Evidence for Multiple, ras-like, Guanine Nucleotide-binding Proteins in Swiss 3T3 Plasma Membranes

STIMULATION OF GTPase ACTIVITY BY CYTOSOLIC FACTORS

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Alan Wolfman†, Adriana Moscucci, and Ian G. Macara
From the Environmental Health Sciences Center, Department of Biophysics, University of Rochester Medical Center, Rochester, New York 14620

A novel method for the analysis of putative G-proteins has been developed that reveals the existence of a large family of GTP/GDP-binding proteins with similar characteristics to those of p21 ras in 3T3 cell plasma membranes. In the presence of Mg**, exchange of [a-32P]GDP with prebound ligand was very slow, but, as with p21 ras, exchange was dramatically accelerated by excess EDTA. In the presence of Mg**, three classes of binding sites were distinguishable. However, no p21 ras was detected in the membranes with the pan-reactive anti-ras antibody, Y13-259. Gel filtration analysis resolved two peaks of binding activity centering at 60 and 21 kilodaltons. High resolution anion exchange chromatography separated at least 11 unique GDP-binding proteins from 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate-solubilized membranes, none of which cross-reacted with a pan-G, antiserum. Analogous to p21 ras, the binding activities of 9 of the 11 species were sensitive to the thiol reagent N-ethylmaleimide, and six peaks possessed detectable GTPase activity in the absence of extrinsic factors. Addition of cytosol activated the GTPase activity of four of the peaks. We infer that the 11 peaks represent novel, small molecular weight guanine nucleotide-binding proteins, similar to those recently described in brain membranes.

The classical G-proteins are transducing elements that couple hormone receptors to intracellular signal generators. They have low intrinsic GTPase activity, and the exchange of GDP for GTP is controlled by receptor-ligand interactions. Classical G-proteins couple hormone receptors to intracellular signal generators. They have low intrinsic GTPase activity, and the exchange of GDP for GTP is controlled by receptor-ligand interactions (Fleischman et al., 1986; Wakelam et al., 1986; Wolfman and Macara, 1987; Laical et al., 1987). The three known p21 ras proteins (Harvey, Kirsten, and N-) bind GDP and GTP, possess intrinsic GTPase activity, exhibit very slow off-rates for GDP in the presence of Mg**, (Hall and Self, 1986; Feuerstein et al., 1987; Poe et al., 1985) and can substitute for yeast G-proteins that regulate adenylate cyclase (Broek et al., 1985). However, they do not regulate this system in mammalian cells (Birchmeier et al., 1988), and the evidence supporting their role as coupling factors for polyphosphoinositides is unconvincing. Recently, a large class of ras-like G-proteins has been discovered in brain tissue (Yamamoto et al., 1988; Kikuchi et al., 1988). It is possible that members of this class function to couple or modulate the transmission of signals generated by receptor-ligand interactions. One approach to the search for members of this family has been to screen cDNA libraries at low stringency with G-protein or ras-related probes. Such studies have uncovered a number of new ras-related genes, designated rab, ral, rap, rho, and R-ras (Touchot et al., 1987; Chardin et al., 1987; Madaule and Axel, 1985; Lowe et al., 1987; Pizon et al., 1988). Proteins homologous to the genes rho and rab3 have recently been isolated from bovine brain plasma membranes (Yamamoto et al., 1988; Kikuchi et al., 1988; Matsui et al., 1988).

Utilizing the unusually slow off-rates for GDP of these ras-like proteins in the presence of Mg**, we have been able to rapidly separate and detect multiple GDP/GTP-binding proteins in Swiss 3T3 membranes. The use of [a-32P]GDP rather than another nonphysiological affinity labels, such as [35S]GTP-gamma-S (Yamamoto et al., 1988; Katada et al., 1987; Uhing et al., 1987) provides the opportunity for detailed kinetic analyses of physiological relevance and provides the potential for studying guanine nucleotide exchange and GTPase activity after stimulation with growth factors, mitogens, or regulatory molecules such as GTPase activating protein (GAP)1 (Trahey and McCormick, 1987; Adari et al., 1988; Cales et al., 1988).

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† To whom correspondence should be addressed: Environmental Health Sciences Center, 601 Elmwood Ave., University of Rochester Medical Center, Rochester, NY 14620. Tel.: (716) 275-5441.

1 The abbreviations used are: GAP, GTPase-activating protein; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; NEM, N-ethylmaleimide; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; GTP-gamma-S, guanosine 5'-O-(thiotriphosphate).
Guanine Nucleotide-binding Proteins in Fibroblast Membranes

EXPERIMENTAL PROCEDURES

Materials—The [-a(32P)]GTP (~3000 Ci/mmol) was purchased from Amersham Corp., the rabbit anti-rab-ant secondary antibody from Sigma and [3H]polyethyleneimine cellulose (255 Ci/mg) fragments were from Du Pont New England Nuclear. Proteinase inhibitors and CHAPS detergent were from Boehringer Mannheim. The anti-G, , a common antibody, J-882, was kindly provided by the laboratory of A. Gilman (University of Texas, Dallas, TX). The recombinant p21" protein was a generous gift from S. Asasmov and J. C. Lacal (University of Texas, Dallas, TX). Anti-ARF antiserum was kindly provided by Richard Kahn (NIH, Bethesda, MD). An anti-rab3 rabbit antiseraum was generated against a synthetic peptide corresponding to residues 158-172 of the putative rab3 amino acid sequence (Touchot et al., 1987). The Y13-259 ras antibody was purchased from Oncogene Science. The remainder of the reagents were from standard vendors. The GF-250 column was from Du Pont and the Mono Q column was from Pharmacia LKB Biotechnology Inc.

