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G-protein α-subunits in cytosolic and membranous fractions of human neutrophils

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Summary

In plasma membranes of human neutrophils, we identified two major pertussis toxin substrates of 40 kDa M_r with pI values of 5.30 and 5.37. Only the acidic of the two substrates was also present in neutrophil cytosol. Two-dimensional tryptic peptide maps revealed a high degree of homology of cytosolic and particulate substrates. Purified G-protein βγ-complex stimulated pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of membranous and cytosolic substrates of neutrophils less than 2-fold and 6-fold, respectively. Hydrodynamic properties of the cytosolic substrate strongly suggested that it exists as a monomer. Purified G-protein βγ-complex increased the $s_{20,w}$ value of the cytosolic substrate from 3.3 S to 4.0 S. The GTP analogue, guanosine 5′-*O*-(3-thiotriphosphate), promoted the release of pertussis toxin substrates from plasma membranes. An antiserum raised against a sequence specific for the G_{i2} α-subunit reacted with 39–40 kDa proteins in plasma membranes and with an apparently single 40 kDa protein in cytosol. We conclude that neutrophil cytosol contains monomeric G_{i2} α-subunits which — by interacting with hydrophobic βγ-complexes — may reversibly bind to the plasma membrane.

Introduction

Guanine nucleotide-binding proteins (G-proteins) are involved in transmembrane signalling (Casey and Gilman, 1988). They are composed of three different subunits referred to as α-, β- and γ-subunits (Hildebrandt et al., 1984). The α-subunits, specific for the individual G-protein, are substrates for ADP-ribosylating bacterial toxins, i.e. for cholera toxin, pertussis toxin or both, and differ by structural criteria including relative molecular masses on SDS gels (39–54 kDa). Multiple cDNA clones encoding pertussis toxin-sensi-

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Abbreviations: ADP, adenosine diphosphate; GDP, guanosine diphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; SDS, sodium dodecylsulfate; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; IEF, isoelectric focusing; NAD, nicotinamide adenine dinucleotide; DTT, dithiothreitol.

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tive α-subunits of G_i-type G-proteins termed G_{i1}, G_{i2} and G_{i3} α-subunits have been identified; apparent molecular masses on SDS gels are 41, 40 and 41 kDa, respectively. In contrast, cDNA clones encoding the α-subunit of the pertussis toxin-sensitive G-protein, G_o, are essentially identical; the α-subunit of this G-protein migrates as a 39 kDa protein on SDS gels (Graziano and Gilman, 1987).

The major pertussis toxin-sensitive G-protein in plasma membranes of polymorphonuclear neutrophils has been purified by several groups (Dickey et al., 1987; Gierschik et al., 1987) and tentatively been identified as G_{i2} (Goldsmith et al., 1987). This protein may be involved in pertussis toxin-sensitive responses of polymorphonuclear neutrophils, e.g. stimulation of phospholipase C (Omann et al., 1987) and NADPH oxidase (Seifert et al., 1988) by chemotactic peptides.

Several groups including ours have recently reported that cytosol of neutrophils contains easily detectable amounts of a 40 kDa pertussis toxin substrate (Gierschik et al., 1987; Rosenthal et al., 1987; Bokoch et al., 1988; Rotrosen et al., 1988), whereas cytosols of human platelets and erythrocytes do not (Rosenthal et al., 1987). Here we report that the cytosolic substrate of neutrophils corresponds to a G-protein α-subunit (most likely a G_{i2} α-subunit) devoid of βγ-complex. In addition, we identified a G-protein α-subunit in plasma membranes of neutrophils which is neither present in the cytosol of this cell type nor in membranous fractions of other cell types.

Materials and methods

Materials

Staphylococcus aureus V8 protease, porcine pancreas elastase, GDP, GTPγS and marker enzymes with known $s_{20,w}$ values were from Boehringer Mannheim (Mannheim, F.R.G.). Lubrol PX was purchased from Sigma (Deisenhofen, F.R.G.) and deionized with a mixed-bed ion-exchange resin (AG 501-X8, 100–200 mesh, Bio-Rad, Munich, F.R.G.). Sucrose and reagents for SDS-PAGE and 2D PAGE were from Serva (Heidelberg, F.R.G.). Carrier-free [^{32}P]phosphoric acid was obtained from New England Nuclear (Bad Nauheim, F.R.G.). Pertussis toxin was a kind gift of Dr. M. Yajima (Shiga, Japan). The α_{common} peptide and the α_o peptide (see below) were gifts of Dr. A. Herz (Munich, F.R.G.). The $\alpha_{i\,common}$ and the α_{i2} peptide were kindly provided by Drs. H. Gausepohl and R. Frank (Heidelberg, F.R.G.). D_2O was from Isotron (Dusseldorf, F.R.G.). Sources for other materials have been cited (Rosenthal et al., 1986; Hinsch et al., 1988).

Preparation of membranes and cytosol

Buffy coat (as a source for neutrophils) was kindly provided by the local blood bank. Neutrophils were isolated by dextran sedimentation, hypotonic lysis of erythrocytes and Ficoll density gradient centrifugation as described by Markert et al. (1984). Isolated neutrophils were disrupted by nitrogen cavitation, and light membrane fractions were obtained by density gradient centrifugation on a discontinuous Percoll gradient (Borregard et al., 1983). Electron micrography of light membranes revealed that they mainly consisted of plasma membrane vesicles, heterogeneous in size and shape (Seifert and Schultz, 1987). The top layer of the gradient was recentrifuged at 200 000 $\times g$ for 1 h at 4°C and extensively dialyzed against a Mg^{2+}-free buffer consisting of (in mM) KCl 100, NaCl 3, EDTA 1, and Pipes 10 (pH 7.3); this preparation is referred to as cytosol.

