Regulation of c-Fes Tyrosine Kinase Activity by Coiled-Coil and SH2 Domains: Analysis with *Saccharomyces cerevisiae*†

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ABSTRACT: The c-Fes protein-tyrosine kinase regulates the growth and differentiation of diverse cell types, including myeloid hematopoietic cells, vascular endothelial cells, and neurons. Structurally, Fes is composed of a unique N-terminal region with coiled-coil oligomerization motifs, followed by SH2 and kinase domains. Although Fes kinase activity is tightly regulated in cells, the structural basis for its negative regulation is not clear. In this report, c-Fes was expressed in *Saccharomyces cerevisiae* to determine whether regulation is kinase-intrinsic or dependent upon protein factors found in mammalian cells. Wild-type Fes kinase activity was completely repressed in yeast and did not affect cell growth. Mutation or deletion of the more N-terminal c-Fes coiled-coil domain reversed negative regulation, leading to strong kinase activation and suppression of yeast cell growth. Similarly, replacement of the wild-type SH2 domain with that of v-Src induced strong kinase activation and the growth-inhibitory phenotype. Immunoblotting with phosphospecific antibodies shows that activation of Fes by either mechanism induced autophosphorylation of the activation loop tyrosine residue (Tyr 713). These data support the idea that Fes naturally adopts an inactive conformation in vivo, and that maintenance of the inactive structure requires the coiled-coil and SH2 domains.

The c-*fes* proto-oncogene encodes a cytoplasmic protein-tyrosine kinase (Fes) that is expressed primarily in myeloid hematopoietic cells, vascular endothelial cells, and some neurons (*1*, *2*). Fes kinase activity is stimulated by multiple cytokines and growth factors, and may transmit signals for morphological differentiation in these and other cell types. Structurally, c-Fes is composed of three distinct regions. These include a unique N-terminal region with at least two putative coiled-coil motifs, a central SH2 domain, and a C-terminal kinase domain (*1*). While the arrangement and sequence of the Fes SH2 and kinase domains are homologous to c-Src and c-Abl, Fes lacks an SH3 domain, a negative regulatory tail and other elements known to contribute to the regulation of these nonreceptor tyrosine kinases. However, like c-Src and c-Abl, wild-type c-Fes tyrosine kinase activity is tightly regulated in mammalian cells, and induces little or no transforming activity upon ectopic expression in rodent fibroblasts (*3*, *4*). However, the mechanism responsible for the negative regulation of Fes kinase activity in vivo is not clear.

One major unanswered question relates to whether down-regulation of Fes kinase activity is intrinsic to the structure of the kinase, or requires interaction with a host cell factor. Recent data from our laboratory suggest that negative regulation is kinase-intrinsic, and involves coiled-coil (CC)[1] oligomerization domains found in the unique N-terminal region (*5*−*7*). Coiled-coils consist of amphipathic α-helices with a heptad repeat pattern in which hydrophobic residues in the first and fourth positions align to form an interface between the interacting strands (*8*). The N-terminal region of Fes is both necessary and sufficient for oligomerization in vitro (*7*), suggesting that interconversion of monomeric and oligomeric forms of the kinase regulate its autophosphorylation in vivo. Two types of evidence support this model. First, insertion, deletion, or point mutations in the more N-terminal coiled-coil domain (CC1) release Fes from negative regulation in vivo, and lead to strong transforming activity in fibroblasts (*5*, *6*). This observation suggests that CC1 has a negative impact on regulation, possibly by interacting with CC2; disruption of this interaction by CC1 mutation leads to oligomerization via CC2 and activation. Second, overexpression of the isolated Fes N-terminal region suppresses the kinase and transforming activities of an activated Fes mutant, possibly by forming an inactive complex consisting of full-length and truncated Fes mono-

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[1] Abbreviations: SH, Src homology; CC, coiled-coil; SD/-U, synthetic drop-out medium lacking uracil; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride).

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mers (*6*, *7*). However, these data could also be explained by an inhibitory trans-acting factor that binds to the Fes N-terminal region, possibly at CC1. Mutation of the Fes CC1 domain may disrupt interaction with the putative repressor, allowing for activation. Similarly, overexpression of the unique N-terminal region may titrate the factor away from Fes, promoting kinase activation.

In the present study, we describe a *Saccharomyces cerevisiae* expression system for Fes that helps to distinguish between these possibilities. Yeast expression has proven to be a valuable tool for the analysis of cytoplasmic protein−tyrosine kinase structure−activity relationships. Both c-Src and c-Abl have been studied extensively in this eukaryotic, nonmammalian cell background (*9*−*14*). In the case of c-Src, overexpression causes strong growth suppression that correlates with unregulated kinase activity. However, coexpression of c-Src with Csk, the kinase responsible for suppression of Src activity in mammalian cells (*15*), is sufficient to induce c-Src down-regulation and permit yeast cell growth (*12*, *14*). Here we show that the tyrosine kinase activity of wild-type human c-Fes is tightly regulated upon overexpression in yeast, and that yeast cell growth is unaffected. Introduction of mutations in the CC1 or CC2 domains induced a marked stimulation of c-Fes kinase activity which correlated with the appearance of the growth-inhibitory phenotype. In addition, substitution of the Fes SH2 domain with the SH2 domain of v-Src also reversed negative regulation, implicating the SH2 domain in the negative regulatory mechanism as well. These data suggest that Fes normally adopts an inactive conformation in vivo that is independent of mammalian negative regulatory factors, and provide further evidence for control of the Fes kinase domain by its noncatalytic regions.

