Interleukin-4 Induces Association of the c-fes Proto-Oncogene Product With Phosphatidylinositol-3 Kinase

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We have previously demonstrated that interleukin-4 (IL-4) induces tyrosine phosphorylation of a protein closely related or identical to the c-fes proto-oncogene product (FES) and association of this protein with the IL-4 receptor α chain (IL-4Ra). IL-4 is known to induce association of phosphatidylinositol-3 (PI3) kinase with the IL-4Ra. Since FES contains the consensus motifs for PI3 kinase binding, we tested the possibility that FES may associate with IL-4Ra upon IL-4 stimulation. We demonstrate herein that IL-4 stimulation induced rapid association of FES with a related protein with PI3 kinase in mouse T-cell lines. We also show an association of human FES (hFES) with the src homology 2 (SH2) domain of PI3 kinase in a COS7 cell expression system. The in vitro PI3 kinase assay using COS7 cells suggested that hFES partly contributes to the association between the hIL-4Ra and PI3 kinase. We have further identified the important region in the cytoplasmic domain of the hIL-4Ra for association of tyrosine-phosphorylated hFES with the hIL-4Ra and SH2 domain of PI3 kinase using a COS7 cell expression system. These results suggest that FES or a related protein/PI3 kinase pathway may play a role in the pleiotropic effects of IL-4.

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ASSOCIATION OF FES WITH PI3 KINASE BY IL-4

MATERIALS AND METHODS

Cell lines. Mouse IL-2–dependent cell lines CTLL-2 and HT2 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μg/mL streptomycin, 100 U/mL penicillin, 5 x 10^{-5} M β-mercaptoethanol, and recombinant mouse IL-2 (provided by Dr Gerard Zurawski of DNAX Research Institute) as described previously. The mouse IL-3–dependent pro–B-cell line BaF3 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 μg/mL streptomycin, 100 U/mL penicillin, 5 x 10^{-5} M β-mercaptoethanol, and recombinant mouse IL-3 (provided by Dr Atsushi Miyajima of DNAX Research Institute). COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin.

Antibodies and reagents. Polyclonal anti-p85 antibody and monoclonal antiphosphotyrosine antibody (4G10) were purchased from UBI (Lake Placid, NY). Polyclonal antiphosphotyrosine antibody was obtained from Zymed (South San Francisco, CA). Anti-FES antibodies used in the present study were either monoclonal rat antibody raised against the product of v-fps oncogene obtained from Oncogene Science or affinity-purified polyclonal rabbit antibody raised against a bacterially expressed glutathione-S-transferase (GST) fusion protein containing human FES (hFES) amino acids leucine 401 to glutamine 445. Anti–hIL-4Ra antibody directed against the extracellular domain of the hIL-4Ra was provided by Schering-Plough (Dardilly, France). Anti-GST antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sepharose preconjugated with GST fusion protein containing the N-terminal SH2 domain of p85 (GST-SH2-p85) was a gift from Dr Alice Mui (DNAX Research Institute). Sepharose preconjugated with GST protein without fusion of GST-SH2-p85 was generated according to the manufacturer’s instructions (Pharmacia, Uppsala, Sweden).

Immunoprecipitation. GST fusion protein binding assays, and Western blotting. Procedures for immunoprecipitation and Western blotting were performed as previously described. For immunoprecipitation of PI3 kinase, hFES, or hIL-4Ra, either protein A–Sepharose preconjugated with anti-p85 antibody (5 μL), polyclonal rabbit antibody against hFES (5 μL), or protein G–Sepharose preconjugated with anti–hIL-4Ra antibody (10 μL) were used, respectively. For isolation of hFES with GST-SH2-p85, Sepharose preconjugated with GST-SH2-p85 was used. Briefly, HT2, CTLL-2, and Ba/F3 cells were grown in serum-deprived media for 6 hours. Serum-starved cells or COS7 cells were lysed in lysis buffer containing 1% Triton X-100, and the proteins were immunoprecipitated from clarified cell lysates by appropriate antibody-preconjugated Sepharose beads or adsorbed by GST-SH2-p85–preconjugated Sepharose beads and incubated for at least 2 hours at 4°C. Sepharose beads were washed four times with the same lysis buffer, and once with the lysis buffer without detergents. Sepharose-bound proteins were then eluted by boiling with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, applied to SDS-PAGE, and transferred electrophoretically to nitrocellulose filters. Proteins were probed with appropriate antibodies and visualized by enhanced chemiluminescence (ECL; Amersham, Bucks, UK).

