Detection of the major pertussis toxin substrate of human leukocytes with antisera raised against synthetic peptides

Judith Falloon*, Harry Malech*, Graeme Milligan+, Cecilia Unson°, Richard Kahn‡, Paul Goldsmith and Allen Spiegel†

+ *Dept of Biochemistry, University of Glasgow, Glasgow, Scotland,* ° *The Rockefeller University, New York, NY 10021,* * *Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases,* ‡ *Laboratory of Biological Chemistry, National Cancer Institute* and *Metabolic Diseases Branch, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

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Antisera raised against the carboxy-terminal decapeptide (KENLKDCGLF) of transducin-α detected the 40 kDa, major pertussis toxin substrate of human neutrophils. The antisera also detected this protein in undifferentiated HL-60 and U937 cells, and revealed an approx. 2-fold increase in protein/mg membrane protein with differentiation into mature phagocytic cells. The results provide direct immunochemical evidence for the presence of a novel, pertussis toxin-sensitive guanine nucleotide-binding protein in human leukocytes.

GTP-binding protein *Signal transduction* *Phosphoinositide breakdown*

1. INTRODUCTION

Neutrophils contain large amounts (at least 20 pmol/mg membrane protein) of an approx. 40 kDa pertussis substrate [1]. This pertussis toxin-sensitive guanine nucleotide-binding protein (G-protein) couples the chemotactic peptide receptor to phosphoinositide (PI) breakdown [2,3]. It was initially assumed that the neutrophil protein is G_i, the G-protein associated with inhibition of adenylate cyclase, since G_i was the first pertussis toxin substrate to be identified [2]. Subsequently, other pertussis toxin substrates have been described, including transducin (TD), the G-protein of photoreceptor cells, and G_o, a G-protein of unknown function discovered in brain [4,5]. Using specific antisera directed against TD and G_o and a TD antiserum that crossreacts with G_i in brain, we found that these G-proteins are present in insufficient amounts to account for the pertussis toxin substrate in neutrophils [1]. On this basis, we suggested that neutrophils contain a novel G-protein as the major pertussis toxin substrate. With newly developed antisera raised against synthetic peptides, we now provide direct immunochemical evidence for the presence of this novel G-protein in neutrophils and cultured leukocyte cell lines.

†To whom correspondence should be addressed at Bldg 10, Rm 9C101

Abbreviations: G-protein, guanine nucleotide-binding protein; G_s and G_i, the G-proteins associated with stimulation and inhibition, respectively, of adenylate cyclase; G_o, a G-protein of unknown function discovered in brain; TD, transducin, the G-protein of retinal rod outer segments; PI, phosphoinositide; fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Cells*

Human neutrophils were purified from peripheral venous blood of normal volunteers by

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Ficoll-Hypaque density centrifugation and dextrose sedimentation with hypotonic lysis of residual erythrocytes [6]. HL-60, a human promyelocyte line [7], and U937, a human monocyte line [8], were maintained in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Both cell lines were induced to differentiate into more mature phagocytic cells by the addition of 750 μM N^6,O^2-dibutyryladenosine 3′,5′-monophosphate to the culture medium for 3 days [9,10].

2.2. *Membrane preparations*

A neutrophil plasma membrane-enriched fraction was prepared by nitrogen cavitation of neutrophils, low-speed centrifugation to remove nuclei, and ultracentrifugation into a Percoll gradient [11]. Washed uninduced and induced HL-60 and U937 cells were subjected to nitrogen cavitation, and low-speed centrifugation as for neutrophils [11]. The supernate, however, was centrifuged at 100 000 × g for 45 min to obtain a particulate fraction which was used without further purification. A crude plasma membrane preparation from human cerebral cortex was prepared as described [12].