MATERIALS AND METHODS

Fes Expression Constructs. Fes mutants used in this study include a kinase-defective mutant with a Glu for Lys substitution in the ATP-binding site (K590E) (*16*), deletion mutants lacking either the first or second coiled-coil motifs (ΔCC1 and ΔCC2) (*6*), and Leu to Pro substitution mutants that disrupt either the first coiled-coil (L145P), the second coiled-coil (L334P), or both (2LP) (*5*). Chimeric Fes contructs in which the wild-type SH2 domain was replaced with the SH2 domain of v-Src (Fes/Src SH2), v-Fps (Fes/Fps SH2), or the p120 Ras GTPase-activating protein (Fes/Gap SH2) have been described elsewhere (*17*). All Fes constructs used in this study have a C-terminal Flag epitope tag. The mutant Fes cDNAs, as well as wild-type Fes, were subcloned into the yeast expression plasmid pESC−Ura (Stratagene) under the control of the galactose-inducible GAL1 promoter. As a positive control, we also created a pESC-v-Src construct.

Yeast Expression System. All experiments employed the *S. cerevisiae* strain YPH 499 (Stratagene). Yeast cells were transformed with the pESC−Ura expression constructs by electroporation, and selected on standard synthetic drop-out medium lacking uracil (SD/-U) with glucose as carbon source to repress protein expression. All liquid cultures were grown at 30 °C with shaking. To monitor the effect of Fes protein expression on yeast cell growth in liquid culture, transformed yeast colonies were first grown in SD/-U medium with raffinose as carbon source, which neither represses nor induces the GAL1 promoter. Cultures were grown to an OD_{600} of 1.0, and resuspended in fresh SD/-U in the presence of either glucose (to repress protein expression) or galactose (to induce protein expression). Cultures were incubated for an additional 6 h, diluted to an OD_{600} of 0.2, and OD_{600} readings were taken every 3 h thereafter for 15 h. For the plate-based growth assay, transformed colonies were started in SD/-U medium in the presence of raffinose as described above, and grown to an OD_{600} of 1.0. Cultures were serially diluted in SD/-U medium and spotted onto SD/-U agar plates containing either glucose or galactose. Plates were incubated at 30 °C until growth of the cultures spotted from the highest dilution was clearly visible on the glucose plates (usually 3 days). All experiments were repeated a minimum of three times with comparable results; representative examples are shown.

Yeast Lysate Preparation and Immunoblotting. Transformed yeast colonies were started in SD/-U medium in the presence of raffinose as described above, and grown to an OD_{600} of 1.0. Cultures were resuspended in fresh SD/-U in the presence of either glucose or galactose and incubated for an additional 6 h. Cultures were then diluted in fresh medium and incubated for 13 h. Equal amounts of cells were harvested by centrifugation (equivalent of a 30 mL culture with an OD_{600} of 1.0), and resuspended directly in 500 μL of SDS−PAGE sample buffer. Samples were vortexed for 30 s and incubated at 95 °C for 5 min. Lysates were clarified by centrifugation, and proteins were separated on 4−12% SDS−polyacrylamide gradient gels. Proteins were transferred to PVDF membranes, and probed for Fes protein expression with either the M2 anti-Flag monoclonal antibody (Sigma), or with an anti-Fes polyclonal anti-serum (generously provided by Dr. Peter A. Greer, Queen's University, Ontario, Canada). Src protein expression was detected with an anti-Src polyclonal antibody (Santa Cruz). Kinase activity was monitored by probing immunoblots with the anti-phosphotyrosine monoclonal antibody, PY20 (Transduction Laboratories). To detect Fes autophosphorylation, phosphospecific antibodies were raised in rabbits against a synthetic phosphopeptide based on the Fes activation loop sequence EADGVY^{p}AASGGLR, which contains the major autophosphorylation site, Tyr 713 (*18*). Phosphopeptide synthesis, immunization, and antibody production were performed by ResGen, Huntsville, AL.

RESULTS AND DISCUSSION

Wild-Type Fes Expression Does Not Affect Yeast Cell Growth. Previous studies have shown that Fes tyrosine kinase activity is tightly regulated in mammalian cells, even upon high-level overexpression (*3*, *5*, *6*, *18*, *19*). These findings suggest that Fes may naturally fold into an inactive conformation, interact with negative regulatory host cell factors, or undergo posttranslational modifications that suppress kinase activity. To distinguish between these possibilities, we turned to a yeast expression system, which lacks regulatory factors for mammalian tyrosine kinases (see introduction). We first compared the effect of Fes expression to that of v-Src, which has previously been shown to inhibit yeast cell growth because of its constitutive tyrosine kinase activity (*12*, *14*). Wild-type and kinase-defective Fes (Figure 1) as well as v-Src were cloned into a galactose-inducible yeast expression plasmid, which was used to transform *S.*

