GTP-binding proteins in rat liver nuclear envelopes

(GTP-dependent nuclear envelope reassembly/nuclear transport/photoaffinity labeling)

JEFFREY B. RUBINS, JOSHUA O. BENDITT, BURTON F. DICKEY, AND NORBERT RIEDEL*

Pulmonary Center, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118

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ABSTRACT Nuclear transport as well as reassembly of the nuclear envelope (NE) after completion of mitosis are processes that have been shown to require GTP and ATP. To study the presence and localization of GTP-binding proteins in the NE, we have combined complementary techniques of [α-32P]GTP binding to Western-blotted proteins and UV crosslinking of [α-32P]GTP with well-established procedures for NE subfractionation. GTP binding to blotted NE proteins revealed five low molecular mass GTP-binding proteins of 26, 25, 24.5, 24, and 23 kDa, and [α-32P]GTP photoaffinity labeling revealed major proteins with apparent molecular masses of 140, 53, 47, 33, and 31 kDa. All GTP-binding proteins appear to localize preferentially to the inner nuclear membrane, possibly to the interface between inner nuclear membrane and lamina. Despite the evolutionary conservation between the NE and the rough endoplasmic reticulum, the GTP-binding proteins identified differed between these two compartments. Most notably, the 68- and 30-kDa GTP-binding subunits of the signal recognition particle receptor, which photolabeled with [α-32P]GTP in the rough endoplasmic reticulum fraction, were totally excluded from the NE fraction. Conversely, a major 53-kDa photolabeled protein in the NE was absent from rough endoplasmic reticulum. Whereas Western-blotted NE proteins bound GTP specifically, all [α-32P]GTP photolabeled proteins could be blocked by competition with ATP, although with a competition profile that differed from that obtained with GTP. In comparative crosslinking studies with [α-32P]ATP, we have identified three specific ATP-binding proteins with molecular masses of 160, 78, and 74 kDa. The localization of GTP- and ATP-binding proteins within the NE appears appropriate for their involvement in nuclear transport and in the GTP-dependent fusion of nuclear membrane vesicles required for reassembly of the nucleus after mitosis.

The nuclear envelope (NE) is a distinct intracellular structure, consisting of an outer membrane continuous with the endoplasmic reticulum (ER), an inner membrane facing the nucleoplasm, the lamina, and pore complexes. As a barrier between the nuclear and the cytoplasmic compartments, the NE is involved in the regulated transport of protein and RNA. These transport processes have been shown to require energy and to depend on specific receptor and transport proteins associated with the NE (2–9). Workers in several laboratories have described rather nonspecific adenosine triphosphatases (ATPases) in the NE, which can bind and hydrolyze both ATP and GTP, and, to a lesser extent, also UTP, CTP, and deoxyadenosine triphosphates (10–12).

In most cells, the NE disassembles and reassembles during mitosis, and the recent development of in vitro systems has permitted a more detailed study of the assembly process (13–16). In these systems, the initial event in the re-formation of the NE at the end of mitosis involves the targeting of nuclear membrane vesicles to the surface of chromatin. Subsequent fusion of these membrane vesicles requires both GTP and ATP, and results in the formation of a double-membrane complex that is apparently identical to that surrounding normal nuclei (17). In this regard, the process of nuclear membrane fusion resembles GTP-dependent fusion processes in other cellular membrane systems, such as the ER and the Golgi, and may be regulated by GTP-binding proteins. GTP-binding proteins may also be involved in processes of nuclear protein association with the NE (18). Takeda et al. (19) have UV-crosslinked [α-32P]GTP to whole nuclei from Swiss 3T3 mice, but no detailed study of GTP-binding proteins in the NE has been performed.

The family of GTP-binding proteins encompasses the heterotrimeric G proteins, which have been well-established as mediators of signal transduction at the plasma membrane (20), as well as a group of elongation and initiation factors involved in protein synthesis (21), and tubulin (22). More recently, low molecular mass GTP-binding proteins related to the ras protooncogene have been implicated in regulating membrane vesicle budding and fusion involved in transport from ER (23) and through the Golgi complex (24, 25), as well as in processes of endocytosis (26) and exocytosis (27, 28). Ras-related GTP-binding proteins also appear to be involved in cytoskeletal polymerization (29). Finally, secretory protein transport across the ER membrane is mediated by the signal recognition particle receptor, which has been shown to be a GTP-binding protein (30).

