Translocation of the FGR protein-tyrosine kinase as a consequence of neutrophil activation
(protooncogene/SRC family/granule membrane/exocytosis)

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ABSTRACT Recent studies of the FGR protooncogene have shown that expression of its mRNA is limited to mature peripheral blood granulocytes, monocytes, and tissue macrophages. In the present study, we have investigated p55c-fgr expression in normal human neutrophils [polymerphonuclear leukocytes (PMN)] and have found enzymatically active p55c-fgr to be abundant in lysates of PMN and murine fibroblasts transfected with a FGR expression plasmid but not control cells. Fractionation studies revealed that neuronal p55c-fgr was present in plasma membrane-enriched fractions as well as fractions containing secondary and tertiary granules. Little change in the distribution of p55c-fgr or FGR kinase activity was observed under conditions favoring tertiary granule release. In contrast, when secondary granule secretion was induced with the chemoattractant peptide, formyl-Met-Leu-Phe, a marked decrease in p55c-fgr and FGR kinase was observed in fractions depleted of secondary granules. Concomitantly, the relative concentration of p55c-fgr and its enzymatic activity were increased in fractions containing plasma membrane. From these findings we conclude that p55c-fgr is associated with functional secretory granules and is redistributed within normal neutrophils in response to their activation.

Roughly half of the protooncogenes identified to date encode a kinase activity specific for tyrosine residues. Structural considerations delimit two groups of these enzymes, the polypeptide growth factor receptors and kinases such as p60c-src and p55c-fgr, which lack the extracellular and transmembrane domains characteristic of the receptor kinases. Whereas roles in regulating normal cell growth have been established for growth factor receptors, normal functions for the SRC family of proteins are not known. Recent findings that one SRC-like protein, p56c-k, is activated by association with the CD4 transmembrane protein in T lymphocytes have strongly suggested that p56c-k kinase is involved in transducing signals across the plasma membrane (1). In addition, detection of maximal p60c-src levels in postmitotic cells (2–4) has suggested that its physiologic role is nonproliferative in nature.

Recent studies of the FGR protooncogene have shown that expression of its mRNA is limited to peripheral blood granulocytes, monocytes, and tissue macrophages (5) and that FGR mRNA (5) as well as its protein product, p55c-fgr (6), accumulate during differentiation of myelomonocytic cell lines. In the present study, we have directly addressed the question of p55c-fgr expression in peripheral blood neutrophils (polymerphonuclear leukocytes [PMN]). We show that the FGR protooncogene product is abundant in normal human granulocytes and demonstrate that enzymatically active p55c-fgr is associated with the plasma membrane as well as specific intracellular membrane compartments. Furthermore, we show that during exocytosis of secondary granules, p55c-fgr is translocated from its intracellular location to a compartment containing plasma membrane. These findings establish mobilization of a nonreceptor protein-tyrosine kinase (EC 2.7.1.112) in response to signals from the extracellular environment.

MATERIALS AND METHODS

Cells. NIH 3T3 murine fibroblasts (7) were maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum. Human neutrophils (PMN) from normal donors were prepared from leukophoresis packs provided by the Department of Transfusion Medicine of the National Institutes of Health. Purified PMN were obtained by sedimentation in 3% dextran at room temperature, density centrifugation on Ficoll/Hypaque, and hypotonic lysis of erythrocytes (8).

Expression of the Human c-fgr Gene in Murine Fibroblasts. To construct a c-FGR expression plasmid, a previously described human FGR cDNA clone, pc41/22 (9), was inserted into the pDOL expression vector (10) in the sense orientation. The resulting plasmid, designated pDOL-c-fgr, also contained a neomycin phosphotransferase gene and expressed simian virus 40 middle-sized tumor antigen. Plasmid DNA transfection of NIH 3T3 cells was performed by the calcium phosphate precipitation technique (11), as modified by Wigler et al. (12). Mass populations expressing the transfected gene were selected for their ability to grow in the presence of geneticin (G418) (GIBCO).

