Studies on protein kinases involved in regulation of nucleocytoplasmic mRNA transport

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The rate of energy-dependent nucleoside triphosphatase (NTPase)-mediated nucleocytoplasmic translocation of poly(A)-containing mRNA [poly(A)*mRNA] across the nuclear envelope is thought to be regulated by poly(A)-sensitive phosphorylation and dephosphorylation of nuclear-envelope protein. Studying the phosphorylation-related inhibition of the NTPase, we found that phosphorylation of one polypeptide of rat liver envelopes by endogenous NI- and NII-like protein kinase was particularly sensitive to poly(A). This polypeptide (106 kDa) was also phosphorylated by nuclear-envelope-bound Ca2+-activated and phospholipid-dependent protein kinase (protein kinase C). Activation of kinase C by tumour-promoting phorbol esters resulted in inhibition of nuclear-envelope NTPase activity and in a concomitant decrease of mRNA (actin) efflux rate from isolated rat liver nuclei. Protein kinase C, but not nuclear envelope NI-like or NII-like protein kinase, was found to be solubilized from the envelope by Triton X-100, whereas the presumable poly(A)-binding site [the 106 kDa polypeptide, representing the putative carrier for poly(A)*mRNA transport] remained bound to this structure. RNA efflux from detergent-treated nuclei lost its susceptibility to phorbol esters. Addition of purified protein kinase C to these nuclei restored the effects of the tumour promoters. Protein kinase C was found to bind also to isolated rat liver nuclear matrices in the absence but not in the presence of ATP. The NII-like nuclear-envelope protein kinase co-purified together with the 106 kDa polypeptide which specifically binds to poly(A) in an ATP-labile linkage.

INTRODUCTION

There is a large body of evidence suggesting gene expression in eukaryotic cells to be regulatable not only on the levels of transcription, post-transcriptional processing and translation but also on the level of transport of mRNA from nuclear compartment to cytoplasmic compartment across the nuclear envelope (for reviews, see Agutter, 1985; Schröder et al., 1987a). Factors controlling nucleocytoplasmic mRNA transport have been shown to include hormones (Schum & Webb, 1981; Bernd et al., 1983), lectins (Baglia & Maul, 1983; Kljačić et al., 1987), mRNA-transport stimulatory proteins (Moffett & Webb, 1981; Schröder et al., 1986c), ageing (Bernd et al., 1983; Schröder et al., 1986a), nutrition (Murty et al., 1977) and cancer (Shearer, 1974; Clawson et al., 1980). These factors were found to influence mRNA transport by interaction either with the release of the mature mRNA from the nuclear matrix (Clawson & Smucker, 1982; Schröder et al., 1987b), or the translocation apparatus in the nuclear envelope (Schröder et al., 1986c, 1987a). The energy-producing nuclear-envelope constituent for ATP-dependent efflux of poly(A)-containing mRNA [poly(A)*mRNA] from nuclei in vitro was found to be a nuclear envelope NTPase (Agutter et al., 1976; Schröder et al., 1987a). This enzyme has been purified to homogeneity (Schröder et al., 1986b). Evidence for the involvement of the NTPase in mRNA transport are as follows: (1) stimulation of the enzyme in whole envelopes by poly(A) or poly(A)*mRNA (Bernd et al., 1982a); (2) the same kinetics, substrate specificity, metal requirement and sensitivity to inhibitors (Agutter et al., 1976; Schröder et al., 1987a); (3) the same alterations during development and hormone treatment (Bernd et al., 1983; Schröder et al., 1986a); (4) parallelism between NTPase activity and transport rate after addition of transport stimulatory proteins (Schröder et al., 1986a,c); and (5) inhibition by antibodies decreasing RNA transport in vitro (Baglia & Maul, 1983) (however, both events can be uncoupled by other antibodies; see Schröder et al., 1988). Some of these effects can be explained by a nuclear-envelope protein kinase-dependent phosphorylation that down-regulates the NTPase (McDonald & Agutter, 1980; Bachmann et al., 1984; Agutter, 1985; Schröder et al., 1986c), and by the action of a nuclear-envelope-associated phosphoprotein phosphatase (Steer et al., 1979a; Bachmann et al., 1984; Schröder et al., 1986c). These interactions do not occur by phosphorylation of the NTPase itself as shown for both the purified

Poly nucleotides and nucleotides are abbreviated according to the recommendations of the Commission on Biochemical Nomenclature. Abbreviations used: poly(A)*mRNA, poly(A)-containing mRNA; NTP, nucleoside triphosphate; protein kinase C, Ca2+-activated and phospholipid-dependent protein kinase; protein kinases NI and NII, nuclear protein kinases types I and II in the nomenclature of Desjardins, Lue, Liew & Gornall (1972) Can. J. Biochem. 50, 1249–1258; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol-12,13-dibutyrate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulphonyl fluoride.

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(Schröder et al., 1986b) and the envelope-bound enzyme (Clawson et al., 1984) but by phosphorylation of a further nuclear-envelope component not yet identified. This component might be related to the mRNA-binding site within the envelope [the putative poly(A)*mRNA carrier; see Bachmann et al., 1984; Agutter, 1985].

In the present report we established both an NII-like nuclear-envelope protein kinase and Ca**+**-activated and phospholipid-dependent protein kinase (protein kinase C; for reviews, see Nishizuka, 1984, 1986) to be involved in regulating NTPase activity. In addition, the phosphorylatable polypeptide representing the poly(A)-recognizing mRNA-binding site in the envelope was identified and partially purified.

Protein kinase C has been shown to translocate between the cytoplasm and the plasma membrane and to be dependent on Ca**+** concentration (Wolf et al., 1985b; Bell, 1986). The enzyme is activated by diacylglycerol generated from breakdown of membrane phosphoinositides (Berridge & Irvine, 1984; Berridge, 1986) or by tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) or phorbol-12,13-dibutyrate (PDBu) (Nishizuka, 1984; Bell, 1986). Here we demonstrate that protein kinase C can reversibly bind to the envelope nuclear and nuclear matrix. Binding of protein kinase C resulted in inhibition of ATP-dependent efflux of mRNA from isolated nuclei, a decrease of NTPase activity and an increase of poly(A)*mRNA binding to isolated envelopes.

EXPERIMENTAL

Materials

The materials were obtained as follows: [*γ*-*32P*]ATP (sp. activity, 3 Ci/mm), [*γ*-*35S*]GTP (10 Ci/mm), [*α*-*32P*]dTPP (3000 Ci/mm), [*32P*]phosphate (200 mCi/mm), [*H*]ATP (11 Ci/mm), [*H*]poly(A) (600 mCi/mm of nucleoside residue), [*H*]poly(U) (600 mCi/mm), [*H*]myo-inositol (71 Ci/mm), [20(n)-*H*]-phorbol-12,13-dibutyrate (3H-PDBu; 20 Ci/mm), phosphatidyleholine, phosphatidylinositol (PI) and its derivatives were from Amersham International (Amersham, Buckinghamshire, U.K.); [*H*]poly(A) [37.4 mCi/mm of phosphate; with an average Mr of 250000, as determined by Studier (1965) from Miles Laboratories (Slough, Berks., U.K.)]; poly(A) (single-stranded, with an average Mr of 248000), poly(U), DNA polymerase I (Escherichia coli; 11388 units/mg), DNAase I (bovine pancreas, grade I, 2000 units/mg), RNAase A (bovine pancreas; 50 Kunitz units/mg), RNAase T1, (Aspergillus oryzae; 280000 Egami units/mg), RNasin, unlabelled DNAs and RNAs from Boehringer (Mannheim, Germany); casein, phosvitin, histone III-S, diolein, O-phospho-d1-serine, PMA and yeast RNA (RNA content greater than 97%) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); poly(A)-Sepharose 4B and DEAE-Sepharose (Fast Flow) from Deutsche Pharmacia (Freiburg, Germany); oligo(dT)-cellulose from Collaborative Research (Waltham, MA, U.S.A.); phenylmethane-sulphonyl fluoride (PMSF) and Triton X-100 from E. Merck (Darmstadt, Germany); nitrocellulose sheets (BA85) from Schleicher & Schuell (Dassel, Germany); Kodak X-Omat XAR-5 X-ray film from Eastman Kodak (Rochester, NY, U.S.A.).