2.3. *Pertussis toxin labeling*

Pertussis toxin (kindly provided by Dr J. Munoz, Rocky Mountain Laboratories, NIAID) was activated by incubating 1 mg/ml pertussis toxin in 100 mM Tris buffer, pH 8.0, with 50 mM dithiothreitol for 15 min at room temperature. A volume of membrane protein (2–5 mg/ml) was mixed with an equal volume of a reaction mixture containing 70 μg/ml activated pertussis toxin, 200 mM Tris buffer, pH 8.0, 4 mM ATP, 2 mM GTP, 2 mM ADP-ribose, 20 mM nicotinamide, and 10 μM NAD containing [α-^{32}P]NAD at a specific activity of 22.8 Ci/mmol (New England Nuclear) and incubated for 1 h at 37°C. Controls were incubated with the same reaction mixture minus pertussis toxin. The reaction was terminated by addition of SDS sample buffer in preparation for SDS-PAGE [6]. Gels were stained with Coomassie blue, dried and subjected to autoradiography using image intensification screens with Kodak X-AR film at −70°C.

2.4. *Immunoblots*

The following antisera were used as first antibodies: RV3, specific for G_o-α and -β [12]; CW6, raised against holotransducin but crossreacting with G_i-α in brain and erythrocyte [13]; A-569 raised against a synthetic peptide common to all known G-α subunits [14]; AS6, 7 and 8, each raised against the carboxy-terminal decapeptide of TD (KENKLDCGLF), conjugated to keyhole limpet hemocyanin with glutaraldehyde. Immunoblotting and affinity purification of antibodies were performed as in [1,12]. For M_r estimations on immunoblots, prestained M_r markers (Bethesda Research Labs) were run in parallel with test samples.

3. RESULTS

Both brain and neutrophils are known to contain high concentrations of pertussis toxin substrates [1]. In brain these are largely accounted for by G_o and G_i, present in a ratio of about 5:1 [12]. Antisera RV3 and CW6 readily detect the α-subunits of G_o and G_i, respectively, in brain. Both antisera detect the common β-subunit [1,12]. In neutrophils, despite the high concentration of pertussis toxin substrate measured by toxin-catalyzed incorporation of [^{32}P]ADP-ribose, we failed to detect immunoreactive G_o- or TD-α [1]. Immunoreactive G_i-α was detected with CW6 but in amounts insufficient to account for the total concentration of neutrophil pertussis toxin substrate. We therefore tested both human neutrophil and cerebral cortical plasma membranes with new antisera raised against the carboxy-terminal decapeptide of TD-α, KENLKDCGLF. These antisera react strongly with TD-α and with G_i-α subunits purified from several tissues. This presumably reflects the similarity in sequence between TD-α and G_i-α (KNNLKDCGLF) [15]. The antisera react poorly if at all with G_o-α which diverges by 5/10 amino acids of the decapeptide [16].

Fig.1 shows that RV3 detects a broad, approx. 39 kDa band representing G_o-α in brain but shows no reactivity in neutrophil. CW6 detects a band with slightly slower migration in both brain and neutrophil. The common β-subunit is detected by both antisera in both tissues. Affinity-purified anti-peptide antibodies (AS7) were diluted to give comparable reactivity to that obtained with CW6

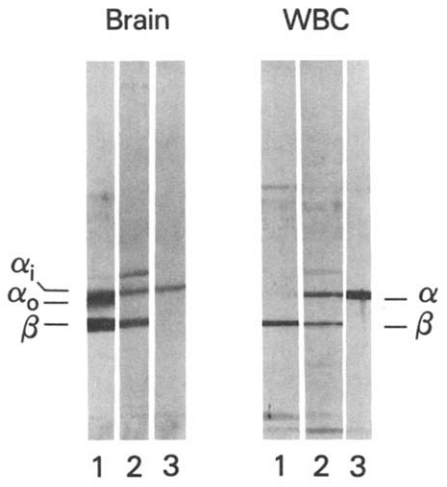

Fig.1. Immunoblots of human cerebral cortical (brain) and human neutrophil (WBC) membranes. 100 μg/lane of plasma membranes were loaded on a 10% SDS gel, separated by SDS-PAGE, and immunoblotted with antisera: (1) RV3, specific for G_o-α and -β; (2) CW6, specific for G_i-α and -β; (3) AS7, raised against the carboxy-terminal decapeptide of transducin-α. For RV3 and AS7, affinity-purified antibodies, diluted 1/20 and 1/10, respectively, were used. For CW6, a 1/100 dilution of crude antiserum was used. The band above G_i-α in lane 2 represents nonspecific reactivity of the crude antiserum.

