [Patton et al., 1993], was found also in other parts of the cell nucleus [Meissner et al., 2000]. Like PTB, it preferentially binds to polypyrimidine tracts in the nascent RNA transcript [Patton et al., 1993]. PSF is a developmentally regulated protein [Chanas-Sacre et al., 1999] which has been shown to bind protein phosphatase 1 [Hirano et al., 1996] and DNA-topoisomerase I [Straub et al., 1998]. Recently PSF was identified as a tyrosine-phosphorylated protein [Otto et al., 2001].

Although the identification of the PKC-binding proteins was achieved after partial purification by ion-exchange chromatography, it cannot entirely be excluded that other, yet unidentified proteins were comigrating within the same band in the 1D-SDS-gel and were responsible

for PKC-binding. Therefore, additional assays were performed for PSF, on which we focussed our interest. Using GST-pull down assays, we could demonstrate that GST-PKCα could precipitate PSF from nuclear extracts, strongly suggesting that there is indeed an interaction between PKC and PSF which can also take place in solution. Similar to the observations in the overlay assays, binding was enhanced in the presence of PKC-activating substances. The observation that the addition of staurosporine enhanced binding is at first sight surprising, but it was shown earlier that staurosporine can induce a change in PKC conformation similar to that induced by phorbol ester and can thus also induce a translocation of PKC from the soluble to the particulate fraction ([Buchner et al., 1999] and references cited therein).

Phosphorylation experiments revealed that, at least under our conditions, PSF is a poor substrate in vitro. Although the sequence of mouse PSF [Shav-Tal et al., 2001] contains three sites for possible PKC phosphorylation (KTYT(286)QR; RGRS(402)T(403)GK), the presence of these sequences alone certainly would not be sufficient to conclude that PSF is a PKC substrate. Proteins containing a consensus phosphorylation site are not necessarily substrates for the respective kinase, because, for example, the phosphorylation site may be buried in the interior of the protein. The poor phosphorylation of PSF found by us indicates that PSF may belong to the same group of PKC-binding proteins as for example RACK1 which is not phosphorylated by PKC [Ron et al., 1994]. For p68 [Buelt et al., 1994] and hnRNP A3, which was identified during a screen for nuclear substrates (Buchner, K. unpublished observations), phosphorylation by PKC has been observed in vitro, which confirms our observations of interaction in the overlay assays and strongly suggests that these proteins are indeed PKC-binding proteins. Whether the interaction occurs via recognition of their substrate region and the catalytic region of PKC, or whether they interact with PKC in the same way as the STICKs, which bind to PKC via a site different from the site phosphorylated by PKC [Jaken and Parker, 2000], remains to be clarified. For hnRNP L additional data from phosphorylation experiments or other assays are not yet available, so that we are less confident than in the other cases of the other proteins that it is a nuclear PKC-binding protein.

The physiological function of the interaction of the proteins with PKC is not yet clear. One possible function is anchoring of PKC in the nucleus. Since the distribution within the nucleus varies among cell types and PKC isoforms [Buchner, 1995; Martelli et al., 1999], transport into the nucleus alone is not sufficient to determine the exact nuclear location of PKC. One way to explain the specificity in PKC targeting may be a cooperative effect of nuclear import and nuclear retention by specific proteins at defined locations.

Another possibility is that upon activation PKC binds to proteins such as PSF which may serve as a scaffolding protein in a similar way as, for example, AKAP79 which tethers PKC, PKA, and calcineurin in proximity to their substrates [Klauck et al., 1996]. PSF, like AKAP79, can bind PKC (this study) as well as a protein phosphatase [Hirano et al., 1996].

As all nuclear PKC-binding proteins, which we identified, interact with RNA and are involved in splicing, we tested a possible involvement of PKC itself in splicing. The influence of phosphorylation on the splice-site selection [Cardinali et al., 1994] or on the assembly of spliceosomes [Mermoud et al., 1994] has been previously observed. However, there are only few reports on the involvement of PKC in splicing processes [Collet and Steele, 1993; Walter et al., 2000]. Collet and Steele [1993], for example, observed the switch from "normal" src to the neuronal splice variant in embryos of *Xenopus laevis*. This switch, which happens in the embryo during the formation of the neural plate, can be mimicked in dissected cells from the blastoderm by the addition of phorbol ester. The authors could rule out any participation of protein synthesis in this switch, but they did not show that this effect is due to a direct phosphorylation by PKC of proteins involved in splicing. The fact that we did not detect a change in either the splicing efficacy or the preferential splice site in the alternative splicing of src argues against a direct role of PKC in this process. However, we cannot rule out that PKC influences splicing only under certain conditions or in certain cell types.

Taken together, the nuclear proteins we have identified are good candidates for physiologically relevant anchor proteins of PKC in the nucleus. It should be noted that, since anchoring of proteins in a specific compartment is also a means to achieve its redistribution to this

compartment, the anchoring of PKC in the nucleus might also be a part of or contribute to the nuclear transport mechanism of PKC. Since up to now, we characterized only those proteins which showed enhanced PKC-binding under activating conditions, we expect still more nuclear PKC-binding proteins to be identified. Further analysis of such protein will probably also lead to a better understanding of the function of PKC in the cell nucleus.

ACKNOWLEDGMENTS

We thank Doris Krück for excellent support in cell culture and the preparation of subcellular fractions. We thank J.G. Patton for the antibody against PSF, G. Dreyfuss for the antibody against hnRNP A1, and Stefan Wagner for supplying us with the GST-PKCα construct.

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