Expression of the *fgr* Protooncogene Product as a Function of Myelomonocytic Cell Maturation

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Abstract. The *fgr* protooncogene is a member of the *src* family of protein tyrosine kinases. Recent studies have shown that normal myelomonocytic cells and tissue macrophages are the major sites of *fgr* mRNA expression. In the present study, we have identified the *fgr* protooncogene protein product in HL60 cells and have examined its expression as a function of HL60 cell maturation. Whether induced toward monocytic or granulocytic lineages, $p55^{c\text{-}fgr}$ accumulated in HL60 cells during maturation. In differentiated cells, the protein was active as a protein tyrosine kinase and was localized to peripheral cell membranes. Demonstration that a myristyl group was covalently bound to the protein probably accounted for its subcellular distribution. These findings establish developmental regulation of $p55^{c\text{-}fgr}$ in a lineage that represents its natural site of expression.

NEARLY half of the retrovirus oncogenes described to date either encode protein tyrosine kinases or share structural homology with genes specifying such enzymes. Discoveries identifying a small number of these oncogenic tyrosine kinases as altered versions of growth factor receptors provided impetus for the idea that dysregulation of pathways normally controlled by growth factors—such as platelet-derived growth factor (10, 33), colony-stimulating factor 1 (28), and epidermal growth factor (11)—can be important steps in the oncogenic process. It would appear, however, that not all oncogenic protein tyrosine kinases represent transmembrane growth factor receptors. Tyrosine kinases specified by the *src* protooncogene (31) as well as the closely related products of cellular *fgr* (c-*fgr*)[1] (15, 17) and *yes* (30) genes do not possess hydrophobic domains able to span the plasma membrane and therefore lack extracellular ligand-binding domains.

Although the *src* family of oncogenic proteins exhibits known enzymatic and transforming activities (for a recent review see 16), functions for their normal cellular counterparts have been elusive. The ubiquitous expression of cellular *src* (c-*src*) has implied the importance of this protooncogene for a variety of cell types but has provided little information concerning its possible function. In contrast, expression of *fgr* protooncogene mRNA is limited in humans to normal circulating granulocytes, monocytes, and tissue macrophages (19). Thus, restricted c-*fgr* expression suggests that its function must represent a feature common to those cell types comprising the myelomonocytic lineage. Previous findings have shown that *fgr* mRNA accumulates during differentiation of a monocytic cell line (19). In the present study, using HL60 cells as a model for myelomonocytic cell maturation, we have detected $p55^{c\text{-}fgr}$ in maturing cells and have observed its accumulation during the HL60 differentiation process. Our findings establish developmental regulation of $p55^{c\text{-}fgr}$ in a lineage that represents its natural site of expression.

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1. *Abbreviations used in this paper*: c-*fgr*, cellular *fgr*; c-*src*, cellular *src*; *fgr* C, peptide that represents the carboxy-terminal region of the putative protein; *fgr* N, peptide that represents the amino-terminal region of the putative protein; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

Materials and Methods

Cells

HL60 cells (6) were maintained in RPMI 1640 medium containing 10% FBS. For differentiation experiments, exponentially growing cells were subcultured at a density of 2.5×10^5 cells/ml, and inducers were added to the medium at the following concentrations: 10^{-6} M retinoic acid (Sigma Chemical Co., St. Louis, MO) (1), 1.25% DMSO (Sigma Chemical Co.) (7), and 20 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma Chemical Co.) (24). After induction, cells were washed twice with PBS before use. NIH 3T3 cells expressing chicken $p60^{c\text{-}src}$ (27) or $p21^{ms}$ (29) have been described. Mononuclear cells isolated from normal human donors were also used.

RNA Preparation and Analysis

RNA was purified from cellular extracts prepared by homogenization in guanidinium thiocyanate as described (5). A genomic fragment from the human c-*fgr* gene (32), encompassing exon 2 and portions of introns 1 and

2, was subcloned into M13mp19, and a uniformly ^{32}P-labeled probe complementary to the primary c-*fgr* transcript was synthesized. Conditions for nucleic acid hybridization and S_1 nuclease protection analysis have been previously reported (19).

Immunoprecipitation and Kinase Assays

Around 10^7 cells were labeled for the last 3 h of their scheduled exposure to inducer in 4 ml of (*a*) methionine-free RPMI 1640 containing 100 μCi [^{35}S]methionine/ml (1,200 Ci/mmol); (*b*) RPMI 1640 containing 500 μCi [^{3}H]palmitic acid (23 Ci/mmol); or (*c*) RPMI 1640 containing [^{3}H]myristic acid (13 Ci/mmol). Radiolabeled 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, 1% Trasylol, and 1 mM PMSF) per petri dish, clarified at 100,000 *g* for 30 min, and divided into five equal portions. Samples were incubated with antisera for 60 min at 4°C. Antibodies against peptides that represent amino- and carboxy-terminal regions of the putative protein (anti-*fgr* N and anti-*fgr* C, respectively) (19), mAb 327 (20), and mAb 259 (12) have been described previously. Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) and analyzed by SDS-PAGE. Immune complex kinase assays using acid-treated rabbit muscle enolase (Sigma Chemical Co.) as an exogenous substrate were performed as described (8).