in brain (fig.1, left, lanes 2,3). The identical dilution of AS7, in contrast, showed substantially more immunoreactivity in neutrophil than did CW6 (fig.1, right, lanes 2,3). Comparable results were obtained with the two other anti-decapeptide sera (AS6,8) that we produced. An antiserum (A-569) against a peptide common to all G-α subunits [14] also showed strong reactivity with a 40 kDa band in neutrophils (not shown).

Certain cultured leukocyte lines such as HL-60 and U937 can be induced to differentiate into mature phagocytic cells responsive to the chemotactic peptide, fMet-Leu-Phe. Pertussis toxin inhibits chemotactic peptide stimulation in differentiated HL-60 cells [17]. This suggests that these cells contain a G-protein functionally equivalent to that in mature neutrophils. The uninduced cells used in our study did not express detectable fMet-Leu-Phe receptors, either by binding assays with tritiated peptide or by photoaffinity labeling, nor did the cells respond chemotactically to fMet-Leu-Phe [6,10,18]. Differentiated cells showed a decrease in size, a marked increase in spontaneous motility in culture, expressed fMet-Leu-Phe receptors, and responded chemotactically to this peptide. Differentiated U937 cells showed an increase in nonspecific esterase activity consistent with maintenance of a monocyte phenotype, while the differentiated HL-60 cells remained free of nonspecific esterase activity.

For pertussis toxin labeling and immunoblotting experiments, we loaded plasma membranes from equivalent numbers of uninduced and induced cells on each SDS gel lane. Since cells induced to differentiate decreased in size, lower amounts of membrane protein from induced cells were loaded per lane compared with uninduced cells (see fig.2 legend). A number of bands were seen on autoradiograms of HL-60 and U937 cell membranes exposed to [^{32}P]NAD, but a single band of about 40 kDa (labeled α in fig.2) was labeled in a toxin-dependent manner in each set of membranes. The

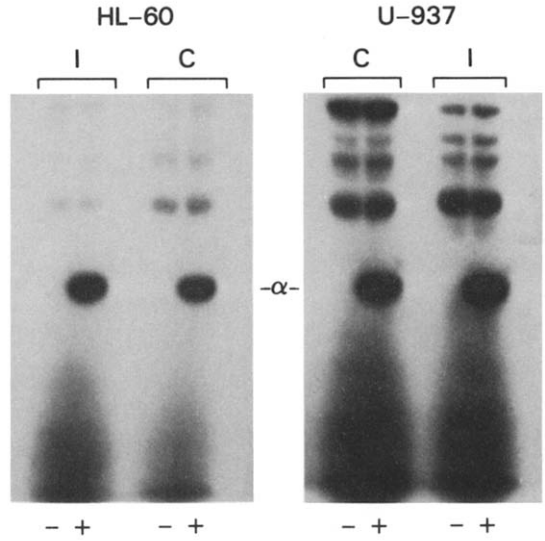

Fig.2. Detection of substrates for pertussis toxin-catalyzed ADP-ribosylation in HL-60 and U937 cell membranes. Plasma membranes from undifferentiated (C) cells, and from cells induced to differentiate (I) were incubated with [^{32}P]NAD and with (+) or without (−) pertussis toxin as described in section 2. Membranes were then loaded on a 10% SDS gel (HL-60, I = 77 μg/lane, C = 139 μg/lane; U937, I = 105 μg/lane, C = 185 μg/lane). An autoradiogram of the dried gel is shown.

