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Fes Mediates the IL-4 Activation of Insulin Receptor Substrate-2 and Cellular Proliferation

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Although Jak kinases are essential for initiating cytokine signaling, the role of other nonreceptor tyrosine kinases in this process remains unclear. We have examined the role of Fes in IL-4 signaling. Examination of Jak1-deficient cell lines demonstrates that Jak1 is required for the activation of Fes by IL-4. Experiments studying signaling molecules activated by IL-4 receptor suggest that IL-4 signaling can be subdivided into Fes-dependent and Fes-independent pathways. Overexpression of kinase-inactive Fes blocks the IL-4 activation of insulin receptor substrate-2, but not STAT6. Fes appears to be a downstream kinase from Jak1/Jak3 in this process. Further examination of downstream signaling demonstrates that kinase-inactive Fes inhibits the recruitment of phosphoinositide 3-kinase to the activated IL-4 receptor complex and decreases the activation of p70S6k kinase in response to IL-4. This inhibition correlates with a decrease in IL-4-induced proliferation. In contrast, mutant Fes does not inhibit the activation of Akt by IL-4. These data demonstrate that signaling pathways activated by IL-4 require different tyrosine kinases. This differential requirement predicts that specific kinase inhibitors may permit the disruption of specific IL-4-induced functions. The Journal of Immunology, 2001, 166: 2627–2634.

The cytokine IL-4 is an important regulator of immune function. For example, IL-4 plays a central role in promoting the differentiation of Ag-stimulated naive T cells to develop into Th2 cells and is also responsible for the induction of B cell Ig class switching to IgE (1). In addition, IL-4 acts as a comitogen for T and B cell growth (2, 3). Like other cytokines, IL-4 induces the activation of signal transduction pathways that are used to regulate cell growth and differentiation (4). In hemopoietic cells, IL-4 signaling pathways are initiated by the oligomerization of the IL-4 receptor α and γ subunits. This juxtaposes their cytoplasmic domain-binding protein tyrosine kinases (PTKs) Jak1 and Jak3, which results in the activation of these kinases. As a result of Jak activation, the α-chain of the IL-4 receptor (IL-4Rα) is tyrosine phosphorylated. Phosphotyrosyl motifs on IL-4Rα serve as docking sites for the recruitment of signal molecules such as insulin receptor substrate-1 or -2 (IRS-1/2) and STAT6 (5–7). Others and we have reported that STAT6 is phosphorylated and activated in response to IL-4 (8–10). Jak1, and in some cases Jak3, is required for maximal activation of STAT6 (11, 12). IRS-1/2 can be recruited to Tyr-495 (Y1) of IL-4Rα after IL-4 stimulation and is subsequently tyrosine phosphorylated (6). Although it has been reported that Jak1 is essential for the cytokine-mediated activation of IRS-1 in fibroblast cells (13–15), whether IRS is a direct substrate of Jak1 remains unclear.

Recent studies on cytokine signaling have suggested that tyrosine kinases other than Jaks are activated by cytokines and may play a role in transducing signals (16). One of these kinases is c-Fes, a Src-related fps PTK member. c-Fes expression is confined to hemopoietic cells, including immature myeloid progenitor cells (17) and lymphocytes (18). The high levels of Fes protein present in human myeloid leukemia are thought to reflect the importance of Fes in the regulation of proliferation and differentiation during myelopoiesis (19, 20). Furthermore, oncogenic v-fps/fes alleles have been frequently isolated as retroviral transforming genes (21). Structurally, fps/fes is quite distinct from cytoplasmic PTKs of the Src family because it lacks a negative regulatory tyrosine phosphorylation site in the carboxyl-terminal region. Fes is also not modified by N-terminal myristylation, suggesting that it is not targeted to the cell membrane (22). These distinct features of fps/fes suggest that it may use a distinct regulatory mechanism to regulate its kinase activity.

c-Fes has also been linked to signaling by cytokines, including GM-CSF, Epo, IL-3, IL-6, and IL-4 (23–26). Each of these cytokines induces the association of Fes with its respective receptor. Thereafter, Fes becomes tyrosine phosphorylated and activated. The mechanism by which Fes associates with these cytokine receptors and is activated upon stimulation with cytokines is currently unclear. In addition, the downstream signaling events mediated through Fes remain unidentified.

