The Fes Protein-Tyrosine Kinase Phosphorylates a Subset of Macrophage Proteins That Are Involved in Cell Adhesion and Cell-Cell Signaling*

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The c-fps/fes proto-oncogene encodes a 92-kDa protein-tyrosine kinase that is expressed at high levels in macrophages. We have previously shown that overexpression of c-fps/fes in a CSF-1-dependent macrophage cell line (BAC1.2F5) partially released these cells from their factor dependence and that this correlated with the tyrosine phosphorylation of a subset of proteins in a tissue-specific manner. We have now identified one of the macrophage substrates of Fes as the crk-associated substrate (Cas) and a second substrate as a 130-kDa protein that has been previously described as a T cell activation-dependent substrate and is unrelated to Cas. Both of these proteins, which have optimal consensus sequences for phosphorylation by Fes, were tightly associated with this kinase through its SH2 domain, suggesting that they were direct substrates of Fes. Remarkably, when the Fes SH2 domain was used as an affinity reagent to identify potential substrates of endogenous Fes in control BAC1.2F5 cells, the phosphotyrosyl proteins that were recognized were the same as those that were specifically phosphorylated when Fes was overexpressed in the same cells. We conclude that these substrates were identified by their SH2 domain, which is structurally related to the physiological targets of this kinase in macrophages. The known functions of Cas and p130 suggest that Fes kinase may play a role in signaling triggered by cell adhesion and cell-cell interactions during immune responses of macrophages.

The c-fps/fes proto-oncogene encodes a non-receptor protein-tyrosine kinase (p92c-fes) (1, 2) that has been repeatedly transduced by RNA tumor viruses (3). In the adult, c-fps/fes is preferentially expressed in hematopoietic cells of the myeloid lineage and in endothelial cells (1, 4–6), whereas in the embryo, a wider pattern of expression has been observed (7).

The tissue specificity of Fes expression has suggested that this kinase may play a role in myelopoiesis or in specialized functions of myeloid cells (1, 5, 6). This idea is supported by several reports describing the involvement of Fes in cytokine receptor signaling (8, 9), in myeloid differentiation (6, 10), and in inhibition of apoptosis during granulocytic differentiation (11). However, the mechanism of action and biological role of this kinase in its target tissues is not well understood.

Since identification of the substrates of Fes kinase is one of the keys to elucidate its biological role, efforts have been made to uncover these substrates. One approach has been to express this kinase in different cell types and to correlate biological activity with phosphorylation of specific cellular targets. In established murine fibroblasts, where Fes is normally not present, expression of this kinase at high levels causes tumorigenic transformation (12, 13). This is mediated by tyrosine phosphorylation or activation of several well known mitogenic targets such as GTPase-activating protein and its associated proteins p62 and p190 (12–14), Bcr/Grb2 (15), She/Grb2 (16, 17), and phosphatidylinositol (PI) 3-kinase (18), suggesting that activation of the ras and PI 3-kinase pathways is involved in the mechanism of transformation by Fes.

By contrast, the biological and biochemical activity of ectopically expressed p92c-fes in macrophages, a cell type where this kinase is normally expressed at the highest levels, was more restricted. Fes expression in the CSF-1-dependent BAC1.2F5 cell line resulted in only partial relief of factor dependence, and Fes phosphorylated a limited subset of proteins on tyrosine, including a 130-kDa (p130) and a 75-kDa (p75) protein, which have so far not been implicated in major mitogenic pathways (19). The lack of a strong proliferative effect of Fes in macrophages and the failure to phosphorylate or activate targets frequently involved in mitogenic and oncogenic signaling suggest that, in contrast to its effect on cell proliferation in fibroblasts, in macrophages Fes may participate in a specialized function of these cells.

In this paper we have identified Cas and p130 as two distinct tissue-specific substrates of Fes in macrophages. Their known functions suggest a possible role of Fes during signaling in response to cell adhesion and interactions with other cells of the immune system.