Cell Cultures—All cell cultures were maintained in Dulbecco's modified essential medium supplemented with 10% calf serum at 37 °C and 5% CO2. Prior to membrane preparation, cell cultures (50% confluent) were serum starved (0.5% serum) for 24-36 h. Membrane Isolation—Cell cultures were washed with phosphate-buffered saline, scraped off their dishes with a rubber spatula, and pelleted by centrifugation. To stabilize the GDP-binding Mg2+ (10 mM), GDP (1 mM), and DTTP (1 mM) were added to all solutions. The cell pellets were resuspended in an equal volume of lysis buffer (1 mM sodium bicarbonate, 0.5 mM CaCl2, pH 7.5), allowed to swell on ice for 10 min, then ruptured by 50-75 strokes of a Dounce homogenizer. The homogenate was then added to a mixture as described by Brusette and Till (1971) except that the upper phase was equilibrated with 1 mM CaCl2. The samples were centrifuged at 10,000 X  g for 10 min. Membranes at the interface were collected, the pellet material resuspended, recentrifuged, and the two fractions of membranes combined. These were then diluted with ice-cold water (20 X the volume of membranes) and centrifuged at 10,000 X  g for 70 min. The pellet material was resuspended in 300 mM sucrose, 20 mM Hepes, pH 7.4, 1 mM EDTA, snap frozen in liquid nitrogen and stored at -80 °C. Once thawed, samples were not refrozen. Protein was determined by the method of Bradford (1976). Typically, 50 μg of membrane protein were obtained from 106 cells. Membrane preparations were resuspended at approximately 2.5 mg/ml protein.

Conversion of [-a(32P)]GTP to [-a(32P)]GDP—The [-a(32P)]GTP was evaporated to dryness under a stream of nitrogen. The reaction mixture (100 μl) consisted of 20 mM Hepes, pH 7.4, 2 mM MgCl2, 5 μg/ml nucleotide diphosphokinase, 2 mM uridine diphosphate, 0.25 mM DTTP, 10% glycerol, and 0.1 mM EDTA. The reaction was allowed to proceed for 30 min at 30 °C. The nucleotide diphosphokinase was inactivated by adjusting the pH to 12 with 4 μl of 1 N NaOH. After 10 min on ice, the reaction mixture was neutralized with 4 μl 1 N HCl, heated to 65 °C for at least 95% and analyzed by thin layer chromatography on polyethyleneimine cellulose using 0.75 M Tris, 0.45 M HCl, and 0.5 M LiCl as the solvent system (Bochner and Ames, 1982). [-a(32P)]GDP Binding Assay—Binding was performed in 20 ml Hepes, pH 7.4, 1 mM EDTA, 50 mM KCl, 10 mM DTTP, 5 mM MgCl2, 1.67 mM CaCl2, 500 mM GDP and 0.5-25 μCi [32P]GDP with 10-100 μg of membrane protein. Generally, 0.5 μg/50 μl [32P]GDP was used for whole membrane binding studies, with 20-25 μg/50 μl being used for labeling membranes to be chromatographically analyzed. Fifty μl reaction volumes were incubated at 30 °C for 10 min. When appropriate, 1 μl of 50 mM MgCl2 was added to stabilize the binary complexes. GDP binding was assessed by a nitrocellulose (0.45 μm) filter assay (Hall and Self, 1986). Each reaction mixture tube was washed twice (1.5 ml each) with ice-cold quenching solution (50 mM Tris, 10 mM MgCl2, 1 mM DTTP, and 1 mM ATP, pH 6.8). Each filter was then washed three times (1.5 ml each) with quenching solution. The radioactivity remaining on the filters was a measure of GDP binding. Typically, background counts were about 10% of total bound [32P] in the presence of 1 μg of membrane protein. Chase experiments were performed by first preloading the membranes with radiolabeled GDP, adding Mg2+ to stabilize the complexes, then adding an excess of unlabeled GDP. Controls to correct for the loss of binding activity as a consequence of time-dependent protein denaturation were performed by the addition Mg2+ and without the addition of unlabeled GDP. Control samples were measured for every point in the time course. The data were fitted by a nonlinear least squares equation describing three independent binding sites. Membranes were solubilized at a final CHAPS concentration of 3% (w/v) after Mg2+ addition in preparation for fast protein liquid chromatography analysis. Samples were incubated on ice for 20 min followed by centrifugation (4 °C) in a microcentrifuge at 10,000 X  g for 8 min. The supernatant was then directly applied to the appropriate column for analytical purposes.

