Subcellular Localization and Quantitation of the Major Neutrophil Pertussis Toxin Substrate, G_n

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Abstract. The subcellular distribution of G protein subunits in the neutrophil was examined. Cells were nitrogen cavitated and subcellular organelles fractionated on discontinuous sucrose gradients. The presence of GTP-binding regulatory protein (G protein) alpha and beta/gamma subunits in each organelle was determined using three methods of analysis: specific binding of guanine nucleotide, ADP ribosylation by pertussis toxin, and immunoblot analysis with subunit-specific G protein antibodies. Both plasma membrane and cytosolic G protein components were detected. In contrast, neither the specific nor the azurophilic granules contained detectable G protein. Based on the ability of exogenous G protein beta/gamma subunits to increase the ADP ribosylation of the cytosolic form of G protein and upon the hydrodynamic behavior of the cytosolic protein, it is likely that this represents an uncomplexed G protein alpha subunit. Proteolytic mapping with *Staphylococcus aureus* V8 protease suggests the soluble alpha subunit is from G_n, the major pertussis toxin substrate of human neutrophils. Using quantitative analysis, the levels of the 40-kD G protein alpha subunit and of the 35/36-kD beta subunit in the neutrophil membrane were determined.

THE GTP-binding regulatory proteins (G proteins)[1] consist of a family of highly homologous proteins which serve to couple various membrane-bound hormone, neurotransmitter, and chemotactic factor receptors to their cellular effector systems. At least five such proteins have been purified and characterized, including the following: G_s and G_i, the stimulatory and inhibitory regulators of adenylate cyclase (5, 38); transducin, which couples rhodopsin to the cGMP phosphodiesterase in rod outer segments (18); G_o, a G protein abundant in brain whose function is as yet unclear but which has been demonstrated to interact with muscarinic cholinergic receptors (9, 37); and G_n, a G protein found in neutrophils which may couple chemotactic factor receptors to a phospholipase C (12, 29). In addition, several other potential G proteins have been described (7, 16, 41). The G proteins are structurally similar, being heterotrimers consisting of distinct alpha and very similar, if not identical, beta/gamma subunits. The alpha subunits in each G protein contain the sites for binding guanine nucleotides and fluoride (5, 26), as well as sites for ADP ribosylation by various bacterial toxins (4, 27). Alpha subunits of at least two G proteins (G_i and G_o) have been shown to behave as soluble proteins in vitro, maintaining behavior as monomeric species in the absence of detergents (36). The beta/gamma subunits form a relatively hydrophobic complex which may be involved in coupling the alpha subunits to membrane receptors (9) and, potentially, the membrane itself. The ability of beta/gamma to promote the association of the alpha subunit with artificial phospholipid vesicles has been demonstrated (36).

While the G proteins largely appear to exist as membrane proteins, requiring detergents to extract them from membranes, there are indications that exceptions to this generalization exist. In particular, transducin has been shown to behave as a peripherally bound membrane protein at physiologic ionic strength (19). The transducin alpha subunit can be released from the membrane and become soluble by treatment with light (i.e., the receptor "agonist") and GTP. Under conditions of low ionic strength, both light and GTP are individually effective in releasing the holoprotein complex from the membrane. The regulation of a soluble cGMP phosphodiesterase by transducin may require formation of the soluble, activated species of Tn-alpha (39). Several other reports suggest the possibility that other G proteins may be able to exist in a soluble form, including G_s (21, 32) and some of the pertussis toxin substrates (12, 23).

In this report, we analyze in detail the subcellular distribution of G protein subunits in the human neutrophil. A cytoplasmic pertussis toxin substrate is described and characterized. Proteolytic mapping identifies the cytosolic protein as identical to the 40-kD alpha subunit of G_n that exists in neutrophil membrane and clearly distinct from the 41-kD alpha subunit of G_i. The cytoplasmic form of G_n appears to represent the alpha subunit of the protein uncomplexed from the beta/gamma subunit. Quantitative estimates of the levels

1. *Abbreviations used in this paper*: DIFP, diisopropylfluorophosphate; G proteins, GTP-binding regulatory proteins; GTPγS, guanosine 5'-gamma (3-0-thio) triphosphate.

