Construction of a cDNA for the Human c-*fes* Protooncogene Protein-Tyrosine Kinase and Its Expression in a Baculovirus System†

Thomas E. Smithgall,‡ Biswendu B. Goswami,§ Zohreh Nagashfar, Shakeel Ahmad, and Robert I. Glazer*
Department of Pharmacology, Georgetown University Medical Center, Washington, D.C. 20007
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ABSTRACT: Previous studies have established that the 93-kDa protein-tyrosine kinase (PTK) encoded by the human c-*fes* protooncogene plays an active role in the induction of terminal myeloid differentiation. However, this enzyme is expressed at very low levels in myeloid cells, making isolation of sufficient quantities for detailed biochemical analysis difficult. To overcome this problem, we used the polymerase chain reaction to construct a full-length c-*fes* cDNA from overlapping 5′ and 3′ partial cDNA sequences. The c-*fes* cDNA was expressed at high levels in a baculovirus system, and the catalytically active recombinant c-*fes* gene product p93$^{c\text{-}fes}$ was partially purified by DEAE-Sepharose and tyrosine–agarose chromatography. Recombinant p93$^{c\text{-}fes}$ was indistinguishable from the native protein in terms of its apparent molecular weight following SDS–PAGE, catalytic activity, K_m for poly(Glu,Tyr)$_{4:1}$, antigenicity, and phosphopeptide pattern generated with *Staphylococcus aureus* protease.

The human c-*fes* locus encodes a protein-tyrosine kinase (PTK)[1] of 93 kDa (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). Previous studies have shown that c-*fes* message levels (Liebermann & Liebermann, 1989) and tyrosine kinase activity (Glazer et al., 1986, 1987; Yu et al., 1988; Yu & Glazer, 1987; Chapekar et al., 1986) increase during the terminal differentiation of myeloid cells, suggesting that this protooncogene may directly regulate the maturation process. Further support for this hypothesis comes from gene-transfer studies using the human myeloid leukemia cell line K-562 (Lozzio et al., 1981), which does not express c-*fes* and is resistant to myeloid differentiation inducers (Koeffler et al., 1981). Transfection of K-562 cells with the human c-*fes* gene resulted in a significant reduction in the cellular growth rate and the expression of functional properties of mature granulocytes (Yu et al., 1989). This result indicates that p93$^{c\text{-}fes}$ tyrosine kinase activity alone is sufficient to trigger terminal differentiation in an appropriate cell type and suggests that it may represent an important effector mechanism for physiological regulators of myelopoiesis such as the colony-stimulating factors (Nicola, 1989). A more detailed analysis of the structural features of p93$^{c\text{-}fes}$ that regulate its tyrosine kinase activity and interaction with other cellular proteins is required to clarify the role of this protooncogene product in myeloid differentiation.

p93$^{c\text{-}fes}$ is a member of a unique subfamily of cytoplasmic PTKs encoded by protooncogenes and shares a number of structural features with a larger subfamily of PTKs 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* kinases. A second region of homology is located directly N-terminal to the catalytic domain and has been termed the SH2 domain for *src* homology region 2 (Koch et al., 1991; Sadowski et al., 1986). An SH2 domain is also present in other nonreceptor protein-tyrosine kinases and their transforming viral counterparts (Koch et al., 1991; Sadowski et al., 1986), and in several cellular signaling molecules including phospholipase C-γ (Rhee et al., 1989) and the *ras* GTPase activator protein (GAP; McCormick, 1989). Recent evidence has shown that the SH2 domain of phospholipase C-γ, GAP, and p60$^{c\text{-}src}$ may play a critical role in mediating protein–protein interactions with the EGF and PDGF receptors following stimulation with their respective ligands (Moran et al., 1990; Anderson et al., 1990). In addition, cellular transformation by p130$^{gag\text{-}fps}$, a viral homologue of c-*fes*, is associated with tyrosine phosphorylation of GAP (Ellis et al., 1990), and mutations in the SH2 domain of v-*fps* impair both transformation and tyrosine phosphorylation. These results suggest that GAP may represent a substrate for p93$^{c\text{-}fes}$ as well, and raise the intriguing possibility of a biochemical link between *fes* and *ras*.

In addition to regulating complex formation between PTKs and substrate proteins, the SH2 domains found in cytoplasmic PTKs appear to regulate the kinase activity of the adjacent catalytic region. Genetic and biochemical studies of p130$^{gag\text{-}fps}$ have suggested that an intramolecular association occurs between the SH2 domain and the adjacent kinase domain that is required for both kinase activation and substrate recognition (Koch et al., 1989). Mutations in the v-*fps* SH2 domain were shown to disrupt this association, and compromise both tyrosine kinase activity and the ability of p130$^{gag\text{-}fps}$ to transform cells (Koch et al., 1989). This finding suggests that stimulation of p93c-*fes* by an upstream signal may cause a conformational change that transiently juxtaposes these two regions, resulting in kinase activation and substrate recognition.

