Effect of the Mutation of Tyrosine 713 in p93$^{c\text{-}fes}$ on Its Catalytic Activity and Ability To Promote Myeloid Differentiation in K562 Cells†

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ABSTRACT: The protooncogene protein-tyrosine kinase c-*fes* plays an active role in the induction of terminal myeloid differentiation in myeloid leukemia cells. Although p93$^{c\text{-}fes}$ contains two autophosphorylation sites, it is not known what role they play in its catalytic or biological activities. To address this question, the major autophosphorylation site at tyrosine 713 was mutated to phenylalanine (YF713), and the mutated cDNA was expressed in a baculovirus system to assess catalytic activity, as well as in an inducible retrovirus to determine its biological activity. The major phosphopeptide in p93$^{c\text{-}fes}$ *in vitro* contained Y713 and was absent in the YF713 mutant, which exhibited an 85% loss of autophosphorylation activity. The catalytic activity of p93$^{c\text{-}fes\text{YF713}}$ with either RCM-lysozyme or poly(Glu,Tyr)$_{4:1}$ as substrate was reduced by 85 and 78%, respectively, in comparison to p93$^{c\text{-}fes}$. Retroviral infection of K562 cells with the c-*fes* cDNA under the control of the mouse metallothionein promoter increased superoxide formation, phagocytosis, CD13 and CD33 antigen expression, and doubling time 4–6 days after induction. Cells infected with c-*fes*YF713 exhibited 40% less superoxide formation but similar levels of phagocytosis, CD13/CD33 antigen, and doubling time in comparison to cells infected with c-*fes*. The level of phosphotyrosine-containing proteins did not markedly differ between K562 cells expressing either *neo*, c-*fes*, or c-*fes*YF713, with the exception of a reduction in the level of a 210-kDa protein specifically in both c-*fes*-expressing cell lines. The p210 was tentatively identified as *bcr-abl*, whose level was also reduced in cells expressing c-*fes* or c-*fes*YF713. These results indicate that loss of the major autophosphorylation site at Y713 in p93$^{c\text{-}fes}$ attenuates some, but not all, properties of differentiated myeloid cells. In addition, the differentiated phenotype induced by c-*fes* is associated with a reduction in *bcr-abl*, which may be associated with induction of differentiation and suppression of oncogenesis.

The human c-*fes* protooncogene encodes a plasma membrane-associated cytoplasmic protein-tyrosine kinase (PTK[1]), p93$^{c\text{-}fes}$, that is expressed exclusively in hematopoietic cells of the granulocytic and monocytic lineages (Feldman *et al.*, 1985; Glazer *et al.*, 1991; MacDonald *et al.*, 1985; Smithgall *et al.*, 1988). Terminal differentiation of myeloid cells is associated with increased levels of c-*fes* mRNA (Liebermann & Hoffman-Liebermann, 1989) as well as increased levels and increased catalytic activity of p93$^{c\text{-}fes}$ (Glazer *et al.*, 1986, 1987; Yu *et al.*, 1988; Yu & Glazer, 1987; Chapekar *et al.*, 1986; Smithgall *et al.*, 1988). Moreover, transfection of the myeloblastic leukemia cell line K562 with the genomic c-*fes* sequence induces functional maturation of these cells to the granulocytic phenotype (Yu *et al.*, 1989). Conversely, an antisense oligodeoxynucleotide against the translation start site in the c-*fes* mRNA inhibited the ability of HL-60 cells to differentiate to the granulocytic phenotype by treatment with dimethyl sulfoxide (Ferrari *et al.*, 1990). These results suggest that p93$^{c\text{-}fes}$ may serve as an important effector for initiating differentiation and perhaps also serve in this capacity for physiological regulators of myelopoiesis such as the colony-stimulating factors (Nicola, 1989). In this regard, the differentiation of early myeloblast progenitor cells in response to GM-CSF coincides with the early expression of c-*fes* before the appearance of other PTKs such as c-*fgr* and c-*fms* (Liebermann & Hoffman-Liebermann, 1989). Thus, c-*fes* appears to play a pivotal role in the recruitment of early myeloblasts into the differentiation pathway.

p93$^{c\text{-}fes}$ shares a number of structural features with a larger subfamily of PTKs that is exemplified by p60$^{c\text{-}src}$. The primary region of homology is the catalytic domain, which is located in the C-terminal portion of both the *fes* and *src* PTKs. Within the catalytic domain, phosphopeptide analysis of autophosphorylated p93$^{c\text{-}fes}$ from either HL-60 cells (MacDonald *et al.*, 1985; Yu *et al.*, 1987) or insect cells infected with a recombinant baculovirus (Smithgall *et al.*, 1992) indicated two autophosphorylation sites. Y713 has been putatively designated as one of the autophosphorylation sites in p93$^{c\text{-}fes}$ by analogy with the viral homolog v-*fps* (Weinmaster *et al.*, 1984), but it has not been established experimentally. Similarly, the effect of autophosphorylation on the catalytic or biological activities of c-*fes* has not been investigated, but by analogy with p130$^{gag/fps}$, it would be expected to play a positive regulatory role (Meckling-Hansen *et al.*, 1987).

To begin to understand the complex regulation of this enzyme, we have constructed a recombinant baculovirus that expresses catalytically active p93$^{c\text{-}fes}$ in insect cells (Smithgall

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[1] Abbreviations: PTK, protein-tyrosine kinase; SH2, *src* homology 2; GAP, *ras* GTPase activator protein; HPLC, high-pressure liquid chromatography; PCR, polymerase chain reaction; RCM-lysozyme, reduced carboxyamidomethylated lysozyme; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GM-CSF, granulocyte/macrophage colony-stimulating factor; Y, tyrosine.

