Identification and Biochemical Characterization of an 80 Kilodalton GTP-Binding/ Transglutaminase from Rabbit Liver Nuclei†

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ABSTRACT: The primary aim of these studies was to identify and biochemically characterize GTP-binding proteins in the nucleus. We found that an 80 kDa protein was responsible for the majority of the GTP-binding activity detected in rabbit liver nuclear preparations as assayed by photoaffinity labeling with [α-^{32}P]GTP. The GTP-binding activity was partially extracted only after treatment of nuclear envelope preparations with 0.5 M NaCl and 1% Triton-X 100, which suggested that this GTP-binding protein was a component of the nuclear pore/lamina fraction. The Triton X-100/NaCl-solubilized 80 kDa protein was purified by a series of steps that included DEAE-Sephacel, Mono-Q, and Ultrogel AcA34 chromatographies. Microsequence analysis of two peptides generated by trypsin digestion of the 80 kDa protein indicates that it shares sequence similarity with the tissue transglutaminases. Purified preparations of the 80 kDa protein show a Ca^{2+}-stimulated transglutaminase activity, as assayed by the incorporation of [^{3}H]putrescine into caesin, which is strongly inhibited by GTP but not by GDP. A 36 kDa GTP-binding protein copurified with the 80 kDa GTP-binding protein through all of the chromatography steps and sequence analysis suggests that the 36 kDa protein represents a proteolytic fragment of the amino-terminal half of the 80 kDa protein and thus serves to mark the GTP-binding domain within the 80 kDa protein. The 36 kDa fragment has a significantly higher efficiency of [α-^{32}P]GTP incorporation compared to the 80 kDa protein, suggesting that the carboxyl-terminal half of the GTP-binding protein/ transglutaminase imparts a negative constraint on GTP-binding activity or on the subsequent incorporation of radiolabeled GTP. Indirect immunofluorescence experiments using a specific anti-transglutaminase monoclonal antibody that cross-reacts with the purified 80 kDa nuclear protein show strong nuclear staining in NIH 3T3 fibroblasts, similar to that observed when using a specific monoclonal antibody raised against a nuclear pore-specific protein, p62 [Davis, L. I., & Blobel, G. (1986) *Cell 45,* 699−709]. In addition, the anti-transglutaminase antibody immunoprecipitates the 80 kDa GTP-binding protein from rabbit liver nuclear extracts together with the p62 protein, raising the possibility that the 80 kDa nuclear GTP-binding protein/transglutaminase is present in a complex with other nuclear pore components.

GTP-binding proteins play a number of key roles in cell biology signaling processes. Perhaps the best known are the heterotrimeric GTP-binding proteins (designated G proteins). These were first identified in the hormone-regulated adenylyl cyclase system (Lefkowitz & Caron, 1988; Gilman, 1987) and the vertebrate vision system (Stryer, 1991; Khorana, 1992) and represent the prototype transducer molecules that mediate receptor/effector coupling. In these systems, the GTP-bound state of the G protein α subunit represents the activated conformation that is primed to interact with the biological effector, whereas the GDP-bound state, which is generated following GTPase activity, represents the inactive state. A second family of GTP-binding proteins are those for which the ras proteins are prototypes (Hall, 1990; Bourne et al., 1990). During the past few years, various members of the ras superfamily have received a great deal of attention because of their possible roles in cell growth, differentiation, protein trafficking, and cytoskeletal rearrangement (Hall, 1990, 1992; Bourne et al., 1990; Barbacid, 1987; Balch, 1990). It appears that in each of these families, the GTP-binding/GTPase cycles of the proteins are critical regulatory features for specific biological functions.

Various studies (Rubins et al., 1990; Seydel & Gerace, 1991) have suggested the presence of GTP-binding proteins in the nucleus. One of these described the identification of a 28 kDa protein that may function in the nuclear pore (Rubins et al., 1990). There are a number of nuclear events that might be under the control of GTP-binding activities, including nuclear transport processes (Dingwall & Laskey, 1986; Gerace & Burke, 1988; Hinshaw et al., 1992) and the assembly and disassembly of the nuclear envelope that occurs during the cell cycle (Gerace & Blobel, 1980; Peter et al., 1990). Among the putative GTP-binding proteins that appear to be present in the nuclear envelope, as detected through assays monitoring the incorporation of radiolabeled GTP (Rubins et al., 1990), are proteins with molecular masses (58−80 kDa) that are larger than the heterotrimeric G proteins and the ras-like molecules. Since it seemed possible that these nuclear proteins might represent members of a new family of GTP-binding proteins, we set out to establish conditions for their purification and biochemical characterization.

In the present study, we describe the isolation of an 80 kDa, high-affinity GTP-binding protein from preparations of rabbit liver nuclei. We find that this nuclear GTP-binding protein shares a high degree of sequence similarity with tissue transglutaminases and is capable of transglutaminase activity that is inhibited by GTP but not GDP. A monoclonal

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antibody raised against the liver tissue transglutaminase cross-reacts with the purified 80 kDa nuclear GTP-binding protein. Indirect immunofluorescence experiments using this antibody yield strong nuclear staining in fibroblasts, and immunoprecipitation experiments from rabbit liver nuclear extracts indicate that the 80 kDa GTP-binding protein/transglutaminase is complexed with a nuclear pore-specific protein.

EXPERIMENTAL PROCEDURES

Preparations of Rabbit Liver Nuclei. Rabbits were sacrificed, and livers were quickly removed and chilled immediately in several volumes of ice-cold 0.25 M sucrose in 50 mM Tris-HCl, pH 7.5, 25 mM KCl, and 5 mM $MgCl_2$ (TKM buffer). All subsequent procedures were performed at 4 °C and in the presence of 100 μM phenylmethanesulfonyl fluoride (PMSF) and 10 μg/mL each of leupeptin, pepstatin, and aprotinin. The livers (~300 g) were homogenized in a Potter–Elvehjem homogenizer, and then the homogenate was filtered through four layers of cheesecloth. The filtrate was centrifuged at 4000*g* for 15 min. The pellet was resuspended in 0.25 M sucrose in TKM buffer, and nuclei were isolated by sucrose density centrifugation by the procedure of Blobel and Potter (1966). The isolated nuclei were suspended in 0.25 M sucrose in TKM buffer and then centrifuged in (~50 mL) aliquots at 1000*g* for 10 min. The pellets were frozen at −70 °C until further use. The nuclear pore–lamina fractions were obtained as described by Dwyer and Blobel (1976), except that the DNase I and RNase A concentrations were 5 and 2 μg/mL, respectively. Briefly, the nuclear suspension was treated with DNase I and RNase A and then centrifuged for 10 min at 4 °C (20000*g*). The nuclear envelope (pellet) obtained after this step was treated with 1% Triton X-100 and recentrifuged. This step was repeated, and then the pellet was resuspended in 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, and 0.5 M NaCl. This suspension was incubated for 30 min at 4 °C and then centrifuged for 10 min at 20000*g*; the supernatant was used for further purification.