To begin to understand the complex regulation of this enzyme, development of a system for the expression of cata-

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* Address correspondence to this author at the Georgetown University Medical Center, Cancer Pharmacology Section, 4 Research Court, Rockville, MD 20850.
‡ Present address: Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 600 S. 42nd St., Omaha, NE 68198-6805.
§ Present address: Food and Drug Administration, 200 C St. S.W., Washington, D.C. 20204.

[1] Abbreviations: PTK, protein-tyrosine kinase; SH2, *src* homology 2; GAP, *ras* GTPase activator protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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lytically active recombinant protein is essential, as native p93$^{c\text{-}fes}$ is expressed at low levels in myeloid leukemia cell lines (Smithgall et al., 1988; Yu & Glazer, 1987). In this report, we describe the cloning of a full-length c-*fes* cDNA using the polymerase chain reaction and the expression of high levels of catalytically active p93$^{c\text{-}fes}$ from Sf-9 insect cells infected with a recombinant baculovirus containing this cDNA. We also provide evidence that the recombinant protein is biochemically and immunologically indistinguishable from its native counterpart.

Experimental Procedures

Materials. A partial c-*fes* cDNA clone and polyclonal antibodies against recombinant c-*fes* peptides were generously provided by Dr. Dennis Slamon, UCLA School of Medicine, Los Angeles, CA. The baculovirus transfer vector pVL1392 and wild-type *Autographica californica* nuclear polyhedrosis virus (baculovirus) were provided by Dr. Max Summers, 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 and [^{35}S]methionine were obtained from Dupont–New England Nuclear, Boston, MA. Bacteriophage T7 RNA polymerase, plasmid vector pGEM-4Z, RNasin, rabbit reticulocyte lysate, and other reagents for cell-free translation were purchased from Promega Biotech, Madison, WI. Oligonucleotide primers for PCR were synthesized by the Macromolecular Sequencing/Synthesis Core Facility, Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, D.C. Other reagents for PCR were purchased from Perkin-Elmer Cetus, Norwalk, CT. Sequenase and other DNA sequencing reagents were purchased from United States Biochemical Corp., Cleveland, OH. Poly-(Glu,Tyr)$_{4:1}$ and tyrosine–agarose were purchased from Sigma Chemical Co., St. Louis, MO. Protein G–Sepharose 4FF and DEAE-Sepharose were obtained from Pharmacia–LKB Biotechnology, Piscataway, NJ. MMLV reverse transcriptase was obtained from Life Technologies Inc., Gaithersburg, MD and m^{7}GpppG and *Staphylococcus aureus* V-8 protease were obtained from Boehringer-Mannheim, Indianapolis, IN.

Production of a Full-Length c-fes cDNA by Overlap Extension PCR. The first strand of a 5′ c-*fes* fragment was obtained by annealing 50 pmol of the primer P-1 (5′-GCA-GCC-CGC-ACG-CCC-AGC-ACA-TAG-3′), that is complementary to nucleotides 555–578 of the c-*fes* open reading frame (Roebroek et al., 1985), to 10 μg of total RNA from human monocytes that was prepared as described previously (Smithgall et al., 1991). The first-strand reaction was conducted in 20 μL of PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (w/v) gelatin] containing 1.0 mM dNTPs and 2.0 units/μL RNasin. MMLV reverse transcriptase (200 units) was added, and the reaction was incubated for 1 h at 37 °C and terminated by heating to 95 °C for 5 min. *Taq* polymerase (2.5 units) and 50 pmol of the forward primer P-2 were added, and the reaction was brought to a final volume of 100 μL. Primer P-2 (5′-GGG-AAG-CTT-AAC-AGC-ACT-ATG-GGC-TTC-TCT-TCC-GAG-3′) encodes the first 18 nucleotides of c-*fes* exon 2 containing the translation initiation codon (Roebroek et al., 1985) as well as a restriction site for *Hin*dIII. Amplification was performed for 40 cycles with each cycle consisting of 1 min at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The resulting 600 bp fragment was isolated by agarose gel electrophoresis, and 10 ng was combined with 10 ng of an overlapping partial c-*fes* cDNA clone containing the balance of the c-*fes* coding sequence in 100 μL of PCR buffer containing 0.2 mM dNTPs and 20 pmol each of primers P-2 and P-3 (5′-TCT-AGA-GAA-TTC-TTT-ATT-GTT-TCT-GCC-CGG-3′). Primer P-3 is complementary to the 3′ end of c-*fes* exon 19 that is downstream from the termination codon and contains the polyadenylation signal sequence (Roebroek et al., 1985) as well as a restriction site for *Xba*I. *Taq* polymerase (2.5 units) was added to this reaction mixture, and amplification was carried out for 25 cycles, each consisting of 1 min at 94 °C, 30 at 50 °C, and 3 min at 72 °C. The resulting 2.7-kb fragment was gel-purified and subcloned into the vector pGEM-4Z, and the sequence of the first 600 bases was determined using a primer complementary to the T7 promoter (Promega), primer P-1, Sequenase, and standard dideoxynucleotide sequencing techniques.