In the present study, we describe the identification and localization of GTP- and ATP-binding proteins in rat liver NEs by using the complementary techniques of filter binding assays and UV crosslinking. We propose that these NE-associated GTP- and ATP-binding proteins may be involved in the important cellular processes of mitosis and nucleocytoplasmic transport.

MATERIALS AND METHODS

Protein Quantitation and Separation. Protein was assayed by the modification of the Lowry method described by Peterson (31), with bovine serum albumin used as a standard. Proteins were separated by discontinuous SDS/PAGE according to the method of Laemmli (32), using the following molecular mass standards: phosphorylase B, 95.5 kDa; glutamate dehydrogenase, 55 kDa; lactate dehydrogenase, 36 kDa; carboxylic anhydrase, 29 kDa; lactoglobulin, 18.4 kDa (Diversified Biotech, Newton, MA).

Isolation of Rat Liver Nuclei and NEs. Nuclei were isolated from male Sprague-Dawley rats (body weight, 150–200 g) by the method of Blobel and Potter (33). NEs were prepared by the Dwyer and Blobel protocol (34) as described (35). All buffers were supplemented with 100 μM phenylmethylsulfonyl fluoride and 10 μM leupeptin.

Abbreviations: NE, nuclear envelope; ER, endoplasmic reticulum.

*To whom reprint requests should be addressed.
Subfractionation of Rat Liver NEs. NEs were subfractionated as described (35) with slight modifications. When citric acid extraction was included, NEs were initially resuspended at a concentration of 2 × 10^6 NEs per ml in 1% citric acid and incubated at 4°C for 10 min, a procedure described to preferentially remove the outer nuclear membrane of whole nuclei (36) and of isolated NEs (P. Agutter, personal communication). After centrifugation at 5000 × g for 10 min, the pellet was resuspended in 0.25 M STKM buffer (0.25 M sucrose/50 mM Tris-HCl, pH 7.4/25 mM KCl/5 mM MgCl2/3.3 mM CaCl2). Subsequent fractionation included treatment with 1% Triton X-100 in 0.25 M STKM for 10 min at 4°C to remove the residual inner nuclear membrane (34, 37-39), a 1 M NaCl extraction to remove residual histones (34), and treatment with 2 M urea to preferentially extract components of the pore complexes (40). Extractions were performed in equal volumes, and protein content per fraction relative to total NEs was 7% in citric acid supernatant, 20% in Triton X-100 supernatant, 28% in NaCl supernatant, and 7% in urea supernatant. All fractions were dialyzed against 0.25 M STKM buffer.

Isolation of Plasma Membranes and Rough ER from Rat Liver Cells. Fractions enriched for these membranes were prepared from rat liver cells exactly as described by Carey and Hirschberg (41).

[a-32P]GTP Binding. Total NEs, NE subfractions, and fractions enriched in rough ER (20–25 μg of protein) were separated by 14–18% SDS/PAGE and then electrophoretically transferred for 3500 mA·h to BA 83 nitrocellulose (Schleicher & Schuell). Blotted proteins were assayed for GTP binding by a slight modification of the method of Lapetina and Reep (42). Briefly, blots were equilibrated in 10 mM Tris-HCl, pH 7.5/150 mM NaCl/0.05% Tween/5% skim milk solids for 60 min and then washed for 30 min with binding buffer (50 mM Tris-HCl, pH 7.5/0.3% Tween 20/5 mM MgCl2/1 mM EGTA). Blots were then incubated in binding buffer containing 0.66 mM [a-32P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq) with or without added unlabeled nucleotides for 60 min at room temperature, washed three times for 20 min each in blotting buffer, air-dried, and exposed to Kodak XAR-5 film without an intensifying screen for 16–48 hr. The intensity of the autoradiographic bands was quantitated by transmission laser densitometry (Ursotran XL, LKB) after determining that the exposure was within the linear range of both the film and the densitometer.