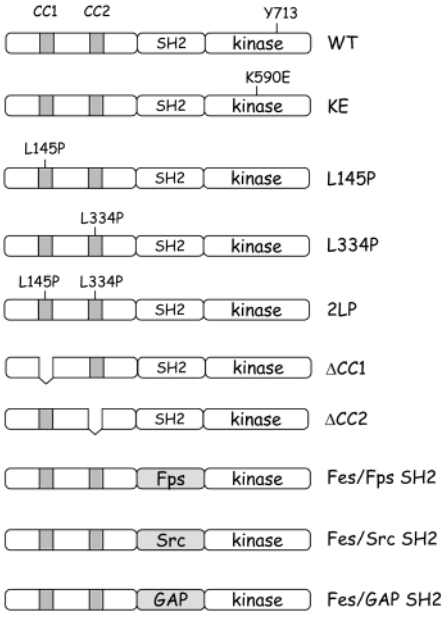

FIGURE 1: Fes constructs used in this study. The structure of wild-type human Fes is shown at the top, which includes a unique N-terminal region, an SH2 domain and a C-terminal kinase domain. The position of the major tyrosine autophosphorylation site is indicated (Y713). The kinase-defective mutant (KE) has a Glu substitution for the conserved Lys in the ATP-binding site (K590E). The locations of the two coiled-coil homology regions are indicated as the shaded boxes (CC1 and CC2). Coiled-coil mutants include Pro substitution of Leu 145 in the first coiled-coil domain (L145P), Pro substitution of Leu 334 in the second coiled-coil domain (L334P), and a double mutant with both Pro substitutions (2LP) (*5*). Coiled-coil deletion mutants are also shown, lacking either CC1 (ΔCC1) or CC2 (ΔCC2) (*6*). The three SH2 domain chimeras are shown at the bottom, in which the wild-type Fes SH2 domain is replaced with the SH2 of v-Fps (Fes/Fps SH2), v-Src (Fes/Src SH2), or Ras Gap (Fes/Gap SH2) (*17*). All Fes constructs encode a C-terminal FLAG epitope tag (not illustrated).

cerevisiae. Following selection, transformed colonies were grown in liquid culture in the presence of galactose to induce protein expression, and growth was followed as the increase in optical density over time. As shown in Figure 2, wild-type Fes, kinase-defective Fes, and cells carrying the empty expression plasmid all produced identical growth curves, suggesting that Fes does not affect yeast cell growth. In contrast, induction of v-Src expression produced marked growth suppression, consistent with previous studies. As a control, the same yeast clones were grown on glucose as a sole carbon source, which represses expression from the GAL1 promoter. As expected, all four transformed strains produced identical growth curves in the presence of glucose (Figure 2, bottom panel).

To verify the result obtained in liquid culture, the experiment was repeated on minimal agar plates containing either glucose or galactose as the sole carbon source. Yeast transformed with the same Fes and Src expression plasmids were grown in liquid culture in the presence of raffinose, which neither induces nor represses the GAL1 promoter, and serial dilutions of each culture were plated as shown in Figure 3. Cells expressing either form of Fes grew to an identical extent as the vector control on galactose plates, while cells expressing Src showed very little growth, even at the lowest dilution. In contrast, all four cultures grew to the same extent in the presence of glucose, in agreement with the results from liquid culture (Figure 2).

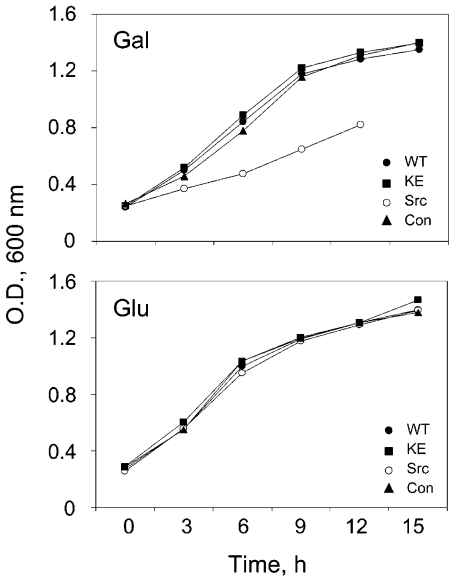

FIGURE 2: Expression of wild-type c-Fes does not affect yeast cell growth in liquid culture. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), kinase-dead c-Fes (KE), v-Src (Src), or the parent expression plasmid as a negative control (Con). Single colonies were started in liquid culture with raffinose as the carbon source, which neither represses nor induces the GAL1 promoter. Cultures were then resuspended in fresh medium in the presence of either glucose (to repress protein expression) or galactose (to induce protein expression), and OD_{600} readings were taken every 3 h for 15 h.

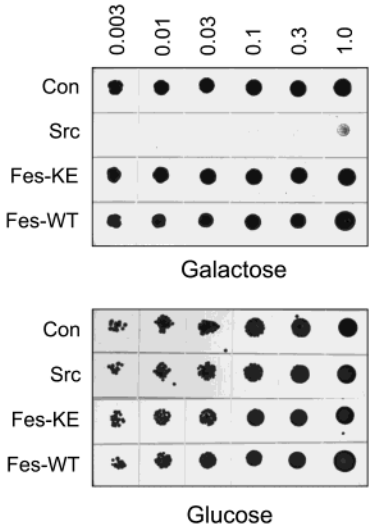

FIGURE 3: Expression of wild-type c-Fes does not affect yeast cell growth in a plate-based growth assay. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), kinase-dead c-Fes (KE), v-Src (Src), or the parent expression plasmid as a negative control (Con). Single colonies were started in liquid culture with raffinose as the carbon source, and grown to an OD_{600} of 1.0. Half-log serial dilutions (shown at the top) were then spotted onto agar plates containing either glucose or galactose, and incubated until growth of the cultures spotted from the highest dilution (0.003) was clearly visible on the glucose plates.