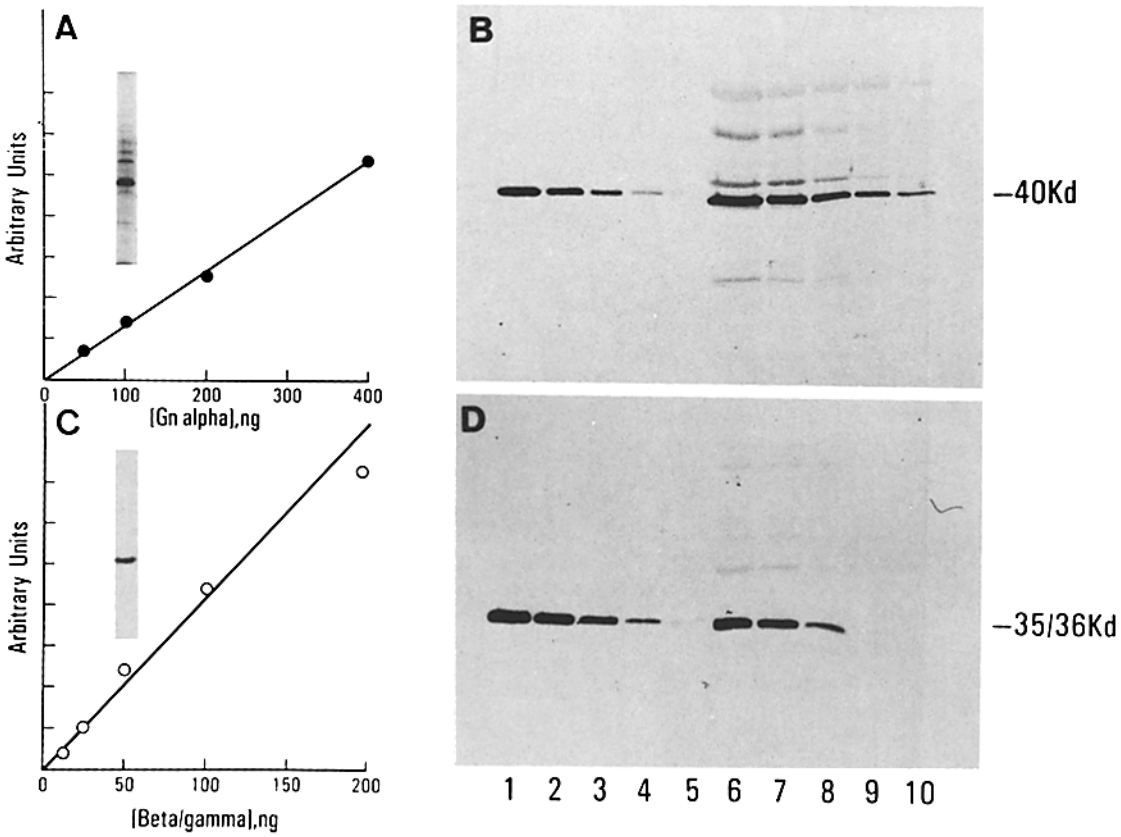

Figure 1. Quantitative immunoblots of neutrophil membrane-associated G protein subunits. Quantitative immunoblotting was carried out as described in Materials and Methods. *A* and *C* show the standard curves for G_n alpha subunit and beta subunit, respectively, plotted as arbitrary densitometric units vs. quantity of standard subunit. The purity of the protein subunits used as standards are indicated by the silver-stained gels (*inset*). The position of the relevant standard on the immunoblots is indicated by the molecular mass of the subunit (G_n alpha = 40,000 and bovine brain beta = 35,000/36,000). Lanes *1–5* contain the purified protein standard at 400, 200, 100, 50, and 25 ng for G_n (*B*); and 200, 100, 50, 25, and 12.5 ng for beta subunit (*D*), respectively. Lanes *6–10* contain serially diluted (1:2) samples of purified neutrophil membrane. The amount of membrane protein loaded in lane *6* was 25 μg in this experiment. The alpha subunit blot (*top*) was with 1:250 dilution of R16,17; the beta subunit blot (*bottom*) was with 1:500 dilution of R3,4. The blot used to prepare *B* was obtained with a 1:250 dilution of R16,17. The presence of the large molecular mass immunoreactive bands in this blot is apparently due to the presence of low levels of nonspecific antibodies in the R16,17 preparation. These bands are not seen at 1:2,000 dilutions of antibody R16,17, even though the intensity of the 40-kD band remains the same. We have subsequently gone to routine use of 1:2,000 dilutions of R16,17 for quantitative Western blotting.

of G protein subunits in neutrophil membrane are also presented.

Materials and Methods

Analysis of the Subcellular Distribution of G Protein Subunits

Neutrophils (8×10^8 cells) were isolated from human blood as described (1, 15) and were ≥90% polymorphonuclear leukocytes. Cells were treated for 5 min on ice with 2.5 mM diisopropylfluorophosphate (DIFP), and then pelleted and resuspended at 1×10^8 cells/ml. Cells were cavitated in an N_2 cavitation chamber in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 U/ml aprotonin by pressurizing them to 450 psi for 15 min at 4°C and then collecting the homogenate into 1 mM EDTA and 1 mM EGTA. Cell debris was pelleted at 250 *g* for 10 min and the supernatant was applied to a discontinuous sucrose gradient in 25 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM EGTA (10 ml 60%, 10 ml 40%, and 4 ml 15% sucrose). Gradients were centrifuged at 45,000 rpm in a fixed angle rotor (model TV850; Beckman Instruments, Fullerton, CA) for 30 min at 2°C and then fractions were collected from top to bottom in 25 1.3-ml portions.

The localization of the major neutrophil subcellular organelles on such gradients has been determined in detail by Jesaitis et al. (15) using various enzymes characteristic of these organelles as markers. We have made use of these, or similar, markers in the present study assayed by the methods described in references 1 and 31.

G Protein Assay

The binding of [^{35}S]guanosine 5′-gamma(3-0-thio) triphosphate (GTPγS) to G protein in sucrose gradient fractions was determined essentially as described (25). Briefly, 20 μl of each fraction was added to 80 μl of a 1.25 × solution of 25 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% lubrol, 100 mM NaCl, 30 mM $MgSO_4$, and 1 μM GTPγS (10,000 cpm/pmol). The mixture was incubated at 30°C in a shaking water bath for 60 min, and then the reaction was terminated by addition of 1 ml of ice cold 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM DTT plus 0.1 mg/ml BSA, and the binding assayed by vacuum filtration as in reference 25.

The conditions used for the ADP ribosylation of G protein by pertussis toxin in the presence of [^{32}P]NAD were as described in reference 5. Analysis of ADP-ribose incorporation into protein was done by SDS-PAGE and autoradiography (4).

Purification of G Protein Standards

G_n alpha subunit was purified from human neutrophils by modification of the methods previously used for purification of rabbit liver G_i (5). Nearly

