The KRAB-associated co-repressor KAP-1 is a coiled-coil binding partner, substrate and activator of the c-Fes protein tyrosine kinase

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INTRODUCTION

The human c-Fes locus is the cellular homologue of the transforming oncogenes expressed by several avian and feline retroviruses and encodes a 93 kDa non-receptor PTK (protein tyrosine kinase) [1–8]. The c-Fes kinase is expressed in granulocytic and monocytic cell lineages, vascular endothelial cells and various neuronal cell types where it is activated by a variety of cytokines and growth factors [6–18; reviewed in 19,20]. A number of studies have suggested an important role for Fes in the differentiation, survival and proliferation of these diverse cell types. For example, Fes antisense oligonucleotides blocked PMA-induced differentiation of HL-60 human promyelocytic leukaemia cells and FDC-P1/MAC-11 murine myeloid precursor cells [21]. In addition, previous studies have shown that constitutively active forms of Fes induce macrophage differentiation in human promonocytic U937 cells and promote survival and differentiation of the myeloid leukaemia cell line, TF-1 [22,23]. Furthermore, a human CML (chronic myelogenous leukaemia)-derived cell line (K-562) that lacks Fes expression can be induced to undergo growth arrest and terminal differentiation following the re-introduction of Fes, implicating this kinase as a suppressor of CML growth arrest and terminal differentiation following the re-introduction of Fes. Together, these data identify a novel Fes–KAP-1 interaction, and suggest a dual role for KAP-1 as both a Fes activator and downstream effector.

Several studies have identified important roles for the CC domains in the regulation of Fes kinase and biological activities. For example, mutations within CC1 increase Fes kinase and differentiation-inducing activities, suggesting an inhibitory role for CC1 in vivo [22,24]. On the other hand, mutation of CC2 partially reverses the increase in Fes activity that follows mutation of CC1, implicating the CC2 domain as a positive regulator [22,24]. Gel-filtration studies suggest a role for the CC domains in the inter-conversion of Fes between inactive monomeric and active oligomeric states [22]. These motifs however, may also mediate novel heterotypic interactions with upstream Fes activators and/or downstream Fes substrates.

In the present study, to identify novel Fes CC binding partners, a yeast two-hybrid screen was initiated, with the Fes CC2 domain serving as bait. A clone encoding the B boxes and CC domain of KAP (Kruppel-associated box-associated protein)-1 was identified as a Fes CC2 interacting partner from a K-562 CML cell cDNA library. KAP-1 is a transcriptional co-repressor that is recruited to DNA through association with KRAB (Kruppel-associated box) domains found in a variety of Kruppel-class Cys2-His2 zinc finger proteins [29–31]. Once recruited to DNA, KAP-1

Key words: c-Fes, coiled-coil, KAP-1, non-receptor protein tyrosine kinase, protein–protein interaction, yeast two-hybrid system.
Myc tag, respectively. CC1 includes the Fes CC1 domain (Met1–Leu176), CC2 includes the Fes CC2 domain (Leu 291–Gly392), whereas GCN4–CC encompasses the CC domain of the yeast transcription factor GCN4 was PCR-amplified and inserted into pGBK7. The coding region for full-length wild-type Fes was also PCR-amplified and inserted into pGBK7 to express DBD–Fes. The Fes CC1 domain coding region was also subcloned into pGADT7 to express AD–CC1. All clones expressed from pGBK7 included a Myc tag, while clones expressed from pGADT7 included the HA epitope. Nucleotide sequences of all PCR-derived cDNA clones were confirmed by DNA sequence analysis.

**Yeast two-hybrid analysis**

The yeast two-hybrid screen employed the Matchmaker Gal4 Two-Hybrid System 3 (Clontech) in which Saccharomyces cerevisiae strain AH109 was co-transformed with the bait plasmid DBD–CC2 and the K-562 cell cDNA library (AD-library) according to the manufacturer’s protocol. For the initial library screen, yeast co-transformed with DBD–CC2 and AD-library plasmids were plated on minimal synthetic dropout medium lacking leucine, tryptophan, adenine and histidine (SD–L–W–A–H) to select for interacting clones. Fifty putative Fes CC2 interacting clones were initially identified out of approximately 4.5 × 10^6 co-transformed yeast cells. Following three additional rounds of selection, CC2-interacting clones were recovered and re-tested to eliminate false-positive interactions with the Gal4 DBD alone. One of these clones was sequenced and identified as a partial KAP-1 clone (AD–KAP-1 114–357). All subsequent yeast-two-hybrid analyses were initially plated onto solid minimal synthetic dropout medium lacking leucine and tryptophan (SD–L–W) to select for the presence of both plasmids. Three or four independent colonies were then replated onto SD–L–W medium and SD–L–W–A–H medium. All yeast were grown at 30°C.

