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EXPRESSION OF *src* FAMILY GENES DURING MONOCYTIC DIFFERENTIATION OF HL-60 CELLS

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It has been reported that *src* family protein-tyrosine kinases were expressed specifically in a certain lineage or differentiation stage of hematopoietic cells. To understand the molecular basis for differentiation and function of monocyte/macrophage, we investigated the expressions of *src* family genes by the HL-60 cells stimulated with differentiation-inducing agents. TPA and vitamin D_3 (D_3) were used as stimulants for monocytic development, since each agent has been known to induce phenotypically specific differentiation of HL-60 cells. The *fyn*, *fgr*, and *lyn* genes were characteristically expressed concomitantly with phenotypic changes and expressions of nuclear proto-oncogenes, whereas *src*, *lck*, *hck*, and *yes* genes were not. In TPA-induced differentiation of HL-60 cells, both *fyn* and *lyn* genes, but not *fgr* gene, were expressed. In contrast, both *fgr* and *lyn* genes, but not *fyn* gene, were expressed in D_3-induced differentiation of the cells. The independent and characteristic expressions of these genes were observed in the further advanced differentiation of HL-60 cells induced by TPA plus D_3 or D_3 plus human transforming growth factor-β1. The granulocytic differentiation of the cells treated with retinoic acid was accompanied by intense expression of *fgr*, but weak or no expression of *lyn* and *fyn* gene. These data indicate that each protein-tyrosine kinase encoded by *src* family genes may play distinct roles in development and/or functions of monocyte/macrophage-lineage cells.

Macrophages participate in the host defense mechanisms by affecting various aspects of immune responses. To name a few, they are essential for presenting Ag to immunocompetent cells, recognizing and killing microbes and malignant cells, and secreting numerous biologically active substances (1). On the other hand, they play a significant role in a variety of pathogenic processes including inflammation, tissue degeneration such as atherogenesis, and even carcinogenesis (2). However, it has been known that macrophages at possibly different stages of maturation showed different functional capabilities. For example, it was reported that peripheral monocytes and tissue macrophages not only differentially responded to extracellular signals, sometimes inducing completely opposite (enhancing or suppressive) effects on biologic responses such as immune reactions (3–5). The molecular basis for the differentiation and function of these cells largely remains to be elucidated.

The promyelocyte-like leukemia cell line HL-60 can be induced by various agents to differentiate in vitro mainly into two cell types of the myeloid lineage: 1) monocyte and/or macrophage-like cells, or 2) granulocytes (6). TPA[2] and D_3 are typical inducers for monocytic differentiation of HL-60 cells. The TPA-induced phenotype partly shares the D_3-induced phenotype, whereas several phenotypic characteristics clearly distinguish the TPA-treated cell from the D_3-treated one (6). TPA-treated HL-60 cells markedly adhere to each other, whereas D_3-treated ones do not (7, 8). On the other hand, chemotactic peptide receptors, nitroblue tetrazolium-reducing ability, and antibody-dependent cytotoxicity activity are all readily demonstrated in D_3-treated HL-60 cells, whereas these characteristics are not induced in the TPA-treated cells (9, 10). It was previously demonstrated that there were differences in the expression of surface functional receptors between TPA-induced and D_3-induced HL-60 cells (11, 12). These previous findings indicate that the HL-60 cells stimulated with various agents may be a good experimental model for studying a molecular basis for expressions of distinct functions during monocytic differentiation.

The *src* gene family includes eight well-characterized elements: *fyn* (13), *fgr* (14), *hck* (15), *lck* (16), *lyn* (17), *src* (18), *yes* (19), and *blk* (20). All encode membrane-associated PTK, which do not possess a membrane-spanning domain, and bind to the cellular plasma membrane exposed to the cytosol. The 60 to 90 residues (unique region) of an NH_2-terminal domain are quite divergent and are believed to contain the recognition sequences for interaction with regulatory proteins or extracellular receptors, and determine the substrate specificity, intracellular location, and specific functions of the encoded proteins (21). The *src* family genes seem to be regulated independently from each other and are expressed in distinct cell lineages at specific developmental stages (22–28). The biologic function and regulation of any member

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[2] Abbreviations used in this paper: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; 1α, 25-dihydroxyvitamin D_3; PTK, protein-tyrosine kinases; RA, retinoic acid; TGFβ, human transforming growth factor-β1; RIPA buffer, 0.15 M NaCl/10 mM Tris-HCl, pH 7.4/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/2 mM EDTA/14 mM 2-ME/20 μg/ml aprotinin/50 mM NaF/0.2 mM Na_3VO_4.

of the *src* family in various cell types including macrophages have been elusive.

A previous report suggested that the *fgr* and *hck* were regulated differentially during macrophage activation by LPS, and might subserve distinct functions (29). In this study, to assess the role of *src* family PTK in monocyte/macrophage differentiation, we investigated the expressions of *src* family genes (*lck, fyn, lyn, yes, src, fgr, hck*) during the differentiation of HL-60 cells induced by TPA, D_3 or RA. The characteristic expression of *fyn, fgr,* and *lyn* were at certain differentiation stages of different cell lineages after the treatments with these reagents. Our results indicate that these closely related gene products participate in functionally distinct signaling pathways in monocytes and/or macrophages.

MATERIALS AND METHODS

Cells. HL-60 cells were maintained in RPMI 1640 medium containing 10% FCS. For differentiation experiments, exponentially growing cells were subcultured at a density of 2×10^5 cells/ml, and inducers were added to the medium at the following concentrations: 10 ng/ml for TPA (Sigma Chemical Co., St. Louis, MO), 10^{-7} M for D_3 (Chugai Pharmaceutical Co.), 3 ng/ml for RA (Sigma), and 1 ng/ml for TGFβ (R&D Systems, Inc. Minneapolis, MN). After incubation of HL-60 cells with these inducers, the cells were washed twice with PBS before use.

Assay for monocyte/macrophage differentiation markers. FITC-conjugated mAb directed against an epitope on the C3bi complement component (CR3) was purchased from Becton Dickinson (Oxnard, CA). Mouse anti-human ICAM-1 antibody was obtained from the culture supernatant of a hybridoma line that was previously established in our laboratory (M. Yamanoha, Y. Koyama, and T. Yoshida, manuscript in preparation). Cells incubated with anti-ICAM-1 antibody were treated with fluoresceneited goat F(ab')2 anti-mouse IgG (Cappel Laboratories, Cochranville, PA). The cells were analyzed on a FACS440 (Becton Dickinson). Anti-CR3 (Cosmo Biological Co.,) and anti-ICAM-1 antibodies were also used at the concentration of 1 μg/ml to inhibit the adhesion of the TPA-treated HL-60 cells.

