Up-regulation of the amount of $G_{i\alpha 2}$ associated with the plasma membrane in human neutrophils stimulated by granulocyte–macrophage colony-stimulating factor

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Preincubation of human neutrophils with the human cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) results in an increase in the amount of α-subunit of G_{i2} ($G_{i\alpha 2}$) associated with the plasma membrane and a corresponding decrease in the amount associated with the granule fractions. Similar results are obtained with interleukin-8. GM-CSF has no effect on the distribution of $G_{i\alpha 3}$. The effect of GM-CSF on $G_{i\alpha 2}$ is time-dependent, and, although a significant effect can be observed after incubation for 5 min with GM-CSF, the enhancement increases with increasing time. Genistein, a protein tyrosine kinase inhibitor, and 1,2-bis-(*O*-aminophenoxyl)ethane-*NNN'N'*-tetra-acetic acid (BAPTA), an intracellular Ca^{2+} chelator, decrease the stimulatory effect of GM-CSF. On the other hand, the protein-synthesis inhibitor cycloheximide does not affect the action of GM-CSF. Also, although preincubation of human neutrophils with GM-CSF increases the levels of $G_{i\alpha 2}$ in the plasma membrane it does not alter the total amount of cellular $G_{i\alpha 2}$. In addition, the level of $G_{i\alpha 2}$ mRNA, unlike that of the proto-oncogene c-*fos*, is not increased in cells treated with GM-CSF. This indicates that the observed increase in the amount of $G_{i\alpha 2}$ associated with the plasma membrane is not due to the synthesis of new $G_{i\alpha 2}$. These data provide insight into the mechanism by which GM-CSF may prime human neutrophils for increased responsiveness to subsequent stimulation by G-protein-dependent agonists.

INTRODUCTION

The human cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) which is released by several activated cells is a haematopoietic growth factor which supports the formation of granulocyte and monocyte colonies from bone-marrow-derived precursors [1]. It plays an important role in the commitment of bone-marrow stem cells, directing the proliferation and differentiation into specific precursors of the different lineages, and enhancing the survival of both progenitors and mature cells [2,3]. This cytokine is also able to potentiate the activation of mature phagocytes such as neutrophils, macrophages and eosinophils, and under certain experimental conditions it can directly activate mature phagocytes [4–9]. The *N*-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe)-induced superoxide production, for example, can be greatly increased in GM-CSF-treated human neutrophils. Thus GM-CSF may play an important role in host defence.

In spite of its importance, the biochemical steps that are involved in mediating the actions of GM-CSF have not been identified. For example, although it is known that GM-CSF, in the absence of a second stimulus, activates the Na^+/H^+ antiporter and increases tyrosine phosphorylation in several proteins, it is not known whether it affects any of the early events in the excitation–response sequence of neutrophil activation.

There are several lines of evidence which support the idea that a G-protein is involved either directly and/or indirectly in the actions of GM-CSF. First, some of the effects of GM-CSF are inhibited by pertussis toxin [8]. Second, the GTPase activity in membranes isolated from GM-CSF-treated neutrophils is higher than that found in membranes prepared from control cells [8]. Third, the α-subunit of G_{i2} ($G_{i\alpha 2}$) is found in specific granules [10]. Fourth, GM-CSF stimulates degranulation [11,12]. Fifth, incubation with GM-CSF enhances the production of superoxide induced by guanosine 5'-[α-thio]triphosphate and NaF [13].

The present studies were undertaken to answer three questions. First, does GM-CSF, in the absence of a second stimulus, up-regulate the amount of the guanine-nucleotide-binding protein G_{i2} associated with the plasma membrane? The rationale behind concentrating on G_{i2} is that most of the known chemotactic factors mediate their actions through this pertussis-toxin-sensitive G-protein. Second, if so, is this up-regulation due to the synthesis of new G-protein or to the redistribution of pre-existing G-protein? Third, what are the parameters that regulate this up-regulation of the G-protein?

MATERIALS AND METHODS

Isolation of human neutrophils

Human neutrophils were isolated from heparinized peripheral blood on Ficoll/Hypaque gradients as described previously [14]. The remaining red blood cells were lysed by hypotonic shock. The neutrophils were suspended in Hanks' Balanced Salt Solution containing 10 mM Hepes (pH 7.4), 124 mM NaCl, 4.9 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 0.1% dextrose and 0.1% BSA.