[^{32}P]ADP-ribosylation of membranous and cytosolic proteins

Pertussis toxin-catalyzed [^{32}P]ADP-ribosylation was performed as described (Rosenthal et al., 1987). Preactivated toxin was diluted with BSA-containing buffer to a final concentration of 1.7 μg/ml. Control samples received dilution buffer without toxin. The final NAD concentration was 1 μM with 37–74 kBq [^{32}P]NAD/assay tube. The assay volumes were 60 and 120 μl for membranous and cytosolic preparations, respectively. Samples were incubated for 30 min at 30°C.

Gel electrophoresis

Prior to gel electrophoresis, proteins were precipitated by acetone; the precipitate was washed with trichloroacetic acid and subsequently with chloroform or methanol. SDS-PAGE was performed according to Laemmli (1970) and 2D PAGE according to O'Farrell (1975) with modifi-

cations described previously (Rosenthal et al., 1986).

Peptide mapping by limited proteolysis was performed according to Cleveland et al. (1977). The procedure was adapted from 2D PAGE with modifications. Following IEF, rod gels were placed on top of the second dimension SDS slab gel (15% acrylamide) and embedded with agarose. After this, the slab gel was transferred into the gel apparatus. The upper chamber was filled with electrode buffer and the gel (1.5 × 200 × 250 mm) carefully overlayed with 1 ml of equilibration buffer supplemented with 30 μg/ml of *Staphylococcus aureus* V8 protease or 16 μg/ml of elastase. Gels were run as described (Rosenthal et al., 1986).

If autoradiography was intended, stained gels were dried and juxtaposed to Kodak XAR 5 or 3M R2 films with or without enhancing screens for various times at −75°C.

Production and characterization of antisera

Antisera were generated against synthetic peptides corresponding to confined regions of G-protein α-subunits. Coupling of peptides to keyhole limpet hemocyanin and immunization of rabbits have been described elsewhere (Rosenthal et al., 1988). The sequence of the α_{common} peptide (C-GAGESGKSTIVKQMK), identical with the one used by Mumby et al. (1986), is highly conserved in all known G-protein α-subunits except the G_z α-subunit (Fong et al., 1988). An antiserum raised against this peptide recognized α-subunits of G_s, G_i-type G-proteins, G_o and the retinal G-protein, transducin (Hinsch et al., 1988; Rosenthal et al., 1988). The sequence of the α_o peptide (C-NLKEDGISAAKDVK), also identical with the one used by Mumby et al. (1986), is specific for the G_o α-subunit. The antiserum raised against this peptide recognized the G_o α-subunit but not other G-protein α-subunits (Hinsch et al., 1988). The sequence of the $\alpha_{i\,common}$ peptide (C-NLREDGEKAAREV) is found in the α-subunits of G_{i1} and G_{i2} and differs from the corresponding sequence in the α-subunit of G_{i3} in only one amino acid. The antiserum raised against this peptide recognized 40–41 kDa α-subunits of G_i-type G-proteins purified from various tissues, which presumably represent G_{i1}, G_{i2} and G_{i3} (Hinsch et al., 1988). The sequence of the α_{i2} peptide (C-TGANKYDEAAS) is specific for the G_{i2} α-subunit. The antiserum generated against this peptide recognized a single 40 kDa protein in preparations of purified G_i-type G-proteins and all tissues tested so far; this immunoreactive protein most likely represents the G_{i2} α-subunit (Hinsch et al., 1988).

Immunoblotting

Proteins were acetone-precipitated, dissolved in sample buffer and loaded onto an SDS slab gel, composed of 8% (w/v) acrylamide, 0.21% (w/v) bisacrylamide and 4 M urea. Electrophoresis was performed at a constant current of 20 mA for 3.5 h. Immunoblotting (using ^{125}I-protein A for the detection of filter-bound antibodies) and autoradiography of filters were performed as described (Rosenthal et al., 1986).

Sucrose density gradient centrifugation

Hydrodynamic properties of the cytosolic α-subunit were analyzed by centrifugation of the protein through H_2O or D_2O (94%) sucrose density gradients (5–20%, 6 ml), containing (in mM) EDTA 1, DTT 1, NaCl 100, GDP 0.05, Hepes 20 (pH 8.0) and Lubrol PX (0.1%). Employed marker enzymes with known $s_{20,w}$ values (S) and $\bar{v}$ values (cm^3/g) were catalase (11.3, 0.730), fumarase (9.1, 0.738), malic dehydrogenase (4.3, 0.732) and cytochrome *c* (1.7, 0.728). Cytosol (120 μl, corresponding to 120 μg of protein) was kept at 30°C for 30 min. When βγ-complex purified from porcine brain was added (480 ng per 120 μg of cytosolic protein), a 14 h incubation at 4°C preceded the 30 min incubation at 30°C. Marker enzymes (30 μl) and cytosol were mixed and loaded on a gradient. Centrifugation was for 55 min at 30°C in a Sorvall TV-865 rotor (400 000 × *g*). The α-subunit was assayed by pertussis toxin-catalyzed [^{32}P]ADP-ribosylation followed by SDS-PAGE (10% gels) and either excision of 40 kDa gel bands and counting in scintillation fluid or densitometric scanning of autoradiographs.

