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Identification of novel GTP-binding proteins in the human neutrophil

Gary M. Bokoch and Charles A. Parkos

Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

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We describe here the existence of previously undescribed GTP-binding proteins within the human neutrophil. These proteins specifically bind guanine nucleotides under conditions in which the previously characterized G-proteins are unable to bind. We have partially purified these proteins and present both functional and immunologic data which indicate that they are unrelated to G_n, the major neutrophil pertussis toxin substrate. An additional protein, of apparent molecular mass 22 kDa, may be related to the ras G-protein family. Analysis of the structural and functional characteristics of these novel proteins will promote a better understanding of the process of neutrophil activation.

GTP-binding protein; Neutrophil; Signal transduction

1. INTRODUCTION

The generation of intracellular signals in response to the occupation of membrane receptors has been found in an increasing number of systems to involve GTP-binding proteins (G-proteins) which regulate the ability of the receptor to interact with various enzymatic activities, ion channels, etc., of the cell [1]. In the neutrophil, a G-protein able to serve as a specific substrate for pertussis toxin has been implicated in the process of neutrophil activation through its ability to couple receptors to phospholipases [2–5]. This protein has recently been purified and characterized as a novel G-protein with an α-subunit of about 40 kDa [6,7]. While the role of this protein in receptor-mediated phospholipase activation seems evident based on data obtained with pertussis toxin [2–5], the possibility that other G-proteins might be involved in the activation process remains. Indeed, in many cell types which contain receptor-activated phospholipase activity, this activation process has been demonstrated to involve an as yet unidentified pertussis toxin-insensitive G-protein [8,9]. There are, additionally, data that indicate that in the neutrophil (as well as in other secretory cells) exocytotic secretion can be initiated by guanine nucleotides in a pertussis toxin-insensitive manner and in the absence of phospholipase C activity or Ca^{2+} mobilization [10–12]. In the light of these data, we have attempted to identify the G-protein composition of the human neutrophil. In this report we describe the presence of GTP-binding proteins in addition to G_n in human neutrophils.

Correspondence address: G.M. Bokoch, Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

2. MATERIALS AND METHODS

2.1. *Preparation of neutrophil membranes*

A highly enriched preparation of neutrophils (85–95% polymorphonuclear cells) was prepared as described [13,14]. Care was taken to minimize red blood cell contamination by repeated lysis in 0.83% ammonium chloride. After diisopropylfluorophosphate treatment, the cells were disrupted by N_2 cavitation and membranes prepared as described in [14], then stored frozen in 25 mM Hepes, pH 7.0/0.2 M sucrose with 1250 units of aprotonin and 1 mM phenylmethylsulfonyl fluoride. The membranes obtained were examined for the

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presence of potentially contaminating platelet membranes using monoclonal antibody 22C4 in a quantitative ELISA [15]. This antibody is specific for the IIIa subunit of the platelet membrane associated adhesion protein GPIIb, IIIa. Comparison of the neutrophil membranes with platelet membranes on a protein-protein basis indicated that less than 1% of our neutrophil membrane preparation could be contaminating platelet membrane. This value is likely to be an overestimate since the platelet membranes used to construct the standard curve in the ELISA were somewhat less purified than the neutrophil membrane preparation.

2.2. *Blotting with [α-^{32}P]GTP*

Samples of membrane or purified proteins (prepared by the procedures described in [25]) were suspended in electrophoresis sample buffer for SDS-polyacrylamide gel electrophoresis by the method of Laemmli [16]. Proteins were then transferred to nitrocellulose for 3 h at 500 mA by the method of Towbin et al. [17].

Transfers which were to be labeled with [α-^{32}P]GTP were immersed in 10 ml of 50 mM Tris-HCl, pH 7.5/0.3% Tween/0.5 mM EDTA/0.5 mM $MgCl_2$ and 1 nM [α-^{32}P]GTP (spec. act. ~2000 dpm/nmol), as described in [18].

The blots were incubated for 30 min at room temperature, then washed three times with 10 ml of 50 mM Tris-HCl, pH 7.5/0.3% Tween/2 μM $MgCl_2$. Blots were dried and bound [α-^{32}P]GTP detected by autoradiography with Kodak XRP film for 5–24 h at −70°C with intensifying screen.