Transfection of plasmids encoding hFES and hIL-4Ra. Plasmids encoding hFES and hIL-4Ra in mammalian expression vector pMIE188 were constructed as described previously.25 COS7 cells were transfected by electroporation as previously described and harvested with phosphate-buffered saline containing 5 mMol/L EDTA after 3 days in culture.

In vitro PI3 kinase assay. The in vitro PI3 kinase assay was performed as previously described. Briefly, cells were lysed in the lysis buffer containing 1% Brij 97 instead of 1% Triton X-100. Proteins immunoprecipitated with antibody against hIL-4Ra or protein G–Sepharose were washed twice with the lysis buffer, twice with 10 mmol/L Tris hydrochloride (pH 7.5) containing 20 mmol/L MgCl₂ and 0.5 mmol/L LiCl, and finally twice with 10 mmol/L Tris hydrochloride (pH 7.5) containing 20 mmol/L MgCl₂ and 100 mmol/L NaCl. An in vitro PI3 kinase assay was performed on immune complexes. Products of PI3 kinase were separated by thin-layer chromatography and visualized with autoradiography.

RESULTS

Association of a tyrosine-phosphorylated 92-kD protein with PI3 kinase. We have previously demonstrated that IL-4 induces association of PI3 kinase with the IL-4Ra, which does not have the consensus motifs for PI3 kinase binding. To explore the possibility that PI3 kinase associates with the ligand-engaged IL-4Ra through tyrosine-phosphorylated intermediate molecules, we stimulated HT2 and CTLL-2 cells with IL-4, immunoprecipitated PI3 kinase using anti-p85 antibody (anti-p85), and then probed the immunoprecipitates with polyclonal antiphosphotyrosine antibody (anti-PY).

A 92-kD tyrosine-phosphorylated protein was detected in both T-cell lines after IL-4 stimulation (Fig 1A). The kinetics of p92 association with PI3 kinase were not identical in the two cell lines: the peak level of association was observed at 1 minute after IL-4 stimulation in HT2 cells, whereas association of p92 with PI3 kinase in CTLL-2 cells was more sustained and peaked at 5 minutes after IL-4 stimulation. It has been shown that a 170-kD protein termed 4PS/IRS-2, which appears to be functionally and structurally similar to IRS-1, is tyrosine-phosphorylated and associates with PI3 kinase upon IL-4 stimulation. However, tyrosine phosphorylation of 4PS/IRS-2 was not detected in CTLL-2 cells and HT2 cells by either polyclonal anti-PY or a monoclonal anti-PY, 4G10, whereas 4PS/IRS-2 was recognized by 4G10 in Ba/F3 cells (Fig 1A, data not shown). As we described in a previous study, tyrosine phosphorylation of FES was not detected by 4G10(25) (Fig 1A). These results suggest that involvement of 4PS/IRS-2 in IL-4–mediated signal transduction is not required in certain cell types. Blotting these immunoprecipitates with anti-p85 demonstrated that invariant levels of PI3 kinase were recovered during IL-4 stimulation (Fig 1B). Tyrosine phosphorylation of the p85 subunit was not detected under these conditions, but IL-2 induced tyrosine phosphorylation of p85 and 90-kD proteins (p90) in both T-cell lines (Fig 1A). Association of a p90 with PI3 kinase could be detected even 15 minutes after IL-2 stimulation, indicating that the association kinetics of PI3 kinase with the IL-2–induced p90 were different from those of the IL-4–induced p92.