Our interest has been in studying the mechanisms leading to Fes activation and the importance of this activation in IL-4 signal transduction. Our data indicate that the activation of c-Fes, in response to IL-4, is mediated through Jak1 in response to IL-4. Remarkably, signals further downstream can be categorized into Fes dependent (IRS-1/2, phosphoinositide 3-kinase (PI3-kinase), and p70 ribosomal protein S6 kinase (p70S6k)) and Fes independent (STAT6 and Akt) pathways. The differing requirement for this
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kinase in downstream signaling pathways suggests a novel paradigm for IL-4 signaling, which may allow the development of inhibitors that effect a subset of IL-4-induced cellular events.

Materials and Methods

Cell culture

Murine B lymphoma cell line M12.4.1 was maintained in RPMI 1640 medium (Mediatech, Washington, DC) containing 10% heat-inactivated FCS (Sigma; St. Louis, MO), 50 μM β-ΜΕ, and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY). Pro-B BA/F3 and myeloid 32D cells were maintained in RPMI 1640 medium containing 10% FCS, 5% WEHI-3B-conditioned medium, 50 μM β-ΜΕ, and 1% penicillin-streptomycin. B2C5 cells (HeLa cell deficiency of Jak1) were cultured in IMDM supplemented with 10% FCS and 1% penicillin-streptomycin. HEK293T cells were maintained in DMEM containing 10% FCS and 1% penicillin-streptomycin.

Plasmid construction

Fes expression vectors were generated by removing Fes-Flag (using Bgl II and EcoRI sites) from pSF70-Fes.Flag (provided by T. Smithgall, University of Pittsburgh, Pittsburgh, PA) and subcloning to pRSV11 and EcoRI sites of pcDNA3. Jak1 and Jak3 were constructed by removing Jak1 (SalI and NotI sites) and Jak3 (NotI site) from BlueScript (provided by J. Krolewski, Columbia University, New York, NY) and subcloning to XhoI and NotI sites of pcDNA3.1 for Jak1 and NotI site of pcDNA3.1 for Jak3 construct. Point mutation of Fes(Y713F), Fes(Y811F), and Fes(R483L) were generated by site-directed mutagenesis using the Quick-change kit (Stratagene, La Jolla, CA).

Transient transfection

Transient transfections were conducted by means of LipofectAMINE (Life Technologies) as described by the manufacture with modification. HeLa or 293T cells were seeded in six-well plates (4 × 10^5 cells/well). After overnight culture, the cells were placed in DMEM serum-free medium and incubated with a DNA-LipofectAMINE complex for 6 h in the absence of serum. After transfection, the transfected medium was aspirated and the cells were washed twice with DMEM containing 10% serum followed by cultured in the same medium for 48 h.

Establishment of stable transfectants

M12.4.1, BA/F3, or 32D/IRS-1 cells (10^6) were transfected with 20 μg of expression plasmids pcDNA3-Fes.Flag (wild-type) or pcDNA3-Fes(Y713F)(Flag) (kinase-inactive) by the electroporation method setting at 960 MHz and 250 V. The cells were cultured in the RPMI 1640 medium containing 10% FCS overnight. The transfected cells were recovered over-night and were distributed to a 96-well plate. M12.4.1 cells were selected in the RPMI 1640 with 10% serum containing 800 μg/ml of G418 (Life Technologies), where BA/F3 or 32D cells were maintained in the RPMI 1640 with 10% serum and 5% WEHI-3B-conditioned medium containing 1 mg/ml of G418. Transfectants were isolated for 14–20 days after selection and screened for the expression of Fes by Western blot analysis using an anti-flag Ab. The similar expression levels of either wild-type or kinase-inactive Fes were chosen for studies. Myeloid 32D/IRS-1 transfectants were first established as described (6). Briefly, 32D cells were transfected as described above with IRS-1 expression construct and selected in the medium containing 1 M Histidinol (Sigma). The transfectants were verified for the expression of IRS-1 and further transfected with expression plasmids pcDNA3-Fes.Flag or pcDNA3-Fes(Y713F)(Flag). The Fes(Y713F)(Flag) expressing cell lines were only used within ten passages.