Gel filtration analysis

Gel filtration was performed on a 7.5 mm × 600 mm (26.5 ml) TSK G3000SW column (Pharmacia/LKB) at 30°C; the flow rate was 0.5 ml/min. The column was equilibrated with (in

mM) EDTA 1, DTT 1, NaCl 100 and Hepes 20 (pH 7.4). Cytosol (130 μl) corresponding to 120 μg of protein was loaded on the column after mixing with 20 μl marker proteins. Fractions (200 μl) were collected and analyzed in the same manner as were the gradient fractions. The Stokes' radii of the employed markers are: fumarase, 5.27 nm; catalase, 5.21 nm; malic dehydrogenase, 3.69 nm and cytochrome *c*, 1.87 nm.

Miscellaneous

[α-^{32}P]ATP was synthesized according to Johnson and Walseth (1979) and [^{32}P]NAD according to Cassel and Pfeuffer (1978). ^{125}I-Protein A was synthesized using Iodo-Gen as oxidizing agent (Salacinski et al., 1981). βγ-Complex of G-proteins was purified from porcine brain (Rosenthal et al., 1986). Protein was assayed according to Lowry et al. (1951) as modified by Peterson (1983), using BSA as a standard.

The results shown are typical for those obtained in two or more experiments performed under identical conditions.

Results

When plasma membranes and cytosol of neutrophils were incubated at increasing concentrations of pertussis toxin with [^{32}P]NAD, the toxin [^{32}P]ADP-ribosylated proteins of 40 kDa M_r in a concentration-dependent manner; radiolabeling of these proteins was not observed in the absence of toxin (data not shown).

[^{32}P]ADP-ribosylation of both membranous and cytosolic 40 kDa proteins was reduced by Mg^{2+} and GTPγS as has been reported for the α-subunits of pertussis toxin-sensitive G-proteins (Tsai et al., 1984; Ribeiro-Neto et al., 1985; Mattera et al., 1987) (data not shown). To further substantiate that the 40 kDa substrates in both preparations represent α-subunits of G-proteins, we examined the influence of the G-protein βγ-complex purified from porcine brain on pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. Some of the βγ-complex preparations used contained small amounts of G-protein α-subunits which were invisible on stained SDS gels but detectable by pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. To avoid radiolabeling of exogenously added G-protein α-subunits, the respective βγ-complex preparations were incubated with non-radioactive NAD at mM concentrations in the presence of the toxin. After this pretreatment and extensive dialysis (for removal of NAD), α-subunits were no longer detectable by subsequent toxin-catalyzed [^{32}P]ADP-ribosylation. In plasma membranes, the detergent Lubrol PX, a constituent of G-protein-containing solutions, stimulated [^{32}P]ADP-ribosylation catalyzed by the toxin 2- to 3-fold (Fig. 1). Addition of βγ-complex at a concentration of 3 μg/ml in Lubrol PX-containing buffer caused a less than 2-fold stimulation as compared to Lubrol PX. Heated βγ-complex preparations were as effective as was Lubrol PX. The detergent stimulated the [^{32}P]ADP-ribosylation of the cytosolic 40 kDa protein to a similar extent as that of the membranous protein. In contrast to its relatively weak stimulatory effect on the membranous substrate, the βγ-complex enhanced [^{32}P]ADP-ribosylation of the cytosolic substrate about 6-fold as compared to Lubrol PX; the effect depended on native βγ-complex, as heated preparations were not more effective than was Lubrol PX. Thus, the pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of both membranous and cytosolic substrates is stimulated by βγ-complex, as has been reported for G-protein α-subunits (Neer et al., 1984). The finding that the effect of βγ-complex is more pronounced in cytosol than in plasma membranes suggests that the cytosolic substrate is at least to a major portion not associated with βγ-complex.

Fig. 2 shows the 2D PAGE analysis of pertussis toxin substrates of neutrophils. In plasma membranes, two major substrates and one minor substrate of 40 kDa M_r were identified with p*I* values of about 5.30, 5.37 and 5.50, respectively. In cytosol, a major and a minor substrate of 40 kDa M_r with p*I* values of about 5.30 and 5.50, respectively, were observed. If a mixture of both preparations, plasma membranes and cytosol, was applied, the cytosolic substrates were indistinguishable from the major acidic (p*I* 5.30) and minor basic (p*I* 5.50) membranous substrate, respectively (data not shown). [^{32}P]ADP-ribosylation of cytosolic substrates was considerably stimulated by βγ-complex plus Lubrol PX. (To yield comparable signals on X-ray films, gels had

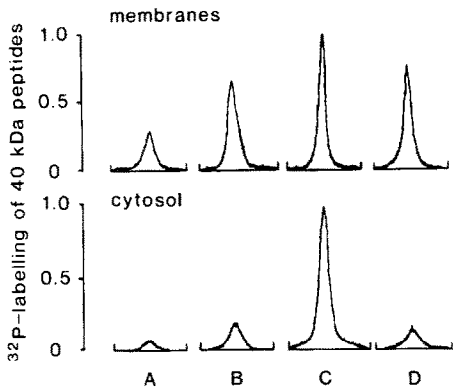

Fig. 1. Stimulation of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation by Lubrol PX and $\beta\gamma$-complex. [^{32}P]ADP-ribosylation of plasma membranes and cytosol of neutrophils (10 μg of protein of each) was carried out in the absence (*A*) or presence of 0.016% Lubrol PX (*B*), 3 μg/ml of $\beta\gamma$-complex plus 0.016% Lubrol PX (*C*) or in the presence of heated (5 min, 95 °C) $\beta\gamma$-complex plus Lubrol PX (*D*). After incubation, membranes or cytosol were applied to a 10% SDS gel. Graphs show radioactivity scans of the 40 kDa region. No radioactivity was detected when pertussis toxin was omitted from the reaction mixture. Radioactivity is given in arbitrary units. The pretreatment of $\beta\gamma$-complex with pertussis toxin and non-radioactive NAD is described under Results.