The finding that wild-type Fes, unlike Src, does not affect yeast cell growth suggests that either Fes kinase activity is tightly repressed or that its kinase activity does not produce the growth inhibitory phenotype observed with c-Src and c-Abl. To distinguish between these two possibilities, we performed anti-phosphotyrosine immunoblot analysis on

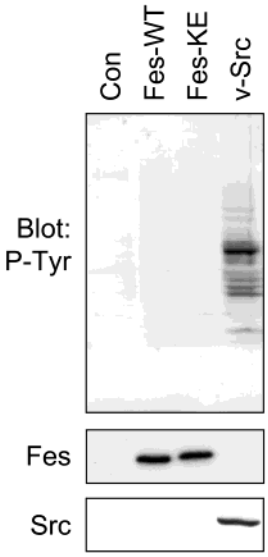

FIGURE 4: Wild-type Fes kinase activity is repressed in yeast. Yeast cells transformed with pESC−Ura expression plasmids carrying wild-type c-Fes (WT), kinase-dead c-Fes (KE), v-Src (Src), or the parent vector (Con) were grown in minimal medium containing galactose to induce protein expression. Protein extracts were prepared by lysing equal cell numbers directly in SDS−PAGE sample buffer. Proteins were separated by SDS−PAGE, transferred to PVDF membranes, and immunoblotted for total phosphotyrosine content with anti-phosphotyrosine antibodies (P-Tyr), Fes protein with an anti-Fes polyclonal antibody (Fes), or v-Src expression with a Src-specific anti-peptide antibody (Src).

whole cell protein extracts from the Src- and Fes-transformed yeast cultures. As shown in Figure 4, extracts from yeast cells expressing wild-type Fes showed undetectable levels of tyrosine phosphoproteins. In contrast, protein extracts from Src-expressing cells showed many tyrosine-phosphorylated bands, indicative of unregulated tyrosine kinase activity. As expected, extracts from cells transformed with kinase-defective Fes or the vector control also showed no evidence of protein-tyrosine phosphorylation. Control blots show that Fes proteins as well as Src were expressed in the yeast cells following induction with galactose (Figure 4). In contrast, no Fes or Src expression or evidence of tyrosine phosphorylation was observed in cells grown in the presence of glucose (data not shown). Taken together, the growth kinetics and anti-phosphotyrosine immunoblots suggest that Fes adopts an inactive conformation in yeast that does not affect cell growth.

Coiled-Coil Mutations Release Fes Tyrosine Kinase Activity and Induce Growth Arrest in Yeast. A major distinguishing feature of the c-Fes tyrosine kinase is the presence of two coiled-coil homology domains in its N-terminal region (*7*) (Figure 1). These domains control the state of Fes oligomerization, which may contribute to Fes activation in vivo (*1*, *2*). Recent work from our laboratory has shown that the first coiled-coil domain (CC1) is essential for the negative regulation of Fes tyrosine kinase activity in mammalian cells. Deletion, insertion, or point mutations in the first coiled-coil substantially increase the tyrosine kinase activity of Fes in fibroblasts, leading to cellular transformation (*5*, *6*). These findings led to the hypothesis that intramolecular interaction of CC1 with CC2 may suppress Fes activation by holding the molecule in an inactive, monomeric conformation. Such a model predicts that mutation of CC1 or CC2 would disrupt the proposed intramolecular interaction, and permit oligomerization and autophosphorylation via the remaining coiled-coil domain. Because wild-type Fes kinase activity is suppressed in yeast, this system provides an excellent system to test this model.

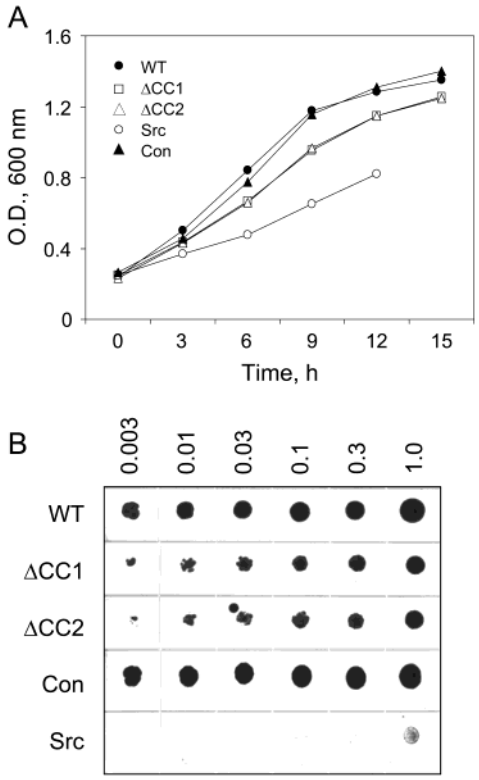

FIGURE 5: Fes coiled-coil domain deletion mutants induce growth arrest in yeast. (A) Analysis in liquid culture. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), deletion mutants lacking either the first (ΔCC1) or second (ΔCC2) coiled-coil domains, v-Src (Src) or the parent plasmid as a negative control (Con). Single colonies were started in liquid culture with raffinose as the carbon source, and then resuspended in fresh medium in the presence of galactose to induce protein expression. OD_{600} readings were taken every 3 h for 15 h. (B) Plate assay. Single colonies of the transformed cultures described in panel A were started in liquid culture with raffinose as carbon source, and grown to an OD_{600} of 1.0. Half-log serial dilutions (shown at the top) were then spotted onto minimal agar plates containing galactose as sole carbon source, and incubated until growth of the control culture spotted from the highest dilution (0.003) was clearly visible. Equivalent growth of all cultures was observed on glucose medium (data not shown).

