Transport of protein kinase C α into the nucleus requires intact cytoskeleton while the transport of a protein containing a canonical nuclear localization signal does not

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SUMMARY

Protein kinase C undergoes a redistribution from the cytosol into the nucleus upon various stimuli. Since protein kinase C does not contain any known nuclear localization signal, the exact pathway and mechanism of the translocation into the nucleus is not known.

We used immunofluorescence microscopy to investigate the role of the cytoskeleton in this process, and to detect the subcellular distribution of protein kinase C α in NIH 3T3 fibroblasts. In these cells protein kinase C α is translocated into the nucleus after stimulation with phorbol ester. We observed that cells treated with the cytoskeleton disrupting agents cytochalasin B or colchicine do not show the nuclear translocation of protein kinase C α after stimulation. In contrast, the nuclear accumulation of a nuclear localization signal containing reporter protein in an in vitro nuclear transport assay is not affected by these drugs. This observation has been confirmed for intact cells by microinjection experiments: cells which have been incubated with cytochalasin B or colchicine prior to microinjection of the reporter protein show the same accumulation in the nucleus as untreated cells.

Our data show that intact cytoskeleton plays an important role in the translocation of protein kinase C α into the nucleus but not in the nuclear import of a karyophilic reporter protein.

Key words: Protein kinase C, Cytoskeleton, Cell nucleus, Nuclear transport, Microinjection, Nuclear localization signal

INTRODUCTION

Transport of signals from the plasma membrane to the nucleus may use passive diffusion of ions like Ca²⁺ (Bachs et al., 1992; Allbritton et al., 1994), or translocation of proteins like transcription factors (Karin, 1994; Vandromme et al., 1996) and of protein kinases such as MAP kinase (Lenormand et al., 1993), the catalytic subunit of protein kinase A (PKA) (Nigg et al., 1985), and some isozymes of protein kinase C (PKC) (Buchner, 1995). Translocation is triggered by activation at the plasma membrane after binding of a ligand to a receptor, followed by intracellular phosphorylation. Targets of the translocated protein kinases are a variety of nuclear proteins including, for example, structural proteins like lamins (Fields et al., 1988; Peter et al., 1990) and transcription factors (CREB, cFos, cJun etc.) (Meek and Street, 1992; Buchner, 1995).

Targeting of proteins to the nucleus requires a signal contained in its primary structure, which marks which protein goes to this compartment. In addition means of transportation are required from the cytosol to the nucleus, through the nuclear envelope to the interior, and within the nucleus to specific subcompartments. For several nuclear proteins nuclear localization sequences (NLS) have been identified (Dingwall and Laskey, 1991). For others unidentified signal sequences or transport via carrier proteins having such signals are postulated. Specific localization of proteins at their target compartment may be accomplished by binding to an ‘anchor’, which, in the case of protein kinases, may or may not be its substrate.

Protein kinase C (PKC) is long known as an example of a protein kinase conveying intracellular signals by moving between compartments (for review see Nishizuka, 1995). Localized in its inactive state in the cytoplasm, PKC translocates upon stimulation (e.g. by phorbol esters) to the plasma membrane and/or to the nucleus. With PKC the problem of signal-dependent targeting is complicated by the existence of many (presently twelve mammalian) isoforms (Dekker and Parker, 1994). The intracellular distribution of PKC isoforms varies among tissues and cells, and no general pattern can be seen. As an example, whereas PKC ε in differentiated NG 108-15 cells is translocated upon stimulation by the phorbol ester phorbol 12-myristate 13-acetate (PMA) to the plasma membrane (Beckmann et al., 1994), in 3T3 cells movement to the nucleus was observed (Leach et al., 1989). PKC ε, on the other hand, was localized to the Golgi in overexpressing 3T3 cells (Lehel et al., 1995), while in NG 108-15 cells we observed it in the nuclear membrane (more specifically in the nuclear pore complex)(Beckmann et al., 1994).