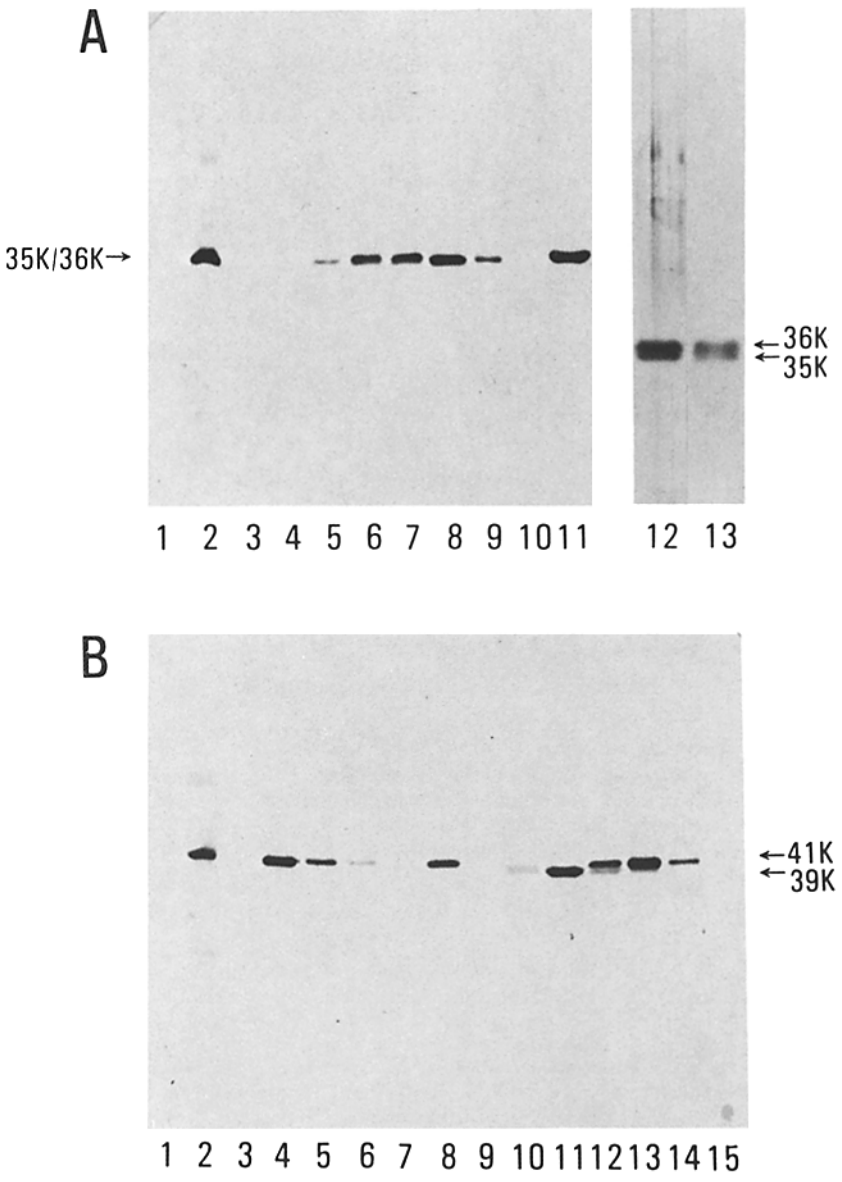

Figure 2. Immunoblots demonstrating reactivity of anti-G protein antibodies with various G proteins. Antibodies prepared against bovine brain beta/gamma subunits (R3,4) or a G_i-specific peptide (R16,17) were tested for reactivity with various G proteins or neutrophil membrane as described in Materials and Methods. Samples were subjected to SDS-PAGE on 10% gels and immunoblotted as described. The arrows indicate the positions of the relevant subunits of the G proteins used. *A* (lanes *1–11*) was with 1:500 dilution of anti-beta antibody R3,4. *B* was with a 1:250 dilution of anti-alpha antibody R16,17. The contents of each lane are as follows: lanes *1*, *3*, and *15* (*A* and *B*), blanks; lane *2* (*A* and *B*), 30 μg human neutrophil membrane; lanes *4–7* (*A* and *B*), G_n samples selected from various fractions obtained after purification. These samples contained varying amounts of G_n alpha and beta/gamma subunits, proceeding from pure alpha subunit in lane *4* to pure beta/gamma subunit in lane *7*. The estimated amount of G_n protein loaded in lanes *4–7* was ~150 ng; lane *8* (*A* and *B*), 180 ng rabbit liver G_i; lane *9* (*A* and *B*), 150 ng bovine brain beta subunit; lane *10* (*A* and *B*) 150 ng bovine brain G_o alpha subunit; lane *11* (*A* and *B*), 150 ng transducin. Lane *12* (*A*) is a silver-stained lane showing purified bovine brain beta subunit (50 ng) and demonstrating the 35- and 36-kD forms of beta. Resolution of the two forms of beta subunit was achieved by using a much longer gel than those depicted in the remainder of *A* and *B*; lane *13* (*A*), immunoblot demonstrating the reactivity of R3,4 (1:1,000 dilution) with both the 35- and 36-kD forms of beta subunit shown in the sample of lane *12*; lane *12* (*B*), ~350 ng G_o/G_i alpha subunits from bovine brain; lane *13* (*B*), ~500 ng G_o/G_i (alpha/beta/gamma) from bovine brain; lane *14* (*B*), ~400 ng bovine brain G_i. The blots in *A*, lanes *1–11* and *B*, lanes *1–15* were obtained with peroxidase-conjugated goat anti-rabbit IgG secondary antibody, while that of *A*, lane *13* was with ^{125}I-labeled goat anti-rabbit secondary antibody.

homogenous G_n alpha subunit was obtained (see Fig. 1) and characterized as a 90% pure 40-kD alpha subunit using immunological methods, as well as two-dimensional gel electrophoresis and *Staphylococcus aureus* V8 protease mapping. Homogenous bovine brain 35/36-kD beta subunit (Fig. 1) was prepared by the method of Sternweis and Robishaw (37) up until the heptylamine-Sepharose chromatography. The beta/gamma subunits were then further purified to homogeneity by a second DEAE chromatography, concentrated by hydroxylapatite column chromatography, and switched to 25 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.1% lubrol buffer, as described previously (5).

Preparation of G Protein Antibodies

Antisera that reacted against G protein subunits were prepared using purified beta subunit (antibody R3,4) or a peptide corresponding to the 9-amino acid carboxyl-terminal sequence of G_i–NNLKDCGLF (antibody R16,17). This sequence is highly conserved in G_i (type I and type II) sequences that have been reported, as well as in transducin (14, 28, 40).

Rabbits (2–2.5 kg, New Zealand White Strain) were injected in multiple subcutaneous sites with antigens suspended in Freund's incomplete adjuvant at an initial dose of 50 μg (proteins) or 200 μg (Keyhole limpet hemocyanin-conjugated peptide). Injections were repeated at 2-wk intervals (50 μg protein, 100 ug peptide) and antibody titers checked by ELISA (6). Antisera obtained were fractionated with 55% saturated ammonium sulfate, followed by ion exchange chromatography to prepare an IgG-enriched fraction. Antiserum R16,17 was further purified by passage over a column of Sepharose 4B to which peptide had been coupled. Antibody retained by the column was eluted with 0.2 M glycine-HCl, pH 3.0, and then neutralized.

Reactivity of R3,4 and R16,17 with various G protein subunits is indicated in Fig. 2. R3,4 (Fig. 2 *A*) reacted specifically with beta subunits from all G proteins, detecting both 35- and 36-kD forms of beta. R16,17 reacted very well with G_n alpha, G_i alpha, and transducin alpha, but only marginally reacted with G_o alpha, and not at all with G_s alpha (not shown directly here) (Fig. 2 *B*). While R16,17 is thus not specific for G_n alpha, it strongly reacts with this protein and we used purified G_n alpha as the standard for quantitation of G_n protein in the intact membrane. Immunoblotting was performed essentially as described by Towbin et al. (42), using either peroxidase-conjugated or ^{125}I-labeled goat anti-rabbit IgG as secondary