RNA preparation. Total cellular RNA from the cultured cells was prepared by guanidine isothiocyanate-CsCl gradient centrifugation. The washed cells were resuspended in solution A (4 M guanidine isothiocyanate/25 mM sodium citrate/0.1 M 2-ME/0.5% sarcosyl, pH 7.0), and layered onto solution B (5.7 M CsCl/25 mM sodium acetate, pH 5.0). RNA was collected after centrifugation at 36,000 rpm for 16 h, dissolved in diethylpyrocarbonate-treated water, and precipitated with 3 M sodium acetate and 95% ethanol. The amount of RNA was determined spectrophotometrically, and the concentration was adjusted to 1 mg/ml and stored at −80°C.

DNA probes. The human *c-myc* probe was provided by Japanese Cancer Research Resource Bank, and the 2.2-kb *Eco*RI fragment of pSPT18 was used as a *c-myc* specific probe. Human c-*myb* cDNA was kindly provided by Dr. Hiroyoshi Ariga (Hokkaido University) and the 0.6-kb *Eco*RI fragment of pUC18 was used as a c-*myb*-specific probe. The 0.6-kb *Bgl*II-*Hin*cII fragment of SN-2 (13) was used as a *fyn*-specific probe. The 1.7-kb *Eco*RI fragment of NT18 (16), kindly donated by Dr. Roger M. Perlmutter (Howard Hughes Medical Institute, Seattle, WA), was used as a *lck*-specific probe. The *fgr* (14), *lyn* (17), *src* (18), *yes* (19), and *hck* (15) probes were prepared as described before. The quantity of mRNA loaded per gel lane was assessed by reprobing stripped blots with human β-actin cDNA, kindly provided by Dr. Kiyoshi Nose (Tokyo University).

Northern blot analysis. RNA samples (10 μg/lane) were denatured in 1 M deionized glyoxal/50% (v/v) DMSO/10 mM sodium phosphate buffer, pH 7.0, at 50°C for 1 h, and size-fractionated by electrophoresis in 1% agarose gels (0.1 M sodium phosphate buffer, pH 7.0). They were transferred to a nylon membrane filter for blotting and hybridized to ^{32}P-radiolabeled DNA probes as described previously (30). Hybridization with ^{32}P-labeled cDNA (10^6 cpm/ml) was performed at 42°C for 24 h in Denhardt's solution/5× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate)/40% (v/v) deionized formamide/100 μg/ml sonicated denatured salmon sperm DNA/10% (w/v) dextran sulfate. The filters were washed with 2× SSC-0.1% SDS to 0.1× SSC-0.1% SDS at 42°C.

Immunoblot analysis of the fyn product. fyn-specific rabbit antiserum was generated against a synthetic peptide corresponding to amino acid residues 25 to 141 of the human *fyn* protein sequence, which was expressed in *Escherichia coli* using Pho-S as a fusion protein. We prepared the positive control cells described previously (28). Ten million of the TPA-induced or uninduced HL-60 cells were solublized in 1 ml of RIPA buffer. Each 5 μl of lysates were subjected to electrophoresis on 8.5% polyacrylamide/SDS gels. Protein blotting, binding of anti-*fyn* antibody and of peroxidase-conjugated anti-rabbit IgG antibody, and the color reaction were performed as described previously (28).

Immune-complex kinase assay. Cells (1×10^7) were solublized in 1 ml of RIPA buffer at 4°C, and the clarified supernatant was immunoprecipitated with anti-*fyn* antiserum. Immunoprecipitates were then incubated with 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 giga-Bq), boiled for 5 min, and subjected to electrophoresis on 8.5% polyacrylamide/SDS gels. The gel was treated with 1 M KOH at 55°C, dried, and exposed to Kodak X-AR film.

Immunofluorescent staining of the fyn product. Cells were collected by centrifugation onto 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 then washed and incubated for 30 min with anti-*fyn* serum. After incubation with rhodamine-conjugated goat F(ab')$_2$ anti-rabbit IgG (Tago Inc., Burlingame, CA) for 30 min, staining was visualized by fluorescent microscopy.

RESULTS

Phenotypic characteristics of HL-60 cells treated with TPA or D_3. Figure 1*A* shows the morphology of HL-60 cells before and after the incubation with TPA (10 ng/ml) or D_3 (10^{-7} M). Morphologically, the cells looked like mature macrophages after the TPA treatment while they were like monocytes after the D_3 treatment. TPA-treated cells exhibited intense adherence to the plastic culture dish surface and markedly adhered to each other, whereas D_3-treated HL-60 cells were much less adherent (Fig. 1*A*, *b* and *c*).

Next, the expression of differentiation markers (or cell surface Ag) on TPA- and D_3-treated HL-60 cells was examined by flow cytometry (Fig. 1*B*). The CR3 Ag (the receptor for C3bi) was detected clearly on TPA- and D_3-treated HL-60 cells. On the other hand, the expression of a cell-adhesion molecule, ICAM-1, was induced more markedly by the TPA treatment than by the D_3 treatment (Fig. 1*B*). LeuM3 Ag was observed equally on both TPA- and D_3-treated HL-60 cells, whereas LeuM5 and LFA-1 Ag were expressed more on TPA-treated HL-60 cells than on D_3-treated HL-60 cells (data not shown).

Expressions of c-myc and c-myb mRNA in differentiation of HL-60. Previously, it was shown that differentiation of HL-60 cells was concomitant with a drastic reduction in the mRNA levels of protooncogenes, c-*myc* and c-*myb* (31, 32). Confirming this first in our experimental system, we have observed that the expressions of c-*myc* and c-*myb* mRNA decreased when HL-60 cells were treated with either TPA or D_3. Interestingly, the levels of c-*myc* and c-*myb* mRNA reduced more quickly and intensely in TPA-induced differentiation than in D_3-induced ones (Fig. 1*C*).