Photoaffinity Labeling with [α-32P]GTP and [α- and γ-32P]-ATP. For photolabeling, fractions were incubated in 100 μl of 0.25 M STKM buffer containing 10 nM [α-32P]GTP or [γ-32P]ATP (both 3000 Ci/mmol; Amer sham) for 15 min at 30°C. All samples were made 1% with Triton X-100 prior to incubation to control for detergent effects on photoaffinity labeling and on migration in SDS/polyacrylamide gels. Samples were placed in a 96-well tissue culture plate on ice and irradiated with a UV source (Oriel, Stamford, CT) at 180 W at 10 cm for 12 min. Proteins were precipitated in 8% trichloroacetic acid, washed with acetone, and then separated on SDS/10% polyacrylamide gels. Labeled proteins were identified by autoradiography and quantitated by densitometry as described above.

Enzyme Assays. 5’-Nucleotidase (EC 3.1.3.5) was assayed as described by Segal and Brenner (43). Glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of Nordlie and Arion (44).

RESULTS

Characterization of Rat Liver Membrane Fractions. Rat liver nuclei prepared by the Blobel and Potter procedure (33) are highly purified by morphological criteria (33, 35). To biochemically characterize the purity of the nuclear fractions used for these studies, we measured the activity of two membrane enzyme markers. The plasma membrane enzyme 5’-nucleotidase had an activity of 274 ± 27.5, 105 ± 2.0, and 6.3 ± 0.1 nmol of substrate hydrolyzed per min per mg of protein in plasma membranes, rough ER, and nuclei, respectively, whereas the activity of the ER marker enzyme glucose-6-phosphatase was 128 ± 11 and 11.9 ± 1.5 nmol of substrate hydrolyzed per min per mg of protein in the rough ER and nuclei, respectively. Thus, the nuclear fraction appears to be essentially free of significant contamination by these cellular membranes.

Identification of Low Molecular Mass GTP-Binding Proteins in NEs. Low molecular mass GTP-binding proteins have been implicated in regulating processes of membrane vesicle fusion. To identify and localize these proteins within the NE, aliquots (20 μg of protein) of whole rat liver nuclei, chromatin, total NEs, and NE subfractions at each step of fractionation were analyzed by assaying the binding of [α-32P]GTP at a concentration of 0.66 nM to proteins that were separated by 14% SDS/PAGE and transferred to nitrocellulose (Fig. 1). Several GTP-binding proteins were identified in rat liver nuclei (lane a), and remained associated with the total NE (lane c) after removal of chromatin (lane b). After preferential extraction of the outer nuclear membrane by 1% citric acid (lane d), the major portion of [32P]GTP-binding activity remained insoluble (lane e). However, extraction of the residual inner nuclear membrane by 1% Triton X-100 in 0.25 M STKM buffer solubilized nearly all GTP-binding proteins, as indicated by a significant increase in the specific activity of GTP binding in the detergent extract (194%, 253%, and 305% increase, respectively, by densitometric scanning of the 26-, 24-, and 23-kDa bands in Fig. 1, lane f). The residual insoluble GTP-binding activity after Triton X-100 extraction (lane g) remained associated with the lamina (lane i) after extraction with 1 M NaCl (lane h). Only faintly labeled bands remained in the pellet (lane k) after extraction with 2 M urea (lane j), presumably due to a denaturing effect of urea on GTP binding. Based on these results, the low molecular mass GTP-binding proteins identified in rat liver nuclei seem to be exclusively localized in the NE, where they appear to be associated with the inner nuclear membrane lamina fraction.

To demonstrate the specificity of GTP binding, aliquots of the NE fraction (25 μg of protein) were separated on 18% polyacrylamide gels, Western-blotted, and incubated with