Synthesis of Capped c-fes Transcripts. The c-*fes* cDNA was subcloned into the plasmid vector pRc/CMV (InVitrogen, San Diego, CA) downstream from the bacteriophage T7 RNA polymerase promoter. The resulting construct was linearized with *Xba*I and used as a template for capped c-*fes* mRNA synthesis. Transcription reactions were conducted in a volume of 50 μL containing Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 100 units of RNasin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.05 mM GTP, 0.5 mM m^{7}GpppG, 2.5 μg of linearized template DNA, and 40 units of T7 RNA polymerase. The reactions were incubated at 37 °C for 1 h and terminated by incubating with 5 units of RNase-free DNase at 37 °C for 15 min. RNA was extracted with phenol/$CHCl_3$ and precipitated with ethanol, and RNA integrity was confirmed by formaldehyde–agarose gel electrophoresis and ethidium bromide staining.

Translation of c-fes RNA Using Rabbit Reticulocyte Lysate and Immunoprecipitation. A large-scale translation mixture (200 μL) was prepared that contained 140 μL of rabbit reticulocyte lysate, 30 μL of water, 160 units of RNasin, 20 μM amino acids (except methionine), 1 μg of c-*fes* RNA, and 160 μCi of [^{35}S]methionine (1200 Ci/mmol) that was incubated at 30 °C for 1 h. Aliquots of 25 μL were combined with 5 μL of c-*fes* antiserum and incubated on ice for 1 h. Protein G–Sepharose was added (20 μL of a 50% w/v suspension in RIPA buffer), and reactions were incubated on a rocker platform for 30 min at 4 °C. Immune complexes were washed 3 times with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), and the final pellet was resuspended in 50 μL of SDS–PAGE sample buffer. Samples were heated to 95 °C for 5 min, separated on 12% polyacrylamide minigels, and visualized by autoradiography.

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 (Summers & Smith, 1987). 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 0.5 mL of extraction buffer (50 mM Tris, pH 7.5, 10 mM dithiothreitol, 2 mM EGTA, 1% Triton X-100, 50 μg/mL aprotinin, 10 μM pepstatin, 1 mM phenylmethanesulfonyl fluoride, 400 μg/mL soybean trypsin inhibitor, and 200 μg/mL leupeptin). Cellular debris was pelleted by centrifugation for 10 min at 10000*g*, and the supernatant was used for further purification. In some experiments, Triton

X-100 was omitted from the extraction buffer, and the volume of extraction buffer was increased to 3.0 mL. The cell extract was applied to a 5-mL DEAE-Sepharose column preequilibrated in DEAE buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol), and $p93^{c\text{-}fes}$ was eluted with a 60-mL linear gradient of 0–0.5 M NaCl in DEAE buffer. Column fractions (1.0 mL) were assayed using poly(Glu,Tyr)$_{4:1}$ (see below), and active fractions were pooled and dialyzed against tyrosine–agarose buffer (40 mM Hepes–NaOH, pH 7.2, 1 mM dithiothreitol, 10% glycerol, 0.5% Triton X-100, and protease inhibitors as listed above for DEAE buffer). Ammonium sulfate was added to a final concentration of 0.8 M, and the sample was applied to a 5.0-mL tyrosine–agarose column. Proteins were eluted with a linear gradient of 0.8–0 M ammonium sulfate in tyrosine–agarose buffer (Yu & Glazer, 1987), and active fractions were pooled and dialyzed against tyrosine–agarose buffer. Fractions were stained with "Fast Stain" (Zoion Research Inc.), and immunoblotting with monoclonal antibody 127-53F8 raised against a C-terminal c-*fes* peptide (Yu & Glazer, 1987) was performed 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, 200 μM poly(Glu,Tyr)$_{4:1}$ and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Reactions were initiated by 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 GFC glass fiber filters and washed with TCA/PP_i and ethanol. Filter-bound radioactivity was quantitated by liquid scintillation counting.

Immune Complex Kinase Assay. Aliquots of crude Sf-9 cell extracts or column fractions were incubated with 2 μL of monoclonal antibody 127-53F8 and precipitated with protein G–Sepharose as described above. Immune complexes were washed 5 times with RIPA buffer followed by a final wash in kinase buffer (50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 5 mM $MnCl_2$). Autophosphorylation reactions were initiated by adding 40 μL of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) to the immunoprecipitate and incubated for 10 min at 30 °C. Reactions were terminated by adding SDS–PAGE sample buffer and heating to 95 °C for 5 min. Phosphorylated proteins were separated by SDS–PAGE and visualized by autoradiography.

