Phosphorylation of the *ras* GTPase-Activating Protein (GAP) by the p93$^{c\text{-}fes}$ Protein–Tyrosine Kinase *in Vitro* and Formation of GAP–*fes* Complexes via an SH2 Domain-Dependent Mechanism†

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ABSTRACT: The protein–tyrosine kinase encoded by the human c-*fes* protooncogene (p93$^{c\text{-}fes}$) plays a direct role in myeloid differentiation, but downstream substrates for this kinase have not been identified. Here we report that the human *ras* GTPase-activating protein (GAP) is a substrate for p93$^{c\text{-}fes}$ *in vitro*. Purified, recombinant GAP was readily phosphorylated on tyrosine residues by bacterially-expressed p93$^{c\text{-}fes}$. Two-dimensional tryptic mapping revealed a single GAP phosphopeptide, consistent with specific phosphorylation of GAP by p93$^{c\text{-}fes}$ on one or several closely-spaced tyrosine residues. Autophosphorylated p93$^{c\text{-}fes}$ also formed a stable complex with GAP. Complex formation is likely to involve the *src* homology 2 (SH2) domains of GAP and autophosphorylated tyrosine residues of p93$^{c\text{-}fes}$, as deletion of the *fes* SH2 domain did not abolish complex formation. Furthermore, immobilized recombinant fusion proteins containing either or both of the GAP SH2 domains were able to precipitate p93$^{c\text{-}fes}$ with an affinity equal to that observed with a monoclonal antibody against the recombinant *fes* protein. Fusion proteins containing the GAP N-terminal, C-terminal catalytic, or SH3 domains did not bind to p93$^{c\text{-}fes}$. Interaction of the GAP SH2 domains with p93$^{c\text{-}fes}$ is phosphorylation-dependent, as the recombinant SH2 domain proteins were unable to bind to a kinase-defective c-*fes* mutant and showed reduced binding of a mutant in which one of the two tyrosine autophosphorylation sites was replaced with phenylalanine. Stimulation of c-*fes* autophosphorylation *in vivo* may induce interaction with GAP, resulting in altered p21^{ras} function.

The human c-*fes* gene encodes a 93-kDa PTK[1] (p93$^{c\text{-}fes}$) that is expressed predominantly in myeloid cells of the granulocytic and monocytic lineages (Feldman *et al.*, 1985; MacDonald *et al.*, 1985; Smithgall *et al.*, 1988). p93$^{c\text{-}fes}$ tyrosine kinase activity is greatly enhanced during the differentiation of human myeloid leukemia cell lines *in vitro*, suggestive of an active role for p93$^{c\text{-}fes}$ during myeloid development (Chapekar *et al.*, 1986; Glazer *et al.*, 1986). Further support for this hypothesis comes from gene-transfer studies with K562 myeloid leukemia cells, a differentiation-resistant cell line that does not express c-*fes* (Lozzio *et al.*, 1981). Transfection of K562 cells with the c-*fes* genomic sequence resulted in a marked reduction in the cellular growth rate and the expression of functional properties of mature phagocytes (Yu *et al.*, 1989). These data indicate that p93$^{c\text{-}fes}$ tyrosine kinase activity alone is sufficient to induce terminal differentiation in an appropriate recipient cell line. Although p93$^{c\text{-}fes}$ can profoundly influence myeloid growth and differentiation, the identities of the substrates phosphorylated by p93$^{c\text{-}fes}$ that ultimately mediate these effects are currently unknown.

The *ras* protooncogenes encode small (21-kDa) GTP-binding proteins that exhibit weak intrinsic GTPase activity (Barbacid, 1987; Grand & Owen, 1991). Several lines of evidence strongly implicate p21^{ras} as an essential component of growth-regulatory signal transduction by both physiological and transforming PTKs. For example, stimulation of growth factor receptors or transformation with oncogenes that encode PTKs is associated with elevated cellular levels of p21^{ras} in its active, GTP-bound form (Satoh *et al.*, 1990; Gibbs *et al.*, 1990). Conversely, microinjection of antibodies to p21^{ras} blocks mitogenic responses to EGF and PDGF as well as cellular transformation by PTK oncogenes (Mulcahy *et al.*, 1985; Smith *et al.*, 1986). These data strongly suggest that p21^{ras} is an essential downstream effector of PTK function.

Because p21^{ras} is active in the GTP-bound form, factors that influence the rate of *ras* GTP hydrolysis or GDP/GTP exchange can significantly alter *ras* function. One of these factors is the *ras* GTPase-activating protein (GAP), which significantly enhances GTP hydrolysis by p21^{ras}, thus promoting its conversion to the inactive, GDP-bound form (Trahey & McCormick, 1987; McCormick, 1989). Previous studies have shown that GAP is phosphorylated in cells transformed with oncogenes that encode PTKs and that GAP forms stable complexes with the oncogenic kinases (Ellis *et al.*, 1990; Brott *et al.*, 1991). In particular, transformation of Rat-2 fibroblasts with the v-*fps* oncogene, an avian homolog of c-*fes*, is associated with enhanced phosphorylation of GAP and its associated proteins p190 and p62. This finding suggests that GAP may serve as a substrate for p93$^{c\text{-}fes}$ as well.

