Disruption of Coiled-coil Domains in Fer Protein-tyrosine Kinase Abolishes Trimerization but Not Kinase Activation*

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The protein-tyrosine kinase Fer and the highly homologous proto-oncoprotein Fps/Fes are implicated in signaling from a variety of growth factor and cytokine receptors. Here we examine the molecular basis of Fer kinase activation with an emphasis on the role of oligomerization. We show that Fer forms trimers in vivo and that disruption of either the first or second coiled-coil domain abolishes oligomerization, suggesting a cooperative interaction between these two domains. Although Fps/Fes also forms homotypic oligomers, probably via homologous coiled-coil domains, no heterotypic interactions were observed between Fer and Fps/Fes. Incorporation of catalytically inactive Fer peptides into the oligomeric complex caused only mild reduction of wild type Fer kinase activity, suggesting that kinase-inactive Fer would not behave as a potent dominant negative. Although oligomerization of Fer can potentiate autophosphorylation in trans at three major phosphorylation sites, these residues can likely also be phosphorylated in cis. In contrast, the testis-specific Fer-T isomer does not oligomerize and is able to autophosphorylate in cis at two of the three residues autophosphorylated in Fer. These results suggest that although oligomerization potentiates autophosphorylation in trans, this is apparently not necessary for Fer activation.

Fer is a cytoplasmic protein-tyrosine kinase (PTK) with close structural similarity to the product of the fps/fes proto-oncogene (1). Indeed, Fer was first observed as one of two PTKs detected in myeloid cell lines using antibodies raised against viral Fps/Fes peptides. Peptide mapping analysis revealed these to be distinct, yet closely related PTKs (2, 3). Human and rat cDNAs encoding Fer were subsequently cloned, and a comparison with the genes encoding cellular and viral Fps/Fes proteins confirmed the close structural similarity between p92 Fps/Fes (hereafter referred to as Fps) and p94 Fer (4–6). Although dozens of novel tyrosine kinases have been identified in recent years, Fps and Fer remain the only two known members of a distinct subclass within the nonreceptor PTK family. They each consist of a C-terminal tyrosine kinase domain, a central Src homology 2 (SH2) domain, and an N-terminal region, which contains three predicted coiled-coil (CC) motifs (7, 8). Unlike members of the Src family, Fps and Fer do not possess N-terminal myristoylation sites, SH3 domains, or C-terminal negative regulatory tyrosine phosphorylation sites. It has been suggested that interactions between the SH2 and catalytic domains of Fps might serve a regulatory role (9, 10); however, a clearer understanding of this awaits detailed structural determination.

By analogy with the receptor PTKs, activation of nonreceptor kinases may also be stimulated by oligomerization followed by autophosphorylation in trans. It has recently been suggested that homotypic CC interactions may mediate oligomerization of both Fer (7), and Fps (8). The conserved putative CCs in these kinases could also mediate heterotypic interactions between Fer and Fps. This raises the possibility of functional or regulatory interactions between these two kinases. Alternatively, the CC domains could mediate interactions of these kinases with other CC domain-containing proteins. In this regard, the N-terminal domain of Fer has been shown to mediate association with the catenin family member, p120CAS (7). In addition to the central armadillo repeats, p120CAS also contains a predicted N-terminal CC domain (11, 12); however, it is not known which domain which domain confers interaction with Fer and whether this interaction is direct (13).

Fer has also been detected in association with the activated EGFR and PDGF receptors in fibroblasts (7), as well as the Fc receptor in mast cells (14). In each of these cases, ligand stimulation of the receptor resulted in elevated Fer kinase activity. Associations of Fps with a number of cytokine receptors have also been described, including those for interleukin-3 (15), granulocyte-macrophage colony-stimulating factor (15, 16), interleukin-4 (17), interleukin-6, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and interleukin-11 (18). Due to the close similarity between Fps and Fer, many of the antisera currently in use are unable to distinguish between these two PTKs. This raises the possibility that the PTKs seen in association with some of these cytokine receptors may have been Fer rather than Fps. Alternatively, both kinases could be involved, just as different members of the Jak kinase family may be involved in signaling from some cytokine receptors (19).