Chromatography—All fast protein liquid chromatography analyses were performed at 4 °C. Standard buffers used were 10 mM Tris, pH 7.0 at room temperature, 8 mM CHAPS (0.5%, w/v), 1 mM MgCl2. Buffer A contained no NaCl, while buffer B was made 1 M NaCl. Proteinase K (1-2 mM) was added to each of the above buffer sets. Filter binding assays were immediately performed on each of 10 fractions as they were eluted off their respective columns.

The Mono Q column has a 2-ml bed volume. The column was eluted at a flow rate of 1 ml/min, and 0.65-ml fractions were collected. The GF-250 gel filtration column (12-ml bed volume) was eluted with 10 mM Tris, pH 7.0, 1 mM DTTP, 1 mM MgCl2, 400 mM NaCl at a flow rate of 0.2 ml/min. To collect small fractions accurately, a 20-μl glass pipette was stretched out to a fine point and inserted into the fraction collector tubing. In this way 50-μl fractions (2.5 μl) could be collected reproducibly.

Production of GAP-containing Extracts—Cytosolic extract was prepared from Swiss 3T3 cells grown to confluence. All procedures were done at 4 °C. Cells were washed with phosphate-buffered saline containing EDTA (1 mM) and washed on their dishes in the same buffer and centrifuged at 400 x  g for 10 min at 4 °C. Cells were resuspended in 1 M NaCl. Following a Dounce homogenization, in hypotonic Tris buffer (10 mM, pH 7.5) containing MgCl2 (5 mM), DTTP (1 mM), pepstatin A (5 μg/ml), trypsin inhibitor (80 μg/ml), and para-nitrophenol phosphate (5 mM), and the resulting suspension was centrifuged for 30 min at 15,000 x  g. The supernatant was snap-frozen and stored at -80 °C. As a control, cytosolic extracts were assayed for GAP activity directed toward recombinant p21" as described by Trahey and McCormick (1987).

Determination of GAPase Activity—Plasma membranes (100 μg) were labeled in the absence of Mg2" with [-a(32P)]GTP (500 nM, 3000 Ci/mmol) for 90 min at 30 °C in 50 mM Tris, 10 mM MgCl2, 1.67 mM CaCl2, 500 nM GDP and 0.5-25 μCi [32P]GTP with 10-100 μg of membrane protein. Generally, 0.5 μg/50 μl [32P]GDP was used for whole membrane binding studies, with 20-25 μg/50 μl being used for labeling membranes to be chromatographically analyzed. Fifty μl reaction volumes were incubated at 30 °C for 10 min. When appropriate, 1 μl of 50 mM MgCl2 was added to stabilize the binary complexes. GDP binding was assessed by a nitrocellulose (0.45 μm) filter assay (Hall and Self, 1986). Each reaction mixture tube was washed twice (1.5 ml each) with ice-cold quenching solution (50 mM Tris, 10 mM MgCl2, 1 mM DTTP, and 1 mM ATP, pH 6.8). Each filter was then washed three times (1.5 ml each) with quenching solution. The radioactivity remaining on the filters was a measure of GDP binding. Typically, background counts were about 10% of total bound [32P] in the presence of 1 μg of membrane protein. Chase experiments were performed by first preloading the membranes with radiolabeled GDP, adding Mg2+ to stabilize the complexes, then adding an excess of unlabeled GDP. Controls to correct for the loss of binding activity as a consequence of time-dependent protein denaturation were performed by the addition Mg2+ and without the addition of unlabeled GDP. Control samples were measured for every point in the time course. The data were fitted by a nonlinear least squares equation describing three independent binding sites. Membranes were solubilized at a final CHAPS concentration of 3% (w/v) after Mg2+ addition in preparation for fast protein liquid chromatography analysis. Samples were incubated on ice for 20 min followed by centrifugation (4 °C) in a microcentrifuge at 10,000 X  g for 8 min. The supernatant was then directly applied to the appropriate column for analytical purposes.

Guanine Nucleotide-binding Proteins in Fibroblast Membranes

10821
Detection of Guanine Nucleotide-binding Proteins by GTP Blotting—GTP blotting of membrane proteins was performed by a modification of the procedure described by Bhullar and Haslam (1987). Following electrophoretic transfer, the nitrocellulose was washed once in buffer (50 mM sodium phosphate, pH 7.3, 10 μM MgCl₂, 0.3% Triton X-100). The nitrocellulose was then incubated for 30 min with the same buffer plus 1 μCi/ml [α-32P]GTP, followed by three washes with the same buffer. GTP-binding proteins were detected by autoradiography at -70°C using a Kodak intensifying screen.