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et al., 1992) and is essentially identical to the native protein (Yu & Glazer, 1987). In this report, we describe the baculovirus expression of a recombinant form of p93$^{c\text{-}fes}$ mutated at its major autophosphorylation site at Y713, as well as inducible retroviral constructs containing the wild-type and mutated c-*fes* cDNA, to investigate the biological role of this gene in differentiation.

EXPERIMENTAL PROCEDURES

Materials. Construction of the c-*fes* cDNA was as described previously (Smithgall *et al.*, 1992). The baculovirus transfer vector pVL1392 and wild-type *Autographica californica* nuclear polyhedrosis virus (baculovirus) were provided by Dr. Max Summers of Texas A&M University (College Station, TX). *Spodoptera frugiperda* (Sf-9) insect cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). [γ-^{32}P]ATP was obtained from Du Pont-New England Nuclear (Boston, MA). Retroviral vector OT1529 was kindly provided by Dr. MaryLou McGeady Cutler of the National Cancer Institute. Oligonucleotide primers for PCR were synthesized by the Macromolecular Sequencing/Synthesis Core Facility, Lombardi Cancer Research Center, Georgetown University Medical Center. Other reagents for PCR were purchased from Perkin-Elmer Cetus (Norwalk, CT). Sequenase and other DNA sequencing reagents were purchased from United States Biochemical Corporation (Cleveland, OH). Poly(Glu,Tyr)$_{4:1}$ was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to phosphotyrosine and c-*abl* were purchased from Boehringer-Mannheim and Oncogene Science, respectively. RCM-lysozyme was a generous gift from Dr. Curt Heinrich of Life Technologies, Inc. (Gaithersburg, MD). The PTK inhibitor halenaquinone was generously provided by Dr. Phillip Crews of the University of California at Santa Cruz.

Construction of the c-fes cDNA Mutated at Y713. Mutation of Y713 to phenylalanine in the c-*fes* cDNA (c-*fes*YF713) was performed by site-directed mutagenesis using the Altered Sites *in vitro* Mutagenesis System (Promega). The mutagenic oligodeoxynucleotide was the sequence 5′-TGAGGCTGCAAAGACCCCATC-3′, which directs replacement of Y713 with phenylalanine. The presence of the mutation was confirmed by DNA sequencing using the Sequenase protocol (U.S. Biochemicals).

Preparation of Recombinant Baculovirus. The c-*fes* cDNA was cloned into the *Bgl*II and *Xba*I sites of the baculovirus transfer vector pVL1392 downstream from the viral polyhedrin promoter (Smithgall *et al.*, 1992). The recombinant baculovirus was selected and plaque-purified as described in detail elsewhere (Goswami & Glazer, 1991).

Partial Purification of Recombinant p93$^{c\text{-}fes}$ from Infected Sf-9 Cells. Sf-9 cells were grown to a density of 10^6 cells/mL in 100-mL spinner cultures and infected with 5 pfu/cell of recombinant baculovirus containing the c-*fes* cDNA. Cells were incubated for 72 h, washed free of serum, and lysed by sonication in 3.0 mL of extraction buffer (50 mM Tris (pH 7.5), 10 mM dithiothreitol, 2 mM EGTA, 5 μg/mL aprotinin, 10 μM pepstatin, 1 mM phenylmethanesulfonyl fluoride, 400 μg/mL soybean trypsin inhibitor, and 200 μg/mL leupeptin). Cellular debris was removed by centrifugation for 60 min at 100000*g*, and the supernatant was used for further purification. The cell extract was applied to a DEAE Mem-Sep 100 cartridge (Millipore) connected to a Pharmacia FPLC and was preequilibrated with buffer A (20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol). p93$^{c\text{-}fes}$ and p93$^{c\text{-}fes\text{YF}713}$ were eluted at a flow rate of 1 mL/min with a linear gradient of 0–1.0 M NaCl in buffer A over 30 min. Column fractions (1.0 mL) were assayed using poly(Glu,Tyr)$_{4:1}$ (see below), and active fractions were pooled, diluted in 5 vol of buffer A, and immediately applied to a Mono Q column (0.5 × 5 cm, Pharmacia). Elution was carried out in the same manner as that described for DEAE chromatography. Fractions were pooled, concentrated in a Centricon 30 microconcentrator (Millipore), and separated by SDS–PAGE in 8% gels and stained with Fast Stain (Zoion Research Inc.). Immunoblotting was conducted with monoclonal antibody 127-53F8 raised against a C-terminal c-*fes* peptide (Yu & Glazer, 1987), as described previously (Smithgall *et al.*, 1988).

Solution Assay for Tyrosine Kinase Activity. PTK activity was measured in an assay volume of 100 μL containing 20 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM ATP, 10 mg/mL poly(Glu,Tyr)$_{4:1}$ or RCM-lysozyme, and 1 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Reactions were initiated by the addition of protein, incubated at 30 °C for 10 min, and terminated by adding 500 μL of 10% trichloroacetic acid containing 2 mM sodium pyrophosphate (TCA/PP$_i$). Reaction mixtures were filtered on Whatman No. 3 paper filters and washed with TCA/PP$_i$ and ethanol. Filter-bound radioactivity was quantitated by liquid scintillation counting. Autophosphorylation was conducted under the same assay conditions, except that the exogenous substrate was omitted.

Phosphopeptide Analysis. p93$^{c\text{-}fes}$ and p93$^{c\text{-}fes\text{YF}713}$ were autophosphorylated and resolved by SDS–PAGE. The band corresponding to the 93-kDa protein was excised, cut into small pieces with a sterile scalpel, and incubated with 50 μg/mL of TPCK-treated trypsin in ammonium bicarbonate buffer (pH 8.0) (Yu & Glazer, 1987). Phosphopeptides were separated by reversed-phase HPLC using a Beckman C-18 column (0.46 × 25 cm) and a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid (pH 2.0) (Yu & Glazer 1987). Phosphopeptides were detected using an on-line Radiomatic β-detector.