Within the cytoplasm interactions of PKC with actin filaments, microtubules and intermediate filaments have been observed (for review see Jaken, 1992). Docking to sites on
cytoskeletal elements may confer specific subcellular localization to PKC isozymes. In the present investigation we wanted to know whether an intact cytoskeleton has functions with respect to PKC beyond anchoring it in a given location. We observed that PKC α upon stimulation was not transported to the nucleus when the cytoskeleton was disrupted. In contrast, the transport of a reporter protein containing a canonical NLS was not affected by agents depolymerizing the cytoskeleton. This indicates that nuclear transport of PKC α and of NLS-proteins via the nuclear pore complex may possibly use different mechanisms.

MATERIALS AND METHODS

Tissue culture and drug treatment

The NIH 3T3 fibroblasts were a kind gift from Katrin Saar (Institut für Humangenetik, Berlin). Cells were maintained in Dulbecco’s modified Eagle’s Medium (Cytogen), containing sodium pyruvate, 1.0 g/l glucose, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (Gibco) in a humidified atmosphere of 5% carbon dioxide at 37°C. Cells were passaged every three days using trypsin/EDTA (Gibco). They were incubated in serum-free medium for approximately 4-6 hours prior to use in indirect immunofluorescence, microinjection or in vitro transport assays.

Exposure to phorbol ester (PMA, 160 nM for ten minutes), colchicine (10 μM, one hour), vinblastine (0.2 μM, one hour) or cytochalasin B (2 μM, one hour) was carried out in serum free medium (all drugs were purchased from Sigma).

Immunocytochemistry

To perform indirect immunocytochemistry, cells were seeded on multilayer glass slides. Cells were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes and permeabilized with 80% methanol in PBS at –20°C for twenty minutes. Blocking was performed using 3% bovine serum albumin in PBS for 15 minutes. Antibodies were diluted in PBS with 3% BSA at the following dilutions: anti-PKC-α (Upstate Biotechnology Incorporated, Lake Placid) at 1:100 for 30 minutes or anti-β-tubulin (Sigma) at 1:20 for one hour. To monitor the integrity of actin polymers, TRITC-phalloidin (Sigma) was used at a dilution of 1:200. Goat anti-mouse Fc-Cy3 (Dianova, Hamburg) was used as the secondary antibody at a dilution of 1:400. Cells were mounted in Fluoromount G (Serva) and viewed with a Leica DMRB inverted fluorescence microscope. Photographs were taken with Kodak T-Max 400 film.

In vitro transport assay

Transport assays were performed using digitonin-permeabilized cells essentially as described by Adam et al. (1990). Fibroblasts were grown on glass slides for approximately 24 hours, rinsed three times with ice-cold transport buffer (TB, 110 mM potassium acetate, 20 mM Hepes, pH 7.3, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol and 1 μM each leupeptin and aprotinin) and then permeabilized on ice with 50 μg/ml digitonin in TB for 5 minutes. After three additional washings with cold TB, the transport mix (rabbit reticuloocyte lysate (Promega), dialyzed against TB, 50 mg/ml protein, 1 mM ATP, 5 mM creatine phosphate and 20 U/ml creatine phosphokinase), together with a fluorescent reporter conjugate (final concentrations 80 μg/ml), was added to the cells. Slides were kept in a humidified atmosphere at 30°C for 30 minutes. Following this incubation, the cells were again rinsed with TB, fixed with 3% paraformaldehyde/PBS for 15 minutes and mounted in Fluoromount G.