RESULTS

GDP Binding to Swiss 3T3 Plasma Membranes—We first examined the general characteristics of [α-32P]GDP binding to partially purified plasma membranes from serum-starved Swiss 3T3 cultures. As shown in Fig. 1A, the rate of GDP association with the membranes was rapid in the absence of Mg²⁺, whereas equilibrium in the presence of millimolar Mg²⁺ (30°C) required at least 1 h, as if the [α-32P]GDP were slowly exchanging with an unlabeled pool of bound nucleotide. Similar effects of Mg²⁺ on GDP binding have been described previously for p21ras (Hall and Self, 1986; Feurestein et al., 1987; Poe et al., 1985). The possibility that exchange is the rate-limiting step for association is supported by the observation that the absence of Mg²⁺ dramatically accelerates [α-32P]GDP release from the membranes (Fig. 1B). However, if Mg²⁺ is added subsequent to the prebinding period, three classes of GDP-binding sites are distinguishable. The data were fit by an iterative nonlinear least squares procedure using equations describing multiple exponential decay. The best fit to the data was obtained for a three-term model with the following parameters for the percent contribution to total binding and first order rate constants: 1) 15% of total binding activity with a kₐ of 4.7 × 10⁻⁴ s⁻¹, 2) 26% with a kₐ of 3.3 × 10⁻³ s⁻¹, and 3) 59% with a kₐ of 2.7 × 10⁻² s⁻¹. These rate constants correspond to t₀ values at 30°C of 0.25 min, 3.5 min, and 7.1 h, respectively. In the presence of Mg²⁺ at 4°C, the t₀ of the rapidly exchanging pools are considerably increased (Fig. 1B). The kₐ for membranes in the presence of Mg²⁺ at 30°C is about 4 × 10⁻² M⁻¹ s⁻¹. In our experiments control binding (without addition of unlabeled GDP) diminished slowly over time (by 35% at 1 h; 55% at 3 h), suggesting that significant denaturation of the proteins occurs with extended incubation at 30°C. The large amount of total binding lost through protein denaturation also complicates the binding measurements in the presence of Mg²⁺ (Fig. 1A).

Specific high affinity binding of [α-32P]GDP to the membranes saturates at roughly 500 nM GDP, with an apparent overall Kₐ value of approximately 50 nM (Fig. 1C). The total specific binding capacity of the membranes for GDP averaged 100 fmol/μg membrane protein. To ensure that the observed binding constants were not being skewed by hydrolysis of the [α-32P]GDP to GMP, we examined the rate of [α-32P]GDP hydrolysis by 3T3 fibroblast membranes. No appreciable breakdown (10% or less) of the [α-32P]GDP was observed after 20 min at 30°C in the presence of membranes (15 μg) (data not shown).

The high specificity of this [α-32P]GDP binding to 3T3 plasma membranes is illustrated by the nucleotide competition data shown in Table 1. Of the related nucleotides tested, only GTP-Y-S efficiently inhibited [α-32P]GDP binding.

Detection of Multiple GDP-binding Proteins by High Resolution Chromatography—We performed gel filtration analysis to assess the amount of GDP-binding to the plasma membranes that was originating from the α subunits of the trimeric G-proteins, G₁α and G₁β. On gel filtration analysis, these guanine nucleotide-binding proteins possess a reported mobility of approximately 115 kDa (Strittmatter and Neer, 1980). The 25-kDa ras-related rab3 protein has also been reported to

FIG. 1. Binding kinetics of GDP to Swiss 3T3 plasma membranes. All assays were performed in duplicate with each sample being within 5% of its match. A, on-rates were measured in the presence (closed circles, ○) or absence (open circles, □) of Mg²⁺ (10 mM). At each time point, samples were immediately filtered through nitrocellulose. B, off-rates were determined by first preloading the samples with radiolabeled ligand in the absence of magnesium for 5 min at 30°C. At time zero, magnesium was added (open circles, ○; closed circles, ●; 30°C) or not (open squares, □; 30°C) along with 2 μl of 1 mM GDP (final concentration of 40 μM). The amount of radiolabeled GDP remaining bound was assessed at the specified times. C, the binding capacity of the membranes was assessed by increasing the concentration of GDP and holding the amount of membrane protein constant (3 μg). Values are means of triplicate measurements ± 1 SD. The line through the data (B, closed circles) was determined by an iterative nonlinear least squares procedure using the following equation for a multieponential decay: A(t)/A₀ = Σ(xᵢ-εᵢ)*e⁻ᵏᵢᵗ, where A(t)/A₀ = proportion of total GDP remaining bound at time t, xᵢ = proportional contribution to total binding capacity and kᵢ = first order rate constant. The minimum number of terms that provided a good fit to the data was n = 3. The best fit parameters are: x₁ = 0.15, k₁ = 4.7 × 10⁻² s⁻¹; x₂ = 0.26, k₂ = 3.3 × 10⁻³ s⁻¹; and x₃ = 0.59, k₃ = 2.7 × 10⁻⁵ s⁻¹.
peaks of GDP binding activity. To rule out proteolysis as an explanation for the heterogeneity of the GDP-binding proteins, 3T3 plasma membranes were labeled with nucleotides and resolved by high resolution chromatography. The resulting elution profile revealed no significant alterations in either GDP concentrations between 500 nM [α-32P]GDP, solubilized and applied to the Mono Q column as described under "Experimental Procedures." The column was eluted at 1 ml/min with 0.65-ml fractions being collected. Each set of 10 fractions was immediately filtered. The closed circles (●) represent the amount of [α-32P]GDP retained on the nitrocellulose filters for each fraction, and the solid line represents the salt gradient used for the elution profile.