Retrovirus Construction. The c-*fes* or c-*fes*YF713 cDNAs were amplified by PCR to produce *Eco*RI and *Xho*I restriction sites at the 5′- and 3′-termini, respectively. The forward primer was 5′-GGGAATTCAACAGCACTATGGGCTTC-3′, and the reverse primer was 5′-GGCTCGAGATTTATTGTTTCTGCCCGG-3′. PCR was carried out for 30 cycles, and each cycle consisted of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 3 min with a final extension cycle of 7 min. The cDNA constructs were digested with *Eco*RI and *Xho*I, gel-purified, and ligated into the *Eco*RI and *Xho*I site 3′ to the mouse metallothionein promoter in retroviral vector OT1529 containing the *neo* locus of resistance (McGeady *et al.*, 1990). Plasmids OT1529, OT1529/fes, and OT1529/YF713 were purified using a Qiagen Midiprep kit according to the manufacturer's directions.

Amphotropic retroviral stocks were produced by cocultivating GP + E86 ecotropic packaging cells and PA317 amphotropic packaging cells, the latter cell line containing the hygromycin locus of resistance (Muenchau *et al.*, 1990). PA317 and GP + E86 cells were each plated at 1 × 10^5 cells per 60-mm dish in Iscove's Minimal Essential Medium (Mediatech) supplemented with 10% fetal calf serum (Biofluids), 4 mM glutamine, 15 mM Hepes (pH 7.4), and 50 μg/mL gentamicin. After 24 h, the medium was replaced with 4 mL of fresh medium, and cells were transfected by calcium phosphate precipitation for 5 h (Eglitis & Anderson, 1988). Cells were then washed, fresh medium was added, and after 2 days 1 mL of virus-containing cell-free medium

was used to infect a fresh culture of cocultivated PA317 and GP+E86 cells. Infected PA317 cells were isolated by selection in medium containing 1.5 mg/mL G418 (Gibco/BRL) and 350 μg/mL hygromycin (Calbiochem). The medium from G418 and hygromycin-resistant colonies was used to determine the virus titer ((2–4) × 10^4 pfu/mL) in 3T3 cells (Cepko, 1992). Virus stocks were assayed for helper virus and found to be negative.

Retroviral Infection of K562 Cells. K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 40 mM Hepes (pH 7.4), and 50 μg/mL gentamicin. Cells were subcultured twice weekly and were maintained at a density of 10^5–10^6 cells/mL. When producing cells reached 50–75% confluence, the medium was replaced with fresh medium, the virus-containing medium was collected 24 h later, and 1.5 × 10^6 K562 cells were added to 6 mL of virus-containing medium and incubated at 37 °C for 6 h. Cells were then transferred to virus-free medium, and after 2 days, 2 mg/mL G418 was added. After 10 days, cells were cultured in complete RPMI 1640 medium with 0.2 mg/mL G418.

DNA Analysis. Genomic DNA extractions were carried out using standard methodology (Strauss, 1987). PCR was used to detect the c-*fes* and c-*fes*YF713 cDNAs. The PCR reaction mixture (50 μL) consisted of 1× *Taq* polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.1 mM each dNTP, 10 pmol of each primer described under Retrovirus Construction, 0.5 μg of genomic DNA, and 2.5 units of *Taq* polymerase. PCR was carried out for one cycle at 94 °C for 4 min, 55 °C for 30 s, and 72 °C for 3 min, followed by 28 cycles of 94 °C for 80 s, 55 °C for 30 s, and 72 °C for 3 min, and the last cycle was 94 °C for 80 s, 55 °C for 30 s, and 72 °C for 10 min.

Detection and Quantification of mRNA. Total cellular RNA was prepared according to the procedure described by Wilkinson (1988). Two micrograms of total RNA was heated to 65 °C for 10 min and incubated for 1 h at 37 °C in a reaction mixture containing, in a total volume of 20 μL, 50 mM Tris-HCl (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, 25 μg/mL bovine serum albumin, 0.1 mM each dNTP, 1 mM DTT, 10 pmol of exon 18 primer (5′-CCTTCTCCA-CAAACTCCCGTG-3′), 5 units of RNasin, and 100 units of MuMLV reverse transcriptase (Promega). Four microliters of this reaction mixture was heated to 100 °C for 3 min and added to a PCR reaction mixture containing 1× *Taq* polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 10 pmol of exon 17 primer (5′GGACCTGGCT-GCTCGGAACTG-3′), and 2.5 units of *Taq* polymerase in a total volume of 45 μL. Forty-nine microliters of PCR mixture containing equal amounts of RNA was added to each of 10 tubes containing a c-*fes* genomic DNA fragment ranging from 3 fg to 1.6 pg per tube. Forty cycles of PCR were performed, with each cycle consisting of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min. The c-*fes* cDNA fragment encoding exons 17 and 18 is 281 bp, whereas the genomic c-*fes* cDNA fragment encoding exon 17, intron 17, and exon 18 is 405 bp.

Immunoblotting of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$. Cell extracts were prepared in buffer containing 1% Triton X-100 as previously described (Yu *et al.*, 1989). Extracts were resolved by SDS–PAGE in an 8% minigel (Novex), and proteins were transferred to nitrocellulose paper as described previously (Smithgall *et al.*, 1988). Immunoreactive p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$ were visualized using monoclonal antibody 127-53F8 to a C-terminal p93$^{c\text{-}fes}$ peptide (Smithgall *et al.*, 1992) and the Protoblot detection system (Promega Biotec) as described by the manufacturer.