The expression patterns of Fps and Fer are quite distinct. Fps is more restricted, with relatively high levels seen in a limited subset of cell types including myeloid, vascular endothelial, and some epithelial and neuronal cells (20, 21). On the other hand, Fer is widely expressed (4, 5), and levels comparable with that of Fps in myeloid cells are observed in most tissues. A further distinctive characteristic of fer is the expression of a testis-specific isoform, FerT, which likely arises from an internal tissue-specific promoter and alternative splicing (22). Murine FerT lacks most of the N-terminal domain of p94

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†The abbreviations used are: PTK, protein-tyrosine kinase; SH2 and SH3, Src homology 2 and 3, respectively; CC, coiled-coil; PAGE, polyacrylamide gel electrophoresis; EGS, ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester).

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Fer and contains a unique 43-amino acid N terminus, followed by the common SH2 and kinase domains. ferT mRNA accumulates transiently in primary spermatocytes during the pachytene stage of meiotic prophase (23). In *Drosophila melanogaster*, cDNAs encoding both the larger DFER and the shorter DFER isoforms have been isolated (24). In *Caenorhabditis elegans*, a putative ferT homolog was identified on chromosome III (25). However, there do not appear to be additional upstream coding sequences that would produce a larger Fer gene product, suggesting that sequences encoding the N-terminal domain of Fer had not yet evolved in *C. elegans*.

There is currently little information regarding the molecular function of Fer, although a role in signaling downstream of activated platelet-derived growth factor, epidermal growth factor, and FcRI receptors has been proposed (7, 14). Recent studies suggest roles for Fer in regulation of the actin cytoskeleton via interaction with cortactin (13) and control of cell adhesion (26). While *fps* was originally identified as a retroviral oncogene (27, 28), the involvement of fer in malignancy or other diseases is not yet established. However, the human fer locus maps to chromosome 5q21, in a region which is frequently deleted or rearranged in myeloid leukemia (29), and recently the in vitro transforming potential of Fer was demonstrated (24). These observations suggest a role for Fer in cell signaling and growth control and a potential involvement in myeloid leukemia or other diseases. Here we examine the role of oligomerization in Fer kinase activation. We show that disruption of either the first or second CC domain completely abolishes oligomerization, and chemical cross-linking indicates that Fer forms trimers. Although both Fer and Fps exist as oligomers in *vivo* and they share conserved CC motifs, no heterotopic association between Fer and Fps was observed. Furthermore, we show that autophosphorylation of Fer probably involves both cis and trans phosphorylation mechanisms. Tryptic phosphopeptide mapping revealed differences in autophosphorylation sites between Fer and FerT that may reflect differences in signal transduction between these isoforms.

**Materials and Methods**

*Plasmid Constructions—*Fer proteins were produced in COS-1 cells using the vector pXN1, which is a modified version of pECE (30) in which the XbaI cloning site was converted to a NotI site by digestion with NotI and ligation with an annealed NotI adapter oligonucleotide (XNot, 5′-CTAGCGGCCGCCG-3′). The 2.7-kilobase pair murine fer cDNA from the pP2c-8 plasmid was excised with ApoI and DraI, blunted with Klenow, and subcloned into the Smal site of pECE. The resulting pECEnFer plasmid was kindly provided by K. Letwin and T. Lawson. The construction of wild-type and Ferglycosylated expression constructs was as described previously (31).

Expression plasmids encoding Myc epitope-tagged wild type and FerTglycosylated were as described previously (31). Mutations within the CC domains were generated using the unique site elimination method (32). Fer proteins were expressed in COS-1 cells using the eukaryotic expression plasmid pEF4, which was generated by the insertion of an EcoRI fragment encoding the human fps cDNA from pFL5.13 (33) into the EcoRI site of pECE (30). The expression plasmid for Myc-Fps was constructed by replacing the green fluorescence protein-encoding portion of fps-green fluorescence protein (20) with six copies of the epitope recognized by the antibodies 9E10, as described monoclonal antibody 9E10, as described for Kpn1 (the latter digestion released a 1.5-kilobase fragment encoding the N-terminal CC domain of Fer), and following gel purification were ligated together. The resulting plasmid allows for initiation of translation at methionine 412 and the production of an N-terminally truncated Fer protein (Myco-FerXN) that resembles the testis-specific FerT, Fer and contains a unique 43-amino acid N terminus, followed by the common SH2 and kinase domains. ferT mRNA accumulates transiently in primary spermatocytes during the pachytene stage of meiotic prophase (23). In *Drosophila melanogaster*, cDNAs encoding both the larger DFER and the shorter DFER isoforms have been isolated (24). In *Caenorhabditis elegans*, a putative ferT homolog was identified on chromosome III (25). However, there do not appear to be additional upstream coding sequences that would produce a larger Fer gene product, suggesting that sequences encoding the N-terminal domain of Fer had not yet evolved in *C. elegans*.