Preparation of nuclei, nuclear envelopes, pore-complex laminae and nuclear matrices

Nuclei were isolated from liver of male Wistar rats (2–3 months old) as described by Blobel & Potter (1966), except that 5 mm-2-mercaptoethanol and 1 mm-PMSF were added to all the buffers used. The nuclear envelopes were prepared by the method of Kaufmann et al. (1983). Pore-complex laminae were obtained from the envelopes by treatment with 2% Triton X-100 for 10 min at 0°C in buffer A [50 mm-Tris/HCl (pH 7.4)/5 mm-MgCl2/5 mm-2-mercaptoethanol/1 mm-PMSF]. The nuclear envelopes and the pore-complex laminae were stored at −70°C in buffer A supplemented with 2.1 m-sucrose. Nuclear matrices were prepared from the isolated nuclei as described by Comerford et al. (1985) and were stored at −20°C in 50 mm-Tris/HCl (pH 7.4)/5 mm-MgCl2/1 mm-EGTA/250 mm-sucrose/1 mm-PMSF. The nuclear-matrix preparations obtained by this method showed an elaborate fibrous network when viewed in electron micrographs (Figs. 1a and 1b), while the pore-complex laminae were merely composed of the peripheral lamina layer interconnecting the pore complexes (Fig. 2b). In some experiments, demembranated nuclei were used, which were obtained by treatment of nuclei with 1% Triton X-100 in ATP-free transport medium (see below) for 10 min at 4°C and found electron microscopically to be obviously membrane-free (Fig. 2a).

**RNA efflux measurements in vitro**

Isolated rat liver nuclei were used for measurement of the ATP-dependent efflux of a specific mRNA (actin).

**Fig. 1.** Electron micrographs of rat liver nuclear matrix

(a) Bar = 3 μm; (b) bar = 0.1 μm.
Nuclei were isolated as described above and were incubated for 0–60 min at 30 °C in transport medium [25 mM-Tris/HCl (pH 7.6)/250 mM-d-glucose/2 mM-MgCl₂/0.5 mM-CaCl₂/0.3 mM-MnCl₂/5 mM-spermidine/5 mM-2-mercaptoethanol, and yeast RNA (300 µg/ml) or 10³ RNasin (units/ml)] supplemented with 2 mM-EDTA (for controls) or 2 mM-ATP plus an ATP-regenerating system [pyruvate kinase (35 units/ml), 5 mM-phosphoenolpyruvate, and 5 mM-Na₂HPO₄] (Schröder et al., 1986a). Transport of actin mRNA was measured by dot-blot hybridization of RNA in the efflux supernatant (see below). The efficiency of the additives used to suppress ribonuclease activity in the postnuclear supernatant was checked by addition of [³H]poly(A) (4 × 10⁶ d.p.m./ml) or [³H]poly(U) (4 × 10⁶ d.p.m./ml). After incubation for different time periods, portions were taken and analysed by gel chromatography on Sephadex G-50, as described previously (Schröder et al., 1980). After incubation for 1 h, > 90% of these polynucleotides were found to be undegraded.

Northern- and dot-blot hybridization

The actin DNA probe used was prepared from the plasmid p41, which contains the mouse β-actin coding sequence cloned into the PstI site of pBR322 (Alonso et al., 1986). This clone was a kind gift from Dr M. Buckingham (Institut Pasteur, Département de Biologie Moleculaire, Paris, France). The actin-specific probe, excised with PstI from the p41, was labelled with [α-³²P]dATP by nick translation (Rigby et al., 1977) to a specific activity of 9.2 × 10⁹ c.p.m./µg of DNA. Electrophoresis of the denatured RNA samples on agarose gels, blot transfer to nitrocellulose and hybridization to the ³²P-labelled, actin-specific probe was performed as described previously (Messer et al., 1986). The dot hybridization assay was a modification (Kindas-Mügge & Sauermann, 1985) of the method of White & Bancroft (1982). The RNA in the postnuclear supernatant was denatured by treatment with 7.5% formaldehyde in 6 × SSC (Maniatis et al., 1982) at 56 °C for 20 min. The RNA samples were then adjusted to 13 × SSC and spotted onto nitrocellulose sheets. Hybridization of the baked sheets with the ³²P-labelled probe was performed as described by Maniatis et al. (1982). The dried filters were exposed to Kodak XAR-5 film with one intensifying screen at -70 °C for 12 to 48 h.

Binding of purified protein kinase C to isolated nuclei, nuclear envelopes and nuclear matrices

Rat liver nuclei, nuclear envelopes or nuclear matrices were suspended in buffer A supplemented with 250 mM-sucrose at a final concentration of 3.5 × 10⁷ nuclei/ml, 0.19 mg of nuclear envelope protein/ml, or 0.34 mg of matrix protein/ml. After addition of 2.3 units of purified protein kinase C/ml from rat brain the suspensions were incubated for 15 min at 22 °C in the presence of 100 µM-CaCl₂, phosphatidylserine (PS) (20 µg/ml), and PMA (0.02 µg/ml). The nuclei, nuclear envelopes, and nuclear matrices were then pelleted by centrifugation (11 000 g, 10 min, 4 °C), washed with buffer A and bound protein kinase C activity was determined. Portions of pelleted nuclei, nuclear envelopes, or nuclear matrices were treated with 1 mM-EGTA or 1% Triton X-100 in buffer A (5 min, 22 °C) and solubilized protein kinase C activity was measured.

Binding of PDBu

Nuclei (6 × 10⁷/ml), nuclear envelopes (0.12 mg of protein/ml), or nuclear matrices (0.31 mg of protein/ml) were incubated in the presence of 10 nM [³H]PDBu (4.4 × 10⁶ d.p.m./nmol) at 22 °C for 20 min in binding mixture (final vol. 100 µl) consisting of 25 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/50 µM-CaCl₂/PS (50 µg/ml)/bovine serum albumin (100 µg/ml). Reaction mixtures were then sucked through Whatman GF/C glass filters and filter-bound radioactivity was measured (Wolf et al., 1985a).

Binding of poly(A) to pore-complex laminae

The poly(A) binding studies were performed essentially as described previously (McDonald & Agutter, 1980; Schröder et al., 1986c) with the following modifications. Rat liver nuclear envelopes were phosphorylated for 5 min at 30 °C by endogenous kinase C with unlabelled ATP as outlined under Protein kinase C assay. In controls, Ca²⁺ and PS were omitted, and 1 mM-EGTA was added to the reaction mixtures. Pore-complex laminae were prepared by treatment of the envelopes with 2% Triton X-100 at 0 °C for 10 min (Aaronson & Blobel, 1975). After centrifugation at 15 000 g for 20 min, the pore-complex laminae were washed twice with binding buffer [25 mM-Tris/HCl (pH 7.6)/1 mM-MgCl₂/0.5 mM-2-mercaptoethanol]. Incubation of the pore-complex laminae with various concentrations of [³H]poly(A) in binding buffer was performed for 10 min at 22 °C. The suspension was then applied on a 30% sucrose cushion in binding buffer and centrifuged at 50 000 g for 15 min at 6 °C in an A 100/30 rotor of a Beckman Airfuge. The amounts of bound and unbound poly(A) were determined by counting the radioactivity in the pellet and in the upper phase.
Solubilization of the nuclear-envelope poly(A) binding site

The envelopes were washed in buffer B [10 mM-Hepes (pH 7.5)/250 mM-sucrose/4 mM-mercaptoethanol] and then suspended in the 3.5-fold volume of buffer A supplemented with 1% Triton X-100. The suspension was incubated with moderate shaking for 10 min at 22°C and the resulting pore-complex laminae, were collected by centrifugation (1600 g, 20 min, 4°C). The pellet was then suspended in 25 mM-Tris/HCl buffer (pH 8.0) containing 5 mM-urea, 2.5 mM-MgCl₂, 150 mM-NaCl, 5 mM-2-mercaptoethanol and 1 mM-PMPSF by repeated suction through a Pasteur pipette. The suspension was incubated for 20 min at 22°C under vigorous shaking, and then centrifuged (11000 g, 4°C, 10 min). During this procedure, the poly(A) binding site within the envelopes was found to be solubilized (Agutter, 1985).