Phosphopeptide Analysis. $p93^{c\text{-}fes}$ from the tyrosine–agarose step was immunoprecipitated and autophosphorylated as described above. The labeled protein was resolved by SDS–PAGE, excised from the gel, and digested with *S. aureus* V-8 protease (Yu & Glazer, 1987). Phosphopeptides were separated by reverse-phase HPLC using a Brownlee RP18 column (4.6 × 100 mm) 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 β-detector.

Results and Discussion

Construction of a Full-Length c-fes cDNA by Overlap Extension PCR. A full-length c-*fes* cDNA has been difficult to isolate from cDNA libraries due to the low abundance of this mRNA. To circumvent this problem, the 5′-terminal 578-nucleotide sequence was synthesized by reverse transcription and PCR and annealed to a partial 3′ cDNA lacking 378 bp of the 5′-terminal sequence (Figure 1). The nucleotide sequence of the first 600 base pair region in the 5′ end of the cDNA was determined by standard dideoxynucleotide techniques, and although this region was subjected to 65 amplification cycles, its sequence was in complete agreement with the published sequence for c-*fes* (Roebroek et al., 1985).

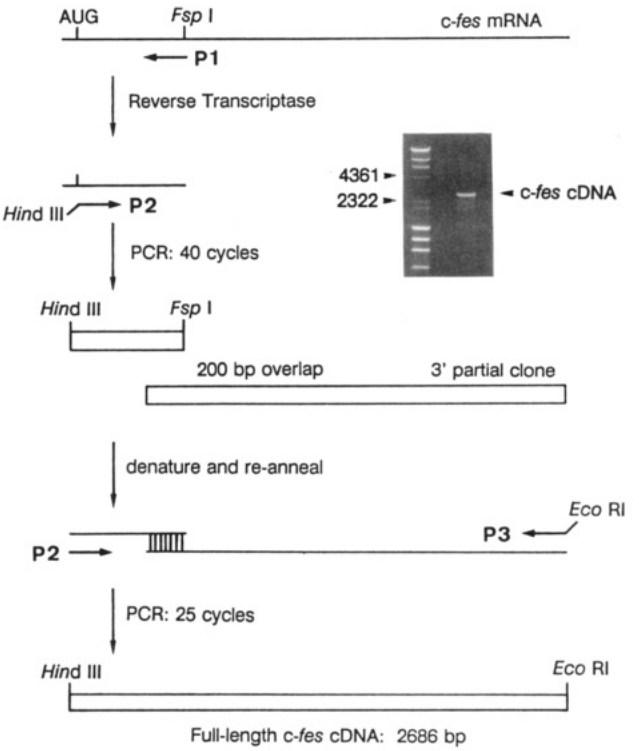

FIGURE 1: Cloning of a full-length c-*fes* cDNA by overlap extension PCR. A 5′ c-*fes* fragment was obtained from monocyte total RNA using primer P-1 and reverse transcriptase, followed by 40 cycles of PCR with the forward primer P-2. This 5′ cDNA fragment was annealed to a second partial clone that contained the balance of the coding sequence and overlapped the 5′ fragment by 200 nucleotides. An additional 25 amplification cycles in the presence of the 3′ primer P-3 yielded the expected 2.7-kb c-*fes* cDNA clone (inset). Specific PCR conditions and primer sequences are described under Experimental Procedures.

Characterization of the c-fes cDNA by Translation in Vitro and Immunoprecipitation. To verify that the c-*fes* cDNA contained the entire $p93^{c\text{-}fes}$ open reading frame prior to generation of a recombinant baculovirus, RNA was transcribed from the cDNA in vitro and expressed using the rabbit reticulocyte lysate system. Following translation, $p93^{c\text{-}fes}$ was immunoprecipitated and analyzed by SDS–PAGE and autoradiography (Figure 2). The rabbit polyclonal antibodies used in the immunoprecipitation experiments were raised against a series of c-*fes* peptides that span 90% of the $p93^{c\text{-}fes}$ sequence (Smithgall et al., 1988). All of the antibodies immunoprecipitated a protein of 93 kDa, indicating the likelihood that the c-*fes* cDNA contains the entire open reading frame for $p93^{c\text{-}fes}$.