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Chemical Cross-linking—Transfected COS-1 cells were harvested in X-linking buffer (20 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) and clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. Extracts were incubated on ice in the absence or presence of 1 mM ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (EGS) for 15 min (EGS from Sigma was prepared as a 100 mM stock solution in MeSO). The reactions were terminated by the addition of 1 µl of 50× TE (500 mM Tris-HCl (pH 8), 50 mM EDTA) and 50 µl of SDS-loading buffer. Samples were heated at 100 °C for 4 min, followed by SDS-PAGE, and prepared for Western blotting as described above.

Tryptic Phosphopeptide Mapping—COS-1 cells were transfected and harvested, and immune complex kinase assays performed as above. The sources of native Fer and FerT were mouse liver and testis, respectively, which were homogenized in KLB. Fer was immunoprecipitated with anti-Fer polyclonal antisera (5 µl), while FerT was immunoprecipitated with anti-Fps/Fer polyclonal antisera (5 µl), and Myc epitope-tagged proteins were immunoprecipitated with monoclonal antibody 1-9E10. Following the kinase reactions, samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and autoradiography was performed. Portions of the membrane containing the radiolabeled proteins were excised, placed in separate 1.5-ml tubes containing 1 ml of 0.5% polyvinyl pyrolidone-360 (in 100 mM acetic acid), and incubated for 30 min at 37 °C. Following aspiration, the membranes were washed five times with 1 ml of water and once with 1 ml of 100 mM HCO3. Ten micromolars of diphenylcarbamyl chloric acid-treated trypsin (Sigma) in 200 µl of buffer (50 mM NH4HCO3, (pH 8.3)) was added to each sample and incubated overnight at 37 °C. Following vortexing, reactions were supplemented with 1 µg of trypsin for 1 h at 37 °C; this step was then repeated. Water was added (300 µl), and samples were centrifuged at 10,000 rpm for 5 min and then lyophilized. Oxidation was performed as follows. Pellets were dissolved in 100 µl of formic acid, and then 25 µl of methanol and 40 µl of peracetic acid (made fresh by combining 0.9 ml of formic acid with 0.1 ml of 30% H2O2) were added; samples were incubated at 0 °C for 2 h. Each sample was divided into two 1.5-ml tubes, and 1.4 ml of water was added prior to lyophilization. Pellets were resuspended in 10 µl of pH 1.9 buffer (50 µl of formic acid (88%, w/v), 156 µl of glacial acetic acid, 1794 ml of water) and spotted on 25 × 20 cm cellulose TLC plates (Selecto Scientific) by the repeated addition of 0.5 µl. TLC plates were electrophoresed using a Hunter Thin Layer Electrophoresis System (C.B.S. Scientific Co.) in pH 1.9 buffer for 25 min at 1000 V. Following evaporation of the buffer, plates were rotated 90° and subjected to chromatography in phosphochromatography buffer (150 µl of n-butanol, 100 µl of pyridine, 30 ml of glacial acetic acid, 120 ml of water). Phosphopeptides were visualized by autoradiography.

RESULTS

Fer is a 94-kDa protein that contains three predicted N-terminal CC motifs (CC1, CC2, and CC3), an SH2 domain, and a C-terminal protein-tyrosine kinase domain (Ref. 6; shown schematically in Fig. 1). In order to explore the role of the SH2 and N-terminal domains in Fer kinase regulation, we generated a series of expression constructs including two separate missense mutations within either catalytic subdomain II (K592R or D743R). Two proline insertion mutations were generated to disrupt the α-helical structure of CC1 (KL134RP) and CC2 (ML322RP). A C-terminal deletion construct allowed expression of the N-terminal CC domain of Fer (Fer-N; amino acids 1–462), and an N-terminal deletion of the CC motifs allowed expression of a protein called Myc-FerAN (encoding amino acids 412–823) resembling the testis-specific FerT isofrom. Fps was expressed in the absence and presence of a C-terminal Myc epitope fusion and also with or without the inactivating missense mutation K592R or D743R. Two proline insertion mutations were generated to disrupt the α-helical structure of CC1 (KL134RP) and CC2 (ML322RP). A C-terminal deletion construct allowed expression of the N-terminal CC domain of Fer (Fer-N; amino acids 1–462), and an N-terminal deletion of the CC motifs allowed expression of a protein called Myc-FerAN (encoding amino acids 412–823) resembling the testis-specific FerT isofrom. Fps was expressed in the absence and presence of a C-terminal Myc epitope fusion.

from usage of an internal testis-specific promoter located in fer (22).