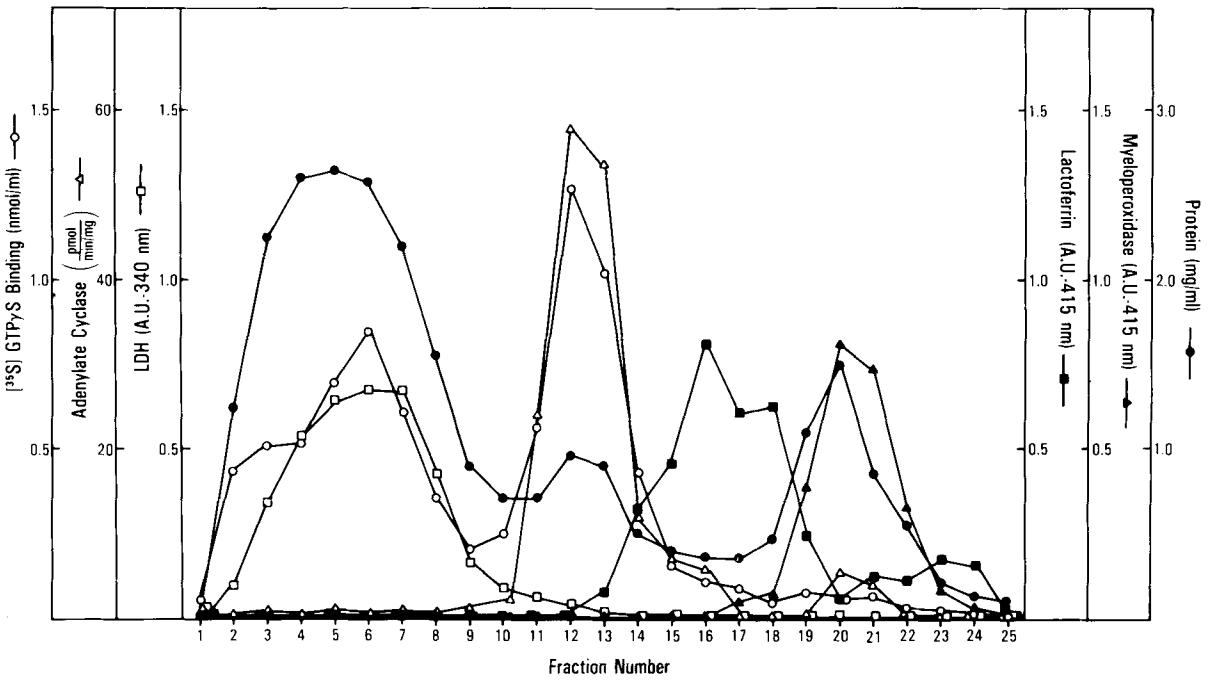

Figure 3. Sucrose gradient analysis of the subcellular distribution of neutrophil GTP-binding protein. 8×10^8 nonactivated neutrophils were applied to each gradient and analyzed as described in Materials and Methods. *LDH*, lactate dehydrogenase.

antibody. For quantitation, standards were serially diluted by a factor of two and analyzed with similarly diluted samples of individual neutrophil membrane preparations (see Fig. 1). Protein levels were assessed by densitometric analysis of immunoblots. Standard curves were obtained for each membrane sample quantitated and the subunit concentrations were determined for the serially diluted samples that fell into the linear range of the assay. Standard curves for each subunit were generally linear between 25 and 200 ng of purified protein, as determined by Amido black assay (33).

Radioimmunoprecipitations

Aliquots of neutrophil cytosol (10 μl) or purified G_n (~0.2 μg) were ADP ribosylated in the presence of [^{32}P]NAD and pertussis toxin for 60 min, as described in reference 5. The reaction was stopped on ice and 50 μl of each sample was incubated on ice overnight with 50 μl of the antiserum to be tested in the presence of 0.3% sodium cholate, 25 mM Tris-HCl, pH 8.0, and 12% sucrose (total volume = 130 μl). 75 μl of a 1:1 mixture of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and Dulbecco's medium was then added to the samples and allowed to incubate on ice for 3 h. The samples were transferred to 1.4-ml microfuge tubes and pelleted at full speed for 2 min in a table top microfuge (Beckman Instruments, Inc.) at 4°C. Supernatants from the immune precipitates were removed for analysis and the pellets resuspended and washed three times with cold 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% sodium cholate. Immune precipitates were then suspended in 100 μl Laemmli sample buffer, allowed to sit for 15 min at room temperature, and then pelleted and the supernatants applied to 11% polyacrylamide gels for analysis.

Proteolytic (Cleveland) Mapping

Samples of purified G_n or G_i (2 μg) or of neutrophil cytosol were ADP ribosylated in the presence of [^{32}P]NAD (~15,000 cpm/pmol) as described previously (5). The reaction was terminated by the addition of an equal volume of 0.25 M Tris-HCl, pH 6.8, 1.0% SDS, 20% glycerol, and 0.0002% bromphenol blue, and heated for 2 min at 100°C. 1 μg of the ADP-ribosylated pure protein or 50 μl of the cytosolic fraction was incubated with the indicated amounts of *S. aureus* V8 protease for 30 min at 37°C. Samples were then analyzed by PAGE on 15% gels and autoradiographic analysis was performed by exposure of Kodak XRP film for 5–7 d with intensifying screen.

Hydrodynamic Analysis of Cytoplasmic G_n

Analysis was essentially as described in reference 4 for nonactivating conditions except that the current studies were performed in the absence of detergent. G protein was detected by ADP ribosylation in the presence of exogenous beta/gamma subunits at 10 μg/ml (cytoplasmic G_n alpha) or by analysis of prebound [^{35}S]GTPγS (G_i alpha) (5).

Miscellaneous

PAGE was performed according to the procedures of Laemmli (20). Protein was assayed by an Amido Black staining procedure (33). [^{35}S]GTPγS and [^{32}P]NAD were from New England Nuclear, Boston, MA. Pertussis toxin was from List Biological Laboratories, Campbell, CA. Peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad Laboratories, Richmond, CA, while ^{125}I-labeled goat anti-rabbit IgG was from New England Nuclear. *S. aureus* V8 protease was from Sigma Chemical Co., St. Louis, MO. Antibody K-521 was obtained from S. Mumby, University of Texas Health Sciences Center, Dallas, TX, and was prepared as described (10).

Results

The fractionation of human neutrophil homogenates on discontinuous sucrose gradients enabled us to clearly resolve several major subcellular compartments of the cell, including the cytosol, plasma membrane, specific (or secondary) granules, and azurophil (or primary) granules (Fig. 3). Analysis of such a gradient for the binding of [^{35}S]GTPγS demonstrated the existence of binding activity in two peaks—one associated with the cytosol (marker enzyme, lactate dehydrogenase) and one associated with plasma membrane (marker enzyme, adenylate cyclase, as well as alkaline phosphatase, not shown). Neither the specific (marker enzyme, lactoferrin) nor azurophil (marker enzyme, myeloperoxidase) granules had significant GTPγS-binding activity associated with them.

