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The regulation of actin polymerization in differentiating U937 cells correlates with increased membrane levels of the pertussis-toxin-sensitive G-protein G_i2

Bhav SHETH, Peter BANKS, Dennis R. BURTON and Peter N. MONK
Department of Molecular Biology and Biotechnology, University of Sheffield, P.O. Box 594, Firth Court, Western Bank, Sheffield S10 2UH, U.K.

Undifferentiated U937 cells appear to lack a capacity to increase cellular F-actin. However, electropermeabilized cells gain the ability to respond in this way to a guanine nucleotide analogue, guanosine 5′-[γ-thio]trisphosphate (GTP[S]) after 1 h of treatment with dibutyryl cyclic AMP (db-cAMP). The results reported here show that the levels of membrane association of the G-protein $G_i2\alpha$ increase with a time course identical with that of the GTP[S]-sensitivity of electropermeabilized cells. These results suggest that $G_i2\alpha$ may be involved in the signal-transduction pathway leading to actin polymerization in db-cAMP-differentiated U937 cells.

INTRODUCTION

The ability of monocytes and neutrophils to accumulate at sites of infection, and to engulf invading micro-organisms, is dependent on rearrangements of the peripheral cytoskeleton (Stossel, 1988). In particular, the microfilamental system, composed largely of polymerized actin (F-actin), undergoes substantial reorganization on stimulation of the cell by chemotactic factors. The signal-transduction mechanisms that regulate this process are still not clearly understood.

Signalling in neutrophils has been studied extensively by using the chemotactic peptide *N*-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe). The evidence for the involvement of a G-protein (or G-proteins) in the signalling pathways stimulated by this agent is strong. Pertussis toxin, which catalyses ADP-ribosylation and consequent inactivation of a class of G-proteins, can abolish fMet-Leu-Phe-induced changes in the actin polymerization state (Shefcyk *et al.*, 1985). More recently, electropermeabilized neutrophils have been used to investigate the effects of a variety of G-protein activators on actin polymerization. AlF_4^- and the metabolically stable GTP analogue guanosine 5′-[γ-thio]trisphosphate (GTP[S]) have both been shown to stimulate incorporation of actin monomers into the cellular cytoskeleton (Bengtsson *et al.*, 1990; Downey *et al.*, 1989; Therrien & Naccache, 1989).

In this study, we have measured the levels of the α-subunits of two G-proteins, G_i2 and G_i3, thought to be associated with chemotactic receptors in neutrophils (Gierschik *et al.*, 1989; Pollock *et al.*, 1990), during differentiation of a human monocyte-like cell line (U937) in the presence of dibutyryl cyclic AMP (db-cAMP). This agent has previously been shown to induce significant numbers of C5a receptors on U937 cells, enabling them to respond to C5a by chemotaxis, production of reactive oxygen intermediates (ROI) and increased expression of a phagocytosis-associated receptor (CR3bi) (Sheth *et al.*, 1988; Monk & Banks, 1991*a*). C5a also stimulates actin recruitment to the cytoskeleton in differentiated U937 cells in a pertussis-toxin-sensitive manner (Banks *et al.*, 1988). The aim of the present study is to compare the changes that occur in the levels of G_i2 and G_i3 α-subunits during differentiation with the induction of actin polymerization and ROI production in response to C5a. This is the first report to suggest that G_i2 rather than G_i3 is involved in the signalling pathway for actin polymerization and that U937 cells become competent for cytoskeletal reorganization before expression of detectable levels of C5a receptors.

MATERIALS AND METHODS

Preparation of differentiated U937 cells

U937 cells were stimulated to differentiate by treatment with 1 mM-db-cAMP (Sigma, Poole, Dorset, U.K.) for 1–48 h as described previously (Banks *et al.*, 1988). Cells were washed and resuspended in balanced salts solution (BSS; 150 mM-NaCl, 5 mM-KCl, 10 mM-D-sorbitol, 13 mM-K_2HPO_4,$3H_2O$, 1 mM-KH_2PO_4 and 10 mM-Hepes, pH 7.4).

Measurement of ROI production

ROI production was assessed as maximal luminol-dependent chemiluminescence as described previously (Monk & Banks, 1991*b*).

Measurement of actin polymerization

Staining of lysophosphatidylcholine-permeabilized cells with rhodamine–phalloidin was performed as described by Banks *et al.* (1988), except that the stained cytoskeletons were not centrifuged and resuspended before flow-cytometric analysis.