Expressions of fyn, fgr, and lyn mRNA in differentiation of HL-60. To determine if the *src* family gene (*fyn, fgr, lyn, src, yes, lck, hck*) expression is associated with the HL-60 cellular differentiation toward macrophage-like cells, these mRNA were assayed in the cells treated with TPA or D_3. Total RNA was prepared from the cells exposed to TPA or D_3 for 0, 3, 24, or 48 h. No *src* gene family was expressed in RNA prepared from HL-60 cells before the treatment. The *fyn, fgr,* and *lyn* mRNA remarkably increased during the cellular differentiation process induced by TPA or D_3 (Fig. 2). The *src, yes, hck,* and *lck* mRNA were not detected during these differentiations. The *fyn, fgr,* and *lyn* genes were apparently

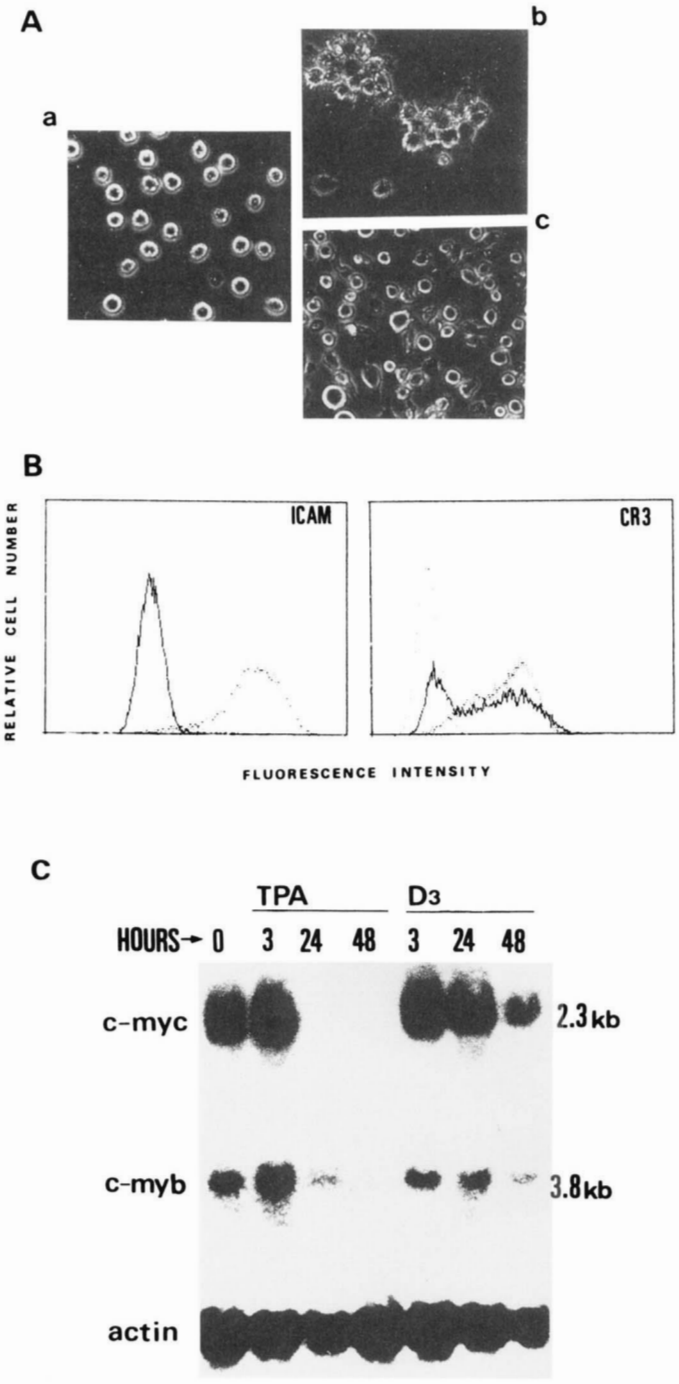


Figure 1. *A*, Morphologic changes of HL-60 cells untreated (*a*) and treated for 48 h with TPA (10 ng/ml) (*b*) or D_3 (10^{-7} M) (*c*). *B*, Immunofluorescence profiles of the untreated (·····), TPA-treated (·····) and D_3-treated (——) HL-60 cells, labeled with anti-ICAM-1 antibody or anti-CR3 antibody. *C*, Reduction of c-*myc* and c-*myb* mRNA levels in the treated HL-60 cells. RNA was harvested from HL-60 cells before (0 h) or 3, 24, or 48 h after treatment with TPA or D_3. Total cellular RNA was analyzed by Northern blot for c-*myc* and c-*myb* mRNA. A β-actin cDNA probe was used as a control for the amount of RNA loaded.

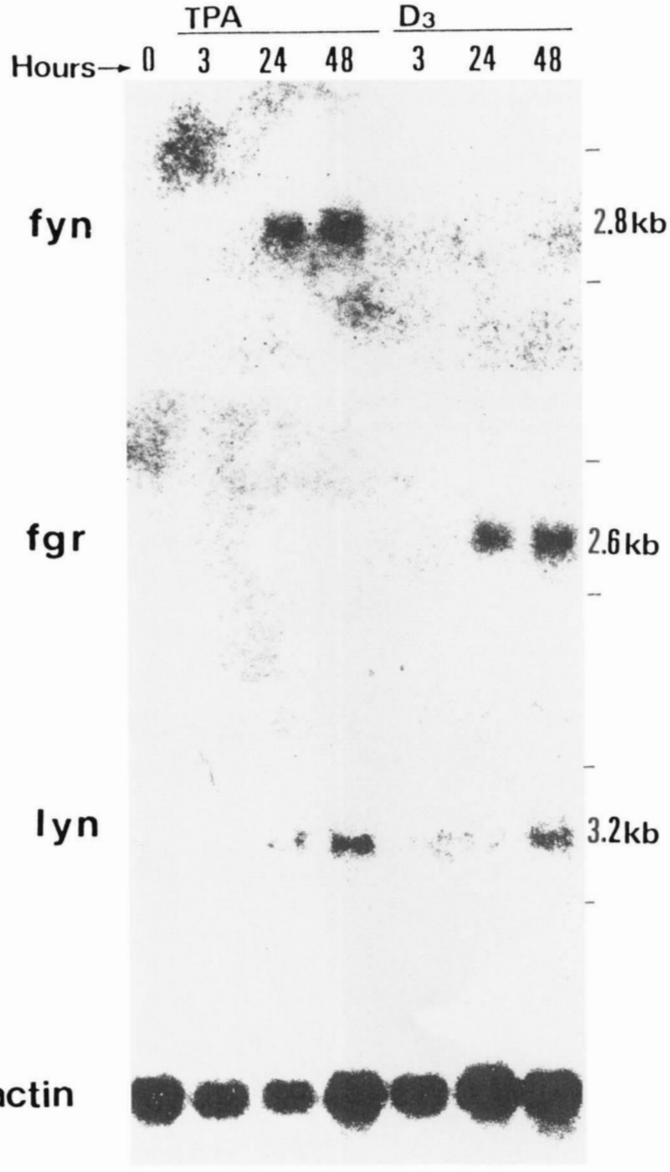
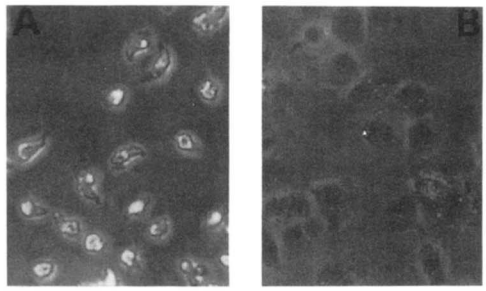