Purification of Nuclear GTP-Binding Proteins. All of the purification steps were performed at 4 °C unless otherwise stated. The extract of the nuclear pore/lamina fraction was dialyzed overnight in 2 L of 25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 100 μM PMSF (TXED buffer). It was then centrifuged at 12000*g* for 60 min. The supernatant was collected and loaded (at 25 mL/h) on a 15 mL DEAE-Sephacel column that was preequilibrated with TXED buffer. After being loaded, the column was thoroughly washed with 5 volumes of TXED buffer, and then the GTP-binding proteins were eluted with a gradient (100 mL) of 0–0.5 M NaCl in TXED buffer. Fractions of 1 mL each were collected. An aliquot (20 μL) of each fraction was used for affinity labeling with [α-^{32}P]-GTP (see below) and for protein (silver) staining and autoradiography. Fractions that contained the 80 kDa GTP-binding protein (as monitored by affinity labeling) were pooled and dialyzed overnight in TXED buffer. The dialyzed sample then was centrifuged at 12000*g* for 60 min and loaded onto a Mono-Q FPLC column that was preequilibrated with TXED. The protein was eluted with a 0–0.5 M NaCl gradient in TXED buffer, and 0.5 mL fractions were collected. The peak fractions containing the 80 kDa GTP-binding protein were pooled and concentrated 5× by ultrafiltration. The concentrated protein fractions were then loaded onto an Ultrogel AcA34 gel filtration column (1.8 × 45 cm) that was preequilibrated with TXED buffer containing 100 mM NaCl. One milliliter fractions were collected, and GTP binding was assayed (on 20 μL aliquots from the fractions) by affinity labeling with [α-^{32}P]GTP. The peak fractions containing GTP binding were pooled and dialyzed against TXED and used for the biochemical studies described under Results.

In order to obtain the peptide sequence for the 36 kDa protein, the peak fractions from Ultrogel AcA34 that were obtained from three preparations were pooled and applied to a (10 mL) hydroxylapatite column. The 36 kDa GTP-binding protein was eluted with KP_i (0–200 mM gradient) in TXED buffer.

Photoaffinity Labeling of GTP-Binding Proteins. Photoaffinity labeling of GTP-binding proteins with [α-^{32}P]GTP was carried out in a buffer that contained 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP. Samples (20 μL) were incubated with 2–3 μCi of [α-^{32}P]GTP (0.02 μM) in the above buffer (20 μL final volume) in tissue culture plates (96 wells) for 10 min at room temperature. The samples then were placed in an ice bath and irradiated with UV light (254 nm) for 15 min. After irradiation, the samples were mixed with 5× Laemmli buffer (Laemmli, 1970) and boiled for 5 min. SDS–polyacrylamide gel electrophoresis was performed using a 10% gel. Gels were dried and exposed (typically for 30 min) on Kodak X-OMAT XAR-5 film using DuPont image intensifying screens.

Indirect Immunofluorescence and Immunoprecipitation with Anti-transglutaminase Antibody. Immunolocalization of transglutaminase in NIH 3T3 cells was performed following cell growth on glass coverslips (Corning) in DMEM supplemented with 10% calf serum. Prior to fixation, the cells were gently washed in 0.1% Triton X-100 in PBS for 4 min at room temperature. Cells were then fixed in incubation in 3.7% formaldehyde, PBS for 6 min. Following rinsing in PBS, coverslips were incubated for 1.5 h at room temperature in primary antibody [dilutions: control IgG, undiluted; anti-transglutaminase (CUB7402), undiluted; anti-p62, 1:50]. After additional washes (3×, 2 mL for 5 min each), fluorescently labeled secondary antibody was added, and the cells were incubated for an additional 1 h. Coverslips were mounted with anti-fade medium (Kirkegaard and Perry, Gaithersburg, MD) and viewed with a Zeiss Axioplan fluorescence microscope. Fluorescent images were recorded on TMA 400 film.

Nuclear DNA was visualized by incubation with Hoest dye #33342 (Molecular Probes) diluted 1:1000 in PBS prior to final rinsing with PBS. Immunoprecipitation experiments were performed as follows. Samples (20 μL) of the Ultrogel AcA34-purified 80 kDa protein or nuclear envelope proteins extracted with 1% Triton X-100 and 0.5 M NaCl were incubated with the anti-transglutaminase monoclonal antibody (100 μg/mL), or with the control antibody, for 2 h at 4 °C. Protein G–Sepharose (4 mg/mL lysates) was then added to the mixture and incubated for 2 h at 4 °C. These mixtures were pelleted and washed (3×) with 137 mM NaCl, 15 mM NaH_2PO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4, containing 1% NP-40 (buffer 1), and then (2×) with 0.1 M Tris, 0.5 M LiCl, pH 7.4, and finally (2X) with 10 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.5.

The pellets were then resuspended in buffer 1. Aliquots (20 μL) of the resuspended pellets were diluted with an equal volume of 2× Laemmli sample buffer, and boiled for 5 min. The samples were subjected to electrophoresis on a 10% SDS−polyacrylamide gel. Proteins were transferred to 0.2 μm nitrocellulose filters, and the filters were blocked using TBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.4) plus 2% BSA for 1 h. The filters were washed in TBS/0.2% Tween-20 (TTBS) at 15 min intervals (3×). The blots were then exposed to primary anti-transglutaminase antibody (100 μg/mL) in TTBS and then to secondary antibody (anti-mouse HRP; Amersham Corp., Arlington Heights, IL) at a 1:5000 dilution in TTBS/1% BSA for 1 h. Filters were washed (3×) with TTBS and then with TBS (2×) and visualized using chemiluminescence (ECL, Amersham).

Transglutaminase Activity Assays. Transglutaminase activity was measured as the Ca^{2+}-dependent incorporation of [^{14}C]putrescine (Amersham Corp.) into *N,N*-dimethylcaesin by a modification of the method described by Nara et al. (1989). Samples from column fractions were added to reaction mixtures consisting of 25 mM HEPES-Na (pH 7.4), 100 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 1 mM $NaHCO_3$, 5 mM glucose, 1 mM $CaCl_2$, 1 mg/mL dimethylcaesin, 20 mM dithiothreitol, 0.8% (v/v) glycerol, and 250 μM [^{14}C]putrescine (0.5 μCi) in a final volume of 300 μL. The reaction mixtures were incubated for 1 h at 30 °C and then stopped by the addition of 100 μL of 40% trichloroacetic acid (TCA). The suspension was centrifuged at 12000*g* for 30 min. The precipitates were washed with 12% TCA twice and then dissolved in 100 μL of H_2O and 10 μL of 1 N NaOH. The radioactivity was measured by liquid scintillation counting. Transglutaminase activity was expressed as nanomoles of putrescine incorporated into caesin in 1 h at 30 °C per milligram of protein.