![Fig. 1. Low molecular mass GTP-binding proteins in NE subfractions. Aliquots were blotted onto nitrocellulose after SDS/PAGE and incubated with [α-32P]GTP. Lanes: a, whole rat liver nuclei; b, solubilized chromatin; c, total NEs; d, citric acid-extracted outer nuclear membranes; e, citric acid-insoluble fraction; f, Triton X-100-extracted inner nuclear membranes; g, Triton X-100-insoluble fraction; h, 1 M NaCl-extracted residual nuclear proteins; i, remaining pore complex–lamina; j, 2 M urea-extracted pore complex proteins; k, residual lamina. Lanes a–i contain 20 μg of protein each; lanes j and k contain 13 μg of protein each.](image-url)
0.66 nM [α-32P]GTP in the presence of increasing concentrations of unlabeled GTP or ATP (Fig. 2A). Binding of [α-32P]GTP to NE proteins was reduced 95% in the presence of 0.1 μM GTP, whereas an ATP concentration of 1 mM was needed to achieve this degree of competition. Of note, [α-32P]GTP binding to the 26- and 23-kDa NE proteins was competed preferentially by coincubation with unlabeled GTP, whereas unlabeled ATP competed equally well for [α-32P]GTP binding to all four proteins.

The pattern of GTP-binding proteins enriched in the nuclear membrane fraction was similar but not identical to that identified by [α-32P]GTP blotting of preparations of rat liver rough ER (Fig. 2B). For comparison of these fractions, membrane proteins were separated by 18% SDS/PAGE on 24-cm plates to increase resolution. By this method, five GTP-binding proteins could be resolved in the total nuclear membrane fraction: more intense bands at ≈26 and ≈24.5 kDa (bands 1 and 3), and less intense bands at ≈25, ≈24, and ≈23 kDa (bands 2, 4, and 5, respectively). The purified rough ER fraction contained six GTP-binding proteins, five of which comigrated with those of the nuclear membrane fraction. However, subtle differences in the relative labeling by [α-32P]GTP binding could be distinguished. For example, the relative intensity of bands 2 and 4 was greater in the rough ER, whereas band 5 was more intense in the nuclear membrane fraction. Furthermore, a GTP-binding protein of ≈19 kDa identified in the rough ER fraction (band 6) was entirely absent from the Triton X-100 extract of NEs.

**Photoaffinity Labeling of NE Proteins.** [α-32P]GTP binding to blotted proteins preferentially identifies a subset of low molecular mass GTP-binding proteins (45). To also examine the presence of other GTP-binding proteins that might be implicated in the processes of nuclear protein transport and NE assembly, aliquots (25 μg of protein) of total NEs, Triton X-100 NE extract, and rough ER fractions were UV crosslinked with 10 nM [α-32P]GTP in the presence or absence of 1 μM unlabeled GTP (Fig. 3). Under these conditions, no proteins were labeled without UV irradiation (data not shown). Multiple proteins were photolabeled in the NE fractions, with enrichment of a prominent 53-kDa protein and less distinct proteins of 140, 33, and 31 kDa in the 1% Triton X-100 extract compared with total NEs. The less intense 140-, 33-, and 31-kDa proteins were more clearly revealed after longer autoradiograph exposures, as shown in Figs. 4A and 5. In contrast to the NE, prominent 68- and 30-kDa proteins were labeled in rough ER (arrowheads in Fig. 3), which presumably correspond to the photolabeled proteins identified as the signal recognition particle receptor subunits by Connolly and Gilmore (30). In addition, a minor band at ≈55 kDa was only observed in the rough ER. These ER proteins were completely absent from the whole NE and NE Triton X-100 extract fractions. Moreover, the prominent 53-kDa protein found in the NEs was absent from the rough ER. Coincubation with 100-fold molar excess of unlabeled GTP (1 μM) during UV crosslinking reduced labeling of the proteins in the total NEs and their corresponding Triton X-100 extract fractions by ≈50%. Labeling of the 55-kDa ER protein was completely blocked by competition at this GTP concentration. However, the labeling of several other proteins was unaffected by competition with a 100-fold GTP excess, including an intense band at 47 kDa present in all

**Fig. 2.** Specificity of [α-32P]GTP binding to blotted NE proteins. (A) Western-blotted NE proteins (25 μg of protein) were incubated with 0.66 nM [α-32P]GTP either without added unlabeled nucleotide (lane –) or in the presence of increasing concentrations of unlabeled GTP or ATP. Autoradiographs were deliberately heavily exposed to contrast the effects of micromolar concentrations of competing nucleotides on the photoaffinity labeling reaction. (B) Aliquots (25 μg of protein) of rough ER (RER) and NE Triton X-100 extract (TXS) were separated by 18% SDS/PAGE on 24-cm plates and blotted for [α-32P]GTP binding.