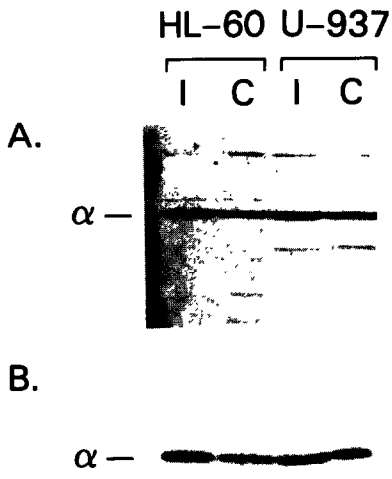

Fig.3. Immunoblots of plasma membranes from differentiated (I) and undifferentiated (C) HL-60 and U937 cells. Membranes (protein amounts identical to those in fig.2) were loaded on a 10% SDS gel and separated by SDS-PAGE. Immunoblotting was performed with: (A) a 1/100 dilution of antiserum AS7; (B) a 1/1000 dilution of antiserum A-569, raised against the peptide common to G-α subunits. In (B) only, radioiodinated goat anti-rabbit Ig was used as second antibody and an autoradiogram (overnight exposure) of the exposed blot is shown.

amount of [^{32}P]ADP-ribosylated toxin substrate was comparable in induced and uninduced U937 cells and only slightly increased in induced HL-60 cells when compared on a cell equivalent basis. Per mg membrane protein, however, there was an approximate doubling in toxin substrate concentration with differentiation in both cell types.

Antisera RV3 and CW6 both detected β-subunit but minimal G_o- or G_i-α subunit reactivity in induced and uninduced HL-60 and U937 cells. In contrast all three anti-decapeptide antisera (AS6,7,8) reacted strongly with a 40 kDa band (not shown). Fig.3 shows the results of an immunoblot with one of the decapeptide antisera (AS7) and with the common G-α peptide antiserum. Each antiserum reacts with the identical, approx. 40 kDa band (labeled α in fig.3). The amount of immunoreactivity in equivalent numbers of induced vs uninduced cells is not clearly different in U937 cells, but in HL-60 cells there is a definite increase with differentiation. When expressed per mg membrane protein, detectable immunoreactivity increases by at least a factor of 2 with differentiation of both cell types.

4. DISCUSSION

Our results represent the first immunochemical detection of pertussis toxin substrate in neutrophils and cultured leukocyte cell lines. The ability of an antiserum raised against a peptide common to all known G-α subunits to detect the neutrophil pertussis toxin substrate is not surprising, since all specific pertussis toxin substrates identified to date are G-α subunits. Detection of the neutrophil protein with antisera raised against the carboxy-terminal decapeptide of TD-α, however, provides direct evidence for the existence of a novel pertussis toxin substrate in neutrophils. The neutrophil substrate presumably contains a sequence identical to, or highly homologous to, the KENLKDCGLF or KNNLKDCGLF sequence with which the antisera strongly react. The comparable sequence of G_o-α, ANNLRGCGLY, is poorly recognized by the decapeptide antisera. Thus G_o cannot account for the immunoreactivity detected with AS6-8. Although TD-α does contain the appropriate sequence, the neutrophol substrate must differ from TD-α since several TD-specific antisera fail to react with neutrophil membranes [1]. Our results clearly show that decapeptide antisera recognize a form of pertussis toxin substrate in neutrophils different from the 41 kDa form of G_i-α in brain recognized by antiserum CW6. Thus, the neutrophil protein (or possibly proteins) shares a highly homologous decapeptide sequence with TD- and 41 kDa G_i-α but must differ from these proteins in one or more other regions, including the epitope recognized by antiserum CW6.