were prepared in the presence or absence of multiple inhibitors to different classes of proteases, as follows: Aprotinin (20 μg/ml) to inhibit serine protease; leupeptin (10 μM) to inhibit trypsin-like proteases; trypsin inhibitor (20 μg/ml) to inhibit trypsin-like proteases; betatin (50 μg/ml) to inhibit aminopeptidases; pepstatin A (2.5 μg/ml) to inhibit cell acid proteases; α,β-macroglobulin (0.1 unit/ml) to inhibit general endopeptidases; EDTA (0.1 mM) to inhibit metallo-proteases; and phosphoramidon (50 μg/ml) to inhibit metallo-endoprotease. When appropriate, all solutions used for membrane preparations, including the buffered saline for washing the cells, contained this protease inhibitor mixture. Moreover, all procedures were performed with ice-cold solutions, and the elapsed time from harvesting the cells to completion of the membrane preparation was no more than 2.5 h. The membranes prepared in the absence of protease inhibitors were warmed during the initial homogenization. The elution profiles for the two membrane preparations revealed no significant differences in the positions or relative abundance of the individual GDP-binding proteins (data not shown). Nonetheless, protease inhibitors were used in all subsequent preparations.

The stabilization of GDP binding at 4°C (Fig. 1B) is sufficient to allow, in principle, the detection of proteins belonging to the three different kinetic classes of GDP-binding proteins described earlier. Binding profiles were assessed on [32P]GDP labeled membranes before and after a 10 min (30°C) chase with an 80-fold excess of unlabeled GDP (plus a 15-min chase at 4°C during solubilization of the membranes). The data (Table II) indicate that at least four peaks of binding activity (8, 9, 10, and 11) possess GDP off-rates of less than ten min. Four other peaks of GDP binding activity (1, 3, 6, and 7) possess t1/2 values between 10 min and 1 h while peaks 2, 4, and 5 exhibit t1/2 values much greater than 1 h. In all cases it is possible that each peak may represent more than one GDP-binding protein, each having different intrinsic off-rate kinetics. Therefore, the values presented in Table II for the individual peaks may represent an average value for multiple GDP-binding proteins eluted at each position.

**Sensitivity of GDP Binding Activity to N-Ethylmaleimide—**

The p21 oncogene family possesses NEM-sensitive cysteine residues which when modified block guanine nucleotide binding (Hattori et al., 1986). We therefore tested the NEM-sensitivity of the various GDP-binding proteins resolved by

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**Table 1**

**Specificity of GDP binding to Swiss 3T3 plasma membranes**

Membranes (1 μg) were incubated in the absence of Mg2+ and the presence of the above nucleotides in addition to 500 nM [α-32P]GDP for 5 min. Samples were filtered and the retained radioactivity measured by liquid scintillation counting. Duplicate samples were within 5% on each other. The extent of inhibition for all the above compounds was obtained on two separate membrane preparations.

<table>
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<th>Added agent</th>
<th>Conc. (µM)</th>
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<tr>
<td>GTPγS</td>
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**Fig. 1**

**GF-250 analysis of native molecular weights of plasma membrane GDP-binding proteins.** Prelabel solubilized membranes (30 μg) were injected on to the gel filtration column and eluted (open circles, ○) as described under "Experimental Procedures." The molecular weight markers (inverted solid triangles, ▲) used were β-amylase (200,000), alcohol dehydrogenase (150,000), carbonic anhydrase (29,000), and cytochrome c (12,400). Calibration of the column was performed under identical conditions used for sample analysis.

elute at an anomalously high apparent molecular mass of approximately 50 kDa (Kikuchi et al., 1988). To determine the apparent molecular weights of the GDP-binding proteins in Swiss 3T3 membranes, CHAPS-solubilized preparations were analyzed on a GF-250 gel filtration column. Control experiments revealed no significant alterations in either GDP binding capacity (Table I) or GDP off-rates when membrane fragments were solubilized, either before or after labeling with [α-32P]GDP (data not shown). A typical profile is shown in Fig. 2. Two major molecular mass classes centered at 60 and 21 kilodaltons were resolved.

To determine the extent of heterogeneity of the GDP-binding proteins, 3T3 plasma membranes were labeled with [α-32P]GDP, solubilized and analyzed by high resolution anion exchange chromatography. The resulting elution profile (Fig. 3) illustrates the clear resolution of at least 11 unique peaks of GDP binding activity. To rule out proteolysis as an explanation for the heterogeneity of the GDP-binding proteins resolved by high resolution chromatography, membranes

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**Fig. 2.** GF-250 analysis of native molecular weights of plasma membrane GDP-binding proteins. Prelabel solubilized membranes (30 μg) were injected on to the gel filtration column and eluted (open circles, ○) as described under "Experimental Procedures." The molecular weight markers (inverted solid triangles, ▲) used were β-amylase (200,000), alcohol dehydrogenase (150,000), carbonic anhydrase (29,000), and cytochrome c (12,400). Calibration of the column was performed under identical conditions used for sample analysis.