Immunoprecipitation and Kinase Assays. Subconfluent cultures of around 107 cells were disrupted with 1 ml of lysing buffer (10 mM sodium phosphate, pH 7.5/100 mM NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1 mM leupeptin/1% Trasylol/1 mM phenylmethylsulfonlfy fluoride) per Petri dish. Neutrophil suspensions (5 x 107 cells) were pretreated for 30 min with 5 mM diisopropyl fluorophosphate at 4°C, washed once with cold phosphate-buffered saline, and lysed in 1 ml of lysing buffer. Samples were incubated with 10 µl of antiserum for 60 min at 4°C. Anti-fgr C and anti-fgr N have been described (6, 9). Immunoprecipitates were recovered with the aid of Staphylococcus aureus protein A bound to Sepharose beads (Pharmacia). Immune complex kinase assays using acid-treated rabbit muscle enolase (Sigma) as an exogenous substrate were performed as described (13).

Immunoblotting. Lysates containing 40 µg of cellular protein were fractionated by sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis (SDS/PAGE) as described (6, 14). Proteins were transferred to nitrocellulose filters, and incubated with peptide antibodies as described (6, 14). Immunocomplexes were visualized by autoradiography after treatment with 125I-labeled protein A (Amersham). Protein size was estimated by comparison with 14C-labeled protein molecular weight standards (Bethesda Research Laborato-

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Abbreviations: PMN, polymorphonuclear leukocytes; fMLP, formyl-Met-Leu-Phe.
RESULTS

Detection of Enzymatically Active p55c-fgr in Human Neutrophils. To determine whether p55c-fgr was expressed in normal neutrophils, lysates from purified (>94%) human PMN, as well as NIH 3T3 cells transfected with a FGR expression plasmid, were subjected to electrophoretic transfer blotting (Western blotting) analysis using p55c-fgr sequence-specific antibodies as probes (9). As shown in Fig. 1, both amino-terminal (anti-fgr-N) and carboxyl-terminal (anti-fgr-C) sera detected a protein of M<sub>r</sub> 55,000 in lysates of PMN and transfectants expressing FGR but not control transfected or untreated NIH 3T3 cells. The identity of this protein as p55c-fgr was further demonstrated by the lack of its detection with either antibody in the presence of homologous peptide (Fig. 1). The slight difference in size observed for human p55c-fgr expressed in mouse fibroblasts as compared to human PMN was reproducible and suggested greater phosphorylation of the neutrophil species.

To examine whether neutrophil-derived p55c-fgr was enzymatically active, lysates from purified PMN or NIH 3T3 cells expressing FGR were tested in immune complex kinase assays. As shown in Fig. 2, p55c-fgr was autophosphorylated in the assay, and phosphate was also transferred to enolase, an exogenous substrate that was included in the reaction mixture. In contrast, antibody preincubated with homologous peptide did not precipitate kinase activity. By phosphoamino acid analysis of labeled p55c-fgr (Fig. 2C) and enolase (data not shown), only phosphotyrosine was detected, establishing neutrophil p55c-fgr as a protein-tyrosine kinase.

Neutrophil p55c-fgr Enriched in Plasma Membrane and Secondary Granule Compartments. In an effort to define possible cellular locations where the tyrosine kinase function of p55c-fgr might be exerted, we fractionated human neutrophils into cytosol and particulate membrane compartments, including primary, secondary, and tertiary granules as well as plasma membrane. Conditions previously established for separation of these cell components were employed (15, 22, 23). Enzymatic activities or protein constituents present in each fraction served as markers for subcellular organelles. Only 12%, 7%, and 5% of total markers for primary, secondary, and tertiary granules, respectively, were secreted from unstimulated neutrophils prior to cell disruption. Plasma membrane (Fig. 3A) as well as primary (Fig. 3B), secondary (Fig. 3C), and recently identified (24) tertiary (Fig. 3D) granule compartments were effectively resolved by this procedure. As judged by lactate dehydrogenase assays, cytosol was confined to gradient fractions 1 (data not shown).