MATERIALS AND METHODS

Cells—The CSF-1-dependent murine macrophage cell line BAC1.2F5 has been described (20). BAC1.2F5 cells and derived subclones were maintained in α-minimum essential medium supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.) (α-minimum essential medium–fetal calf serum) and 36 ng/ml human recombinant CSF-1 (Chiron Corp., Emeryville, CA). BAC1.2F5 cells that overexpress c-fps/fes...
fes (BAC1-Fes) were obtained by introduction of the retroviral expression vector pFF as described previously (19).

**DNAs and Recombinant DNA Methods**—The retroviral expression vector pFF, which encodes human p92c-Fes, has been described (2, 12). The bacterial pGEX-3X vector (21), which allows the expression of bacterial proteins as glutathione transferase (GST) fusion proteins, was obtained from Pharmacia Biotech Inc. The GST-SH2 fusion construct encoding the SH2 domain of Fes has been described (19).

**Purification of Bacterial GST-SH2 Protein**—pGEX-3X control and pGEX-SH2 bacteria were induced with 0.1 mM isoprropyl-1-thio-β-D-galactopyranoside as described (21). Bacteria were lysed by sonication in a buffer containing 50 mM HEPES-KOH, pH 7.4, 1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, and 2% (v/v) Trasylol (FBA Pharmaceuticals, NY), and the crude bacterial extracts were clarified by centrifugation at 15,000 × g for 10 min at 4 °C. Control GST and GST-SH2 fusion proteins were purified by adsorption to glutathione-agarose (Sigma) as described (19, 21).

**Antibodies**—Polyclonal and monoclonal antibodies to Fes proteins have been described (2, 19). The rabbit polyclonal antiserum directed against p130 has been described (22). Monoclonal antibodies directed against Cas were obtained from Transduction Laboratories (Lexington, KY), and rabbit polyclonal antibodies directed against Cas were obtained from H. Hirai (Tokyo) (23), and from Amy Bouton (University of Virginia, Charlottesville). Monoclonal antibodies directed against phosphorylated Cas (4G10) were obtained from UBI (Lake Placid, NY). Purification of bacterial proteins as glutathione transferase (GST) fusion proteins, was obtained using the bacterial pGEX-3X vector (21), which allows the expression of bacterial proteins as GST fusion proteins, was obtained from Pharmacia Biotech Inc. The GST-SH2 fusion construct encoding the SH2 domain of Fes has been described (19).

**Preparation of Cell Lysates and Protein Analysis**—Preparation of cell lysates for protein analysis was carried out as described (19) in a buffer containing 50 mM HEPES-KOH, pH 7.4, 1% (v/v) Triton X-100, 150 mM NaCl, 2.5 mM EDTA, 10% (v/v) glycerol, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate, and 2% (v/v) Trasylol. The analysis of proteins by immunoprecipitation, the in vitro protein kinase assay, electrophoresis in 8.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and Western blot analysis by enhanced chemiluminescence (Amersham Corp.) have been described (1, 13, 19). For immunoprecipitation, cell lysates containing 200 μg of protein were immunoprecipitated with the indicated antisera, and the immunoprecipitates were collected on Protein A-Sepharose (Pharmacia) as described (19). For adsorption to bacterial fusion proteins, cell lysates containing 200 μg of protein were incubated with 1–2 μg of bacterial protein immobilized on glutathione-agarose for 2 h at 4 °C. Adsorbed pellets were washed five times in cell lysis buffer and analyzed by SDS-PAGE, followed by Western blot analysis using the indicated antibodies.