Figure 2. Accumulation of *fyn*, *fgr*, and *lyn* mRNA during differentiation of HL-60 cells. Total cellular RNA was harvested from HL-60 cells before (0 h) or 3, 24, 48 h after treatment with TPA or D_3, and analyzed by Northern blot for *fyn*, *fgr*, and *lyn* mRNA. The positions of ribosomal RNA markers are indicated to the right of the blots. A β-actin cDNA probe was used as a control for the amount of RNA loaded.

under separate regulatory mechanisms. Thus, the *fyn* transcripts (2.8 kb) were detected when the cells were treated by TPA, but not when treated by D_3. In contrast, the *fgr* transcripts (2.6 kb) were detected after the exposure to D_3, but not after the TPA exposure. The *lyn* transcripts (3.2 kb) were detected after either TPA or D_3 exposure. Maximum levels of these gene expressions were reached at 48 h after the exposure and remained essentially unchanged until 96 h (data not shown). To establish that the differences observed were not due to the variation of a total amount of RNA blotted, the same blots were re-hybridized with a probe for an irrelevant gene, β-actin. Almost an equal level of β-actin mRNA was detected in different RNA preparations used.

Changes in the expression of fyn, fgr, and lyn mRNA in further differentiated HL-60 cells. TGFβ has been shown to modify the differentiation of HL-60 cells induced by D_3 (33). Thus, D_3 and TGFβ had synergistic effects of augmenting morphologic changes, of inducing mature monocytic Ag (CR3, LeuM3), and of inducing functional properties such as phagocytosis and antibody-dependent cytotoxicity, although the cells treated with D_3 and TGFβ did not show either intense cellular adhesion or the adhesion to plastic surface (Fig. 3*A*). As reported previously (34), upon simultaneous exposure to TPA and D_3, HL-60 cells became flattened, spread out, and adherent firmly on plastic (Fig. 3*B*). Since these combined



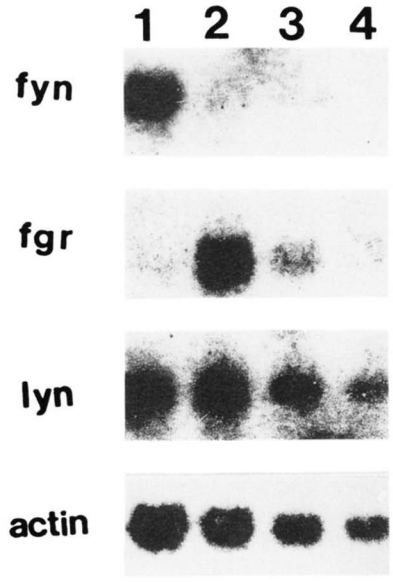
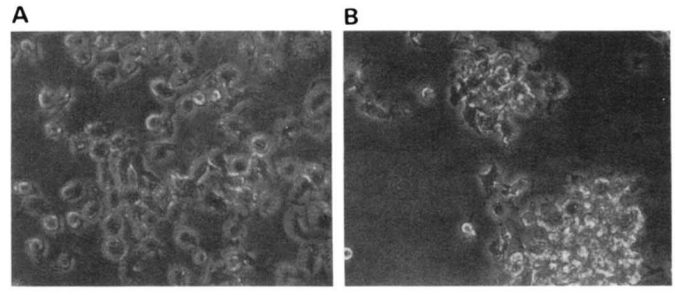


Figure 3. Morphologic changes, and expression of *fyn*, *fgr*, and *lyn* mRNA of HL-60 cells treated with D_3 (10^{-7} M) plus TGFβ (1 ng/ml) (*A*), or TPA (10 ng/ml) plus D_3 (10^{-7} M) (*B*). Total cellular RNA was harvested from HL-60 cells treated for 48 h with TPA alone (*lane 1*), D_3 and TGFβ (*lane 2*), D_3 alone (*lane 3*), and TPA and D_3 (*lane 4*), and analyzed by Northern blot for *fyn*, *fgr*, and *lyn* mRNA. A β-actin cDNA probe was used as a control for the amount of RNA loaded.



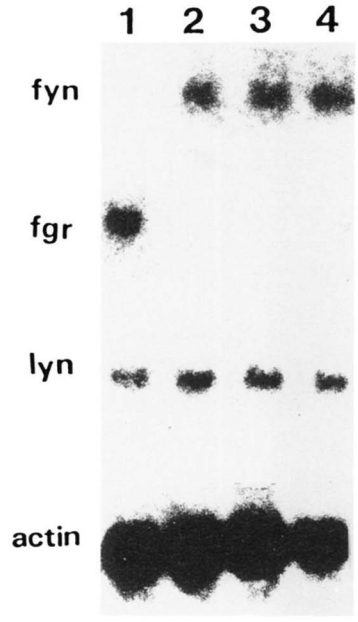


Figure 4. HL-60 cells were cultured with TPA in the presence of anti-ICAM-1 antibody (1 μg/ml) (*A*) or anti-CR3 antibody (1 μg/ml) (*B*). The cellular aggregation induced by TPA was inhibited by anti-ICAM-1 (*A*), but not by anti-CR3 (*B*). The expression of *fyn*, *fgr*, and *lyn* mRNA in these cells was examined. Total cellular RNA was harvested from HL-60 cells cultured for 48 h with D_3 alone (*lane 1*), TPA in the presence of anti-CR3 (*lane 2*), TPA in the presence of anti-ICAM-1 (*lane 3*), and TPA alone (*lane 4*), and analyzed by Northern blot for *fyn*, *fgr*, and *lyn* mRNA. A β-actin cDNA probe was used as a control for the amount of RNA loaded.

treatments of HL-60 cells seemed to push the cells to differentiate further than the single agent treatment (either D_3 or TPA alone), we examined the expression of the *fyn*, *fgr*, and *lyn* genes in the cells under such conditions. Interestingly, *fyn* mRNA was not detectable in the cells treated for 48 h either with D_3-TGFβ or with the TPA-D_3 combination (Fig. 3). On the other hand, *fgr* mRNA was increased in the cells treated with D_3-TGFβ, but not detectable in HL-60 cells treated with the TPA-D_3 combination. The level of *lyn* mRNA was essentially unchanged in the cells treated with either of these combinations. TGFβ alone did not induce any differentiation-associated phenotypic changes, nor any expressions of these mRNA in HL-60 cells.