Immunoblotting

Cells were disrupted in lysing buffer supplemented with 0.1% SDS. Lysates containing 40 μg of protein were fractionated by SDS-PAGE and transferred electrophoretically to nitrocellulose filters. Immunodetection using peptide antibodies was performed as previously described (18). Briefly, filters were incubated for 2 h at room temperature with antibody, washed, and treated with ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, IL). Protein size was estimated by comparison with ^{14}C-labeled protein molecular mass standards (Bethesda Research Laboratories, Gaithersburg, MD). Autoradiography was performed with the aid of an intensifying screen at −80°C.

Cell Fractionation

A modification of the procedure of Lutton et al. (21) was used. Briefly, metabolically labeled cells were incubated for 30 min at 4°C in hypotonic buffer containing 1 mM Tris, pH 7.5, 1 mM PMSF, and 1% Trasylol. Swollen cells were disrupted by 30 strokes in a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, NJ) until >95% of the nuclei were released as judged by phase microscopy. Postnuclear supernatants were separated into soluble and particulate fractions by centrifugation at 100,000 *g* for 60 min at 4°C. The soluble fraction was lyophilized, and each fraction was resuspended in an equal volume of lysing buffer for analysis by immunoprecipitation.

Immunofluorescent Staining

Cells were collected by centrifugation onto gelatin-coated glass slides, fixed with 3% paraformaldehyde, and permeabilized in the presence of PBS containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% Triton X-100. Cells were washed and incubated for 45 min with anti-*fgr* C serum in the presence or absence of 10 μg *fgr* C peptide/ml. After incubation with fluorescein-conjugated goat anti-rabbit IgG for 45 min, staining was visualized by fluorescent microscopy.

Results

Induction of fgr Protooncogene mRNA in HL60 Cells

Previous studies have shown that normal sources of *fgr* protooncogene mRNA are limited to peripheral blood granulocytes, monocytes, and alveolar macrophages. Furthermore, *fgr* mRNA levels were shown to increase upon induction of monocytic differentiation by the phorbol ester TPA in U937 cells (19). To determine whether c-*fgr* expression also responded to myelocytic differentiation, we assayed for the c-*fgr* transcript in HL60 cells induced to differentiate toward the myelocytic state with retinoic acid. Maturation was assessed by the nitroblue tetrazolium reduction assay (2). At 1, 2, 3, and 4 d of retinoic acid exposure, 31, 67, 84, and 96%, respectively, of treated HL60 cells reduced nitroblue tetrazolium as judged by the formation of intracellular formazan precipitates. Only 1–2% of untreated cells demonstrated this property. When cultures were examined for expression of c-*fgr* mRNA, no c-*fgr* mRNA expression was detected in RNA prepared for HL60 cells before treatment (Fig. 1). In contrast, mature c-*fgr* transcript, represented by a band of 239 nucleotides, increased in abundance during retinoic acid exposure. Maximum levels of c-*fgr* mRNA were achieved at 3–4 d, the time at which myelocytic differentiation was nearly complete (Fig. 1). Thus, *fgr* protooncogene mRNA accumulates during maturation of HL60 cells induced to differentiate toward the granulocytic lineage.

In addition to the 239-nucleotide band representing c-*fgr* exon 2 sequences (19), bands of 278 and 622 nucleotides were protected by RNA from control mononuclear cells as well as from treated HL60 cells (Fig. 1). Based upon the intron–exon composition of the probe used, we concluded that precursor RNA molecules containing exon 2 plus intron 1 gave rise to the 622-nucleotide band. Similarly, exon 2 and only intron 2 sequences protected the 278-nucleotide band from digestion. Thus, c-*fgr* RNAs containing introns 1 and 2 are detectable in maturing HL60 and normal mononuclear cells. These findings are consistent with previous studies demonstrating introns 2, 4, and 7 within 10–20% of c-*fgr* cDNAs cloned from mononuclear cell mRNA templates (17) and the presence of intron 7 in 20% of the *fgr* transcripts detected in TPA-treated U937 cells (19).

Accumulation of p55$^{c\text{-}fgr}$ during the Course of HL60 Cell Differentiation

The primary structure of the *fgr* protooncogene product predicted from nucleotide sequence analysis of c-*fgr* cDNA has recently been reported (15, 17). By using anti-*fgr* N or anti-*fgr* C, it has been possible to identify p55$^{c\text{-}fgr}$ as the primary translational product of the c-*fgr* gene (17). These same antibodies were used in immunoblotting assays to attempt detection of p55$^{c\text{-}fgr}$ in HL60 cells exposed to retinoic acid. As shown in Fig. 2, both anti-*fgr* N and anti-*fgr* C detected a protein of 55 kD in lysates of treated but not untreated HL60 cells. The identity of this protein as p55$^{c\text{-}fgr}$ was further demonstrated by lack of its detection with either antibody in the presence of homologous peptide (Fig. 2). In addition to p55$^{c\text{-}fgr}$, bands with molecular masses of <55 kD were specifically detected with anti-*fgr* C but not anti-*fgr* N sera. These bands may represent p55$^{c\text{-}fgr}$ proteins that have lost portions of their amino-terminal regions or may represent related proteins. In any case, when examined over a 3-d time course, p55$^{c\text{-}fgr}$ accumulated at a steady state in response to retinoic acid treatment, reaching the highest levels at 3 d of induction. Thus, p55$^{c\text{-}fgr}$ expression parallels that of its mRNA in retinoic acid–treated HL60 cells.