Extraction of nuclear envelopes with Triton X-114

Nuclear envelopes were extracted with Triton X-114 according to the method of Bordier (1981), with the following two modifications (Glenney & Glenney, 1984; Tiruppathi et al., 1986). (1) The envelopes were suspended at a protein concentration of 0.9 mg/ml in 500 μl of buffer C [20 mM-Tris/HCl (pH 7.4)/140 mM-NaCl/1 mM-PMPSF] containing 1% precondensed (Bordier, 1981) Triton X-114. After incubating for 10 min at 4°C under moderate shaking, insoluble material was removed by centrifugation (11000 g, 10 min, 4°C) and the supernatant was applied onto a 300 μl cushion of 6% sucrose in buffer C containing 0.6% Triton X-114. Before centrifugation clodung was allowed to occur by incubating the tube for 5 min at 32°C. After separation at 3000 g for 10 min at 22°C, the upper aqueous phase was removed, mixed with 0.5% Triton X-114 at 0°C, and then centrifuged again, after clodung, through a sucrose cushion. This operation was repeated once. Finally the aqueous phase was washed with 2% Triton X-114. The lower detergent phase was rinsed with buffer C without Triton X-114. (2) The envelopes were extracted first with 1% Triton X-100 in 20 mM-Tris/HCl (pH 7.5)/0.2 mM-dithiothreitol/1 mM-PMPSF. After centrifugation (11000 g, 10 min, 4°C) the pellet was resuspended and washed in the same buffer containing 0.25% Triton X-100 and 0.05% SDS. The pellet was then subjected to a second extraction with 0.25% Triton X-114, 0.05% SDS and 10 mM-ATP in the Tris/HCl buffer for 10 min at 4°C. For better solubilizing, the suspension was sonicated for 2 x 15 s at 50 W (Branson Sonifier model B-12). After centrifugation (11000 g, 10 min, 4°C) the supernatant was supplemented with 1% Triton X-114 at 0°C and subsequent phase separation was performed as described above.

Purification of nuclear envelope-associated NI-like and NII-like protein kinases

Nuclear envelopes were treated with 1% Triton X-100 in 10 mM-Hepes (pH 7.5)/250 mM-sucrose/4 mM-2-mercaptoethanol/1 mM-PMPSF for 10 min at 22°C. After centrifugation, the resulting pellet was extracted for 20 min at 22°C with 5 mM-urea buffer D [25 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂/0.1 mM-EDTA/50 mM-NaCl/5 mM-2-mercaptoethanol/1 mM-PMPSF/25% (v/v) glycerol]. Endogenous NI-like and NII-like protein kinase activities were separated by chromatography on a DEAE-Sepharose column (Yutani et al., 1982) that had been equilibrated with buffer D containing 5 mM-urea. During elution with equilibration buffer, protein kinase NI appeared in the flow-through fraction. Protein kinase NII was eluted in the presence of 0.5 mM-NaCl. Fractions containing protein kinase NII activity were pooled and dialysed overnight against buffer D supplemented with 1% Triton X-100, and then applied to a 2 ml column of poly(A)–Sepharose 4B equilibrated with the same solution. Elution was performed with 1 mM-NaCl. Final purification was achieved by binding to a Sepharose 4B sebacic acid hydrazide β,γ-methylene ATP column (1.2 ml; for preparation see Schröder et al. 1986b) and elution with equilibration buffer (buffer D plus 1% Triton X-100) in the presence of 1 mM-NaCl.

Enzyme preparations

Protein kinase C was purified to apparent homogeneity from rat brain by the procedure described by Wolf et al. (1985a). The human erythrocyte inside-out vesicles used during this procedure were prepared as described by Steck & Kant (1974). Final purification was achieved by phenyl-Sepharose chromatography (Wolf et al., 1985a).

Nuclear-envelope NTPase activity was purified to homogeneity by the method of Schröder et al. (1986b).

Protein kinase NI and NII assay

Endogenous NI- and NII-like protein kinase activity was measured essentially as previously described (Bachmann et al., 1984) in 100 μl reaction mixtures containing 25 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂/150 mM-NaCl/10 mM-NaF/10 mM-O-phospho-DL-serine/1 mM-2-mercaptoethanol/40 μM-[γ-32P]ATP (2 x 10⁵ d.p.m./pmol), and 50 μg of envelope protein. In some experiments, reactions were performed in the presence of [γ-32P]GTP (2 x 10⁵ d.p.m./pmol) instead of ATP. In this case, only protein kinase NII activity is measured, which utilizes both ATP and GTP (Thornburg et al., 1979). The activity of protein kinase NI which only effectively uses ATP was estimated by subtraction of the rate of incorporation of phosphate in the presence of GTP from that determined with ATP as substrate. To determine the phosphorylation of exogenous substrate, the reaction mixture was supplemented with casein (2.5 mg/ml) (NI-type activity) or phosvitin (2.5 μg/ml) (NII-type activity). Incubations were performed for 0–30 min at 22°C, and the trichloroacetic acid-precipitable radioactivity incorporated into protein was determined.

Protein kinase C assay

The mixture to measure protein kinase C activity consisted, in a final volume of 150 μl, of 25 mM-Tris/HCl (pH 7.5)/1 mM-MgCl₂/0.5 mM-CaCl₂/PS (50 μg/ml)/diolein (0.5 μg/ml)/40 μM-[γ-32P]ATP (2 x 10⁵ d.p.m./pmol) with or without histone type III-S (1 mg/ml) as exogenous substrate. Control assays were performed under the same conditions but in the presence of 1 mM-EGTA and in the absence of CaCl₂ and phospholipid. After incubating for 5 min at 30°C, the incorporation of 32P into protein was determined as described above.

Other enzyme assays

Nuclear-envelope NTPase activity and phospho-protein phosphatase activity were determined essentially
as described previously (Schröder et al., 1986b). In some experiments the NTPase activity was measured in the presence of 20 μM-poly(A); at this concentration the NTPase was found to be stimulated about half-maximally (Agutter et al., 1977).

Radiolabelling and identification of phospholipids

L5178y cells were incubated for 1 h at 37 °C with [32P]phosphate (50 μCi/ml) and the nuclear envelopes were isolated as described above. The nuclear-envelope phospholipids were extracted essentially as described by Agranoff et al. (1983). In brief, the sample was mixed with 0.5 ml of ice-cold methanol, followed by addition of 0.25 ml of 2.4 M-HCl and 0.75 ml of chloroform. After mixing, phase separation by centrifugation, re-extraction of the resulting aqueous phase with chloroform again, and extraction of the chloroform phases with 0.25 ml of 2.4 M-HCl and 2 ml of methanol/water (1:1, v/v), the final chloroform phases were collected and the solvent was removed by evaporation. Separation of the obtained phospholipids was performed by t.l.c. using the following two systems (Carney et al., 1985): (1) silica-gel 60 plates (Merck, Darmstadt, Germany) which were developed in chloroform/methanol/acetic acid/water (65:43:1:3, by vol.); and (2) oxalate-impregnated silica-gel 60 plates which were developed in chloroform/methanol/4 M-NH4OH (9:7:2, by vol.) (Gonzales-Sastre & Folch-Pi, 1968). The separated radiolabelled lipids were detected by autoradiography, using the authentic compounds as references.