RESULTS

Identification of an 80 kDa GTP-Binding Protein and a 36 kDa GTP-Binding Protein in Rabbit Liver Nuclei. Previous studies have indicated that the nuclear envelope contains a number of GTP-binding activities (Rubins et al., 1990; Seydel & Gerace, 1991). Some of these proteins appear to be significantly larger than the members of the well-characterized heterotrimeric "G protein" family or ras-superfamily (i.e., M_r > 50 000) and could constitute a new family of G proteins. Thus, we have begun to purify and characterize these "large" nuclear G proteins. To do this, rabbit liver nuclei were prepared essentially as described by Dwyer and Blobel (1976) and then treated with DNase [or with DNase and RNase (Gerace et al., 1982)], 1% Triton X-100, or a combination of 1% Triton X-100 and 0.5 M NaCl. We have tested the different nuclear extracts for the presence of contaminating cytosol by assaying for the cytosolic marker lactate dehydrogenase (Neilands, 1955). We determined that rabbit liver cytosol contained 8400 units of activity (nmol of NADH formed per min) per milligram of protein. The DNase I/RNase A-treated nuclear extract and the Triton X-100-treated extract contained 48 units of activity per milligram of protein, while no detectable lactate dehydrogenase activity was measured in the Triton X-100/NaCl extract. Western blot analysis using a specific histone antibody indicates that histones (which serve as an intranuclear marker) are present in the DNase/RNase and Triton X-100 extracts, but are not detected in the Triton X-100/NaCl extract (data not shown). Proteins that remain in the pellet after Triton X-100 extraction have been previously defined as components of the nuclear pore complex−lamina fraction (Seydel & Gerace, 1991; Davis & Blobel, 1986).

When these nuclear preparations were exposed to [α-^{32}P]-GTP, followed by irradiation with ultraviolet light and then autoradiography, a number of radiolabeled bands were detected, as originally described by Seydel and Gerace (1991). In order to facilitate the purification and characterization of the radiolabeled proteins (M_r > 50K), we initially focused on those proteins that were most effective at incorporating radiolabeled GTP, i.e., proteins that were detected after only a short autoradiographic exposure. Figure 1A shows the autoradiogram obtained after a brief (15 min) exposure of different extracts from the nuclear envelopes of rabbit liver. Under these conditions, two predominant bands were observed corresponding to proteins with apparent sizes of 80 and 36 kDa, and a third, relatively weaker band corresponding to a protein of ~65 kDa. Detergent treatment of the nuclear envelopes proved to be ineffective for solubilizing these GTP-binding proteins. Specifically, we have examined a variety of detergents including CHAPS, octyl glucoside, sodium cholate, and Triton X-100 and found that in all cases only 10% (or less) of the total amount of the 80 kDa protein present in the nuclear envelopes was solubilized (see lane 2 in Figure 1A which represents the results obtained with 1% Triton X-100). The addition of NaCl (0.5−1 M) together with 1% Triton X-100 significantly increased the yields of both the 80 and 36 kDa proteins (compare lanes 2 and 3 in Figure 1B), with 0.5 M NaCl providing a maximal effect (i.e., identical to the results obtained with 1 M NaCl). Still, greater than 50% of the GTP-binding proteins typically remained in the nuclear envelope pellets.

The resistance of these GTP-binding proteins to solubilization by a combination of detergent and high salt argued against these proteins being contaminants from the cytosol or the (soluble) nuclear contents, but rather suggested that they were part of the nuclear pore/lamina complex (Dwyer & Blobel, 1976; Gerace et al., 1982; Snow et al., 1987). It is interesting that the signal recognition particle (SRP) receptor, which mediates the transport of proteins across mammalian endoplasmic reticulum (ER) and upon its purification shows two bands that incorporate radiolabeled GTP, molecular masses ~72 and 30 kDa (Connolly & Gilmore, 1989), also is not readily solubilized from the ER. However, while this pair of GTP-binding proteins bears some resemblance to the 80 kDa/36 kDa species that we have found in the nuclear envelope preparations, antibodies raised against the SRP receptor GTP-binding subunits (kindly provided by Dr. P. Walters, University of California, San Francisco) showed no detectable cross-reactivity with the 80 and 36 kDa GTP-binding proteins (data not shown). In addition, the incorporation of radiolabeled GTP into the SRP receptor 72 and 30 kDa subunits was blocked in the presence of ~25 μM ATP, as well as being inhibited by 25 μM GTP. The 80 and 36 kDa proteins from the rabbit liver nuclear envelopes appeared to bind GTP with a higher degree of selectivity (relative to the SRP receptor subunits). Figure 1B shows that GTP, at levels of 10 μM, inhibited the incorporation of radiolabeled GTP into the 80 and 36 kDa proteins by at least 50% (and a greater than 90% inhibition was observed at 30 μM), whereas ATP was a relatively

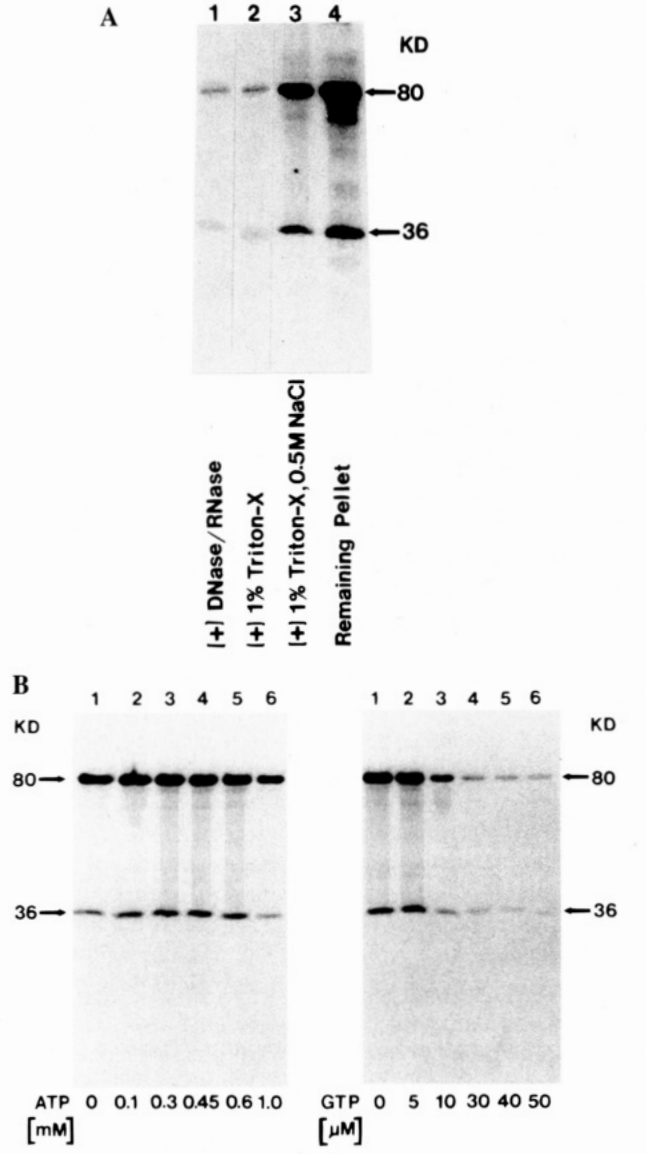


FIGURE 1: Fractionation and photolabeling of rabbit liver nuclei with [α-^{32}P]GTP. Rabbit liver nuclei were digested with nucleases and solubilized with 1% Triton X-100, or 1% Triton X-100 plus 0.5 M NaCl, as described under Experimental Procedures. (A) Aliquots of protein (100 μg) were photolabeled with [α-^{32}P]GTP and then analyzed by SDS−polyacrylamide gel electrophoresis and autoradiographed. The arrows mark the positions of proteins with apparent molecular masses of 80 and 36 kDa, based on the mobility of known standards. (B) The supernatant obtained from rabbit liver nuclear envelopes solubilized with 0.5 M NaCl and 1% Triton X-100 was photolabeled with [α-^{32}P]GTP in the presence and absence of the indicated amounts of nonradioactive ATP and GTP. Samples were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography. The arrows mark the positions of proteins with apparent molecular masses of 80 and 36 kDa, based on the mobility of known standards.