GAP and p93$^{c\text{-}fes}$ share a common structural feature that may serve to link them in a myeloid differentiation signal

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[1] Abbreviations: PTK, protein–tyrosine kinase; SH2, *src* homology 2; PLC, phospholipase C; GAP, GTPase-activating protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; GST, glutathione *S*-transferase.

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transduction cascade. This feature is known as the SH2 domain, as it is was first identified in $p60^{src}$ and other cellular and transforming PTKs [reviewed by Pawson (1988), Pawson and Gish (1992), and Koch *et al.* (1991)]. Since then, SH2 domains have been found in a number of other proteins involved in growth regulation, including GAP (Trahey & McCormick, 1987; McCormick, 1989), PLC-γ (Rhee, 1991), and phosphatidylinositol 3′-kinase p85 subunit (Escobedo *et al.*, 1991; Skolnik *et al.*, 1991; Otsu *et al.*, 1991). SH2 domains share the common function of binding tightly to peptide sequences containing phosphotyrosine residues such as autophosphorylated growth factor receptors or cytoplasmic PTKs. Thus, tyrosine phosphorylation has been proposed as a key molecular signal that stimulates the formation of protein complexes between autophosphorylated kinases and downstream effectors with SH2 domains (Pawson & Gish, 1992; Koch *et al.*, 1991).

In this report, we show that *ras* GAP is a substrate for $p93^{c\text{-}fes}$ and demonstrate that GAP can form stable complexes with the autophosphorylated c-*fes* protein. In addition, we show that recombinant fusion proteins containing either of the GAP SH2 domains bind tightly to $p93^{c\text{-}fes}$ in a phosphorylation-dependent manner. These findings suggest that *fes* and GAP may form part of a signal transduction cascade in myeloid cells in which upstream signals for differentiation are coupled to $p21^{ras}$ via GAP–*fes* interaction.

EXPERIMENTAL PROCEDURES

Bacterial Expression and Immunoprecipitation. Mutagenesis of the c-*fes* consensus sequences for autophosphorylation (Tyr 713 → Phe) and ATP binding (Lys 590 → Glu) as well as deletion of the c-*fes* SH2 domain is described in detail elsewhere (Hjermstad *et al.*, 1993). Wild-type and mutant c-*fes* cDNAs were expressed as fusion proteins in *Escherichia coli* using the pFLAG-1 expression vector (Kodak/IBI, New Haven, CT). This vector directs the fusion of a unique eight amino acid FLAG sequence (DYKDDDDK) to the N-terminus of the *fes* proteins, allowing for efficient immunoprecipitation and immunoblotting with an anti-FLAG monoclonal antibody (M2 antibody). FLAG–*fes* fusion proteins were expressed in *E. coli* DH5α cells by induction of 50-mL cultures with 1.7 mM IPTG for 4 h at 37 °C. Bacterial cells were pelleted, washed once with ice-cold PBS, and lysed by sonication in 1.0-mL ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 10 mM DTT, 1% Triton X-100, 1 mM PMSF, and 50 μg/mL aprotinin). Lysates were clarified by centrifugation at 12000*g* for 5 min and incubated with 1.0 μg of purified M2 monoclonal antibody (Kodak/IBI) and 20 μL of protein G-Sepharose (Pharmacia, Piscataway, NJ) for 1 h at 4 °C. Immunoreactive c-*fes* proteins were precipitated by centrifugation and washed three times with 1.0 mL of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and once with 1.0 mL of kinase buffer (50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, and 5 mM $MnCl_2$) prior to phosphorylation assays.

Phosphorylation of GAP and Enolase. Purified recombinant human GAP was the generous gift of Drs. Frank McCormick and Gideon Bollag, Onyx Pharmaceuticals, Richmond, CA. GAP (M_r = 120 000) was expressed using a baculovirus/insect cell system and undergoes partial proteolysis during purification to 110- and 95-kDa forms (Halenbeck *et al.*, 1990). Phosphorylation of GAP by immunoprecipitated $p93^{c\text{-}fes}$ was conducted in 40 μL of kinase buffer containing 1 μg of GAP (M_r = 120 000; 208 nM final concentration) and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; DuPont/New England Nuclear, Boston, MA). Some assays contained 2.5 μg of purified rabbit muscle enolase (M_r = 85 000; 735 nM final concentration; Boehringer-Mannheim, Indianapolis, IN) which was denatured with acetic acid prior to use (Cooper *et al.*, 1984). Phosphorylation reactions were incubated for 10 min at 37 °C and stopped by heating in SDS–PAGE sample buffer. Phosphoproteins were resolved by SDS–PAGE and visualized by autoradiography. To test for the presence of GAP–*fes* protein complexes, immune-complex kinase reactions were washed with three 1.0-mL aliquots of various buffers after phosphorylation and prior to SDS–PAGE (see legend to Figure 2).