To determine the generation of inositol phosphates, under conditions applied during RNA efflux experiments, L5178y cells (4.4 × 10⁶ cells/ml) were incubated for 24 h with 20 μCi of [3H]inositol/ml. Subsequently nuclei were isolated, as described by Blobel & Potter (1966), and incubated for 20 min at 30 °C in transport medium containing 2 mM-ATP and an ATP-regenerating system (see above). The reaction was stopped by addition of ice-cold 10% trichloroacetic acid. The trichloroacetic acid was removed by extraction with ethyl ether and soluble inositol phosphates in the aqueous phase were identified and quantified by binding to a 3 ml Dowex 1×8-50 (Sigma) column and stepwise elution with ammonium formate, as described previously (Vincentini & Villereal, 1984; Carney et al., 1985).

Analytical methods

Gel electrophoresis was performed in 10% polyacrylamide slab gels in the presence of 0.1% SDS, as described by Laemmli (1970). To determine incorporation of [32P]phosphate into nuclear-envelope polypeptides, the envelopes were incubated for 5 min at 30 °C with [γ-32P]ATP under the conditions used in the protein kinase NII or protein kinase C assay. The reactions were terminated by addition of ice-cold 15% trichloroacetic acid. The pelleted proteins were boiled in SDS sample buffer (95 °C, 10 min) and then subjected to electrophoresis as described above. Autoradiography of the dried gels was for 2 days at −70 °C. In parallel experiments, the denatured protein samples were electrophoresed in cylindrical SDS/10% polyacrylamide gels. After each run, gels were sliced into 2 mm sections using a Gilson gel slicer (no. 37182), and counted for radioactivity. Protein was measured as described by Lowry et al. (1951) using bovine serum albumin as standard.

Electron microscopy

For electron microscopy, nuclear-matrix pellets were fixed in 2% (v/v) glutaraldehyde in 0.1 M-phosphate (pH 7.0) for 45 min at 22 °C. Postfixation was performed in 2% (w/v) OsO4 in phosphate buffer. After dehydration in ethanol and propylene oxide, the specimens were embedded in Araldite and sliced. Sections were stained with uranyl acetate and lead citrate, and studied with a Zeiss EM 9A electron microscope.

RESULTS

Nuclear-envelope poly(A)-binding site

From Scatchard analyses it is known that pore-complex laminae contain apparently only one class of mRNA binding sites with a high affinity for poly(A) (McDonald & Agutter, 1980; Schröder et al., 1986c). The dissociation constant (Kd) of this site for poly(A) was found to be 0.21 μM (with respect to phosphate content) (Fig. 3). Phosphorylation of nuclear envelopes under conditions optimal for endogenous NII- and NII-like kinase activities has been shown to result in a significant increase in the poly(A) binding affinity without altering the total number of poly(A) binding sites (McDonald & Agutter, 1980; Schröder et al., 1986c). In Fig. 3 it is shown that phosphorylation of nuclear envelopes by endogenous protein kinase C also resulted in a significant decrease of the dissociation constant for the complex formation of the corresponding

![Fig. 3. Enhancement of poly(A)-binding complex laminae by protein kinase C-dependent phosphorylation](image-url)

Nuclear envelopes were phosphorylated by endogenous protein kinase C for 10 min at 22 °C and pore-complex laminae were prepared from the envelopes by Triton treatment as described in the text, but in the presence of 10 mM-NaF and 10 mM-O-phospho-DL-serine. Binding of [3H]poly(A) to phosphorylated pore-complex laminae (●) and to unphosphorylated pore-complex laminae (○; prepared from envelopes that had been incubated with protein kinase C reaction mixture in the presence of 1 mM-EGTA and no PS). Results were evaluated according to Scatchard and are the means of two experiments, each done in triplicate.
phosphorylated pore-complex lamina sites with poly(A) ($K_d$ 0.09 $\mu$M). The total number of poly(A)
binding sites was not changed by phosphorylation and was approx. 2.4 nmol of poly(A) phosphate/mg of pore-
complex lamina protein.

When pore-complex laminae were treated with 5 M
urea, all poly(A) binding sites were found to be lost
(results not shown), consistent with the results of Agutter
(1985); the resulting supernatant was used for purification
of the poly(A) binding site, presumably representing
the postulated poly(A) recognizing carrier for mRNA
transport (Bachmann et al., 1984; Agutter, 1985;
Schröder et al., 1986c), and of nuclear-envelope-
associated cAMP-independent protein kinases (NI- and
NII-like; see below).

Properties of nuclear-envelope protein kinases

The nuclear-envelope of nuclei from rat liver cells
(Lam & Kasper, 1979; Steer et al., 1979b, 1980;
McDonald & Agutter, 1980; Smith & Wells, 1983a;
Schröder et al., 1986a,c) or other tissues (Bachmann et al.,
1984; Schröder et al., 1986a) contains a considerable
amount of protein kinase activity assumed to be involved
in important functions of this organelle, e.g. RNA
binding and translocation (McDonald & Agutter, 1980;
Bachmann et al., 1984; Schröder et al., 1986a,c). The
specific endogenous protein kinase activity, measured
under protein kinase NI/NII conditions (see under
Experimental), in nuclear envelopes prepared according
to the procedure described by Kaufmann et al. (1983)
was 0.035 nmol of phosphate incorporated/min per mg of
nuclear-envelope protein or, with casein as exogenous
substrate, 1.16 nmol/min per mg. Because phosphoryl-
ation of endogenous nuclear-envelope proteins seems not
to be catalysed by a single type of kinase (Smith & Wells,
1983a; Schröder et al., 1988) we tried to separate these
enzymes by differential extraction and chromatographic
procedures.

Protein kinase NI- and NII-like activities were
purified from rat liver as described in the Experimental
section. After extraction with 1% Triton X-100, most of
the nuclear envelope-associated NI- and NII-like protein
kinase activity (distinguished by their different substrate
specificity) remained bound to the resulting pore-complex
lamina pellet (86% and 98%, respectively). However,
both protein kinases were found to be dissociated from
the pore-complex laminae in the presence of 5 M-urea.

The protein kinase activity appearing in the flow-
through of the DEAE-Sephadex column exhibited similar
properties to the NI-type protein kinase described in the
literature (Yutani et al., 1982; Delpech et al., 1986),
such as specificity for ATP (but not GTP), higher
phosphorylation of $\alpha$-casein and phosphoehistone than
of histone, optimal activity at 10 mM-MgCl$_2$ and pH 7–8,
no sensitivity to spermene, heparin or poly(A), and no
dependence on cAMP or cGMP (1 or 10 $\mu$M).

Properties of the protein kinase that was bound to the
DEAE column were similar to those reported for protein

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**Fig. 4. Autoradiographs showing the pattern of phosphorylated nuclear-envelope polypeptides after phosphorylation with the endogenous protein kinases NI and NII (a) or with the endogenous Ca$^{2+}$- and phospholipid-dependent protein kinase (protein kinase C; b)**

(a) Autophosphorylation of nuclear envelopes under conditions used to measure protein kinases NI and NII (see the Experimental section) was performed in the absence (lanes a and b) and in the presence (lanes c and d) of 65 $\mu$M-poly(A) (5 min;
22 °C). Lanes a and c, 50 $\mu$g, and lanes b and d, 10 $\mu$g, of phosphorylated envelope protein were subjected to electrophoresis in SDS/polyacrylamide (10 %) slab gels. Relative molecular masses are given on the left side. (b) Phosphorylation of nuclear envelope protein (50 $\mu$g) by endogenous protein kinase C was done both in the presence (lane a) and in the absence (lane b) of
1 mM-EGTA (5 min; 22 °C). The polypeptide band of the putative "carrier" protein (p106) is indicated at the right side.
Protein kinases involved in mRNA transport

Fig. 5. Inhibition of incorporation of $^3$P into nuclear-envelope polypeptides by poly(A), determined in sectioned cylindrical SDS/polyacrylamide (10%) gels

Phosphorylation of nuclear envelopes by endogenous protein kinase NI and NII in the absence (open columns) or presence (shaded columns) of 65 $\mu$M-poly(A); electrophoresis and sectioning of gels were performed as described in the Experimental section. The molecular masses (in kDa) of the main phosphorylated bands are indicated.

kinase NII (Thornburg et al., 1979; Yutani et al., 1982). The purified enzyme was optimally active at a pH value of 8.0 and at an Mg$^{2+}$ concentration of 10 mm. It utilized both ATP and GTP with a similar efficacy. The $K_m$ for ATP with phosphovitin (preferred substrate) was 19 $\mu$M and the $K_m$ for GTP, 22 $\mu$M. The purified enzyme was strongly inhibited by poly(A) (to 61% of that of control at a concentration of 65 $\mu$M) and by heparin (29% at a concentration of 1 $\mu$g/ml), and was stimulated by spermine (to 216% at a concentration of 2 mM). Neither cAMP nor cGMP (at final concentrations of 1 and 10 $\mu$M) showed any effect on the phosphorylation of exogenous phosphovitin (by the purified enzyme) and endogenous nuclear-envelope proteins (non-purified enzyme).

