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FES kinase participates in KIT-ligand induced chemotaxis

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ABSTRACT

FES is a cytoplasmic tyrosine kinase activated by several membrane receptors, originally identified as a viral oncogene product. We have recently identified FES as a crucial effector of oncogenic KIT mutant receptor. However, FES implication in wild-type KIT receptor function was not addressed. We report here that FES interacts with KIT and is phosphorylated following activation by its ligand SCF. Unlike in the context of oncogenic KIT mutant, FES is not involved in wild-type KIT proliferation signal, or in cell adhesion. Instead, FES is required for SCF-induced chemotaxis. In conclusion, FES kinase is a mediator of wild-type KIT signalling implicated in cell migration.

Introduction

FES and FER are closely related cytoplasmic kinases first identified as the oncogene product of avian and feline viruses [1]. Their physiological functions remain incompletely understood despite many reported studies including the generation of knock-in and knock-out mice. In particular, deficient mice showed subtle phenotypes in the haematopoietic compartment with slight homeostasis phenotype for c-*fes* deficiency and defects in innate immunity responses for both c-*fes* and *fer* deficient mice [2–5]. At the molecular level, the two proteins seem to be involved in the regulation of proteins implicated in cytoskeleton and adhesion-complexes dynamics. For instance, FER was implicated in phosphorylation of the actin-binding protein cortactin [2,6], in the phosphorylation and function of β-catenin, in the interaction of β-catenin with α-catenin [7], in the formation of synapses in neurons [8] and in the function of N-cadherin [9,10]. On the other hand, FES has been implicated in microtubule assembly [11,12], interaction with ezrin in epithelial cells [13] and focal adhesions turnover [14].

A link between FER and KIT receptor was established in a study of mouse mast cell primary cultures [15]. In the latter study, FER-deficient mast cells showed higher cell adhesion and reduced chemotaxis upon SCF stimulation. More recently, we found that FES was phosphorylated by an oncogenic form of KIT receptor and reported that FES was necessary for G1/S transition of the cell cycle and for KIT-mutant induced cell proliferation [16]. Here we addressed whether FES participates in wild-type KIT signalling and whether it is required for well characterised KIT cellular functions such as proliferation, adhesion to fibronectin or chemotaxis.

Materials and methods

Cells and transfections. All TF-1 cells were grown in RPMI medium supplemented with 10% FBS and 5 ng/ml GM-CSF (Berlex, Seattle). TF-1/FesR and TF-1/SRα cells were provided by Yates. They are stable populations of TF-1 cells transfected with a kinase-defective mutant of FES (a point mutation K590R, named FesR) and the control SRα vector, respectively [17]. HMC-1 cells provided by Butterfield were grown in RPMI and 10% FBS. COS-7 cells were grown in DMEM, supplemented with 10% FBS. COS-7 cells were transfected using Fugene reagent. For RNA interference experiments, electroporation conditions for TF-1 cells and siRNA sequences were as described [16].

Yeast two-hybrid. Yeast two-hybrid screen was carried out as described previously [18–20]. In brief, KIT was expressed as LexA fusion proteins using pBTM116 vector in the yeast strain L40. A

Abbreviations: SCF, stem cell factor; FBS, foetal bovine serum; SH2, Src-homology domain-2; WT, wild-type; HBSS, Hanks' balanced salt solution.

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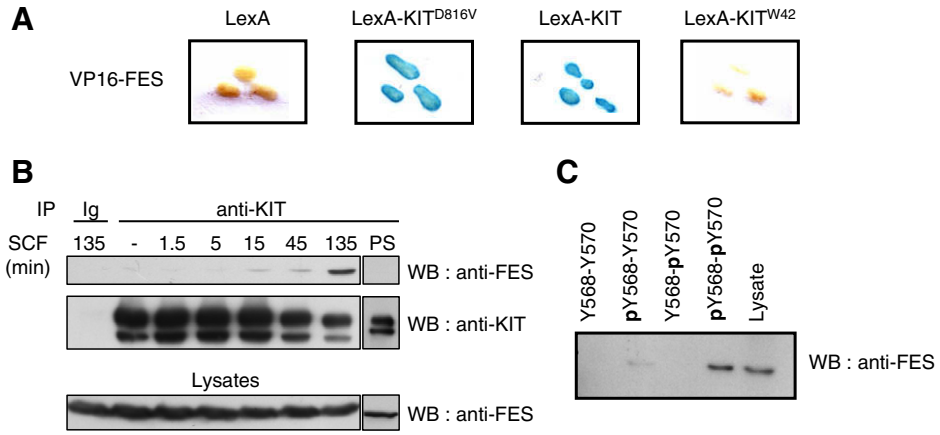