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis. Dried gel slices containing ^{32}P-labeled GAP were rehydrated, and the SDS was removed by sequential washing with acetone and water. The gel slices were then incubated in 300 μL of 0.25% ammonium bicarbonate, pH 8.0, containing 10 μg of N^{α}-(tolylsulfonyl)phenylalanine chloromethyl ketone (TPCK)–trypsin (Worthington Biochemicals, Freehold, NJ) for 16 h at 37 °C. Tryptic digests were lyophilized, redissolved in pH 1.9 electrophoresis buffer (2.5% formic acid plus 7.5% acetic acid), and spotted on thin-layer cellulose plates (E.M. Science, Gibbstown, NJ). Tryptic phosphopeptides were separated in the first dimension by electrophoresis in pH 1.9 buffer for 30 min at 1000 V, followed by chromatography at a right angle to the electrophoresis dimension in 1-butanol/pyridine/acetic acid/water (75:50:15:60). Phosphopeptides were visualized by autoradiography. For phosphoamino acid analysis, GAP phosphopeptides were heated in 6 N HCl at 110 °C for 1 h. Samples were diluted with water, lyophilized, and separated by two-dimensional thin-layer electrophoresis. The first dimension was run in pH 1.9 buffer at 1500 V for 30 min; the second dimension was run in pH 3.5 buffer (5% acetic acid and 0.5% pyridine) at 1300 V for 20 min. Phosphoamino acid standards were added to the samples prior to electrophoresis to permit localization with ninhydrin. Radiolabeled phosphoamino acids were visualized by autoradiography. Details of these procedures have been reviewed elsewhere (Boyle *et al.*, 1991).

Expression of GAP–GST Fusion Proteins in E. coli. DNA fragments encoding the GAP N-terminal domain (amino acids 1–181), N-terminal SH2 domain (amino acids 181–273), SH3 domain (amino acids 272–351), C-terminal SH2 domain (amino acids 351–441), both SH2 domains and the intervening SH3 domain (amino acids 181–441), and the C-terminal domain (amino acids 441–1047) were amplified by PCR (see Figure 3). SH2 domain boundaries were defined by sequence homology to SH2 domains from other proteins (Koch *et al.*, 1991). The PCR primers incorporated *Bam*HI and *Eco*RI sites at the 5′ and 3′ ends of the PCR-amplified fragments to facilitate cloning into the *E. coli* expression vector pGEX-2T (Pharmacia). The resulting recombinant plasmids were used to express GAP domains as fusion proteins with glutathione *S*-transferase (GST). Mid-log cultures (250 mL) of *E. coli* DH5α transformed with either the parent vector or the recombinant plasmids were induced with 0.1 mM IPTG at 22 °C for 4 h. The cultures were lysed by sonication, and the recombinant proteins were recovered from the clarified supernatants with glutathione–agarose beads (Sigma Chemical Co., St. Louis, MO). Following washing to remove contaminating proteins, the amount of each fusion protein bound to the beads was estimated by two-dimensional laser densitometry (Molecular Dynamics) of Coomassie-stained SDS gels. Expression of recombinant fusion proteins using

the pGEX system is described in more detail elsewhere (Smith & Johnson, 1988).

Association of GAP–GST Fusion Proteins with p93$^{c\text{-}fes}$. Bacterial cultures expressing FLAG–*fes* proteins were induced and lysed as described above, except that 50 μM ATP, 5 mM $MgCl_2$, and 5 mM $MnCl_2$ were added to the lysis buffer. Aliquots of the clarified supernatants containing the recombinant c-*fes* proteins were incubated with 2.5 μg of each immobilized GAP–GST domain fusion protein or GST alone. Each reaction was brought to a final volume of 1.0 mL with the modified lysis buffer and incubated at 4 °C for 1 h. Following incubation, the beads were washed with three 1.0-mL aliquots of RIPA buffer. Proteins bound to the beads were eluted by heating in SDS–PAGE lysis buffer, resolved by SDS–PAGE, and transferred to nitrocellulose membranes. Membranes were probed for the presence of FLAG–*fes* fusion proteins by immunoblotting with the anti-FLAG monoclonal antibody, M2 (Kodak/IBI). Alternatively, blots were probed with the anti-phosphotyrosine antibody, PY-20 (ICN, Santa Clara, CA). Immunoreactive proteins were visualized using goat anti-mouse IgG coupled to alkaline phosphatase and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer's protocol (Promega, Madison, WI). Relative levels of immunoreactive proteins were quantitated by two-dimensional laser densitometry.

RESULTS

Phosphorylation of Recombinant Human GAP by p93$^{c\text{-}fes}$ in Vitro. Transformation of fibroblasts with the v-*fps* oncogene, an avian homolog of c-*fes*, is associated with enhanced phosphorylation of *ras* GAP (Ellis *et al.*, 1990). To investigate the possibility that GAP is a substrate for p93$^{c\text{-}fes}$ as well, purified recombinant human GAP was added to an immune-complex kinase assay containing bacterially-expressed p93$^{c\text{-}fes}$ (see Experimental Procedures). As shown in Figure 1A, lane 4, GAP was readily phosphorylated by p93$^{c\text{-}fes}$. To determine how efficiently GAP was utilized as a substrate, we compared the phosphorylation of GAP to that of the model substrate enolase under identical conditions. Enolase is an excellent substrate for p93$^{c\text{-}fes}$, with a K_m of approximately 100 nM (Hjermstad *et al.*, 1993). GAP was phosphorylated at least as well as enolase, as approximately equal amounts of ^{32}P were incorporated into both proteins (Figure 1A, lanes 2 and 4). Note that the concentration of enolase in this experiment was approximately 700 nM, and that of GAP was approximately 200 nM. In addition, only one major site is phosphorylated on GAP (see below), whereas multiple tyrosine residues are likely to be phosphorylated on enolase by p93$^{c\text{-}fes}$ (Cooper *et al.*, 1984). We also investigated whether the presence of GAP had any effect on c-*fes* PTK activity toward enolase, as previous studies with the *src*-related tyrosine kinase p56^{lck} suggested that GAP may stimulate its PTK activity (Amrein *et al.*, 1992). However, GAP did not alter the phosphorylation of enolase by p93$^{c\text{-}fes}$ under these conditions (Figure 1A, lane 3). This result is consistent with the idea that GAP is a downstream effector for p93$^{c\text{-}fes}$. No phosphorylation was observed when GAP was incubated with [γ-^{32}P]ATP in the absence of p93$^{c\text{-}fes}$ (Figure 1A, lane 5).