to be exposed for 1–2 days or 2–3 weeks, depending on whether or not $\beta\gamma$-complex plus Lubrol PX were present during toxin-catalyzed [^{32}P]ADP-ribosylation.) In addition, incorporation of radioactivity into the major basic substrate (p*I* 5.37) was overproportionally increased; control experiments with heated $\beta\gamma$-complex revealed that this effect was caused by Lubrol PX, whereas $\beta\gamma$-complex enhanced [^{32}P]ADP-ribosylation without changing the pattern of radiolabeled proteins qualitatively (data not shown). We did not detect a cytosolic equivalent of the major basic membranous substrate (p*I* 5.37).

In order to reveal structural differences between the various pertussis toxin substrates of neutrophils, we separated substrates by IEF and allowed limited proteolysis during SDS-PAGE, using *Staphylococcus aureus* V8 protease or elastase from porcine pancreas. Probably due to the small difference in charge, we were unable to distinguish between the radiolabeled fragments of the two major toxin substrates (p*I* 5.30 vs. 5.37) identified in neutrophil membranes. Therefore, [^{32}P]ADP-ribosylation was performed under conditions, under which either of the these substrates was the preferential toxin substrate, i.e. in the absence and presence of $\beta\gamma$-complex plus Lubrol PX (see Fig. 2). Using *Staphylococcus aureus* V8 protease, the same electrophoretic pattern was obtained under both conditions (Fig. 3). Partial digests of elastase also yielded almost identical patterns under both conditions (data not shown). This suggests that both major membranous substrates are structurally very similar. Peptide maps of the cytosolic substrates were essentially identical with those of the membranous substrates, independent of whether or not $\beta\gamma$-complex plus Lubrol PX were present during the incubation with toxin. The data suggest that membranous and cytosolic substrates are highly homologous.

For further characterization of G-protein α-subunits in neutrophils, we performed immunoblots, using high resolution (urea-containing) SDS gels and antisera raised against synthetic peptides (see Materials and Methods). Cholate extract from porcine brain, which contains various G-proteins including G_s, G_{i1}, G_{i2} and G_o (Hinsch et al., 1988; Mumby et al., 1988), was run as a control. An antiserum raised against the α_{common} peptide recognized various proteins of 39–41 kDa M_r in neutrophil plasma membranes and an apparently single 40 kDa protein in neutrophil cytosol (Fig. 4, panel *A*). An antiserum raised against the $\alpha_{i\,common}$ peptide detected a relatively broad band (39–40 kDa) in the plasma membrane preparation but a narrow band (40 kDa) in the cytosol (panel *B*). Similarly, an antiserum raised against the α_{i2} peptide reacted with 39–40 kDa proteins in plasma membranes and with an apparently single 40 kDa protein in cytosol (panel *C*). An antiserum raised against the α_o peptide recognized the 39 kDa G_o α-subunit and a 40 kDa protein in brain cholate extract. The 40 kDa protein, also present in some preparations of purified G_o, may be identical with the one described by Goldsmith et al. (1988); it did not correspond to one of the G_i α-subunits which were not recognized by this antiserum (Hinsch et al., 1988). The α_o peptide antiserum failed to detect an immunoreactive protein of 39–41 kDa in membranous or cytosolic preparations of neutrophils (panel *D*), indicating that neutrophils do not contain G_o. The absence of G_o from plasma membranes of neutrophils has also