**RESULTS**

**Fes Kinase Phosphorylates the crk-associated Substrate (Cas)**—To identify potential physiological substrates of Fes in macrophages, we have overexpressed this kinase in BAC1.2F5, a CSF-1-dependent macrophage cell line (20) capable of carrying out specialized immune functions such as activation in response to bacterial lipopolysaccharide and interferon-γ. Human p92c-Fes was introduced into these cells by retroviral-mediated gene transfer using the retroviral expression vector pFF (12, 19). As previously shown, this vector induced the expression of p92c-Fes at levels 30–50 times higher than background levels found in control cells (19). Ectopically expressed Fes protein was enzymatically active as shown by its in vitro kinase activity (Fig. 1A) and by the induction of tyrosine phosphorylation of several proteins that were largely undetectable in control BAC1.2F5 cells (Fig. 1B). The most prominent were two phosphorysyl protein of 130-kDa (p130) and a 75-kDa (p75) that we have previously described (19). p75 did not cross-react with an antibody (4P4) directed to a known substrate of v-src (19, 24–26). This v-src substrate has been cloned and named crk-associated substrate (Cas) because of its association with the v-crk oncoprotein in v-crk-transformed cells (23, 27). Since the original antibody used to characterize p130 also recognized other tyrosine-phosphorylated proteins (19), we sought additional evidence that p130 and Cas were the same protein. To this end we used specific antibodies raised to the bacterially expressed Cas protein.
two putative tyrosine phosphorylation sites (YD
Fes SH2 domain. This association and the presence in Cas of
was able to precipitate Cas, suggesting that the tight associa-
were unrelated to Cas (22). In addition, DNA sequence analysis
substrate, this T cell protein was also recognized by 4F4 anti-
expressed in lymphoid and myeloid cells and becomes tyrosine-
protein was a direct substrate of Fes in BAC1-Fes cells.
phosphotyrosyl p130 protein we originally described (19). Therefore we
sought to identify p130 using antisera to known signaling
proteins in the same molecular weight range. One of the can-
sion kinase (31), phospholipase C-γ (32), and the pp120 v-src
substrate (33) were not detectably tyrosine-phosphorylated in
BAC1-Fes cells and that therefore these were not primary
substrates of Fes kinase in these cells (19, data not shown).
However, we cannot rule out that there are other substrates of
Fes in this molecular weight range in BAC1-Fes cells, which
were not identified in this study.
We then examined whether phosphotyrosyl p130 was recog-
nized by the Fes SH2 domain. Cell lysates from BAC1-Fes cells
were adsorbed with bacterially expressed GST-SH2 fusion pro-
tein, and the adsorbed proteins were analyzed by anti-phospho-
tyrosine immunoblotting. GST-SH2 but not control GST vector
protein was able to precipitate several tyrosine-phosphorylated
proteins including p130 and p75 from cell lysates of Fes over-
expressing cells (Fig. 5, lanes 1 and 2). Immunoblotting of
GST-SH2 precipitates with anti-p120/130 antibody confirmed the
identity of the phosphotyrosyl p130 protein recognized by the
Fes SH2 domain as p120/130 (Fig. 5, lane 5). Fig. 5, lane 3,
3, lanes 2 and 3, GST-SH2 but not control GST vector protein
was able to precipitate Cas, suggesting that the tight associa-
tion of Fes with phosphorylated Cas was mediated through the
Fes SH2 domain. This association and the presence in Cas of
of two putative tyrosine phosphorylation sites (YDXV) (23) that
are close to the optimal consensus sequence for Fes phospho-
ylation and binding to its SH2 domain (YEXV) suggest that
Cas may be a direct substrate of Fes in BAC1-Fes cells.
From these results we conclude that Cas or a closely related
protein is one of the substrates phosphorylated by Fes in BAC1-
Fes cells and that this substrate remains tightly associated
with Fes kinase through the Fes SH2 domain.
p130 Cross-reacts with a 120–130-kDa Protein Phosphoryl-
ated on Tyrosine During T Cell Activation—The results pre-
presented above indicated that Cas was distinct from the phospho-
tyrosyl p130 protein we originally described (19). Therefore we
sought to identify p130 using antisera to known signaling
proteins in the same molecular weight range. One of the can-
didates we tested was a 120–130-kDa protein that is selectively
expressed in lymphoid and myeloid cells and becomes tyrosine-
phosphorylated during T cell activation (22). Like our p130
substrate, this T cell protein was also recognized by 4F4 anti-
body (28), but the use of specific polyclonal antibodies raised
against purified p120/130 has recently revealed that p120/130
was unrelated to Cas (22). In addition, DNA sequence analysis
of cloned p120/130 cDNA confirmed that this protein had no
homology to Cas or any other protein in the data bank. Therefore,
we used this specific anti-p120/130 antiserum to determine if
the 120–130-kDa T cell protein and the p130 substrate of
Fes were related proteins. As shown in Fig. 4, anti-p120/130
precipitated a prominent tyrosine-phosphorylated 130-kDa
protein from lysates of BAC1-Fes cells, which co-migrated with
the phosphotyrosyl p130 present in anti-Fes immunoprecipi-
tates (Fig. 4). This suggested that the 120–130-kDa protein
identified in T cells was the same or related to the p130 protein
phosphorylated in BAC1-Fes cells. Using specific antibodies to
other proteins in the same molecular weight range of p130 and
Cas, we also determined that the β subunit of granulocyte/ macrophage-CSF receptor (29), JAK kinases (30), focal adhe-