**Yeast protein extraction**

Yeast co-transformations were performed as described for the yeast two-hybrid analyses and protein extraction was performed as described previously [37] with minor modification. Briefly, independent colonies exhibiting growth on solid SD–L–W medium were used to inoculate liquid SD–L–W medium and grown overnight. The resulting culture was diluted to an OD_600 of 0.2 in liquid YPAD (1% yeast extract, 2% peptone, 0.004% adenine and 2% dextrose) medium and grown until the OD_600 reached 0.6. Yeast (7.5 × 10^9 units) were collected by centrifugation, resuspended in 300 μl of water and combined with 300μl of 0.2M NaOH. Following incubation at room temperature (22°C) for 5 min, yeast cells were pelleted and resuspended in 150 μl of 2× SDS-SB (SDS-sample buffer).

**Recombinant baculovirus generation and protein expression in SF-9 insect cells**

Recombinant baculoviruses were generated by co-transfection of baculovirus expression plasmids with pVl-GST vectors in SF-9 cells.

**MATERIALS AND METHODS**

**Yeast expression plasmids**

The yeast two-hybrid fusion proteins used in this study are illustrated in Figure 1. The pGBK7 vector (Clontech) encodes the Gal4 DBD (DNA binding domain) and expresses a leucine-selectable marker, whereas the pGADT7 vector (Clontech) expresses the Gal4 AD (activation domain) and confers tryptophan selection. A human K-562 cDNA library, (Clontech) fused in frame to an N-terminal Gal4 AD and a HA (haemagglutinin) epitope tag, was amplified according to the manufacturer’s instructions. Coding regions for the Fes CC1 domain (amino acids Met1–Leu176), CC2 domain (amino acids Leu301–Gly392) and CC2 domain harbouring an L334P mutation [22] were PCR-amplified and subcloned into pGBK7 for expression of DBD–CC1, DBD–CC2 and DBD–L334P respectively. To express DBD–GCN4CC, the coding sequence for the CC domain of the yeast transcription factor GCN4 was PCR-amplified and inserted into pGBK7. The coding region for full-length wild-type Fes was also PCR-amplified and inserted into pGBK7 to express DBD–Fes. The Fes CC1 domain coding region was also subcloned into pGADT7 to express AD–CC1. All clones expressed from pGBK7 included a Myc tag, while clones expressed from pGADT7 included the HA epitope. Nucleotide sequences of all PCR-derived cDNA clones were confirmed by DNA sequence analysis.

**Yeast two-hybrid system**

Wild-type Kap-1 is shown at the top for reference. AD–KAP-1 114–357 was isolated in a yeast two-hybrid screen using the c-Fes CC2 domain as bait. This clone includes amino acids 114–357 of KAP-1 which encompass the B boxes and CC domain. A frameshift mutation (mt) at residue 357 results in a stop codon (TGA) ten amino acids downstream. The AD construct consists of the Gal4 AD and confers tryptophan selection, CC2-interacting clones were recovered and re-tested to eliminate false-positive interactions with the Gal4 DBD alone. One of these clones was sequenced and identified as a partial KAP-1 clone (AD–KAP-1 114–357). All subsequent yeast-two-hybrid analyses were initially plated onto solid minimal synthetic dropout medium lacking leucine and tryptophan (SD–L–W) to select for the presence of both plasmids. Three or four independent colonies were then replated onto SD–L–W medium and SD–L–W–A–H medium. All yeast were grown at 30°C.

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**Recombinant baculovirus generation and protein expression in SF-9 insect cells**

Recombinant baculoviruses were generated by co-transfection of baculovirus expression plasmids with pVl-GST vectors in SF-9 cells.
expression plasmid provided by Dr Frank Rauscher III, The Wistar Institute Cancer Center, Philadelphia, PA, U.S.A.) and inserted into pVL-GST for expression of GST–KAPCC.

Recombinant baculoviruses were generated from the pVL-GST constructs by co-transfection of SF-9 cells with Baculogold DNA (Pharmingen) utilizing CellFectin® Reagent (Invitrogen) according to the manufacturer’s protocol. Following 4 days in culture, the primary viral supernatant was clarified by centrifugation and this primary baculovirus stock was amplified according to methods described previously [38]. For protein expression, 2.5 × 10^6 SF-9 cells were infected with baculoviruses for 1 h at room temperature (22°C). The virus was replaced with fresh medium and cells were incubated at 27°C for 2–3 days.