Induction of p55$^{c\text{-}fgr}$ by a Variety of Differentiating Agents

To investigate the ability of monocytic- as well as myelocytic-inducing agents to affect p55$^{c\text{-}fgr}$ expression, HL60 cells were treated with DMSO, which induces myelocytic differentiation, or the phorbol ester, TPA, which induces mono-

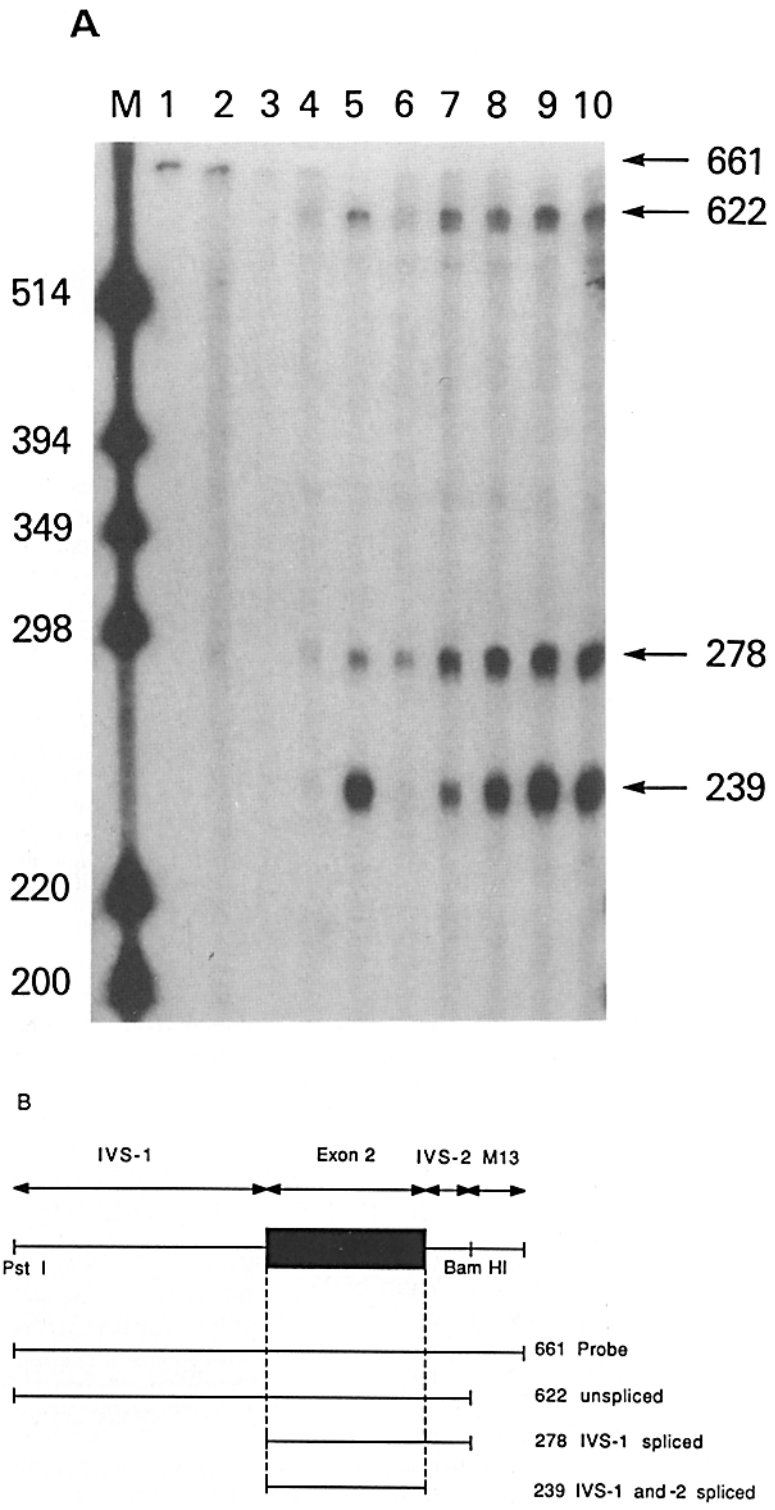

Figure 1. Accumulation of fgr mRNA during differentiation of HL60 cells. RNA was harvested from HL60 cells before (lane *3*) or after treatment with retinoic acid for 6 or 12 h (lanes *4* and *6*, respectively) or 1, 2, 3, or 4 d (lanes *7–10*, respectively). Samples were used to hybridize radiolabeled c-*fgr* probe as described in Materials and Methods. Control samples consisted of probe alone (lane *1*) or probe plus tRNA (lane *2*) or RNA from normal human mononuclear cells (lane *5*). Sizes of molecules surviving S_1 nuclease treatment are indicated on the right in nucleotides. *M*, molecular size standards. (*B*) Schematic representation of probe used to detect c-*fgr* mRNA. Closed box indicates location of c-*fgr* exon 2 within labeled DNA fragment. Sizes of probe molecules that would be protected by various forms of c-*fgr* RNA are shown in nucleotides. *IVS*, intron.