Fig. 1. Interaction of FES and KIT kinases. (A) Interaction in the yeast two-hybrid system. A cDNA clone encoding FES SH2 domain was co-transfected with c-*kit* cDNAs. Protein interactions are revealed following a β-galactosidase assay. c-*kit* cDNAs were KITD816V, a gain-of-function mutant, WT KIT or KITW42, a kinase dead mutant of KIT. (B) Co-immunoprecipitation of endogenous proteins in TF-1 cells. Lysates from TF-1 cells stimulated with SCF for the indicated time were immunoprecipitated either with anti-KIT or isotype control antibodies. PS, permanent stimulation refers to cells grown in SCF. (C) Peptide pull-down assays. Four peptides with the same amino-acid sequence but different phosphorylation modification on tyrosine residues were immobilized on sepharose beads and incubated with cell lysates from HMC-1 cells. FES protein was detected on the purified protein bound fraction. Cell lysate was used as a positive control showing FES protein. Y stands for non-phosphorylated tyrosine; pY indicates phosphorylated tyrosine.

cDNA library cloned in pVP16 was derived from the multipotential hematopoietic cell line EML-c1 (kindly provided by Dr. S. Tsai). The bait was functionally tested against the SH2 domains of p85 and GRB-2 before performing the screen.

Immunoprecipitations and Western-blotting. Cell lysates, immunoprecipitations and Western-blotting were done as previously described [19]. The antibodies used in the study were as follow: anti-FES (Calbiochem), anti-AKT (Cell Signalling Technology), anti-ERK2 and anti-KIT (Santa-Cruz), anti-phosphotyrosine 4G10 (Upstate), anti-Flag (Sigma–Aldrich).

Peptide binding assay. For peptide pull-down experiments, 15-mer peptides (INGNNYVYIDPTQLP) comprising phosphorylated or unphosphorylated KIT Y567–Y569 were coupled to N-hydroxy-succinimide (NHS)-activated sepharose beads (GE Healthcare) and incubated with HMC-1 cell lysates for 2 h at 4 °C. The precipitated proteins were separated by SDS–PAGE and subjected to immunoblotting.

Adhesion to fibronectin. Ninety-six-well plates were incubated overnight at 4 °C with PBS containing 25 μg/ml of human fibronectin (Chemicon International). The coating solution was removed by aspiration, replaced with HBSS containing 5% BSA and incubated for 1 h at 37 °C. The plates were then washed three times with cell adhesion medium (HBSS containing 0.5% BSA). Cells starved overnight in RPMI containing 0.5% FBS without growth factor were harvested, washed and resuspended in cell adhesion medium. Cells (6×10^4) were plated in 100 μl of cell adhesion medium with or without hSCF (100 ng/ml, Peprotech) in triplicate into the fibronectin-coated wells. Plates were incubated at 37 °C for 15 min. After washing, cell adhesion was then quantified using CellTiter-Glo according to the manufacturer's recommendations (Promega). Results are expressed as percentage of adherent cells where 100% corresponds to 6×10^4 cells.

Chemotaxis assay. In vitro migration assays were done using modified Boyden chambers in 24-well plates with 8 μm pore inserts (Becton Dickinson). Cells were starved in RPMI containing 0.5% FBS for 16 h, then 1.5×10^5 cells were plated in the upper chamber in migration medium (starving media plus 0.5% BSA). The lower chamber contained migration medium and SCF (100 ng/ml). The number of cells in the lower chamber was evaluated after 6 h incubation at 37 °C as described [19].