Fps is a 92-kDa protein that is closely related to Fer and possesses a similar domain structure (1, 8). The N-terminal CC domain of human Fps exhibits 37 and 35% identity to human and mouse Fer, respectively, while the SH2 and kinase domains of Fps and Fer display 67 and 68% sequence identity, respectively (6). Fps was expressed both as an untagged protein and with a C-terminal Myc epitope fusion (Myc-Fps).

Homotypic but Not Heterotypic Oligomerization of Fer and Fps in Vivo—Since both Fps and Fer possess N-terminal CC domains that are implicated in oligomerization in vitro (7, 8), we wished to determine whether Fps and Fer form homotypic or heterotypic oligomers in vivo. COS-1 cells were transfected with plasmids expressing Fer, Fps, Myc-Fer, and Myc-Fps. At 48 h post-transfection, soluble cell lysates were collected; a fraction of each lysate was retained for Western blotting, while immunoprecipitations with anti-Myc were performed on the remainder of the lysates. Western blotting with antiserum that recognizes both Fps and Fer (anti-Fps/Fer) and in vitro kinase assays allowed for detection of Fps and Fer in the immunoprecipitates (Fig. 2). Western blotting of soluble cell lysates from mock transfected COS-1 cells identified the endogenous simian Fer protein (top panel, lane 1), which co-migrated with the overexpressed murine Fer protein (lane 2). Fps protein migrated slightly faster than Fer (compare lanes 3 and 2), consistent with its slightly smaller size. The 120-kDa Myc-Fer protein was detected when co-expressed with either Fer (lane 4) or Fps (lane 5). As with the untagged proteins, Myc-Fps migrated slightly faster than Myc-Fer (compare lanes 5 and 6) and was expressed with both Fer (lane 6) and Fps (lane 7). Neither Fer nor Fps was immunoprecipitated by the anti-Myc antibody (middle panel, lanes 1–3). However, in the presence of Myc-Fer, untagged Fer was immunoprecipitated (lane 4). Thus, Fer forms oligomers in vivo. In contrast, no Fps was recovered with Myc-Fer (lane 5), and no Fer was detected in Myc-Fps immunoprecipitates (lane 6), indicating that no protein complexes containing both Fps and Fer were observed. However, we did detect homotypic Fps oligomers (lane 7), which is consistent with a previous study (8).
**FIG. 2.** Homotypic, but not heterotypic, interactions of Fps and Fer in vivo. COS-1 cells were transfected with 2 μg of the expression plasmids indicated at the top. Western blots with anti-Fps/Fer antiserum (which recognizes both Fps and Fer) were performed on soluble cell lysates (top panel) and anti-Myc immunoprecipitates (IP) (middle panel). An in vitro kinase assay was also performed on the same immunoprecipitates (bottom panel). Molecular weight markers are shown on the left, and the migrations of Myc-tagged and untagged Fer and Fps are indicated on the right with arrows.

Immune complex kinase assays were also performed, and the results were consistent with those described above. No kinase activities were observed in anti-Myc immunoprecipitates from mock transfected cell lysate (Fig. 2, bottom panel, lane 1) or Fer- and Fps-expressing cell lysates (lanes 2 and 3, respectively). Two radiolabeled polypeptides were observed in the immunoprecipitate from the Myc-Fer- and Fer-expressing cell lysate (lane 4); these proteins corresponded to the sizes of Fer and Myc-Fer detected by Western blotting (lane 4, top and middle panels). Only Myc-Fer kinase was detected in the immunoprecipitate from the Myc-Fer- and Fps-expressing cell lysate (bottom panel, lane 5). Likewise, no Fer kinase was co-immunoprecipitated with Myc-Fps (lane 6), thus suggesting that no Fps-Fer protein complexes were formed in COS-1 cells. In contrast, Fps kinase was recovered in Myc-Fps immunoprecipitates (lane 7). Therefore, Fer and Fps form homotypic but not heterotypic oligomers in vivo. It is also noteworthy that the amount of 32P incorporated into Fps was lower than that for Fer (compare lanes 4 and 5 with lanes 6 and 7). Fer is also more highly phosphorylated than Fps in vivo, as determined by anti-phosphotyrosine blotting (data not shown), suggesting either that Fps has a lower specific activity than Fer or that Fer has more autophosphorylation sites than Fps.

