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Selective Phosphorylation of a Nuclear Envelope Polypeptide by an Endogenous Protein Kinase†

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ABSTRACT: Incubation of highly purified nuclear envelope with [γ-^{32}P]ATP resulted in the selective phosphorylation of a major membrane polypeptide (M_r 68 000) by an endogenous protein kinase. Phosphorylation of this major polypeptide decreased drastically upon perturbation with low concentrations of Triton X-100 or sodium deoxycholate. Similar results were obtained when the nuclear envelope was sonicated vigorously prior to incubation with [γ-^{32}P]ATP. No stimulation of ^{32}P incorporation was noted in the presence of either cAMP or cGMP. Magnesium ion was required for maximal phosphorylation of the 68 000 molecular weight polypeptide; however, Mn^{2+} was approximately 40% as effective as Mg^{2+}. No phosphorylation occurred in the presence of Ca^{2+} or Cu^{2+}. Optimal phosphorylation was obtained between pH 6 and 9. Studies with the membrane matrix of the endoplasmic reticulum revealed the complete absence of the readily labeled 68 000 molecular weight polypeptide found in the nuclear envelope. Phosphorylation of the microsomal membrane was less specific as evidenced by the incorporation of ^{32}P into at least three proteins, the most prominent having molecular weights of 54 000 and 49 000. Both membranes yielded *O*-phosphoserine and *O*-phosphothreonine in a ratio of 3:1 after acid hydrolysis. Partially purified preparations of pore complexes derived from nuclear envelope showed an enrichment of the 68 000 molecular weight protein, suggesting the possible involvement of this highly selective kinase reaction in the exchange of solutes between the nucleus and cytoplasm.

The nuclear envelope, composed of an inner and outer leaflet, each of which possesses the morphological characteristics of a typical membrane, forms the physical barrier separating the nucleoplasm from the remainder of the cell. A unique structural feature of the envelope is the pore complex which, although it is an integral part of the envelope, does not appear to possess the basic phospholipid bilayer structure common to the remainder of the envelope. The physical and biochemical characteristics of the nuclear envelope have been recently reviewed (Kay & Johnston, 1973; Kasper, 1974a,b; Franke & Scheer, 1974; Wunderlich et al., 1976; Harris, 1978), and it is apparent that an integrated picture of structure and function is slowly beginning to emerge. For example, it is clear that the polypeptide composition of the nuclear envelope differs significantly from that of the microsomal membrane (Bornens & Kasper, 1973) in spite of the fact that the enzymology of these two closely associated membrane systems is qualitatively very similar. Also of functional importance is the fact that the nuclear envelope TPNH

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electron transport chain involving TPNH-cytochrome *c* oxidoreductase, cytochrome P-450, and associated aryl hydroxylase activity is not induced by phenobarbital as is its microsomal counterpart (Kasper, 1974b; Kasper, 1971; Khandwala & Kasper, 1973; Fahl et al., 1978). Phenobarbital administration also reduces the level of nuclear envelope epoxide hydrase to approximately 70% of the control value while stimulating a threefold increase in the level of the microsomal enzyme (Fahl et al., 1978). Nuclear envelope catalyzed benzo[*a*]pyrene oxidation is, however, markedly increased by 3-methylcholanthrene induction (Khandwala & Kasper, 1973; Fahl et al., 1978), up to eleven times the corresponding microsomal activity from similarly induced animals (Fahl et al., 1978). The localization of this enzyme system in the nuclear envelope suggests that the crucial site for metabolic activation of carcinogenic substances that ultimately react with nuclear DNA is the envelope. Although activation also occurs in the endoplasmic reticulum, the short half-life of the activated intermediates and the abundance of nucleophiles within the cell make it less likely that this metabolic route represents the major source of electrophilic reactants responsible for covalent modification of DNA.