Results

FES tyrosine kinase interacts with KIT via its SH2 domain

To identify new intracellular proteins that interact with KIT receptor, a yeast two-hybrid screen was performed using KITD816V intracellular domain fused to LexA DNA binding domain

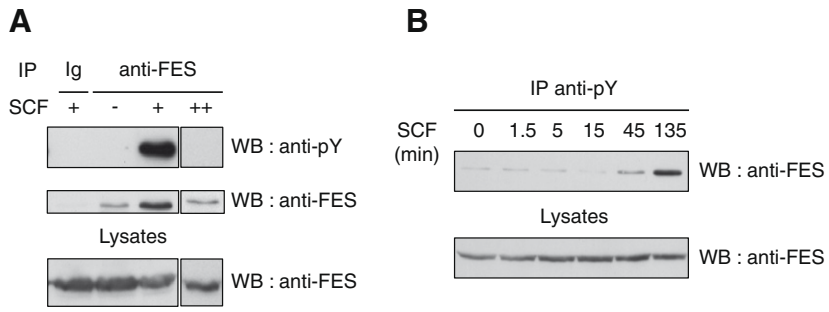

Fig. 2. Tyrosine phosphorylation of FES following KIT stimulation. (A) Phosphorylation of FES following KIT activation. TF-1 cells were stimulated (+) or not (−) with SCF for 2 h. "++" are cells grown (i.e. stimulated permanently) with SCF. Proteins in cell lysates were then immunoprecipitated either with an anti-FES rat monoclonal antibody or with an isotype control antibody. Phosphorylation of FES was revealed with anti-phosphotyrosine antibody (pY) (upper panel). Expression of FES protein in the cell lysates used for immunoprecipitation is shown in the lower panel. (B) Kinetics of FES phosphorylation upon SCF stimulation. Lysates from TF-1 cells were stimulated for the indicated time. The phosphorylated form of FES was revealed by immunoprecipitation with anti-phosphotyrosine antibody and detection with a specific anti-FES monoclonal antibody. Expression of FES in the cell lysates are shown in the lower panel.

as bait and a cDNA library from EML-c1 cells. KITD816V is a gain-of-function mutation. The bait was functionally tested against the SH2 domains of p85 and GRB-2 before the screen. Nine independent clones were isolated from the library. They all interacted with KITD816V and WT KIT but not with the control bait LexA-lamin and KIT W42, a loss of function mutant [21]. Four clones corresponded to proteins known to interact with KIT: the regulatory subunit p85 of PI3-kinase, SHC-2, GRB-2 and SOCS-1. The other clones were the adapter proteins SH2-B, VAV 1 and 3BP2 and the tyrosine kinases ABL and FES (Fig. 1A). Since all nine clones were partial cDNAs containing SH2 domains and since the interactions were not seen with the kinase dead mutant KITW42, these associations presumably depend on phosphotyrosine/SH2 domain interactions.

To determine whether the endogenous FES protein interacted with KIT receptor, we used the human erythroleukemia cell line TF-1 which expresses both proteins. TF-1 cells were stimulated with KIT–ligand SCF, then KIT receptor was immunoprecipitated and the presence of FES was looked for in the immuno-complex. FES was found in association with KIT only after SCF treatment