We first examined the effect of Fes CC1 and CC2 deletion mutants on yeast cell growth, both in liquid culture and on plates. As shown in Figure 5, Fes mutants lacking either CC1 or CC2 markedly suppressed yeast cell growth following induction of protein expression with galactose in both assays. In contrast, cells expressing wild-type Fes grew with kinetics identical to those transformed with the empty expression vector, and are consistent with the data presented in Figures 2 and 3. Although these Fes mutants induced a growth-suppressive phenotype, the magnitude of the effect was not as strong as that observed with Src. The significance of this difference is not clear, and may represent qualitative differences in substrate selection related to growth suppression or simple differences in the expression levels of the two kinases.

We next examined the impact of Fes coiled-coil domain point mutants in the yeast growth assay. Coiled-coil domains consist of a heptad repeat structure, in which the first and

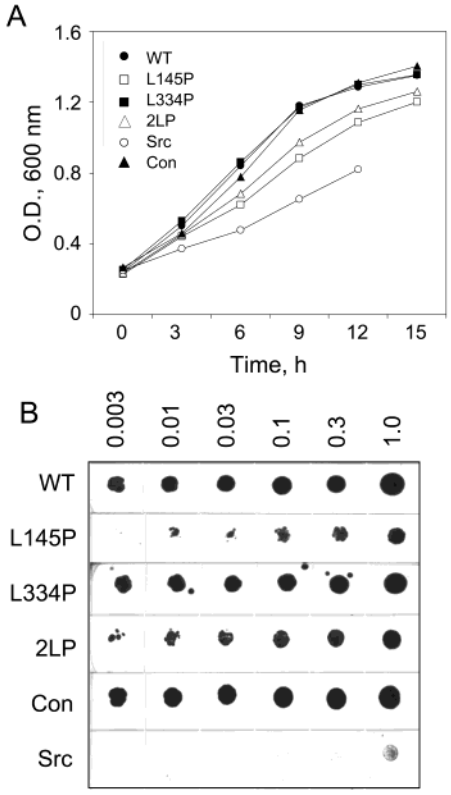

FIGURE 6: A Fes mutant with a point mutation in the first coiled-coil domain induces growth arrest in yeast. (A) Analysis in liquid culture. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), Fes mutants with Pro substitutions for Leu 145 in the first coiled-coil domain (L145P), or Leu 334 in the second coiled-coil domain (L334P), a double mutant with both coiled-coil Pro substitutions (2LP), v-Src (Src), or the parent plasmid as a negative control (Con). Single colonies were started in liquid culture with raffinose as sole carbon source, and then resuspended in fresh medium in the presence of galactose to induce protein expression. OD_{600} readings were taken every 3 h for 15 h. (B) Plate assay. Single colonies of the transformed cultures described in panel A were started in liquid culture with raffinose as carbon source, and grown to an OD_{600} of 1.0. Half-log serial dilutions (shown at the top) were then spotted onto minimal agar plates containing galactose as sole carbon source, and incubated until growth of the control culture spotted from the highest dilution (0.003) was clearly visible. Equivalent growth of all cultures was observed on glucose medium (data not shown).

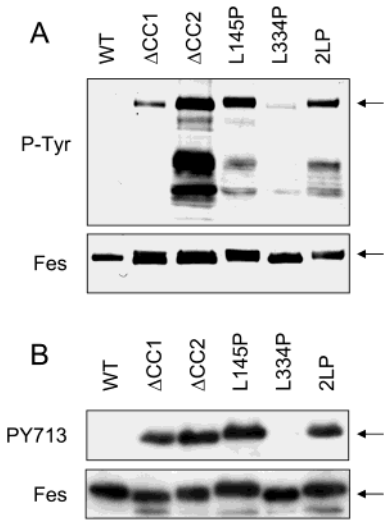

FIGURE 7: Analysis of Fes coiled-coil domain mutant kinase activity following expression in yeast. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), deletion mutants lacking either the first (ΔCC1) or second (ΔCC2) coiled-coil domains, point mutants with Pro substitutions for Leu 145 in the first coiled-coil domain (L145P) or Leu 334 in the second coiled-coil domain (L334P), or a double mutant with both Pro substitutions (2LP). Transformed cultures were grown in minimal medium containing galactose to induce expression. Yeast proteins were solubilized directly in SDS−PAGE sample buffer, separated by SDS−PAGE, and transferred to PVDF membranes. (A) Membranes were probed for total phosphotyrosine content with anti-phosphotyrosine antibodies (P-Tyr) or Fes protein using the M2 anti-FLAG monoclonal antibody (Fes). (B) Membranes were probed with phosphospecific antibodies raised against a synthetic phosphopeptide corresponding to the Fes activation loop sequence surrounding the autophosphorylation site at Tyr 713 (PY713), or Fes protein using an anti-Fes polyclonal antibody (Fes). The positions of the Fes proteins are indicated by the arrows.