Immunoprecipitation and Western blotting

Cells were suspended in regular lysis buffer (50 mM Tris-HCl pH 7.5, 0.5% Nonidet P-40, 100 mM NaCl, 0.1 mM EDTA, 10 mM Na_2VO_4, 1 mM PMSF, 5 μg/ml apronin, and 2 μg/ml leupeptin) or in membrane lysis buffer (regular lysis buffer plus 1% Triton X-100) for 30 min at 4°C. The lysates were cleared off debris by centrifugation at 12,000 × g for 15 min, and the supernatants were incubated with desired Abs at 4°C for 2–3 h after normalization of total proteins (Bio-Rad protein-assay kit; Bio-Rad, Richmond, CA). Immune complexes were captured by incubating with either protein A- or G- (agarose conjugated) for 1–2 h, and followed by washing with the lysis buffer three to four times. Proteins were eluted and electrophoresed on SDS-polyacrylamide gels, then transferred onto nitrocellulose membranes. The membranes were probed with Abs and visualized with the ECL detection system (Amersham, Arlington Heights, IL) as described by the manufacture’s instruction.

Proliferation assay

M12.4.1 (4 × 10^5 cell/well) or BA/F3 (2 × 10^5 cells/well) transfectants were seeded in 96-well plates and incubated in RPMI 1640 containing 0.1% serum. After 2 h, serum starvation, the cells were cultured alone or with IL-4 for an additional 48 h. The cells were labeled with [3H]thymidine (1 μCi/ml; NEN, Boston, MA) for the last 16 h and were then meshed and collected using cell harvester (PHD; Brandel, Bethesda, MD). The cellular contents captured on the membrane were subjected to scintillation counting.

Results

IL-4-activation of Fes requires Jak1 and correlates with receptor association

An early event in the initiation of cytokine signal transduction is the activation of tyrosine kinases. As with other tyrosine kinases, the activation of Fes depends upon the phosphorylation of a tyrosine residue located within the kinase catalytic domain (27, 28). To assess the activity of Fes following IL-4 stimulation, the kinetics of Fes tyrosine phosphorylation in response to this cytokine was examined. Ba/F3 is a murine pro-B cell line that expresses high levels of endogenous Fes. In these cells, Fes is phosphorylated as rapidly as 1 min following IL-4 treatment. Tyrosine phosphorylation of Fes reaches a maximum at 5 min and lasts for at least 1 h (Fig. 1A). To gain insight into the mechanism by which Fes is phosphorylated, the association of Fes with IL-4Rα-chain was examined. In extracts from cells cultured with IL-4, Fes co-immunoprecipitates with the IL-4Rα-chain (Fig. 1B). The time...
course of Fes association with IL-4Rα correlates with Fes phosphorylation, suggesting that phosphorylation of Fes may occur upon recruitment to IL-4Rα.

To determine whether the phosphorylation of Fes in response to IL-4 is due to autophosphorylation, we examined the IL-4-induced phosphorylation of kinase-inactive Fes (which lacks the ability to autophosphorylate). Stable transfectants expressing either wild-type Fes or kinase-inactive Fes (K90E) were generated. M12.4.1 B lymphoma cell lines, which express low levels of endogenous Fes, were used. Wild-type Fes expressed in M12.4.1 transfectants is phosphorylated in response to IL-4 (data not shown), with a phosphorylation pattern similar to that observed in BA/F3 cells. Interestingly, IL-4 also induces the tyrosine phosphorylation of kinase-inactive Fes (Fig. 2A). In these cells, the kinetics of Fes phosphorylation was similar to that of wild-type Fes (Fig. 1A and data not shown). This observation suggests that other kinase(s) might play a role in phosphorylating Fes. Jak1 and Jak3, two tyrosine kinases involved in the IL-4-activated STAT6 pathway, were examined in M12 cells for their phosphorylation patterns upon stimulation of IL-4. The kinetics of phosphorylation for both Jak1 and Jak3 are similar to that observed for Fes (data not shown). These data indicate a correlation between Fes and Jak kinases activation downstream of IL-4.