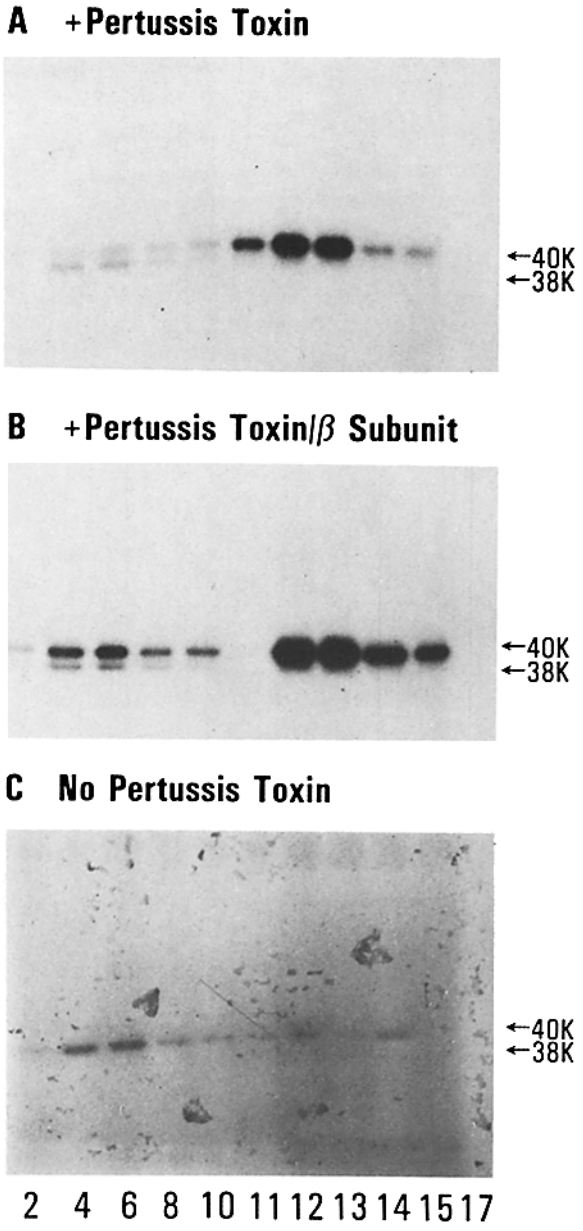

Figure 4. Distribution of pertussis toxin substrate in subcellular fractions of the neutrophil. The indicated fractions (10 μl) from the gradient shown in Fig. 3 were analyzed for pertussis toxin substrate as described in Materials and Methods under the following conditions: (*A*) in the presence of 10 μg/ml pertussis toxin; (*B*) in the presence of 10 μg/ml pertussis toxin plus 10 μg/ml of bovine brain beta/gamma subunit complex purified as described in Materials and Methods; (*C*) in the absence of pertussis toxin. The labeled band in this panel is the 38-kD one observed in *A* and *B*. Autoradiography was with Kodak XRP film exposed for 72 h or 120 (*C*) with intensifying screen at −70°C. (The fraction *11* lane in *B* is a blank, accidently receiving no protein from the gradient fraction 11.)

Analysis of gradients for the presence of pertussis toxin substrate is shown in Fig. 4. A 40-kD protein that was a specific toxin substrate was observed in both the cytoplasmic and the plasma membrane-containing fractions, while no toxin substrate was apparent in either granule. An additional protein of ~38 kD was labeled in the neutrophil in the presence of [^{32}P]NAD (Fig. 4, *A* and *B*), but this labeling occurred even in the absence of pertussis toxin (Fig. 4 *C*).

Since the ability of G proteins to serve as pertussis toxin substrates can be markedly influenced by the subunit composition of the substrate (17, 24), we examined the effect of addition of exogenous beta/gamma subunit complex on the extent of ADP ribosylation of various gradient fractions (Fig. 4 *B*). The presence of beta/gamma subunits markedly enhanced the degree of ADP-ribose incorporation into the cytosolic substrate. The plasma membrane-associated protein demonstrated only modest increases in the extent of labeling with beta/gamma present. Densitometric analysis of peak cytosolic fractions indicate an average increase in the extent of labeling of 12 ± 5-fold, while the peak membrane fractions only increased by an average of 2.0 ± 0.5-fold (n = 6). The relative lack of enhancement of the labeling of the membrane G protein is not likely to be due to problems with accessibility of the added beta/gamma subunits to the toxin substrate, since similar results are seen with cholate extracts of the membrane (not shown). These data suggest that the cytosolic G protein might exist largely in the form of the alpha subunit uncomplexed from the beta/gamma subunit.

Using antibodies that specifically recognize alpha and beta subunits of the G proteins, we examined the distribution of subunits on the discontinuous sucrose gradients. The immunoblots in Fig. 5 show the results with the indicated fractions from the gradient used in Fig. 3. Beta subunit was readily detected by immunoblotting in the plasma membrane-containing fractions (Fig. 5 *C*) and its position exactly correlates with that of the plasma membrane-associated alpha subunit (Fig. 5 *A*). We did not detect any cytoplasmic nor granule-associated beta subunit with this antibody (R3,4), even though it can detect as little as 10 ng of purified beta subunit in immunoblots. Cholate extracts of specific and azurophil granules contained no detectable beta subunit either (not shown). The cytosolic and granule membrane levels of beta subunit are thus either below the detection limit of our immunoblots or nonexistent.

Alpha subunit was also clearly detected by immunoblotting in the plasma membrane-containing peak fractions (Fig. 5 *A*). The granule-containing fractions and cholate extracts of these fractions did not show any alpha subunit immunoreactivity. We were unable to detect the cytosolic alpha subunit in immunoblots with antibody R16,17. This may be partially due to the low levels of alpha subunit in the cytosol and partially due to a protein of ~40 kD in the cytoplasm that reacted even with the preimmune serum (Fig. 5 *B*) and which may obscure detection of low levels of alpha subunit. This protein is visualized with several samples of nonimmune rabbit serum and does not appear to represent G protein alpha subunit. To determine if the cytosolic pertussis toxin substrate would indeed be recognized by antibody R16,17, we immunoprecipitated the [^{32}P]ADP ribosylated protein, which allowed us to detect the interaction of the much lower cytoplasmic levels of this protein with anti-alpha antibody. As shown in Fig. 6, the cytoplasmic pertussis toxin substrate was readily immunoprecipitated by R16,17, but was not precipitated by preimmune serum (compare lanes *1* and *2* with *5* and *6*). Precipitation of purified G_n by the same antiserum is shown as a control (compare lanes *3* and *4* with