In a continuing effort to identify important functional aspects of the nuclear envelope, phosphorylation of intrinsic envelope polypeptides has been examined. Earlier studies have pointed out that intranuclear phosphorylation of histones (Balhorn et al., 1972) and nuclear acidic proteins (Allfrey et al., 1973) correlates closely with DNA synthesis and gene activation, respectively. Recently, considerable attention has been given to the phosphorylation of membrane proteins by endogenous protein kinases (Rubin & Rosen, 1975; Hosey & Tao, 1977). Such reactions have been described for plasma membranes isolated from a variety of sources: mammary gland (Majumder & Trukington, 1972), red blood cells (Avruch & Fairbanks, 1974; Guthrow et al., 1972; Rubin & Rosen, 1973; Hosey & Tao, 1976), fat cell (Chang et al., 1974), 3T3 cells (Mastro & Rozengurt, 1976)), rat liver microsomal membranes (Jergil & Ohlsson, 1974; Sharma et al., 1976; Sharma et al., 1978), chloroplast membrane (Bennett, 1977), pancreatic zymogen granules (MacDonald & Ronzio, 1974; Lambert et al., 1974), mitochondrial membranes (Moret et al., 1975), sarcoplasmic reticulum (Wray et al., 1973), muscle membrane (Andrew et al., 1975; Casnelle & Greengard, 1974; Andrew et al., 1973; Sulakhe & Drummond, 1974), and synaptic membranes (Ueda et al., 1973). Many of the above reactions exhibit cyclic nucleotide dependency (Guthrow et al., 1972; Rubin & Rosen, 1973; Chang et al., 1974; Lambert et al., 1974; Wray et al., 1973; Casnelle & Greengard, 1974; Sulakhe & Drummond, 1974; Ueda et al., 1973), while others do not (Majumder & Turkington, 1972; Mastro & Rozengurt, 1976; Sharma et al., 1976; MacDonald & Ronzio, 1974; Moret et al., 1975).

In this paper, we report for the first time on the selective phosphorylation of a specific nuclear envelope protein catalyzed by an endogenous protein kinase and compare this specificity with that exhibited by the endoplasmic reticulum.

Experimental Procedures

Preparation of Nuclear Envelope and Microsomal Membrane. Highly purified nuclear envelope was prepared according to the method of Kasper (1974a) with a slight modification. After sonication, the nuclear lysate was layered over a cushion of sucrose–buffer TKM[1]–10% citrate (density 1.22 g/mL at 23 °C) and centrifuged at 100000*g* for 1 h. The partially purified nuclear envelope collected on the sucrose cushion was further purified in a discontinuous sucrose–citrate gradient as previously described (Kasper, 1974a). The purified nuclear envelope was then washed with buffer TKM, followed by buffer TG,[2] by centrifugation at 7000*g* for 30 min. The final membrane pellet was homogenized in buffer TG. The concentration of the membrane used for phosphorylation studies was about 1 mg of protein/mL.

Microsomal membrane was purified by density gradient centrifugation on a discontinuous sucrose–citrate gradient by the method of Blackburn et al. (1976). The purified membrane was washed twice with buffer TKM–10% citrate and once with buffer TG by centrifugation at 180000*g* for 70 min. The membrane was homogenized and sonicated between washes to remove extraneous material trapped inside the membrane vesicles. The final concentration of the membrane suspension was about 1–2 mg of protein/mL in buffer TG. Protein was determined by the following procedure. The membrane suspension (0.2 mL) was added to 1 mL of 0.1% sodium dodecyl sulfate (w/v) and dialyzed against the same sodium dodecyl sulfate solution for about 10 h. The dialyzed sample was diluted to about 2 mL, and protein was determined by the Folin procedure (Bailey, 1972) using two-times crystallized ovalbumin as the standard.

Preparation of [γ-^{32}P]ATP. [γ-^{32}P]ATP with a specific activity of about 1 Ci/μmol was prepared according to the method of Schendel & Wells (1973) with modifications (Miller & Burgess, 1978). Ammonium acetate (1 M, pH 7.0) was used as the final elution buffer. The product was lyophilized and washed with doubly distilled water. The purity of [γ-^{32}P]ATP was checked by ascending thin-layer chromatography on polyethylenimine impregnated cellulose (Brinkmann Instruments, Inc.) in 1.8 M LiCl, 0.01 M EDTA, pH 6.5. For use, [γ-^{32}P]ATP was diluted to the desired specific activity with unlabeled ATP (Sigma Chemical Co.).