**FIG. 3.** Disruption of CC1 or CC2 abrogates oligomerization but not autophosphorylation. Lysates were prepared from COS-1 cells that were either mock transfected (lane 1) or transfected with the following expression plasmids: 2 μg of Fer (lane 2), 1 μg of Myc-Fer (lane 3), 2.5 μg of Myc-Fer and 3 μg of Fer (lane 4), 2.5 μg of Myc-Fer and 2 μg of Fer-N (lane 5), 1 μg of Myc-FrKL134RP and 3 μg of Fer (lane 6), 3 μg of Myc-FrKL134RP and 5 μg of Fer (lane 7), 5 μg of FpsKL322RP and 3 μg of Fer (lane 8), 1 μg of Myc-FrKL134RP (lane 9), 3 μg of Myc-FrKL134RP (lane 10), and 5 μg of Myc-FrKL322RP (lane 11). Immunoprecipitations (IP) were carried out with monoclonal antibody 1-9El0, followed by either Western blotting with anti-Fer antibody or in vitro kinase assays. Molecular weight markers are shown on the left, and the positions of Myc-tagged and untagged Fer proteins, Fer-N, and enolase are indicated on the right with arrows.

was efficiently co-immunoprecipitated with Myc-FrK592R (lane 6) but was not recovered with Myc-FrKL134RP (lane 7) or Myc-FrKL322RP (lane 8). Thus, disruption of either CC1 or CC2 domains completely abrogates co-immunoprecipitation of Fer, suggesting that both α-helical domains participate in oligomerization. Kinase activity or phosphorylation is not required for oligomerization, since we have observed strong association between Myc-FrK592R and FerKL413R in similar experiments (data not shown).

Immune complex kinase assays were performed in the presence of denatured enolase, which is an in vitro substrate of Fer. Myc-Fer autophosphorylation (Fig. 3, bottom panel, lane 3) was similar when complexed with either Fer (lane 4) or Fer-N (lane 5). In contrast, enolase phosphorylation was higher in the presence of two active kinases (Myc-Fer and Fer) compared with Myc-Fer alone (lanes 3 and 4). The presence of stoichiometric amounts of inactive Fer-N in the complex had little effect on either Myc-Fer autophosphorylation or enolase phosphorylation (lane 5). Phosphorylation of the inactive Myc-FrKL134RP protein in the presence of Fer (lane 6), but not with its absence (lane 9), suggests that phosphorylation of Fer can occur in trans. Although the mutations to CC1 and CC2 disrupted oligomerization, both proteins retained kinase activity (Myc-FrKL134RP, lanes 7 and 10; Myc-FrKL322RP, lanes 8 and 11). Autophosphorylation of these mutants was similar to that of wild type Fer (compare with lane 3); however, enolase phos-
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Fer Protein Forms Trimers in Vivo—To address the stoichiometry of Fer oligomerization, chemical cross-linking was performed on lysates from COS-1 cells expressing various Fer proteins (Fig. 5, indicated at the top). Soluble cell lysates were either loaded directly (lanes 1–7) or following incubation with the cross-linking reagent EGS (lanes 8–14). Following SDS-PAGE, Western blotting was performed sequentially with anti-Fer (top panel) and anti-Myc antibodies (bottom panel). Fer-N migrated as a 65-kDa protein in the absence of EGS (top panel, lane 2), while upon cross-linking, a portion of the protein migrated at ~190 kDa (lane 9, indicated by the arrow at the right). An additional ~170-kDa complex was present in lower amounts and may be due to partial proteolysis of Fer-N. Taken together, these results indicate that the N-terminal domain of Fer forms a trimer in vitro and are consistent with a previous in vitro study (7). Cross-linking of Myc-Fer resulted in a shift in mobility from ~120 kDa (lanes 3 and 4), ~350 kDa (lanes 10 and 11, indicated by the upper arrow at the right). In the presence of both Myc-Fer and Fer-N, three additional cross-linked species were observed (lane 11), including the aforementioned ~190-kDa product (probably arising from trimers of Fer-N) and two species migrating slightly faster than the ~350-kDa product (indicated by two arrows at the right). The latter probably correspond to incorporation of either one or two Fer-N subunits in the Myc-Fer oligomers. In agreement with the results described above, disruption of either CC1 or CC2 abolished oligomerization (compare lanes 5 and 6 with lanes 12 and 13). Taken together, these results indicate that both CC1 and CC2 domains mediate trimerization of Fer in vivo. These results do not exclude additional protein-protein interactions, for example via the SH2 domain and phosphotyrosine residues.