2.3. *Immunoblotting*

Sample proteins were transferred to nitrocellulose as described above and immunoblotting performed essentially as described by Towbin et al. [17]. Antibodies utilized were: R5,6, a rabbit polyclonal antibody prepared against bovine brain G_i and which reacts with both the α- and β-subunits of G_n, G_i and G_o, as described in [19]. R16,17, a rabbit polyclonal prepared against the peptide NNLKGCDLF, representing the 9 carboxyl-terminal amino acids of the G_i sequence described in [20]. This latter antibody will recognize G_i, G_n, and transducin α-subunits, while reacting very poorly with the α-subunit of G_o [21]. 142-24E05, a mouse monoclonal antibody which was raised against a synthetic peptide comprising amino acids 96–118 of H-ras [22], although the exact epitope recognized is unknown. A portion of this sequence is necessary for guanine nucleotide binding by ras proteins and is very highly conserved amongst H, N, and K-ras, all of which are reactive with this antibody.

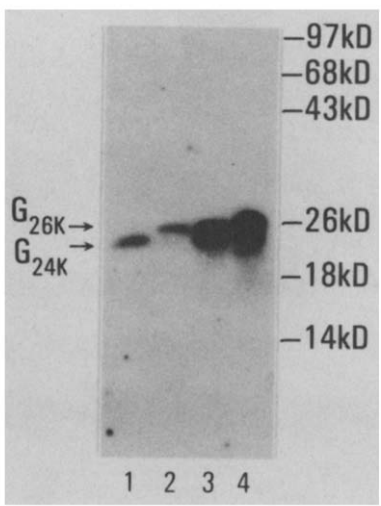

Fig.1. Binding of [α-^{32}P]GTP to neutrophil and platelet proteins analyzed by blot analysis. Protein was electrophoresed on 15% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with [α-^{32}P]GTP as described in section 2. Lanes: 1, 180 ng total protein of G_{24K} pool depicted in fig.3; 2, 300 μg total protein of G_{26K} pool depicted in fig.3; 3, 22 μg human neutrophil membrane; 4, 22 μg human platelet membrane.

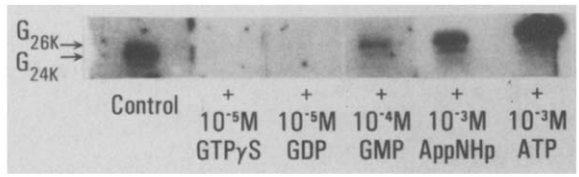

Fig.2. Competition by various nucleotides of the binding of [α-^{32}P]GTP to neutrophil membranes. Neutrophil membrane (22 μg) was analyzed as described in section 2 in the presence of the indicated concentrations of unlabeled nucleotides. 'Control' indicates incubation with [α-^{32}P]GTP only.

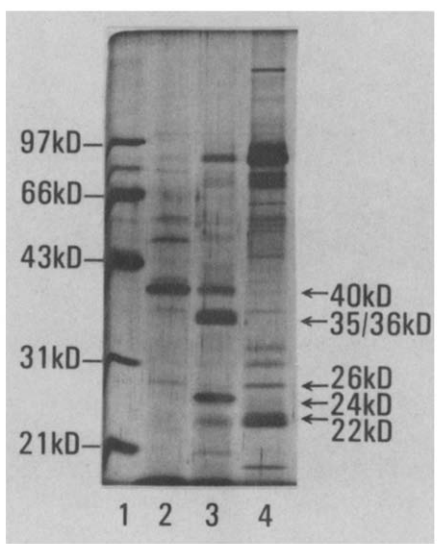

Fig.3. Silver stain visualization of human neutrophil GTP-binding proteins. Human neutrophil GTP-binding proteins were purified by modifications of the methods described in [25]. Molecular masses of relevant G-protein subunits are indicated on the right of the figure. Lanes: 1, molecular mass standards; 2, 500 ng of the 40 kDa α-subunit of G_n; 3, 500 ng of G_{24K} pool, also containing some G_n α-subunit (40 kDa) and β-subunit of 35/36 kDa; 4, 500 ng of G_{22K} and G_{26K} pool, also containing a contaminant at ~90 kDa.

2.4. *Miscellaneous*

Protein was assayed by an amido black procedure [23]. Silver staining of gels was performed as in [24]. [α-^{32}P]GTP was from ICN Radiochemicals.

3. RESULTS

We utilized the technique of binding [α-^{32}P]GTP to nitrocellulose blots of proteins separated by SDS-PAGE [18] to examine human neutrophils for the presence of GTP-binding proteins. Data obtained with human neutrophil membranes are shown in fig.1, lane 3. Neutrophil membranes contained at least two proteins able to interact with [α-^{32}P]GTP under these conditions. These proteins had apparent molecular masses of 24 and 26 kDa. No bands in the 40–50 kDa molecular mass range (i.e. G_n or G_s) were detected by this method. This binding technique thus distinguishes the G_n and G_s proteins from the 24 and 26 kDa proteins in terms of their ability to retain guanine nucleotide binding after exposure to SDS-PAGE. Platelet membranes have been reported to contain a similar class of proteins [18] and fig.1, lane 4 shows that two of the platelet bands migrate identically with those of the neutrophil. The platelet may also contain additional [α-^{32}P]GTP-binding proteins at about 23 and 27 kDa.