cytic differentiation. As determined by nitroblue tetrazolium reduction assays for DMSO or by morphologic criteria (2) for TPA, exposed cultures followed a maturation time course nearly identical to that observed with retinoic acid (see above). Thus, after 4 d of exposure, 91, 83, or 81%, respectively, of cells treated with retinoic acid, DMSO, or TPA were fully differentiated and viable. Lysates of treated cultures were evaluated for $p55^{c\text{-}fgr}$ expression by immunoblotting. As shown in Fig. 3, levels of $p55^{c\text{-}fgr}$ increased during differentiation induced by each of these agents. However, maximum induction by DMSO or TPA was ~40% of the $p55^{c\text{-}fgr}$ abundance observed in retinoic acid–treated cells. These findings demonstrated that the *fgr* protooncogene product accumulated in HL60 cells that were induced to differentiate toward either monocytic or myelocytic lineages.

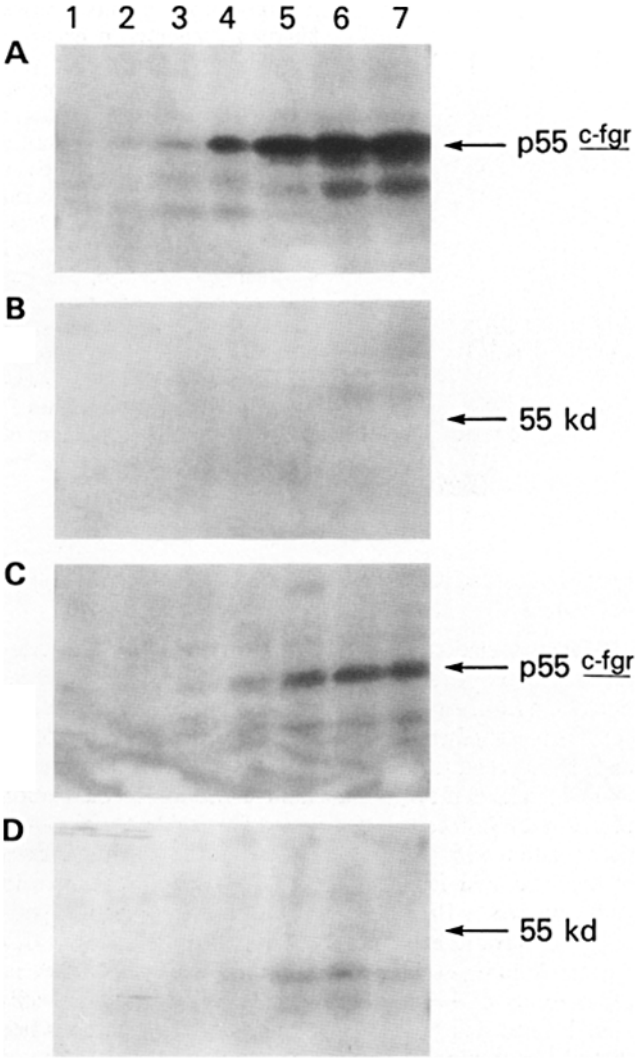

Figure 2. Expression of p55$^{c\text{-}fgr}$ during HL60 differentiation. Protein extracts were prepared from HL60 cells before (lanes *1*) or after treatment with retinoic acid for 6 or 12 h (lanes *2* and *3*, respectively) or 1, 2, 3, or 4 d (lanes *4–7*, respectively). Samples were fractionated by SDS-PAGE and transferred to nitrocellulose filters. Filters were incubated with anti-*fgr* C (*A* and *B*) or anti-*fgr* N (*C* and *D*). In some cases, antibodies were preincubated with homologous peptide (*B* and *D*). Immune complexes were labeled using iodinated protein A and visualized by autoradiography. The location of p55$^{c\text{-}fgr}$ is indicated.