Preparation of NLS-TRITC-BSA fluorescent conjugate

Synthetic peptides containing the SV40 large-T antigen wild-type nuclear localization signal (NLS; sequence: CGTGPKKKRRKGG) were obtained from Bachem (Heidelberg) and as a kind gift from Victor Tsetlin of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry in Moscow, Russia. Conjugation with TRITC-BSA (Sigma) was performed as described (Adam et al., 1990). In short, peptides were resuspended in 50 mM Hepes, pH 7.0, reduced with 50 mM dithiothreitol and desalted on a Sephadex G-10 column. TRITC-BSA was activated with a 20-fold molar excess of the sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfos- SMCC) crosslinking reagent (Pierce). Following the activation, a 50-fold molar excess of the reduced peptides was added and the mixture was incubated at 4°C in darkness overnight. The NLS-TRITC-BSA conjugates were desalted on a Sephadex G-25 column, dialyzed against TB and the protein concentration was adjusted to 1 mg/ml using the method of Bradford (1976).

Microinjection

Microinjection was used to investigate the accumulation of fluorescent conjugates in intact cells. Cells were grown on coverslips and injected with NLS-TRITC-BSA or TRITC-BSA using a manual system (Eppendorf, Germany) with pipets pulled from borosilicate glass. The outlet tip diameter of the pipet was approximately 0.5 μm. The injection pressure was 150-250 hPa and the injection time was 300 milliseconds. Approximately 10^{-15} to 10^{-14} liters of the protein solution (1 mg/ml in TB) were injected into each cell. After injection, cells were allowed to sit for 10 minutes at 5% carbon dioxide and 37°C in a humidified atmosphere. Fixation, mounting, viewing and taking photographs were carried out as described above.

RESULTS

In order to investigate the role of cytoskeletal elements in the nuclear accumulation of PKC, we destroyed the actin and microtubule network by using cytochalasin B and vinblastine or colchicine, respectively. As shown in Fig. 1, incubation with 2 μM cytochalasin B for one hour led to the complete disruption of the actin filaments, which were visualized by probing filamentous actin with fluorescence-labeled phalloidin (TRITC-phalloidin). The photographs for Fig. 1A and 1B were taken under identical conditions.

As shown in Fig. 1D, also taken under identical conditions, incubation with 10 μM colchicine for one hour led to depolymerization of the microtubules. In this case a monoclonal antibody against β-tubulin was used to detect microtubules. Treatment with 0.2 μM vinblastine had very similar effects (data not shown).

In resting 3T3 cells PKC α is localized almost completely in the cytoplasm as revealed by immunocytochemistry with the isofrom specific monoclonal antibody against PKC α (Fig. 2A). As already observed earlier (Leach et al., 1989), incubation with phorbol ester leads to a translocation of the enzyme into the nucleus (Fig. 2B).

Fibroblasts which were incubated with cytochalasin B show the normal, primarily cytoplasmic, localization of PKC α (Fig. 2C). However, the phorbol ester-induced translocation of PKC α from the cytoplasm to the nucleus was abolished after incubation with cytochalasin B (Fig. 2D).

Disrupting the microtubules with vinblastine or colchicine also inhibits the PMA-induced translocation of PKC α (Fig. 3). After incubation with the microtubule-depolymerizing agents, localization of PKC α remained cytoplasmic even after stimulation with PMA (Fig. 3).
Taken together, our observations show that the disruption of both the actin filaments and the microtubules inhibits the translocation of PKC α from the cytosol into the nucleus.

Since PKC isoforms do not contain sequences that resemble canonical NLSs, we investigated whether this cytoskeleton-dependence of nuclear transport is shared by the nuclear import of NLS-bearing proteins. For that we performed in vitro transport assays using permeabilized fibroblasts (Adam et al.,...
Cells were permeabilized with digitonin and incubated with a mixture of reticulocyte lysate, ATP-regenerating system and a fluorescent reporter protein as described in Materials and Methods. Whereas a reporter protein containing no NLS showed no nuclear accumulation but is, rather, washed out after the incubation (Fig. 4B), the NLS-containing reporter protein NLS-BSA-TRITC accumulated in the nucleus (Fig. 4C). This accumulation even took place when the cells were treated with cytochalasin B (Fig. 4D), vinblastine (Fig. 4E) or colchicine (Fig. 4F). These observations show that the disabling of both actin filaments and microtubules has no effect on the nuclear uptake of the fluorescent, NLS-bearing conjugate.