Electropermeabilization of U937 cells

Cells were permeabilized with a Bio-Rad Gene Pulser by using a single discharge (0.6 kV, with a 25 μF capacitance setting, and 0.4 cm electrode-gap cuvettes). This procedure was optimized for U937 cells (at 10^6/ml, in BSS + 0.1 mM-$MgCl_2$) to ensure maximal permeabilization [measured by ethidium bromide-associated fluorescence (Therrien & Naccache, 1989)] with the viability of the cells at > 90% (measured after 30 min on ice, by using Trypan Blue dye exclusion). Ethidium bromide uptake was maximal within 2 min after discharge, and addition of ethidium bromide after 5 min incubation on ice showed no further uptake of the dye by the cells, indicating that pore formation had ended. Production of ROI in electropermeabilized cells in response to 10 nM-C5a decreased only slightly (< 40%) in comparison with that observed in non-permeabilized cells.

Abbreviations used: ROI, reactive oxygen intermediates; db-cAMP, dibutyryl cyclic AMP; BSS, balanced salts solution; GTP[S], guanosine 5′-[γ-thio]triphosphate; fMet-Leu-Phe, *N*-formylmethionyl-leucylphenylalanine; PK-A, cyclic-AMP-dependent protein kinase; TBS, Tris-buffered saline.

G-protein studies

Cells treated for various periods of time with db-cAMP were harvested, washed and re-pelleted. The cells were then disrupted by hypo-osmotic shock; 0.5 ml of water was added to the cell pellet, followed by several passages through a fine needle. After disruption, the solution was made up to 50 mM-Tris, pH 7.4, 50 mM-NaCl, 1 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride, 1 μg of DNAase I/ml and 10 μM each of aprotinin, iodoacetamide and NaN_3. Intact cells and nuclei were removed by centrifugation at 1000 *g* for 10 min at 4 °C; supernatants were then further centrifuged at 100000 *g* for 10 min at 4 °C, to obtain crude membranes. Pelleted membranes were resuspended in Tris-buffered saline (TBS; 150 mM-NaCl/10 mM-Tris/HCl, pH 8) at 1–2 mg of protein/ml. Membrane proteins (20–75 μg) were separated by SDS/PAGE (10% acrylamide) and electroblotted on to nitrocellulose (Schleicher and Schuell). After blocking with 5% (w/v) skimmed-milk powder in TBS for 1 h, the blots were incubated for 1 h with rabbit antisera to $G_i2\alpha$ and $G_i3\alpha$ (Bio-Mac, Glasgow, U.K.). Immunoreactivity was detected using a horseradish peroxidase-linked goat anti-rabbit IgG (Serotec, Oxford, U.K.). Band density was assessed with a Hoeffer GS300 densitometer, in the reflectance mode.

RESULTS

Western blotting with anti-peptide antisera revealed that the amount of α-subunit of G_i2 associated with the membrane fraction increased after treatment with db-cAMP (Fig. 1). Within 1 h the level of $G_i2\alpha$ had risen markedly and continued to rise for several hours before reaching a plateau, which persisted for 48 h in the continued presence of db-cAMP. The rapidity of this response indicates that it is unlikely to be due solely to synthesis *de novo*, but may also represent G-protein translocation, perhaps from the cytoplasm. This hypothesis was tested by treating U937 cells with the protein-synthesis inhibitor cycloheximide (at 25 μg/ml for 5 h in all cases) during incubation with db-cAMP. The increase in $G_i2\alpha$ observed after 1 h of db-cAMP stimulation in cycloheximide-treated cells was 81 ± 12% of the increase in the absence of cycloheximide; however, at 5 h membrane-associated $G_i2\alpha$ in cycloheximide-treated cells had decreased to 27 ± 6% of controls (means ± S.E.M. of three determinations). The level of $G_i2\alpha$ in cytosolic fractions was also measured; in control cells db-cAMP treatment for 1 and 5 h caused small decreases (to 88 ± 1% and 95 ± 13% of control respectively; means ± S.E.M., $n = 3$). In contrast, cells treated with cycloheximide have slightly lower levels of cytosolic $G_i2\alpha$ at these time points (75 ± 10% and 72 ± 6%; means ± S.E.M., $n = 3$). Cycloheximide treatment had no significant effect on total cellular protein levels (results not shown).