**SF-9 cell lysis and GST-pull-down analyses**

Infected SF-9 cells were washed twice with ice-cold PBS and pelleted by centrifugation. Cell pellets were re-suspended in RIPA (radio-immunoprecipitation assay) buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM PMSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 2 mM sodium orthovanadate and 25 mM sodium fluoride] and sonicated for 10 s. The lysate was clarified by micro-centrifugation and a portion was combined with an equal volume of 2× SDS-SB, heated to 95°C for 5 min and stored at −80°C. The remaining lysate was incubated with 20 μl of GSH (glutathione)–agarose beads (50% slurry) for 2 h at 4°C. The GSH–agarose precipitate was washed three times with RIPA buffer, re-suspended in 2× SDS-SB, heated to 95°C for 5 min and stored at −80°C.

**293T cell transfection, lysis and immunoprecipitation**

Mammalian cell expression vectors for Fes, Fes-L145P and Fes-KE include C-terminal FLAG epitope tags and have been described previously [22]. KAP-1 was expressed from the pC3 FLAG–KAP-1 expression vector, which encodes KAP-1 amino acids Pro295–Pro371 plus an N-terminal FLAG epitope tag. For transient transfections, 293T cells were plated in 60 mm (1.0 × 10^6 cells) or 100 mm (2.5 × 10^6 cells) plates and transfected 1 day later according to the calcium phosphate method as described previously [24]. At 2–4 days post-transfection, cells were lysed in Fes lysis buffer [50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM MgCl₂, 2 mM PMSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 2 mM sodium orthovanadate and 25 mM sodium fluoride] and sonicated for 10 s. The lysate was clarified by micro-centrifugation and a portion was combined with an equal volume of 2× SDS-SB, heated to 95°C for 5 min and stored at −80°C. For immunoprecipitation, the remaining lysate was incubated with 5 μl of a Fes-specific rabbit polyclonal antiserum (provided by Dr Peter Greer, Queen’s University Cancer Research Institute, Ontario, Canada) or Fes-specific goat polyclonal antiserum at 1 μg/ml (Fes-N-19, Santa Cruz Biotechnology) with 20 μl of protein G–Sepharose (1:1 w/v slurry) for 2 h at 4°C. Alternatively, FLAG-tagged Fes was immunoprecipitated with 20 μl of the anti-FLAG-M2 agarose affinity gel (Sigma). KAP-1 was also immunoprecipitated with the anti-FLAG-M2 agarose affinity gel. All immunoprecipitates were washed three times with RIPA buffer, re-suspended in 2× SDS-SB, heated to 95°C for 5 min and stored at −80°C.

**Immunoblot analyses**

Antibodies anti-Myc (9E10), anti-GST (B-14) and anti-phosphotyrosine (PY99) were obtained from Santa Cruz Biotechnology. Antibodies anti-HA (HA-7) and anti-FLAG (M2) were obtained from Sigma. All antibodies were used at 1 μg/ml except for the anti-HA antibody that was used at a 1:10000 dilution. Aliquots of cell lysates or immunoprecipitates were separated by discontinuous SDS/PAGE (10% or 12% gels). Proteins were transferred to PVDF membranes, blocked overnight at 4°C or for 1 h at room temperature (22°C) in TBST–BSA [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20 and 1.5% BSA], and probed for 1 h at room temperature (22°C) or overnight at 4°C with primary antibodies diluted in TBST–BSA.

Following incubation with primary antibodies, membranes were probed for 1 h at room temperature (22°C) with the appropriate alkaline phosphatase-conjugated secondary antibodies (Southern Biotechnology) diluted 1:10000 in TBST–BSA. All blocking and antibody incubations were followed by three TBST washes for 5 min at room temperature (22°C). Detection was performed using the CDP-STAR® Chemiluminescence Reagent detection system (PerkinElmer) or with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro Blue Tetrazolium). In some instances, blots were stripped by a 30 min incubation at 50°C in strip buffer (62.5 mM Tris/HCl, 100 mM 2-mercaptoethanol and 2% SDS) and re-probed.