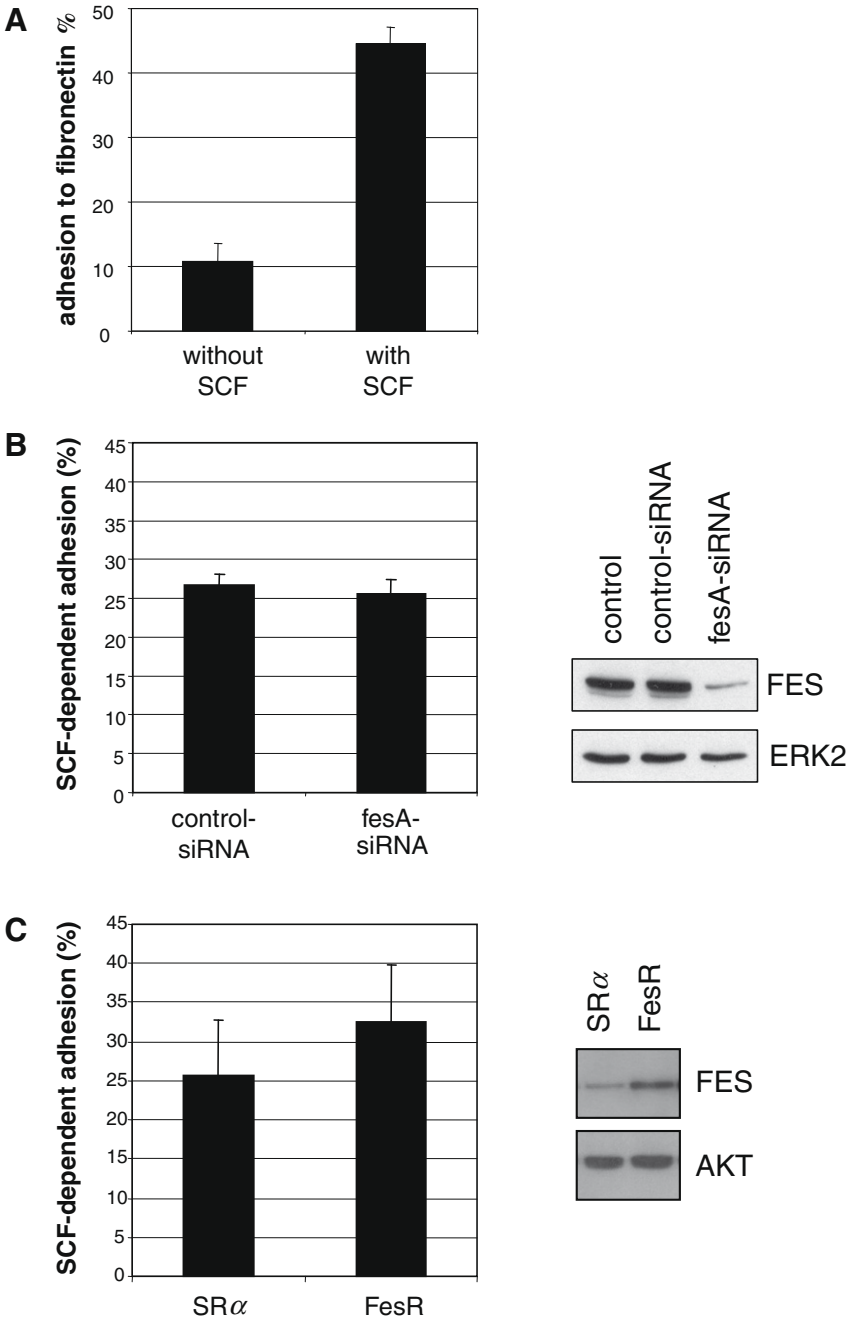

Fig. 3. Cell adhesion to fibronectin in response to SCF is not dependent on FES. (A) Effect of SCF treatment on adhesion to fibronectin of TF-1 cells. Starved TF-1 cells were plated on fibronectin coated plates in the presence or absence of SCF and the percentage of adherent cells was estimated as indicated in Materials and methods. (B) Effect of reduced FES expression on adhesion. Adhesion assay were done following electroporation of cells either with control, fes or no siRNA as indicated. The results are expressed as SCF-dependent adhesion (% of cells that adhere with SCF minus the % without SCF). The Western-blot shows the effect of siRNAs on FES protein expression. (C) Adhesion of TF-1 cells stably transfected with a kinase-dead FES mutant. The assay was performed using a population of TF-1 cells stably transfected either with a dominant negative FES (FesR) or with the control vector (SRα).

(Fig. 1B). The interaction was transient, detected after 15 min and peaked at 135 min. The interaction was also found in transfected COS cells (Fig. S1).