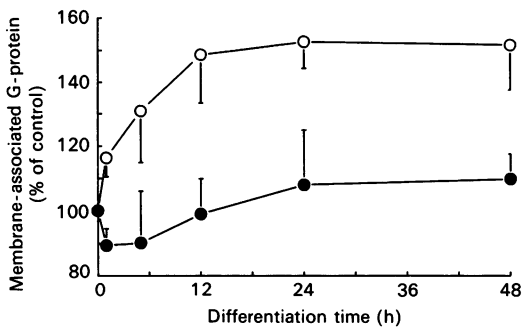

Fig. 1. Levels of $G_i2\alpha$ and $G_i3\alpha$ in membranes prepared from U937 cells

Membranes were prepared from U937 cells treated with 1 mM-db-cAMP for the indicated times. Proteins were separated by SDS/PAGE and electroblotted on to nitrocellulose. The blots were incubated with antisera to $G_i2\alpha$ (○) or $G_i3\alpha$ (●), and bands were made visible with peroxidase-conjugated goat anti-rabbit IgG. The relative quantity of G-protein per band was estimated by densitometry. Results shown are the means ± S.E.M. of four separate experiments.

Membrane-associated $G_i3\alpha$ clearly declines over the first 5 h of db-cAMP treatment (Fig. 1), although the levels recover and appear to be slightly increased at 48 h.

The C5a-responsiveness of U937 cells is induced at a relatively late period of the differentiation process (Fig. 2). Actin polymerization is not stimulated by C5a until 12–18 h after db-cAMP addition, but then continues to increase up to 48 h. C5a-stimulated ROI production is similar, beginning at 12 h of differentiation and continuing to increase up to 48 h (Fig. 2).

The F-actin content of undifferentiated cells did not change markedly after electropermeabilization in the presence of 1 mM-GTP[S] (Fig. 2). However, after only 1 h of db-cAMP treatment, the cells became responsive to the guanine nucleotide analogue, and F-actin levels increased. The distribution of fluorescence caused by rhodamine–phalloidin binding indicated that an homogeneous population of cells was involved. The early time course for the appearance of this response was similar to that obtained for $G_i2\alpha$ levels (Fig. 1).

DISCUSSION

By treatment with db-cAMP, the pre-monocytic U937 cell line can be induced to differentiate into monocyte-like cells, which respond to chemotactic stimulation by C5a (Sheth *et al.*, 1988). During the time course of differentiation, some or all of the components of the responsive pathways, not present in immature cells, must be synthesized or otherwise activated.

The results obtained here suggest that $G_i2\alpha$ is part of the signal-transduction pathway involved in the stimulation of actin polymerization. The initial increase in $G_i2\alpha$ associated with the membrane preparation at 1–5 h of differentiation correlates well with the onset of responsiveness of electropermeabilized cells to GTP[S], a direct activator of G-proteins. The mechanism by which this increase occurs is not clear; at the earliest time point, synthesis *de novo* of $G_i2\alpha$ is unlikely to be the main contributor. This was shown by the failure of cycloheximide [known to inhibit protein synthesis in neutrophils at the concentration used (Woodman *et al.*, 1988)] to alter membrane-associated levels of $G_i2\alpha$ after 1 h of db-cAMP treatment, but at 5 h cycloheximide typically inhibited the response to db-cAMP by some 73%. Similarly, the decreases observed in cytoplasmic levels of $G_i2\alpha$ were exaggerated in the presence of cycloheximide, indicating that resynthesis of translocated protein was inhibited. G_i2 redistribution rather than synthesis *de novo* was also demonstrated by using whole cells instead of membrane/cytosol preparations for PAGE; after 18 h of db-cAMP treatment, the normal increase in $G_i2\alpha$ was observed, but after only 1 h no such increase occurred (results not shown).

It is possible that initially $G_i2\alpha$ is translocated from the cytoplasm to a membrane site as a consequence of increased cyclic-AMP-dependent protein kinase (PK-A) activity, which may increase the affinity of the α-subunit for $\beta\gamma$ complexes in the plasma membrane. Alternatively, membrane attachment may be increased by *N*-myristoylation of the α-subunit (Buss *et al.*, 1987) in a PK-A-dependent manner. Whatever the mechanism, membrane association is apparently essential for the proper functioning of G-proteins; all successful reconstitutions of hormone-responsive systems have been in lipid vesicles (Neer & Clapham, 1988). $G_i2\alpha$ is present in membranes from undifferentiated cells, but apparently cannot mediate actin polymerization. There are three possible explanations for the induction of sensitivity to