Abbreviations used: GM-CSF, granulocyte–macrophage colony-stimulating factor; fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine; BAPTA/AM, 1,2-bis-(*O*-aminophenoxyl)ethane-*NNN'N'*-tetra-acetic acid acetoxymethyl ester; IL-8, interleukin-8; PMSF, phenylmethanesulphonyl fluoride; DFP, di-isopropyl fluorophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Cell fractionation

Neutrophils were suspended at 10^7 cells/ml in buffered sucrose solution containing 10 mM Hepes (pH 7.4), 0.25 M sucrose, 1 mM EGTA, 1 mM di-isopropyl fluorophosphate (DFP), 1 mM phenylmethanesulphonyl fluoride (PMSF) and 50 μg/ml leupeptin. Cells were disrupted by nitrogen cavitation at 1725 kPa (250 lb/in^2) for 15 min at 4 °C as described previously [15]. To remove intact cells, the homogenate was centrifuged at 450 ***g*** for 5 min and the pellet discarded. The post-nuclear fractions were layered on sucrose step gradients (1.5 ml each of 30% and 50% sucrose). The gradients were centrifuged at 200000 ***g*** for 75 min at 4 °C in a Beckman TL-100 ultracentrifuge. The cytosol fractions were recovered from the top, the plasma membrane from the interface (30/50%) [16], and the granules [17] were found in the pellet. We have shown previously that the fraction recovered at the interface contains the Na^+/K^+-ATPase activity and binds the chemotactic factor fMet-Leu-Phe [15].

Western blotting

Immunoblotting was carried out as described previously [18–20]. Briefly, 30 μl protein samples (1 μg/ml) were mixed with 10 μl of Laemmli stopping solution. The stopping solution contains 9% (w/v) SDS, 6% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and a trace amount of Bromophenol Blue in 0.196 M Tris/HCl, pH 6.7. The mixture was boiled and electrophoresed through an SDS/10%-polyacrylamide gel. The proteins were then transferred to Immobilon-P transfer membrane and probed with antiserum (AS7) against $G_{i\alpha 2}$ protein (1:500 dilution) generously provided by Dr. Allen Spiegel (N.I.H.) [10]. Antiserum (EC/2) against $G_{i\alpha 3}$ (1:500 dilution) was purchased from NEN Dupont (Boston, MA, U.S.A.). Bound antibodies were detected with ^{125}I-labelled Protein A (1 μCi/ml, final concn. in 5% w/v BSA). Blots were exposed to Kodak X-Omat films overnight at −70 °C. The developed autoradiographs were then scanned on an LKB laser densitometer.

Northern blots

Neutrophils (2×10^7/ml) were incubated with GM-CSF and total RNA was prepared as previously described [21]. Briefly, total RNA isolated from the different donors was pooled and poly(A)$^+$ RNA was then prepared by using mRNA purification kits (Pharmacia, Montreal, Quebec, Canada) according to the manufacturer's instructions. The mRNA was electrophoresed on an agarose gel, transferred to Hybond-N membranes and hybridized with the $G_{i\alpha 2}$ cDNA probe. The filters were washed and exposed to Kodak X-ray film at −80 °C. The human $G_{i\alpha 2}$ cDNA probe in this study, which was labelled by the random-primer method, was generously given by Dr. Randall Reed (Howard Hughes Medical Institute Research Laboratories, Johns Hopkins University, Baltimore, MD, U.S.A.). Equal loading of mRNA in each lane was verified by washing the filters and rehybridizing with a cDNA for GAPDH (purchased from the American Type Tissue Collection, Rockville, MD, U.S.A.). The c-*fos* probe was generously given by Dr. Raymond Vincent (Centre Hospitalier Robert Giffard, Quebec, Canada).

Chemicals

Recombinant GM-CSF and interleukin-8 (IL-8) were purchased from Amgen Biologicals (Thousand Oaks, CA, U.S.A.). Hepes, DFP, EGTA, PMSF, fMet-Leu-Phe and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