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**Fig. 3.** Detection of multiple GDP-binding proteins by high resolution anion exchange chromatography. Membranes were prelabeled with [α-32P]GDP, solubilized and applied to the Mono Q column as described under "Experimental Procedures." The column was eluted at 1 ml/min with 0.65-ml fractions being collected. Each set of 10 fractions was immediately filtered. The closed circles (●) represent the amount of [α-32P]GDP retained on the nitrocellulose filters for each fraction, and the solid line represents the salt gradient used for the elution profile.
TABLE II

<table>
<thead>
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<th>Peak</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<th>8</th>
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<tr>
<td>% GDP lost after 10-min chase</td>
<td>29</td>
<td>0</td>
<td>37</td>
<td>0</td>
<td>0</td>
<td>10</td>
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<td>63</td>
<td>87</td>
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<td>&gt;60</td>
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<td>&gt;60</td>
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Fig. 4. NEM-sensitivity of the resolved GDP-binding proteins. Membranes (25 µg) were pretreated with 10 mM NEM for 10 min at 30 °C. The membranes were then added to the reaction mixture containing 20 mM DTT, incubated at 30 °C for an additional 10 min, solubilized, and the GDP-binding profile obtained as previously described (open triangles, Δ). The control membranes were incubated at 30 °C for 10 min with a premixed solution of NEM and DTT equivalent to the final concentrations described above (closed circles, ○).

Fig. 5. Immunoblot analysis of the trimeric G-proteins. A sample of Swiss membranes (100 µg) identical to the material (300 µg) separated by anion exchange chromatography was separated in lane M. Approximately one-third of each fraction containing guanine nucleotide binding activity (1–11) was separated by 12% polyacrylamide gel electrophoresis and immunoblotted for trimeric G-proteins using the polyclonal antibody J-882.

Identification of Guanine Nucleotide-binding Proteins by GTP and Immunoblotting—Immunoblotting analysis using a polyclonal antibody (J-882) which recognizes all proteins belonging to the trimeric G-protein α subunit family (Mumbey et al., 1986) was performed to determine whether any of the GDP-binding proteins resolved by the Mono Q column are related to the 35–45-kDa G-protein family. As expected, the unseparated plasma membrane preparation (lane M, Fig. 5) possessed an immunoreactive protein of about 41 kDa. However, samples of GDP-binding proteins obtained by Mono Q chromatography, corresponding to the amount of protein used in lane M, did not cross-react detectably with the antibody, indicating that these GDP-binding proteins are not related to the Gα/Gi class of α subunits.

We also used the pan-reactive p21ras monoclonal antibody Y13-259 to try to identify p21ras in the Swiss 3T3 membrane preparation. Surprisingly, however, as shown in Fig. 6A, no immunoreactive 21-kDa protein was detectable. A parallel blot of 1 µg of purified p21ras (Fig. 6B) illustrates the efficacy of the antibody. By using goat-anti-rabbit F(ab)2 fragments at 5.3 μCi/μg, the calculated sensitivity of the immunoblot is approximately 1000 dpm 125I/fmol of antigen for a 12-h exposure with an intensifying screen. However, no 21-kDa band was observed even after 4 days exposure at −70 °C with an intensifying screen (data not shown). There were also no immunoreactive proteins detected with polyclonal antisera directed against either the ARF or ralB proteins (data not shown).

To further test the hypothesis that the observed proteins do not represent forms of p21ras, solubilized membranes were immunoprecipitated with Y13-259 under conditions known to quantitatively precipitate recombinant p21ras (Bhullar and Haslam, 1987). No significant decrease in GDP binding was detected (data not shown). We therefore conclude that p21ras does not contribute significantly to the GDP binding activity observed in Swiss 3T3 membranes.

Transfer to nitrocellulose from SDS gels appears to allow the partial renaturation of certain GTP-binding proteins (Bhullar and Haslam, 1987). By using phosphate buffer rather than Tris buffer, as described by Bhullar and Haslam, we were able to distinguish from rat brain membranes at least five bands possessing GTP binding activity (Fig. 7A, 4 h exposure) ranging in molecular masses from about 20 to 30 kDa. When plasma membranes from Swiss 3T3 cells were...
approximately one-third of each GDP-binding peak was analyzed for p21'" (0.5 and fractions possessing GDP binding activity as well as purified renatured guanine nucleotide binding activity.

Following 12% SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose as described under "Experimental Procedures." Approximately one-third of each GDP-binding peak was analyzed for renatured guanine nucleotide binding activity. A and B were exposed for 4 h with C being exposed for 4 days with an intensifying screen at -70 °C.

analyzed in a similar manner, two bands of about 27 and 29 kDa were detected in the intact membranes (Fig. 7B; 4 h exposure). These proteins did not bind to the Mono Q column and were eluted in peak 1 (Fig. 7C). Longer exposure of the nitrocellulose resolved several other GTP-binding proteins of 27 kDa (peaks 8, 9, and 10), 25 kDa (peaks 6, 7, and 8), and 17 kDa (peaks 6 and 7). Note that all fractions analyzed in this fashion were separated from one another by at least three other fractions. Recombinant p21'" (0.5 μg) was not detectable by this assay (Fig. 7C). These data support the hypothesis that the GDP-binding proteins resolved by anion exchange chromatography are novel small guanine nucleotide-binding proteins and are not simply post-translationally modified forms of p21'".