The kinetic studies of the phosphorylation of Jak1/3 and Fes suggest a potential link between these tyrosine kinases. As both Jak1 and Fes have been shown to associate with IL-4Rα (29–31), we asked whether Jak1 is required for the phosphorylation of Fes. A Jak1-deficient HeLa cell line (B2C6) was used to study the Jak1 dependence of Fes activation (Fig. 2B). We have previously used this cell line to study the requirement of Jak1 for the activation of STAT6 (12). Cotransfection of Fes (Flag-tag) with control vector in B2C6 cells does not result in the phosphorylation of Fes from either IL-4-stimulated or unstimulated cells. In contrast, Fes is tyrosine phosphorylated when Jak1 is reconstituted in the same cells. The phosphorylation of Fes is enhanced upon IL-4 stimulation. These results suggest that Jak1 is required for the phosphorylation of Fes in response to IL-4.

To examine the mechanism by which Jak1 is required for Fes activation, the possible physical association of Fes and Jak1 was examined. When epitope-tagged (Flag) Fes is immunoprecipitated, Western blotting demonstrates coimmunoprecipitation of Jak1 (Fig. 2C). The coimmunoprecipitation of Jak1 and Fes is observed in the absence of IL-4 treatment, and was slightly increased upon IL-4 stimulation. A similar pattern for coimmunoprecipitation of kinase-inactive Fes and Jak1 was also observed (data not shown). These data demonstrate that Fes and Jak1 can interact in M12.4.1 cells. This interaction, along with the requirement of Jak1 for Fes phosphorylation in response to IL-4, suggests that Fes may be a substrate for Jak1. Indeed, Jak1 can phosphorylate kinase-inactive Fes when these proteins are coexpressed in 293T cells (data not shown). Interestingly, Jak1 is unable to phosphorylate a double mutation of Fes (FesKE-Y713F) if these proteins are coexpressed. Because the phosphorylation of tyrosine 713 of Fes has been shown to be critical for Fes kinase activity (27, 28), these results further suggest that Jak1 can activate Fes.

Kinase-inactive Fes inhibits the phosphorylation of IRS-2 induced by IL-4

Because Fes is phosphorylated and activated in an IL-4-inducible, Jak1-dependent fashion, we investigated the involvement of Fes in IL-4-induced signaling. Previous reports have shown that IRS-1/2 can be recruited to Tyr-495 (Y1) of the activated IL-4Rα (6). Following this association with the activated IL-4 receptor complex, IRS-1/2 is tyrosine phosphorylated. Although it has been reported that Jak1 is required for the IL-4-induced phosphorylation of IRS-1 (32, 33), it remains unclear whether Jak1 is the kinase that directly phosphorylates IRS-1/2. BA/F3 cells express IRS-2, and its basal levels of phosphorylation remain high even after serum starvation. M12.4.1 cells express IRS-2, and culture of these cells with IL-4 induces the phosphorylation of IRS-2. M12.4.1 cell lines that overexpress control vector, wild-type (FesWT), FesKE (14), or kinase-inactive Fes (FesKE 8, FesKE 14) were used to examine the importance of Fes in the activation of IRS-2. When these transfectants are examined, the phosphorylation of IRS-2 in response to IL-4 is greatly decreased in cells expressing kinase-inactive Fes. In contrast, phosphorylation of IRS-2 is enhanced in cells expressing wild-type Fes (Fig. 3A). These data suggest that Fes performs a role in mediating IL-4-induced phosphorylation and activation of IRS-2.
The activation of STAT6 in IL-4 signaling requires the activation of Jak1 (12, 32). In contrast, the phosphorylation of STAT6 in response to IL-4 is not inhibited by overexpression of kinase-inactive Fes (Fig. 3B). These data suggest that Fes is not required for the activation of STAT6 in response to IL-4. To determine whether the overexpression of Fes alters the activation of JAK kinases, the tyrosine phosphorylation of Jak1 or Jak3 in response to IL-4 was examined. We observe that the IL-4-induced phosphorylation of Jak1 and Jak3 is not significantly different in the cell lines expressing wild-type and kinase-inactive Fes (data not shown). Taken together with prior results demonstrating the requirement of Jak1 for the activation of IRS-2, these results suggest that Fes acts downstream of Jak1 and Jak3 in mediating IL-4-induced phosphorylation of IRS-2.