The specificity of the guanine nucleotide-binding site of the 24 and 26 kDa neutrophil proteins is demonstrated in fig.2. 10 μM GTP (not shown in fig.2), GDP or GTPγS were able to effectively compete with [α-^{32}P]GTP for this binding site, while GMP was only partially effective at concentrations of 100 μM. Both ATP and its non-hydrolyzable analog, AppNHp, did not compete even at concentrations as high as 1 mM. The binding site of these proteins is thus specific for guanine nucleotides.

Using a modification of the methods of G-protein purification described in [25], which includes ion-exchange, gel-filtration and hydrophobic chromatography of cholate-extracted neutrophil membranes, we have succeeded in substantially purifying the major G-proteins of neutrophils (submitted), and silver stained gels of these proteins are shown in fig.3.

Three proteins were obtained in significant quantities: G_n, with a 40 kDa α-subunit; G_{24K}, a

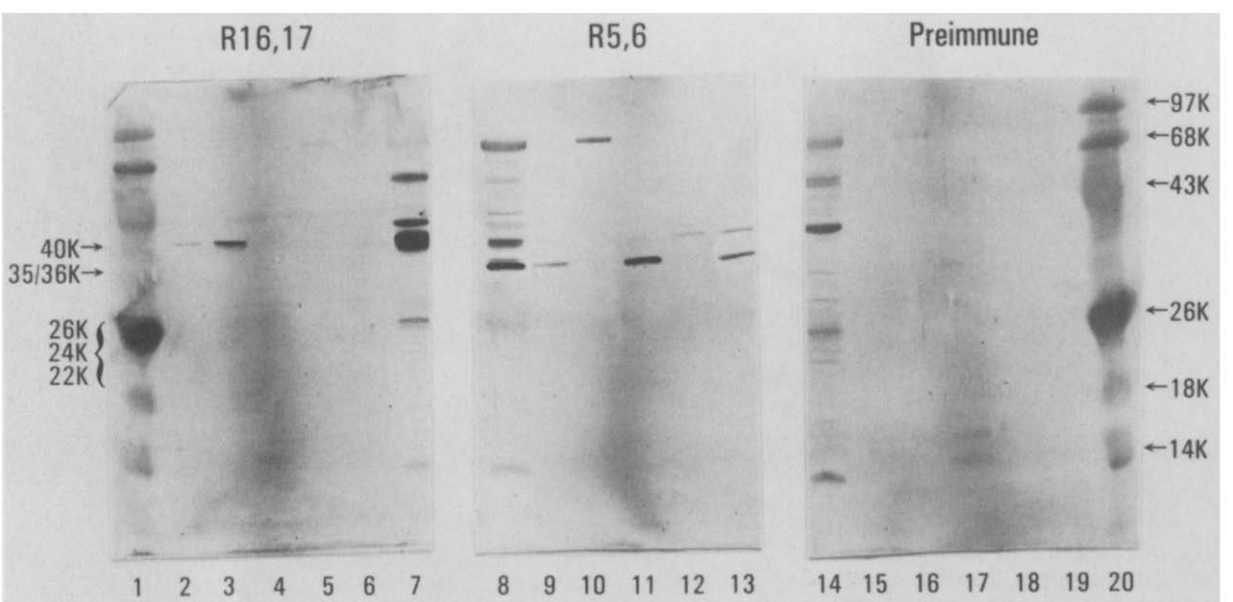

Fig.4. Western blot analysis of the immunological crossreactivity of $G_{22K-26K}$ with antibodies that recognize G_n. Western blots were performed as described in section 2. R16,17 was utilized at 1:250 dilution; R5,6 was at 1:250 dilution; preimmune serum was at 1:200 dilution. Lanes: 1,20, prestained molecular mass standards; 2,13,19, 300 ng rabbit liver G_i ($\alpha\beta\gamma$); 3,12,18, 400 ng human neutrophil G_n α-subunit depicted in fig.3; 4,11,17, 380 ng human neutrophil G_{24K} pool depicted in fig.3; 5,10,16, 600 ng human neutrophil G_{22K}/G_{26K} pool depicted in fig.3; 6,9,15, 300 ng human neutrophil β-subunit; 7,8,14, 60 μg human neutrophil membrane.