Measurement of Phosphorylation. Phosphorylation was carried out according to Johnson & Allfrey (1972) with modifications. The standard assay mixture (final volume of 0.20 mL) contained 5 mM Tris-HCl buffer (pH 7.0), 0.01 M magnesium acetate, 0.01 M NaF, 0.01 M magnesium chloride, 20% (v/v) glycerol, 50 μM [γ-^{32}P]ATP, and 50 μL of membrane suspension (50–100 μg of protein). In certain cases, 10 μM cyclic nucleotide was incorporated into the reaction mixture. [γ-^{32}P]ATP was added to start the reaction. Mixtures were incubated at 33 °C for 40 min unless otherwise specified, and reactions were stopped by the addition of 4 mL of cold 5% trichloroacetic acid containing 1.5% sodium pyrophosphate and 1% monobasic sodium phosphate. After at least 16 h at 0 °C, the mixtures were filtered on Millipore Type HA (0.45-μm pore size filter) and washed eight times each with 4 mL of the same cold trichloroacetic acid solutions to ensure minimal background. The filters were dried in an oven and the radioactivity was measured in 10 mL of scintillation fluid (160 mL of rpi Scintillator PPO–POPOP/gal of toluene; Research Products International Corp., IL) by a Nuclear Mark III 6880 liquid scintillation system (Searle Analytic, Inc.).

Gel Electrophoresis and Autoradiographic Analysis. Phosphorylation reactions were terminated by one of the following methods, both of which yielded comparable results. (1) Sodium dodecyl sulfate (10% w/v) solution was added to the reaction mixture to make up a final detergent concentration

[1] Buffer TKM, 50 mM Tris-HCl, pH 7.5, containing 25 mM KCl and 5 mM $MgCl_2$.

[2] Buffer TG, 50 mM Tris-HCl, pH 7.0, containing 10 mM $MgCl_2$ and 20% glycerol (v/v).

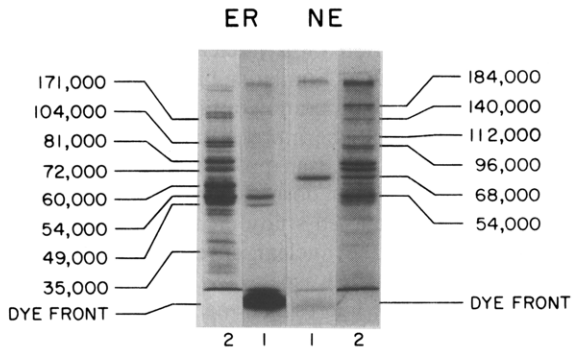

FIGURE 1: Autoradiogram (channel 1) and Coomassie brilliant blue stained electrophoretogram (channel 2) of nuclear envelope (NE) and endoplasmic reticulum (ER) after incubation with [γ-^{32}P]ATP.

of 2%. The mixture was then dialyzed against sample buffer (0.08 M Tris-HCl, pH 6.8, containing 6% (v/v) β-mercaptoethanol and 2.5% sodium dodecyl sulfate) overnight with several changes of the same buffer to remove as much free radioactivity as possible. (2) Sodium dodecyl sulfate (20% w/v, 100 μL) and 50 μL of β-mercaptoethanol were added to 0.9 mL of reaction mixture and incubated at 33 °C for about 0.5 h prior to electrophoresis.

Phosphorylated membranes were analyzed by stacking sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8.75% acrylamide) according to the procedure of Laemmli (1970). Glycerol (10–15%) was added to each sample and electrophoresis was carried out using a slab gel apparatus (Hoefer Scientific Instruments) in which the gel measured 138 × 90 × 1.5 mm. Gels were stained with 0.75% Coomassie blue in methanol–acetic acid–water (v/v, 7:3:30) for 2–4 h and destained in methanol–acetic acid–water (v/v, 7:3:30) by diffusion.

Destained slab gels were dried on filter paper under vacuum for about 10–15 h. The filter paper, containing the dried gel, was placed next to Kodak X-Omat R or Kodak X-ray film N-S and exposed for varying lengths of time (usually 24–48 h), depending on the radioactivity in the dried gel.

Measurement of ^{32}P Incorporation into the 68 000 Molecular Weight Polypeptide. For quantitative analysis of specific labeled proteins, the region of the dried gel corresponding to the labeled protein was carefully excised with a pair of scissors and the sample prepared for counting by one of the following two procedures: (1) the cut gel was incubated with 0.8 mL of 30% H_2O_2 at 60 °C overnight and counted in 10 mL of RIA-Solve II (Research Products International Corp.) or (2) the cut gel was allowed to swell in 0.1 mL of H_2O prior to the addition of 0.5 mL of 95% Protosol (v/v) (New England Nuclear). After incubating at 45 °C overnight in a tightly capped vial, 0.5 mL of 1 M Tris-HCl (pH 7.0), followed by 10 mL of RIA-Solve II, was added. The samples were counted by a Nuclear Mark III 6880 liquid scintillation system (Searle Analytic Inc.).