Expression of Recombinant $p93^{c\text{-}fes}$ Using the Baculovirus Expression System. Since previous isolation procedures for $p93^{c\text{-}fes}$ required very large amounts of HL-60 leukemia cells (Yu & Glazer, 1987), the baculovirus system provided an attractive alternative method for the isolation of high yields of catalytically active $p93^{c\text{-}fes}$. Recombinant $p93^{c\text{-}fes}$ was partially purified from Sf-9 cells infected with a recombinant baculovirus encoding the c-*fes* cDNA by sequential DEAE-Sepharose and tyrosine–agarose chromatography (Table I, procedure 1). The initial purification procedure utilized extraction with a buffer containing Triton X-100 since we found previously that this detergent was required to solubilize $p93^{c\text{-}fes}$ from differentiated HL-60 cells (Yu & Glazer, 1987). Analysis of the tyrosine–agarose fraction by SDS–PAGE revealed that the protein was approximately 20% pure at this

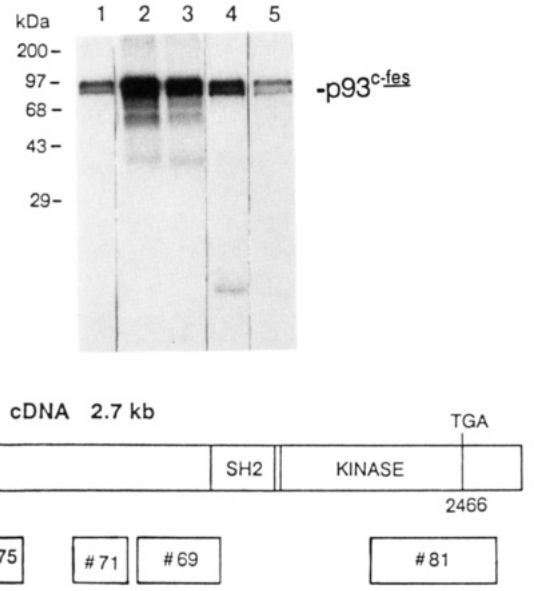

FIGURE 2: Characterization of PCR-derived c-*fes* cDNA by in vitro translation and immunoprecipitation. The c-*fes* cDNA was subcloned into the plasmid vector pRc/CMV downstream from the bacteriophage T7 RNA polymerase promoter. The resulting construct was linearized with *Xba*I and used as a template for in vitro transcription of capped c-*fes* mRNA. The c-*fes* mRNA was translated in vitro using rabbit reticulocyte lysate and [^{35}S]methionine, immunoprecipitated, and visualized by SDS–PAGE and autoradiography. Rabbit antibodies used to immunoprecipitate p93$^{c\text{-}fes}$ were raised against recombinant c-*fes* peptides and are numbered 75, 71, 69, and 81 (Smithgall et al., 1988). The relative locations of the restriction fragments used to generate these peptides are shown below the autoradiograph with the respective antibodies. Lane 1, antibody 75; lane 2, antibody 71; lane 3, antibody 69; lane 4, antibody 81; lane 5, 5 μL of the rabbit reticulocyte translation mixture containing p93$^{c\text{-}fes}$.

Table I: Partial Purification of p93$^{c\text{-}fes}$ from Sf-9 Cells Infected with Recombinant Baculovirus[a]

fraction	protein (mg)	total act. (pmol/min)	sp act. (pmol min^{-1} mg^{-1})	yield	purification (*x*-fold)
procedure 1					
Triton extract	50.0	33800	676	100	1
DEAE-Sepharose	10.4	23850	2293	71	3.3
tyrosine–agarose	3.8	12630	3324	37	5.0
procedure 2					
cell extract	2.4	2310	963	100	1
DEAE-Sepharose	0.26	4020	15460	174	16

[a] PTK activity was assayed with poly(Glu,Tyr)$_{4:1}$ as substrate as described under Experimental Procedures. A total of 10^8 cells were extracted with a buffer containing 1% Triton X-100 (procedure 1), or a total of 10^7 Sf-9 cells were extracted with the same buffer without Triton X-100 (procedure 2) as described under Experimental Procedures.

stage (Figure 3A), although this represented only a 5-fold purification relative to the crude cell extract. The major contaminating protein was a 55-kDa viral protein with similar chromatographic properties as p93$^{c\text{-}fes}$ (Figure 3A). Subsequently, we found that this contaminant could be virtually eliminated by omission of Triton X-100 from the extraction buffer (Table I, procedure 2). The detergent-free extract exhibited a 7-fold increase in specific activity after DEAE-Sepharose and was judged to be 80–90% pure by staining (results not shown). These results suggest that p93$^{c\text{-}fes}$ represents approximately 5% of the total protein present in the recombinant baculovirus-infected Sf-9 cells. This level of expression is in marked contrast to that observed in HL-60 leukemia cells where a 2700-fold purification of p93$^{c\text{-}fes}$ required 20 L of cells and yielded 35 μg of purified material (Yu & Glazer, 1987). The identity of p93$^{c\text{-}fes}$ at each stage of the purification was confirmed by immunoblot analysis with