**Detection of Fes–KAP-1 complexes in HL-60 cells**

HL-60 cells (a gift of Dr Richard Steinman, University of Pittsburgh Cancer Institute, Pittsburgh, PA, U.S.A.) were grown in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biological) and 50 μg/ml gentamicin (Gibco). Cells were maintained at a density of 10⁶–10⁷ cells/ml. To induce granulocytic differentiation, cells were seeded at a density of 5.0 × 10⁶ cells/ml and treated with DMSO (Sigma) to a final concentration of 1.25% or all-trans retinoic acid (1 μM; a gift of Dr Neil Hukriede, University of Pittsburgh School of Medicine, Pittsburgh, PA, U.S.A.). Differentiation was assessed after 4 days as the percentage of cells able to reduce NBT. To detect Fes–KAP-1 complexes, cells (2.5 × 10⁷) were sonicated in 0.5 ml of 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 50 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 2 mM PMSF, 25 μM sodium orthovanadate, 25 mM sodium fluoride and Protease Inhibitor Cocktail Set III (Calbiochem). Lysates were clarified and aliquots were incubated with 2 μg of a Fes-specific goat polyclonal antiserum (Fes-C-19; Santa Cruz Biotechnology) for 1 h at 4°C, followed by an additional hour with 30 μl protein G–Sepharose beads (AP Biotech; 1:1 w/v slurry) at 4°C. The beads were washed three times with lysis buffer and protein complexes were eluted by heating in SDS-SB. Immunoprecipitates and clarified lysates were subjected to immunoblot analysis with antibodies to Fes (C-19; 1 μg/ml) and KAP-1 (Bethyl Laboratories, A300-274A; 1:1000 dilution). Lysates were also blotted with actin antibodies (Chemicon MAB1501; 1:10000 dilution) as a loading control.

**RESULTS**

**Identification of KAP-1 as a Fes CC domain binding protein**

The Fes CC2 domain has been identified as an important modulator of Fes kinase and biological activities, suggesting that this motif may recruit binding partners involved in the regulation of kinase activity and downstream signalling [22,24]. To identify Fes CC2-interacting proteins, the CC2 domain was used as bait to screen a K-562 cell cDNA library by yeast two-hybrid analysis. K-562 cells were originally derived from a patient with CML [39] and are devoid of detectable Fes expression [6,25]. Re-expression of Fes, however, promotes growth arrest and terminal
**Figure 2** KAP-1 selectively interacts with the Fes CC2 domain

Yeast strain AH109 was co-transformed with the indicated pairs of plasmids and grown either on plates lacking leucine and tryptophan (−L−W) or on plates lacking leucine, tryptophan, adenine and histidine (−L−W−A−H; upper panel). Growth on −L−W plates selects for the presence of both plasmids and growth on −L−W−A−H plates is indicative of protein–protein interaction. Results from four independent transformants are shown for each combination. Yeast colonies from −L−W plates in the upper panel were grown in liquid medium and cell lysates were immunoblotted with Myc (middle panel) or HA antibodies (lower panel) to detect the DBD and AD fusions respectively. The positions of the fusion proteins are indicated on the left. Data are representative of three independent experiments.

**Figure 3** KAP-1 interacts with full-length Fes

Yeast strain AH109 was co-transformed with the indicated pairs of plasmids. Colonies were replated on −L−W plates to verify transformation of both plasmids and on −L−W−A−H plates to test for protein–protein interaction (upper panel). Results from four independent transformants are shown for each combination. Colonies from −L−W plates were grown in liquid medium and clarified lysates were subjected to immunoblot analyses utilizing Myc antibodies to detect DBD-fusion proteins and HA antibodies to visualize AD-fusion proteins as indicated (lower panels). The positions of the AD and DBD proteins are indicated on the left. Data are representative of three independent experiments.

**KAP-1 CC domain interacts with the Fes N-terminal region**

We next investigated whether the results observed in the yeast two-hybrid system suggest that KAP-1114-357 is selective for the Fes CC2 motif and is not a general CC interacting protein. Despite the lack of interaction between Fes DBD–CC1 and AD–KAP-1114-357, DBD–CC1 did interact with a Fes AD–CC1 fusion protein, indicating that the Fes CC1 domain is functional when expressed in yeast. Mutation of a Leu134 to proline within the Fes CC2 domain (DBD–CC2 L334P) eliminated interaction with KAP-1114-357. Previous studies have established that Leu134 occupies a critical position within the heptad repeat predicted to form the CC structure [22,28,40]. Clarified yeast cell lysates were immunoblotted with antibodies against the Myc and HA epitopes to verify expression of the DBD and AD fusion proteins, respectively (Figure 2, lower panels).