The fyn mRNA in TPA-treated HL-60 cells is not induced secondarily by cellular adhesion. The results mentioned above seem to indicate that the *fyn* mRNA could be induced secondarily by cellular aggregation or adherence to plastic in the TPA-treated cells. To clarify this point further, HL-60 cells were treated with TPA in the presence of anti-ICAM-1 antibody or anti-CR3 antibody. As shown in Figure 4, *A* and *B*, anti-ICAM-1 antibody prevented cellular aggregation and anti-CR3 antibody inhibited the adhesion to a plastic surface. The levels of *fyn* mRNA expressed in the TPA-treated HL-60 cells were not affected by the inhibition of cellular aggregation and adhesion (Fig. 4). The levels of *lyn* mRNA were also unchanged even after the abrogation of cellular adhesion or aggregation (Fig. 4).

Accumulation and localization of p59^{fyn} during TPA-induced differentiation of HL-60 cells. Next, Western blot analysis was performed using anti-*fyn* antiserum to assess the expression of p59^{fyn} in the TPA-treated HL-60 cells. As shown in Figure 5*A*, the band corresponding to p59^{fyn} was detected in the lysate of the HL-60 cells treated with TPA for 48 h, but not in the untreated cell-lysate. The result indicates that p59^{fyn} is generated concomitantly with the expression of *fyn* mRNA induced in TPA-treated HL-60 cells. Furthermore, a concomitant increase in PTK activity of p59^{fyn} in TPA-treated HL-60 cells was demonstrated by immunoprecipitation followed by in vitro phosphorylation (Fig. 5*B*). We could also detect the induced p59^{fyn} in the cells using indirect immunofluorescence staining. As expected, the bright fluorescence was apparent around the cell periphery with diffuse staining of the cytoplasma (Fig. 5*C*).

Expression of fyn is not induced during granulocytic differentiation of HL-60 cells. To investigate *fyn*, *fgr*,