fourth positions are occupied by Leu or other hydrophobic residues that pack together to form the interface between the coiled strands (*8*). In previous studies, we found that substitution of a central Leu in CC1 with Pro (L145P) released Fes from negative regulation in fibroblasts as well as myeloid cells, a physiological site of Fes expression (*5*). A similar Leu-to-Pro substitution in the second coiled-coil (L334P) was without effect. However, combining the first and second coiled-coil point mutations (2LP) reduced the transforming activity of Fes by 50% in fibroblasts and completely blocked Fes kinase activity and differentiation signaling in myeloid cells. Expression of the Fes coiled-coil point mutants in yeast produced results remarkably similar to those observed previously in fibroblasts. As shown in Figure 6, the CC1 point mutant (L145P) strongly inhibited growth both in liquid culture and the plate assay. The CC2 point mutant (L334P) had no detectable effect on cell growth by itself, but led to an intermediate growth-inhibitory phenotype when combined with the CC1 mutation (2LP mutant).

The kinase activity of all of the Fes coiled-coil domain mutants was compared by anti-phosphotyrosine immunoblotting. Yeast cultures expressing each of the mutants described above as well as wild-type Fes were induced with galactose, and whole-cell protein extracts were analyzed by anti-phosphotyrosine immunoblotting. As shown in Figure 7A, the relative level of protein-tyrosine phosphorylation observed with each of the mutants correlates with its effect on growth. Protein extracts from cultures expressing ΔCC1 and ΔCC2 showed tyrosine phosphorylated bands of approximately 92 kDa, which corresponds to the apparent molecular masses of these deletion mutants. Autophosphorylation of the CC1 point mutant, L145P, was also readily detected, consistent with its growth-inhibitory phenotype. In contrast to the deletion mutants and L145P, wild-type Fes and the CC2 point mutant (L334P) were essentially devoid of activity, which agrees with their inability to inhibit yeast cell growth. The phosphotyrosine content of the 2LP double point mutant was intermediate between that observed with L145P and L334P, which is consistent with the partial reversion of the L145P growth-inhibitory phenotype that occurred following introduction of the second coiled-coil mutation (Figure 6). Control blots show that each Fes protein was expressed to about the same level in each culture (Figure 7A, lower panel).

To confirm that Fes autophosphorylation was occurring within the c-Fes activation loop on Tyr 713, the major site of Fes autophosphorylation (*18*), duplicate blots were probed with antibodies raised against a synthetic phosphopeptide corresponding to this sequence. As shown in Figure 7B, the Fes phosphospecific antibody failed to detect wild-type Fes, consistent with the result shown in Figure 7A and the idea that wild-type Fes adopts an inactive conformation in yeast. Both coiled-coil deletions as well as the CC1 point mutation (L145) all strongly induced autophosphorylation of Tyr 713, consistent with the anti-phosphotyrosine immunoblots. The CC2 single point mutation (L334P) was without effect, while the double mutant (2LP) produced a reduced level of autophosphorylation relative to L145P. These results provide direct evidence that the Fes coiled-coil domains regulate kinase activity by affecting autophosphorylation of the activation loop tyrosine.

Results presented in Figures 5−7 support previous models in which the coiled-coil domains cooperate to control Fes kinase activity in vivo. The CC1 domain clearly functions in the negative regulation of kinase activity, as deletion or proline substitution within this region led to a strong increase in kinase activity and induced growth suppression. These findings agree with our previous observations in both rodent fibroblasts and human myeloid leukemia cell lines (*5*, *6*), and argue that negative regulation by CC1 is intrinsic to the structure of the kinase and is not dependent upon mammalian host cell factors. Interestingly, deletion of CC2 also led to a marked increase in kinase activity and growth suppression similar to that observed with the CC1 mutants. The finding that deletion of CC1 or CC2 induces kinase activation in vivo supports the possibility that CC1 and CC2 may interact in cis to maintain an inactive monomeric state. In such a model, deletion of either CC1 or CC2 removes this putative regulatory interaction, allowing for oligomerization and subsequent autophosphorylation through the remaining coiled-coil. The finding that CC2 deletion strongly activates Fes in yeast is one important difference relative to fibroblasts, where CC2 deletion induced a smaller increase in kinase activity and transforming function (*6*). One possible explanation for this difference is that CC2 may be required for subcellular localization or recruitment of substrates related to full kinase activation and transformation in fibroblasts.

Although the level of Fes autophosphorylation correlates with growth suppression in yeast, tyrosine phosphorylation of endogenous yeast proteins by the Fes CC domain mutants correlates less well (Figure 7). For example, deletion of either CC1 or CC2 results in strong Fes autophosphorylation, whereas ΔCC2 induces stronger tyrosine phosphorylation of yeast proteins than ΔCC1. One explanation for this difference is that an intact CC1 domain may be essential for recruitment of substrate proteins, whereas both CC domains are required for negative regulation of the kinase domain through a mechanism such as the one described above. A similar argument could be applied to a comparison of the CC1 deletion mutant relative to L145P (CC1 point mutant). The L145P mutant shows greater phosphorylation of endogenous yeast proteins than ΔCC1, whereas both mutants undergo autophosphorylation to a similar extent. In this case, changing a single CC1 domain residue may be sufficient to deregulate kinase activity to the same extent as CC1 deletion while maintaining some substrate recruitment function. Indeed, phosphorylation of endogenous substrates by L145P appears to be intermediate between that of ΔCC2 and ΔCC1.