We have also demonstrated that IL-4 induces tyrosine phosphorylation of FES or a FES-related protein, and association of this protein with the IL-4Ra. To examine whether the IL-4–induced p92 that associates with p85 is FES or a FES-related protein, anti-p85 immunoprecipitates were probed with an antibody against FES (anti-FES). Anti-FES recognized a 92-kD protein in immunoprecipitates of PI3 kinase (Fig 2), and the association kinetics of this anti-FES reactive protein in IL-4–stimulated HT2 and CTLL-2 cells were identical to those of the anti-PY reactive p92 species (Fig 1A). These results indicate that IL-4 induces association
Fig 1. Western blotting of immunoprecipitates with anti-p85 antibody. Immunoprecipitates with anti-p85 antibody from CTLL-2 and HT2 cells and Ba/F3 cells (1 x 10^7 cells) stimulated by IL-4 (10 ng/mL) or IL-2 (10 ng/mL) for the indicated times were probed with polyclonal anti-PY antibody (CTLL-2 and HT2), monoclonal anti-PY antibody (Ba/F3) (A), or anti-p85 antibody (B). Molecular masses of marker proteins are indicated (kDa).

of FES or a FES-related protein with PI3 kinase in HT2 and CTLL-2 cells. The IL-2–induced p90 that associates with PI3 kinase was not recognized by anti-FES (Fig 2). Although IL-2 is known to induce association of PI3 kinase with tyrosine phosphoproteins, these results indicate that distinct phosphotyrosine-containing proteins are involved in IL-2– and IL-4–mediated activation of PI3 kinase.

Association of hFES with PI3 kinase in COS7 cells. To demonstrate that FES can bind to the p85 subunit of PI3 kinase, we used GST-SH2-p85 to examine the association of hFES expressed in COS7 cells. hFES did associate with GST-SH2-p85, and interestingly, this association was only seen when hFES was coexpressed with the hIL-4Ra (Fig 3A). Although association of hFES with GST-SH2-p85 was observed in unstimulated cells, stimulation of COS7 cells with hIL-4 clearly augmented the association of hFES with GST-SH2-p85. Expression levels of hFES in COS7 cells transfected with hFES cDNA alone or cotransfected with
ASSOCIATION OF FES WITH PI3 KINASE BY IL-4

A. Blot a FES Ab

E. Blot a hIL-4R Ab

Immun. Pt. %P85

Gsr Imm. PL

Blot a GST Ab

Fig 3. Association of FES with the N-terminal SH2 domain of p85. COS7 cells were transfected with hFES cDNA alone, hIL-4Ra cDNA alone, or a combination of hIL-4Ra cDNA and hFES cDNA. After cells were stimulated or not with hIL-4 (10 ng/mL), GST-SH2-p85-Sepharose (SH2-p85)- or GST-Sepharose (GST)-adsorbed proteins (A and C) or anti-hIL-4Ra antibody (B) or immunoprecipitates with anti-FES antibody (D) from lysates of COS7 cells (1 x 10⁶ cells) were probed with either monoclonal anti-FES antibody (A and D), anti-hIL-4Ra antibody (B), or anti-GST antibody (C).

hFES cDNA and hIL-4Ra cDNA were invariable (Fig 3D), as were expression levels of the hIL-4Ra in COS7 cells transfected with hIL-4Ra cDNA alone or cotransfected with hFES cDNA and hIL-4Ra cDNA (Fig 3B). The amount of GST-SH2-p85 used to adsorb hFES in these experiments was also constant (Fig 3C). The GST alone did not associate with hFES (Fig 3A). Thus, association of hFES with GST-SH2-p85 was dependent on the presence of the hIL-4Ra, and stimulation of COS7 cells with hIL-4 augmented this association.