24 kDa protein and G_{22K}, a 22 kDa protein. Also present in the same pool as the 22 kDa protein was a less abundant protein band at 26 kDa (G_{26K}). The ability of the substantially purified 24 and 26 kDa proteins to bind [α-^{32}P]GTP after transfer and blotting is shown in fig.1, lanes 1,2. Pure $G_n\alpha$ does not bind GTP under these conditions (not shown).

An additional difference between G_n and the lower molecular mass proteins was that G_n served as an excellent substrate for pertussis toxin in the presence of exogenously added β/γ-subunit, while neither the 22, 24 nor the 26 kDa proteins were labeled by pertussis or cholera toxins (not shown).

We next examined the ability of the various proteins to interact with several G-protein antibodies (fig.4). R16,17 is a polyclonal antibody that reacts with the 9 carboxyl-terminal amino acids of G_n, G_i and transducin (Tn) [21]. R5,6 is another polyclonal antibody that was prepared against bovine brain G_i and which reacts with the α- and β-subunits of G_i, G_n, and G_o [19]. As indicated in fig.4, neither of these antibodies reacted with any

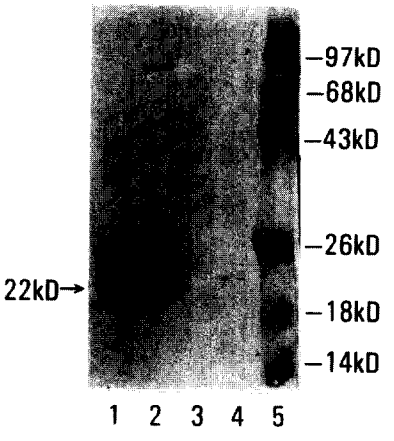

Fig.5. Recognition of human neutrophil 22 kDa protein by a monoclonal anti-ras antibody. Western blots were performed as described in section 2. Anti-ras antibody 142-24E05 was utilized at 1:350 dilution. Lanes: 1, H-ras standard; 2, 500 ng G_{22K}/G_{26K} pool depicted in fig.3; 3, 400 ng G_{24K} pool depicted in fig.3; 4, 400 ng G_n α-subunit depicted in fig.3; 5, prestained molecular mass standards, as indicated on the right of the figure. The apparent crossreactivity of 142-24E05 with the contaminant at ~90 kDa appeared to be non-specific and was likely due to the high concentration of this contaminant in the protein preparation. Note: the H-ras standard in lane 1 contained urea, resulting in some smearing of the protein band on the gel.

of the 22, 24 or 26 kDa proteins. This suggests that these proteins are antigenically unrelated to G_n or the other previously described members of the G-protein family [1].

As shown in fig.5, lane 2, however, the 22 kDa protein purified from neutrophils crossreacts with 142-24E05, a monoclonal antibody specific for a highly conserved portion of the GTP-binding site in ras and ras-like proteins [22]. The 24 or 26 kDa proteins did not react with this antibody (fig.5, lanes 2,3); neither did the 40 kDa α-subunit of G_n (fig.5, lane 4). The neutrophil 22 kDa protein had the same mobility on SDS-polyacrylamide gels as purified H-ras (fig.5, lane 1).

4. DISCUSSION

We have identified 24 and 26 kDa proteins in the human neutrophil which may represent novel GTP-binding proteins. These proteins have a guanine-nucleotide-specific binding site which exhibits properties distinct from G_n (as well as G_s, G_i, G_o and Tn) of being able to bind guanine nucleotide after the denaturing conditions of SDS-PAGE – transfer to nitrocellulose. These proteins do not serve as pertussis or cholera toxin substrates as purified, nor do they react with antibodies able to recognize a variety of G α-subunits. These proteins thus appear to represent a distinct class of membrane associated GTP-binding proteins. A class of proteins with similar GTP-binding characteristics has been described in platelets [18]; however, it is unlikely that the presence of these proteins in the neutrophil preparation is due to platelet contamination, as less than 1% contamination was noted (see section 2).

An additional 22 kDa neutrophil membrane protein identified here may be related to the G-proteins referred to as the ras proteins. The neutrophil G_{22K} protein comigrates with H-ras on SDS-polyacrylamide gels. (We obtain an apparent molecular mass of 22 kDa for the sample of Harvey ras protein in our gel system, even though it is usually described as having a molecular mass of 21 kDa.) Additionally, the neutrophil G_{22K} protein crossreacts with 142-24E05, a monoclonal antibody directed against the GTP-binding domain of known ras proteins in both vertebrate and invertebrate species [22]. The presence of these novel proteins in the neutrophil suggests the possibility

that they may be involved in the process of signal transduction in these cells. We are in the process of further characterizing the properties of this group of G-proteins.

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