Immunoblotting of c-abl and Phosphotyrosine-Containing Proteins. Cell extracts were prepared from cells that were preincubated for 20 min at 37 °C in RPMI 1640 medium containing 2 mM H_2O_2 and 0.1 mM sodium vanadate (Bushkin *et al.*, 1991), and extracts were prepared in a buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM EGTA, 10 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, and 1 mM sodium vanadate. Samples were separated by SDS–PAGE in an 8% minigel, transferred to nitrocellulose, and developed using monoclonal antibodies against c-*abl* (Oncogene Science) or phosphotyrosine (Boehringer-Mannheim) with a chemiluminescence detection system (Tropix). Some experiments utilized monoclonal antiphosphotyrosine antibody PY20 (ICN), and similar results were obtained. Addition of 2 mM phospho-L-tyrosine to the blocking buffer before the addition of antiphosphotyrosine antibody completely eliminated the signal, whereas 2 mM phospho-L-serine had no effect.

Histochemical Assays. The ability of K562 cells to reduce NBT to formazan and phagocytize sheep erythrocytes was assessed by the methods described previously (Yu *et al.*, 1989). CD13 and CD33 were measured by fluorescence-activated cell sorting as also described previously (Yu *et al.*, 1991).

RESULTS

Baculovirus Expression of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$. The c-*fes* and c-*fes*YF713 cDNAs were cloned into baculovirus expression vector pVL1392 and used to produce recombinant baculoviruses for expressing the respective PTKs encoded by these genes in Sf-9 insect cells. The wild-type and mutated p93$^{c\text{-}fes}$ were purified by FPLC using DEAE and Mono Q chromatography and found to be 90% homogeneous by staining with Fast Stain (results not shown). The specific activity of p93$^{c\text{-}fesYF713}$ was 22 and 15% of the activity of p93$^{c\text{-}fes}$ with saturating concentrations of poly(Glu,Tyr)$_{4:1}$ and RCM-lysozyme as substrates, respectively (Figure 1). The K_m values for poly(Glu,Tyr)$_{4:1}$ for p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$ were 18 and 4 μM, respectively, and for RCM-lysozyme 43 and 15 μM, respectively.

Autophosphorylation of native p93$^{c\text{-}fes}$ involves two tyrosine residues *in vitro* (Yu & Glazer, 1987; Smithgall *et al.*, 1992). Tryptic phosphopeptide analysis of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$ by HPLC revealed the complete absence of the major autophosphorylation site associated with Y713 (Figure 2), and SDS–PAGE analysis confirmed that autophosphorylation was reduced by 85% in p93$^{c\text{-}fesYF713}$ in comparison to p93$^{c\text{-}fes}$ (Figure 3). Thus, mutation of Y713 reduced catalytic activity by 80–85%, depending on the substrate, and autophosphorylation by 85%.

To assess the biological functions of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$, retroviruses were constructed using the cocultivation procedure of Muenchau *et al.* (1990), which employed ecotropic and amphotropic packaging cells. The resulting retroviruses encoded the c-*fes* and c-*fes*YF713 cDNAs under the control of the heavy metal-inducible mouse metallothionein promoter and the resistance selection marker *neo* under the control of the viral long terminal repeat. K562 cells were batch-infected with the retroviruses expressing only the *neo* gene (K562/neo), the c-*fes* (K562/fes), or the c-*fes*YF713 (K562/YF713), and selection was carried out in G418. After 2 weeks in selection medium, genomic DNA was prepared and amplified with a pair of primers delineating exon 2 through exon 19 in the c-*fes* cDNA (Figure 4). Polyclonal cell lines K562/fes and K562/YF713 both contained the integrated cDNA sequence that was absent in control K562/neo cells.

The growth of K562 cells after retroviral infection was assessed in the absence and presence of $CdCl_2$ and $ZnSO_4$,

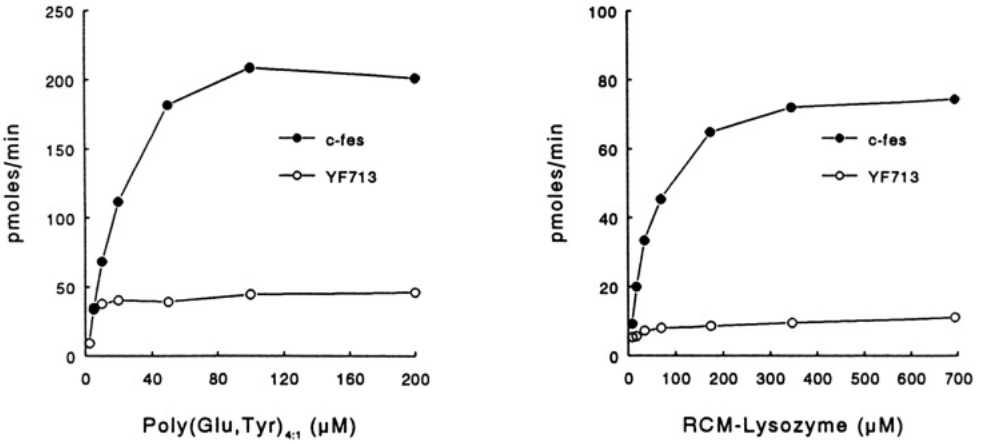

FIGURE 1: Activity of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fes\text{YF}713}$ with poly(Glu,Tyr)$_{4:1}$ (left) and RCM-lysozyme (right) as substrates. Each value is the mean of triplicate determinations.