Two-dimensional tryptic phosphopeptide mapping experiments were performed on GAP following phosphorylation by p93$^{c\text{-}fes}$. As shown in Figure 1B, a single major phosphopeptide was observed, consistent with the phosphorylation of GAP by p93$^{c\text{-}fes}$ on a single or several closely-spaced tyrosine residues. Both p60^{src} and the EGF receptor kinase have recently been

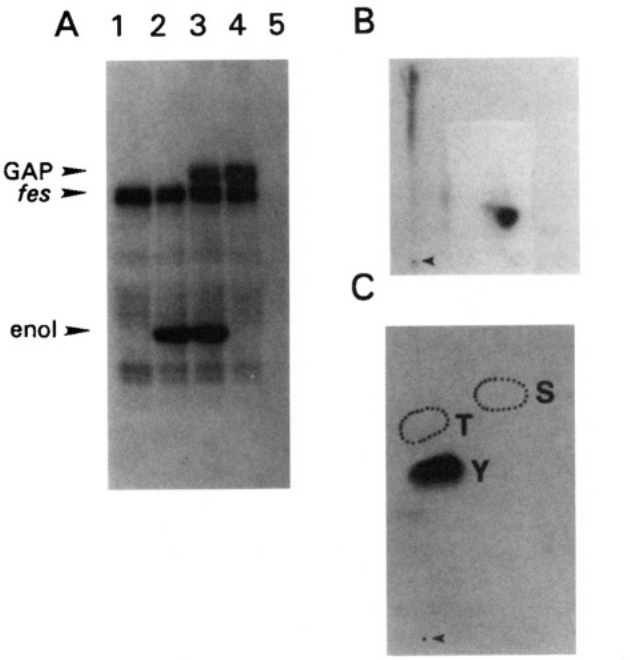

FIGURE 1: Phosphorylation of p120 *ras* GAP by p93$^{c\text{-}fes}$. (A) Immune-complex kinase assay. Recombinant p93$^{c\text{-}fes}$ was expressed in *E. coli* using the FLAG system and immunoprecipitated with an anti-FLAG monoclonal antibody and protein G-Sepharose as described in the text. The resulting p93$^{c\text{-}fes}$ immune complexes were washed and incubated with [γ-^{32}P]ATP in the absence or presence of substrate proteins: lane 1, no substrate; lane 2, enolase; lane 3, GAP and enolase; lane 4, GAP. Following incubation, labeled proteins were resolved by SDS–PAGE and visualized by autoradiography. Lane 5 shows the result of incubation of GAP with [γ-^{32}P]ATP in the absence of p93$^{c\text{-}fes}$. (B) Two-dimensional tryptic phosphopeptide mapping. Labeled GAP was excised from the gel and digested to completion with trypsin, and tryptic peptides were resolved by two-dimensional thin-layer analysis. Electrophoresis was from left to right (cathode on the right), and chromatography was from bottom to top. The arrow indicates the origin. (C) Phosphoamino acid analysis. GAP phosphopeptides were hydrolyzed with HCl, and the resulting phosphoamino acids were resolved by two-dimensional electrophoresis and visualized by autoradiography. The positions of the phosphoserine and phosphothreonine standards are outlined. The arrow indicates the origin.

shown to phosphorylate GAP on Tyr 460 *in vitro* and *in vivo* (Liu & Pawson, 1991; Park *et al.*, 1992a). Whether or not this site is utilized by p93$^{c\text{-}fes}$ as well is currently under investigation. Phosphoamino acid analysis demonstrated that phosphorylation of GAP by p93$^{c\text{-}fes}$ occurred exclusively on tyrosine residues (Figure 1C).

Formation of p93$^{c\text{-}fes}$–GAP Complexes. Phosphorylation of GAP by both cytoplasmic and receptor-linked PTKs is often associated with GAP–PTK complex formation, which may represent an important regulatory interaction (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990; Bouton *et al.*, 1991; Brott *et al.*, 1991; Park *et al.*, 1992b). To investigate the possibility of *fes*–GAP interaction, immune-complex kinase assays identical to those shown in Figure 1 were conducted, except that the immune-complex pellets were washed with various buffers after the phosphorylation reaction. As shown in Figure 2A, GAP remained bound to p93$^{c\text{-}fes}$ regardless of the composition of the buffer used for the second wash step. Control experiments show that the anti-FLAG (M2) antibody used to precipitate the FLAG–*fes* fusion protein does not cross-react with GAP (Figure 2B,C). These results indicate that GAP and *fes* form stable complexes *in vitro* that may be dependent upon phosphorylation.

