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, \( p55^{c-fgr} \) 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 \( p55^{c-fgr} \) in a lineage that represents its natural site of expression.

Naturally 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-\( fgr \)) (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 to 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 \( p55^{c-fgr} \) in maturing cells and have observed its accumulation during the HL60 differentiation process. Our findings establish developmental regulation of \( p55^{c-fgr} \) in a lineage that represents its natural site of expression.

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 \( 2.5 \times 10^5 \) cells/ml, and inducers were added to the medium at the following concentrations: 10\(^{-5} \) 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\(^{c-src} \) (27) or p21\(^{mu} \) (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
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\(\alpha\) 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\(\alpha\) as the primary translational product of the c-fgr gene (17). These same antibodies were used in immunoblotting assays to attempt detection of p55\(\alpha\) 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\(\alpha\) was further demonstrated by lack of its detection with either antibody in the presence of homologous peptide (Fig. 2). In addition to p55\(\alpha\), 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\(\alpha\) 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\(\alpha\) accumulated at a steady state in response to retinoic acid treatment, reaching the highest levels at 3 d of induction. Thus, p55\(\alpha\) expression parallels that of its mRNA in retinoic acid–treated HL60 cells.

Induction of p55\(\alpha\) by a Variety of Differentiating Agents

To investigate the ability of monocytic- as well as myelocytic-inducing agents to affect p55\(\alpha\) expression, HL60 cells were treated with DMSO, which induces myelocytic differentiation, or the phorbol ester, TPA, which induces mono-
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 p55c-fgr expression by immunoblotting. As shown in Fig. 3, levels of p55c-fgr increased during differentiation induced by each of these agents. However, maximum induction by DMSO or TPA was ~40% of the p55c-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.
Detection of p55-c-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 \times 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-fgr is indicated.

Figure 2. Expression of p55-c-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-fgr is indicated.

p55-c-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-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-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-fgr, only phosphotyrosine was detected, establishing p55-c-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-fgr accumulates as an active protein tyrosine kinase during the maturation of HL60 cells.

Localization of p55-c-fgr in Induced HL60 Cells

In an effort to define possible cellular locations where the tyrosine kinase activity of p55-c-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-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-fgr was membrane associated.

To confirm the localization of p55-c-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-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-fgr and enolase are indicated.

Modification of p55-c-fgr by Posttranslational Addition of Fatty Acid

The posttranslational addition of a myristyl group to cytoplasmic p60-c-fgr 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-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-fgr was detected in lysates of myristic acid–labeled cells but was barely observed in cells exposed to [3H]palmitic acid (Fig. 7). Control experiments showed, as expected, that p60-c-fgr (3) was more readily detectable in lysates of myristic acid–labeled cells, whereas the intensity of the p21 (26) band was greater when cells were labeled with palmitic acid. These findings demonstrate that p55-c-fgr is modified by the posttranslational addition of fatty acid, most likely a myristyl group, and suggest that p55-c-fgr associates with membrane components by virtue of this modification.

Discussion

The present study has examined expression of the fgr protooncogene product, p55-c-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-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-fgr in HL60 cells probably reflects the relatively advanced stage of U937 cell differentiation. Furthermore, the time course of p55-c-fgr induction in HL60 cells in response to TPA is nearly identical to that previously described for p60-c-fgr (13). Abundant c-fgr mRNA is present in fully mature human (19) and murine (34) monocytes, and p55-c-fgr is expressed at high levels in normal granulocytes purified from human blood (13a). Taken together, the evi-
The extent of p55c-af 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 p55c-af in HL60 cells exposed to TPA (19), p55c-af clearly accumulated during differentiation by TPA. Similar low levels of p55c-af were also observed in DMSO-treated cells, suggesting no relationship between the extent of p55c-af 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 p55c-af 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 delineate 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, p55c-af 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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References

Figure 7. Metabolic labeling of p55--src 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-src (lanes 1 or p21-ras (lanes 4), were immunoprecipitated with mAb 327 (lanes 1), anti-src C (lanes 2 and 3), or anti-p21 sera (lanes 4). In some cases, anti-src C was preincubated with src 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.

Notario et al. Developmental Regulation of p55-src Expression

3135