The two major sites of recruitment of SH2-containing proteins in KIT are Y568–Y570 motif in KIT juxtamembrane domain and Y721 in the kinase insert region. Y721 is the site of recruitment of p85, the regulatory subunit of PI3-kinase, while Y568–Y570 has been shown to interact with many intracellular proteins in various cells [22]. We tested whether FES could interact with the juxtamembrane motif using a combination of four peptides which differ in the phosphorylation state of the two tyrosines. In pull-down assays using the peptides and cell lysates containing endogenous FES protein, FES did not interact with non-phosphorylated peptide nor with the pY570 mono-phosphorylated peptide. By contrast, a weak interaction with pY568 peptide and strong interaction with the di-phosphorylated peptide pY568pY570 were detected (Fig. 1C). This suggests that the Y578Y570 motif of KIT is a site of recruitment of FES SH2 domain.

FES is phosphorylated following of KIT receptor activation

We next investigated whether FES was activated downstream of KIT. Tyrosine-phosphorylated FES is considered as the activated form of FES kinase [23–25]. TF-1 cells were stimulated with SCF and FES was immunoprecipitated using a specific monoclonal antibody. FES was phosphorylated on tyrosine only following KIT stimulation (Fig. 2A). However, FES activation downstream of KIT is transient since FES is not phosphorylated following prolonged stimulation with KIT ligand (Fig. 2A). The kinetics of FES phosphorylation was similar to that of KIT/FES interaction (Fig. 2B). These data indicate that FES kinase is phosphorylated downstream of KIT and suggest that FES is activated following SCF stimulation.

FES is dispensable for SCF-induced adhesion to fibronectin in TF-1 cells

KIT and its ligand SCF induce pleiotropic cellular responses such as proliferation, survival, chemotaxis and adhesion. In a previous study, we have observed that FES was dispensable for WT KIT evoked cell proliferation and survival in TF-1 cells [16]. We therefore challenged cell adhesion and chemotaxis responses in the absence of FES. To this end, we used both RNA interference to reduce FES protein expression and a population of TF-1 cells stably transfected with a dominant negative mutant of FES [17].

Activation of KIT resulted in increased cell adhesion to fibronectin but not to other matrix proteins such as collagens, laminin or vitronectin [26] (Fig. 3A). Adhesion to fibronectin in response to SCF stimulation was tested in TF-1 cells treated either with control- or fes-siRNAs (Fig. 3B). In these experiments, reduction of FES expression did not alter the percentage of adherent cells (26.7% vs 25.6%). Similar results were obtained with TF-1 cells stably transfected with the dominant negative FESK590R mutant (Fig. 3C). We concluded that FES was dispensable for adhesion to fibronectin downstream of KIT.

Impaired SCF-induced chemotaxis in the absence of FES in TF-1 cells

In parallel to adhesion experiments, an SCF-dependent chemotaxis assay was performed with the various TF-1 cells. We used a Transwell assay to measure cell migration towards SCF. In our experiments, control cells showed a chemotactic response in the presence of 100 ng/ml of SCF (Fig. 4A, Fig. S2). As a positive control in this assay, we inhibited the expression of FER protein which was previously shown to be required for SCF-dependent chemotaxis in mast cells [15]. Two independent siRNAs targeting c-*fes* showed a twofold reduced migration of FES-deficient TF-1 cells towards the gradient of SCF (Fig. 4A).