Both p93$^{c\text{-}fes}$ and GAP contain SH2 domains, and both proteins become phosphorylated on tyrosine residues during

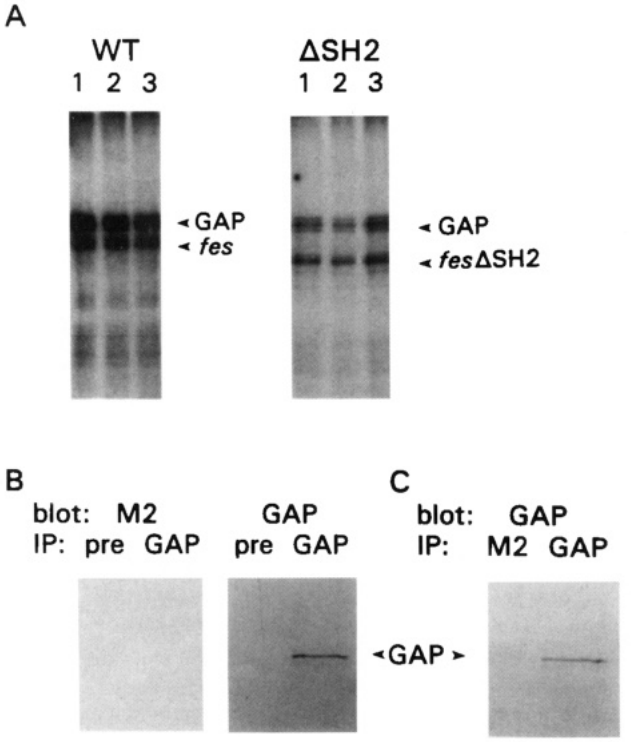

FIGURE 2: Analysis of GAP–p93$^{c\text{-}fes}$ complexes. (A) Immune-complex kinase assays identical to those described in Figure 1 were conducted using wild-type p93$^{c\text{-}fes}$ (WT) or a mutant in which the SH2 domain was deleted (ΔSH2). Following phosphorylation, the immune complexes were washed a second time prior to electrophoresis and autoradiography. The buffers used in the second wash step contained the following: 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1% sodium deoxycholate, and 0.1 % SDS (lane 1); 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 150 mM NaCl (lane 2); or 50 mM Tris-HCl, pH 7.4, and 1% Triton X-100 (lane 3). (B) The anti-FLAG monoclonal antibody (M2) does not cross-react with GAP on immunoblots. Lysates of HL-60 cells were prepared as described elsewhere (Smithgall *et al.*, 1988) and incubated with rabbit anti-GAP or preimmune serum (pre) and protein G-Sepharose. Precipitated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with either an anti-FLAG antibody (M2) or a mouse monoclonal antibody to GAP (Santa Cruz Biotechnology) as a positive control. (C) The M2 antibody does not immunoprecipitate GAP. Immunoprecipitates were prepared from HL-60 lysates using M2 or GAP monoclonal antibodies, and subsequent blots were probed with rabbit anti-GAP serum. The position of p120 GAP in the positive control lanes is indicated.

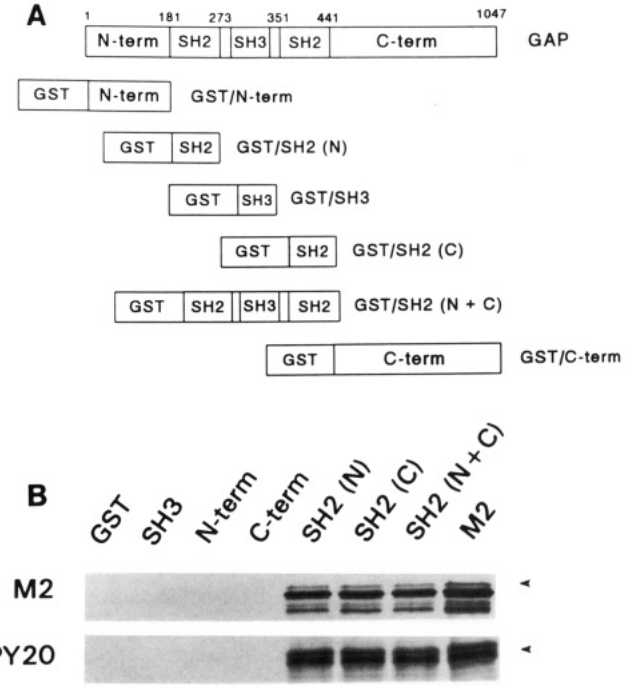

FIGURE 3: Mapping of GAP domains involved in association with p93$^{c\text{-}fes}$. (A) cDNA fragments encoding various regions of GAP were amplified by PCR and cloned into the bacterial expression vector pGEX-2T. GST–GAP fusion proteins were expressed from the recombinant plasmids and immobilized on glutathione–agarose. The primary structure of GAP is shown at the top, and the numbers above the figure indicate the amino acid boundaries of the SH2 domains. The structures of the six GST–GAP fusion proteins are also shown. GST was also produced for use as a negative control. (B) Binding of p93$^{c\text{-}fes}$ to GST–GAP fusion proteins. Bacterial cultures expressing FLAG–*fes* proteins were lysed, and aliquots of the clarified supernatants were combined with the immobilized GST–GAP fusion proteins or with immobilized GST alone. As a positive control for protein expression, aliquots were immunoprecipitated with an anti-FLAG monoclonal antibody (lane marked M2) and protein G-Sepharose. Reactions were incubated in the presence of ATP, Mg^{2+}, and Mn^{2+} for 1 h at 4 °C. Precipitates were washed extensively, and bound proteins were eluted by heating in SDS–PAGE sample buffer, resolved on polyacrylamide gels, and transferred to nitrocellulose. Identical blots were probed either with the M2 antibody to determine protein levels or with an anti-phosphotyrosine antibody (PY20) to determine phosphotyrosine content. The position of p93$^{c\text{-}fes}$ is indicated by the arrows.