2 A. J. da Silva, submitted for publication.
also shows that in BAC1.2F5 cells, p130 consists of two closely migrating species.

We conclude that the 120–130-kDa substrate identified in T cells and the p130 substrate of Fes are the same or related proteins and that p130 and Cas are two different substrates of Fes in BAC1-Fes cells. DNA sequence analysis of full-length p120/130 cDNA clones revealed the presence of two phosphotyrosine motifs (YDDV) that are close to the optimal consensus sequence for Fes phosphorylation, which is consistent with the idea that p130 may be a direct substrate of Fes. Further analysis will be required to identify the Fes tyrosine phosphorylation sites in p130.

The Fes SH2 Domain Recognizes the Same Subset of Tyrosine-phosphorylated Proteins in Control and Fes-overexpressing BAC1.2F5 Cells—Since the Fes SH2 domain appears to have high specificity for the substrates of this kinase (19), we reasoned that this SH2 domain might have affinity for potential physiological targets of endogenous p92c-fes in control macrophages. To determine if the Fes SH2 domain recognized any phosphotyrosyl proteins in BAC1.2F5, cell lysates were adsorbed with GST-SH2 fusion protein, and the adsorbed proteins were analyzed by anti-phosphotyrosine immunoblotting. As shown in Fig. 6A, lanes 3 and 4, GST-SH2 but not GST recognized a number of phosphotyrosyl proteins from control BAC1.2F5 cells. Remarkably, these phosphotyrosyl proteins had the same electrophoretic mobilities as those that were identified as the major substrates of Fes in BAC1-Fes cells (Fig. 6A, lanes 1, 2, 4, and 5). Immunoblotting of the Fes-SH2-adsorbed proteins from control BAC1.2F5 cells with anti-p120/130 antibody confirmed that p120/130 was one of the proteins recognized by the Fes SH2 domain (Fig. 6B, lanes 3 and 5).

Taken together, these results are highly suggestive that the Fes substrates we identified are structurally related or identical to the physiological substrates of Fes kinase in BAC1.2F5 macrophages.

DISCUSSION

In this study we have identified Cas and p130 as two different tissue-specific substrates of Fes in macrophages.