Recent cloning of cDNAs for multiple G-α subunits suggests the existence of as many as three different forms of G_i-α ([15,16]; Reed, R. and Jones, D., personal communication). Two of these contain the KNNLKDCGLF sequence and one the KENLKDCGLF sequence, so that all three should be detected with our decapeptide antisera. The tissue distribution of these distinct forms of G_i is not known, but our data suggest that qualitative and/or quantitative differences in distribution may occur in brain and neutrophil. The finding that the

pertussis toxin substrate in various types of white cells is also sensitive to cholera toxin may represent another distinguishing feature between the neutrophil G-protein and the pertussis toxin substrates of brain [19]. The functional significance, if any, of subtle differences in amino acid sequence between different forms of G_i-α is not known. Kikuchi et al. [20] have found that both G_i and G_o, purified from brain, could reconstitute fMet-Leu-Phe stimulated PI breakdown in pertussis toxin treated HL-60 cells. This may indicate that any form of G_i and indeed even G_o can substitute for the endogenous neutrophil pertussis toxin substrate, but it remains possible that functional differences do exist.

The immunoblot data indicate that a protein identical or very closely related to the pertussis toxin substrate of mature neutrophils is present in cultured leukocyte cell lines such as HL-60 and U937. Interestingly, this protein is already detectable in undifferentiated cells. With differentiation there is at least a 2-fold increase in concentration of the protein/mg membrane protein as shown by both toxin labeling and immunoblotting. At present it is not clear if this increase represents de novo synthesis of protein, altered turnover, or both. Further studies with differentiating leukocyte lines may prove useful in helping to identify the pertussis toxin substrate of neutrophils.

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REFERENCES

[1] Gierschik, P., Falloon, J., Milligan, G., Pines, M., Gallin, J.I. and Spiegel, A. (1986) J. Biol. Chem. 261, 8058-8062.
[2] Okajima, F. and Ui, M. (1984) J. Biol. Chem. 259, 13863-13871.
[3] Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875-5878.
[4] Gilman, A. (1984) Cell 36, 577-579.
[5] Spiegel, A. (1986) Mol. Cell. Endocrinol., in press.
[6] Malech, H.L., Gardner, J.P., Heiman, D.F. and Rosenzweig, S.A. (1985) J. Biol. Chem. 260, 2509-2514.
[7] Collins, S.J., Ruscetti, F.W., Gallager, R.E. and Gallo, R.C. (1979) J. Exp. Med. 149, 969-974.
[8] Sundstrom, C. and Nilsson, K. (1976) Int. J. Cancer 17, 565-577.
[9] Chaplinski, T.J. and Niedel, J.E. (1982) J. Clin. Invest. 70, 953-964.
[10] Kay, G.E., Lane, B.C. and Snyderman, R.C. (1983) Infect. Immun. 41, 1166-1174.
[11] Ohno, Y., Seligmann, B.E. and Gallin, J.I. (1985) J. Biol. Chem. 260, 2409-2414.
[12] Gierschik, P., Milligan, G., Pines, M., Codina, J., Klee, W. and Spiegel, A. (1986) Proc. Natl. Acad. Sci. USA 83, 2258-2262.
[13] Pines, M., Gierschik, P., Milligan, G., Klee, W. and Spiegel, A. (1985) Proc. Natl. Acad. Sci. USA 82, 4095-4099.
[14] Mumby, S.M., Kahn, R.A., Manning, D.R. and Gilman, A.G. (1986) Proc. Natl. Acad. Sci. USA 83, 265-269.
[15] Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Haga, K., Haga, T., Ichiyama, A., Kangawa, K., Hiranaga, M., Matsuo, H. and Numa, S. (1986) FEBS Lett. 197, 305-310.
[16] Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. and Kaziro, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 3776-3780.
[17] Brandt, S.J., Dougherty, R.W., Lapetina, E.G. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 3277-3280.
[18] Heiman, D.F., Gardner, J.P., Apfeldorf, W.J. and Malech, H.L. (1986) J. Immunol. 136, 4623-4630.
[19] Verghese, M., Uhing, R.J. and Snyderman, R. (1986) Biochem. Biophys. Res. Commun. 138, 887-894.
[20] Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558-11562.