ineffective inhibitor of GTP incorporation and showed less than 50% inhibition of GTP incorporation into the 80 kDa protein at concentrations of ~1 mM.

Purification of the 80 and 36 kDa GTP-Binding Proteins. We have used the incorporation of [α-^{32}P]GTP as an assay to purify the 80 and 36 kDa proteins. The nuclear pore/lamina extract was solubilized with 1% Triton X-100 and 0.5 M NaCl and subjected to DEAE Sephacel chromatography (see Experimental Procedures). The majority of the protein that was applied to the DEAE-Sephacel column was eluted with ~200 mM NaCl; however, the peak fractions

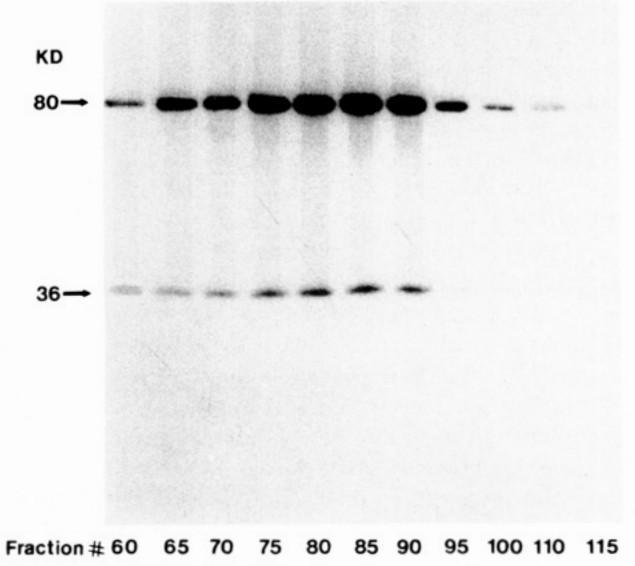


FIGURE 2: DEAE-Sephacel chromatography of the nuclear pore/lamina fraction from rabbit liver. The extract of the nuclear pore/lamina fraction from rabbit liver was prepared as described under Experimental Procedures, dialyzed versus 25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 100 μM PMSF, and then applied to a DEAE-Sephacel column that was equilibrated with the same buffer. 20 μL of the indicated fractions was photolabeled with [α-^{32}P]GTP as indicated under Experimental Procedures.

for [α-^{32}P]GTP incorporation into the 80 and 36 kDa proteins eluted at 300−400 mM NaCl (data not shown). At this stage of the purification, it was not possible to identify the protein bands (at 80 and 36 kDa) that were responsible for GTP binding. However, as shown in Figure 2, the peak fractions for [α-^{32}P]GTP incorporation into the 80 kDa protein (fractions 75−90) were the same as those for the incorporation of the radiolabeled GTP into the 36 kDa protein. This was the case throughout the course of the purification of these GTP-binding proteins (see below).

The peak fractions containing [α-^{32}P]GTP binding were pooled, dialyzed to remove NaCl, and then applied to a Mono-Q column. Approximately 50% of the total protein was eluted from the Mono-Q resin with 200−300 mM NaCl; the majority of the remainder of the protein was then eluted at >400 mM NaCl (Figure 3A). The 80 and 36 kDa GTP-binding proteins were eluted between fractions 72 and 90. Two peaks of GTP-binding activity (as monitored by [α-^{32}P]-GTP incorporation) were observed; one peak occurred at fraction 74 and the second peak at fraction 82 (Figure 3B).

Since the second peak of GTP-binding activity appeared to contain significantly more (total) protein than the first, we used the first peak for further purification. These peak fractions were concentrated by Amicon filtration and applied to an Ultrogel AcA34 gel filtration column (see Experimental Procedures). The first major protein peak eluted between fractions 64 and 70. The silver stain profile of the SDS−PAGE of these fractions showed a single predominant protein band corresponding to molecular mass ~80 kDa (Figure 4, upper panel). The relative intensities of the silver stain profiles containing this 80 kDa protein matched the relative intensities for [α-^{32}P]GTP incorporation into an 80 kDa protein (Figure 4, lower panel). Given the correspondence between this major protein band and the peak fractions for the incorporation of radiolabeled GTP, we suspected that the 80 kDa, silver-stained protein represented the GTP-binding