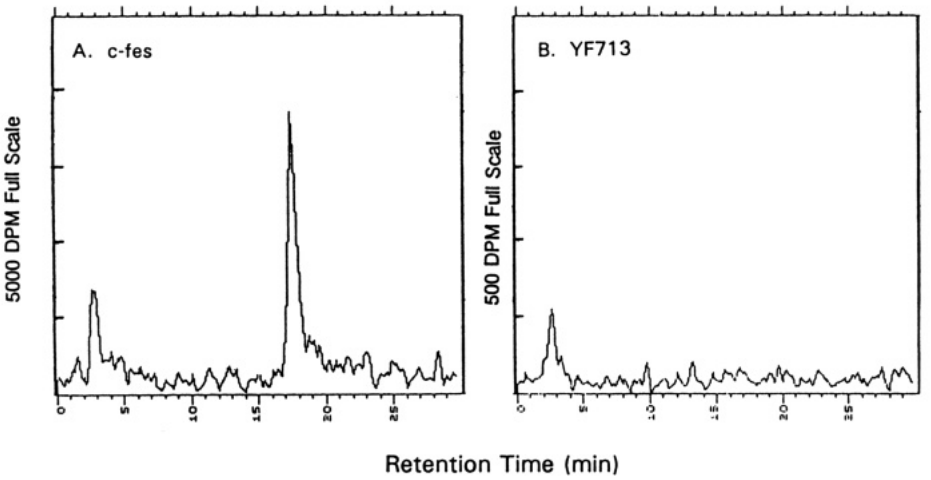

FIGURE 2: Tryptic phosphopeptide analysis of p93$^{c\text{-}fes}$ (A) and p93$^{c\text{-}fes\text{YF}713}$ (B). Baculovirus-expressed p93$^{c\text{-}fes}$ and p93$^{c\text{-}fes\text{YF}713}$ were resolved by SDS–PAGE in 8% gels, and the band was excised and incubated with trypsin. Tryptic phosphopeptides were separated by reversed-phase HPLC as described under Experimental Procedures.

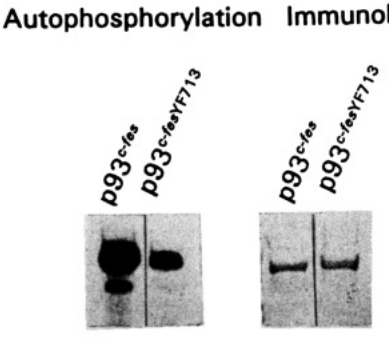

FIGURE 3: Autophosphorylation of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fes\text{YF}713}$. Purified enzyme (1 μg) following Mono Q FPLC was autophosphorylated with [γ^{32}P]ATP and separated by SDS–PAGE in 8% gels. Samples were transferred to nitrocellulose and visualized by autoradiography or immunoblotting with monoclonal antibody 127-53F8.

FIGURE 4: PCR analysis of genomic DNA for integration of the c-*fes* or c-*fes*YF713 cDNA after retrovirus infection. The c-*fes* and c-*fes*YF713 cDNA were amplified from genomic DNA with primers for exon 2 and exon 19 as described in the Experimental Procedures. DNA was obtained from the cells 2 weeks after retroviral infection by selection in G418. The PCR products were separated by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide. Each lane contained 0.5 μg of DNA. The markers represent 2.3- and 2.0-kbp fragments of *Hin*dIII-digested λ-DNA.

inducers of transcription from the metallothionein promoter (Figure 5). Over the course of 11 days, and in the absence of $CdCl_2$ and $ZnSO_4$, K562/fes and K562/YF713 cells demonstrated a slight increase in doubling time from 24 to 25.5 h, while K562/neo cells did not exhibit changes in growth (results not shown). In the continuous presence of $CdCl_2$ and $ZnSO_4$, the doubling time of K562/fes and K562/YF713 cells was increased to 43 h in both cell lines after 5–7 days and to a lesser extent at 1–4 and 8–11 days after induction, whereas no change in cell growth occurred in K562/neo cells (Figure 5).

Expression of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fes\text{YF}713}$ was assessed after treatment with $CdCl_2$ and $ZnSO_4$ by immunoblotting (Figure 6). Although a basal level of p93$^{c\text{-}fes}$ was present in K562/fes and K562/YF713 cells, respectively, exposure to $CdCl_2$ and $ZnSO_2$ for 2 or 6 days resulted in noticeable increases in protein levels. Induction of c-*fes* expression was also corroborated by quantitative competitive PCR analysis of mRNA levels in K562 cells after induction (Figure 7). The point of equivalency between the genomic and cDNA fragments indicates the message level present. In K562/fes or K562/fesYF713 cells, the equivalency point was reached between 50 and 100 fg of

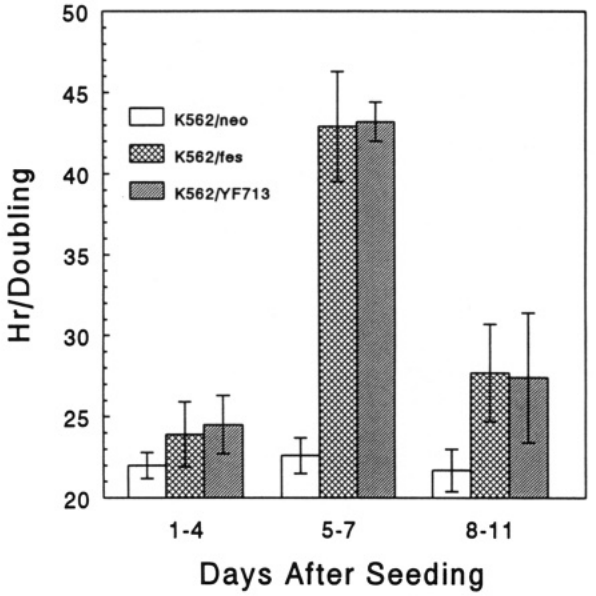

FIGURE 5: Doubling time of K562 cells after retrovirus infection. K562/neo, K562/fes, and K562/YF713 cells were grown in the continuous presence of 5 μM $CdCl_2$ and 60 μM $ZnSO_4$, and the doubling time was determined during the time periods shown. Each value is the mean ± standard error of five separate experiments.