To exclude the possibility that the uptake of NLS-TRITC-BSA into nuclei even after disruption of the cytoskeleton might be an artifact of the permeabilization procedure, we microinjected the reporter proteins NLS-BSA-TRITC or TRITC-BSA into intact fibroblasts. As Fig. 5A shows, TRITC-BSA remains completely cytosolic after microinjection. In contrast, the bright nuclear fluorescence in Fig. 5B indicates strong nuclear accumulation of NLS-TRITC-BSA in untreated cells. Furthermore, cells which were treated with cytochalasin B (Fig. 5C), vinblastine (Fig. 5D) or colchicine (Fig. 5E) prior to microinjection showed essentially the same behavior. These observations reveal that the reporter proteins TRITC-BSA and NLS-TRITC-BSA show the expected subcellular distributions not only in permeabilized cells but also when microinjected into intact cells, and that

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**Fig. 3.** Subcellular distribution of PKC α in cells after depolymerization of microtubules. After disabling the microtubule network with either 0.2 μM vinblastine (A,B), or 10 μM colchicine (C,D) for one hour, localization of PKC α was primarily cytoplasmic even in cells which were stimulated with 160 nM PMA for ten minutes (B,D). Treatment of cells with microtubule depolymerizing agents without subsequent stimulation (A,C) does not lead to any alterations of the subcellular distribution of PKC α (compare to Fig. 2A). Bar, 2.5 μm.

**Fig. 4.** Accumulation of reporter proteins in nuclei of permeabilized cells. Fibroblasts were permeabilized with digitonin in transport buffer and incubated with transport mix as described in Materials and Methods. (A) Phase contrast micrograph of permeabilized NIH 3T3 cells. Incubation with TRITC-BSA does not cause any nuclear fluorescence (B), whereas the bright fluorescence of nuclei in C,D,E,F indicates the nuclear accumulation of the reporter protein NLS-TRITC-BSA. Prior to transport experiment cells were treated with 2 μM cytochalasin B (D), 0.2 μM vinblastine (E) or 10 μM colchicine for one hour (F), or were left untreated (C). Bar, 5 μm.
proteins which do not have any canonical NLS but can be
oplasmin (Dingwall and Laskey, 1991). Furthermore there are
bipartite NLSs, which type is exemplified by the NLS of nucleo-
tate (Lenormand et al., 1993) and the PKC (Buchner, 1995). For
these proteins two possibilities are conceivable: either they
have an unknown type of NLS, i.e. a sequence that can bind to
a member of the nuclear import factor family, or they
interact with a carrier protein, which in turn interacts with
nuclear import factors or directly with the nuclear pore
complex (NPC) transport machinery. Which possibility applies
to a given protein remains to be determined for each case.

In the case of PKC, our results indicate that the cytoskele-
ton plays an important role for enabling binding to an NLS
receptor or a carrier protein. After activation, PKC may
associate with the cytoskeleton as a prerequisite to ‘meet’ the
appropriate binding partner for nuclear transport. Although in
the traditional view PKC translocates to membranes after activ-
ation, enhanced interaction with elements of the cytoskeleton
has been demonstrated (for review see Jaken, 1992). All major
components of the cytoskeleton, i.e. actin filaments, micro-
tubules and intermediate filaments, have been described as able
to act as docking sites for PKC (Jaken, 1992). Which
component is the decisive one for PKC α in 3T3 fibroblasts
cannot yet be discerned. It is known that disruption of one kind
of filament affects the integrity of the others. Disruption of
microtubules, for example, leads to a collapse of the inter-
mediate filaments into a ring around the nucleus or a tight knot
near the nucleus (Murti et al., 1992). However, these collapsed
structures around the nucleus do not block the nuclear import
of PKC sterically by blocking the nuclear pores, since NLS-
proteins can be translocated into the nucleus, and there are no
indications for PKC not being transported through the pore
complexes. Rather, our data indicate that the translocation of
PKC from the cytoplasm to the outside of the nuclear pore may
be different from that of the NLS-proteins. Our finding that the
nuclear translocation of an NLS-bearing protein is independent
of an intact cytoskeleton very probably not only applies to the
investigated reporter protein, but also to endogenous proteins.
Rosette and Karin (1995) found that depolymerisation of
microtubules leads to activation of NFκB. The authors do not
discuss the mechanism of NFκB’s nuclear translocation con-
comitant with the activation, but the order of events (the
maximum of NFκB-binding was observed 15 to 45 minutes
after microtubule disruption was detected; Rosette and Karin,
1995) implies that NFκB’s nuclear translocation is indepen-
dent of an intact microtubule network.