Detection of GTPase Activity in the Absence or Presence of GAP—The observation that the resolved GDP-binding proteins can also bind GTP suggested that, like p21'" and some other members of the ras super-family, they might also possess GTPase activity. To test this hypothesis, membranes were preloaded with [α-32P]GTP, separated on the Mono Q column and each peak assayed for the production of GDP. To minimize the level of [α-32P]GDP in the reaction, plasma membranes were preloaded with [α-32P]GTP (80 μCi, 3000 Ci/mmole) at 4 °C for 90 min. Control experiments have shown that in the absence of Mg2+, GTP binding occurs without significant production of GDP (Fig. 8A). After preloading the membranes, Mg2+ was added and unbound GTP was removed by filtration through a glass fiber filter (GF/B). The membrane proteins were then solubilized off the glass fiber filter and analyzed by anion exchange chromatography. This methodology ensured that only bound guanine nucleotides were applied to the Mono Q column. Changes in the GTP/GDP ratio of total guanine nucleotides reflect single turnover GTPase activity, which eliminates kinetic artifacts that could cause changes in the bound guanine nucleotide pools.

Fig. 8B represents the GDP/GTP ratio of the individual peaks as they were eluted from the Mono Q column. Fig. 8C represents the same fractions after a 1-h incubation at 30 °C. The GDP/GTP ratios were quantitated by laser densitometry (Table III). The data clearly demonstrate the depletion of GTP and production of GDP in peaks 1–6, supporting the hypothesis that these proteins possess intrinsic GTPase activity. The data for peaks 7 and 8 and 9–11 were not as clear.

For peaks 7 and 8, GTPase activity could not be distinguished from [α-32P]GDP binding during the initial incubation. Although very small amounts of [α-32P]GDP were present (Fig. 8A) during this incubation, selective binding of GDP versus GTP would also explain these results. Peaks 9–11 appeared to have no detectable catalytic activity.

To determine whether this absence of catalytic activity reflected a loss of regulatory factors (such as GAP) during purification, we reassessed the GTPase activity of the GDP-binding peaks separated by anion exchange chromatography in the presence of cytosol. The activity of each peak was measured in the presence of cytosolic extract by filter binding and thin layer chromatography. Cytosolic GAP activity selectively enhanced GTPase activity of four of these proteins.
Guanine Nucleotide-binding Proteins in Fibroblast Membranes

The relative amounts of GTP and GDP in the autoradiographs (Fig. 7, B and C) were quantitated using an LKB densitometer.

<table>
<thead>
<tr>
<th>Peak</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tbody>
<tr>
<td>GDP/GTP, $t = 0$</td>
<td>1.2</td>
<td>1.2</td>
<td>1.3</td>
<td>1.5</td>
<td>3.4</td>
<td>2.4</td>
<td>14.6</td>
<td>10</td>
<td>0.2</td>
<td>0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>GDP/GTP, $t = 60$ min</td>
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<td>5.8</td>
<td>5.0</td>
<td>3.6</td>
<td>8.1</td>
<td>3.9</td>
<td>12.0</td>
<td>10</td>
<td>0.3</td>
<td>0.3</td>
<td>2.5</td>
</tr>
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</table>

Table III
Quantitation of GTPase activity associated with the GDP-binding peaks resolved by anion exchange chromatography

FIG. 9. Enhanced GTPase activity of multiple guanine nucleotide-binding proteins from Swiss 3T3 membranes. Fractions corresponding to the resolved guanine nucleotide-binding proteins were loaded with $[\alpha^{32}P]GTP$ at 4 °C for 90 min in the absence of Mg$^2+$. Mg$^{2+}$ and unlabeled GTP and GDP (final of 1 mM each) were added with either buffer (-), cytosol (+) (A) or boiled cytosol (B). The reaction was allowed to proceed for 30 min at 30 °C. The reactions were filtered and analyzed for bound guanine nucleotides as described under “Experimental Procedures.” Peak numbers correspond to the guanine nucleotide-binding proteins shown in Fig. 3.

FIG. 9
Enhanced GTPase activity of multiple guanine nucleotide-binding proteins from Swiss 3T3 membranes. Fractions corresponding to the resolved guanine nucleotide-binding proteins were loaded with $[\alpha^{32}P]GTP$ at 4 °C for 90 min in the absence of Mg$^2+$. Mg$^{2+}$ and unlabeled GTP and GDP (final of 1 mM each) were added with either buffer (-), cytosol (+) (A) or boiled cytosol (B). The reaction was allowed to proceed for 30 min at 30 °C. The reactions were filtered and analyzed for bound guanine nucleotides as described under “Experimental Procedures.” Peak numbers correspond to the guanine nucleotide-binding proteins shown in Fig. 3.

associated with the guanine nucleotide-binding proteins. This effect was thermolabile.