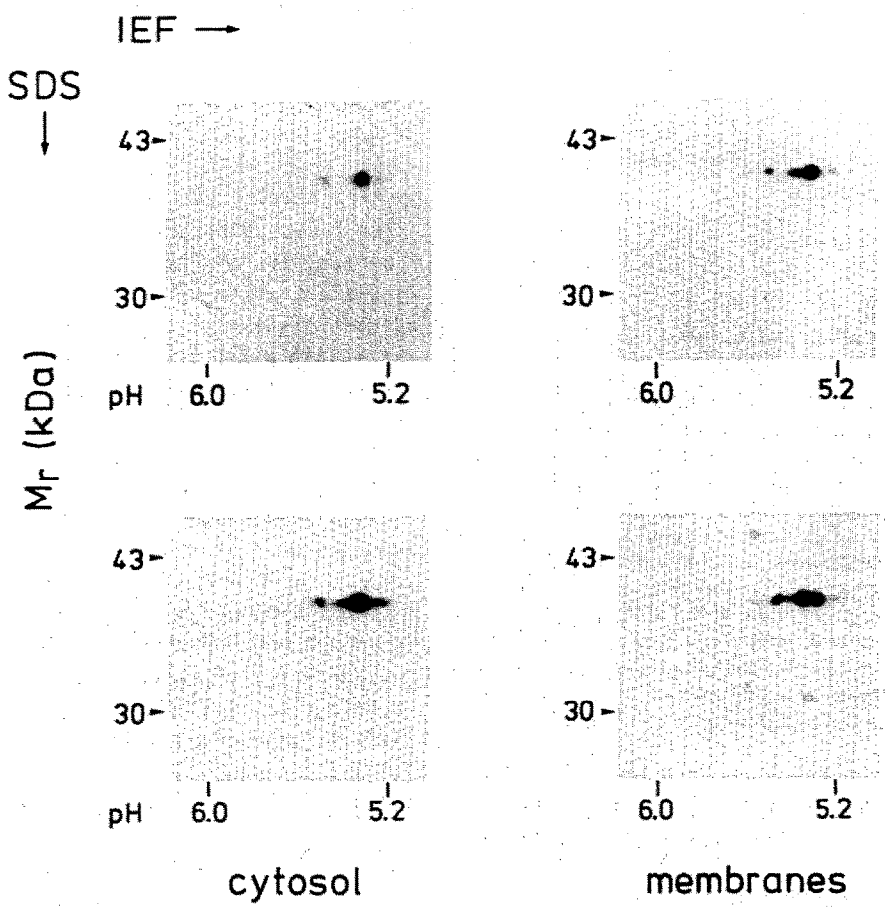

Fig. 2. Comparison of cytosolic and membranous pertussis toxin substrates of neutrophils (2D PAGE analysis). The applied amounts of protein were 10 μg of either preparation. The incubation with pertussis toxin and [^{32}P]NAD was performed in the absence (upper panels) or presence of pretreated βγ-complex (3 μg/ml) plus Lubrol PX (0.016%) (lower panels). Radiolabeling of the 40 kDa proteins was strictly dependent on the presence of pertussis toxin. Shown are autoradiographs of 10% SDS gels.

been reported by others (Goldsmith et al., 1987). The results suggest that, whereas plasma membranes contain at least two subtypes of G_i α-subunits and/or two forms of the G_{i2} α-subunit, the cytosolic α-subunit corresponds to one type of the G_{i2} α-subunit.

The $s_{20,w}$ value of the cytosolic α-subunit was determined by centrifugation through sucrose density gradients. The values obtained in H_2O and D_2O were 3.27 ± 0.16 S ($n = 6$) and 2.47 ± 0.18 S ($n = 4$), respectively (compare Figs. 5A and 6), indicating that a substantial amount of detergent is bound to the protein. Calculations according to the method described by Sadler (1979) revealed that 0.58 mg Lubrol PX were bound per 1 mg of protein (36.7%, w/w). The partial specific volume of the protein-detergent complex was 0.817 ± 0.017 cm^3/g and its corrected $s_{20,w}$ value 3.35 S. The Stokes' radius was determined as 3.80 ± 0.14 nm ($n = 3$) in the absence of detergent (not shown). The molecular mass of the cytosolic α-subunit was calculated according to the Svedberg equation *.

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

where N_{av} is Avogadro's number, $\eta_{20,w}$ the viscosity of water at 20 °C, $\rho_{20,w}$ the density of water at 20 °C, $\bar{v}$ the partial specific volume assumed in this case to be 0.735 ml/g, a the Stokes' radius, and $s_{20,w}$ the standard sedimentation coefficient (see text).

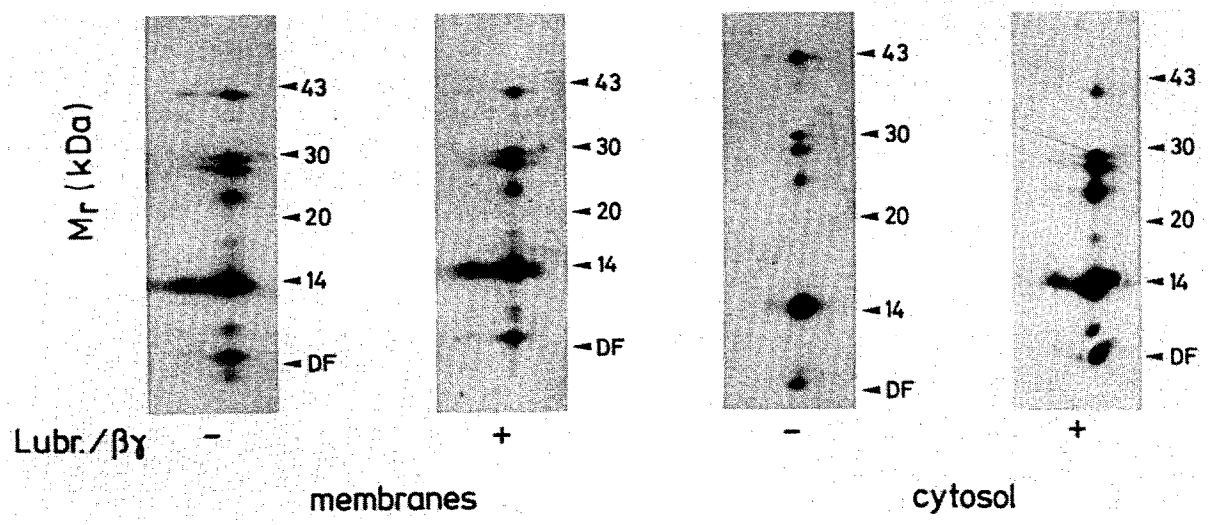

Fig. 3. Partial proteolysis of pertussis toxin substrates of neutrophil plasma membranes and cytosol. Membranes (30 μg of protein) and cytosol (8 μg of protein) were incubated with pertussis toxin and [^{32}P]NAD in the absence or presence of Lubrol PX (0.016%) plus βγ-complex (3 μg/ml; βγ). Limited proteolysis using *Staphylococcus aureus* V8 protease and analysis of digests were performed as described under Materials and Methods. The autoradiographs were obtained after exposure times ranging from a few days to 2 weeks depending on whether βγ-complex was added or not. The pH range shown was from about 6.5 to about 5.2 (from left to right). DF, dye front.