In a final series of experiments, we investigated the interaction of full-length Fes with KAP-1114-357 in the yeast system. Fes was fused to the Gal4 DBD (DBD–Fes) and assessed for association with AD–KAP-1114-357, AD–KAP-1114-357 interacted with DBD–Fes, permitting growth on SD-L-W-A-H medium (Figure 3). Immunoblots of yeast cell lysates demonstrate expression of the DBD–Fes and AD–KAP-1114-357 fusion proteins as well as the DBD and AD controls.

**The KAP-1 CC domain directly interacts with the Fes N-terminal region**

We next investigated whether the results observed in the yeast two-hybrid assay were due to direct interaction between the KAP-1 and Fes proteins. In these studies, Fes and KAP-1 proteins were co-expressed in Sf-9 insect cells using recombinant baculoviruses. The baculoviruses used for these experiments are illustrated in Figure 4. In the first experiment, GST–KAP-1114-357 was co-expressed with FLAG-tagged full-length Fes. GST–KAP-1114-357 was then precipitated with glutathione–agarose beads and associated Fes was visualized by immunoblotting with FLAG-specific antibodies. GST–KAP-1114-357 bound full-length wild-type Fes, whereas no association with Fes was observed with GST alone.
KAP-1 is a substrate and activator of c-Fes

Figure 4 Baculoviruses expression constructs

Recombinant baculoviruses were created for expression of wild-type Fes and a series of deletion mutants in Sf-9 insect cells. The Fes deletion mutants include the Fes N-terminal region alone (Fes N), the Fes SH2 domain plus the kinase domain (Fes ΔSH2), and the Fes SH2 domain plus the kinase domain alone (Fes kinase). All Fes constructs include a C-terminal FLAG epitope tag (F). Baculoviruses were also created for expression of GST fused to the KAP-1 CC domain (GST–KAPCC) or to the B boxes plus the CC domain (GST–KAPCC).

(Figure 5). Because KAP-114−357 contains a CC domain that interacted with the Fes CC2 domain in the yeast two-hybrid assay (Figure 2), we also tested whether the KAP-1 CC domain alone was sufficient to interact with Fes. As shown in Figure 5, a GST–KAP-1 CC domain fusion protein also associated with Fes at levels comparable to those observed for GST–KAP-114−357. This result shows that the KAP-1 CC domain is sufficient for recognition of c-Fes.

To identify the Fes domains required for KAP-1 binding, the Fes deletion mutants illustrated in Figure 4 were co-expressed with GST–KAP-114−357 or GST and assessed for association as described in Figure 5 for full-length Fes. Figure 6 (upper panel) demonstrates that the Fes N-terminal region alone (N) and a Fes deletion mutant lacking the SH2 domain (ΔSH2) exhibited strong interaction with KAP-114−357. Neither Fes deletion construct interacted with GST, demonstrating a dependence on the KAP-1 sequence. In contrast, a construct lacking the N-terminal domain (ΔN) did not bind to KAP-114−357. These results demonstrate that KAP-114−357 interacts with the unique N-terminal region of Fes, which contains the CC2 domain used in the original two-hybrid screen (Figure 2). Surprisingly, a Fes construct containing only the Fes kinase domain also interacted with KAP-114−357. This result suggests the presence of a second KAP-114−357 interacting motif that becomes accessible following deletion of the Fes N-terminal and SH2 domains (see the Discussion section). The blots in the upper panel of Figure 6 were stripped and re-probed with antibodies against GST (Figure 6, middle panel) to demonstrate equivalent levels of GST-fusion proteins. Clarified lysates were blotted for FLAG to verify equal expression of the Fes deletion mutants (Figure 6, lower panel).

To determine whether the KAP-1 CC domain alone was sufficient to interact with the Fes N-terminal region and kinase domain, GST–KAPCC was co-expressed with the Fes deletion constructs and assessed for association. As shown in Figure 6 (upper panel), the KAP CC domain efficiently interacted with Fes mutants that retained the N-terminal region (N and ΔSH2), whereas deletion mutants lacking this region (ΔN and kinase) exhibited low or undetectable association with KAPCC (upper panel). Together, these data suggest that KAP114−357 interaction with the Fes N-terminal region is dependent on the KAP-1 CC domain whereas a secondary contact between KAP114−357 and the Fes kinase domain is independent of the KAP CC domain and may reside in the B-box motifs.