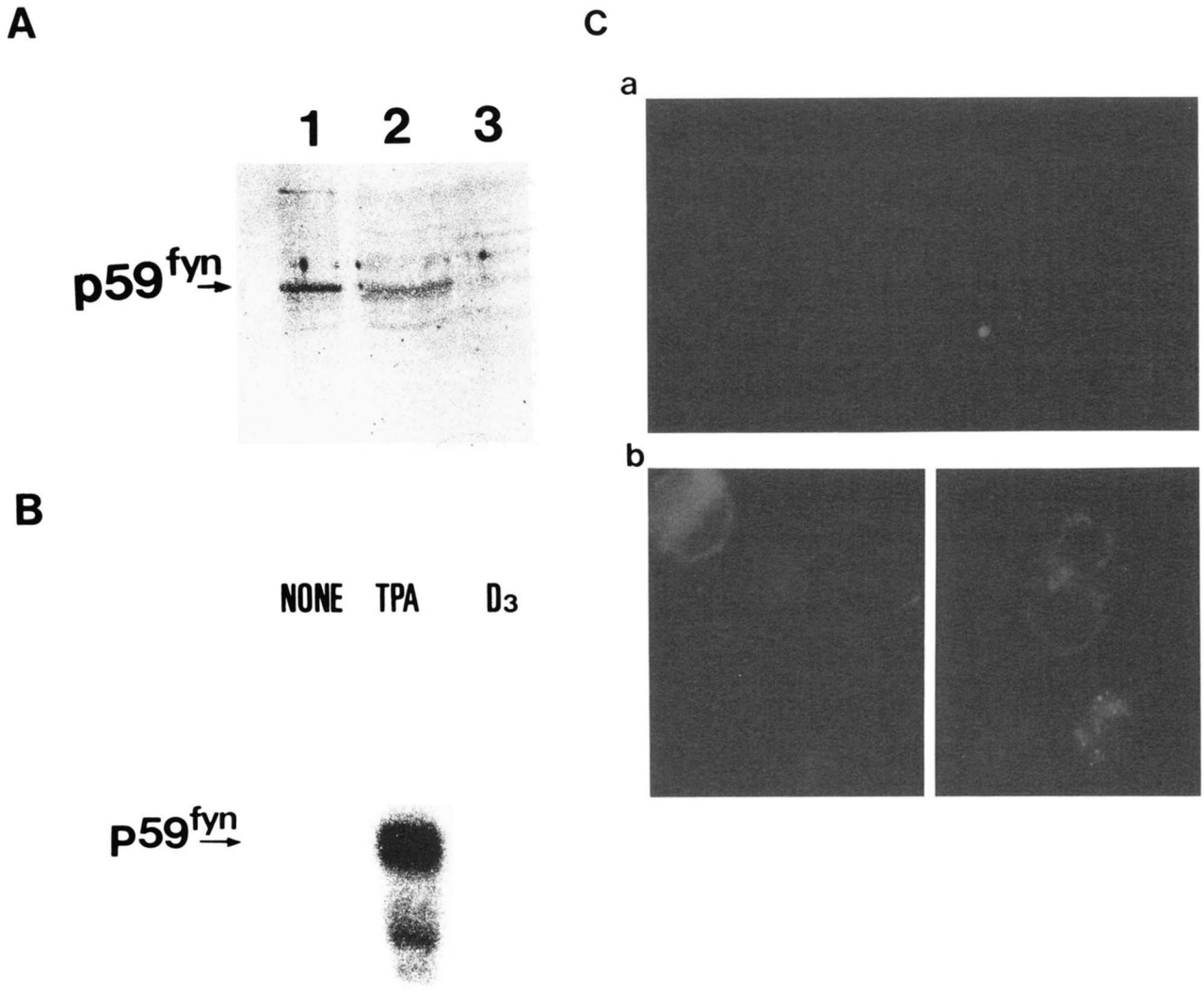


Figure 5. *A*, Immunoblot analysis of $p59^{fyn}$ expression during HL-60 differentiation. Untreated HL-60 cells (*lane 3*), the cells treated for 48 h with TPA (*lane 2*), and pSV-FN-transfected CV1 cells (*lane 1*) as a positive control, were solubilized and analyzed by Western blotting by using *fyn*-specific rabbit antiserum. *B*, Immunocomplex kinase assay of $p59^{fyn}$ from TPA-treated HL-60 cells. Lysates of untreated HL-60 cells (*NONE*), the cells treated for 48 h with TPA (*TPA*) or with D_3 (D_3) were immunoprecipitated with anti-$p59^{fyn}$ antiserum. *C*, Localization of $p59^{fyn}$ by indirect immunofluorescence. HL-60 cells (*a*) or the cells cultured with TPA for 48 h (*b*) were incubated with anti-*fyn* antiserum and stained with rhodamine-coupled goat anti-rabbit IgG.

and *lyn* mRNA expression in granulocytic differentiation, HL-60 cells were treated for 48 h with RA, which is known to induce the cellular differentiation to granulocytes. After the treatment, the *fgr* mRNA increased enormously, whereas the *fyn* mRNA was not detectable (Fig. 6). The *lyn* mRNA was also generated in RA-treated HL-60 cells, but the level was much less than that induced in the D_3-treated or TPA-treated HL-60 cells.

DISCUSSION

The expression of *src* family genes has been observed in some types of cells at a fully differentiated stage (22, 23, 35). Thus, the *src* family kinases have been implicated to be essential in various biologic processes including granule fusion, endocytosis, morphologic changes, and transmembrane molecular diffusion between communicating cells (36, 37). Interestingly, some of *src* family kinases were found expressed cell-lineage-specifically in the cells involved in immune system. Thus, the *lck* and *fyn* mRNA are expressed abundantly in T cells (28). The *lyn* is expressed in B cells and macrophages, but not in T cells (17). Recently, *fgr* and *hck* were characteristically found in mature macrophages and granulocytes (15, 29).

In the present study, we found the expressions of *fyn* and *lyn* at a relatively matured stage in macrophage-like differentiation of HL-60 cells. In contrast to *fgr*, these genes were barely induced in granulocytic differentiation of the cells. Interestingly, the expression of *fyn* and *fgr* in HL-60 cells differentiated with various inducers was mutually exclusive, as were *hck* and *fgr* genes induced in activated macrophages as summarized in Table I. These observations, in addition to the fact that the *src* family genes possess divergent sequences in its N-terminal region (unique domain), indicate that each of the protein-tyrosine kinases encoded by *src* family genes may play a specific and individual role in signal transduction pathways for activation, differentiation, growth, or other functions of the cells involved in the immune system.

In this respect, it is of a great interest that the increased accumulation of *fyn* mRNA and $p59^{fyn}$ is a characteristic feature in HL-60 cells treated with TPA. These cells exhibited strong cellular aggregation and adherence to a

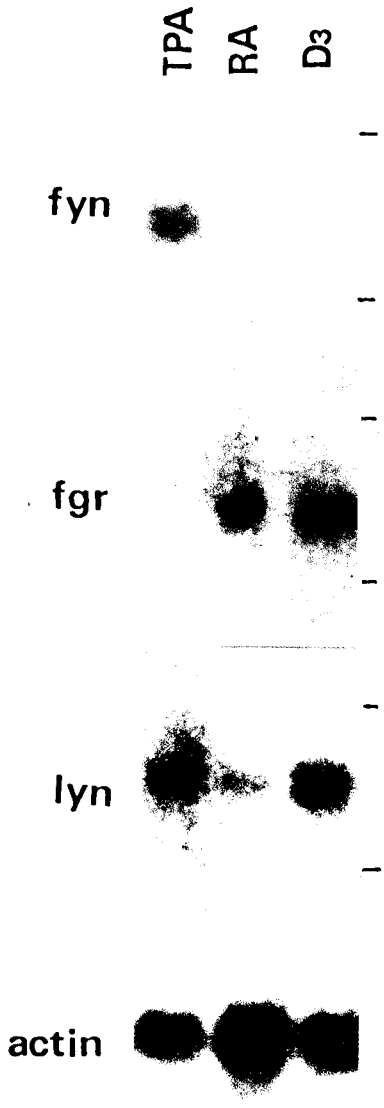


Figure 6. Expression of *fyn*, *fgr*, and *lyn* mRNA in the HL-60 cells treated by RA. Total cellular RNA was harvested from HL-60 cells treated for 48 h with TPA, RA, and D_3, and analyzed by Northern blot for *fyn*, *fgr*, and *lyn* mRNA. A β-actin cDNA probe was used as a control for the amount of RNA loaded.

TABLE I

Summary: Relationship between cellular adhesion and expression of fyn, fgr, and lyn genes during IIL-60 cell differentiation by various inducers

Inducers	Adherence[a] to Plastic	Homotypic[a] Aggregation	Expressions[a] of		
			fyn	*fgr*	*lyn* mRNA
None	−	−	−	−	−
TPA	+	+	+	−	+
D_3	−	−	−	+	+
D_3-TGFβ	±	−	−	++	+
D_3-TPA	++	−	−	−	+
RA	−	−	−	+	±

[a] The extent of the expression is arbitrarily determined as follows: −, not detectable; ±, weakly positive; +, positive; ++, strongly positive.

plastic surface, whereas the cells treated with D_3 alone or a D_3-TGFβ combination did not show such characteristics. On the other hand, the cells treated with a TPA-D_3 combination, in which *fyn* mRNA was not detectable, firmly adhered to the plastic dish surface, but exhibited no cellular aggregation. With these results taken together, we speculate that the cellular aggregation of HL-60 may represent one of specific features associated with the increased expression of *fyn*. A previous report (38) and our present data demonstrate that ICAM-1/LFA-1, which has been suggested to play important roles in a wide variety of adhesion-dependent immune functions (38, 39), directly correlate with the ability for cellular aggregation acquired by the TPA-treated HL-60 cells. In view of the recent finding that the $p56^{lck}$ kinase associates with CD4/CD8 Ag at the cytoplasmic membrane of T cells and transmits a signal for activation by a ligand through these Ag (40–42), $p59^{fyn}$ kinase might be involved in the signal transmission through ICAM-1/LFA-1 Ag in macrophages. This hypothesis is now being studied in our laboratory.