Since, as mentioned above, the disruption of one major
component of the cytoskeleton also affects the integrity of the
others, it is possible that by our treatment the filaments attached
to the cytoplasmic side of the nuclear pore complexes (Panté
and Aebi, 1994) were also compromised. The exact nature of
these filaments has not yet been clarified, but it was shown
recently that a NPC protein, Nup358, is attached to them
(Moore, 1995). Nup358 in its turn can bind Ran/TC4, which
is essential for nuclear protein import (Moore, 1995). It may
be that these filaments, in addition to binding Nup358 and
Ran/TC4, are necessary for bringing PKC toward the NPC.

DISCUSSION

Fig. 5. Distribution of fluorescent reporter proteins after cytosolic
microinjection. Microinjection of TRITC-BSA (A) or NLS-TRITC-
BSA (B,C,D) was performed as described in Materials and Methods.
Ten minutes after the injection, TRITC-BSA remained almost
completely cytosolic (A), whereas NLS-TRITC-BSA accumulated in
the nuclei of the intact cells, even when they were treated with 2 μM
cytoschalasin B (C), or 10 μM colchicine (D) for one hour prior to
microinjection. (B) Untreated control cells. Bar, 2.5 μm.

integrity of the cytoskeleton is not required for the nuclear
accumulation of NLS-bearing reporter proteins.

Nuclear uptake of PKC α into the nucleus of 3T3 fibroblasts requires an intact
cytoskeleton. This property of nuclear PKC transport is different from the nuclear import of an NLS-bearing reporter
protein which accumulated in the nucleus even after the
cytoskeleton was disrupted.

Nuclear uptake of NLS-bearing proteins is a two-step
process: a temperature- and energy-independent binding step
is followed by a translocation step which requires energy (for
a recent review see Melchior and Gerace, 1995). Whereas
rather little is known about the actual translocation of proteins
through the nuclear pore complexes, major progress has been
made in the past few years in understanding the first step. Several nuclear import factors have been identified from yeast,
Xenopus and mammals, which bind NLS-bearing proteins and
dock them onto the nuclear pore complexes (for review see
Sweet and Gerace, 1995). Although presently these proteins
comprise a whole family of homologous proteins, certainly not
all NLS receptors for all kinds of NLSs have been identified
yet.

Most studies are conducted with the NLS of the SV40 large-
T antigen, whereas very little is known about receptors for bipartite NLSs, which type is exemplified by the NLS of nucleo-
plasmin (Dingwall and Laskey, 1991). Furthermore there are
proteins which do not have any canonical NLS but can be
effectively transported into the nucleus under certain condi-
tions. Examples for such proteins are the MAP-kinase
(Lenormand et al., 1993) and the PKC (Buchner, 1995). For
these proteins two possibilities are conceivable: either they
have an unknown type of NLS, i.e. a sequence that can bind to
a member of the nuclear import factor family, or they
interact with a carrier protein, which in turn interacts with
nuclear import factors or directly with the nuclear pore
complex (NPC) transport machinery. Which possibility applies
to a given protein remains to be determined for each case.

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