Fes interaction with full-length KAP-1 in mammalian cells requires an activating mutation in the Fes CC1 domain

To determine whether Fes interacts with KAP-1 in mammalian cells, co-immunoprecipitation analyses were performed. For these studies, 293T cells were co-transfected with a KAP-1 expression plasmid as well as expression vectors for wild-type Fes (WT), a kinase dead form (KE) of Fes containing a glutamate to lysine mutation at residue 590 within the ATP binding site, or a CC1 point mutant (L145P) shown previously to induce strong activation of Fes kinase activity [22]. The KAP-1 and Fes constructs were tagged with the FLAG epitope. Fes was then immunoprecipitated from clarified cell extracts with an antibody directed against the N-terminal region, and associated KAP-1 was identified via
immunoblotting with a FLAG-specific antibody (M2; Figure 7A, upper panel). Surprisingly, full-length KAP-1 interaction with wild-type Fes was low or undetectable. The introduction of a point mutation within the CC1 domain significantly enhanced association of Fes with KAP-1. This mutation has been shown to enhance Fes kinase and biological activities [22] through a mechanism that may expose the CC2 domain for substrate recruitment (see the Discussion section). Control FLAG (M2) immunoblots show equivalent recovery of Fes protein in each immunoprecipitate as well as equivalent expression of KAP-1 under all conditions.

**KAP-1 is a Fes substrate**

The direct interactions between Fes and KAP-1 observed in yeast and mammalian cells suggested that Fes binding may induce KAP-1 tyrosine phosphorylation. To test this hypothesis, 293T cells were co-transfected with Fes and KAP-1 expression plasmids as described for Figure 7(A). Following immunoprecipitation of KAP-1 with FLAG-specific antibodies, phosphotyrosine content was analysed by Western blotting with the anti-phosphotyrosine antibody PY99 (Figure 7B, upper panel). Co-expression of KAP-1 with either wild-type Fes or the active L145P mutant induced strong KAP-1 tyrosine phosphorylation.

In contrast, KAP-1 tyrosine phosphorylation was undetectable in the absence of Fes or upon co-expression of a kinase-defective Fes mutant (KE). The blot in the upper panel of Figure 7(B) was stripped and re-probed with a FLAG-specific antibody to demonstrate equivalent immunoprecipitation of KAP-1. Immunoblots of the clarified lysates demonstrate equivalent expression of the wild-type and L145P forms of Fes in the presence and absence of KAP-1.

To provide further evidence that KAP-1 is a direct substrate for Fes and to map the region of KAP-1 that is phosphorylated, Sf-9 insect cells were infected with the GST–KAP-1114−357 and GST–KAPCC baculoviruses in the absence or presence of a c-Fes baculovirus. GST fusion proteins were precipitated with glutathione–agarose beads and KAP phosphotyrosine content was assessed by immunoblotting (Figure 8). Both GST–KAP-1114−357 and GST–KAPCC were strongly tyrosine phosphorylated upon co-expression with Fes (Figure 8, upper panel). Fes did not induce phosphorylation of GST alone. The blot in Figure 8, upper panel was stripped and re-probed with anti-GST antibodies to verify...
KAP-1 is a substrate and activator of c-Fes

Figure 8 Fes phosphorylates the KAP-1 CC domain

Sf-9 cells were infected with baculoviruses carrying GST, GST–KAP-1 114−357 or GST fused to the KAP-1 CC domain (GST–KAPCC) in the presence or absence of a Fes baculovirus. GST fusion proteins were precipitated with glutathione–agarose beads and immunoblotted with anti-phosphotyrosine antibodies (pTyr; upper panel). This blot was stripped and reprobed with a GST antibody to verify GST fusion protein recovery (middle panel). Cell lysates were immunoblotted with FLAG antibodies (M2) to demonstrate equivalent Fes protein expression (lower panel). Data are representative of three independent experiments.

equivalent recovery of the GST fusion proteins (Figure 8, middle panel), while Fes expression was confirmed in the cell lysates by anti-FLAG immunoblotting (Figure 8, lower panel). Together these data indicate that KAP-1 is a Fes substrate and that at least one site of KAP tyrosine phosphorylation occurs within the KAP-1 CC domain.

A slower migrating tyrosine phosphorylated protein was also observed in the GSH-agarose precipitates when Fes was co-expressed with GST–KAP-1 114−357 and GST–KAPCC but not with GST alone (Figure 8, upper panel). This protein co-migrated with the Fes band identified in the clarified lysates, suggesting that active Fes associates with the KAP-1 CC domain. Note that unlike 293T cells, high-level expression of wild-type Fes in Sf-9 insect cells results in constitutive kinase activity [38].