Since we observed an association of hFES with GST-SH2-p85 in unstimulated cells, we examined whether hFES is constitutively tyrosine-phosphorylated in COS7 cells transfected with hFES alone and a combination of hFES and the hIL-4Ra. hFES was constitutively tyrosine-phosphorylated in COS7 cells, and cotransfection of the hIL-4Ra did not influence the constitutive tyrosine phosphorylation levels of hFES (Fig 4A). The expressed FES was invariable, and the hIL-4Ra was expressed only in COS7 cells cotransfected with both hFES and the hIL-4Ra (Fig 4B and C). Because of a lack of a negative regulatory domain, it is possible that the constitutive tyrosine phosphorylation of hFES is due to transphosphorylation between overexpressed hFESs.

Association of PI3 kinase with the hIL-4Ra in the presence of hFES in COS7 cells. We next performed the in vitro PI3 kinase assay in immunoprecipitates with anti-hIL-4Ra antibody from transfected COS7 cells to test whether hFES is essential for the association between PI3 kinase and the hIL-4Ra. Coexpression of hFES and the hIL-4Ra induced association between PI3 kinase and the hIL-4Ra, and stimulation of these COS7 cells with hIL-4 also increased PI3 kinase activity in these immunoprecipitates (mean of 145% in three experiments as judged by an image analyzer; Fig 5A). Interestingly, expression of the hIL-4Ra alone caused association of PI3 kinase with the hIL-4Ra, although the level of PI3 kinase activity is less than that seen in COS7 cells cotransfected with both hFES cDNA and hIL-4Ra cDNA (mean of 43% in three experiments as judged by an

A. Imm. Pt. α FES Ab

B. Imm. Pt. α hIL-4Ra Ab

C. Imm. Pt. α FES Ab

Fig 4. Tyrosine phosphorylation of FES in COS7 cells. COS7 cells were transfected with hFES cDNA alone or a combination of hIL-4Ra cDNA and hFES cDNA. Immunoprecipitates with anti-FES antibody (A and C) or anti-hIL-4Ra antibody (B) from lysates of COS7 cells (1 x 10⁶ cells) were probed with either polyclonal anti-PY antibody (A), anti-hIL-4Ra antibody (B), or monoclonal anti-FES antibody (C).
image analyzer). Expression levels of the hIL-4Ra and FES were invariant (Fig 5B and C). These results suggest that although hFES does not appear to be essential for this association in COS7 cells, hFES partly contributes to the association of the IL-4Ra with PI3 kinase. Thus far, it is not clear which tyrosine kinase enables PI3 kinase to associate with the hIL-4Ra constitutively in COS7 cells.

**Identification of the critical region of the hIL-4Ra required for association of tyrosine-phosphorylated hFES with the hIL-4Ra.** Since we observed tyrosine phosphorylation of hFES in COS7 cells transfected with hFES cDNA alone and failed to detect an association of hFES with PI3 kinase in this study, tyrosine phosphorylation of FES does not seem sufficient for the association of FES with PI3 kinase. However, the observations that IL-4 induces tyrosine phosphorylation of FES or a FES-related protein and association of this molecule with PI3 kinase in mouse T-cell lines and that constitutively tyrosine-phosphorylated hFES in COS7 cells associates with GST-SH2-p85 suggest that tyrosine phosphorylation of FES is important for the association of FES with PI3 kinase. We therefore sought to identify sequences within the hIL-4Ra that might be required for the association of tyrosine-phosphorylated hFES with the hIL-4Ra. To analyze this, we cotransfected cDNAs encoding a series of truncated hIL-4Ras (Fig 6) and hFES into COS7 cells, immunoprecipitated these hIL-4Ras after hIL-4 stimulation, and then probed them with anti-PY (Fig 7A) or anti-FES (Fig 7C). hFES was detected in association with all four of these IL-4Ras (Fig 7C), consistent with the previous result that a region located between amino acids 353 and 431, numbering from the methionine start of the signal peptide, was sufficient to mediate association of the hIL-4Ra with hFES. Constitutive association of tyrosine-phosphorylated hFES with the
hIL-4Rα was detected in COS7 cells expressing the full-length hIL-4Rα (F) or a mutant lacking the C-terminal 200 amino acids (N), and hIL-4 augmented tyrosine phosphorylation of hFES in these cells (Fig 7A). In contrast, tyrosine phosphorylation of hFES was greatly diminished in COS7 cells cotransfected with hIL-4Rα mutants lacking the C-terminal 305 or 394 amino acids, M-2 and P, respectively (Fig 7A). We also detected IL-4–induced tyrosine phosphorylation of the full-length hIL-4Rα in COS7 cells cotransfected with hFES cDNA, consistent with our previous report. Tyrosine phosphorylation of the hIL-4Rα is much decreased in the N-hIL-4Rα mutant, indicating that the major phosphorylation site of the hIL-4Rα is either tyrosine 631, 713, or 821, which is lacking in the N-hIL-4Rα mutant (Fig 7A). Similar expression levels of hFES were seen in all these COS7 cells (Fig 7D). Although expression levels of different hIL-4Rαs varied, the deletion mutants were all expressed at levels equal to or exceeding that of the full-length hIL-4Rα (Fig 7B). These results indicate that a region within the hIL-4Rα located between amino acids 520 and 625 plays an important role in the association of tyrosine-phosphorylated hFES with the hIL-4Rα.