Sensitivity of phosphorylation of nuclear-envelope polypeptides to poly(A)

As shown in Fig. 4(a), nine major nuclear-envelope proteins with relative molecular masses of 135, 106, 90, 64, 55, 47, 40, 38 and 25 kDa were found to be phosphorylated by the endogenous protein kinase NI/NII-like activity. To quantify the inhibition of phosphorylation by poly(A), the $^3$P incorporated into size-separated envelope polypeptides was counted in sections of sliced SDS gels (Fig. 5). In the presence of 65 $\mu$M-poly(A) with an incubation period of 5 min, protein kinase NI/NII-dependent phosphorylation of total nuclear-envelope protein was inhibited by approx. 15% (Fig. 6). As shown in Fig. 4(a), lanes c and d, and Fig. 5, the phosphorylation of several nuclear-envelope polypeptides was slightly decreased in the presence of poly(A). However, the phosphorylation of one protein showed a pronounced sensitivity against poly(A); the phosphorylation of this protein with a polypeptide $M_c$ of 106000 (p106) was strongly inhibited (by about 50%) in the presence of 65 $\mu$M-poly(A) (Fig. 4(a), lanes c and d). When [$\gamma$-$^3$P]ATP was substituted by [$\gamma$-$^3$P]GTP, a similar phosphorylation pattern was found (results not shown). Under these conditions, only NII-like protein kinase activity is measured because protein kinase NI or C cannot utilize this nucleoside triphosphate (Thornburg et al., 1979; Yutani et al., 1982). The incorporation of $^3$P in the presence of GTP was 37% lower than that in the presence of ATP (Fig. 6); however, the relative phosphorylation of p106 was 2-fold higher than it was with ATP. In addition, two further proteins with 49 and 52 kDa molecular masses were detected, which were phosphorylated only in the presence of GTP (results not shown).

Autophosphorylation of nuclear envelopes under conditions used for measuring protein kinase C activity resulted in labelling of five major phosphoprotein bands with 135, 106, 47, 40 and 25 kDa (Fig. 4b, lane b). Interestingly, no phosphorylation of p106 could be detected after incubation of the envelopes in the presence of 1 mM-EGTA and the absence of Ca$^{2+}$ and PS (Fig. 4b, lane a). After addition of purified protein kinase C to the nuclear envelopes, an increased incorporation of $^3$P into p106 was obtained (see Rottmann et al., 1987). Therefore we conclude that p106 is indeed a substrate for protein kinase C. There are no hints for the presence of a calmodulin-dependent protein kinase in the nuclear envelope (see Smith & Wells, 1983a); these results were confirmed by using highly purified envelopes obtained by the procedure of Kaufmann et al. (1983) (results not shown). In addition, it could be demonstrated that the p106 becomes photolabelled with azido$^3$P]ATP (Schröder et al., unpublished work); this result is consistent with the model proposed by Schröder et al. (1987a).

Purification of the putative carrier p106

The poly(A)-sensitive phosphorylatable polypeptide p106 was purified from rat liver nuclear envelopes without or with prior phosphorylation of the envelopes by endogenous NI- and NII-like protein kinases. In the
latter case a higher yield of p106 was achieved. Nuclear envelopes were phosphorylated for 10 min at 22 °C as described in the Experimental section, except that unlabelled ATP was used. Porcine complex laminae were obtained after 1% Triton X-100 treatment and extracted with 5 mM-urea in 25 mM-Tris/HCl (pH 8.0)/2.5 mM-MgCl₂/150 mM-NaCl/5 mM-2-mercaptoethanol/1 mM-PMSF. The extract was dialysed against equilibration buffer [25 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂/50 mM-NaCl/15 mM-2-mercaptoethanol/1 mM-PMSF] supplemented with 100 μg of poly(C)/ml, and then applied on a 2 ml poly(A)-Sepharose column equilibrated with this buffer plus 100 μg of poly(C)/ml. Elution was performed, first with 1 M-NaCl in equilibration buffer plus poly(C) and second, with equilibration buffer containing 20 mM-ATP and 1 M-NaCl. As shown in Fig. 7 (lane a) three polypeptides could be detected in SDS gels of the last eluate fraction; their molecular masses were 106, 64, and 55 kDa. In some experiments performed under otherwise identical conditions, or after storage of the eluate fraction, only the 64 kDa and the 55 kDa polypeptides were found, but the p106 was not (Fig. 7, lane b). Therefore we conclude that p64 and p55 represent degradation products of p106. After dialysis against equilibration buffer, the poly(A) binding nuclear-envelope protein fraction (ATP eluate) was further purified on a Sepharose 4B sebacic acid hydrazide β,γ-methylene ATP column, equilibrated and eluted with the same buffer. All of the three proteins (p106, p64 and p55) were found to bind to this column and were eluted in the presence of 1 M-NaCl (results not shown).

After phosphorylation with [γ-32P]ATP and treatment of the envelopes with Triton X-100, p106 and, in addition, p64 and p55 which possibly are degradation products of p106 (see above) could not be detected among the solubilized proteins in SDS gels by autoradiographical procedures (not shown). This result is compatible with our previous findings and the results of McDonald & Agutter (1980) showing that the poly(A) binding site in the nuclear envelope is not solubilized by this detergent. Interestingly, the nuclear-envelope-associated protein kinase NII was found to co-purify with the putative carrier protein p106. The binding of this protein kinase to poly(A) also showed some specificity because only 7% of the total poly(A) binding NII-like activity was eluted in the presence of poly(C). Addition of 20 mM-MgATP did not result in dissociation of the poly(A)-bound protein kinase. After only addition of 1 M-NaCl plus 20 mM-MgATP was protein kinase NII eluted, like p106, from the column by ATP.

Modulation of RNA transport by nuclear-envelope-associated protein kinases

As shown in Table 1, the NTPase in rat liver envelopes, responsible for ATP-dependent translocation of poly(A) mRNA, was found to be significantly inhibited by the phorbol esters PDBu and PMA (0.02 μg/ml each) in the presence of 100 μM-Ca²⁺ and 10 μg of PS/ml. In the absence of Ca²⁺ and PS both phorbol esters displayed a lower efficacy. After addition of PS alone, but not of Ca²⁺, PMA was 45% as effective as with both additives. Diblein could partially substitute for PMA (by 34%) in inhibiting NTPase activity. The inactive 4α-phorbol (which lacks any tumour-promoting activity) exhibited no effect on the enzyme. The purified, homogeneous NTPase was not affected by any of these agents (Table 1).

The involvement of nuclear-envelope-associated protein kinase C in regulating nucleocytoplasmic mRNA transport was studied by using a transport system

![Fig. 7. Purified poly(A)-binding proteins from rat liver porcine complex laminae](image)

Coomassie Blue-stained gels of the ATP eluate of the poly(A)-Sepharose column, lane a, and of the same fraction after storage for 12 h at 4 °C, lane b, are shown. Relative molecular masses are given on the left side.