When fractions were assayed for the presence of the FGR protooncogene product by Western blotting and immune complex tyrosine kinase assays, only small amounts of partially degraded FGR gene product were detected in cytosol or in fractions containing primary granules (Fig. 4 A and

Neutrophil Fractionation and Activation. PMN (1.4 × 10<sup>6</sup>) were resuspended at 5 × 10<sup>7</sup> cells per ml in Hanks’ balanced salt solution (HBSS) buffer at 4°C. The cell suspension was divided into three equal fractions. One sample was held at 4°C, another was incubated at 37°C for 15 min with 5 μg of cytochalasin B per ml, and a third sample was preincubated at 37°C for 5 min with 5 μg of cytochalasin B per ml and then exposed to 0.1 μM formyl-Met-Leu-Phe (fMLP) for 10 min. Treated PMN were chilled with 2 volumes of cold HBSS, adjusted to 5 mM disopropyl fluorophosphate, and labeled by incubation with [3H]Con A (Amersham; 60 Ci/mmol, 50 μCi/ml; 1 Ci = 37 GBq) for 20 min on ice. Aliquots were saved to determine the release of enzyme markers for each cytoplasmic granule. After centrifugation, marker concentrations were measured in supernatants and cell pellets to determine the percent of granule release under the various conditions utilized. Cells were washed and disrupted in a Potter–Elvehjem homogenizer as described (13). Postnuclear supernatants were layered onto a 15–40% (wt/wt) sucrose and centrifuged at 76,000 × g for 15 min at 4°C. The first fraction, corresponding to cytosol, was centrifuged for 30 min at 100,000 × g to remove contaminating membrane fragments. The remaining fractions were diluted with 50 mM Tris-HCl, pH 8.0/100 mM NaCl and centrifuged at 100,000 × g for 60 min. The distribution of primary, secondary, and tertiary granules was determined by using assays for β-glucuronidase (16), vitamin B<sub>12</sub>-binding protein (17, 18), and gelatinase (19), respectively.
The use of acid-treated enolase as an exogenous substrate has been reported (20). In some cases (even-numbered lanes), antibodies were preincubated with an excess of homologous peptide. Reaction products were fractionated by SDS/PAGE, dried, and subjected to autoradiography for 24 hr. Lysates of purified PMN (lanes 7 and 8) were analyzed identically except that autoradiography was for 6 hr. Molecular weights are given as Mr x 10^3. (C) The 32P-labeled band corresponding to autophosphorylated neutrophil p55c-fgr was cut from the gel and electroeluted. The sample was acid-hydrolyzed and analyzed by thin-layer electrophoresis (TLE) and chromatography (TLC) in the first and second dimensions, respectively (21). Localizations of markers of phosphorylated serine (P-S), threonine (P-T), and tyrosine (P-Y) are indicated.

B, lanes 1). The absence of p55c-fgr in primary granules (Fig. 4 A and B, lanes 7 and 8) demonstrated a specificity for its association with other cellular components. Abundant p55c-fgr was detected in the plasma membrane-enriched fractions (Fig. 4 A and B, lanes 2 and 3) as well as those containing secondary (Fig. 4 A and B, lanes 4–6) and tertiary (Fig. 4 A and B, lanes 3–6) granules. By comparison of p55c-fgr signal intensities, we determined that at least 40% of the protein cofractionated with secondary and possibly tertiary granules. These findings demonstrated that enzymatically active p55c-fgr was present in neutrophil membranes and that nearly half of the protein was specifically localized to a membrane compartment that included secondary and tertiary intracellular granules.

Mobilization of p55c-fgr During Secretion of Secondary Granules. To investigate whether p55c-fgr was associated with functional secretory organelles, we treated neutrophil suspensions with stimuli known to induce granule exocytosis. Thus, very mild conditions of warming at 37°C in the presence of cytochalasin B (10, 25) induced the release of 44% of the tertiary granules present in the neutrophil preparation (Fig. 3). At the same time, only 22% and 5% of total secondary and primary granule constituents, respectively, were secreted. However, little change in the distribution of p55c-fgr or FGR kinase activity was observed under these conditions (Fig. 4 C and D). Thus, it is unlikely that p55c-fgr is physically associated with tertiary granules.