**Kinase-inactive Fes inhibits the association of IRS-2 and PI-3 kinase**

IRS-1 and -2 are thought to act as an adapter protein that functions in a diverse spectrum of signaling pathways (34), including the activation of PI3-kinase by IL-4 (35). Therefore, we examined the effects of altering Fes activity on pathways downstream of IRS-2. When IL-4 is added to cultures of M12.4.1 cells expressing vector control or wild-type, and kinase-inactive Fes (FesWT 10, FesWT 14), IRS-2 associates with the p85 catalytic a subunit of PI3-kinase. However, the IL-4-induced association of IRS-2 and p85 is greatly diminished in cells expressing kinase-inactive Fes (FesKE 8, FesKE 14) (Fig. 4, A and B). This is likely secondary to the decreased levels of IRS tyrosine phosphorylation found in cells overexpressing kinase-inactive Fes. Therefore, these data suggest a model for an IL-4-induced and Fes-mediated signaling pathway in which Fes activates IRS-1/2 and subsequently triggers the activation of the PI-3 kinase pathway, leading to alteration of downstream cellular signaling.

**Kinase-inactive Fes inhibits the activation of p70\(^{66K}\) but not Akt**

The finding that overexpression of kinase-inactive Fes inhibits the association of IRS-2 with the p85 a subunit of PI3-kinase suggests that pathways regulated by PI3-kinase may be altered in these cells. PtdIns(3,4,5)P3, an enzymatic product of PI3-kinase, is thought to activate two different protein kinases, Akt/PKB and p70\(^{66K}\) (36, 37), although the mechanisms by which PtdIns(3,4,5)P3 regulates these two kinases appear to differ (38). Considerable evidence has suggested that the regulation of p70\(^{66K}\) activity involves a multiple-step phosphorylation (39). The phosphorylation (Thr\(^{421}\)/Ser\(^{424}\)) within an autoinhibitory motif of p70\(^{66K}\) is required for further phosphorylation of catalytic domain and achievement of substantial activation of p70\(^{66K}\). The phosphorylation of Akt on Ser\(^{273}\), presumably by phosphoinositide-dependent protein kinase-1 (PDK1), is required for the activation of Akt/PKB. To further investigate signaling events downstream of Fes, we examined the activation of p70\(^{66K}\) and Akt/PKB in response to IL-4 in cells expressing wild-type and kinase-inactive forms of Fes.

Because the basal activities of p70\(^{66K}\) and Akt/PKB can be deprived in murine myeloid 32D/IRS-1 (2) cells under serum/IL-3 starvation, we generated 32D/IRS-1 stable transfectants expressing vector control or wild-type, and kinase-inactive Fes (FesWT 10, FesWT 14), IRS-2 associates with the p85 catalytic a subunit of PI3-kinase. However, the IL-4-induced association of IRS-2 and p85 is greatly diminished in cells expressing kinase-inactive Fes (FesKE 8, FesKE 14) (Fig. 4, A and B). This is likely secondary to the decreased levels of IRS tyrosine phosphorylation found in cells overexpressing kinase-inactive Fes. Therefore, these data suggest a model for an IL-4-induced and Fes-mediated signaling pathway in which Fes activates IRS-1/2 and subsequently triggers the activation of the PI-3 kinase pathway, leading to alteration of downstream cellular signaling.