the immune-complex kinase reaction, suggesting two possible mechanisms for complex formation: (1) autophosphorylated tyrosine residues in the kinase domain of p93$^{c\text{-}fes}$ interact with one or both of the GAP SH2 domains, or (2) GAP is phosphorylated first by p93$^{c\text{-}fes}$, promoting interaction with the c-*fes* SH2 domain. To distinguish between these two possibilities, we conducted immune-complex kinase assays with a mutant of c-*fes* in which the SH2 domain was deleted (ΔSH2). As shown in Figure 2, the ΔSH2 mutant was able to form complexes with GAP, suggesting that the mode of interaction between the two proteins involves the SH2 domains of GAP and autophosphorylated tyrosines on p93$^{c\text{-}fes}$.

Phosphorylation-Dependent Association of the GAP SH2 Domains with p93$^{c\text{-}fes}$. The observation that protein–protein interaction between GAP and p93$^{c\text{-}fes}$ did not require the c-*fes* SH2 domain suggested that complex formation may involve autophosphorylated *fes* tyrosine residues and the SH2 domains of GAP. To specifically localize the GAP domains involved in this interaction, we expressed GAP as a series of fusion proteins with glutathione *S*-transferase (GST) in bacteria and immobilized these proteins on glutathione–agarose beads. Six different fusion proteins were prepared containing either the GAP N-terminal domain, the N-terminal SH2 domain, the SH3 domain, the C-terminal SH2 domain, both SH2 domains and the intervening SH3 domain, or the C-terminal catalytic domain (see Figure 3A). The purified, immobilized fusion proteins were incubated with bacterial cell lysates containing recombinant FLAG–*fes* protein. Following extensive washing with buffer containing detergent and NaCl, proteins bound to the beads were separated by SDS–PAGE, and p93$^{c\text{-}fes}$ protein levels and phosphotyrosine content were analyzed by immunoblotting. As shown in Figure 3B, only the fusion proteins containing SH2 domains formed stable complexes with p93$^{c\text{-}fes}$. No binding of p93$^{c\text{-}fes}$ was observed with the SH3, N-terminal, or C-terminal fusion proteins or with immobilized GST alone, indicating that the binding reaction requires amino acid sequences derived from the GAP SH2 domains. The apparent affinity of the GAP SH2 domains for p93$^{c\text{-}fes}$ was striking, as the SH2 domain fusion proteins precipitated p93$^{c\text{-}fes}$ as efficiently as the monoclonal antibody that recognizes the FLAG epitope fused to the N-terminus of the *fes* protein (M2 antibody).

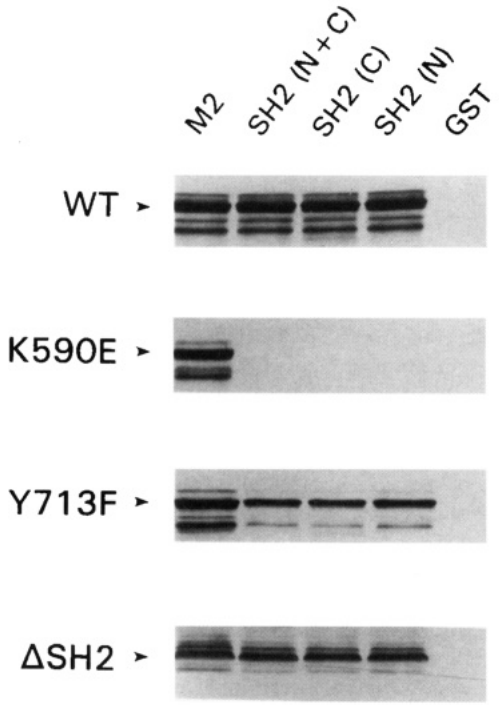

FIGURE 4: Binding of wild-type and mutant c-*fes* proteins to recombinant GAP SH2 domains. Bacterial lysates containing FLAG-*fes* proteins were incubated with immobilized GST fusion proteins containing the GAP SH2 domains, with immobilized GST alone, or with an anti-FLAG monoclonal antibody (M2) and protein G-Sepharose as described in the text and the legend to Figure 3. Following incubation, precipitates were washed and bound proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with the M2 antibody to determine relative protein levels. FLAG–*fes* fusion proteins tested include wild-type p93$^{c\text{-}fes}$ (WT), a kinase-defective mutant (K590E), an autophosphorylation site mutant (Y713F), and the SH2 deletion mutant (ΔSH2).