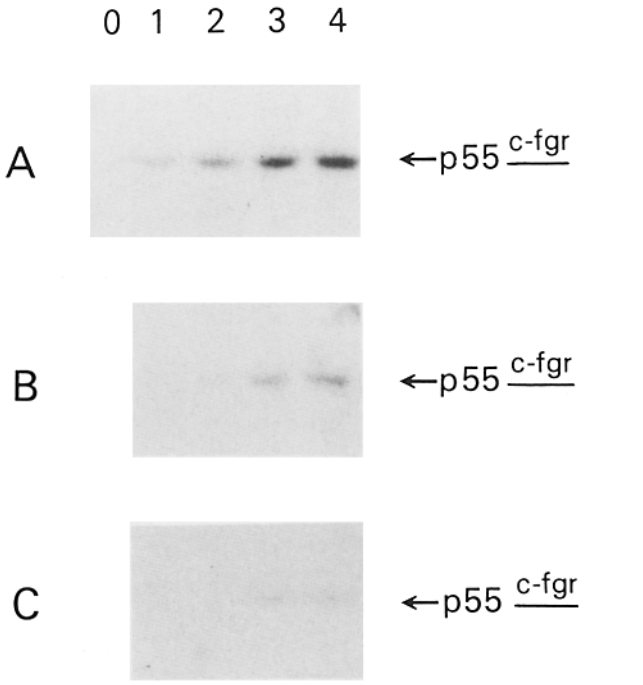

Figure 3. Detection on p55$^{c\text{-}fgr}$ during exposure of HL60 cells to various differentiation agents. HL60 cell extracts were prepared at varying times after treatment with inducing agents. Cells seeded at a concentration of 2.5 × 10^5/ml on day 0 were incubated in the presence of retinoic acid, DMSO, or TPA (*A–C*, respectively) for up to 4 d. Samples were analyzed by immunoblotting using anti-*fgr* C serum as described in Materials and Methods. Immune complexes were labeled using iodinated protein A and visualized by autoradiography. The location of p55$^{c\text{-}fgr}$ is indicated.

p55$^{c\text{-}fgr}$ Is Active as a Protein Tyrosine Kinase

Genes of the *src* protooncogene family encode enzymes with protein tyrosine kinase activity (for review see 14). To examine whether p55$^{c\text{-}fgr}$ also possessed this enzymatic activity, lysates of HL60 cells treated with retinoic acid were tested in immune complex kinase assays. As shown in Fig. 4, p55$^{c\text{-}fgr}$ was autophosphorylated in the assay, and phosphate was transferred to enolase, an exogenous substrate that was included in the reaction mixture. In contrast, antibody preincubated with *fgr* C peptide did not precipitate kinase activity. By phosphoamino acid analysis of labeled p55$^{c\text{-}fgr}$, only phosphotyrosine was detected, establishing p55$^{c\text{-}fgr}$ as a protein tyrosine kinase (data not shown). When examined as a function of HL60 cell differentiation, the abundance of *fgr* kinase increased with time of exposure to retinoic acid (Fig. 4). Thus, p55$^{c\text{-}fgr}$ accumulates as an active protein tyrosine kinase during the maturation of HL60 cells.

Localization of p55$^{c\text{-}fgr}$ in Induced HL60 Cells

In an effort to define possible cellular locations where the tyrosine kinase activity of p55$^{c\text{-}fgr}$ might be exerted, we fractionated retinoic acid–induced HL60 cells into cytosol and particulate membrane compartments and assayed each for the presence of the *fgr* protooncogene product by immunoprecipitation. As shown in Fig. 5, no p55$^{c\text{-}fgr}$ was detected in the cytosolic fraction, but the protein was abundant in crude membranes. By comparison of signal intensities observed in cytosolic and membrane fractions, we concluded that at least 95% of p55$^{c\text{-}fgr}$ was membrane associated.

To confirm the localization of p55$^{c\text{-}fgr}$ and to visualize its distribution among cellular membranes, we attempted to detect the protein using an indirect immunofluorescence staining approach. As shown in Fig. 6, staining was readily observed in HL60 cells fixed after 2 d of retinoic acid exposure but not in uninduced cells. The specificity of the signal was further demonstrated when identically prepared cells were treated with anti-*fgr* C in the presence of homologous peptide (Fig. 6 *B*). As shown in Fig. 6 *C*, bright fluorescence appeared toward the cell periphery with diffuse staining of the cytoplasm. Little if any perinuclear or nuclear signal was observed. The pattern of staining was consistent with the lo-