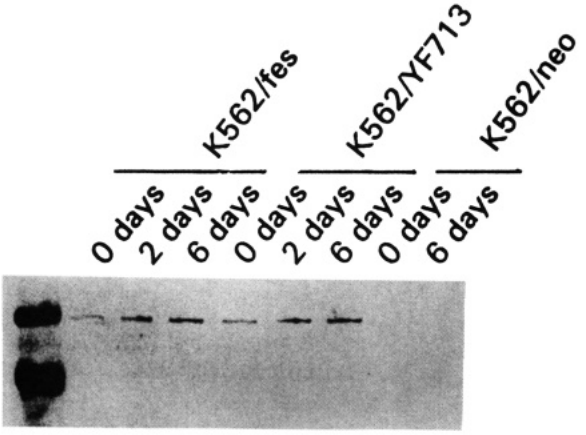

FIGURE 6: Immunoblot of p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$ levels in K562 cells after retrovirus infection. K562/neo, K562/fes, and K562/YF713 cells were grown in the continuous presence of $CdCl_2$ and $ZnSO_4$ for 2 or 6 days. Cells were extracted with Triton X-100, and 70 μg of protein per lane was resolved by SDS–PAGE and transferred to nitrocellulose as described in the Experimental Procedures. p93$^{c\text{-}fes}$ and p93$^{c\text{-}fesYF713}$ were visualized with monoclonal antibody 127-53F8.

mRNA, while the equivalency point after induction was between 200 and 400 fg of mRNA. No c-*fes* mRNA was detectable in K562/neo cells. Therefore, $CdCl_2$ and $ZnSO_4$ induction produced an approximately 4-fold elevation of c-*fes* and c-*fes*YF713 mRNA levels.

To determine whether induced expression of c-*fes* or c-*fes*YF713 resulted in the appearance of the mature myeloid phenotype in K562 cells, two functional properties were determined. K562 cells expressing either the wild-type or mutant forms of c-*fes* were both capable of reducing NBT after induction with $CdCl_2$ and $ZnSO_4$, in contrast to results showing a lack of NBT reduction in wild-type K562 cells or in cells infected with the *neo* gene alone (Figure 8). NBT reduction was approximately 40% lower in cells expressing p93$^{c\text{-}fesYF713}$ in comparison to p93$^{c\text{-}fes}$. Other parameters indicative of the myeloid phenotype were also measured. Seven days after infection K562/neo cells did not exhibit phagocytic activity, while 25% and 19% of K562/fes and K562/YF713 cells, respectively, were capable of phagocytizing sheep erythrocytes. Cells also expressed greater levels of the myelomonocytic surface antigens CD13 and CD33 6 days after induction of c-*fes* or c-*fes*YF713 expression (Figure 9).

Endogenous phosphotyrosine-containing proteins were visualized by immunoblotting in K562 cells expressing either *neo*, c-*fes*, or c-*fes*YF713 after induction with $ZnCl_2$ and $CdCl_2$ (Figure 10). Several phosphotyrosine-containing proteins were evident in either *neo*-, c-*fes*-, or YF713-expressing K562 cells, but with the exception of a 210-kDa protein, there were no major qualitative differences among the three cell lines. K562/fes and K562/YF713 cells displayed slightly lesser amounts of phosphotyrosine-containing proteins as well as lower levels of the 210-kDa protein. Immunoblotting with an antibody against c-*abl* indicated that the p210$^{bcr\text{-}abl}$ PTK was reduced in both c-*fes*-expressing cell lines (Figure 10).

DISCUSSION

The present study demonstrates that retroviral-mediated transfer of the c-*fes* cDNA into undifferentiated K562 leukemia cells results in the acquisition of a more mature myeloid phenotype. Cells infected with c-*fes* exhibited superoxide formation (NBT reduction), phagocytosis, increased expression of the myeloid surface antigens CD13 and CD33, and an increased doubling time. These results are consistent with our previous study, which demonstrated that transfection of K562 cells with the c-*fes* genomic sequence resulted in progression to a granulocytic phenotype. The c-*fes*YF713 mutant possessed a reduced ability to induce superoxide production in K562 cells, but it did not diminish phagocytosis, doubling time, or myeloid antigen presentation. Despite the markedly reduced autophosphorylating and catalytic activities of p93$^{c\text{-}fesYF713}$, these *in vitro* activities were clearly not predictive of its biological activity. This result is somewhat analogous to the transforming activity of v-*fps* mutated at the equivalent position at Y1073 (Weinmaster *et al.*, 1984). In the latter study, the level of fibroblast transformation by the mutant v-*fps* was similar to that of the wild-type oncogene despite an 80% reduction in autophosphorylation and catalytic activity, but it did show an increased latency period for the onset of transformation. Thus, loss of autophosphorylation at Y713 in p93$^{c\text{-}fes}$ does not appear to severely compromise its ability to function in the signaling pathway leading to differentiation.