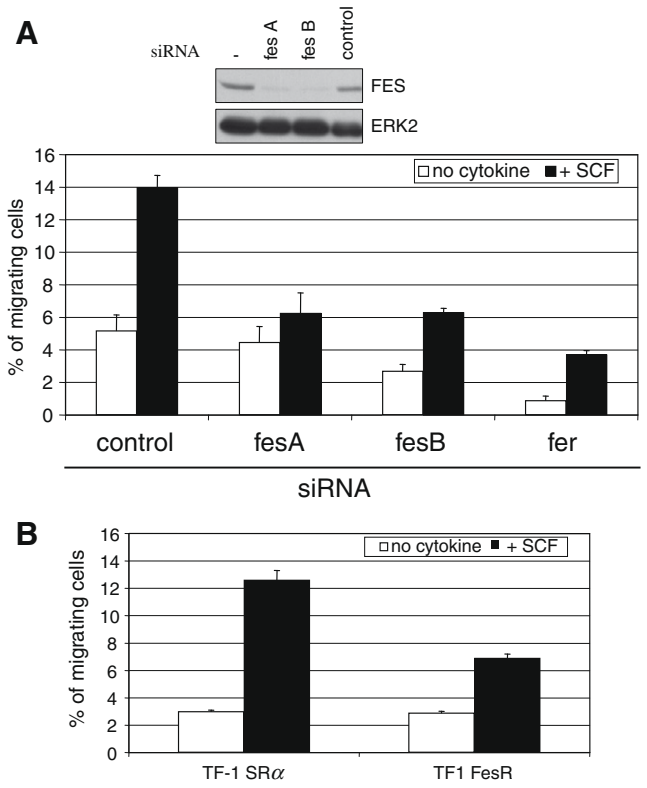

Fig. 4. Reduced SCF-driven chemotaxis in FES deficient cells. (A) Migration of TF-1 cells treated with fes-siRNAs. Cells treated either with control, or fesA, or fesB or fer siRNAs were plated in the upper chamber of a modified Boyden chamber. The lower chamber contained medium with or without SCF (100 ng/ml) as indicated. fesA and fesB are two independent siRNAs. The Western-blot shows the level of FES expression in the siRNA treated cells. (B) Migration of TF-1 cells stably transfected with a kinase-dead FES mutant. The same assay was done using populations of TF-1 cells stably transfected with a dominant negative FES mutant (FesR) or the control plasmid (SRα).

To confirm this result, the same assay was applied to TF-1 cells that express FES mutant K590R. Compared to control cells, migration of TF-1 FESK590R towards SCF was reduced by half (Fig. 4B). This reduction was comparable to that observed with fes-siRNAs. We concluded that SCF-driven chemotaxis was impaired in the absence of FES expression or kinase activity.

Discussion

We report here a link between the cytoplasmic tyrosine kinase FES and KIT receptor, suggesting that FES is a signal transducer downstream of KIT. FES is part of KIT multiprotein signalling complex and becomes activated following KIT activation. Among the cellular responses initiated upon KIT activation, proliferation, survival and adhesion to fibronectin did not depend on FES expression. Instead, FES was required for SCF-induced chemotaxis.

Interestingly, a similar function for the FES related-kinase FER was previously reported in mast cells [15]. Both SCF- and IgE-dependent chemotaxis was largely impaired in cells that expressed a kinase-defective FER mutant [15]. Therefore, both FES and FER seem individually required for a similar function. This suggests that despite their structural similarities, they are involved in two different fundamental steps of the migration process. Alternatively, FES and FER may act at the same level with some redundancy but the expression of these kinases may be rate limiting.

We have initially studied FES function in oncogenic KIT receptor signalling, and our experiments were in part done with TF-1 cells. In the context of mutant receptor, FES is permanently activated

and is absolutely required for cell proliferation. Under WT KIT stimulation, FES phosphorylation is transient and results in a different final outcome: proliferation and survival are not dependent on FES while chemotaxis is. This suggests that the altered regulation of FES activation in the mutant context is responsible for the redirected function of FES in cell proliferation.

Our results do not address the molecular mechanism by which FES exerts its function downstream of KIT. The way FES becomes activated by cell surface receptors is not clear. The fact that FES interacts with KIT suggests that FES is activated by the receptor signalling complex. It remains to be determined whether the receptor or another kinase such as a member of SRC family kinases is the direct upstream FES activator. The positioning of FES in classical signalling pathways is also unclear. Many signalling proteins have been proposed to interact or be substrates for FES kinase but to date no *bona fide* FES pathway has been demonstrated. As stated in Introduction, a possible link between Fes activation and cell migration [13] may be the cytoskeleton and proteins involved in cell adhesion. It remains to be addressed whether proteins such as actin, cortactin, ezrin or tubulin are modified by FES in the context of KIT signalling. Clearly, the study of the molecular mechanism of FES is a challenge to better understand its function in cell biology.

During the preparation of this manuscript, a study of Smith and colleagues reporting a function for FES in cell migration downstream of KIT and β-1 integrin receptors was published online [27]. Their results support a function for FES downstream of KIT in mouse mast cells. In disagreement with our observations in human cells, they also report a function for FES on cell adhesion to fibronectin.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.116.

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