**FIGURE 3.** Inhibitory effects of kinase-inactive Fes on the phosphorylation of IRS-2 induced by IL-4. A and B, Control, wild-type, and kinase-inactive Fes expressing M12.4.1 cells were either unstimulated or stimulated with MuIL-4 (100 ng/ml) for 15 min. Cell lysates were made and used for sequential immunoprecipitation with Abs to IRS-2 (UBI) (A) and to STAT6 (UBI) followed by Western blotting with 4G10 (top) and reprobed with anti-IRS-2 (A) or -STAT6 (B) Abs (bottom).

**FIGURE 4.** Effects of Fes on the association of IRS-2 with p85 of PI3-kinase in response to IL-4. A and B, Wild-type and kinase-inactive Fes expressing M12.4.1 cells were stimulated with MuIL-4 (100 ng/ml) for 15 min or left unstimulated as shown (−). Cell extracts were made and used for immunoprecipitation with anti-IRS-2 (A) or anti-p85 (PI3-kinase) (UBI) (B) Abs. The immunoprecipitates were Western blotted with anti-p85 (A) and reprobed with anti-IRS-2 Abs, or with anti-IRS-2 (B) and reprobed with anti-p85 Abs.
cells and those overexpressing wild-type Fes (Fig. 5B). The lack of alternation of Akt phosphorylation in cells expressing kinase-inactive Fes is in stark contrast to the decreased phosphorylation of IRS-1 in these same cells, because we still observed that the phosphorylation of IRS-1 is inhibited in cells expressing kinase-inactive Fes (FesKE 8, FesKE 14), whereas the phosphorylation of IRS-1 is enhanced in the cells expressing wild-type Fes (Fes WT 7, Fes WT 13) (Fig. 5C). These results suggest that Fes selectively regulates the p70 S6K pathway and does not contribute to the activation of the Akt/PKB pathway in response to IL-4.

Fes mediates IL-4-activated proliferation of BA/F3 and M12.4.1 cells

The data above indicate that Fes can regulate the activation of IRS-2 in response to IL-4. Previous work has demonstrated that expression of either IRS-1 or IRS-2 is required for IL-4-induced proliferation (44, 45). Phosphorylation of IRS-2 and increased activity of PI3-kinase is important for the mitogenic effects of insulin and IGF-1 (46). To determine whether Fes is involved in regulating IL-4-induced cell proliferation, we measured IL-4-induced \[^{[3}H\]thymidine incorporation in M12.4.1 or BA/F3 cells expressing wild-type or kinase-inactive Fes grown under serum starvation or serum/IL-3 starvation conditions, respectively. IL-4 is able to induce 2- to 3-fold cell growth in parental M12.4.1 cells when compared with cells not treated with IL-4 (Fig. 6A). In two cell lines overexpressing wild-type Fes (Fes WT 10, Fes WT 14), enhanced \[^{[3}H\]thymidine incorporation in response to IL-4 (3- to 5-fold) is observed. However, in cell lines expressing the kinase-inactive Fes (FesKE 8, FesKE 14), we observe decreased \[^{[3}H\]thymidine incorporation after culture with IL-4 when compared with parental cells. In addition, we also examined the effect of Fes on \[^{[3}H\]thymidine incorporation in BA/F3 cells. IL-4 induces a 3- to 6-fold increase of \[^{[3}H\]thymidine incorporation in BA/F3 parental cells (Fig. 6B). BA/F3 cells expressing wild-type Fes (Fes WT 7, Fes WT 13) exhibit a 6- to 8-fold increase in \[^{[3}H\]thymidine incorporation after stimulation by IL-4, whereas BA/F3 cells expressing kinase-inactive Fes (Fes KE 10, Fes KE 18) show only a 1- to 2-fold increase in \[^{[3}H\]thymidine incorporation (Fig. 6B). These results suggest that Fes performs a role in mediating IL-4-induced proliferation.