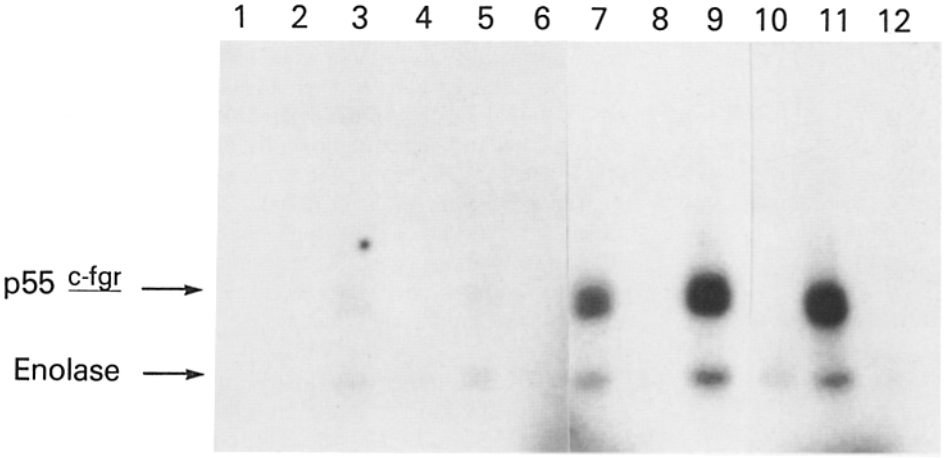

Figure 4. p55$^{c\text{-}fgr}$ protein tyrosine kinase accumulates in maturing HL60 cells. Protein extracts were prepared from HL60 cells before (lanes *1* and *2*) or after treatment with retinoic acid for 6 (lanes *3* and *4*) or 12 h (lanes *5* and *6*) or 2 (lanes *7* and *8*), 3 (lanes *9* and *10*), or 4 d (lanes *11* and *12*). Samples were immunoprecipitated with anti-*fgr* C serum, and immune complexes were assayed for kinase activity as described in Materials and Methods. In some cases, anti-*fgr* C was preincubated with *fgr* C peptide (lanes *2*, *4*, *6*, *8*, *10*, and *12*). Locations of labeled p55$^{c\text{-}fgr}$ and enolase are indicated.

calization of p55$^{c\text{-}fgr}$ to the plasma membrane. Taken together, our findings revealed that the *fgr* protooncogene product is distributed toward the periphery of mature HL60 cells in association with membrane components.

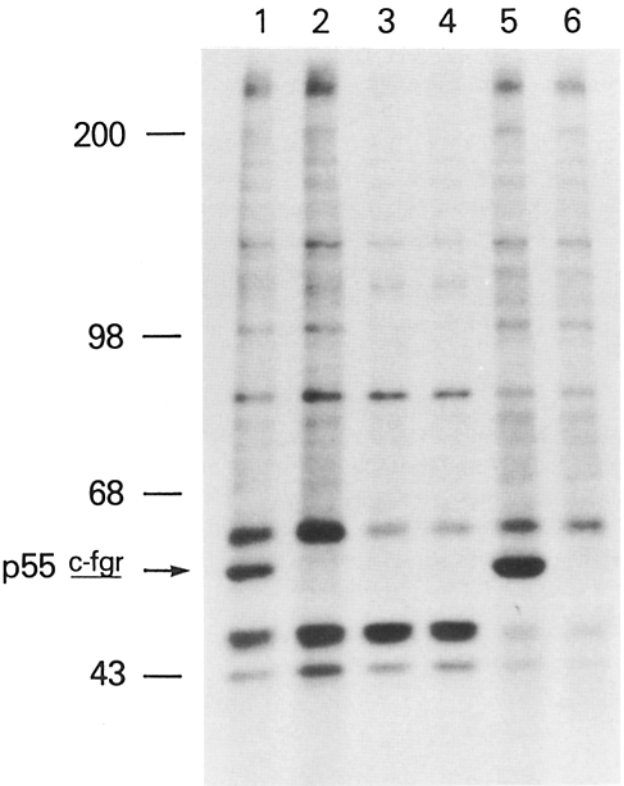

Figure 5. Localization of p55$^{c\text{-}fgr}$ by cell fractionation. HL60 cells, treated with retinoic acid for 48 h and metabolically labeled with [^{35}S]methionine, were fractionated into soluble (lanes *3* and *4*) and particulate (lanes *5* and *6*) components as described in Materials and Methods. Fractions as well as whole-cell lysates (lanes *1* and *2*) were adjusted in volume such that each represented the same number of cells and were analyzed by immunoprecipitation using anti-*fgr* C serum. In some cases antibody was preincubated with *fgr* C peptide (lanes *2*, *4*, and *6*). Location of p55$^{c\text{-}fgr}$ is indicated.

Modification of p55$^{c\text{-}fgr}$ by Posttranslational Addition of Fatty Acid

The posttranslational addition of a myristyl group to cytoplasmic p60$^{c\text{-}src}$ has been shown essential for its accumulation at the inner surface of the plasma membrane (9, 23). To determine whether similar modifications affected p55$^{c\text{-}fgr}$ and thereby contributed to its subcellular location, we attempted to label the c-*fgr* translational product by incubation of induced HL60 cells with tritiated fatty acids. By immunoprecipitation with anti-*fgr* C, p55$^{c\text{-}fgr}$ was detected in lysates of myristic acid–labeled cells but was barely observed in cells exposed to [^{3}H]palmitic acid (Fig. 7). Control experiments showed, as expected, that p60$^{c\text{-}src}$ (3) was more readily detectable in lysates of myristic acid–labeled cells, whereas the intensity of the p21^{ras} (26) band was greater when cells were labeled with palmitic acid. These findings demonstrate that p55$^{c\text{-}fgr}$ is modified by the posttranslational addition of fatty acid, most likely a myristyl group, and suggest that p55$^{c\text{-}fgr}$ associates with membrane components by virtue of this modification.