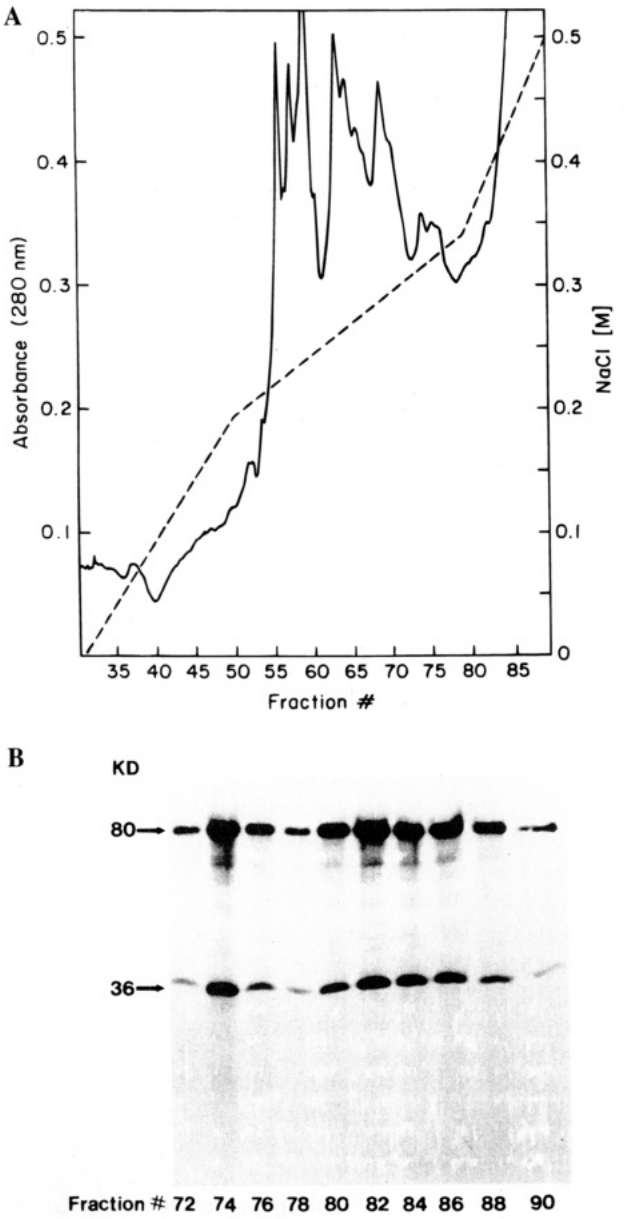


FIGURE 3: Mono-Q chromatography of the 80 kDa GTP-binding protein. (A) Chromatogram: The peak fractions containing the 80 kDa GTP-binding protein were eluted from the DEAE-Sephacel column, dialyzed versus 25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 100 μM PMSF, and then applied to a Mono-Q column that was equilibrated with the same buffer. The protein was eluted from the Mono-Q column using a NaCl gradient (0−0.5 M). The solid line represents the absorbance at 280 nm measured for the eluted protein. The dashed line indicates the NaCl concentrations used to elute the protein. (B) Photolabeling: 20 μL of the indicated fractions was photolabeled with [α-^{32}P]-GTP and applied to a 10% SDS−polyacrylamide gel as indicated under Experimental Procedures. The arrows mark the positions of proteins with apparent molecular masses of 80 and 36 kDa, based on the mobility of known standards.

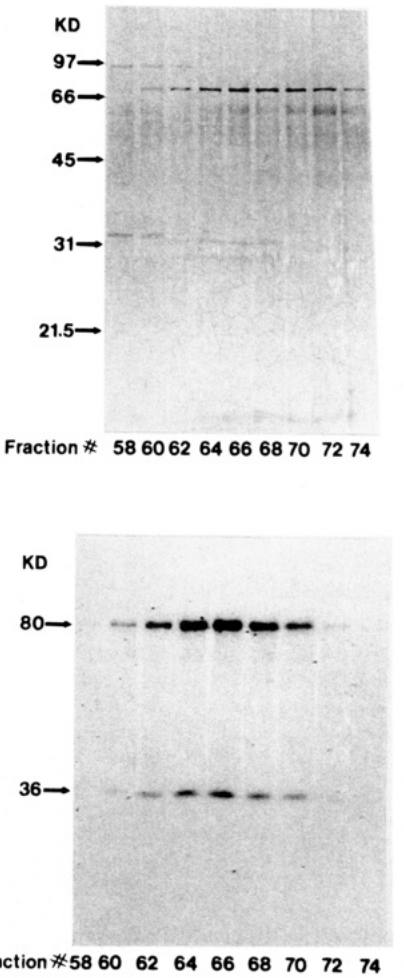
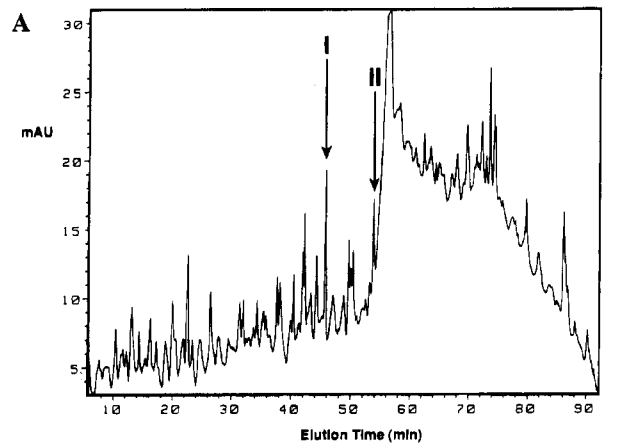


FIGURE 4: Ultrogel AcA34 chromatography of the 80 kDa GTP-binding protein. The fractions from the first peak of the 80 kDa GTP-binding protein, eluted from the Mono-Q column, were concentrated by ultrafiltration and then loaded onto an Ultrogel AcA34 column that was preequilibrated with 25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 100 μM PMSF. (Upper panel) SDS−PAGE: 20 μL of the indicated fractions was applied to a 10% SDS−polyacrylamide gel and silver stained. Molecular masses of standard proteins are shown on the extreme left. (Lower panel) Photolabeling: 20 μL of the indicated fractions was photolabeled with [α-^{32}P]GTP as indicated under Experimental Procedures.

protein, and thus this band was sequenced (see below). Despite the fact that the 36 kDa GTP-binding protein was detected (by incorporation of radiolabeled GTP) in the same fractions as the 80 kDa protein, we were not able to detect this protein by silver staining. Thus, at this stage of the purification, the 80 kDa protein is likely to be present in high excess (i.e., >10-fold) relative to the 36 kDa protein. Since the incorporation of radiolabeled GTP into the 36 kDa protein can be visualized, under conditions where the 80 kDa protein is present in high excess, it appears that the 36 kDa protein is capable of a high efficiency of incorporation of the radiolabeled GTP.

Peptide Sequence Analysis of the 80 and 36 kDa GTP-Binding Proteins. The 80 kDa GTP-binding protein purified by Ultrogel AcA34 chromatography was subjected to trypsin digestion, and the resultant peptide fragments were resolved by reverse-phase HPLC (Figure 5A). Two of these peptides (designated as peptides I and II in Figure 5A) were sequenced and showed a high degree of similarity to the members of the transglutaminase family (Figure 5B). Specifically, the 14 residues of peptide I were identical to sequences in the human and mouse transglutaminase-C enzyme, and 13 of 14 residues were identical to a sequence in the guinea pig liver enzyme. In the case of peptide II, 18 of the 21 residues were identical to a sequence in the guinea pig enzyme, 15 of 21 were identical to a corresponding sequence for the human enzyme, and 14 of 21 were identical to a sequence in the mouse enzyme.

The 36 kDa GTP-binding protein was obtained by pooling three preparations after the Ultrogel AcA34 chromatography



B

Peptide I		V V T N Y N S A H D Q N S N
Guinea Pig Transglutaminase-C	296	V V T N F N S A H D Q N S N 309
Human Transglutaminase-C	297	V V T N Y N S A H D Q N S N 310
Mouse Transglutaminase-C	297	V V T N Y N S A H D Q N S N 310
Peptide II		S Y E A G V D T L T F S A V T G P A P S E
Guinea Pig Transglutaminase-C	48	G Y E A G V D T L T F N A V T G D D P S E 68
Human Transglutaminase-C	49	N Y Q A S V D S L T F S V V T G P A P S Q 69
Mouse Transglutaminase-C	49	G Y Q A S V D S L T F G A V T G P D P S E 69