Discussion

Cytokine receptors lacking intrinsic tyrosine kinase activity must couple with non-receptor PTK to induce intracellular tyrosine phosphorylation (16). Although Jak PTKs have been demonstrated...
to be required in a variety of cytokine signaling pathways, other families of Src and Src-related nonreceptor PTKs have also recently been implicated in cytokine signaling pathways. Syk, a member of the Src-related family PTK, has been found to associate with the IL-2 receptor and is activated in response to IL-2 (47). The involvement of Syk in IL-2 signaling has been linked to the induction of c-Myc by IL-2 (48). Another IL-2-activated tyrosine kinase, Pyk2, a FAK family member, was reported to be a component of the IL-2 signaling pathway and is thought to mediate cell proliferation activated by IL-2 (49). More recently, Pyk2 was shown to mediate the Jak-dependent activation of mitogen-activated protein kinase and STAT1 in IFN-γ signaling, leading to an antiviral effect (50). The mechanisms underlying the association of these nonreceptor PTKs with their respective cytokine receptors are poorly understood. In addition, downstream elements under regulation of these PTKs remain unclear.

In this manuscript, experiments are presented suggesting that the activation of the Fes kinase is required for the activation of a subset of signaling pathways downstream from the IL-4 receptor. The data suggest a model in which the IL-4-activation of Jak kinases leads to the activation of Fes. Fes can then phosphorylate IRS-1/2, which permits the recruitment and activation of PI3-kinase. The activation of PI3-kinase can then lead to the activation of p70S6k and cellular proliferation. The data do not allow us to predict which kinase is directly responsible for the activation of PI3-kinase.

The activation of Jak1, which leads to phosphorylation of IL-4Rα and STAT6, is also required for the phosphorylation of Fes. Thus Jak1 seems to play an essential role in the activation of IL-4-initiated signaling pathways. Harada and his coworkers have shown that an acidic motif located in the membrane proximal region of IL-4Rα is required for the recruitment of Fes (29). It has been proposed that Jak1 can associate with a membrane proximal region of the IL-4Rα-chain that contains a box 1 motif and an acidic region (30, 51). Thus, targeting Jak1 and Fes to IL-4Rα juxtaposes these two kinases and allows them to associate together. We have found that Jak1 associates with Fes and mediates its phosphorylation. This phosphorylation event appears to be accomplished by direct association of Jak1 with Fes as we observed. In addition, the fact that a kinase-inactive Fes failed to alter activation of Jak1 in response to IL-4 further supports the hypothesis that Fes is likely to function downstream from Jak1. Our data demonstrate that Jak1 can phosphorylate and activate Fes during IL-4 signaling and suggest a mechanism by which activation of Fes is regulated through Jak1 in cytokine signaling.

Although the domain of IL-4Rα responsible for Fes binding has been shown, whether Fes associates with IL-4Rα directly or through other intermediates remains unclear. We demonstrate that Jak1 can associate with Fes. Thus Jak1 may serve as an intermediate for Fes and IL-4Rα. Furthermore, we were able to observe coimmunoprecipitation of IL-4Rα with either wild-type or an SH2 domain mutant (SH2-R483L) of Fes in 293T cells (our unpublished observation). These results agree with the previous observation (29) that the membrane proximal region of IL-4Rα responsible for Fes binding contains no tyrosine residues, and suggest that the association is unlikely to be mediated by the interaction of the Fes SH2 domain and a phosphotyrosine of IL-4Rα. Thus, targeting Fes to IL-4Rα may be via a direct association and/or mediated through Jak1.