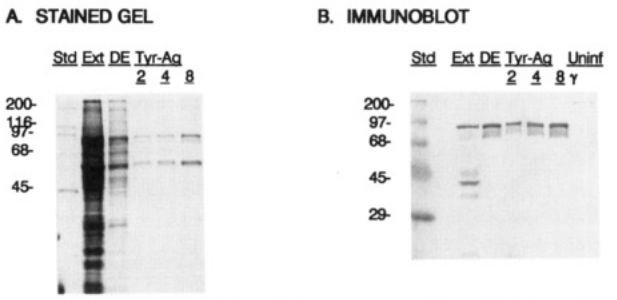

FIGURE 3: Purification of baculovirus-expressed p93$^{c\text{-}fes}$ from Sf-9 insect cells. Sf-9 cells were infected with a recombinant virus encoding the c-*fes* cDNA, and cell extracts were prepared 72 h after infection. Cell extracts (Ext) or either DEAE-Sepharose (DE) or tyrosine–agarose (Tyr-Ag) fractions were resolved by SDS–PAGE in 10% gels and either stained (A) or identified by immunoblotting with monoclonal antibody 127-53F8 (B). The amount of protein in each lane was as follows: cell extract, 40 μg; DEAE-Sepharose fraction, 8 μg; tyrosine–agarose fraction, 2,4, or 8 μg.

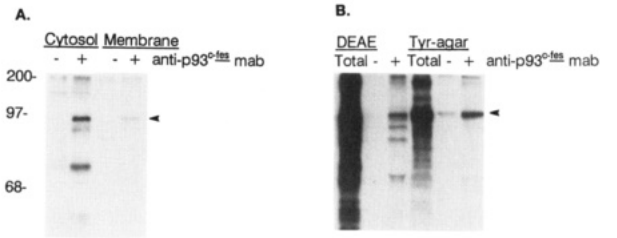

FIGURE 4: Immune complex kinase assay of baculovirus-expressed p93$^{c\text{-}fes}$. Extracts from Sf-9 cells infected with the c-*fes* recombinant virus were sequentially chromatographed on DEAE-Sepharose and tyrosine–agarose, and column fractions were assayed for tyrosine kinase activity using poly(Glu,Tyr)$_{4:1}$ as substrate as described under Experimental Procedures. The active fractions were pooled, and aliquots were incubated with a monoclonal antibody raised against a C-terminal c-*fes* peptide. Immune complex assays of autophosphorylated p93$^{c\text{-}fes}$ were resolved by SDS–PAGE and visualized by autoradiography. The autoradiographs show the results of the immune complex assay performed in the presence and absence of the antibody (lanes marked "+" and "–"). (A) Cytosol (40 μg of protein) and membrane (4 μg of protein) fractions of p93$^{c\text{-}fes}$ partially purified by DEAE-Sepharose chromatography. (B) Cytosol Fractions after DEAE-Sepharose (DEAE) or tyrosine–agarose (Ty-agar) chromatography. Phosphorylation reactions run directly without the immunoprecipitation step are marked Total. The location of the immunoreactive band corresponding to p93$^{c\text{-}fes}$ is indicated by the arrowhead.

monoclonal antibody 127-53F8 raised against a C-terminal p93$^{c\text{-}fes}$ peptide (Yu & Glazer, 1987) (Figure 3B).

Determination of the K_m for poly(Glu,Tyr)$_{4:1}$ for the baculovirus-expressed p93$^{c\text{-}fes}$ (Table I, procedure 2) was 25 μM (results not shown), a value that is similar to that determined previously (20 μM) for p93$^{c\text{-}fes}$ purified from HL-60 cells (Yu & Glazer, 1987).

The subcellular distribution of p93$^{c\text{-}fes}$ in baculovirus-infected Sf-9 cells was evaluated using an immune complex kinase assay. Sf-9 cells infected with c-*fes* were fractionated into cytosol (100000*g* supernatant) and membrane fractions (100000*g* pellet), and the resulting proteins were incubated with monoclonal antibody 127-53F8. The distribution of p93$^{c\text{-}fes}$ in Sf-9 cells was primarily in the cytosol and not in the membrane fraction (Figure 4A). By contrast, differentiated HL-60 cells contained p93$^{c\text{-}fes}$ associated almost exclusively with the plasma membrane fraction (Yu & Glazer, 1987). Possible explanations for this difference are that Sf-9 cells may lack a membrane receptor protein necessary for p93$^{c\text{-}fes}$ association that is present in myeloid cell membranes or that they cannot posttranslationally modify p93$^{c\text{-}fes}$, if indeed this occurs. It is not believed that p93$^{c\text{-}fes}$ is myristylated since