DISCUSSION

We have demonstrated the existence of at least 11 distinct GDP/GTP-binding proteins in 3T3 cell plasma membranes, the properties of which differ significantly from those of the Gs/Gt, $\alpha$ subunit family of proteins. The characterization of the GDP-binding proteins relies on the trapping of $[\alpha^{32}P]GTP$ at 4 °C for 90 min in the absence of Mg$^2+$. Mg$^{2+}$ and unlabeled GTP and GDP (final of 1 mM each) were added with either buffer (-), cytosol (+) (A) or boiled cytosol (B). The reaction was allowed to proceed for 30 min at 30 °C. The reactions were filtered and analyzed for bound guanine nucleotides as described under “Experimental Procedures.” Peak numbers correspond to the guanine nucleotide-binding proteins shown in Fig. 3.

plasma membrane protein with the monoclonal p21<sup>ras</sup> antibody Y13-259 did not detect a specific 21-kDa protein in Swiss 3T3 membranes. The calculated sensitivity of this method suggests that a minimum of 1 fmol/mg of membrane protein could have been detected after 4 days of autoradiography. Since the amount of p21<sup>ras</sup> in these membranes was below this level of detection, we estimate there can be no more than 1,000 copies of total (N, Ki, and Ha) p21<sup>ras</sup>/cell. This result strongly suggests that the observed GTP-binding proteins do not represent different forms of p21<sup>ras</sup>. Moreover, the GTP binding activity was not immunoprecipitable by the anti-p21<sup>ras</sup> antibody.

It is instructive to compare the properties of the proteins described in this report with those of p21<sup>ras</sup> and other known G-proteins. The effects of Mg$^2+$ on GDP binding, for instance are more similar to those described for p21<sup>ras</sup> (Hall and Self, 1986; Feustein et al., 1987) than the Mg$^{2+}$-independent GDP binding observed with the $\alpha$ subunits of Gt and Gs (Ferguson et al., 1986). The apparent overall $K_a$ (5-50 nM) for GDP also agrees quite closely with values obtained with purified p21<sup>ras</sup> proteins (Finkel et al., 1984; Manne et al., 1984; Hattori et al., 1985; Sigal et al., 1986). The $k_{cat}$ value for the slow component is at least an order of magnitude lower than the value reported for purified p21<sup>ras</sup> (Hall and Self, 1986; Hattori et al., 1987). However, it is important to note that whereas in our experiments the decay of binding was corrected for protein denaturation, similar corrections have not been reported for measurements of the $k_{cat}$ for GDP binding to p21<sup>ras</sup>. At 30 °C denaturation contributes significantly to the overall loss of binding, and it is therefore likely that the $k_{cat}$ for p21<sup>ras</sup> has been overestimated. Purified p21<sup>ras</sup> possesses intrinsic GTPase activity and guanine nucleotide binding activity which is sensitive to the thiol reagent N-ethylmaleimide (Hattori et al., 1986). Eight of the GDP-binding proteins described in this report possess detectable GTPase activity, and the GDP binding activity of 9 of the 11 GDP-binding proteins is sensitive to pretreatment with NEM.

We have separated the plasma membrane GDP binding activity into 11 distinct peaks. Each of these proteins appears to be unique, judging from their reproducible mobilities on a high resolution anion exchange column and the large assortment of GDP off-rates measured for the individual proteins (Table II). None of the observed GDP-binding activity in Swiss 3T3 plasma membranes appears to be attributable to p21<sup>ras</sup> which, if present, is below the detectable levels of both GDP binding and immunoblot analysis. Moreover, the data shown in Fig. 5 suggest that $\alpha$ subunit-related proteins do not contribute significantly to the total amount of GDP binding by 3T3 plasma membranes. We have also been unable to detect p21<sup>ras</sup> or p25<sup>ras</sup> by immunoblot assays specific for these proteins. Therefore, the identities of the 11 GDP-binding proteins in the 3T3 membranes remain to be established. GTP blotting indicates that some of them possess molecular weights similar to those of GTP-binding proteins recently purified from brain membranes (Kikuchi et al., 1988; Yaramoto et al., 1988). The observation that recombinant
p21<sup>TM</sup> is undetectable by this assay provides further evidence that the peaks of guanine nucleotide binding activity eluted off the anion exchange column are not p21<sup>TM</sup>. Gel filtration analysis of solubilized membranes revealed two major peaks of activity, of around 50–60 and 20–30 kDa. While all of the ras-like proteins sequenced to date possess subunit molecular masses of about 20–25 kDa, the rab3 protein elutes as a 50-kDa protein on gel filtration analysis (Kikuchi et al., 1988). Therefore, it is possible that the 60-kDa GDP binding activity represents multimers of smaller GDP-binding proteins.

Although the relative abundance of the different GDP-binding proteins appears to vary, the positions of the GDP binding activity eluting off the Mono Q column were identical in membranes prepared from Swiss 3T3, NIH 3T3, and liver cells (data not shown), indicating that this family of proteins, or very similar proteins, are widely distributed.

We have demonstrated also that cytosolic GAP activity is able to stimulate the conversion of GTP to GDP by at least four unique members of a family of membrane-bound guanine nucleotide-binding proteins. This is of particular interest since p21<sup>TM</sup> cannot be detected in our membrane preparations. The existence of a second unique protein possessing GAP activity toward the ras-related rho protein has recently been reported (Garrett et al., 1989). It will clearly be of great interest to characterize the GAP family of proteins so as to determine the specificity of interaction with the large array of available GTP-binding proteins and to determine the mechanisms by which their activity is regulated.

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Guany Nucleotide-binding Proteins in Fibroblast Membranes

10827