Results

Specificity of Membrane Phosphorylation. Phosphorylation of intrinsic membrane proteins of the nuclear envelope and microsomal membrane by endogenous protein kinases was examined (Figure 1). Phosphorylation catalyzed by the nuclear envelope was highly selective; one polypeptide of 68 000 molecular weight was preferentially labeled (Figure 1). In contrast, phosphorylation of microsomal membrane under similar conditions resulted in the incorporation of label into at least three proteins. Molecular weights of the major microsomal phosphoproteins were calculated to be 173 000, 54 000, and 49 000. Both the Coomassie blue stained gels and

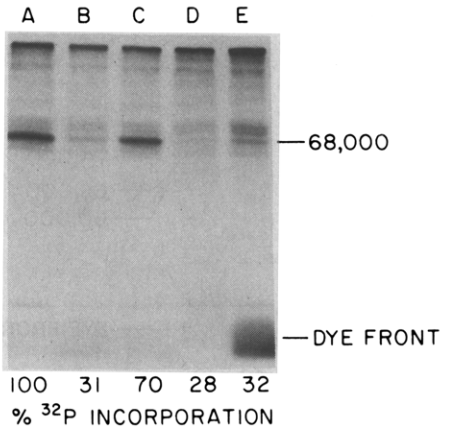

FIGURE 2: Autoradiogram showing the effect of mechanical and detergent treatments on nuclear envelope phosphorylation. All mechanical treatments were performed prior to incubation with [γ-^{32}P]ATP. A, control; B, sonication for 20 s at a power of 6.5 by a sonifier cell disrupter (Branson Sonic Power Co.); C, membrane was frozen and thawed five times using dry ice in 95% ethanol; D, 2.5% Triton X-100 (v/v) was incorporated into the incubation mixture; E, 0.125% sodium deoxycholate (w/v) was incorporated into the incubation mixture. ^{32}P incorporation into the 68 000-M_r protein, as indicated below the autoradiogram, was determined as described under Experimental Procedures.

the autoradiographic patterns revealed the complete absence of the 68 000 molecular weight polypeptide from the microsomal membrane. In the case of each membrane, variable amounts of labeled protein failed to enter the gels. Various methods of solubilization were attempted in order to minimize or eliminate this problem but without success. In spite of the excluded material at the gel surface polypeptide patterns were always reproducible. Another feature of the autoradiographic pattern worthy of comment is the occurrence of radioactivity immediately to the rear of the dye front in the microsomal membrane system. This does not correspond to a Coomassie blue staining region and is present at a markedly reduced level in the nuclear envelope. The identity and significance of this labeled membrane component are unknown; however, based on the location in the gel, lipids and/or small peptides are definite possibilities.

The effect of disrupting membrane organization by either mechanical means or detergent treatment on the specificity of the phosphorylation reaction was also studied. Qualitative evaluation of nuclear envelope preparations that had been subjected to vigorous sonication, exposure to Triton X-100, or exposure to sodium deoxycholate (Figure 2, channels B, D, and E, respectively) indicated that all these treatments reduce phosphorylation of the 68 000 molecular weight protein. Quantitative evaluation of the degree of phosphorylation of the 68 000 molecular weight protein (as shown in the numbers below the autoradiogram in Figure 2) revealed that, after such treatments, there was a 70% reduction of ^{32}P incorporation into this protein, whereas repeated freezing and thawing reduced phosphorylation by about 30% (Figure 2, channel C).

The 2 N HCl hydrolysis of phosphorylated control membranes at 110 °C for 3, 5.5, and 9 h all revealed *O*-phosphoserine and *O*-phosphothreonine in a molar ratio of 3:1.

Divalent Metal Ion Dependency. Endogenous phosphorylation of the nuclear envelope had an absolute dependency on divalent metal ion. The results in Figure 3 illustrate a strong preference for Mg^{2+} and further demonstrate that ^{32}P incorporation into the 68 000 molecular weight polypeptide was diminished by 60% when Mg^{2+} was replaced by Mn^{2+}. However, minor phosphorylation of the polypeptides of molecular weights 78 000, 74 000, and 60 000 remained unchanged