Employing the Stokes' radius of 3.80 nm and the corrected $s_{20,w}$ value of 3.35 S, we calculated a molecular mass of 54.2 kDa. This value is in good agreement with that obtained for the GTPγS-liganded G_i α-subunit (51 kDa, Bokoch et al., 1983). Since the cytosolic α-subunit binds a con-

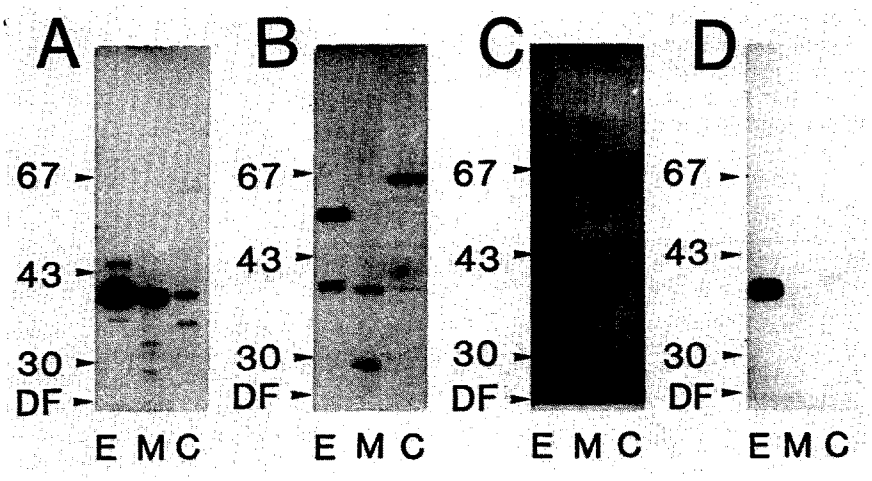

Fig. 4. Immunological characterization of G-protein α-subunits in plasma membranes and cytosol of neutrophils. SDS gels were loaded with cholate extract from membranes of porcine brain (45 μg; E), employed as a positive control (see Results and Hinsch et al., 1988), neutrophil plasma membranes (45 μg; M) and neutrophil cytosol (100 μg; C). Nitrocellulose filters were incubated with the α_{common} peptide antiserum (1 : 500, panel *A*), the $\alpha_{i\,common}$ peptide antiserum (1 : 500, panel *B*), the α_{i2} peptide antiserum (1 : 500, panel *C*) or the α_o peptide antiserum (1 : 300, panel *D*). Shown are autoradiographs of immunoblots. In contrast to the binding of antisera to proteins of $M_r \gg 43$ kDa, the binding of the antisera to proteins of 39–43 kDa M_r in cholate extract from membranes of brain cortex or to α-subunits of purified G-proteins was blocked by preincubation of antisera with the respective synthetic peptide employed as a hapten (not shown).

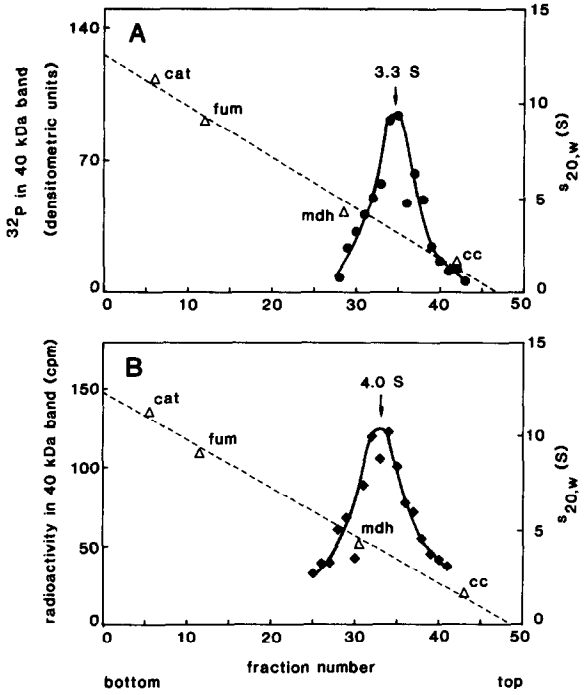

Fig. 5. Influence of added $\beta\gamma$-complex on the sedimentation behaviour of the cytosolic G-protein α-subunit in neutrophils. Prior to centrifugation, cytosol was incubated without (panel *A*) or with $\beta\gamma$-complex purified from porcine brain (panel *B*). The experiments were performed as described under Materials and Methods with the exception that [^{32}P]ADP-ribosylation was performed in the presence of 0.3% Lubrol PX. The open triangles denote the marker enzymes, the closed circles arbitrary densitometric units and the closed diamonds radioactivity incorporated into the 40 kDa gel band. cat, catalase; fum, fumarase; mdh, malic dehydrogenase; cc, cytochrome *c*.