In contrast to the behavior of *fyn*, the level of *fgr* mRNA dramatically increased when the cells were exposed to D_3, and the increment was further augmented by the addition of TGFβ. The increased accumulation of *fgr* mRNA was not observed when the cells were treated with TPA alone or TPA-D_3 combination, as summarized in Table I. The granulocytic differentiation of HL-60 cells treated with RA was also accompanied by the increased expression of *fgr*, which was consistent with the report by others (43). Abundant *fgr* mRNA has been shown to be present in fully mature human and murine monocytes, and human granulocytes (25, 26). Therefore, it may be plausible to consider that the expression of *fgr* is closely associated with basic cellular functions such as chemotaxis, phagocytosis, and respiratory burst reaction, which are certainly shared by matured myeloid lineage cells such as monocytes and granulocytes.

The *lyn* mRNA expression increased as HL-60 cells were incubated with either TPA or D_3. Interestingly, as also summarized in Table I, this gene expression was essentially unchanged when the cells were treated with D_3-TGFβ combination or TPA-D_3 combination. The level of *lyn* mRNA was extremely low in the HL-60 cells differentiated toward granulocytes by the treatment with RA. The murine thioglycolate-induced peritoneal macrophages were shown to express the *lyn* transcripts abundantly (17). It is conceivable, therefore, that a tyrosine kinase encoded by the *lyn* gene may be involved in a signal transduction specific for functions of monocyte/macrophage.

A previous report documented a fivefold increase in *hck* expression in HL-60 cells after treatment of TPA (44). We could not detect the increase of *hck* mRNA in differentiated HL-60 cells. Ziegler et al. (15) showed that activation of human macrophages with LPS and IFN-γ induced the abundant expression of *hck* gene. We guess that the abundant expression of *hck* gene may be associated with activation of fully differentiated macrophages.

In conclusion, the role of the *src* gene family in the monocytic differentiation of HL-60 cells was examined systemically. *Fyn*, *lyn*, and *fgr* were all found to be involved and to play distinct roles in the differentiation processes. Importantly, this may be the first report that *fyn* gene is closely and specifically associated with a macrophage maturation process. A possible function of the newly identified $p59^{fyn}$ kinase is now under investigation. A further study is also warranted on the roles of *src* family kinases in the expression of diversified immunologic functions by macrophages.