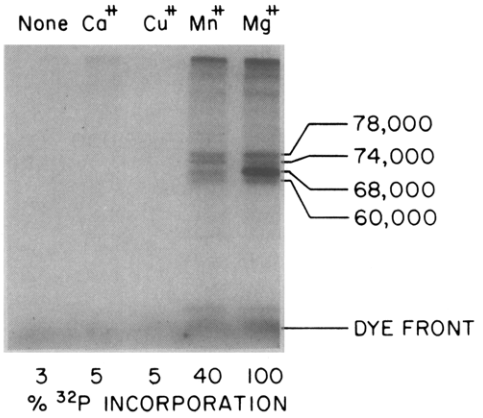

FIGURE 3: Autoradiogram showing the effect of divalent metal ions on nuclear envelope phosphorylation. Nuclear envelope was washed with 20% glycerol before incubation with [γ-^{32}P]ATP. The chloride salt of each metal ion was added to a final concentration of 0.02 M. ^{32}P incorporation into the 68 000-M_r protein, as indicated below the autoradiogram, was determined as described under Experimental Procedures. 50 μg of membrane protein was applied to each gel slot.

Table I: Effect of Inhibitors on ^{32}P Incorporation into the 68 000 Molecular Weight Nuclear Envelope Polypeptide

inhibitor[a]	% ^{32}P incorporation
control	100
dA	51
dAMP	79
AMP	82
EDTA	68

[a] All inhibitors were tested at a final concentration of 0.5 mM.

in the presence of Mn^{2+}. When Mg^{2+} was replaced by Cu^{2+}, no phosphorylation was observed. Similar results were observed with Ca^{2+} except that a low level of ^{32}P incorporation into aggregates retained on the gel surface was noted. Optimum phosphorylation was obtained over a wide range of Mg^{2+} concentrations (5–40 mM).

Factors Affecting Phosphorylation. Various compounds were tested as potential inhibitors of ^{32}P incorporation into the 68 000 molecular weight nuclear envelope protein (Table I). Deoxyadenosine (0.5 mM) inhibited phosphorylation by 51%, and dAMP and AMP inhibited by about 20%. EDTA inhibited approximately 30%, probably by chelating Mg^{2+}.

The pH dependence of ^{32}P incorporation into the 68 000 molecular weight protein was also examined. A typical bell shaped curve was obtained with optimal phosphorylation occurring between pH 6 and 9. Incorporation dropped off rapidly on both the acid and alkaline side of this range.

Time course studies indicated that maximum phosphorylation of the 68 000 molecular weight polypeptide was achieved after an incubation period of 25–30 minutes at 33 °C. Although NaF was usually included in the incubation mixture, the specific radioactivity of the 68 000 molecular weight protein was enhanced approximately 35% when fluoride ion was omitted. In each case, however, no dephosphorylation was observed during the 45-min incubation period.

Neither cAMP nor cGMP in a concentration range of 0–100 μM had an effect on the extent of phosphorylation under the conditions studied. Comparative autoradiographic analysis of polypeptide patterns obtained from membranes phosphorylated in the presence or absence of 10 μM cAMP revealed no changes in the labeling pattern of the membrane proteins.

Discussion

Our studies clearly demonstrate that highly purified nuclear envelope contains a polypeptide of 68 000 molecular weight that is readily and selectively phosphorylated by an endogenous protein kinase in the presence of [γ-^{32}P]ATP. This represents the first demonstration of a nuclear envelope catalyzed phosphorylation reaction. Similar studies with the microsomal membrane revealed incorporation of radioactivity into at least three proteins and, furthermore, established the complete absence of the 68 000-dalton protein characteristic of the nuclear envelope. This observation is also in complete agreement with a detailed study on the comparative polypeptide analysis of the nuclear envelope and microsomal membrane (Bornens & Kasper, 1973). Two prominently labeled proteins of the microsomal membrane with molecular weights of 49 000 and 54 000, which were completely absent in the nuclear envelope autoradiographic pattern, serve as critical and sensitive indicators for possible microsomal contamination of nuclear envelope preparation. The molecular weights of these polypeptides are close to those reported for cytochromes P-450 LM_2 (48 700) and P-450 LM_4 (55 300) by Haugen & Coon (1976). It should be emphasized, however, that nuclear envelope does contain proteins in this molecular weight range, but they are not phosphorylated in the native membrane.