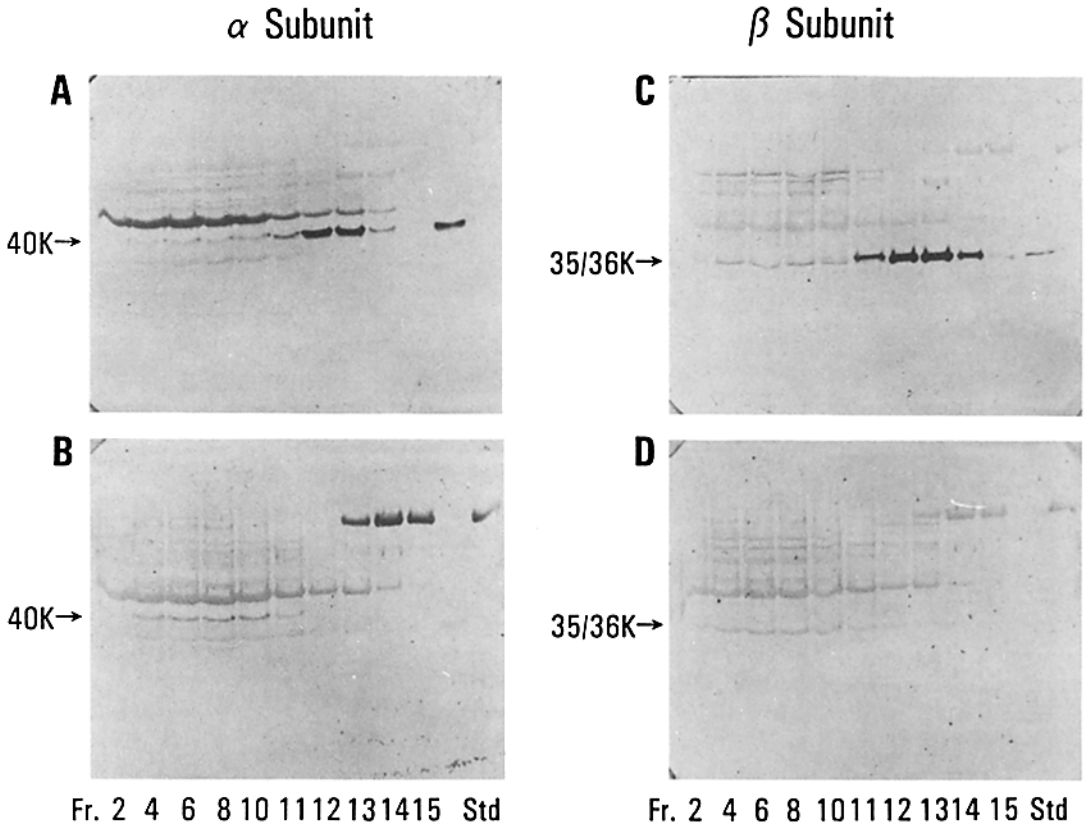

Figure 5. Immunoblot analysis of the subcellular distribution of G protein alpha or beta subunits. The indicated fractions (40 μl) from the gradient of Fig. 3 were analyzed by immunoblotting for the distribution of G protein alpha or beta subunits. (*A*) 1:250 dilution of antibody R16,17 which detects G protein alpha subunit specifically; (*B*) 1:100 dilution of preimmune serum from the rabbits used to produce R16,17; (*C*) 1:500 dilution of antibody R3,4 which specifically reacts with G protein beta subunit; (*D*) 1:100 dilution of preimmune serum from the rabbits which produced R3,4. The arrows indicate the position on the immunoblots of the relevant subunit. *Std* indicates the lane containing a sample of pure rabbit liver G_i (*A* and *B*) or of pure bovine brain beta/gamma subunits (*C* and *D*).

7 and *8*). The unidentified ADP-ribosylated band at ~38 kD was not immunoprecipitated by the anti-alpha antibody (compare lanes *1* and *2*).

Proteolytic Mapping of Cytosolic G Protein

Analysis of the proteolytic fragments of [^{32}P]ADP-ribosylated cytosolic G protein generated by *S. aureus* V8 protease enabled us to compare the digestion patterns with those obtained from purified, membrane-associated G_n and rabbit liver G_i. The results (Fig. 7) demonstrate that the cytoplasmic G protein exhibits a *S. aureus* V8 digestion pattern identical in several diagnostic regions with that of the membrane-associated G_n. Proteolytic fragments at 37/35 kD, 24/22 kD, and in the region from 28 to 32 kD were identical for both membrane G_n (lane *4*) and the soluble pertussis toxin substrate (lane *6*), while G_i clearly differed from both (lane *5*). The evidence suggests that the cytoplasmic G protein represents the same protein that is the major membrane-associated pertussis toxin substrate, G_n.

Hydrodynamic Properties of Cytoplasmic G_n

To determine if the cytoplasmic G_n was indeed a free alpha subunit, as suggested by the effect of exogenous beta/gamma subunits on ADP ribosylation of this protein by pertussis toxin (Fig. 4), we examined the hydrodynamic properties of cytoplasmic G_n alpha subunit. These data are shown in Table I. The calculated molecular mass of the cytoplasmic pertussis toxin substrate under nonactivating conditions and in the absence of detergent was 42,300 D. For comparison, GTPγS-liganded G_i alpha subunit from bovine brain was analyzed. This form should represent fully activated alpha subunit uncomplexed from beta/gamma subunits. It gave a molecular mass of 50,800 D, consistent with previous determinations of activated G_i (4), and clearly distinct from the size of the holoprotein complex of M_r ~82,000 (4). The cytoplasmic G_n alpha subunit thus behaves as a hydrodynamic particle of ~42 kD, consistent with its existence in the cytoplasm as the uncomplexed alpha subunit.

Quantitation of G Protein Subunits

The levels of G protein alpha and beta subunits in isolated washed neutrophil membranes were estimated by quantitative immunoblot analysis (Fig. 2). This procedure has been used in several other membrane systems to analyze G protein levels (11, 22, 43). Using purified G_n alpha subunit as the standard, we obtained a value for G_n alpha subunit in washed neutrophil membranes of 30 ± 7.5 μg/mg of membrane protein (n = 6). This value indicates that G_n alpha is a major protein component of the human neutrophil membrane. Levels of beta subunit, assessed using a bovine brain

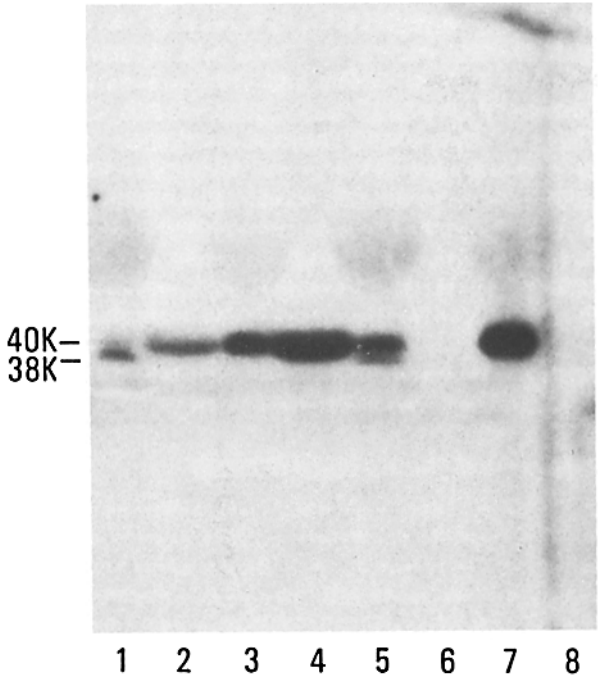

Figure 6. Immunoprecipitation of cytoplasmic G protein with anti-alpha antibody (R16,17). Samples of cytoplasm and purified G_n were ADP ribosylated by pertussis toxin in the presence of [^{32}P]NAD and imunoprecipitated as described in Materials and Methods. Lanes *1–4* were incubated with undiluted serum R16,17 and lanes *5–8* with preimmune serum. Lanes *1* and *5*, cytosolic G protein, supernatant from the immune precipitate; lanes *2* and *6*, cytosolic G protein, immune precipitate; lanes *3* and *7*, G_n, supernatant from the immune precipitate; lanes *4* and *8*, G_n, immune precipitate. The amount of supernatant loaded per lane was equivalent to 50% of the amount of immunoprecipitate loaded. Autoradiography was for 6 d on Kodak XRP film, with intensifying screen at −70°C.