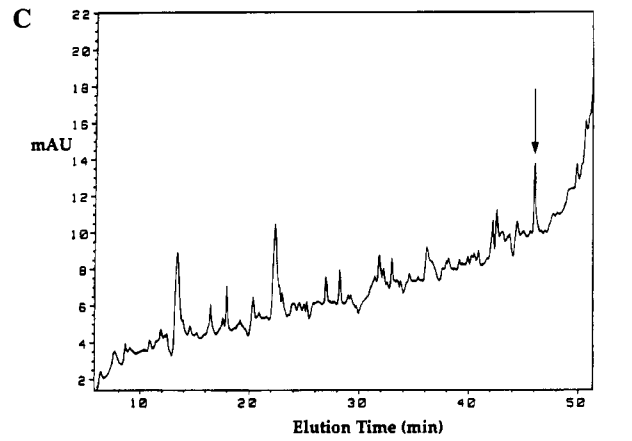


FIGURE 5: Sequence similarities between the 80 and 36 kDa GTP-binding proteins and tissue transglutaminases. (A) Isolation of peptide sequences: The 80 kDa GTP-binding protein was purified by DEAE-Sephacel, Mono-Q, and Ultrogel AcA34 chromatographies as outlined under Experimental Procedures. The Ultrogel-purified 80 kDa protein (which corresponded to the protein photolabeled with [α-^{32}P]GTP) was subjected to SDS−PAGE and then electroblotted onto Problot. The transferred protein was then trypsinized, and the resultant peptides were isolated by reverse-phase HPLC and sequenced by Dr. William Lane (Harvard Medical School Sequencing Facility). (B) Sequence Comparisons: The sequences from peptides I and II (Figure 6A) are aligned with the known sequences of mammalian tissue transglutaminases. Amino acid residues from the different transglutaminases that are identical to the residues of the peptide sequences are shown in boldface type. (C) The 36 kDa GTP-binding protein was purified by DEAE-Sephacel, Mono-Q, Ultrogel AcA34, and hydroxylapatite chromatographies (see Experimental Procedures) and then was sequenced as described in (A).

step and then further purifying the GTP-binding protein by hydroxylapatite chromatography. The 36 kDa GTP-binding protein was then subjected to trypsin digestion, and the peptide fragments were resolved by reverse-phase HPLC (Figure 5C). One of the peptides (see the arrow in Figure 5C) was sequenced (YEAGVDTLTFS) and was found to be identical to a portion of peptide II obtained from the 80 kDa GTP-binding protein (Figure 5B). These results suggest that the 36 kDa GTP-binding protein is likely to be a proteolytic fragment of the 80 kDa protein. This sequence is found within the amino-terminal halves of the tissue transglutaminases (Ikura et al., 1988). Given that the 36 kDa fragment can effectively incorporate [α-^{32}P]GTP, these results suggest that the GTP-binding domain exists within the amino-terminal half of the tissue GTP-binding protein/transglutaminases.

Biochemical Characterization of the 80 kDa GTP-Binding Protein. Overall, the high degree of sequence similarity noted above suggests that the 80 kDa GTP-binding protein represents a rabbit liver form of the general family of tissue transglutaminases. We have analyzed the purified preparation of the 80 kDa protein for GTP-binding, GTPase, and transglutaminase activity. The purified preparation shown in Figure 4 bound [^{35}S]GTPγS with an apparent single class of binding sites with a K_d of ~200 nM. Since the 80 kDa protein represented the majority of the protein present in the preparation (based on silver staining), it is likely that the 80 kDa protein accounted for most of the [^{35}S]GTPγS binding activity. The purified preparation of the 80 kDa GTP-binding protein also was capable of a Mg^{2+}-dependent GTP hydrolytic activity. This activity was linear over a time period of 1 h, and if we assume that the majority of the activity was contributed by the 80 kDa protein, the turnover number was ~1 mol of [^{32}P]P_i released min^{-1} (mol of protein)$^{-1}$ (data not shown).

The transglutaminase activity of the 80 kDa protein was assayed by measuring the incorporation of [^{14}C]putrescine into casein, with the time course of the incorporation being essentially linear through 1 h (data not shown). Figure 6 shows that the transglutaminase activity of the 80 kDa protein is significantly inhibited by GTP binding. Essentially full inhibition of the transglutaminase activity was achieved with ~5 μM GTP when the assays were performed in the presence of 1 mM $MgCl_2$. Interestingly, under identical conditions, GDP showed little or no effect on the transglutaminase activity. These data differ from those reported for the guinea pig liver transglutaminase for which both GDP and GTP were shown to inhibit the enzyme activity (Achyuthan & Greenberg, 1987), although in that case half-maximal inhibition required ~100 μM GTP and ~500 μM GDP. The guanine nucleotide regulation of the nuclear 80 kDa GTP-binding protein/transglutaminase is consistent with a tight coupling of the GTP-binding/GTPase cycle of the 80 kDa protein to its enzyme (transglutaminase) activity.

Anti-Transglutaminase Antibody Recognizes the 80 kDa Nuclear GTP-Binding Protein. Given the high degree of sequence identity between the 80 kDa GTP-binding protein and the guinea pig transglutaminase, we determined if an anti-transglutaminase antibody raised against the guinea pig liver enzyme could immunoprecipitate the Ultrogel-purified 80 kDa GTP-binding protein. The results presented in Figure 7A,B show that this is the case. The monoclonal antibody immunoprecipitates the purified 80 kDa GTP-binding protein

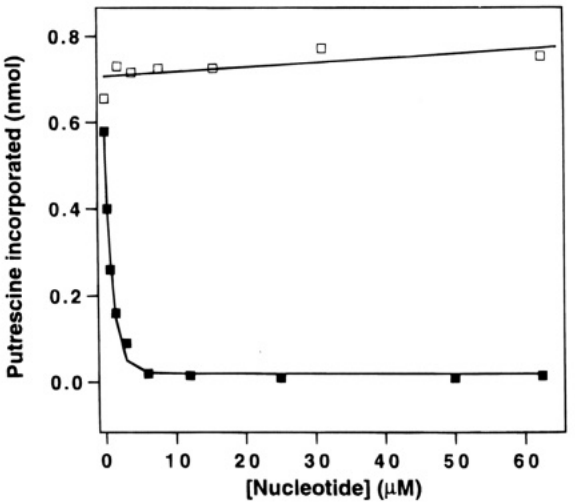


FIGURE 6: Assays of transglutaminase activity of the Ultrogel-purified 80 kDa GTP-binding protein. The 80 kDa GTP-binding protein was purified by DEAE-Sephacel, Mono-Q, and Ultrogel AcA34 chromatographies, incubated with increasing concentrations of GTP (■) and GDP (□), and then assayed (500 ng of protein) for transglutaminase activity by measuring the incorporation of [^{14}C]-putrescine into *N*,*N*-dimethylcaesin for 1 h at 37 °C as indicated under Experimental Procedures.