We next induced secondary granule secretion with the chemoattractant peptide fMLP (26, 27). Thus, 25%, 55%, and 58% of total primary, secondary, and tertiary granules, respectively, were secreted when fMLP was added to the preparation of cytochalasin B-treated neutrophils (Fig. 3). Under these conditions, a marked decrease in p55c-fgr and FGR kinase was observed in fractions depleted of secondary granules. Concomitantly, the concentration of p55c-fgr and its enzymatic activity were increased in fractions containing
plasma membrane (Fig. 4 E and F). From these findings we conclude that p55c-fgr is associated with functional secretory granules and is redistributed within normal neutrophils in response to their activation.

**DISCUSSION**

In the present study, we have identified abundant p55c-fgr in the purified neutrophils obtained from normal donors. The
protein was present in cell membranes but absent from cytosol, findings consistent with the observation that p55c-fgr is posttranslationally myristylated (6). Fractions enriched for plasma membrane and secondary granules but not primary granules contained enzymatically active p55c-fgr. Thus, p55c-fgr was not randomly distributed within neutrophils but was instead specifically associated with certain surface and intracellular membrane. Moreover, the translocation of p55c-fgr during secondary granule exocytosis demonstrated that the protein was associated with functional secretory granules.

Although the protein products of the SRC family of oncoproteins have well-established enzymatic and transforming activities (28), elucidation of physiologic roles for their normal cellular counterparts has been elusive. Enzymatically active p60c-src is highly concentrated in platelet membranes (3), a large portion of which consist of intracellular secretory granules. Other major sources of p60c-src include neurons (2) as well as chromaffin cell granules (4, 29). Like platelets, neurons, and chromaffin cells, neutrophils are highly specialized for rapid exocytosis of intracellular vesicles upon activation through specific receptors (15). Thus, our evidence that p55c-fgr is associated with functional secretory granules in normal neutrophils is consistent with a role for the SRC subfamily of kinases in some aspect of the degranulation process.

At least 40% of neutrophil p55c-fgr and its tyrosine kinase activity was localized to secondary granule-enriched fractions. Secondary granules are secretory vesicles that fuse rapidly with the plasma membrane upon neutrophil activation, releasing their contents to the environment. Granule fusion also plays an important role in chemotaxis and cell adhesion by bringing additional chemotactant and adhesion receptors to the cell surface immediately after activation (20-23, 30, 31). Although we have no direct evidence for a FGR function, our present findings significantly limit the biologic framework in which the FGR protooncogene must act. This protein may play a role in the process of exocytosis perhaps involving rapid cytoskeletal changes that facilitate granule mobilization in cytoskeleton organization are known to occur rapidly upon neutrophil stimulation (32, 33). Alternatively, an increased concentration of p55c-fgr at the cell surface as a consequence of degranulation may aid in sensitizing the neutrophil to the presence of chemotactants or to other environmental stimuli. In any case, our findings strengthen the hypothesis that protein-tyrosine kinases of the SRC protooncogene family perform specialized functions in fully differentiated cells.

Emerging evidence that SRC-like proteins normally function in postmitotic cells also has potentially important implications regarding the mechanisms of transformation by oncogenic versions of SRC family members. It has been established that the growth factor receptor class of protein-tyrosine kinases transforms by a mechanism reflecting its normal mode of action. For example, cells expressing large numbers of epidermal growth factor receptors are transformed in the presence but not the absence of epidermal growth factor (34, 35). If SRC-like kinases follow this paradigm, their transforming activities also might mimic their normal functions. Although their action is unlikely to have a transforming effect in postmitotic cells, expression of their functions in cells that retain proliferative capacity might have very different consequences. Regardless of their mechanisms of action, establishment of normal functions for the SRC family of kinases is likely to provide considerable insight into the molecular events involved in their ability to induce cellular transformation.

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