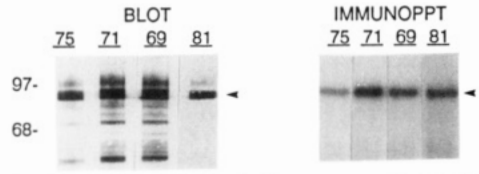

FIGURE 5: Reactivity of baculovirus-expressed p93$^{c\text{-}fes}$ with sequence-specific polyclonal antibodies. Baculovirus-expressed p93$^{c\text{-}fes}$ was purified by DEAE-Sepharose chromatography (procedure 2, Table I) and either immunoblotted (Blot) or autophosphorylated with [^{32}P]ATP, immunoprecipitated with rabbit polyclonal antibodies 75, 71, 69, and 81, and detected by autoradiography (Immunoppt).

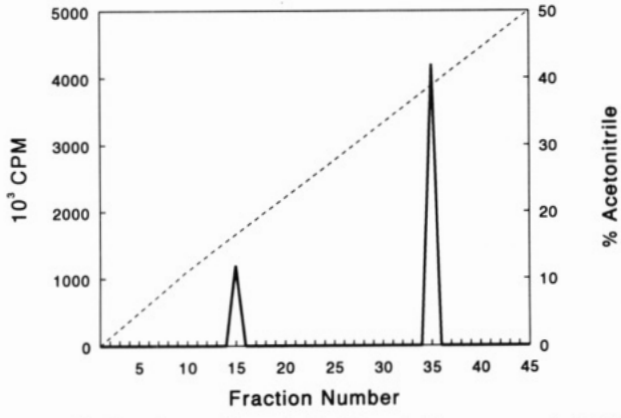

FIGURE 6: Phosphopeptide analysis of baculovirus-expressed p93$^{c\text{-}fes}$. An aliquot of p93$^{c\text{-}fes}$ partially purified from baculovirus-infected Sf-9 cells after tyrosine–agarose chromatography was immunoprecipitated and autophosphorylated as described in the text. The autophosphorylated enzyme was gel-purified and digested with *S. aureus* V-8 protease, and phosphopeptides were resolved by reverse-phase HPLC as described under Experimental Procedures.

an N-terminal peptide (GFSSELCSPQGHGVLQ) does not serve as a substrate for mouse brain *N*-myristoyl transferase (Geahlen and Glazer, unpublished results).

Immune complex kinase assays were also performed on whole cell extracts and on the DEAE-Sepharose and tyrosine–agarose fractions obtained from detergent-extracted cells (Figure 4B). At each stage of the purification, p93$^{c\text{-}fes}$ is clearly visible as an autophosphorylated band of 93 kDa. Similar experiments were carried out with p93$^{c\text{-}fes}$ purified by DEAE-Sepharose (Table I, procedure 2) (Figure 5). Immunoblotting of p93$^{c\text{-}fes}$ indicated strong reactivity with rabbit polyclonal antibodies 75, 71, 69, and 81 that was virtually identical to that reported for the native enzyme from HL-60 cells (Yu & Glazer, 1987). Immunoprecipitation of autophosphorylated p93$^{c\text{-}fes}$ with the same antibodies showed an immunoreactivity similar to p93$^{c\text{-}fes}$ expressed by in vitro translation (Figure 2).

Autophosphorylation of native p93$^{c\text{-}fes}$ involves at least two tyrosine residues in vitro (Yu & Glazer, 1987). To determine whether these sites were phosphorylated in recombinant p93$^{c\text{-}fes}$, phosphopeptide analysis was carried out by reverse-phase HPLC after V-8 protease digestion of the gel-purified autophosphorylated protein obtained after tyrosine–agarose chromatography (Figure 6). The resulting HPLC profile showed two major radioactive peaks with retention times nearly identical to those previously generated from p93$^{c\text{-}fes}$ purified from HL-60 cells (Yu & Glazer, 1987), suggesting that the same tyrosine residues are phosphorylated in the recombinant protein.

In summary, these data demonstrate the utility of the baculovirus system for the high-level expression of recombinant p93$^{c\text{-}fes}$ protein-tyrosine kinase. Many properties of the baculovirus-expressed protein were indistinguishable from those of native p93$^{c\text{-}fes}$ previously isolated from HL-60 promyelocytes (Yu & Glazer, 1987), including apparent molecular weight, antigenicity toward mono- and polyclonal antibodies, K_m for poly(Glu,Tyr)$_{4:1}$ as substrate, and phosphopeptide pattern. Recently, the baculovirus system has been successfully used to express other members of the cytoplasmic class of PTKs, including p60$^{c\text{-}src}$ (Piwnica-Worms et al., 1990) and p56^{lck} (Ramer et al., 1991). Our results further validate the baculovirus system as an appropriate model for structure–function analysis of this class of PTK.