The direct effect of GM-CSF on the amounts of $G_{i\alpha 2}$ associated with the plasma membrane was examined. In these experiments human neutrophils were incubated with 200 pM GM-CSF at 37 °C in a temperature-controlled shaker. After a preset incubation period, the cells were disrupted and the plasma membrane fraction was prepared. Figure 1 shows the results of an immunoblot analysis of the plasma-membrane fraction with an antiserum against $G_{i\alpha 2}$. The data obtained from 30 experiments showed that the amount of $G_{i\alpha 2}$ associated with the plasma membrane increased after incubation with GM-CSF. The means ± S.E.M. of the optical densities relative to the value obtained in the absence of GM-CSF are as follows (30 experiments): control 1.0, GM-CSF 2.2 ± 0.10. A similar increase is found after incubation with IL-8. Since it is thought that the fMet-Leu-Phe receptor also interacts with the $G_{i\alpha 3}$ subtype, we measured the effect of GM-CSF on the distribution of $G_{i\alpha 3}$. It was found that, although GM-CSF specifically alters the distribution of $G_{i\alpha 2}$, $G_{i\alpha 3}$ distribution is not affected. The means ± S.E.M. of the optical densities representing the relative amount of $G_{i\alpha 3}$ associated with the plasma membrane are as follows: control 1.0, GM-CSF 1.07 ± 0.10. As shown in Figure 2, the GM-CSF-induced increase in the amount of $G_{i\alpha 2}$ associated with

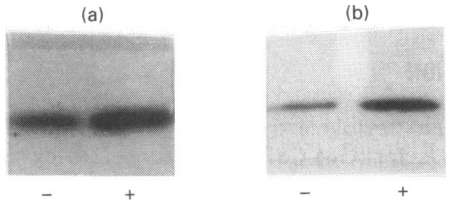

Figure 1 Autoradiograph showing immunoblotting of $G_{i\alpha 2}$ protein in membrane preparations isolated from control and GM-CSF-treated human neutrophils

The cells were incubated with 200 pM GM-CSF (**a**), 10 ng/ml IL-8 (**b**), or diluent at 37 °C for 30 min. The cells were disrupted by nitrogen cavitation as described in the Materials and methods section and plasma membrane was isolated. Plasma-membrane proteins were analysed by SDS/PAGE, followed by immunoblotting with the anti-$G_{i\alpha 2}$ serum AS/7. A representative experiment, typical of at least four different experiments, is shown + and − refer to the presence and absence of GM-CSF (**a**) or IL-8 (**b**).

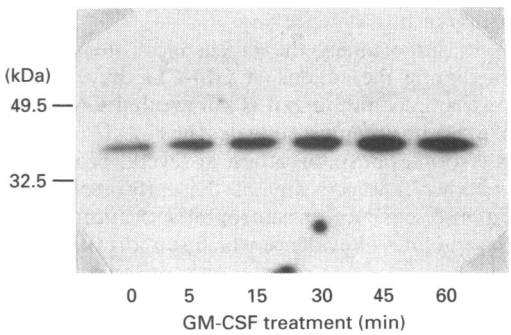

Figure 2 Time course of the effect of GM-CSF on the amounts of $G_{i\alpha 2}$ associated with the plasma membrane

Cells were incubated with GM-CSF (200 pM) for the times indicated before preparation of plasma membrane. A representative experiment, typical of at least three separate experiments, is shown. Positions of protein standards are indicated in kDa at the left.

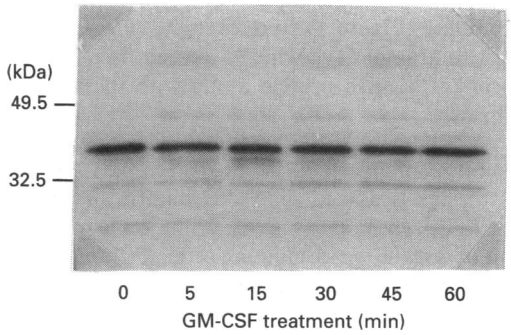

Figure 3 Autoradiograph showing immunoblotting of the amounts of total cellular $G_{i\alpha2}$

Cells were incubated with GM-CSF (200 pM) for the times indicated before $G_{i\alpha2}$ measurement. A representative autoradiograph, typical of at least three separate experiments, is shown. Positions of protein standards are indicated in kDa on the left.

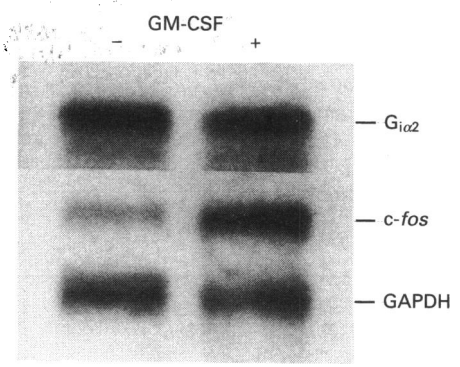

Figure 4 Effect of GM-CSF on the induction of $G_{i\alpha2}$, c-*fos* and GAPDH mRNAs

Cells were incubated with GM-CSF for 30 min at 37 °C. Total RNA isolated from different donors was pooled and poly(A)$^+$ RNA was prepared and subjected to Northern-blot analysis as described in the Materials and methods section. The autoradiograph represents one experiment typical of at least two different experiments.