**Fig. 3.** [α-32P]GTP photolabeling of NEs, NE Triton X-100 extract, and rough ER fractions. Aliquots (25 μg of protein) of total NEs (NE), Triton X-100 extract of NEs (TXS), and rough ER (RER) fractions were UV crosslinked with 10 nM [α-32P]GTP in the presence (lanes +) or absence (lanes –) of 1 μM unlabeled GTP. Arrowheads indicate labeled proteins at ≈68 and 30 kDa in the rough ER fraction.

**Fig. 4.** Competition of GTP and ATP for UV photolabeling of the Triton X-100-extracted NE fraction. (A) [α-32P]GTP photolabeling of aliquots (20 μg of protein) of Triton X-100 NE extract in the presence of increasing concentrations of unlabeled GTP and ATP. Autoradiographs were deliberately heavily exposed to contrast the effects of micromolar concentrations of competing nucleotides on the photoaffinity labeling reaction. (B) [α-32P]ATP photolabeling of Triton X-100 NE extract. Aliquots (20 μg of protein) of Triton X-100 NE extract were UV crosslinked with 10 nM [α-32P]ATP in the absence of competing nucleotide (lane a) and in the presence of 10 μM ATP (lane b) or GTP (lane c), respectively. Solid arrowheads indicate 160- and 78- to 74-kDa proteins labeled by [α-32P]ATP but not [α-32P]GTP; open arrowheads indicate 53-, 47-, and 33- to 31-kDa proteins labeled by both nucleotides.
**Fig. 5.** [α-32P]GTP photoaffinity-labeled proteins in NE subfractions. Aliquots (20 μg of protein) were UV crosslinked with 10 nM [α-32P]GTP. Lanes: a, whole rat liver nuclei; b, solubilized chromatin; c, total NEs; d, citric acid-extracted outer nuclear membranes; e, Triton X-100-extracted inner nuclear membranes; f, Triton X-100-insoluble fraction; g, 1 M NaCl-extracted residual nuclear proteins; h, remaining pore complex-lamina; i, 2 M urea-extracted pore complex proteins; j, residual lamina. Lane k is a longer exposure of lane e.

fractions, and the two prominent 68- and 30-kDa bands in the rough ER.

To rigorously examine the specificity of UV crosslinking at these concentrations, aliquots of the Triton X-100 extract (20 μg of protein) were photoaffinity labeled with 10 nM [α-32P]GTP in the presence of increasing concentrations of unlabelled GTP and ATP (Fig. 4A), and the intensity of labeling was quantitated by densitometry. Labeling of all GTP-binding proteins was reduced in a concentration-dependent fashion by GTP, with a 50% $K_i$ ($K_50$) of about 0.5 μM GTP. Similarly, photoaffinity labeling of the 53-kDa protein, however, could not be abolished even by millimolar concentrations of ATP. In fact, increasing the ATP concentration above 10 μM reproducibly caused a broadening of the 53-kDa band and a paradoxical slight increase in the total incorporation of 32P (Fig. 4A). Identical competition curves with GTP and ATP were observed when total NEs were photolabeled with [α-32P]GTP (data not shown), excluding the possibility that the nucleotide competition was affected by detergent.