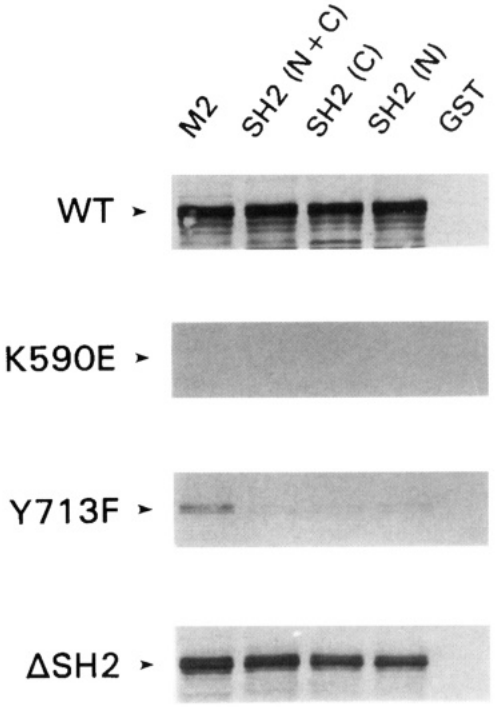

FIGURE 5: Phosphotyrosine content of FLAG–*fes* fusion proteins associated with recombinant GAP SH2 domains. Bacterial lysates containing FLAG–*fes* proteins were incubated with immobilized GST fusion proteins containing the GAP SH2 domains, with immobilized GST alone, or with an anti-FLAG monoclonal antibody (M2) and protein G-Sepharose as described in the text and the legend to Figure 3. Following incubation, precipitates were washed and bound proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against phosphotyrosine (PY20). FLAG–*fes* fusion proteins tested include wild-type p93$^{c\text{-}fes}$ (WT), a kinase-defective mutant (K590E), an autophosphorylation site mutant (Y713F), and the SH2 deletion mutant (ΔSH2).

The *fes* protein that bound to the GAP SH2 domains contained phosphotyrosine as judged by immunoblotting with anti-phosphotyrosine antibodies (Figure 3B), suggesting that the interaction between p93$^{c\text{-}fes}$ and the GAP SH2 domains is phosphotyrosine-dependent. To address this possibility directly, identical experiments were conducted with a c-*fes* mutant in which Lys 590 was replaced by Glu (K590E mutant). This highly conserved lysine residue is critical for ATP binding by most kinases (Hanks *et al.*, 1988), and mutagenesis of this site renders the c-*fes* kinase incapable of autophosphorylation (Hjermstad *et al.*, 1993). As shown in Figure 4, the K590E mutant did not bind to the GAP SH2 domains, despite expression of the mutant protein at levels comparable to those of the wild-type as assessed by immunoprecipitation and blotting with the anti-FLAG (M2) antibody. Immunoblots of duplicate gels from this experiment with anti-phosphotyrosine antibodies revealed no detectable phosphotyrosine in the M2 lane, as expected for this mutant (Figure 5). These data clearly demonstrate that interaction of p93$^{c\text{-}fes}$ with the GAP SH2 domains is autophosphorylation-dependent and have important implications for the mechanism of c-*fes* differentiation signal transduction (see Discussion).

Autophosphorylation of p93$^{c\text{-}fes}$ occurs on at least two tyrosine residues *in vitro* (MacDonald *et al.*, 1985; Greer *et al.*, 1988; Smithgall *et al.*, 1992), one of which we have recently mapped to tyrosine 713 (Hjermstad *et al.*, 1993). To determine which of these sites associates with the GAP SH2 domains, binding experiments were repeated with a c-*fes* mutant in which Tyr 713 was replaced with Phe (Y713F mutant). As shown in Figure 4, mutation of this site reduced binding of the GAP SH2 domains to p93$^{c\text{-}fes}$ by 60% relative to the M2 antibody control as determined by two-dimensional laser densitometry. Parallel blots with anti-phosphotyrosine antibodies show that the SH2-bound Y713F protein contained phosphotyrosine, although at a lower level than observed for the wild-type (Figure 5). This result is consistent with the greatly reduced autophosphorylation capacity of the Y713F mutant (Hjermstad *et al.*, 1993). Whether the decrease in SH2 binding observed with the Y713F mutant is due to loss of SH2 binding at this site or due to decreased phosphorylation at the other site remains to be established. However, demonstration that Y713F can bind to the GAP SH2 domains indicates that the second site of p93$^{c\text{-}fes}$ autophosphorylation (currently unidentified) is involved in SH2 interaction.

SH2 binding was also investigated using the c-*fes* mutant in which the SH2 domain was deleted (ΔSH2). As shown in Figure 4, this mutant bound effectively to all of the GAP SH2 domain fusion proteins. The ΔSH2 mutant that associated with the GAP SH2 domains contained phosphotyrosine (Figure 5), consistent with a role for phosphotyrosine in the association reaction. These results are in good agreement with the data shown in Figure 2, in which complexes were observed between the autophosphorylated ΔSH2 mutant and GAP.

DISCUSSION

Phosphorylation of GAP is emerging as a central theme in cellular signaling by many PTKs, including several involved in hematopoiesis. For example, activation of the colony-stimulating factor-1 and erythropoietin receptors is associated with GAP phosphorylation and a concomitant increase in

cellular p21^{ras}–GTP content (Heidaran *et al.*, 1992; Reedijk *et al.*, 1990; Torti *et al.*, 1992). In addition, GAP may be involved in signal transduction for a number of cytoplasmic PTKs associated with hematopoietic cells. Examples include p56^{lck} (Ellis *et al.*, 1991; Amrein *et al.*, 1992), which has been implicated in CD4, CD8, and interleukin-2 signaling in T-cells (Rudd *et al.*, 1988; Turner *et al.*, 1990; Veillette *et al.*, 1988; Hatakeyama *et al.*, 1991), as well as *lyn*, *fyn*, and *yes*, which form complexes with GAP in thrombin-stimulated platelets (Cichowski *et al.*, 1992). In this report, we demonstrate that recombinant human GAP is a substrate for and complexes with the myeloid-specific PTK encoded by the c-*fes* gene. Previous studies strongly suggest that p93$^{c\text{-}fes}$ plays an active role in the terminal differentiation of myeloid cells (Yu *et al.*, 1989). Demonstration of an interaction between *fes* and GAP suggests that some of the biological effects of p93$^{c\text{-}fes}$ are mediated via the p21^{ras} signaling pathway.