One mechanism by which many cytosolic PTKs function in signal transduction is through the interaction of their autophosphorylated domain with SH2-containing proteins (Koch *et al.*, 1991). This interaction may be intramolecular as well as with exogenous proteins and, therefore, may serve the dual function of modulating both the catalytic and biological activity of the PTK. This was demonstrated with the viral avian homolog of p93$^{c\text{-}fes}$, p130^{gagfps}, where mutational insertion or substitution of amino acids within conserved regions of the SH2 domain abrogated its transformational and catalytic activities (DeClue *et al.*, 1987; Sadowski *et al.*, 1986). Cells transformed by v-*fps* also contain high levels of phosphotyrosyl SH2 domain proteins, such as GAP and its associated 62-kDa protein (Ellis *et al.*, 1990; Moran *et al.*, 1990). Mutation of the SH2 domain results in reduced levels of tyrosine phosphorylation of these substrates (Koch *et al.*, 1991), suggesting that interaction with exogenous SH2 substrates is important for oncogenic activity.

In the present study, mutation of Y713 resulted in a loss of catalytic activity, but its biological activity was compromised

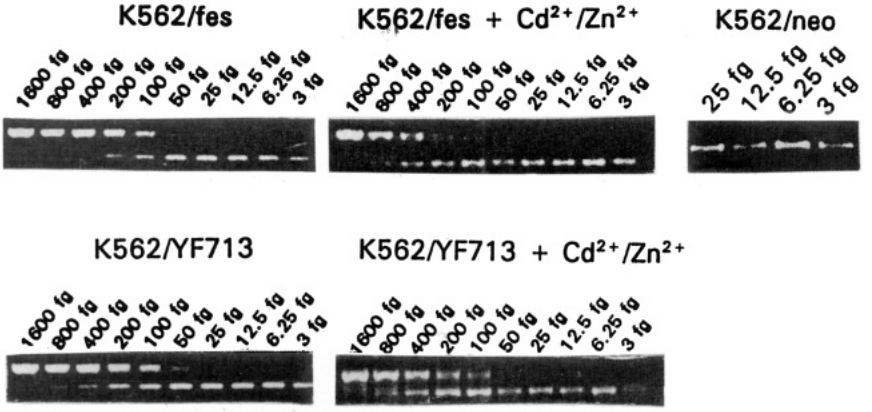

FIGURE 7: Quantitative PCR analysis of c-*fes* and c-*fes*YF713 mRNA levels in K562 cells after retrovirus infection. K562/neo, K562/fes, and K562/YF713 cells were grown in the continuous presence of $CdCl_2$ and $ZnSO_4$ for 6 days. Competitive PCR was carried out with a pair of primers delineating a 405-bp region in the genomic c-*fes* sequence encoding exon 17, intron 17, and exon 18 (upper band) or a 281-bp region in endogenous c-*fes* or c-*fes*YF713 mRNA (lower band). Each lane contained 0.4 μg of total RNA. Note the absence of the lower band in K562/neo cells.

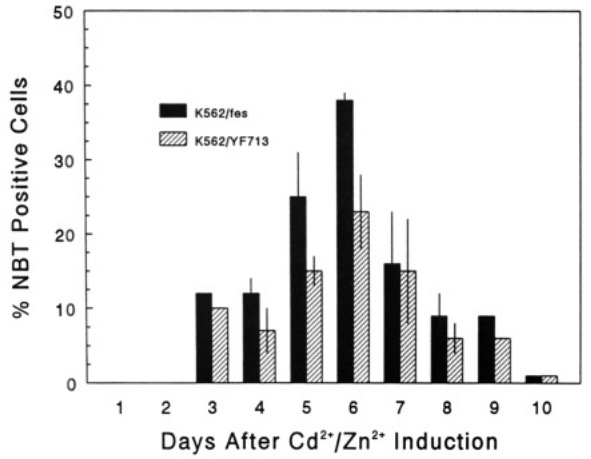

FIGURE 8: NBT reduction in K562 cells after retrovirus infection. K562/neo, K562/fes, and K562/YF713 cells were grown in the continuous presence of $CdCl_2$ and $ZnSO_4$, and cells were assessed for NBT reduction and erythrophagocytosis at the indicated times. Each value is the mean ± standard error of three experiments or the mean of two experiments.

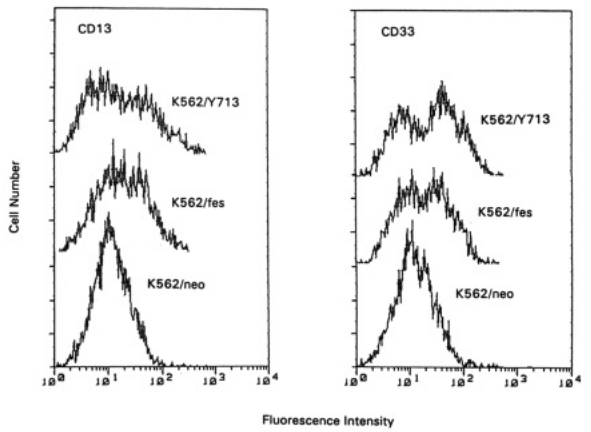

FIGURE 9: Fluorescence-activated cell-sorting analysis of CD13 and CD33 expression in K562 cells after retrovirus infection. Cells were grown in the continuous presence of $CdCl_2$ and $ZnSO_4$ for 6 days. The mean fluorescence of K562/neo, K562/fes, and K562/YF713 cells were 16, 42, and 50 for CD13 and 20, 36, and 50 for CD33, respectively.

to a lesser extent. Thus, it is probable that other phosphorylation sites in p93$^{c\text{-}fes}$ may be more crucial for its biological activity and for its interaction with endogenous SH2 substrates. First, the sequence surrounding Y713, EEADGVYA, does not fit the consensus sequence, V/LXXXXEYL/I, for the