KAP-1 is a Fes kinase activator

Previous studies suggest that Fes is maintained in an inactive state in mammalian cells [22,24]. Fes activation may result from CCM-mediated oligomerization via N-terminal CC domains and subsequent trans-autophosphorylation of the activation loop tyrosine [22,24,27,38]. Because KAP-1 binds to the Fes CC2 domain in the N-terminal region, we reasoned that this interaction could affect Fes kinase activity. To test this hypothesis, full-length KAP-1 was co-expressed in 293T cells with wild-type (WT), active (L145P) or kinase-dead (KE) forms of Fes. Fes proteins were immunoprecipitated and probed for kinase activity via immunoblotting with anti-phosphotyrosine antibodies (Figure 9A, upper panel). In agreement with previous studies, autophosphorylation of wild-type Fes was barely detectable in 293T cells [24,41]. Co-expression with KAP-1 enhanced wild-type Fes autophosphorylation nearly 2-fold (Figure 9B). Disruption of the CC1 domain (Fes L145P) released Fes kinase activity and KAP-1 co-expression had no further effect. KAP-1 did not affect the kinase-dead form of Fes, suggesting that the observed increase in wild-type Fes phosphotyrosine content results from direct KAP-1 binding and does not involve another kinase. The blot in the upper panel of Figure 9(A) was stripped and re-probed with a Fes-specific antibody to demonstrate equivalent recovery of Fes proteins (Figure 9A, middle panel), and clarified lysates were probed with a FLAG-specific antibody to verify KAP-1 expression (Figure 9A, lower panel).

Association of endogenous Fes with KAP-1 in HL-60 leukaemia cells

To determine whether Fes and KAP-1 interact in a physiological setting, we looked for Fes–KAP-1 complexes in HL-60 cells that were induced to differentiate along the granulocytic pathway by treatment with retinoic acid or DMSO. Treatment of HL-60 cells with either of these agents led to the induction of differentiation.
Figure 10 Association of endogenous Fes with KAP-1 in HL-60 cells

(A) HL-60 myeloid leukaemia cells were treated with retinoic acid (RA) or DMSO to induce terminal differentiation or left untreated (Control). The extent of differentiation was assessed 4 days later as the percentage of cells able to reduce NBT. Results are mean ± S.D., n = 6.

(B) Cell lysates from control and differentiated cells in (A) were probed for Fes and KAP-1 protein levels by immunoblotting as described in the Materials and Methods section. An actin blot was also performed as a loading control.

(C) Fes was immunoprecipitated from HL-60 cell lysates and probed for associated KAP-1 as well as Fes by immunoblotting. This experiment was repeated five times, and the level of Fes–KAP-1 interaction shown was observed in two of these experiments. KAP-1 recovery was weaker or absent in the other trials, which is likely to reflect heterogeneity in the level of Fes protein available for immunoprecipitation.

as well as an increase in the level of Fes protein (Figure 10), as has been reported previously [6]. KAP-1 expression was easily detected in lysates of HL-60 cells, although the overall levels of KAP-1 appeared to decline in the differentiated cell lysates. To determine whether endogenous Fes and KAP-1 associate in HL-60 cells, Fes was immunoprecipitated from the cell lysates and probed for associated KAP-1 as well as Fes by immunoblotting. This experiment was repeated five times, and the level of Fes–KAP-1 interaction shown was observed in two of these experiments. KAP-1 recovery was weaker or absent in the other trials, which is likely to reflect heterogeneity in the level of Fes protein available for immunoprecipitation.

**Discussion**

In the present paper, the transcriptional co-repressor KAP-1 was identified as an interacting partner for the non-receptor PTK, Fes. Molecular recognition of KAP-1 by Fes was mediated by mutual CC domain interactions, although other domains may contribute to the association (see below). KAP-1 represents the first protein identified to interact with the Fes CCs, and defines a new mode of substrate recognition for non-receptor protein tyrosine kinases. In addition, KAP-1 binding stimulated Fes autophosphorylation and led to KAP-1 tyrosine phosphorylation, suggesting that KAP-1 is an activator as well as a downstream substrate for Fes.