Although a number of PTKs have been shown to phosphorylate GAP, the effect of this phosphorylation event on GAP activity toward p21^{ras} is unclear. Although tyrosine phosphorylation of GAP may not influence its catalytic activity per se (Liu & Pawson, 1991), formation of complexes between GAP and the autophosphorylated kinase or other phosphoproteins may significantly affect GAP activity. For example, binding of GAP to the activated EGF receptor kinase reduced GAP activity toward p21^{ras} *in vitro* (Serth *et al.*, 1992). EGF stimulation also results in complex formation between GAP and its associated protein, p190 (Settleman *et al.*, 1992). EGF-induced GAP–p190 complex formation is phosphorylation-dependent and results in reduced GAP activity (Moran *et al.*, 1991). In the present study, we observed stable complex formation between p93$^{c\text{-}fes}$ and GAP. Because previous biological data indicate that p93$^{c\text{-}fes}$ has a negative effect on myeloid growth (Yu *et al.*, 1989), p93$^{c\text{-}fes}$ may stimulate GAP activity, possibly by unique interactions with the GAP SH2 domains (see below). Alternatively, the tight association between GAP and p93$^{c\text{-}fes}$ may antagonize interaction between GAP and PTKs transmitting proliferative signals or with p190. Decreased proliferative signaling through p21^{ras} would result in either case.

Because of the possible significance of *fes*–GAP complex formation, we examined the mechanism of this interaction in detail. Data in Figures 3–5 show that GAP–*fes* complexes involve interactions between both GAP SH2 domains and autophosphorylated tyrosines on p93$^{c\text{-}fes}$; other GAP domains are apparently not involved. The affinities of the GAP SH2–*fes* interactions were remarkably high, as the immobilized SH2 domains were able to precipitate p93$^{c\text{-}fes}$ as efficiently as a monoclonal antibody. Autophosphorylation is absolutely required for complex formation to occur, as a kinase-defective mutant of c-*fes* was unable to complex with the GAP SH2 domains. The c-*fes* SH2 domain is apparently not required, as a c-*fes* SH2 deletion mutant formed complexes with the GAP SH2 domains and with full-length GAP. Thus, the *fes* SH2 domain may be available to bind p93$^{c\text{-}fes}$ to a phosphorylated target upstream, such as a hematopoietic growth-factor receptor. Precedent for this idea is provided by p60^{src}, which not only forms stable complexes with GAP (Brott *et al.*, 1991; Park *et al.*, 1992b) but may interact with autophosphorylated growth-factor receptors through its SH2 domain (Anderson *et al.*, 1990; Moran *et al.*, 1990). A recent study has shown that p93$^{c\text{-}fes}$ associates with the β-subunit of the GM-CSF receptor and is activated in response to ligand binding (Hanazono, *et al.*, 1993). Whether or not this association involves the *fes* SH2 domain or leads to association with GAP remains to be determined.

Autophosphorylation of p93$^{c\text{-}fes}$ Tyr 713 appears to be an essential event in the activation of this enzyme, as replacement of this site with phenylalanine results in a dramatic decrease in both autophosphorylation and external substrate phosphorylation (Fang *et al.*, 1993; Hjermstad *et al.*, 1993). However, mutagenesis of this site did not abolish complex formation with the GAP SH2 domains, indicating that the other c-*fes* autophosphorylation site (currently unidentified) is at least partially involved in binding to GAP. Recent data show that mutagenesis of c-*fes* Tyr 713 reduces but does not eliminate the differentiation-inducing activity of p93$^{c\text{-}fes}$ in K562 cells, despite its negative impact on kinase activity *in vitro* (Fang *et al.*, 1993). One possible explanation for this observation is that differentiation signal transduction involving *fes* and GAP or other SH2-containing proteins can still occur via protein–protein interaction with the remaining autophosphorylation site.

Fusion proteins containing either or both SH2 domains of GAP bound to phosphorylated p93$^{c\text{-}fes}$ with equal affinity. This is in marked contrast to the interaction of recombinant GAP SH2 domains with the activated EGF and PDGF receptors, in which the C-terminal SH2 domain is essentially devoid of binding activity (Anderson *et al.*, 1990; Moran *et al.*, 1990). In addition, the amino acid sequences surrounding the c-*fes* autophosphorylation site at Tyr 713 as well as those surrounding candidate tyrosines for the unidentified autophosphorylation site share little similarity to reported sequences involved in GAP SH2 domain binding to the autophosphorylated PDGF receptor (Fantl et al., 1992). These observations suggest that *fes* contains unique binding sites for the GAP SH2 domains. Thus, GAP–*fes* association may produce unique effects on GAP biological activity and/or modify its interactions with other phosphorylated proteins *in vivo*.

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