These competition studies suggested that the [α-32P]GTP-photolabeled proteins have equal or higher affinity for ATP than for GTP. However, the inability to completely block photoaffinity labeling of some proteins with ATP, in contrast to GTP, suggested that ATP might interfere with GTP binding by mechanisms other than a direct competition for a single nucleotide-binding site. Therefore, we investigated whether these same proteins could be photoaffinity labeled with ATP. Aliquots of the 1% Triton X-100 extract (20 μg of protein) were UV crosslinked with either [γ-32P]ATP or [α-32P]ATP. Under these conditions, photolabeling with [γ-32P]ATP revealed no distinct bands (data not shown). However, photoaffinity labeling with [α-32P]ATP revealed several discrete bands (Fig. 4B). Of note, the efficiency of photolabeling of these proteins with [α-32P]ATP was substantially lower than that of labeling with [α-32P]GTP. Proteins with molecular masses apparently identical to the 53- and 47-kDa proteins that were labeled by [α-32P]GTP were also photolabeled by [α-32P]ATP (lane a), and at longer exposures, the 31- to 33-kDa proteins were also detected (open arrowheads). Further, distinct proteins with molecular masses of approximately 160, 78, and 74 kDa were photolabeled by [α-32P]-ATP, but not by [α-32P]-GTP (solid arrowheads). Photoaffinity labeling of all of these proteins with [α-32P]ATP could be completely blocked by incubation with ATP at a concentration of 10 μM (lane b). However, the unique UV crosslinking with [α-32P]ATP and the lack of competition with GTP (lane c) indicated that the three proteins of 160, 78, and 74 kDa bind ATP exclusively under the conditions tested.

The enrichment of the [α-32P]GTP-photolabeled proteins in the Triton X-100 extract was further investigated. Aliquots (20 μg of protein) of whole rat liver nuclei, chromatin, total NEs, and NE subfractions were analyzed by UV crosslinking with 10 nM [α-32P]GTP (Fig. 5). The [α-32P]GTP-crosslinked proteins of whole nuclei (lane a) remained associated with the total NE (lane c) after removal of chromatin (lane b). We observed that the ratio of the labeled 53- and 47-kDa proteins varied in these crosslinking experiments (compare Figs. 3–5). Preferential extraction of the outer nuclear membrane by 1% citric acid solubilized a minor portion of these proteins (lane d). In contrast, extraction of the residual inner nuclear membrane by 1% Triton X-100 in 0.25 M STKMC solubilized nearly all of these proteins (lane e), with a 250–500% increase in autoradiographic intensity compared with total NEs and the revealing of additional labeled proteins with molecular masses of approximately 40, 26, 24, 20, and 18 kDa (see lane k, which is a longer exposure of lane e). Residual photolabeled proteins remained with the pore complex–lamina fraction (lane h) after extraction of the remaining Triton X-100 pellet (lane f) with 1 M NaCl (lane g). Since no labeling was observed in the supernatant (lane i) and pellet (lane j) fractions after urea extraction, we cannot exclude an irreversible denaturing effect of urea on GTP binding. In summary, these results show a preferential extraction of GTP-binding proteins with 1% Triton X-100 in 0.25 M STKMC buffer.

**DISCUSSION**

We have localized GTP-binding proteins in the NE by using a well-characterized sequential extraction of NE components. Our initial studies focused on low molecular mass GTP-binding proteins that have been shown to mediate membrane budding and vesicle fusion in a number of cellular processes (23–28) and would seem likely to mediate the GTP-dependent fusion of nuclear membrane vesicles to reform the NE after mitosis. By binding of [α-32P]GTP to nitrocellulose-blotted proteins, we found five GTP-binding proteins of approximately 26, 25, 24.5, 24, and 23 kDa in NEs (Fig. 2B). Binding of [α-32P]GTP to these proteins was highly specific for GTP as compared to ATP (Fig. 2A). These proteins are mostly insoluble in 1% citric acid but are significantly enriched in the Triton X-100 extract, suggesting that they are mostly associated with the inner nuclear membrane. However, it is possible that these proteins are loosely associated with the pore complex–lamina fraction and are extracted by the combination of Triton X-100 and 0.25 M STKMC buffer. We have previously reported that NE-associated binding proteins for the simian virus 40 large tumor antigen nuclear location signal can be extracted with 1% Triton X-100 in 0.25 M STKMC despite their apparently preferential but transient association with the pore complex (8).

Comparison of NE Triton X-100 extract and purified rough ER fractions reveals a similar but not identical pattern of low molecular mass GTP-binding proteins (Fig. 2B). Considering that >20 GTP-binding proteins of 19–28 kDa have been identified by molecular cloning, it is possible that distinct GTP-binding proteins are present in these two membrane fractions, which cannot be fully resolved by one-dimensional SDS/PAGE. Alternatively, the same GTP-binding proteins...
may serve similar functions in these structurally and biochemically homologous membrane compartments.