Immunoelectron microscopic studies of Myc-Fer autophosphorylation were performed sequentially with anti-phosphotyrosine monoclonal antibody PY99 (top panel) or 1-9E10 (bottom panel). Molecular weight markers are shown on the left, and the position of the IgG heavy chain is indicated on the right.

Myc-Fer
Myc-FerKL134RP
Myc-FerKL134RP
Myc-FerN

FIG. 4. Oligomerization of Fer is not required for autophosphorylation in vivo. Lysates were prepared from COS-1 cells that were either mock transfected (lane 1) or transfected with 5 μg of the following expression plasmids: Myc-Fer (lane 2), Myc-FerKL134RP (lane 3), Myc-FerKL134RP (lane 4), Myc-FerN (lanes 5). Immunoprecipitations (IP) were carried out with anti-Myc monoclonal antibody 1-9E10, followed by Western blotting with either anti-phosphotyrosine monoclonal antibody PY99 (top panel) or 1-9E10 (bottom panel). Molecular weight markers are shown on the left, and the position of the IgG heavy chain is indicated on the right.

Do Inactive Fer Proteins Have Dominant Negative Activity?—We next wished to address the role of oligomerization on Fer activity and the effect of inactive Fer proteins on the activity of associated wild type Fer. COS-1 cells were transfected with various Fer expression plasmids (Fig. 6, as indicated at the top), and lysates were prepared for immunoprecipitation with anti-Myc antibody, followed by either Western blotting or immune complex kinase assays. Western blotting of soluble cell lysates with anti-Fer or anti-Myc antibodies revealed that all Fer proteins were expressed (first and second panels, lanes 2–14). Western blotting of the immunoprecipitates (third and fourth panels) revealed increasing amounts of Myc-Fer when expressed alone (lanes 4–7) or when co-expressed with either Fer-N (lanes 8–10) or FerD743R (lanes 11–13). Myc-FerN was readily detected in the immunoprecipitate (lane 14, fourth panel).

Immune complex kinase assays were performed in the presence of denatured enolase (bottom panel; phosphorylated enolase is indicated on the right). Specific activities of Myc-Fer and Myc-FerN were calculated for both autophosphorylation and substrate phosphorylation via quantification of incorporated 32P in Fer and enolase proteins relative to the amounts of Myc-Fer and Myc-FerN. The specific activity of Myc-Fer when expressed alone (lanes 4–7) was 27.4 ± 5.9 for autophosphorylation and 20.0 ± 2.7 for enolase phosphorylation (the error given is the S.D.). When in a complex with Fer-N (lanes 8–10), Myc-Fer autophosphorylation was 19.6 ± 5.1 and enolase phosphorylation was 14.1 ± 4.5. In the presence of FerD743R, autophosphorylation of Myc-Fer was 14.5 ± 4.5, and enolase phosphorylation was 13.5 ± 3.8. Therefore, the specific activity of Myc-Fer was reduced slightly (25–40%) when complexed with inactive Fer proteins. The N-terminal deletion mutant Myc-FerN had substantially lower specific activity for autophosphorylation than Myc-Fer (4-fold) but showed much higher enolase phosphorylation (2.5-fold). This would suggest that although Myc-FerN does not oligomerize (Fig. 5, and data not shown), it retains robust kinase activity toward substrate in vitro. However, the greatly reduced autophosphorylation signal suggests that efficient autophosphorylation of Fer may require oligomerization.