Discussion

The present study has examined expression of the *fgr* protooncogene product, p55$^{c\text{-}fgr}$ in maturing HL60 cells. The choice of this model was based upon previous studies showing the presence of *fgr* mRNA only in granulocytes, monocytes, and macrophages (19). Demonstration that p55$^{c\text{-}fgr}$ accumulated during maturation of HL60 cells has established that expression of the *fgr*-encoded protein is developmentally regulated in HL60 cells. These findings are consistent with previous studies that have shown increasing *fgr* mRNA abundance during maturation of U937 cells (19). More rapid induction of c-*fgr* mRNA during U937 cell maturation as compared with that of p55$^{c\text{-}fgr}$ in HL60 cells probably reflects the relatively advanced state of U937 cell differentiation. Furthermore, the time course of p55$^{c\text{-}fgr}$ induction in HL60 cells in response to TPA is nearly identical to that previously described for p60$^{c\text{-}src}$ (13). Abundant c-*fgr* mRNA is present in fully mature human (19) and murine (34) monocytes, and p55$^{c\text{-}fgr}$ is expressed at high levels in normal granulocytes purified from human blood (13a). Taken together, the evi-

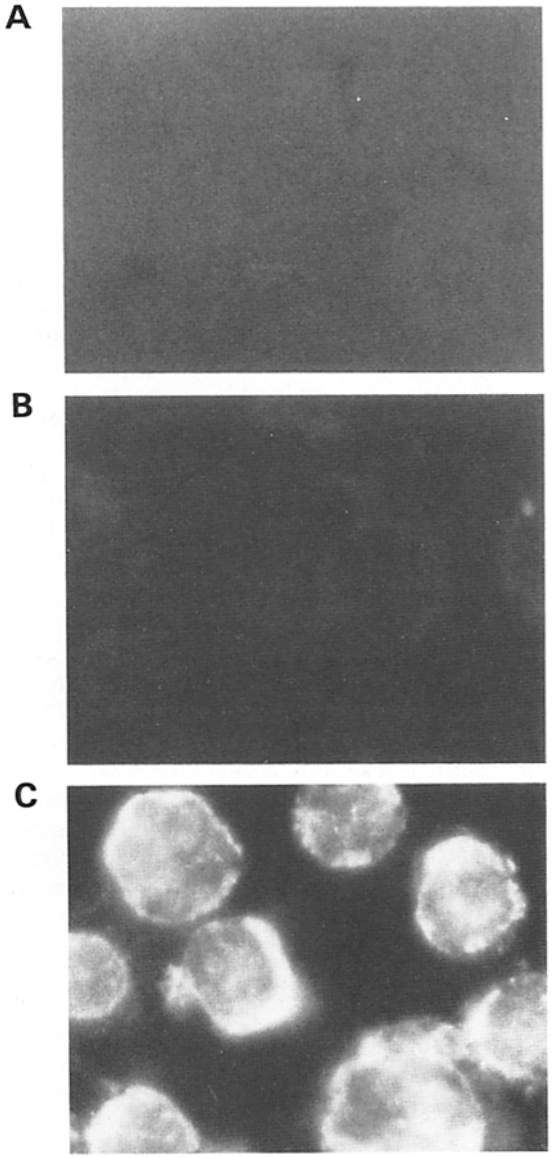

Figure 6. Localization of p55$^{c\text{-}fgr}$ by indirect immunofluorescence. Untreated HL60 cells (*A*) or cultures treated with retinoic acid for 48 h (*B* and *C*) were fixed and incubated with anti-*fgr* C. In one case, antibody was preincubated with *frg* C peptide (*B*). Immune complexes were visualized by staining with fluorescein-conjugated goat anti-rabbit IgG.

dence strongly suggests that accumulation of p55$^{c\text{-}fgr}$ in HL60 cells maturing toward monocytes or granulocytes reflects its normal pattern of expression.

The extent of p55$^{c\text{-}fgr}$ induction was greatest when HL60 cells were exposed to retinoic acid as compared with TPA or DMSO. Although preliminary studies using lower-titered antibodies did not detect p55$^{c\text{-}fgr}$ in HL60 cells exposed to TPA (19), p55$^{c\text{-}fgr}$ clearly accumulated during differentiation by TPA. Similar low levels of p55$^{c\text{-}fgr}$ were also observed in DMSO-treated cells, suggesting no relationship between the extent of p55$^{c\text{-}fgr}$ induction and maturation toward either monocytic or myelocytic lineages. The time course of differentiation induced by all of the agents tested, including retinoic acid, were nearly identical. Thus, the higher levels of p55$^{c\text{-}fgr}$ observed in retinoic acid–treated cells appear to relate specifically to retinoic acid. Preliminary experiments addressing this issue have taken advantage of an earlier observation that Epstein–Barr virus–infected B cells (Ramos-AW), but not uninfected Ramos cells, expressed c-*fgr* mRNA (4). When exposed to retinoic acid, c-*fgr* mRNA levels increased two- to threefold in Ramos-AW cells but remained undetectable in Ramos cells (our unpublished observations). These data would suggest that retinoic acid not only induces c-*fgr* mRNA as a function of HL60 maturation but also has an additional enhancing effect on the *fgr* locus in cells already expressing c-*fgr* mRNA.