### Table 1. Influence of phorbol esters and diolein on nuclear-envelope NTPase activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nuclear-envelope-associated enzyme (%)</th>
<th>Purified enzyme (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺, PS</td>
<td>101.3</td>
<td>102.0</td>
</tr>
<tr>
<td>PMA</td>
<td>86.9</td>
<td>99.1</td>
</tr>
<tr>
<td>PMA, Ca²⁺</td>
<td>74.4</td>
<td>99.9</td>
</tr>
<tr>
<td>PMA, PS</td>
<td>85.4</td>
<td>100.3</td>
</tr>
<tr>
<td>PMA, Ca²⁺, PS</td>
<td>67.2</td>
<td>104.1</td>
</tr>
<tr>
<td>PDBu, Ca²⁺, PS</td>
<td>80.1</td>
<td>102.0</td>
</tr>
<tr>
<td>4α-Phorbol, Ca²⁺, PS</td>
<td>98.9</td>
<td>103.1</td>
</tr>
<tr>
<td>Diolein, Ca²⁺, PS</td>
<td>88.9</td>
<td>98.9</td>
</tr>
</tbody>
</table>
Protein kinases involved in mRNA transport

Fig. 8. Influence of phorbol esters and protein kinase C on ATP-dependent efflux of actin mRNA from isolated rat liver nuclei

Nuclei (a) and demembranated nuclei (b; obtained after a 10-min treatment with 1% Triton X-100) from rat liver were preincubated in ATP-free transport medium for 10 min at 22 °C in the absence (○) or the presence of 0.02 μg of PMA/ml (●), 0.02 μg of PDBu/ml (△), 0.02 μg of 4α-phorbol/ml (▲), 0.8 μg of diceolin/ml (□), 2.3 units of purified protein kinase C/ml plus 20 μg of PS/ml (×), or 2.3 units of purified protein kinase C/ml plus 20 μg of PS/ml plus 0.02 μg of PMA/ml (■). Measurement of ATP-dependent RNA efflux and quantification of the RNA in the postnuclear supernatants by dot-blot hybridization and autoradiography were performed as described in the Experimental section. Evaluations of autoradiograms were done densitometrically. Results are the means ± S.D. of three triplicate determinations.

in vitro. The ATP-induced efflux of one specific mRNA (actin) from isolated rat liver nuclei was measured as described in the Experimental section. The reliability and the relevance in vivo of the system used (nuclear integrity, nuclear restriction of immature messengers, absence of RNA degradation, etc.) had been the subject of a number of reports (e.g. Agutter, 1982). The released actin mRNA was detected by RNA dot-blot hybridization using cloned mouse actin DNA. The results are shown in Fig. 8. Using this probe a single band of 2.1 kb was found by Northern-blot hybridization analysis of the RNA in the efflux supernatant (Fig. 9). Quantification (arbitrary units) of the actin mRNA in the efflux supernatants was performed by densitometric scanning of the autoradiograms using a standard curve. As shown in Fig. 8(a), preincubation of isolated rat liver nuclei with 0.02 μg of PMA or PDBu/ml resulted in a significant decrease of the rate of ATP-dependent efflux of actin mRNA. The inactive 4α-phorbol had no effect while diceolin (0.8 μg/ml) was 57% as effective as PMA (30 min incubation period; Fig. 8a). When nuclei were depleted of nuclear membranes by treatment with 2% Triton X-100 (10 min at 0 °C) efflux of actin mRNA also required the presence of ATP in the external transport medium (Fig. 8b). However, RNA efflux from membrane-depleted nuclei showed no sensitivity against PMA (Fig. 8b). On the other hand, addition of purified protein kinase C (2.3 units/ml) plus 0.02 μg of PMA/ml and 20 μg of PS/ml to the transport assay significantly reduced the release of actin mRNA (by 23%; Fig. 8b). This effect was not observed in the presence of PS (results not shown). Without addition of PMA, protein kinase C and PS produced a small, but also significant, effect on RNA efflux from the Triton-treated nuclei (Fig. 8b). Addition of 2 mM-EGTA to untreated or Triton-treated nuclei (in the presence of protein kinase C and PS) abolished
Table 2. Requirements for association of protein kinase C with nuclei, nuclear matrices and nuclear envelopes

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nuclei</th>
<th>Nuclear matrix</th>
<th>Nuclear envelope</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.08</td>
<td>0.07</td>
<td>1.3</td>
</tr>
<tr>
<td>PKC</td>
<td>0.4</td>
<td>0.2</td>
<td>5.0</td>
</tr>
<tr>
<td>PKC, PMA</td>
<td>0.5</td>
<td>0.4</td>
<td>10.1</td>
</tr>
<tr>
<td>PKC, Ca²⁺</td>
<td>1.4</td>
<td>0.5</td>
<td>16.0</td>
</tr>
<tr>
<td>PKC, PMA, Ca²⁺</td>
<td>2.9</td>
<td>0.4</td>
<td>39.9</td>
</tr>
<tr>
<td>PKC, PMA, Ca²⁺, PS</td>
<td>3.1</td>
<td>3.0</td>
<td>51.0</td>
</tr>
<tr>
<td>PKC, EGTA</td>
<td>0.12</td>
<td>0.06</td>
<td>1.2</td>
</tr>
<tr>
<td>PKC, PMA, EGTA</td>
<td>0.3</td>
<td>0.09</td>
<td>2.8</td>
</tr>
<tr>
<td>PKC, 4α-phorbol, Ca²⁺, PS</td>
<td>1.5</td>
<td>1.1</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Fig. 10. Time-course of association of purified protein kinase C with purified nuclei, nuclear envelopes and nuclear matrices

Protein kinase C was isolated from rat brain and incubated with nuclei (a), nuclear envelopes (b) and nuclear matrices (c), freshly prepared from rat liver, in the presence of 100 μM-CaCl₂ (●, ○, △), or in the absence of Ca²⁺ but the presence of 1 mM-EGTA (▲) with (●, ○, △) or without (●) 20 μg of PS/ml, as described in the Experimental section. Addition of 1 mM-ATP. [³²P]PDBu binding was used to measure bound protein kinase C. Bound [³²P]PDBu at time zero was subtracted from each value.

Table 3. Effect of nucleotides on association of protein kinase C with nuclei, nuclear matrices and nuclear envelopes

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Conc. (mm)</th>
<th>Nuclei</th>
<th>Nuclear matrix</th>
<th>Nuclear envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>1</td>
<td>46.1</td>
<td>23.4</td>
<td>50.1</td>
</tr>
<tr>
<td>GTP</td>
<td>1</td>
<td>87.9</td>
<td>70.0</td>
<td>79.1</td>
</tr>
<tr>
<td>APPCP</td>
<td>1</td>
<td>85.6</td>
<td>72.8</td>
<td>69.1</td>
</tr>
<tr>
<td>APCPP</td>
<td>1</td>
<td>70.4</td>
<td>43.0</td>
<td>58.7</td>
</tr>
<tr>
<td>ADP</td>
<td>1</td>
<td>75.5</td>
<td>70.9</td>
<td>55.7</td>
</tr>
<tr>
<td>AMP</td>
<td>1</td>
<td>94.1</td>
<td>84.2</td>
<td>88.1</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1</td>
<td>97.9</td>
<td>102.0</td>
<td>96.8</td>
</tr>
</tbody>
</table>

The effect of PMA on mRNA transport (results not shown).