IRS-1/2 plays a critical role in the mitogenic effects of insulin, IGF-1, and IL-4 (52). It has been demonstrated that IRS is recruited to these receptors and subsequently phosphorylated by the receptor-containing kinases of insulin and IGF-1. Although the targeting of IRS-1/2 to IL-4Rα via its phosphotyrosine binding domain has been demonstrated (53), the mechanism leading to IRS phosphorylation in response to IL-4 is not fully understood. In a number of studies, Jak1 was found to be required for the phosphorylation of IRS-1. Our present data indicate that Fes appears to be downstream from Jak1 for the tyrosine phosphorylation of IRS-1/2, but not STAT6 in both Ba/F3 and M12 cells. Although previous studies have demonstrated that IRS-1 is phosphorylated when cotransfected with Jak1 in 293T cells (54), our results reveal that one-tenth the amount of Fes can achieve the same phosphorylation level of IRS-1 as that mediated by Jak1 and Jak3 in 293T cells (Fig. 7, lanes 7–9). This demonstrates that Fes is an effective kinase for IRS-1/2. Moreover, kinase-inactive Fes, Jak1, or Jak3 can partially inhibit the phosphorylation of IRS-1 by the wild-type Fes, Jak1, or Jak3 kinases, respectively (Fig. 7, lanes 10–12). More importantly, Fes can efficiently block IRS-1 phosphorylation by Jak1 or Jak3, whereas neither kinase-inactive Jak1 nor kinase-inactive Jak3 can inhibit IRS-1 phosphorylation by Fes (Fig. 7, lanes 1–6). These data suggest that Fes can effectively compete with Jak1 or Jak3 for IRS-1/2, and imply that Fes may be an important contributor to the activation of IRS-1/2 in vivo.

The recruitment of the p85 subunit of PI3-kinase by phosphorylated IRS is important for the activation of PI3-kinase by several cytokines and growth factors. Activation of PI3-kinase leads to several pathways, including the serine/threonine kinases p70S6k (55, 56) and Akt/PKB (57, 58). The PI3-kinase-Akt/PKB pathway has a well-defined role in anti-apoptosis (59), whereas p70S6k is known as a kinase of ribosomal subunit S6 that promotes cell division by up-regulating translation procession (60). Although we have observed a diminished IL-4-induced association of the p85α subunit of PI3-kinase with IRS-2 in the cells expressing kinase-inactive Fes, the phosphorylation of Akt at Ser473 remained unaltered in these cells. Several other reports have shown that activation of Akt can occur without detectable activity of PI3-kinase (61, 62). It was shown that expression of pleckstrin homology (PH) and PTB domain mutants of IRS-1 can still mediate the phosphorylation of Akt (Ser473), inhibition of apoptosis, and proliferation of cells during insulin stimulation (61). This IRS-1 mutation, which lacks the multiphosphorylated sites required for association with PI3-kinase, still activates Akt/PKB. Thus, this result is in agreement with our observation. It is possible that the phosphorylation of Akt can occur in the presence of basal levels of PI3-kinase.
activity, although increasing PI3-kinase activity may further enhance and prolong activation of Akt/PKB. In contrast, regulation of p70S6K activity involves multiple steps of phosphorylation. It has been suggested that the activity of p70S6K, through mediating the phosphorylation of an autoinhibitory motif (including Thr241/Ser242) located in the carboxyl-terminal of p70S6K, is required to allow the subsequent phosphorylation of its catalytic loop (Thr249) by PDK1 to yield a fully active kinase (42). Although a number of studies have suggested that phosphorylation on the autoinhibitory motif does not depend on PI3-kinase, the possible mechanism that Fes mediates the phosphorylation of p70S6K remains to be investigated.

Fes has been shown to play a role in regulating the differentiation of K562 myeloid cells and in cellular transformation (63, 64). A null mutation of murine c-Fes locus was generated and these c-Fes -/- mice reveal reduced numbers of B lymphocytes at all stages of B cell development (65), whereas the excess numbers of monococytes and neutrophils were observed in the same mice. Using a kinase-inactive Fes, we have demonstrated that Fes kinase activity is required for IL-4-induced proliferation in pro-B and B lymphoma cell lines. Together these data suggest that Fes may be an important regulator of B cell growth.

The finding that the activation of signaling pathways downstream of the IL-4 receptor may require different tyrosine kinases is novel. Because kinases are potential targets for therapeutic intervention, these findings suggest that inhibitors may be identified that block a subset of IL-4-induced events. This may be important as the role of IL-4 and IL-13 in allergic immune responses is further defined.

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