Several recent studies have documented the involvement of protein tyrosine kinases in the program of myelomonocytic cell differentiation. Expression of the colony-stimulating factor 1 receptor is induced when HL60 cells differentiate toward monocytes but not granulocytes (25). This receptor is known to play an important role in both proliferation and maturation of monocytes (22). Furthermore, colony-stimulating factor 1 stimulation of bone marrow–derived monocytic cells induces the expression of c-*fgr* mRNA (34), a finding consistent with the presence of c-*fgr* mRNA in resting peripheral blood monocytes (19). Circulating monocytes also express *hck* mRNA, and even higher expression of *hck* is achieved upon activation of these cells (35). The c-*src*–specified kinase accumulates in HL60 cells during differentiation into monocytic or granulocytic cells (1, 13). All of these findings suggest that regulation of tyrosine phosphorylation is of critical importance for the development of myelomonocytic cells.

Although we have no direct evidence for an *fgr* function, our present studies in combination with earlier findings delimit the biologic framework in which *fgr* must normally act. Within the myelomonocytic lineage, c-*fgr* may play a role in signaling the cessation of cell growth in preparation for maturation or may be involved in some aspect of the maturation process itself. Alternatively, in light of its peripheral membrane location, p55$^{c\text{-}fgr}$ may function in mature cells in response to extracellular signals. In this regard, it may be useful to consider response functions—such as chemotaxis, phagocytosis, and respiratory burst reactions—that are shared by mature monocytes, macrophages, and neutrophils. In any case, it is now possible to focus upon myelomonocytic cells in the later stages of their development in search of a physiologic role for the *fgr* protooncogene.

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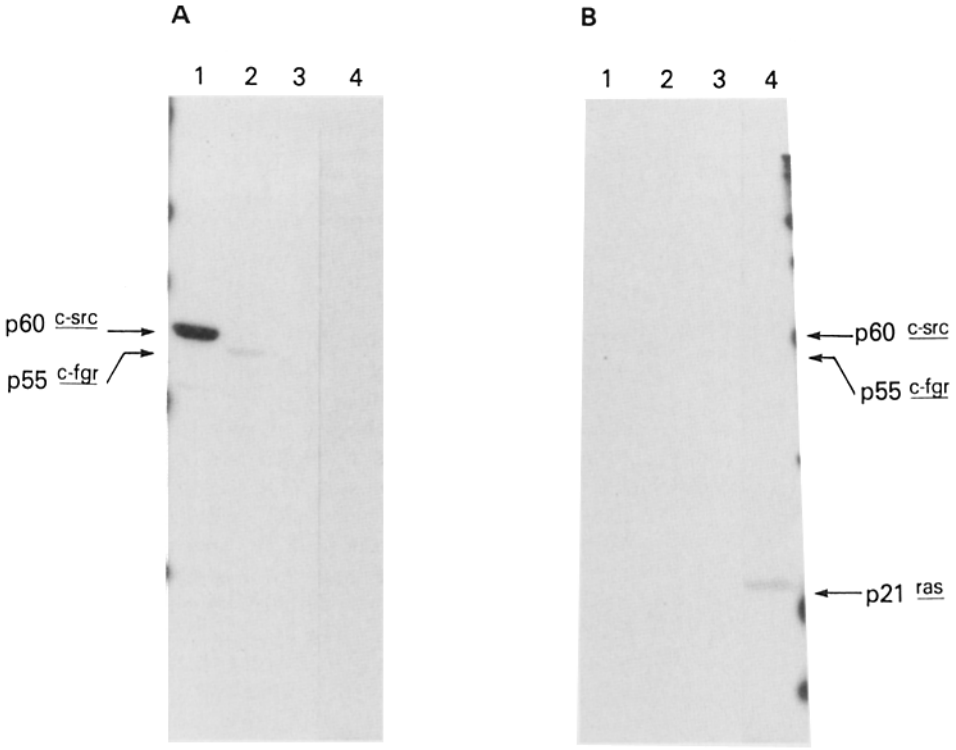

Figure 7. Metabolic labeling of $p55^{c\text{-}fgr}$ with ^3H-fatty acids. Cultures were metabolically labeled with [^3H]myristic acid (*A*) or [^3H]palmitic acid (*B*) as described in Materials and Methods. Lysates, prepared from HL60 cells induced for 48 h with retinoic acid (lanes *2* and *3*) or from NIH 3T3 cells expressing $p60^{c\text{-}src}$ (lanes *1*) or $p21^{ras}$ (lanes *4*), were immunoprecipitated with mAb 327 (lanes *1*), anti-*fgr* C (lanes *2* and *3*), or anti-p21 sera (lanes *4*). In some cases, anti-*fgr* C was preincubated with *fgr* C peptide (lanes *3*). Immune complexes were fractionated by SDS-PAGE and visualized by autoradiography. Locations of c-*src*, c-*fgr*, and c-Ha-*ras* gene products are indicated.

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