Association of protein kinase C with nuclear membranes

It has been previously shown that highly purified nuclear envelopes from rat liver contain a substantial amount of protein kinase C activity (Schröder et al., 1988). The specific activity of the enzyme in the nuclear envelope was determined to be 1.3 pmol of phosphate incorporated/min per mg of nuclear-envelope protein (Table 2). Therefore, we investigated which factors influence association of protein kinase C with nuclear membranes or other nuclear structures. It was found that treatment of nuclear envelopes with 1% Triton X-100 for 10 min at 22°C resulted in a nearly quantitative loss of protein kinase C activity (cf. Table 4). Besides detergent treatment, dissociation of the nuclear-membrane–protein kinase C complex could also be achieved by use of 1 mM-EGTA (by about 90% after 10 min at 22°C). However, purified protein kinase C was found to be able to bind to isolated nuclei, nuclear matrices and nuclear envelopes in the presence of some additives (PMA, Ca²⁺ and PS) (Table 2). It was found that protein kinase C binding was optimal in the presence of all of these three additives. In the absence of PS, protein kinase C bound efficaciously to nuclei and nuclear envelopes, but not to nuclear matrices. 4α-Phorbol could not act as a substitute for PMA (Table 2). As shown in Fig. 10, binding of purified protein kinase C to isolated nuclei, nuclear envelopes or nuclear matrices was already completed after a 10 min incubation at 22°C, when Ca²⁺ and PS were present. The binding could be prevented in the presence of 1 mM-EGTA or 1 mM-ATP (see also Table 3).

It has been shown that the complex formed between protein kinase C and erythrocyte membrane can be dissociated in the presence of ATP and Mg²⁺ (Wolf et al., 1985a). Other nucleotides and a non-hydrolysable analogue of ATP were either less effective or ineffective (Wolf et al., 1985a). Here we found that binding of purified protein kinase C to isolated nuclei, nuclear matrices and nuclear envelopes was strongly inhibited in the presence of ATP if Mg²⁺ was present but not if it was absent (Table 3). ADP and α,β-methylene ATP exhibited a lower efficacy in preventing association of kinase C with nuclear structures compared with ATP while β,γ-
methylene ATP and GTP showed a less significant effect. AMP and adenosine had only a little effect or were ineffective (Table 3).

Identification of phosphoinositides in nuclear envelope

Protein kinase C is assumed to be activated physiologically by diacylglycerol which is produced from phosphatidylinositol 4,5-bisphosphate (PIP_2) by a membrane-bound phosphodiesterase (Berridge, 1986). Therefore we searched for the presence of PI derivatives in the nuclear membranes. Lipids were extracted from nuclei of L-cells that had been preincubated with [32P]phosphate and analysed on oxalate-impregnated or acidic t.l.c. plates. The resulting autoradiograms are shown in Fig. 11. In the acidic system (Fig. 11, lane a), most of the radioactivity co-migrating with authentic phosphatidic acid (PA) and PI on oxalate-impregnated plates (Fig. 11, lane b) banded together with PI rather than PA. In addition, phosphatidylinositol 4-phosphate (PIP) and PIP_2 were clearly detectable. Thus, all three phosphorylated inositol derivatives are indeed present in L-cell nuclear membranes.

To determine if nuclear membranes were able to release inositol phosphates, nuclei were isolated from L-cells that had been preincubated with [3H]inositol, and were incubated in the presence of ATP for 20 min (see Experimental section). The generated inositol mono-, bis- and tris-phosphates were then extracted and separated by ion-exchange chromatography. All three inositol derivatives could be detected by this experiment, in amounts similar to those found in whole cells (Carney et al., 1985). The following amounts of [3H]inositol-derived radioactivity were found (aqueous phase applied to Dowex column, 11200 c.p.m.): PIP, 1027 ± 182; PIP_2, 816 ± 186; inositol trisphosphate, 322 ± 150 c.p.m./10^6 cells (means ± S.D. from four independent experiments).

Phase separation of nuclear-envelope proteins using Triton X-114

Phase separation of membrane-bound proteins extracted with Triton X-114 represents a simple method of distinguishing between hydrophobic integral membrane proteins appearing in the detergent-rich phase and hydrophilic proteins present in the detergent-poor phase (Bordier, 1981). Proteins present in both phases are considered as peripheral membrane proteins (Bordier, 1981). Therefore, phase partitions of polypeptides extracted from nuclear envelopes of rat liver in the presence of Triton X-114 were performed. It has been already previously shown that the nuclear-envelope NTPase is solubilized with 1% Triton X-100 (Smith & Wells, 1984; Schröder et al., 1986b). Now it was found that the NTPase was present in both the detergent-poor and the detergent-rich phases in nearly equal amounts after Triton X-114 extraction (Table 4). In control experiments it was established that 0.1% Triton X-114 present in the assays had no effect on the NTPase activity in whole nuclear envelopes. As shown in Table 4 also,

---

Table 4. Phase separation of nuclear-envelope-associated enzyme activities involved in mRNA translocation using Triton X-114

<table>
<thead>
<tr>
<th>Sample</th>
<th>NTPase (±ATP)</th>
<th>Protein kinase NI (±ATP)</th>
<th>Protein kinase NII (±ATP)</th>
<th>Protein kinase C (±ATP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−ATP (+ATP)</td>
<td>−ATP (+ATP)</td>
<td>−ATP (+ATP)</td>
<td>−ATP (+ATP)</td>
</tr>
<tr>
<td>Supernatant of Triton X-114</td>
<td>61 (74)</td>
<td>19 (25)</td>
<td>3 (5)</td>
<td>98 (99)</td>
</tr>
<tr>
<td>extraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detergent-poor phase</td>
<td>28 (35)</td>
<td>8 (18)</td>
<td>1 (2)</td>
<td>86 (98)</td>
</tr>
<tr>
<td>Detergent-rich phase</td>
<td>33 (39)</td>
<td>11 (7)</td>
<td>2 (3)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>Pore-complex lamina pellet</td>
<td>39 (26)</td>
<td>81 (75)</td>
<td>97 (95)</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

---

Fig. 11. Incorporation of 32P into nuclear envelope lipids

L5178y cells were incubated in the presence of 32P (50 µCi/ml) for 1 h. Nuclei were then isolated as described by Blobel & Potter (1966) and the nuclear lipids were extracted and separated by t.l.c. on silica-gel plates using chloroform/methanol/acetate acid/water, lane a, or on oxalate-impregnated silica-gel plates using chloroform/methanol/ammonia, lane b, as described in the Experimental section. The radiolabelled phospholipids were detected autoradiographically. The positions of standard lipids which were run in parallel are indicated at the right. O, origin; PA, phosphatidic acid; PC, phosphatidylcholine.
nuclear-envelope-associated protein kinase C was soluble in 1% Triton X-100 or 1% Triton X-114. This result is in line with the opinion that protein kinase C represents a Ca²⁺-dependent membrane surface-bound protein. The extracted protein kinase C was nearly exclusively present in the detergent-poor phase (Table 4). In contrast with protein kinase C, only a small amount of the nuclear-envelope-bound NF- and NII-like protein kinase was solubilized in the presence of Triton X-114 or Triton X-100 (Table 4). However, most of these enzymes were dissociated from the nuclear envelope (pore-complex lamina) structure in the presence of 5 M-urea (NI, 75%; NII, 95%).

**DISCUSSION**

One crucial step of nucleocytoplasmic mRNA transport is assumed to be the ATP-dependent passage of this macromolecule through a nuclear-envelope pore complex. This step is mediated by an energy-delivering NTPase which is regulatable, in its envelope-bound form, by poly(A) binding and protein kinase-dependent phosphorylation of nuclear-envelope constituents obviously not identical with this enzyme. In the present report we identified both a NII-like and a C-type protein kinase in nuclear envelopes from rat liver, which were able to phosphorylate a nuclear-envelope (pore-complex lamina) polypeptide that binds poly(A) in an ATP-labile linkage. The two types of protein kinases could be separated by extraction with Triton X-100 (X-114). The influence of both kinases on the mRNA translocation apparatus was studied.