Analysis of Autophosphorylation Sites within Monomeric and Oligomeric Fer Proteins—To identify the number of autophosphorylation sites in Fer, and the effect of oligomerization on

Oligomerization and Autophosphorylation of Fer in Vivo—To assess the state of autophosphorylation of oligomeric and monomeric Fer proteins in vivo, Myc-tagged Fer proteins expressed in COS-1 cells were immunoprecipitated with anti-Myc monoclonal antibody 1-9E10, followed by Western blotting with an anti-phosphotyrosine monoclonal antibody (Fig. 4, top panel). Myc-Fer displayed higher phosphotyrosine levels than Myc-FerKL134RP and Myc-FerKL134RP (compare lanes 2–4); however, this coincided with reduced protein levels for the CC mutants (bottom panel, compare lanes 2–4). Likewise, the truncated protein Myc-FerN, which lacks all CC domains, was tyrosine-phosphorylated in vivo (top panel, lane 5). Thus, oligomerization is not obligatory for autophosphorylation of Fer in vivo.

Oligomerization-independent Autophosphorylation of Fer in Vivo—To address the state of autophosphorylation of oligomeric and monomeric Fer proteins in vivo, Myc-tagged Fer proteins expressed in COS-1 cells were immunoprecipitated with anti-Myc monoclonal antibody 1-9E10, followed by Western blotting with an anti-phosphotyrosine monoclonal antibody (Fig. 4, top panel). Myc-Fer displayed higher phosphotyrosine levels than Myc-FerKL134RP and Myc-FerKL322RP (compare lanes 2–4); however, this coincided with reduced protein levels for the CC mutants (bottom panel, compare lanes 2–4). Likewise, the truncated protein Myc-FerN, which lacks all CC domains, was tyrosine-phosphorylated in vivo (top panel, lane 5). Thus, oligomerization is not obligatory for autophosphorylation of Fer in vivo.

phorylation was slightly reduced (30–50%). Therefore, oligomerization is not a prerequisite for Fer autophosphorylation in vitro, but it can potentiate phosphorylation in trans (i.e. with one subunit contributing to the phosphorylation of another).
Fer phosphorylation, tryptic phosphopeptide mapping was performed. Soluble cell lysates from COS-1 cells expressing Fer or co-expressing Myc-FerKL134RP and Fer were subjected to immunoprecipitation with anti-Fer antibody, followed by in vitro kinase reactions. Myc epitope-tagged proteins were immunoprecipitated with anti-Myc antibody, followed by in vitro kinase reactions. The radiolabeled products were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and visualized by autoradiography. Portions of the membrane corresponding to autophosphorylated Fer, Myc-FerKL134RP, Myc-FerML322RP, Myc-FerΔN, and Myc-FerK592R (which was phosphorylated in trans by Fer), were excised and digested with trypsin. Peptides that were released from the membrane were oxidized and then spotted on cellulose plates for electrophoresis followed by thin layer chromatography (Fig. 7). Subsequent autoradiography resulted in the identification of three tryptic phosphopeptides for Fer (Fig. 7A). The peptide corresponding to spot 1 was most highly labeled, followed by spots 2 and 3. Three major tryptic phosphopeptides were also described for Fps (36), although the mobilities of the phosphopeptides are distinct from those observed here for Fer. The ability of Fer to phosphorylate an inactive Fer protein in trans (as shown in Fig. 3, lane 6) allowed for analysis of the sites within Myc-FerK592R that were phosphorylated by Fer in trans (Fig. 7B). Again, three major phosphopeptides were detected that co-migrated with those described for Fer (labeled 1–3). However, the stoichiometry of phosphorylation was quite different, in that spot 1, which corresponds to the major phosphopeptide in Fer (Fig. 7A), became the minor phosphopeptide in Myc-FerK592R (Fig. 7B). The monomeric Myc-FerKL134RP and Myc-FerML322RP mutants also displayed all three tryptic phosphopeptides; however, the stoichiometry was distinct from wild type Fer in that spot 1 was the minor phosphopeptide (Fig. 7, C and D, respectively). This would suggest that all of the major autophosphorylation sites in Fer can be phosphorylated in cis but that spot 1 is less efficiently phosphorylated in the monomeric mutants. Since Myc-FerΔN does not oligomerize (Fig. 5, lane 14) and displayed reduced autophosphorylation (Fig. 6, lane 14), some differences in the phosphorylation state were expected. Indeed, the tryptic phosphopeptide map of Myc-FerΔN revealed only two major phosphorylation sites, which correspond to spots 2 and 3 of Fer (Fig. 7E). In contrast, there was no evidence of spot 1. The loss of this phosphorylation site might indicate that an N-terminal phosphorylation site is lost with this deletion; however, Fer-N was not phosphorylated when complexed with Fer (Fig. 3, lane 5, and Fig. 6, lanes 8–10). We are currently attempting to identify the autophosphorylation site that corresponds to spot 1.