The KAP-1 clone (KAP-1_{114-357}) isolated from the K562 library screen exhibited selectivity toward the Fes CC2 domain, as it did not interact with the Fes CC1 domain or the CC domain from the yeast transcription factor GCN4 (Figure 2) [42]. Interaction of KAP-1 with the Fes CC2 domain was eliminated upon mutation of the CC2 Leu344 to a proline residue, a substitution previously shown to destabilize CC2 function [22,43]. Moreover, the KAP-1 CC domain alone was sufficient to interact with the Fes N-terminal region, which contains the CC domains. In contrast, the KAP-1 CC domain failed to associate with Fes deletion mutants lacking the N-terminal sequences. Together with the yeast data, these studies strongly suggest that the KAP-1 CC domain interacts directly with the Fes CC2 domain.

Although the Fes CC2 domain is sufficient for KAP-1 binding, experiments in Sf-9 insect cells suggest additional points of contact. This second KAP-1 interaction motif may lie in the Fes kinase domain as this domain alone interacted with KAP-1_{114-357} (Figure 6). In contrast, the KAP-1 CC domain alone did not interact with the Fes kinase domain. As KAP-1_{114-357} contains only the B-boxes and CC domain of KAP-1, this result suggests that the KAP-1 B-boxes are most likely to be responsible for interaction with the Fes kinase domain. Accessibility to this second KAP-1 interaction domain may be important for stable interaction of full-length KAP-1 and Fes in mammalian cells as well, as association was only detected following the introduction of a destabilizing leucine to proline mutation in the Fes CC1 domain (Figure 7A). Interestingly, this same mutation released Fes transforming activity in Rat-2 cells and promoted Fes-induced differentiation and survival of TF-1 myeloid leukaemia cells [22]. In light of these observations, it will be interesting to determine whether KAP-1 or other CC2-binding proteins are required for the biological effects of Fes.

Co-expression of full-length Fes with KAP-1 in mammalian cells resulted in KAP-1 tyrosine phosphorylation (Figure 7B). Phosphorylation was not detected in the absence of Fes or following co-expression with a kinase inactive form of Fes (Fes-KE), suggesting that KAP-1 is a direct substrate for the Fes kinase domain. Similar studies in Sf-9 cells demonstrated strong tyrosine phosphorylation of KAP-1_{114-357} and the KAP-1 CC domain following co-expression with Fes (Figure 8). Our results also identify KAP-1 Tyr^{242} as a phosphorylation site for Fes, as this is the only tyrosine residue present in the KAP-1 CC domain. Tyrosine phosphorylation within the CC domain could potentially alter KAP-1 oligomerization and interactions with other transcription factors.

In addition to serving as a Fes substrate, KAP-1 also stimulated Fes autophosphorylation (Figure 9). The mechanism by which KAP-1 association activates Fes tyrosine kinase activity is not entirely clear. KAP-1 is oligomeric [44], suggesting a mechanism in which KAP-1 oligomers recruit multiple Fes monomers, promoting Fes oligomerization and autophosphorylation. Interestingly, the breakpoint cluster region protein has also been identified as a Fes N-terminal domain binding protein and Fes activator, suggesting that Fes activation via substrate association with its N-terminal region may represent a general activation mechanism [45]. Like KAP-1, the breakpoint cluster region protein has an N-terminal CC domain that mediates tetramerization [46].

KAP-1 represents a new member of a growing list of transcription factors that are modulated by Fes and the closely related FER kinase. Fes has been shown to activate STAT3 (signal transducer and activator of transcription 3) by direct tyrosine phosphorylation in human 293T cells. Macrophages derived from mice in which the endogenous fes gene was replaced with a kinase inactive mutant exhibited reduced STAT3 activation in response to GM–CSF (granulocyte/macrophage colony-stimulating factor) [47,48]. More recently, Fes has been shown to enhance PU.1 and C/EBPα activity in myeloid cell lines [23,49]. STAT3,
PU.1 and C/EBPα together with Fes have been implicated in myeloid cell differentiation suggesting that these transcription factors may act downstream of Fes [21,22,24,25,50–52]. In the present study we show that Fes stably associates with KAP-1 in both control and terminally differentiated HL-60 cells (Figure 10), supporting a connection between these signalling proteins in myeloid differentiation. Other work has shown that differentiation of HL-60 cells promotes localization of Fes to the nucleus [53], where it may come into contact with KAP-1 and other transcription factors that regulate the differentiation programme. This possibility will be the subject of future investigation. Fer associates with and phosphorylates the nuclear transcription factor TGF (TATA element modulatory factor) [54]. TGF binds to the TATA element of RNA polymerase II promoters where it represses transcription by competing for TATA binding protein recruitment to the TATA element. Binding and phosphorylation of transcriptional regulators such as TGF and KAP-1 by the Fes and Fer kinases suggests a more general function for this kinase family in the regulation of transcription.

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