A final comment regards the CC2 domain point mutant, L334P. In all systems tested to date (fibroblasts, myeloid cells, and now yeast), this mutant displayed little or no evidence of kinase activity (*5*, *6*). This is particularly clear in the present study, where use of the phosphospecific antibody revealed no detectable autophosphorylation of the activation loop in the L334P mutant. This observation is consistent with the very low level of endogenous protein-tyrosine phosphorylation in yeast cells expressing this mutant. These observations suggest that unlike CC2 deletion, the CC2 point mutation may not be sufficient to disturb negative regulatory interactions with CC1 or possibly the SH2 domain. However, it does at least partially reverse the activating effect of the CC1 point mutation in all three systems in terms of biological activity (fibroblast transformation, myeloid differentiation, and growth suppression in yeast), suggesting that it may affect recruitment of substrate proteins relevant to the biological response.

The Fes SH2 Domain Regulates Kinase Activity in Yeast. In addition to the coiled-coil domains, previous studies in mammalian cells have established a role for the SH2 domain in the regulation of Fes tyrosine kinase activity. Early studies with v-Fps, an avian transforming homologue of c-Fes, suggested that the SH2 domain interacts with the kinase domain as part of the active kinase structure (*20*, *21*). Deletion of the c-Fes SH2 domain greatly reduces kinase activity in vitro, consistent with this earlier work (*16*). More recently, we found that substitution of the Fes SH2 domain with that of v-Src released Fes from negative regulation in fibroblasts, leading to constitutive tyrosine kinase activity and fibroblast transformation (*17*). This mutant also induced differentiation of myeloid leukemia cells, implying that the change in SH2 specificity primarily affected kinase regulation in vivo without disrupting normal signaling functions. However, the possibility remains that SH2 substitution may cause recruitment of an activating protein, resulting in the observed phenotypes. Therefore, we employed the yeast system to address whether activation of Fes by Src SH2 substitution was due to a change in the conformation of the modified kinase protein or dependent upon protein factors unique to mammalian cells.

For these experiments, we constructed yeast expression vectors for Fes proteins in which the wild-type SH2 domain was substituted with those of v-Fps, v-Src, and p120 Ras GAP (*17*). The SH2 sequence of v-Fps is very similar to that of c-Fes, with only four amino acid substitutions. The SH2 sequence of v-Src is more divergent from that of c-Fes, although the phosphopeptide recognition specificity of these two SH2 domains is very similar. The final SH2 chimera was constructed using the N-terminal SH2 domain of Ras Gap. Unlike the v-Fps and v-Src SH2 domains, the Gap SH2 domain displays a different tyrosine phosphopeptide binding specificity from that of c-Fes. In addition, substitution with this Gap SH2 domain has been previously shown to release the tyrosine kinase and transforming activities of c-Abl in fibroblasts (*22*), although a similar effect was not observed with c-Fes (*17*). Yeast cultures were transformed with each of these vectors and tested for effects on cell growth both in liquid culture and in the plate-based assay. As shown in Figure 8, the Fes/Src SH2 domain chimera produced a

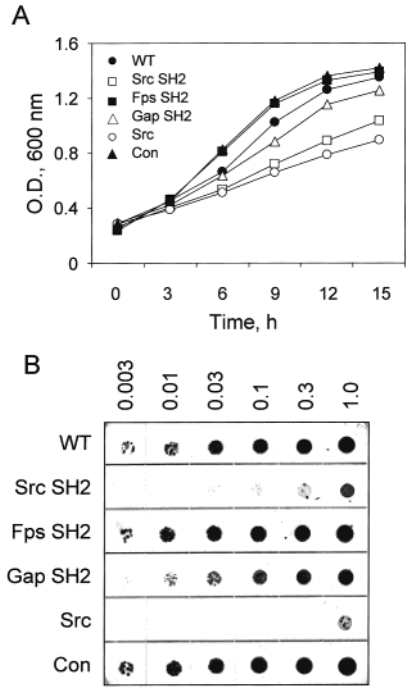

FIGURE 8: Analysis of Fes SH2 domain chimeras on yeast cell growth. (A) Analysis in liquid culture. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), or Fes SH2 domain chimeras in which the wild-type SH2 domain was replaced with the SH2 of v-Fps (Fes/Fps SH2), v-Src (Fes/Src SH2), or Ras Gap (Fes/Gap SH2). pESC-v-Src (Src) or the parent plasmid (Con) were included as controls. Single colonies were started in liquid culture with raffinose as the carbon source, and then resuspended in fresh medium in the presence of galactose to induce protein expression. OD_{600} readings were taken every 3 h for 15 h. (B) Single colonies of the transformed cultures described in panel A were started in liquid culture with raffinose as carbon source, and grown to an OD_{600} of 1.0. Half-log serial dilutions (shown at the top) were then spotted onto minimal agar plates containing galactose as sole carbon source, and incubated until growth of the control culture spotted from the highest dilution (0.003) was clearly visible.