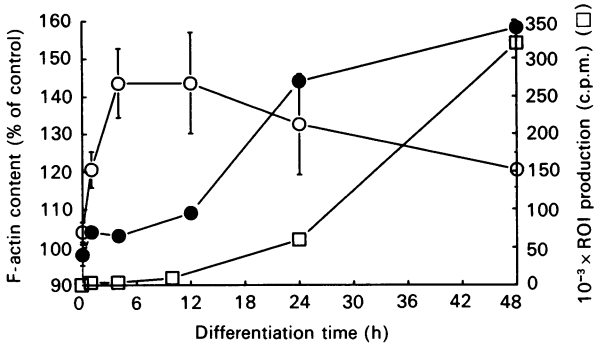

Fig. 2. Effects of C5a and GTP[S] on actin polymerization in U937 cells

U937 cells were treated with 1 mM-db-cAMP for the indicated times. C5a-stimulated (●) and GTP[S]-stimulated (○) actin polymerization was assessed by rhodamine–phalloidin binding to lysophosphatidylcholine-permeabilized cells, by using flow cytometry. Electropermeabilization was performed under the conditions stated in the Materials and methods section, in the presence of 1 mM-GTP[S]. After 3 min, cells were analysed for rhodamine–phalloidin binding as a measure of actin polymerization. Results shown are means ± S.E.M. of three to five separate experiments. Cells were stimulated with 1 nM-C5a for 90 s before F-actin determination; results shown are means from one typical experiment (of three) conducted in triplicate. ROI production in response to 10 nM-C5a (□) was determined as maximal luminol-dependent chemiluminescence. The results of one typical experiment (of two) are shown.

GTP[S]. (1) The levels of G-proteins associated with the membrane have been shown to be a limiting factor in signal transduction (Mei *et al.*, 1989); therefore a simple increase in $G_i2\alpha$ membrane attachment could be responsible, if the pre-existing G_i2 is sequestered by other receptors in membrane domains where it is not available for the stimulation of actin polymerization. (2) The translocated $G_i2\alpha$ may be of a different sub-type from that already attached to the membrane. (3) An activated (e.g. phosphorylated) form of $G_i2\alpha$ may be translocated which is able to participate in the stimulation of actin polymerization. At later time points (> 12 h) the responsiveness of the differentiating cells to GTP[S] appears to decline slightly, although $G_i2\alpha$ levels remain high. This may be explained in similar terms; for instance, the synthesis *de novo* which apparently occurs after the initial phase of db-cAMP treatment may of several sub-types of $G_i2\alpha$, only one of which can participate in the stimulation of actin polymerization. Alternatively, the type of activation of $G_i2\alpha$ may change during the differentiation time course. Further work is necessary to distinguish between these possibilities.

The other pertussis-toxin substrate present in these cells, $G_i3\alpha$, does not appear to be involved in F-actin formation; over a similar early time period, the level of $G_i3\alpha$ associated with the membrane fraction decreases. This G-protein has been implicated previously as an activator of inositol phospholipid hydrolysis in neutrophils stimulated by both GTP[S] and fMet-Leu-Phe (Verghese *et al.*, 1989). In C5a-stimulated U937 cells, we have found that inositol lipid hydrolysis can be dissociated from actin polymerization responses (Monk & Banks, 1991*b*).

The possible involvement of either of these G-proteins in C5a-induced signalling is less clear. Although detectable C5a-receptor expression occurs only after 12 h of differentiation (Barker, 1987), this does not rule out the possible presence of small numbers of competent receptors on less mature or undifferentiated U937 cells. Indeed, Sklar *et al.* (1985) have suggested that only a few tens of fMet-Leu-Phe receptors on neutrophils need to be occupied to stimulate actin polymerization. It is difficult, or perhaps impossible, to detect the low levels of ligand binding that are essential for this response. However, C5a does not appear to stimulate either increased F-actin formation or ROI production in differentiating cells until a detectable increase in ^{125}I-C5a binding occurs (Barker, 1987). This is in contrast with the results of Rao *et al.* (1988), who showed that db-cAMP-treated HL-60 promyelocytes could respond to fMet-Leu-Phe with a significant increase in cytoskeletal F-actin before a detectable increase in receptors for fluorescently labelled ligand occurred. Unlike HL-60 cells, undifferentiated U937 cells may possess no functional chemotactic-ligand receptors. However, the effects of GTP[S] suggests that other elements of the signal-transduction pathway involved in the stimulation of actin polymerization are present and active after only 1 h of db-cAMP treatment, including $G_i2\alpha$.

In conclusion, we have demonstrated that differentiation of U937 cells with db-cAMP rapidly confers the ability to undertake actin polymerization. This is correlated not with the expression of C5a receptors, but with increases in membrane-associated $G_i2\alpha$. This G-protein may therefore be a part of the signalling mechanism that leads to cytoskeletal reorganization.

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