**DISCUSSION**

PI3 kinase is thought to play an important role in mitogenic signaling. We and others have demonstrated that IL-4 induces association of PI3 kinase with the IL-4Rα, which causes translocation of PI3 kinase from the cytosol.
to the plasma membrane, where substrates are available.\(^{12,13}\)

Since the IL-4Ra does not appear to contain consensus binding motifs for SH2 domains of the p85 adapter subunit of PI3 kinase, it is conceivable that PI3 kinase associates with the IL-4Ra indirectly. Others have demonstrated that the IRS-1-like molecule 4PS/IRS-2 provides a link between the IL-4Ra and PI3 kinase.\(^{23}\) They also demonstrated that binding of 4PS/IRS-2 to the IL-4Ra involves a tyrosine-containing motif located between the hIL-4Ra (residues 488 to 502, termed the insulin/IL-4R motif [I4R motif]).

In our previous study, we demonstrated that IL-4 induces tyrosine phosphorylation of a protein tyrosine kinase closely related or identical to FES, as well as association of hFES with the hIL-4Ra.\(^{25}\) In the present study, we provide evidence that IL-4 induces association of FES or a FES-related protein with PI3 kinase in two T-cell lines (Figs 1 and 2). Our findings also imply that FES is involved in the association mechanism between the IL-4Ra and PI3 kinase. Using a COS7 cell expression system, we show that the presence of the hIL-4Ra is essential for association of hFES with GST-SH2-p85 (Fig 3).

Furthermore, the in vitro PI3 kinase assay indicated that hFES augments the association of PI3 kinase with the hIL-4Ra, although the FES is not essential for this association in the COS7 cell expression system (Fig 5). These observations suggest that FES or a FES-related protein partly contributes to association of PI3 kinase with the IL-4Ra in COS7 cells. It is possible that another mechanism for association of the hIL-4Ra with PI3 kinase in COS7 cells is mediated by 4PS/IRS-2. However, in T-cell lines in which 4PS/IRS-2 is not involved, FES may act solely as an adapter molecule between PI3 kinase and the hIL-4Ra.