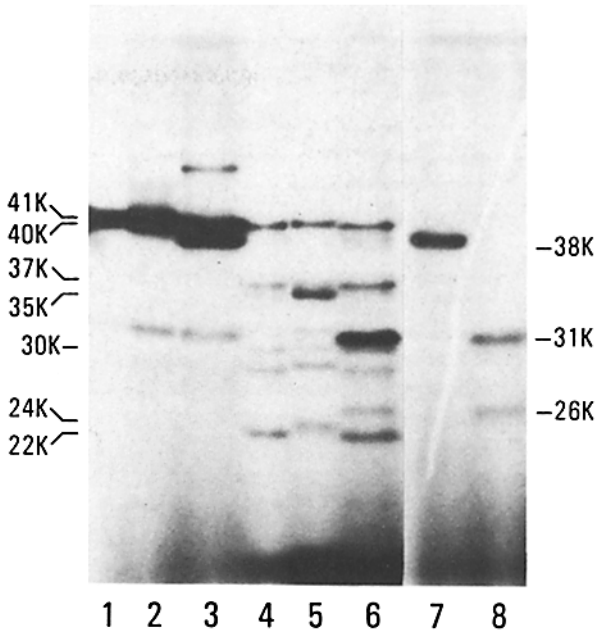

Figure 7. Proteolytic mapping with *S. aureus* V8 protease of cytoplasmic G protein, G_n, and G_i. Proteolytic digestions were performed as described in Materials and Methods. Molecular masses of diagnostic peptides generated are indicated at the sides of the figure. Lanes *1–3* and *7* had no protease added, while lanes *4–6* and lane *8* had 20 μg/ml and 50 μg/ml, respectively, of *S. aureus* V8 protease for 30 min at 37°C. Lanes *1* and *4*, G_n; lanes *2* and *5*, G_i; lanes *3* and *6*, cytoplasmic G protein; lanes *7* and *8*, cytoplasmic G protein sample with no pertussis toxin included in the labeling reaction. Lanes *7* and *8* serve as controls for the unidentified labeled band at ~38 kD in the cytoplasm samples and show proteolytic fragments derived from this protein at 31 and 26 kD. The relative intensities of the 31- and 26-kD fragments derived from the 38-kD protein at a trypsin concentration of 20 μg/ml (not shown here) were identical to those seen in lane *6*. The band at ~31 kD in lanes *1–3* represents a labeled subunit of pertussis toxin. This band does not contribute to any of the proteolytic fragments seen in the presence of *S. aureus* V8 (control not shown). Autoradiography was performed with Kodak XRP film exposed for 1 wk with intensifying screen at −70°C.

beta subunit as the standard, were only 8.0 ± 1.6 μg/mg of membrane protein (n = 6). This value was confirmed using an additional antibody, K-521, which has been described by Gao et al. (10) and shown to detect both 35- and 36-kD forms of beta subunit (8.1 ± 0.7 μg/mg, n = 4). This is surprisingly lower than the value obtained for G_n alpha. Quantitative estimates for each subunit were obtained on six distinct membrane preparations and the values for beta subunit were consistently less than those for alpha subunit within each membrane preparation. The assay of beta subunit by quantitative immunoblotting was not compromised by the existence of some substance in the membrane able to interfere with the reaction of the R3,4 antibody with endogenous beta subunit, since the levels obtained in the beta subunit assay for membranes that had been "spiked" with a known quantity of purified beta protein reflected the quantity of standard added at each sample dilution (data not shown).

Discussion

Intracellular G Protein Distribution

We have presented data obtained using GTPγS binding, pertussis toxin labeling, and immunoblotting that demonstrate the presence of G protein subunits in at least two subcellular sites: one associated with the plasma membrane and one existing in the cytosol. In contrast, we detected no G protein

Table I. Hydrodynamic Parameters of Cytosolic Gn

Parameter	Cytosolic G_n	GTPγS-liganded G_i
Stokes radius (a)	3.40 nm	3.64 nm
Sedimentation coefficient ($s_{20,w}$)	2.90	3.25
M_r*	42,300 D	50,800 D

The values given are the averages of duplicate determinations.
* Calculated according to the equation

$$M_r = \frac{s_{20,w} 6\pi\eta_{20,w} N_{av} a}{1 - V\rho_{20,w}},$$

where N_{av} is Avagadro's number; $\eta_{20,w}$, the viscosity of water at 20°C; $\rho_{20,w}$, the density of water at 20°C; V, the partial specific volume, assumed in this case to be 0.735 ml/g; and a and $s_{20,w}$ are the values shown in the above table.

alpha or beta subunits in the neutrophil specific or azurophil granules. The distribution of human neutrophil subcellular fractions on analytical sucrose gradients has been well characterized by Jesaitis et al. (15, 31). Previous data (12) had indicated the presence of a pertussis toxin substrate in a light membrane fraction and its absence from a heavy membrane fraction, but neither of these membranes was characterized. The absence of detectable G protein associated with the specific granule markers is interesting in light of the data that indicate that this granule or one of similar properties is a source of formyl peptide receptors which can translocate to the plasma membrane under the influence of degranulating stimuli (8). Our preliminary data indicate that ligand binding to formyl peptide receptors in these granules is insensitive to guanine nucleotides, consistent with the absence of functional G proteins as determined herein.

The cytoplasm-associated G protein alpha subunit we have identified appears to represent a truly soluble form of G protein, as evidenced by its distribution with cytoplasmic markers on analytical sucrose gradients, its failure to sediment after 1 h at 165,000 *g*, and its ability to pass through 0.25-μm pore filters (not shown). The presence of G_n alpha in cytosolic fractions indicates that this subunit was either present in soluble form in the intact cell or that it was displaced from the membrane during the process of cell homogenization/centrifugation. Detergent is clearly not required for this process to occur. Whether release of alpha subunit occurs under physiologically relevant activating conditions is as yet undetermined.