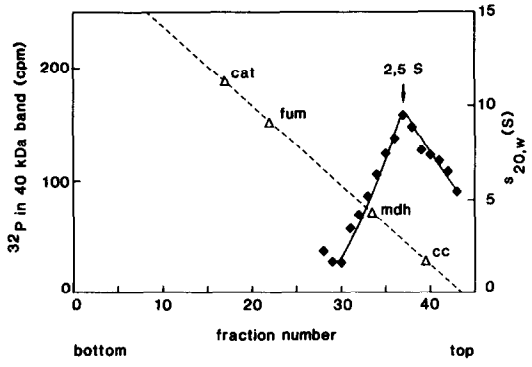

Fig. 6. Sedimentation of the cytosolic G-protein α-subunit in a D_2O-containing sucrose density gradient. The experiment is equivalent to the one shown in Fig. 5*A* except that D_2O was used as solvent.

siderable amount of detergent, a calculated molecular mass of 54.2 kDa is likely to represent an overestimate. As even this value is substantially lower than those reported for heterotrimeric G_i (82 kDa, Bokoch et al., 1983; 96 kDa, Codina et al., 1984), it is most likely that the cytosolic α-subunit exists as a monomer. The amount of deter-

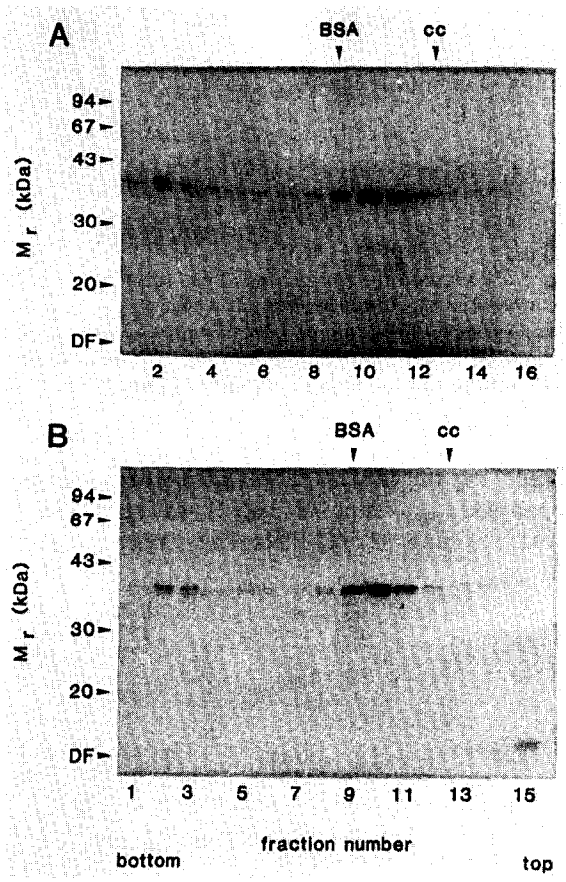

Fig. 7. GTPγS-induced release of G-protein α-subunits from plasma membranes of neutrophils. Plasma membranes (20 μg of protein) were [^{32}P]ADP-ribosylated as described under Material and Methods. Subsequently $MgCl_2$ (1 mM) and guanine nucleotides (1 mM GDP, panel *A*; 100 μM GTPγS, panel *B*) were added to the reaction mixture. Following an incubation at 30 °C for 15 min, samples were centrifuged through a 5–20% sucrose density gradient. Shown are autoradiographs of SDS gels to which aliquots of gradient fractions were applied. The positions of marker proteins (bovine serum albumin, BSA, 4.3 S; cytochrome *c*, cc, 1.7 S) are indicated. The 40 kDa regions of SDS gels were excised and counted for radioactivity in liquid scintillant. Panel *A*: fractions 1–7, 299 cpm; fractions 8–13, 495 cpm; panel *B*: fractions 1–7, 231 cpm; fractions 8–13, 594 cpm.

gent bound to the cytosolic α-subunit was higher than that reported for the G_o α-subunit by Huff et al. (1985), who estimated that 0.1 mg of Lubrol PX is bound per 1 mg of protein. This finding indicates that the G_{i2} α-subunit is a more hydrophobic protein than the G_o α-subunit.

Upon addition of βγ-complex prior to centrifugation, an $s_{20,w}$ value of 4.04 ± 0.14 S ($n = 5$) was obtained in H_2O-containing sucrose density gradients (Fig. 5*B*); this value is similar to that observed for heterotrimeric G-proteins (Codina et al., 1984; our own unpublished data). This finding suggests that the monomeric cytosolic α-subunit associates with added βγ-complex to form a heterotrimeric G-protein.

In seven experiments we found that the GTP analogue, GTPγS, promoted the release of pertussis toxin substrates from neutrophil plasma membranes in the absence of detergent. Membranes were [^{32}P]ADP-ribosylated with pertussis toxin as described under Materials and Methods and subsequently incubated with $MgCl_2$ and GDP or GTPγS in a hypotonic buffer (15 min at 37 °C; see legend to Fig. 7). GDP was included in the control experiments in order to prevent denaturation (loss of activity, aggregation) of G-proteins in the presence of Mg^{2+} (Codina et al., 1984). Membrane-associated and released pertussis toxin substrates were separated by centrifugation of samples through sucrose density gradients at 4 °C or centrifugation of samples at room temperature at 165 000 × *g*. In the experiment shown in Fig. 7, GTPγS caused an increase in pertussis toxin substrates found in the light fractions (8–13); pertussis toxin substrates in the heavy fractions (1–7) decreased correspondingly. The ratio of the amount of radioactivity found in the heavy fractions to that found in the light fractions was 0.60 and 0.39 in the presence of GDP and GTPγS, respectively.