Overexpression of Fes kinase in BAC1.2F5 cells resulted in tyrosine phosphorylation of a subset of proteins, which were different from known adapter proteins associated with mitogenic signaling. These proteins were tightly bound to Fes through its SH2 domain and contained optimal consensus sequences for phosphorylation by Fes kinase and binding to its SH2 domain, suggesting that they were direct substrates of this kinase.
Using peptide library technology it was previously shown that the optimal consensus sequences for phosphorylation by non-receptor tyrosine kinases were very similar to the optimal consensus sequences for binding to the SH2 domains of the corresponding kinases (34, 35). Thus, the catalytic and SH2 domains of tyrosine kinases have co-evolved so that some of the phosphorylated substrates can be retained by their SH2 domains. This mechanism may allow for phosphorylation of additional sites in the retained substrates or in other proteins present in the phosphotyrosyl complex. Our results are consistent with some of the predictions of this model. We showed that some of the Fes substrates in BAC1-Fes cells remain associated with Fes after phosphorylation and that this association was mediated through the Fes SH2 domain. Although the Fes tyrosine phosphorylation sites in Cas and p130 have not yet been mapped, these two proteins contain optimal Fes tyrosine phosphorylation sites, which may be phosphorylated first and mediate subsequent binding to the Fes SH2 domain. Cas contains several additional tandem YDPY motifs that do not conform to the optimal sequences for Fes phosphorylation. Yet, the detection of multiple Cas forms in the Fes-Cas complex, which may represent different phosphorylated forms of Cas, suggests that some of these non-canonical sites may also be phosphorylated by Fes, perhaps facilitated by the formation of the initial complex between Fes and its substrate. Further analysis using mutants of Fes and its substrates should clarify the mechanism used by Fes to select and phosphorylate its targets.

Our previous work (12, 13, 19) and the results presented here suggest that the biological and biochemical activity of Fes is tissue-specific. In established murine fibroblasts, Fes expression led to cell transformation mediated through phosphorylation or activation of proteins involved in mitogenic and oncogenic signaling such as the GTPase-activating protein-p62-p190 complex (12–14, 36), Shc (16), and PI 3-kinase (18). On the other hand, overexpression of p92E fes had only a modest effect on the proliferative capacity of BAC1 cells, and Fes kinase phosphorylated a subset of proteins which so far have not been implicated in mitogenic pathways (19). This is in contrast to the action of other tyrosine kinases present in the same cells. In BAC1.2F5, the CSF-1 receptor is involved in the control of cell proliferation, and this is mediated through activation of the Shc/Grb2/Ras and PI 3-kinase pathways (19, 37). Thus, in macrophages Fes may not be involved in mitogenic signaling but participate in specialized signaling pathways that operate in these cells.

One of the most interesting findings of this study was the observation that the phosphotyrosyl proteins recognized by the SH2 domain of Fes in control BAC1.2F5 cells were very similar to the substrates identified by overexpression of Fes in the same cells. Thus the Fes SH2 domain, which does not recognize the substrates of other tyrosine kinases present in BAC1.2F5 (19), appears to be a very specific reagent for Fes substrates. Taken together, our results are consistent with the idea that some of the substrates phosphorylated by ectopically expressed Fes in macrophages may be structurally related or identical to its physiological substrates.

The identity of the two substrates described in this study provides insight into the possible site of action of Fes kinase in macrophages. Cas has an SH3 domain that is a binding site for focal adhesion kinase (38),1 a tyrosine kinase that is activated by integrin engagement (39). Cas also binds tensin (40) and is believed to play a role in reorganization of the actin cytoskeleton and other signaling events induced by cell adhesion and cell-cell interactions. Moreover, Cas is phosphorylated on tyrosine following integrin engagement (41–43). This suggests that in macrophages and leukocytes, which carry out functions that involve close interactions with other cells of the immune system and with the cell matrix (e.g., cell adhesion during inflammatory responses), Fes may relay some of the signals generated during these processes. Similarly, the identity of p130 as a Fes substrate also suggests a role for Fes during cell-cell interactions. The expression of p130 in T cells and in myeloid cells and its tyrosine phosphorylation during engagement of receptors during T cell activation (22) suggest that this protein may play a role in the generation of bi-polar signals in interacting immunocompetent cells. In T cells p130 is believed to be phosphorylated by Fyn (22), whereas in macrophages Fes may be one of the kinases involved in the phosphorylation of this protein. The identity of the macrophage substrates uncovered in this study suggests that Fes kinase may play a role in signaling triggered by cell adhesion and cell-cell interactions during immune responses of macrophages.

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