Endogenous phosphorylation catalyzed by both membrane systems was found to be cyclic nucleotide independent. Microsomal phosphorylation has been reported to be both dependent upon (Jergil & Ohlsson, 1974) as well as independent of (Sharma et al., 1976) cAMP. The exact reason for this discrepancy is unclear; however, one cannot rule out that the regulatory subunit(s) of the protein kinase might somehow be removed during the membrane isolation procedure leading to a loss of cAMP dependency. Also, the occurrence of an intrinsic adenyl cyclase in the membranes would result in the formation of cAMP from the added ATP thus obscuring any stimulatory effect due to cAMP in the incubation mixture. There have been reports that high levels of cAMP actually inhibit protein kinase activity (Iwai et al., 1972). For both the nuclear and microsomal systems, no inhibition was noted with cAMP concentrations up to 0.1 mM. The membrane-associated protein kinases of nuclear envelope and microsomal membrane are capable also of phosphorylating exogenous substrates such as various histones and α-casein. However, incorporation of ^{32}P into histone (H-1), but not α-casein, was markedly stimulated by 10 μM cAMP (K. S. Lam and C. B. Kasper, unpublished results).

Various factors were found to affect the specificity of membrane phosphorylation. In the case of nuclear envelope, physical disorientation of the membrane brought about by excessive sonication resulted in a substantial decrease in ^{32}P incorporation into the 68 000 molecular weight polypeptide. Similar results were obtained when 2.5% Triton X-100 (v/v) or 0.125% sodium deoxycholate (w/v) was incorporated into the incubation mixture. Nuclear envelope, unlike microsomal membrane, was highly resistant to solubilization by nonionic detergents such as Triton X-100. For example, electrophoretic analysis of the membrane pellet and supernatant after treatment with 2% Triton X-100 (v/v) revealed the solubilization of only a few minor polypeptides in the molecular weight range of 49 000 to 54 000. The 68 000 molecular weight polypeptide was not solubilized under these conditions. This is in agreement with the results of other investigators (Dwyer & Blobel, 1976) who have shown the insolubility of those proteins associated with the dense lamina in Triton solutions.

A possible explanation of this reduction in net phosphorylation is that there may exist a close topological relationship between the kinase and its 68 000 molecular weight protein substrate and that this unique spacial relationship is disrupted

upon such perturbations. The results may also be due to the inactivation of the kinase by such treatments. Furthermore, at this stage of our studies it is not possible to state whether the phosphorylated protein is in fact the product of autophosphorylation of the kinase.

NaF has been shown to be an inhibitor of phosphoprotein phosphatase (Ueda et al., 1973; Maeno & Greengard, 1972; Khandelwal, 1977) and ATPase. Consequently, one would expect to find a higher level of ^{32}P incorporation in the presence of NaF. This enhancement was not observed, however. Incorporation studies performed in the presence of 10 mM NaF revealed a slight but significant inhibition of ^{32}P incorporation into the 68 000 molecular weight protein. The reason for this unexpected result is unclear although F^- may disrupt the microenvironment of the kinase and substrate to bring about the observed effect. In the case of the microsomal membrane, however, a higher level of phosphorylation was obtained in the presence of NaF. This provides an additional distinction between the endogenous phosphorylation of these two membrane systems.

Nuclear envelope phosphorylation showed a definite cation preference. Mg^{2+} yielded an optimal response while Mn^{2+} was only 40% as effective. It is of interest to note that substitution of Mn^{2+} for Mg^{2+} did not reduce the minor phosphorylation of three nuclear envelope proteins having molecular weights of 60 000, 74 000, and 78 000. This result suggests that more than one kinase may be involved, each having a different cation dependence. Virtually no phosphorylation was obtained in the absence of divalent cation or in the presence of Ca^{2+} or Cu^{2+}. On the other hand, studies on phosphorylation of endogenous substrates by erythrocyte membrane protein kinases have demonstrated that Mn^{2+} is as effective as Mg^{2+} (Avruch & Fairbanks, 1974; Hosey & Tao, 1976).

Efforts are underway to further characterize the 68 000 molecular weight protein and to determine the biochemical significance of its preferential phosphorylation. The fact that this protein is absent from the microsomal membrane suggests that it may be associated with some structural feature unique to the nuclear envelope such as the pore complex. Preparation of a pore complex–dense lamina fraction from the nuclear envelope by the method of Dwyer & Blobel (1976) reveals that this fraction is enriched in the 68 000 molecular weight phosphoprotein. Although the evidence at this stage is circumstantial it seems appropriate on the basis of the foregoing discussion to consider the kinase and the 68 000-dalton protein as possible components of the pore complex and that they may play a regulatory role in nucleocytoplasmic transport.

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