Table 1 Effect of GM-CSF and IL-8 on the subcellular distribution of $G_{i\alpha2}$

Cells were treated with the indicated stimuli for 30 min at 37 °C. Cells were then fractionated on sucrose gradients as described in the Materials and methods section and the proteins were analysed by immunoblot analysis with anti-$G_{i\alpha2}$ antiserum. Data are means ± S.E.M. of the optical densities relative to the value obtained in the control samples (arbitrarily taken as 1.0). Autoradiographs of $G_{i\alpha2}$ immunoblots were scanned on a laser densitometer.

Condition	Plasma membrane	Granule fraction
Control	1.0	1.0
GM-CSF (200 pM)	2.2 ± 0.12	0.6 ± 0.08
IL-8 (10 ng/ml)	2.1 ± 0.10	0.6 ± 0.10

the plasma membrane is time-dependent (optical densities relative to the value obtained in the absence of GM-CSF are as follows: 5 min, 1.59; 15 min, 1.82; 30 min, 2.5; 45 min, 2.8; 60 min, 2.4). The amounts of $G_{i\alpha2}$ associated with plasma-membrane preparations isolated from control cells did not vary with time (results not shown). As shown, a significant ($P < 0.01$) increase can be found as early as 5 min after addition of GM-CSF.

The level of G-protein in the membrane can be increased either by stimulating the translocation of G-proteins from storage granules and/or activating synthesis *de novo* of $G_{i\alpha2}$. To distinguish between these two possibilities, we carried out four sets of experiments. In the first set, the total amount of $G_{i\alpha2}$ in the cells was measured before and after stimulation with 200 pM GM-CSF. The data summarized in Figure 3 show that the total cellular amount of $G_{i\alpha2}$ is unchanged after treatment with GM-CSF. In the second set of experiments, the amounts of $G_{i\alpha2}$ associated with plasma membrane, cytosolic and granules fractions were determined. In these experiments, human neutrophils were incubated with 200 pM GM-CSF for 30 min at 37 °C. The cells were then fractionated on a sucrose gradient. The results are summarized in Table 1. Note that the amount of $G_{i\alpha2}$ associated with the plasma-membrane fraction increases and that associated with the granules fraction decreases. Very small amounts of $G_{i\alpha2}$ were found in the cytosolic fraction (results not shown), and for simplicity will be considered negligible. The total protein amount assayed (30 μg) was equal for both the plasma-membrane and granules fractions as well as for control and GM-CSF-treated cells. The combined relative amounts of $G_{i\alpha2}$ in the plasma-membrane and granules fractions is equal to that of GM-CSF-treated samples, and is taken arbitrarily as 100%. In fractions from control cells 66.7% of the $G_{i\alpha2}$ is associated with the plasma membrane and 33.3% is within the granules fraction, whereas fractions from GM-CSF-treated cells show 80.3% in the plasma-membrane fraction and 16.7% in the granules fraction. Thus the gain of $G_{i\alpha2}$ in the plasma membrane in response to GM-CSF is proportional to the loss of granule $G_{i\alpha2}$ (see the Discussion section for more detail). In the third set of experiments, we examined the effect of GM-CSF on the resting level of $G_{i\alpha2}$ mRNA. Cells were incubated with GM-CSF or its diluent for 30 min at 37 °C, and total RNA was analysed by Northern blotting to detect $G_{i\alpha2}$ mRNA. This time point was chosen in view of the kinetic responses observed for both the increase in membrane-associated $G_{i\alpha2}$ as well as maximal priming by GM-CSF. No mRNA for $G_{i\alpha2}$ was observed under these conditions. We therefore purified poly(A)$^+$ RNA from the total RNA and performed Northern-blot analysis. As shown in Figure 4, a single band of approx. 2.4 kb was detected in both control and GM-CSF-treated cells after hybridization with the $G_{i\alpha2}$ cDNA probe. However, GM-CSF had no detectable effect on the level of $G_{i\alpha2}$ mRNA in neutrophils. The filters were washed and rehybridized with a cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading of the mRNA. The results of this hybridization showed that equal amounts of mRNA were loaded in the two lanes. As a positive control, the filters were also hybridized with cDNA probe for the proto-oncogene c-*fos*. As expected [21], it can be clearly seen that incubation of neutrophils with GM-CSF enhanced the level of expression of c-*fos* mRNA, indicating that GM-CSF was active under these conditions. In the fourth set of experiments, we measured the effect of cycloheximide on the GM-CSF-induced increase in $G_{i\alpha2}$ protein in the plasma membrane. As shown in Table 2, cycloheximide had no effect on the observed increase.