In the present study, we show that hFES expressed in COS7 cells was constitutively tyrosine-phosphorylated, and hIL-4 augmented tyrosine phosphorylation of hFES to some extent (Figs 3 and 4). Under the same condition, hFES did not associate with GST-SH2-p85 in COS7 cells transfected with hFES cDNA alone, even after hIL-4 stimulation (Fig 5A). Thus, the association mechanism of hFES with GST-SH2-p85 cannot be explained solely by tyrosine phosphorylation of hFES. The presence of the hIL-4Ra appears to be essential for association of hFES with GST-SH2-p85. It is therefore tempting to speculate that tyrosine-phosphorylated hFES may associate first with the hIL-4Ra, and this may induce a conformational change in hFES that allows it to associate with p85. It is also possible that hFES association with the hIL-4Ra induces phosphorylation of specific tyrosine residues on hFES that are required for association with p85. A potential binding site for the p85 SH2 domains is located around tyrosine 633 in hFES.\(^{22,28}\) However, it is not known if this tyrosine residue becomes phosphorylated in hFES, since this tyrosine is not accompanied by upstream acidic residues typical of tyrosine phosphorylation sites. A study of hFES in which another amino acid is substituted for tyrosine 633 would be useful.

We found that a membrane distal portion of the cytoplasmic domain (residues 520 to 625) of the hIL-4Ra plays a major role in IL-4-induced association of tyrosine-phosphorylated hFES with the hIL-4Ra and SH2 domain of PI3 kinase (Fig 7). Although the mechanism by which this region regulates association of tyrosine-phosphorylated hFES with the hIL-4Ra and SH2 domain of PI3 kinase is unknown, it is possible that this region somehow facilitates hFES auto-phosphorylation. Alternatively, another tyrosine kinase that interacts with this region may be involved. Interestingly, Keegan et al\(^{21}\) have demonstrated that a GST fusion protein containing amino acids 424 to 561 of the hIL-4Ra precipitates tyrosine kinase activity. Our study demonstrated that coexpression of the P-hIL-4Ra mutant with hFES in COS7 cells greatly decreased the association of hFES with GST-SH2-p85. However, the role of this region in the proliferation signal is controversial.\(^{23,27,33,34}\) Although the reasons for this discrepancy are unclear, it is possible that this region may be important for the long-term growth or optimum growth response induced by IL-4. This hypothesis is supported by the fact that Ba/F3 transfectants expressing the mutated hIL-4Ra lacking the I4R motif showed a decreased response to hIL-4.\(^{27}\)

Recently, it has been shown that \(\gamma_c\) is shared with other cytokine receptors, including IL-4R, IL-7R, IL-9R, and IL-15R\(^{6,8,35,36}\) and that both IL-2 and IL-4 induce tyrosine phosphorylation of JAK1 and JAK3,\(^{14,15}\) suggesting that IL-2 and IL-4 may transduce common signals through \(\gamma_c\). However, we have previously shown that IL-2 and IL-4 induce different patterns of tyrosine phosphorylation in HT2 cells, even though both cytokines promote cellular proliferation.\(^{13}\) Similarly, our present study indicates that distinct tyrosine phosphoproteins associate with PI3 kinase after IL-2 or IL-4 stimulation, and that the p85 subunit of PI3 kinase is tyrosine-phosphorylated in IL-2– but not in IL-4–stimulated cells. Therefore, it is conceivable that IL-4 and IL-2 have
their own distinct signal pathways that correlate with the specific biologic activities of these two cytokines. It will be important to investigate the specific signaling pathways for each cytokine to understand how each exerts its different biologic responses.

Involvement of FES in the signal transduction pathways of IL-3, granulocyte-macrophage colony-stimulating factor, erythropoietin, and IL-6 has been suggested, and more recently, others have proposed that granulocyte-macrophage colony-stimulating factor--induced tyrosine-phosphorylated protein is similar to but distinct from FES. Thus, FES seems to be involved in several cytokine signaling processes. However, the physiologic significance of FES in these cytokine signalings is unclear. FES has been reported to play a role in differentiation toward myeloid cells, and a recent study using transgenic mice showed a direct involvement of FES in the regulation of angiogenesis, suggesting that FES may have various biologic functions in different cell types. Although our results suggest that FES is involved in the association between the IL-4Ra and PI3 kinase, we cannot exclude the possibility that FES is also involved in other IL-4-mediated signaling pathways by phosphorylating or associating with other signaling molecules.

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