Discussion

In this report, we provide evidence that the pertussis toxin substrate of neutrophil cytosol is a monomeric G_{i2} α-subunit. While this paper was in preparation, Bokoch et al. (1988) reported that the pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of a 40 kDa protein in neutrophil cytosol was stimulated by G-protein βγ-complex. Using an antiserum raised against a peptide highly conserved in G_i-type G-proteins (G_{i1-3}) and transducin, this group, by applying the immunoblot technique, detected G-protein α-subunits in neutrophil plasma membranes but not in cytosol. They showed, however, that the [^{32}P]ADP-ribosylated cytosolic substrate was precipitated by the antiserum. We show here that neutrophil cytosol contains a 40 kDa protein which is not only detected by antisera raised against the α_{common} and $\alpha_{i\ common}$ peptides but also by an antiserum raised against the α_{i2} peptide. Thus, the cytosolic substrate appears to represent a G_{i2} α-subunit. The occurrence of a cytosolic G_{i2} α-subunit is consistent with the observation that this G-protein α-subunit — similar to the G_o α-subunit (Sternweis and Robishaw, 1984) — dissociates from βγ-complex in the absence of activating agents during chromatography on heptylamine-Sepharose (unpublished data). Using antisera raised against the α_{common} peptide, we also detected an immunoreactive 40 kDa protein in the cytosol of myeloid differentiated HL-60 cells (unpublished data).

In contrast to the easily detectable cytosolic α-subunit, β-subunits were undetectable in the cytosol, as determined with an antiserum raised against purified βγ-complex or an antiserum raised against the retinal G-protein (transducin) which strongly crossreacted with β-subunits of non-retinal G-proteins (Rosenthal et al., 1986; unpublished data). Similar findings have been reported by Bokoch et al. (1988). Thus, at least the major portion if not all of the cytosolic α-subunits are not associated with β-subunits. This notion is supported by the finding that [^{32}P]ADP-ribosylation of the cytosolic pertussis toxin substrate is greatly enhanced by added βγ-complex and that purified βγ-complex increases the protein's sedimentation coefficient to a value consistent with that of a heterotrimeric G-protein. Finally, the calculated (probably overestimated) molecular mass of the cytosolic substrate (54.2 kDa) is consistent with a monomeric G-protein α-subunit but inconsistent with a heterotrimeric G-protein.

We observed that 40 kDa pertussis toxin substrates, which by several criteria represent G-protein α-subunits (see Figs. 1–4; Gierschik et al., 1987), are released from neutrophil plasma mem-

branes and that the release is stimulated by the poorly hydrolyzable GTP analogue, GTPγS; the sedimentation coefficient of the GTPγS-activated released substrate was about 3 S at 4°C; this value is essentially identical with that obtained for the cytosolic substrate under similar conditions (unpublished observation). Our findings are in contrast to a report by Eide et al. (1987), who — under experimental conditions different from those we have chosen — did not observe a release of G-protein α-subunits after a 15 min incubation at 37°C in the absence or presence of 100 μM GTPγS. Milligan et al. (1988) reported a slow release of a G_{i2} α-subunit from membranes of rat glioma C6 BU1 cells and of unspecified G_i α-subunits from membranes of both human platelets and the neuroblastoma × glioma hybrid cell line NG 108-15. In the latter cell line, a guanine nucleotide-mediated release of the G_o α-subunit has also been observed (McArdle et al., 1988). Lynch et al. (1986) demonstrated the release of the G_s α-subunit from rat liver plasma membranes upon activation with cholera toxin. It is not clear at present whether the release of G-protein α-subunits is related to receptor-mediated activation of G-proteins. However, these findings may support the idea that α-subunits are released from the plasma membrane, thereby rendered able to modulate the activity of effectors which are not associated with the plasma membrane (Rodbell, 1985). Our finding that cytosolic α-subunits are capable of interacting with purified βγ-complexes indicates that G-protein α-subunits are not only released from the plasma membrane but are also able to reassociate with the plasma membrane by binding to the permanently membrane-bound βγ-complex. Functional evidence for the occurrence of a G-protein in soluble fractions has been provided by Bhat et al. (1980), who demonstrated that a 300 000 × *g* supernatant fraction from liver conferred guanine nucleotide and fluoride activation to adenylyl cyclase of a mouse lymphoma cell line (S49) that lacks the stimulatory G-protein, G_s (*cyc*$^-$ mutant).

2D PAGE analysis showed that plasma membranes of neutrophils contain a pertussis toxin substrate (M_r 40 kDa, p*I* 5.37) which — in contrast to the other two substrates with p*I* values of 5.30 and 5.50 — is neither present in the cytosol of this cell type (see Results) nor in membranous fractions of human erythrocytes and platelets (Koesling et al., 1988). [^{32}P]ADP-ribosylation of this neutrophil-specific membranous substrate was overproportionally increased by Lubrol PX, suggesting that the accessibility of this substrate for preactivated pertussis toxin was hampered in the absence of detergent. The assumption that the substrate represents a cell type-specific G-protein α-subunit is supported by the finding that the antiserum raised against the α_{i2} peptide recognized an additional protein of 39 kDa in neutrophil plasma membranes, whereas in all other preparations, i.e., purified G-proteins from brain, liver and erythrocytes as well as membranous fractions of various cell types, the antiserum, under identical experimental conditions, detected an apparently single protein of 40 kDa, presumably the G_{i2} α-subunit (Hinsch et al., 1988). As this antiserum did not crossreact with the 39 kDa G_o α-subunit and antisera specific for the G_o α-subunit failed to detect immunoreactive proteins in both neutrophil plasma membranes and cytosol, the immunoreactive 39 kDa protein in neutrophil plasma membranes may represent an α-subunit homologous to the G_{i2} α-subunit or a posttranslationally modified form of the G_{i2} α-subunit.

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