Although the GM-CSF receptor does not contain the consensus sequences characteristic of tyrosine kinase domains, its occupancy by GM-CSF activates one or more protein tyrosine

Table 2 Effect of various compounds on the GM-CSF-induced increase in plasma-membrane-associated $G_{i\alpha 2}$ levels

Cells were pretreated with the indicated compounds for 60 min (cycloheximide and genistein) or for 30 min (BAPTA/AM). Cells were then incubated with GM-CSF (200 pM) for a further 30 min before plasma membrane was prepared. Data are means ± S.E.M. of the optical densities relative to the value obtained in the absence of GM-CSF (arbitrarily taken as 1.0). Autoradiographs of $G_{i\alpha 2}$ immunoblots were scanned on a LKB laser densitometer.

Condition	Plasma membrane
Control → GM-CSF	2.09 ± 0.10
Cycloheximide (100 μg/ml) → GM-CSF	1.93 ± 0.09
Genistein (10 μg/ml) → GM-CSF	1.07 ± 0.08
BAPTA/AM (30 μM) → GM-CSF	1.40 ± 0.10

kinases and/or inhibits tyrosine phosphatases, resulting in the phosphorylation of several proteins on tyrosine residues in human neutrophils [22–24]. In order to examine if a tyrosine kinase step is involved in the observed increase in the amount of $G_{i\alpha 2}$ associated with the membrane in response to stimulation by GM-CSF, we tested the effects of inhibitors of protein tyrosine kinase on this increase. In these experiments, cells were incubated with genistein (10 μg/ml) for 60 min at 37 °C, followed by GM-CSF treatment for 30 min. This concentration of genistein was found to inhibit the GM-CSF-induced protein phosphorylation (results not shown). Genistein alone has no effect on the basal level of $G_{i\alpha 2}$ associated with the plasma membrane, but inhibited the increase produced by GM-CSF (Table 2). Similar results were found by using erbstatin, another tyrosine kinase inhibitor, which has also been shown to inhibit protein tyrosine phosphorylation in human neutrophils stimulated with GM-CSF ([24]; results not shown).

The intracellular concentration of free Ca^{2+} is an important parameter in the initiation and/or regulation of many cellular events. Although GM-CSF does not act directly to increase the intracellular concentration of free Ca^{2+}, the basal level of this ion may be important to the action of GM-CSF. To investigate this point, we determined the effect of the Ca^{2+} chelator 1,2-bis-(*O*-aminophenoxyl)ethane-*NNN'N'*-tetra-acetic acid acetoxymethyl ester (BAPTA/AM) on the observed GM-CSF-induced increase in the amount of $G_{i\alpha 2}$ associated with the membrane. In these experiments, the cells were incubated with BAPTA/AM (30 μM) for 30 min before the addition of GM-CSF. The results summarized in Table 2 show that the increase in amount of $G_{i\alpha 2}$ associated with the membrane induced by GM-CSF is decreased in BAPTA/AM-treated cells.

DISCUSSION

The amount of $G_{i\alpha 2}$ associated with the plasma membrane increased by more than 2-fold in membrane preparations isolated from GM-CSF-treated human neutrophils (Figure 1). The increase was evident at 5 min, and increased with increasing time of incubation with the cytokine (Figure 2). The previous observations [20] that the basal GTPase activity is significantly increased in membrane preparations isolated from GM-CSF-treated human neutrophils and that the stimulation requires time is consistent with this present finding, as is the observation that GM-CSF increased responsiveness to guanosine 5'-[γ-thio]triphosphate and NaF [13]. IL-8 treatment of human neutrophils also caused an increase in the amount of $G_{i\alpha 2}$ associated with the plasma membrane (Figure 1). Interestingly, GM-CSF did not affect the distribution of $G_{i\alpha 3}$, which is present in neutrophils as the less abundant 41 kDa protein along with the 40 kDa $G_{i\alpha 2}$ subtype [10].