It has been previously shown that some effectors of NTPase activity in the intact envelope, which do not bind to the enzyme itself, such as poly(A) (Bernd et al., 1982a; Agutter, 1985) or mRNA-transport stimulatory proteins (Schröder et al., 1986c), alter the maximum catalytic rate of the NTPase without affecting its apparent $K_m$. A possible explanation for this is that these effectors act through an inhibitor protein in the envelope which inactivates, when it is phosphorylated, part of the NTPase (Schröder et al., 1986c). The anomalous kinetic behaviour of the envelope-bound NTPase also has been attributed to its interaction with this phosphorylatable protein (Agutter, 1985; Schröder et al., 1986c; the purified NTPase shows normal Michaelis–Menten kinetics). Because protein kinase-dependent modulation of NTPase activity was uncoupled by a monoclonal antibody apparently recognizing neither the NTPase nor the nuclear-envelope poly(A) binding site itself (Schröder et al., 1988), this interaction seems to be mediated by an additional component, not yet characterized. Binding of poly(A) or poly(A)*mRNA to the modulating protein has been shown to increase NTPase activity, while protein kinase-dependent phosphorylation down-regulates this enzyme (Agutter, 1985; Schröder et al., 1986c). On the other hand, phosphorylation of the poly(A) binding site has been shown to enhance the affinity of this site for poly(A) (McDonald & Agutter, 1980; Bachmann et al., 1984). This has been explained by stimulation of the nuclear-envelope phosphoprotein phosphatase activity by the bound poly(A) (McDonald & Agutter, 1980; Bachmann et al., 1984), which results in an increased NTPase activity due to dephosphorylation of the inhibitory protein [poly(A)-binding component] by the phosphatase. The results presented in this paper strongly suggest that the modulatory protein in the envelope is a phosphoprotein with a molecular mass of 106 kDa. First, the phosphorylation of this polypeptide by nuclear-envelope protein kinases was found to be more sensitively inhibited by poly(A) than that of other nuclear-envelope polypeptides (although the phosphorylation of several other polypeptides was also inhibited, albeit to a lesser extent). Secondly, this protein was purified and found to bind to poly(A) [in the presence of poly(C)]. Also, when it was phosphorylated (in accordance with earlier models for mRNA transport) (Agutter, 1985; Schröder et al., 1986c), the 106 kDa polypeptide dissociated from poly(A) in the presence of ATP. Thirdly, from Scatchard analyses of poly(A) binding studies, there is no evidence for an additional class of poly(A) binding site in the envelope (McDonald & Agutter, 1980; Bernd et al., 1982b; Schröder et al., 1986c): the dissociation constant for poly(A) binding to the phosphorylated poly(A) binding site has been found to be nearly identical with the concentration of poly(A) that half-maximally stimulates the NTPase in whole envelopes (Agutter et al., 1977; McDonald & Agutter, 1985), indicating that this site is the modulatory protein. Both the poly(A) binding site (McDonald & Agutter, 1980; Agutter, 1985) and the p106 (this paper) were found not to be soluble in 1% Triton X-100. However, this protein could be solubilized from the nuclear envelope in the presence of 5 M-urea. Besides p106, the urea extract contained two additional polypeptides of 64 and 55 kDa (p64 and p55) which were able to bind to poly(A). Because, after prolonged storage, p106 was found to be degraded to two smaller protein species with the same molecular masses as p64 and p55, we conclude that these two proteins are degradation products of p106. At present it is unknown if p64 and p55 are related to the nuclear poly(A) binding proteins of 55 and 53 kDa from rat liver described by Sachs & Kernberg (1985). It is also not known if there is a relationship between p106 and the 115 kDa polypeptide from brain nuclei described by Wolf & Sahyoun (1986); this protein which is phosphorylated by protein kinase C has been shown to bind only in its unphosphorylated form to protein kinase C. Also, interestingly, incubation of intact cells with phorbol ester has been reported to result in phosphorylation of two nuclear proteins of 64 and 60 kDa (Anderson et al., 1985), possibly identical with p64 and p55.

Also interesting is the fact that the nuclear-envelope-associated NII-like protein kinase was found to co-purify with the putative carrier protein p106. This result suggests a possible involvement of this kinase in mRNA transport. However, p106 was phosphorylated by nuclear-envelope-bound protein kinase C with somewhat higher specificity. Moreover, there are strong indications that protein kinase C is involved in the mRNA-translocation process. Particularly the finding that the phorbol esters PMA and PDBu, which stimulate protein kinase C, inhibit the NTPase within the envelope and NTPase-coupled mRNA transport in vitro, points to a regulatory function of this type of kinase in mRNA translocation across the nuclear envelope. Recently we could demonstrate that the nuclear-envelope-associated topoisomerase II is phosphorylated by protein kinase C resulting in a stimulation of enzyme activity (Rottmann et al., 1987; Schröder et al., 1987a). Interestingly, release of mature mRNA from the intranuclear matrix structure is inhibited
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by topoisomerase inhibitors (Schröder et al., 1987b). A similar enzyme is also assumed (Schröder et al., 1987b) to be responsible for changes in the tertiary structure of the mRNA particle to be transported (Skoglund et al., 1983).

It is well-established that there is an interaction between phosphoinositid metabolism and protein kinase C activity (Nishizuka, 1984, 1986; Bell, 1986). Thus, diacylglycerol formed during PI breakdown acts as a stimulator of protein kinase C activity. From the results presented in this work it appears that it might be possible for nuclear-envelope-associated protein kinase C activity to be linked also with polyphosphoinositide metabolism, as is the case with cell membranes (Berridge & Irvine, 1984; Berridge, 1986). By using the L5178y cell system, we could detect the release of phosphorylated derivatives of inositol from isolated nuclei. In addition, some enzymes involved in polyphosphoinositide metabolism (PI kinase, PIP2 kinase and diacylglycerol kinase) have been identified in isolated envelopes from rat liver (Smith & Wells, 1983b).

The nuclear-envelope-bound protein kinase C was easily soluble in 1% Triton X-100 and was found almost exclusively in the detergent-poor phase after extraction with Triton X-114. Therefore, one can conclude that the nuclear-envelope-associated protein kinase C is a peripheral membrane protein. This result is consistent with the view that protein kinase C is bound to the cytoplasmic side of the cell membrane (Bell, 1986). The binding of this enzyme to the membrane depends on the cytosolic Ca2+ concentration. It is assumed that the Ca2+-mediated binding occurs through the head groups of membrane-integrated phospholipids. Only in the membrane-bound state is the cytosolic enzyme activated (Bell, 1986).

In contrast with protein kinase C, the nuclear-envelope-associated protein kinase NI was found to be solubilized in the presence of 5 M-urea (together with the pore complexes; Aaronson et al., 1982) but not by Triton treatment. These results indicate that protein kinase NI is not an integral component of the nuclear-envelope membranes but rather linked with the residual pore-complex lamina structure. The same might be true for the nuclear-envelope-associated NI-like protein kinase activity.

In a model proposed by Wolf et al. (1985b) protein kinase C can translocate from the cytoplasm, where it is in an inactive state, to the plasma membrane, where it is bound and activated after phospholipase C-regulated (Majerus et al., 1986) production of diacylglycerol in the membrane, in response to external stimuli (Berridge & Irvine, 1984). From our results we assume that protein kinase C also can bind to nuclear membranes (and nuclear matrices) and, after activation, influence, through phosphorylation of regulatory nuclear-envelope proteins, gene expression on the level of mRNA transport out of the nucleus. Thus, phosphorylation of nuclear-envelope proteins by protein kinase C may provide a cytosolic mechanism for control of nuceleocytoplasmic mRNA transport, as opposed to poly(A)-sensitive phosphorylation of the mRNA-binding site within the envelope by endogenous protein kinase NI, representing a nuclear control mechanism which results in down-regulation of the NTPase.

We wish to thank Dr P. S. Agutter (Napier College, Edinburgh, U.K.) for valuable discussion. This work was supported by Grants Schr 277/2-1 and Mu 348/7-6 from the Deutsche Forschungsgemeinschaft.

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Received 18 September 1987/2 February 1988; accepted 23 February 1988.