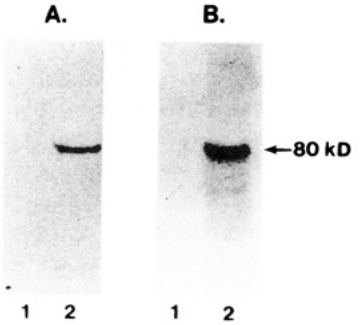


FIGURE 7: Immunoprecipitation with an anti-transglutaminase antibody. An aliquot (20 μL) of the Ultrogel AcA34-purified 80 kDa GTP-binding protein was immunoprecipitated with a monoclonal antibody (CUB 7402) raised against the guinea pig liver transglutaminase (A, lane 2; B, lane 2) or with IgG control antibody (A, lane 1; B; lane 1) as outlined under Experimental Procedures. The proteins were electrophoresed on SDS−PAGE, transferred to nitrocellulose, probed with the anti-transglutaminase antibody, and then visualized with ECL (Amersham) (A), or first labeled with [α-^{32}P]GTP, electrophoresed, and visualized by autoradiography (B).

as visualized either on a Western blot using the transglutaminase antibody (Figure 7A) or when resuspending the precipitate and assaying its ability to photoincorporate [α-^{32}P]-GTP (Figure 7B).

The monoclonal anti-transglutaminase antibody was used in indirect immunofluorescence experiments (Figure 8). Figure 8A,B shows the results obtained for control NIH 3T3 fibroblasts using a control IgG as the primary antibody (Figure 8A) or after DNA staining to identify the nuclear compartment (Figure 8B). As shown in Figure 8C, the anti-transglutaminase antibody shows some general staining of the cytosol, consistent with the known intracellular location of the tissue transglutaminases, and strong nuclear staining (Figure 8D shows the DNA staining of the same cells).

These indirect immunofluorescence results have been complemented by Western blotting of the same nuclear fractions shown in Figure 1A with the anti-transglutaminase antibody. All of the fractions (and not just the Triton X-100/

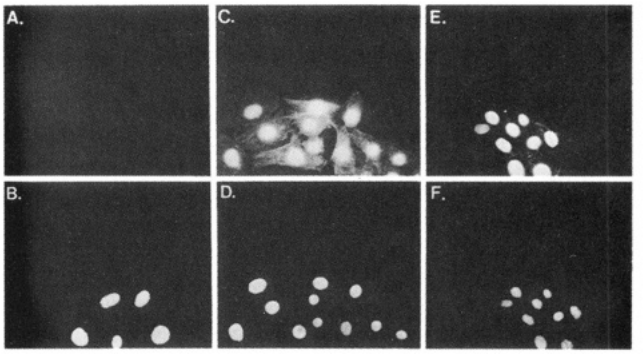


FIGURE 8: Localization of nuclear transglutaminases by immunofluorescence microscopy. Immunolocalization of a transglutaminase to the nucleus in NIH 3T3 cells. Cells were grown on glass coverslips 72 h prior to permeabilization with 0.1% TX-100 in PBS. After being washed thoroughly in cold PBS at 4 °C, cells were fixed and stained as described under Experimental Procedures. (A) Control antibody showing background in the absence of transglutaminase-specific antibody. (B) Anti-transglutaminase (MAb 7402) immunofluorescence pattern. (C) Anti-p62 immunofluorescence showing characteristic staining pattern for this nuclear pore marker. Panels D, E, and F show the DNA staining pattern with Hoest dye #33342 for fields A, B, and C, respectively.

NaCl-solubilized fraction and the insoluble pellet) showed strong immunoreactivity with the anti-transglutaminase antibody (data not shown). This was somewhat unexpected since only the Triton X-100/NaCl-solubilized fraction and the remaining pellet showed measurable [α-^{32}P]GTP binding activity. Thus, these results suggest that while transglutaminase immunoreactivity is present throughout the nucleus, significant GTP-binding activity is only present in the nuclear pore/lamina fraction. These findings further suggest either that a positive regulator for the guanine nucleotide exchange activity of the 80 kDa GTP-binding/transglutaminase is present in the nuclear pore/lamina fraction and/or that a negative regulator is present in other parts of the nucleus.

Since the fractionation data suggested that most of the 80 kDa GTP-binding activity was present in the nuclear pore/lamina, we set out to obtain independent evidence for this suggestion. Given the suggestions that the nuclear pore represented an intricate multiprotein complex, we reasoned that it may be possible to use immunoprecipitation to demonstrate the presence of the 80 kDa GTP-binding protein/transglutaminase in a complex with other nuclear pore-specific marker proteins. We in fact were able to show that one such nuclear pore-specific marker, p62, appears to be present in a complex together with the 80 kDa GTP-binding protein/transglutaminase. The p62 protein was originally identified to be specific for the nuclear pore by Davis and Blobel (1986). Using a specific monoclonal anti-p62 antibody, kindly provided by Dr. L. Davis (Duke University Medical Center), we find that it is present in the same nuclear fractions (and in the same proportion) as those that show the 80 kDa GTP-binding activity (data not shown). Indirect immunofluorescence studies (see Figure 8E,F) also show that the anti-p62 antibody yields a highly specific nuclear staining. The results presented in Figure 9 show that the anti-transglutaminase antibody [that was used to precipitate the 80 kDa GTP-binding protein (Figure 7B)] will specifically precipitate the nuclear pore-specific marker, p62, together with the 80 kDa GTP-binding protein from nuclear envelopes treated with 1% Triton X-100 and 0.5 M NaCl. While these results suggest that the 80 kDa GTP-binding protein/transglutaminase is present in a complex with the nuclear

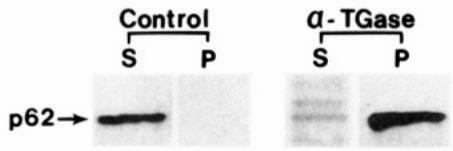


FIGURE 9: Coimmunoprecipitation of the 80 kDa GTP-binding/transglutaminase with the nuclear pore-specific protein p62. Rabbit liver nuclei were solubilized with 1% Triton-X 100 and 0.5 M NaCl (Experimental Procedures), and then 20 μL of the solubilized extract was immunoprecipitated with the anti-transglutaminase monoclonal antibody (CUB 7402) or with nonimmune IgG as a control. Following electrophoresis on SDS−PAGE and transfer to nitrocellulose, Western blotting was performed with an anti-p62 monoclonal antibody (kindly provided by Dr. L. Davis, Duke University Medical Center). S refers to the supernatant fraction and P refers to the pellet, following immunoprecipitation with the anti-transglutaminase antibody.

pore-specific 62 kDa protein, we do not yet know if this represents a direct interaction between these two proteins or if the anti-transglutaminase antibody is precipitating a large complex of nuclear pore proteins such that the association of the 80 and 62 kDa proteins is indirect.