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Alterations in Substrate Utilization in the Reperfused Myocardium: A Direct Analysis by ^{13}C NMR†

A. Dean Sherry,*,‡,§,∥ Craig R. Malloy,§,∥,⊥ P. Zhao,‡ and Jennifer R. Thompson‡

Department of Chemistry, University of Texas at Dallas, P.O. Box 830688, Richardson, Texas 75083-0688, and Departments of Radiology and Internal Medicine and Mary Nell and Ralph B. Rogers Biomedical Magnetic Resonance Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9085

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ABSTRACT: An alternative ^{13}C NMR method which allows direct determination of substrate oxidation in tissue for up to three competing ^{13}C-enriched substrates is presented. Oxidation of competing substrates can be measured by ^{13}C NMR spectroscopy under non-steady-state conditions if the relative areas of the glutamate C3 and C4 resonances can be determined. The accuracy of this measurement is limited during brief exposure to ^{13}C-enriched substrates because of the low enrichment in the C3 carbon. The glutamate C4 resonance from a tissue sample which has oxidized a combination of [1,2-^{13}C]acetate (or a uniformly enriched fatty acid mixture) and [3-^{13}C]lactate appears as a nine-line resonance consisting of four multiplet components: a singlet (C4S), two doublets with differing one-bond coupling constants (C4D34 and C4D45), and a quartet (C4Q). It is shown that the sum of the C4S + C4D34 resonance areas versus the C4D45 + C4Q resonance areas directly reports the relative utilization of [3-^{13}C]lactate versus [1,2-^{13}C]acetate, respectively, regardless of citric acid cycle intermediate pool sizes or carbon flux through anaplerotic reactions. We also show that homonuclear ^{13}C decoupling of the glutamate C2 resonance collapses the C3 resonance multiplet into an apparent triplet (actually, a singlet plus a doublet); the relative area of the singlet component reflects the amount of unlabeled acetyl-CoA entering the cycle. The method has been used to determine the contribution of lactate/acetate/glucose to acetyl-CoA in normoxic and reperfused rat hearts. ^{13}C spectra of freeze-clamped heart extracts show quite directly that lactate oxidation is depressed after a 10-min period of global ischemia while acetate becomes the predominant source of energy (60% of the total substrate oxidized through the citric acid cycle). Substrate utilization returns to basal levels (34% acetate, 28% lactate, and 38% unlabeled sources) during 25 min of reperfusion. Ischemic hearts also switch to acetate as the principle source of energy in the presence of a pyruvate/acetate/glucose mixture but not when presented an acetoacetate/acetate/glucose mixture. These results likely reflect control at the level of the pyruvate dehydrogenase complex, which is known to become phosphorylated in heart mitochondria during ischemia.

^{13}C NMR offers considerable potential for monitoring specific biochemical reactions in vivo. The most widely studied pathways so far have been related to glycogen storage and mobilization (Brainard et al., 1989; Jue et al., 1989a,b; Shulman et al., 1990). Since these may be described in most simple terms as a linear biochemical pathways, the exogenously added ^{13}C-enriched substrate (usually [1-^{13}C]glucose) may be followed by NMR as it accumulates in the storage product glycogen or as glycogenolysis is activated to produce end products of the glycolytic pathway which are easily detected, such as [3-^{13}C]lactate. We have chosen to use ^{13}C NMR to examine a variety of biochemical reactions involving the Krebs citric acid cycle (Sherry et al., 1988; Malloy et al., 1988, 1990a). Entry of ^{13}C into a cyclic pathway such as the citric acid cycle inevitably produces intermediates with enriched ^{13}C nuclei in more than a single site within the same molecule (^{13}C isotopomers). This complicates the ^{13}C NMR spectrum as a result of spin–spin coupling between adjacent ^{13}C nuclei, but it also provides valuable biochemical information. We have reported that a complete isotopomer analysis of a single ^{13}C spectrum obtained under metabolic and isotopic steady-state conditions provides information about the relative utilization of competing substrates and ^{13}C flux through various pathways involving the cycle (Malloy et al., 1988, 1990a). More recently, we reported a non-steady-state ^{13}C NMR method for determining relative substrate utilization which could be applied to in vivo situations where the magnetic field homogeneity may not be sufficient to resolve ^{13}C–^{13}C coupling (Malloy et al., 1990b). We now report another simple method which we

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* Author to whom correspondence should be addressed.
‡ Department of Chemistry, University of Texas at Dallas.
§ Department of Radiology, University of Texas Southwestern Medical Center.
∥ Mary Nell and Ralph B. Rogers Biomedical Magnetic Resonance Center, University of Texas Southwestern Medical Center.
⊥ Department of Internal Medicine, University of Texas Southwestern Medical Center.

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