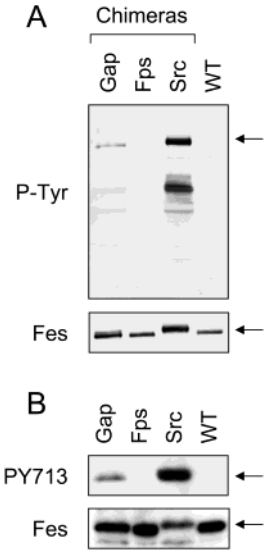

FIGURE 9: Analysis of Fes SH2 chimera kinase activity following expression in yeast. Yeast cells were transformed with the galactose-inducible expression plasmid pESC−Ura carrying wild-type c-Fes (WT), or Fes SH2 domain chimeras in which the wild-type SH2 domain was replaced with the SH2 of v-Fps (Fes/Fps SH2), v-Src (Fes/Src SH2), or Ras Gap (Fes/Gap SH2). Transformed cultures were grown in minimal medium containing galactose to induce expression. Yeast proteins were solubilized directly in SDS−PAGE sample buffer, separated by SDS−PAGE, and transferred to PVDF membranes. (A) Membranes were probed for total phosphotyrosine content with anti-phosphotyrosine antibodies (P-Tyr) or Fes protein using the M2 anti-FLAG monoclonal antibody (Fes). (B) Membranes were probed with phosphospecific antibodies raised against a synthetic phosphopeptide corresponding to the Fes activation loop sequence surrounding the autophosphorylation site at Tyr 713 (PY713), or Fes protein using an anti-Fes polyclonal antibody (Fes). The positions of the Fes proteins are indicated by the arrows.

dramatic suppression of yeast cell growth in both assays, almost as strong as v-Src itself. In contrast, the Fes/Fps SH2 domain chimera had little impact on cell growth, a result not unexpected given the close sequence similarity between the Fes and Fps SH2 domains. The Fes/Gap SH2 chimera produced an intermediate phenotype, producing partial growth suppression in both assays.

We next correlated the effects of Fes SH2 domain substitution on yeast growth with Fes tyrosine kinase activity by immunoblot analysis. Figure 9A shows that all of the SH2 chimeras were expressed at similar levels, and that activity correlated closely with effects on growth suppression. The Fes/Src SH2 domain chimera, which was strongly growth-inhibitory, exhibited very high levels of autophosphorylation and transphosphorylation of yeast cell proteins as well. Immunoblots with the phosphospecific antibody show that autophosphorylation occurred within the activation loop on Tyr 713 (Figure 9B). The striking similarity between the Fes/Src SH2 chimera and v-Src itself in terms of growth suppression and kinase activity suggests that the Src SH2 domain may be involved in recruitment of yeast cell proteins important to the growth inhibitory phenotype. The Fes/GAP SH2 chimera showed some evidence of autophosphorylation, consistent with its partial effect on yeast cell growth. In contrast, the Fps SH2 domain chimera did not reveal evidence of autophosphorylation, consistent with its inability to elicit a growth-inhibitory phenotype. Taken together, these results show that the native SH2 structure is essential for regulation of Fes autophosphorylation and kinase activity in vivo.

Cooperation of Coiled-Coil and SH2 Domains in the Regulation of Fes Kinase Activity. Data presented in this report provide new evidence that the negative regulation of c-Fes is intrinsic to the structure of the kinase and is independent of mammalian host cell factors. These data support a role for both the N-terminal coiled-coils and the SH2 domain in the down-regulation of c-Fes in vivo. As described above, previous work in fibroblasts and myeloid leukemia cells has led to a model in which the coiled-coil domains may interact in cis to suppress Fes oligomerization and autophosphorylation as a possible negative regulatory mechanism (*5*, *6*). Work presented here supports this model, as deletion of either CC1 or CC2 leads to potent Fes activation and the growth-suppressive phenotype. En bloc substitution of the wild-type SH2 domain also released Fes kinase activity in yeast. This effect was most dramatic with the Src SH2 domain, as observed previously in fibroblasts (*17*). These finding raise the question of how the N-terminal coiled-coils and SH2 domain act in concert to down-regulate kinase activity. Previous work has suggested that intramolecular SH2-kinase interaction may be important for full kinase activation (*16*, *20*, *21*). One possibility is that

intramolecular coiled-coil interactions may block SH2 access to the kinase domain. Interestingly, the Src SH2 domain has been shown to bind to the Fes kinase domain in vitro (*16*), suggesting that it may support the formation of the active kinase structure in the chimera without being affected by N-terminal regulation. Molecular modeling of the SH2-kinase region of Fer, a very close homologue of c-Fes, suggests that intramolecular SH2-kinase interaction is supported by an ion pair involving E473 in the αA helix of the SH2 domain and K660 in the αE helix of the kinase domain (*23*). Both of these residues are conserved in Fes, and the Src SH2 domain has the homologous glutamic acid residue, suggesting that this interaction may contribute to activation in the Fes/Src SH2 domain chimera. Activation of Fes by recruitment of N-terminal binding partners may have two effects—to disrupt intramolecular coiled-coil interactions and promote oligomerization, and to remove a constraint on the SH2 domain that permits its association with the kinase domain and subsequent activation. Future studies will address these possibilities.

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