DISCUSSION

In the work described here, we present the purification and characterization of an 80 kDa, high-affinity GTP-binding protein that is present in rabbit liver nuclear envelope preparations and contains sequence identity with the family of mammalian (tissue) transglutaminases. We show that the purified 80 kDa protein is capable of being immunoprecipitated with a specific monoclonol antibody raised against the guinea pig liver transglutaminase and is coprecipitated with a nuclear pore-specific marker, and that the purified nuclear GTP-binding protein possesses transglutaminase activity. These results, together with those from other studies that reported that tissue transglutaminases were capable of binding GTP (Achyuthan & Greenberg, 1987; Takeuchi et al., 1992), indicate that the 80 kDa protein is a dual-function macromolecule that serves as a high-affinity GTP-binding/GTPase and a transglutaminase enzyme. The transglutaminases characterized to date are calcium-dependent enzymes that catalyze the formation of covalent bonds between peptide-bound glutamyl residues and the primary amino groups in a variety of compounds, including the ϵ-amino group of lysine in certain proteins. These enzymes have been shown to be involved in a variety of biological responses that include the stabilization of the structure of fibrin (Sakata & Aoki, 1982), stiffening of the erythrocyte membrane (Siefring et al., 1978), the formation of covalently cross-linked matrices of proteins at sites of cell−cell contacts (Slife et al., 1986), formation of the cornifying envelope in keratinocytes (Rice & Green, 1978), wound healing (Mosher & Schad, 1979), cell growth and differentiation (Higashijima et al., 1987), and in nerve regeneration through the dimerization of interleukin-2 (Eitan et al., 1994). The specific mechanisms by which the transglutaminases participate in these different activities remain to be determined. However, given the plausible role of cellular transglutaminases in the maintenance and regulation of membrane structure, it is tempting to speculate that the nuclear transglutaminase described here may play a role in the biosynthesis or stabilization of the nuclear envelope and/or the nuclear pore (see below).

Throughout the purification of the 80 kDa protein, a 36 kDa GTP-binding protein was present in the peak fractions that contained the 80 kDa protein. The sequence that was determined for a trypsin-generated peptide from the 36 kDa protein was identical to a portion of one of the sequences obtained for the 80 kDa protein. The simplest interpretation of these results is that the 36 kDa protein is a proteolytic fragment of the 80 kDa GTP-binding protein. However, it is not clear why the 36 kDa protein appears to copurify with the 80 kDa protein throughout the purification protocol. One possibility is that the 80 kDa protein is clipped by a protease to yield a 36 kDa fragment, but because of tertiary structural interactions, the clipped 80 kDa protein remains intact and yields the 36 kDa fragment only following its exposure to SDS and denaturation. This also would require that the proteolyzed 80 kDa protein maintains its original tertiary structure and copurifies with the intact 80 kDa protein through various chromatographic steps. Another possibility is that the 36 kDa fragment, which is generated by proteolysis, is capable of binding to a distinct portion of the 80 kDa transglutaminase molecule, such that this binding mimics an intramolecular interaction that normally occurs within the intact protein. On the basis of comparisons with the known amino acid sequences of other tissue transglutaminases, it appears that the 36 kDa protein is derived from the amino-terminal half of the 80 kDa protein. The fact that the 36 kDa protein binds [α-^{32}P]GTP indicates that the guanine nucleotide binding domain is contained within this portion of the 80 kDa protein. A potentially interesting feature of the 36 kDa protein is that it incorporates (through photoaffinity labeling) [α-^{32}P]GTP with significantly higher efficiency than the 80 kDa protein. It is possible that upon proteolysis, the guanine nucleotide binding site of this GTP-binding protein/transglutaminase becomes much more accessible and/or a conformational change occurs so that a side chain of an amino acid residue that incorporates the radiolabeled GTP becomes more susceptible to photoaffinity labeling. In this regard, the enhanced GTP binding by the 36 kDa fragment may be related to the proteolytic activation of the 77 kDa transglutaminase 3 enzyme (Kim et al., 1990).

The binding of GTP to the 80 kDa protein strongly attenuates its transglutaminase activity whereas the binding of GDP has little or no effect. The attenuation by GTP absolutely requires millimolar concentrations of Mg^{2+} (data not shown), suggesting that GTP and Mg^{2+} may induce distinct conformational states within the 80 kDa protein, similar to the case for heterotrimeric G proteins where both Mg^{2+} and GTP are required for activating conformational changes that lead to G protein/effector interactions (Higashijima et al., 1987). This regulation differs from that which has been reported for other tissue transglutaminases where both GDP and GTP (at significantly higher concentrations than used here) have been shown to inhibit the enzyme activity (Achyuthan & Greenberg, 1987). In the latter cases, it is difficult to envisage when the transglutaminase would be active (since presumably the protein would always contain bound guanine nucleotide, i.e., either GDP or GTP), although it is possible that increases in cellular Ca^{2+} may relieve the inhibitory constraint imparted by GDP or GTP (Achyuthan & Greenberg, 1987).

Recently it has been reported that a 70 kDa GTP-binding protein, originally termed G_h (Im & Graham, 1990), which copurified with the rat liver α_1-adrenergic receptor and activates phospholipase C (Im et al., 1992), also shares sequence similarity with the type-C (or type-II) tissue

transglutaminases and contains transglutaminase activity (Nakaoka et al., 1994). As we have observed with the 80 kDa nuclear GTP-binding protein, the transglutaminase activity of the G_h protein is strongly inhibited by GTPγS. Thus, the 80 kDa nuclear protein and the plasma membrane-associated G_h protein appear to be members of a larger family of dual-function G protein/transglutaminases for which the regulation of the GTP-binding/GTPase cycle has direct consequences on the transglutaminase activity.

The cell fractionation studies, as well as the indirect immunofluorescence studies, suggest that the 80 kDa protein is present throughout the nuclear envelope. However, the [α-^{32}P]GTP-binding activity is strongest in the nuclear pore/lamina fractions. These findings raise a number of important questions, namely, what other (regulatory) proteins are associated with the 80 kDa GTP-binding/transglutaminase and influence its activity and what is the role of this GTP-binding protein in the nucleus. It will be of interest to determine if distinct proteins are responsible for the regulation of the GTP-binding/GTPase cycle of the nuclear 80 kDa protein, as is the case for the ras-like proteins (Evans et al., 1991). The identification of such regulatory proteins as well as knowledge of their cellular location may provide important clues regarding the specific physiological role of the 80 kDa GTP-binding protein/transglutaminase in the nucleus. It also will be important to determine whether the presence of an 80 kDa GTP-binding/transglutaminase in the nucleus is related to or involved in the regulation of nuclear phospholipase C enzymes (Martelli et al., 1992; Divecha et al., 1993). Moreover, the nuclear pore, itself, is still relatively poorly understood on a biochemical level, and, in particular, only a small number of the total proteins comprising the nuclear pore have been identified. Although a three-dimensional structure for the nuclear pore has been proposed (Hinshaw et al., 1992), a formidable task lies ahead in terms of understanding how the intricate network of connections comprising the nuclear pore is stabilized and how this complicated structure mediates the passive diffusion of ions and small molecules and the active transport of large molecules and ribonucleoprotein particles. Given the intricacy of the different connections between the subunits of the nuclear pore and the necessity for the pore to continuously assemble and disassemble with each cell cycle, it will be of interest to determine whether the 80 kDa transglutaminase plays an active role in the generation and/or breakdown of this complex structure. The tight guanine nucleotide-dependent influence on the transglutaminase activity of the nuclear 80 kDa protein could represent an important regulatory step in these structural changes.

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