Using the technique of protein labeling by UV crosslinking of $[\alpha^{32}P]GTP$, we have identified photolabeled proteins of 140, 53, 47, 33, and 31 kDa in NE membranes (Fig. 4A), with some corresponding to those observed in a previous report (19). These proteins were completely excluded from the chromatin fraction and could be almost completely solubilized from the NE by extraction with 1% Triton X-100 in 0.25 M STKMC, with sufficient enrichment to reveal additional labeled proteins with apparent molecular masses of 40, 26, 24, 20, and 18 kDa (Fig. 5, lane b). By our NE subtraction, these proteins also appear to be preferentially associated with the inner nuclear membrane (Fig. 5). Perhaps our most striking finding, considering that the nuclear membrane is a continuous and almost impermeable barrier, is the presence of 68- and 30-kDa proteins labeled by $[\alpha^{32}P]GTP$ in ER (30) which are undetectable in the NE fractions.

In contrast to these low molecular mass proteins identified by filter-binding assays (Fig. 2A), the major proteins identified by $[\alpha^{32}P]GTP$ photolabeling have equal or higher affinity for ATP (Fig. 4A). We do not know whether this reflects the native nucleoside triphosphate binding affinity of these two different groups of proteins or whether the observed differences are the result of the different techniques used. It is possible that the photolabeled proteins function as GTP- and/or ATP-binding proteins in vivo. Of note, the $[\alpha^{32}P]GTP$-photolabeled NE proteins can be distinguished from those that photolabel with $[\alpha^{32}P]ATP$ exclusively (Fig. 4B). Furthermore, the competition of ATP for $[\alpha^{32}P]GTP$ photolabeling is incomplete. This is most apparent for the 53-kDa protein, whose ATP-binding protein is a 53- and 40-kDa ATP-binding protein. Although the ATP concentrations increase above 10 $\mu$M, migrates as a doublet, with increased total incorporation of $^{32}P$ label (Fig. 4A). These studies suggest the presence of a second, low-affinity GTP-binding site in the 53-kDa protein, binding to which is facilitated in the presence of ATP.

The identity of the $[\alpha^{32}P]GTP$-photolabeled proteins remains unknown. Berrios et al. (10), who examined ATP/dATP-binding proteins in the nuclear matrix-rod complex-lamina fraction of Drosophila melanogaster embryos, also observed $[\alpha^{32}P]GTP$ photolability of a 50- to 55-kDa protein. In contrast to the studies described here, their protein apparently remained soluble after detergent and high salt extraction. The 5- and 40-kDa GTP-binding proteins identified in our system suggest the presence of the $\alpha$ subunits of transducing guanine nucleotide-binding regulatory proteins (G proteins) G, and Gs (s, stimulatory; i, inhibitory) in the NE fractions; however, we could not detect substrates for either cholera toxin- or pertussis toxin-catalyzed ADP-ribosylation, which specifically label these G proteins (data not shown). One interesting possibility is that the 53-kDa photolabeled protein represents the $\beta$ subunit of tubulin, which is known to be a GTP-binding protein (22) and is associated with the nuclear membrane throughout the cell cycle (47).

UV crosslinking with $[\alpha^{32}P]ATP$ photolabeled the 53- and 47-kDa proteins identified by $[\alpha^{32}P]GTP$ photolabeling and three additional proteins of approximately 160, 78, and 74 kDa that labeled only with ATP (solid arrowheads in Fig. 4B). It is possible that the 160-kDa ATP-binding protein is the ATPase/dATPase described by Berrios et al. (10). We (12) and others (11, 48) have described a Triton X-100-extractable NE-associated nucleoside triphosphatase of 43-47 kDa with equal affinity for ATP and GTP. It is possible that this enzyme, which is presumably involved in mRNA transport, is identical to the photolabeled 47-kDa protein (Figs. 3-5).

In summary, our studies show that several low molecular mass GTP-binding proteins and $[\alpha^{32}P]GTP$-photolabeled proteins of the NE are unique to this organelle and preferentially localize to the inner nuclear membrane, possibly to the interface with the lamina. Purification of these solubilized proteins will be required to explore their role in the regulation of NE reassembly and nucleocytoplasmic transport.

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