The observed increase in the amount of $G_{i\alpha 2}$ associated with plasma-membrane preparations isolated from GM-CSF-treated human neutrophils is due to the redistribution of pre-existing $G_{i\alpha 2}$ and not to its synthesis. This redistribution is probably due to the fusion of certain G_{i2}-containing cellular granules (specific granules) with the plasma membrane. This conclusion is supported by four experimental observations. First, the total amount of $G_{i\alpha 2}$ found in the whole cell does not change in cells pretreated with GM-CSF for 30 min (Figure 3). Second, although the amounts of $G_{i\alpha 2}$ associated with the membrane increases, the amount associated with the granules decreases after pre-treatment with GM-CSF (Table 1). Third, cycloheximide, an inhibitor of protein synthesis does not affect the GM-CSF-induced increase (Table 2). Fourth, although incubation of human neutrophils enhances the level of expression of the proto-oncogene c-*fos* mRNA, it has no effect on the level of $G_{i\alpha 2}$ mRNA (Figure 4). These $G_{i\alpha 2}$-containing granules may also contain certain integrins. GM-CSF is known to up-regulate the amounts of CD11b/18 on the surface of the neutrophils [25].

The GM-CSF-induced increase in the amounts of $G_{i\alpha 2}$ in the plasma membrane is significantly ($P < 0.001$) decreased in cells pre-treated with BAPTA/AM (Table 2). The intracellular concentration of free Ca^{2+} serves as an important signal or regulator in many intracellular events. It is known that GM-CSF does not act directly to stimulate a rise in the cytosolic free Ca^{2+} concentration [24–27]. The inhibition by BAPTA/AM treatment of the GM-CSF action suggests that Ca^{2+} at the basal level plays a permissive role for the observed GM-CSF-induced translocation of $G_{i\alpha 2}$ to the plasma membrane. This is consistent with the idea that the observed increase in membrane-associated $G_{i\alpha 2}$ is due to the fusion of certain granules with the plasma membrane. Also in support of this idea is the presence of $G_{i\alpha 2}$ in neutrophil specific granules [10]. The increase in the amount of $G_{i\alpha 2}$ in the plasma membrane cannot, however, be correlated quantitatively with the decrease in amount of $G_{i\alpha 2}$ in the granules fraction. This is because the identities of the $G_{i\alpha 2}$-containing granules and the concentration of $G_{i\alpha 2}$ in these granules that may fuse with the plasma membrane and the nature of this fusion are totally unknown. Consistent with this, we and others [12] have found that GM-CSF (in the absence of cytochalasin B, conditions similar to our present studies) does not cause a measurable release of any of the known granule contents. This suggests that the observed increase in the amount of $G_{i\alpha 2}$ associated with the plasma membrane comes from unknown granules and/or from a small number of granules with high $G_{i\alpha 2}$ content (relative to total granule proteins).

GM-CSF-induced increase in the amount of $G_{i\alpha 2}$ translocated to the membrane is sensitive to inhibitors of tyrosine kinase (Table 2). Although the GM-CSF receptors do not have intrinsic kinase activity, activation of these receptors by GM-CSF results in the phosphorylation of several proteins on tyrosine residues in human neutrophils [20,22–24]. The observed phosphorylation can be inhibited by tyrosine kinase inhibitors such as genistein and erbstatin [22–24]. The present data suggest that activation of a protein tyrosine kinase and the subsequent phosphorylation of specific proteins on tyrosine is closely involved in the GM-CSF-induced translocation of $G_{i\alpha 2}$ to the plasma membrane. The identities of these specific proteins are not known, and the determination of their identities is the subject of a separate investigation. This translocation is most likely due to the fusion

of certain $G_{i\alpha 2}$-containing cellular granules with the plasma membrane. The present observation that the GM-CSF action is inhibited in cells treated with BAPTA/AM supports the fusion idea. If the fusion idea is correct, then these proteins may be part of the annexins family, which are known to be related to exocytosis. This will be extremely interesting, since it will imply that protein tyrosine phosphorylation is important for the fusion of at least some granules with the plasma membrane.

The demonstrated increase in the amount of $G_{i\alpha 2}$ associated with the plasma membrane may be responsible, in part, for the priming actions of GM-CSF. This increase results in more fMet-Leu-Phe receptor–$G_{i\alpha 2}$ complex being available for transduction. The time course of GM-CSF priming of fMet-Leu-Phe-induced responses such as Ca^{2+} mobilization [4], oxidative burst [6,7] and activation of phospholipases, correlates well with the observed time course of the GM-CSF-induced increase in the amount of $G_{i\alpha 2}$ in the plasma membrane.

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