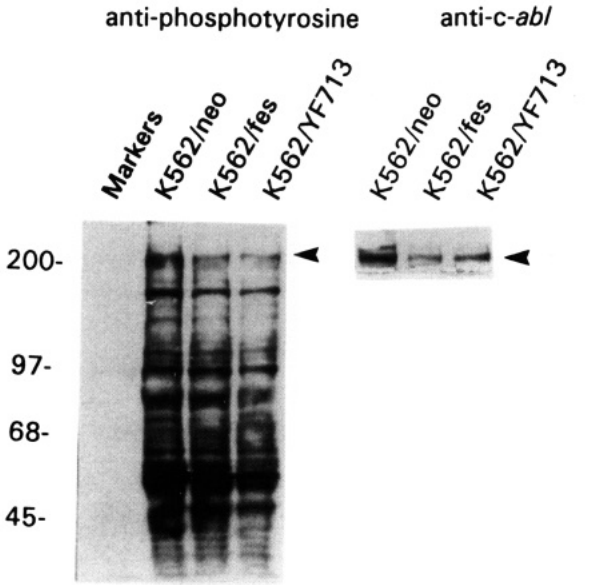

FIGURE 10: Immunoblot of phosphotyrosine-containing proteins and c-*abl* in K562 cells after retrovirus infection. Immunoblotting was carried out with cell extracts prepared from K562/neo, K562/fes, and K562/YF713 cells grown in the continuous presence of $CdCl_2$ and $ZnSO_4$ for 6 days. The primary monoclonal antibody used was either against phosphotyrosine (left) or against c-*abl* (right). Each lane contains 50 μg of protein.

phosphotyrosine domain found in the EGF receptor that interacts with exogenous SH2-containing substrates (Rotin *et al.*, 1992). Secondly, a synthetic 13-mer peptide of the sequence surrounding Y713 in p93$^{c\text{-}fes}$, but containing 4-(phosphonomethylene)phenylalanine in place of tyrosine, did not affect the activity of p93$^{c\text{-}fes}$ *in vitro* (R. I. Glazer, J. Lei, and T. Burke, unpublished results). This suggests that a second autophosphorylation site in p93$^{c\text{-}fes}$ may be responsible for interacting with intramolecular or exogenous SH2 domains. Deletion of the entire SH2 domain in either p93$^{c\text{-}fes}$ (Hjermstad et al., 1993) or p130^{gagfps} (Sadowski *et al.*, 1986) results in a drastic reduction of catalytic and autophosphorylation activities. This is completely opposite to the regulation of p60^{src}, where deletion of the SH2 domain or the mutation of Y527 which interacts with its SH2 domain results in activation of the enzyme (Cantley *et al.*, 1991). Alternatively, phosphoserine or phosphothreonine sequences within p93$^{c\text{-}fes}$ may be more important for intramolecular binding to its SH2 domain in a manner analogous to the interaction of BCR with the ABL SH2 domain in BCR-ABL (Pendergast *et al.*, 1991).

p93$^{c\text{-}fes}$ is phosphorylated in intact cells on serine and threonine (Feldman *et al.*, 1985; MacDonald *et al.*, 1985; Greer *et al.*, 1988), and therefore, the regulation of p93$^{c\text{-}fes}$ via a serine/threonine kinase may also be important for its biological activity.

In regard to potential SH2 substrates for p93$^{c\text{-}fes}$, a novel protein with a single SH2 domain was isolated from a KG-1 myeloid leukemia cell cDNA library by screening with the SH2 domain of c-*fes* (Pellicci *et al.*, 1992). This protein, named SHC, appeared to be involved in coupling activated growth factor receptors to the proliferation potential of the cell; however, this protein is ubiquitously expressed, and it remains to be seen whether SHC is of functional importance to the ability of p93$^{c\text{-}fes}$ to induce myeloid differentiation.

Expression of p93$^{c\text{-}fes}$ or p93$^{c\text{-}fes\text{YF}713}$ did not result in major changes in the level of phosphotyrosine-containing proteins, with the notable exception of a reduction in the 210-kDa PTK, p210$^{bcr\text{-}abl}$. In contrast to myeloid cell lines with a more differentiated phenotype, such as HL-60 and KG-1, K562 cells contain significant levels of phosphotyrosine-containing proteins (Huhn *et al.*, 1987; Giordano *et al.*, 1987). Our results substantiate these findings and show further that overexpression of c-*fes* does not result in a marked qualitative change in the composition of these endogenous phosphotyrosyl proteins, with the single exception of a reduction in the level of p210$^{bcr\text{-}abl}$. Since K562 cells were originally derived from a Philadelphia chromosome-positive patient with chronic myelogenous leukemia (Lozzio & Lozzio, 1979) and contain the 9;22 translocation gene product, p210$^{bcr\text{-}abl}$ (Kloetzer *et al.*, 1985; Naldini *et al.*, 1986), it is not unexpected that the level of phosphotyrosine-containing proteins is so high. Other investigations have found high levels of phosphotyrosine-containing proteins in this cell line (Huhn *et al.*, 1987; Giordano *et al.*, 1987), and this property may be related to the transforming activity of p210$^{bcr\text{-}abl}$ in murine bone marrow stem cells where it initiates a syndrome similar to chronic myelogenous leukemia (Daley *et al.*, 1990; Heisterkamp *et al.*, 1990. It is of interest that the level of p210$^{bcr\text{-}abl}$ is reduced upon myeloid differentiation since this process would be expected to arrest the proliferative capacity of these cells. Reduction of p210$^{bcr\text{-}abl}$ in K562 cells undergoing erythroid differentiation in response to hemin has also been reported (Richardson *et al.*, 1987), and thus, it is likely that this change is a general response to differentiation and not a lineage-specific effect.

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