REFERENCES

1. **Adams, D. O., and T. A. Hamilton.** 1984. The cell biology of macrophage activation. *Ann. Rev. Immunol. 2:283.*
2. **Tracey, E. D.** 1983. Macrophage-mediated injury. In *The Reticuloendothelial System in Immunopathology 4.* N. R. Rose and B. V. Siegel, eds. Plenum Publishing Corp., New York, pp. 77–96.
3. **Lee, S. H., and L. B. Epstein.** 1980. Reversible inhibition by interferon of the maturation of human peripheral blood monocytes to macrophages. *Cell. Immunol. 50:177.*
4. **Lee, K. C., J. Kay, and M. Wong.** 1979. Separation of functionally distinct subpopulations with different activities in promoting immune responses and suppressing tumor cell growth. *J. Immunol. 118:1530.*
5. **Lee, K. C., J. Kay, and M. Wong.** 1979. Separation of functionally distinct subpopulations of *Corynebacterium parvum*-activated macrophages with predominantly stimulatory or suppressive effect on the cell-mediated cytotoxic T cell response. *Cell. Immunol. 42:28.*
6. **Collins, S. J.** 1987. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood 70:1233.*
7. **Rovera, G., D. Santoli, and C. Damsky.** 1979. Human promyelocytic leukemia cell in culture differentiation into macrophage-like cells when treated with phorbol diester. *Proc. Natl. Acad. Sci. USA 76:2779.*
8. **Rovera, G., T. O'Brien, and L. Diamond.** 1979. Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. *Science (Washington DC) 204:818.*
9. **Newburger, P., R. Baker, S. Hansen, R. Duncan, and J. Greenberger.** 1981. Functionally deficient differentiation of HL-60 promyelocytic leukemia cells induced by phorbol myristate acetate. *Cancer Res. 41:1861.*
10. **Harris, P., P. Ralph, J. Gabrilove, K. Welte, R. Karmali, and M. Moore.** 1985. Distinct differentiation-inducing activities of gamma interferon and cytokine factors acting on the human promyelocytic leukemia cell line HL-60. *Cancer Res. 45:3090.*
11. **Graziano, R., E. Ball, and M. Fanger.** 1983. The expression and modulation of human myeloid-specific antigens during differentiation of the HL-60 cell line. *Blood 61:1215.*
12. **Tanaka, H., E. Abe, C. Miyaura, Y. Shiina, and T. Suda.** 1983. 1 Alpha dihydroxyvitamin D_3 induces differentiation of human promyelocytic leukemia cells (HL-60) into monocyte-macrophages but not into granulocytes. *Biochem. Biophys. Res. Commun. 117:86.*
13. **Semba, K., M. Nishizawa, N. Miyajima, M. Yoshida, J. Sukegawa, Y. Yamanashi, M., Sasaki, T. Yamamoto, and K. Toyoshima.** 1986. *Yes*-related protooncogene, *syn*, belongs to the protein-tyrosine kinase family. *Proc. Natl. Acad. Sci. USA 83:5459.*
14. **Nishizawa, M., K. Semba, M. Yoshida, T. Yamamoto, M. Sasaki, and K. Toyoshima.** 1986. Structure, expression and chromosomal location of the human c-*fgr* gene. *Mol. Cell. Biol. 6:511.*
15. **Ziegler, S. F., J. D. Marth, D. B. Lewis, and R. M. Perlmutter.** 1987. Novel protein-tyrosine kinase (*hck*) expression in cells of hematopoietic origin. *Mol. Cell. Biol. 7:2276.*
16. **Marth, J. D., R. Peet, E. G. Krebs, and R. M. Perlmutter.** 1985. A lymphocyte-specific protein tyrosine kinase gene is rearranged and overexpressed in the murine T-cell lymphoma LSTRA. *Cell 43:393.*
17. **Yamanashi, Y., S. Fukushige, K. Semba, N. Miyajima, K. Matsubara, T. Yamamoto, and K. Toyoshima.** 1987. The *yes*-related cellular gene *lyn* encodes a possible tyrosine kinase similar to $p56^{lck}$. *Mol. Cell. Biol. 7:237.*
18. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. *Cell 32:881.*
19. **Semba, K., Y. Yamanashi, M. Nishizuka, J. Sukegawa, M. Yoshida, M. Sasaki, T. Yamamoto, and K. Toyoshima.** 1985. Localization of the c-*yes* gene on the human chromosome and its expression in various tissues. *Science (Washington DC) 227:1038.*
20. **Dymechi, S. M., J. E. Niederhuber, S. V. Desiderio.** 1989. Specific expression of a tyrosine kinase gene, *blk* in B lymphoid cells. *Science (Washington DC) 247:332.*
21. **Hunter, T.** 1987. A thousand and one protein kinases. *Cell 50:823.*
22. **Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane.** 1985. Neurons express high levels of a structurally modified, activated form of $pp60^{c\text{-}src}$. *Nature (Lond) 316:554.*
23. **Golden, A., S. P. Nemeth, and J. S. Brugge.** 1986. Blood platelets express high levels of the $pp60^{c\text{-}src}$-specific tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA 83:852.*
24. **Gee, C. E., J. Griffin, L. Sastre, L. J. Miller, T. A. Springer, H. Piwnica-Worms, and T. M. Roberts.** 1986. Differentiation of myeloid cells is accompanied by increased levels of $pp60^{c\text{-}src}$ protein and kinase activity. *Proc. Natl. Acad. Sci. USA 83:5131.*
25. **Ley, T. J., N. L. Connally, S. Katamine, M. S. C. Cheah, R. M. Senior, and K. C. Robbins.** Tissue-specific expression and developmental regulation of the human *fgr* proto-oncogene. *Mol. Cell. Biol. 9:92.*
26. **Willman, C. L., C. C. Stewart, J. K. Griffith, S. T. Stewart, and T. B. Tomashi.** 1987. Differential expression and regulation of the c-*src* and c-*fgr* protooncogenes in myelomonocytic cells. *Proc. Natl. Acad. Sci. USA 84:4480.*
27. **Marth, J. D., D. B. Lewis, C. B. Wilson, M. E. Gearn, E. G. Krebs, and R. M. Perlmutter.** 1987. Regulation of $pp56^{lck}$ during T cell activation: functional implications for the *src*-like protein kinases. *EMBO J. 6:2727.*
28. **Katagiri, T., K. Urakawa, Y. Yamanashi, K. Semba, T. Takahashi, K. Toyoshima, T. Yamamoto, and K. Kano.** 1989. Overexpression of *src* family gene for tyrosine-kinase $p59^{fyn}$ in $CD4^-CD8^-$ T cells of mice with a lymphoproliferative disorder. *Proc. Natl. Acad. Sci. USA 86:10064.*
29. **Ziegler, S. F., C. B. Wilson, and R. M. Perlmutter.** 1988. Augmented expression of a myeloid-specific protein tyrosine kinase gene (*hck*) after macrophage activation. *J. Exp. Med. 168:1801.*
30. **Katagiri, K., T. Katagiri, S. Yokota, R. A. Eisenberg, P. L. Cohen, and J. K. Ting.** 1988. Two different molecular pathways account for low IL-2 receptor and c-*myc* mRNA expression by *lpr* $Lyt2^-L3T4^-$ T cells. *J. Immunol. 141:2145.*
31. **Reitsma, P., P. Rothberg, S. Astrin, J. Trial, Z. Bar-Shavit, S. Hall, S. Teitelbaum, and A. Kahn.** 1983. Regulation of c-*myc* gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature 306:492.*
32. **Gonda, T. T., and D. Metcalf.** 1984. Expression of *myb*, *myc*, and *fos* protooncogenes during the differentiation of a murine myeloid leukemia. *Nature (Lond.) 310:249.*
33. **Morikawa, M., N. Harada, G. Soma, and T. Yoshida.** 1990. Transforming growth factor β1 can differentiate leukemia cells into macrophage-like cells in the presence of 1α,25-dihydroxyvitamin D_3. *In Vitro Cell. Der. Biol. 26:682.*
34. **Cayre, Y., M. C. Raynal, Z. Darzynkiewicz, and M. H. Dorner.** 1987. Model for intermediate steps in monocytic differentiation: c-*myc*, c-*fms* and ferritin as markers. *Proc. Natl. Acad. Sci. USA 84:7619.*
35. **Sorge, L. K., B. T. Levy, and P. F. Maness.** 1984. $pp60^{c\text{-}src}$ is developmentally regulated in the neural retina. *Cell 36:249.*
36. **Hunter, T., and J. A. Cooper.** 1986. Protein tyrosine kinases. *Annu. Rev. Biochem. 54:897.*
37. **Azarnia, R., S. Reddy, T. E. Kmiecik, D. Shalloway, and W. R. Loewenstein.** 1987. The cellular *src* gene product regulates junctional cell-to-cell communication. *Science (Washington DC) 239:398.*
38. **Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer.** 1987. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol. 137:1270.*
39. **Rothlein, R., and T. A. Springer.** 1986. The requirement for lymphocyte function-associated antigen in homotypic leukocyte adhesion stimulated phorbol ester. *J. Exp. Med. 163:1132.*
40. **Shaw, A. S., K. E. Amerin, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose.** 1989. The ick tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. *Cell 59:627.*
41. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. P. Levin, R. M. Permutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrisine kinase p561ck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell 60:755.*
42. **Veillette, A., J. B. Bolen, and M. A. Bookman.** 1989. Alternations in tyrosine protein phosphorylation induced by antibody-mediated cross-linking of the CD4 receptor of T lymphocytes. *Mol. Cell. Biol. 9:4441.*
43. **Notario, Y., J. S. Gutkind, M. Imaizumi, S. Katamine, and K. C. Robbins.** 1989. Expression of the *fgr* protooncogene product as a function of myeloidmonocytic cell maturation. *J. Cell Biol. 109:3129.*
44. **Quintrell, N., R. Lebo, H. Varmus, J. M. Bishop, M. J. Pettenati, M. M. Le Beau, M. O. Biaz, and J. D. Rowley.** 1987. Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells. *Mol. Cell. Biol. 7:2267.*