Characterization of Cytosolic G Protein

While the cytoplasmic G protein that we observe, and which has been observed by others (12, 23), is a pertussis toxin substrate, it was not clear that it represented the same toxin substrate that existed in association with the plasma membrane. We have shown that an antibody able to recognize membrane-associated G_n alpha cross-reacts with the cytoplasmic alpha subunit. Proteolytic mapping of the cytoplasmic form with *S. aureus* V8 protease distinguishes the cytoplasmic pertussis toxin substrate from G_i purified from rabbit liver and gives an identical pattern of fragments as does the G_n purified from neutrophil membrane. It is likely that the cytoplasmic G protein is thus a form of a G_n and not a distinct protein.

G_i and G_o alpha subunits are able to behave as soluble proteins when uncomplexed from the hydrophobic beta/gamma subunits, which may "anchor" alpha subunits within the phospholipid bilayer (36). This has led to the suggestion that activating conditions, which promote the dissociation of G protein subunits, might be expected to allow a significant amount of alpha subunit to become dissociated from the membrane. Such a process has been shown to occur for the rhodopsin-activated transducin alpha subunit (19). The operation of this type of mechanism would predict that soluble G protein would exist as the free or uncomplexed alpha subunit. This, in fact, seems to be the case: we observe that the degree of pertussis toxin–catalyzed ADP ribosylation of the cytoplasmic form of G_n is markedly enhanced by the addition of exogenous beta/gamma subunits (12-fold increase), whereas that of the membrane-associated G_n is only modestly affected (two- or threefold increase). In addition, the hydrodynamic behavior of the cytoplasmic G_n is similar to that of GTPγS–liganded G_i, which exists as the fully uncomplexed alpha subunit (4). These data support the idea that formation of the soluble state of G_n is associated with the absence of complexed beta/gamma subunits. Release of a form of G_n alpha subunit from the membrane could allow G_n to modulate enzymes or events localized in the cytosol or potentially in nonplasma membrane subcellular organelles. Regulation of the signal transduction process in the membrane merely by changing the levels of G_n subunits available to interact with receptor units is also a possibility. Such an analagous regulatory process has been shown to occur with protein kinase C (44). Certainly, the importance of G protein in the regulation of receptor-mediated neutrophil activation has been demonstrated (2, 30).

Quantitation of Neutrophil G Proteins

Using antibodies that react specifically with either G alpha (R16,17) or beta (R3,4) subunits, we have determined that G protein makes up between 1 and 3% of total membrane protein in the human neutrophil. This level is higher than has been described for G protein levels in several other tissues (11, 37, 43) and is comparable to the relative G protein level found in brain (22, 37). Based on a figure of 50 pg protein/neutrophil (31), of which 5% may be membrane protein, we estimate there are $\sim 1 \times 10^6$ copies of 40-kD protein per cell. This number includes only membrane-associated G_n units; there seem to be significant levels in the cytosol also (see below). The figure of $\sim 1 \times 10^6$ copies of membrane G_n alpha per cell indicates that G proteins exist at membrane levels 10-fold greater than the number of formyl peptide receptors present per cell (50,000–100,000) (35). However, the total number of receptors that may interact with G_n protein may be as much as 1×10^6, if one includes the LTB_4, PAF, IgG, C5a, etc. receptors present on each neutrophil, all of which have been implicated as coupling to a G protein (30, 34). Our quantitative data thus has implications with regard to models of receptor–G protein interaction. There clearly is not a large excess of G units over total receptors, although any individual receptor class may "see" excess G units. Studies on the competitive interactions of multiple neutrophil chemotactic factor receptors for available G protein units may prove informative.

The presence of levels of G_n alpha subunit in the membrane in apparent excess of the levels of G beta subunit is interesting, especially in view of the presence of free G_n alpha subunit in the cytoplasm. While we have not been able to accurately quantitate the levels of cytosolic alpha subunit as yet, the level would seem to be substantial. One can estimate from the pertussis toxin labeling data of Fig. 4 that, while the concentration of G_n alpha in the cytoplasm may be 1/10 or less of that associated with the membrane, the larger cytoplasmic volume would indicate total cytoplasmic levels may be within a factor of 1/3 to 1/2 of those present in the membrane. Our data suggests that the existence of this soluble alpha subunit could reflect the lack of sufficient beta subunit to "anchor" it to the membrane. Several caveats must be made with regard to our quantitation. Our assay assumes that beta subunits (both 35- and 36-kD forms) in the neutrophil are identical with those found in bovine brain, since we used the purified brain protein to generate our standard curves. Although this assumption has not been rigorously tested, it is widely believed that beta subunits from various

sources are largely identical (10, 11, 13). If, however, the reactivity of one (or both) form of the neutrophil beta subunit with our antibody differs significantly, we may be underestimating the total levels of beta subunit present. The fact that two distinct anti-beta antisera, one (R3,4) prepared against the purified beta subunit and one (K-521) prepared against a peptide, give similar estimates of the membrane beta subunit levels is reassuring in this regard.

The levels of G_n alpha subunit we have estimated by quantitative immunoblotting appear to be relatively high when compared with the levels of [^{35}S]GTPγS binding we obtain in the same membranes. These latter values range from 250 to 350 pmol/mg membrane, translating into a level of 10–15 μg/mg membrane if one assumes that all of this binding is due to the 40-kD protein (even though additional GTPγS binding proteins do exist in the neutrophil; see reference 3). It is not clear, however, that such binding estimates are truly estimating the total G protein present. If it is true that uncomplexed G_n alpha subunit only poorly binds GTPγS (12, 29) then the presence of large amounts of uncomplexed alpha subunit would not be readily detected by [^{35}S]GTPγS binding. There is also a discrepancy between the levels of alpha and beta subunits in the membrane reported here and previous biochemical studies. These showed that neutrophil membranes appear to be insensitive to the adenylate cyclase inhibitory effects of exogenously added beta/gamma subunits and that apparently uncomplexed beta subunit activity exists in detergent extracts of unstimulated neutrophil membranes (1). The affinity of G_n alpha for beta/gamma remains to be established, but, if low, could allow significant levels of uncomplexed beta/gamma subunits to exist, probably more than enough to saturate and inhibit the low levels of G_s alpha subunit that are likely to exist in the neutrophil membrane. The situation might thus not be one of excessive levels of beta subunit inhibiting adenylate cyclase, but rather of the relative affinities of each particular type of G protein alpha subunit for beta. The interactions between the various G protein alpha and beta/gamma subunits in the neutrophil membrane require considerably more study.

We have demonstrated the existence of both membrane and cytoplasmic forms of G_n and the absence of G protein subunits in neutrophil granules. The soluble G_n appears to exist as the uncomplexed alpha subunit. Both alpha (40 kD) and beta subunits exist in the neutrophil at relatively high concentrations in terms of total membrane protein, and their levels appear to differ, with the alpha subunit in excess of beta subunit. Our quantitative analysis is an initial step in developing an understanding of how the receptor(s)–G protein interaction is regulated in the neutrophil.

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