To determine whether the phosphorylation sites in Fer protein overexpressed in COS-1 cells were similar to that of native Fer, tryptic phosphopeptide mapping was performed on Fer that was immunoprecipitated from mouse liver extracts using anti-Fer polyclonal antiserum and labeled in vitro (Fig. 7F). Three major phosphopeptides were observed with mobility similar to that described for Fer expressed in COS-1 cells (Fig. 7A). We performed a similar analysis of native FerT, which was immunoprecipitated from mouse testis (Fig. 7G). Two major phosphopeptides were observed corresponding to spots 2 and 3. However, no phosphopeptide corresponding to spot 1 was detected. Since autophosphorylation of Myc-FerΔN resembled that of native FerT (Fig. 7, compare E and G), the unique N-terminal sequence of FerT, which is not present in Myc-FerΔN, probably plays no role in kinase activation. Overall, these results suggest that oligomerization promotes kinase.
activation by potentiating autophosphorylation in trans. However, since monomeric Fer mutants (or the naturally occurring FerT) can autophosphorylate in cis, oligomerization is clearly not required for kinase activation. Finally, the differences in phosphorylation sites between Fer and FerT may reflect distinct roles in signal transduction between the ubiquitous Fer and the testis-specific FerT.

DISCUSSION

The Fer protein-tyrosine kinase and the highly homologous oncprotein Fps both contain an N-terminal domain with three predicted CC motifs that are thought to promote oligomerization (7, 8). In this study, we have examined the requirements of these CC motifs for oligomerization and Fer kinase activation.

Our results show that both CC1 and CC2 are required and probably cooperate in trimerization of Fer in vivo (Figs. 3 and 5). It is also apparent that oligomerization is not required for autophosphorylation in vitro (Figs. 3 and 6) or in vivo (Fig. 4). So what is the role of CC-mediated trimerization of Fer? It clearly distinguishes the ubiquitous p94 Fer from its testis-specific isoform FerT. However, it would appear that activation of Fer, and probably Fps, differs from many protein kinases that require oligomerization for activation (37). Few cytoplasmic PTKs have been shown to oligomerize, with Fer and Fps being notable exceptions. Activation of most cytoplasmic PTKs involves interactions with oligomeric proteins such as activated growth factor, cytokine, or immune recognition receptors (37). These interactions may promote activation through autophosphorylation in trans and induced conformational changes (38, 39). Src family kinases also possess a negative regulatory C-terminal tyrosine phosphorylation...
site that must be dephosphorylated for kinase activation (40). While Fer and Fps do not undergo this mode of negative regulation, intramolecular interactions have been proposed between the SH2 domain and a phosphotyrosine residue in Fps (10, 41). Preliminary evidence indicates that Fer is constitutively oligomeric and that growth factor stimulation has no effect on formation of trimers (data not shown). Another potential role for the CC domains of Fps and Fer may involve their subcellular localization. It will be of great interest to determine whether disruption of CC1 or CC2 causes aberrant localization of Fer. It is also worth noting that a number of serine/threonine kinases possess CC oligomerization domains including p160 ROCK (42), TOUSLED (43), and myosin heavy chain kinase A (44). Fer and Fps may have retained CC domains throughout evolution from their putative serine/threonine kinase ancestor. There are other examples of PTKs that oligomerize by virtue of chromosomal translocations that juxtapose oligomerization domains with tyrosine kinase domains of ABL (45, 46), RET (47), MET (48), and TRKA (49). All of these fusion proteins are hyperactive kinases that possess oncogenic activities.

Although oligomerization is not required for kinase activation, it does promote autophosphorylation in trans (Fig. 3). The significance of this observation is not clear, since incorporation of inactive proteins into the trimer has only mild inhibitory effects on activation of wild type Fer (Figs. 3 and 6), which argues against a prominent role for trans phosphorylation. Also, the ability of monomeric Fer proteins to autophosphorylate (Figs. 3 and 4), suggests that an additional cis-mediated autophosphorylation mechanism probably exists. The relative contributions of these two mechanisms to Fer activation in vivo is not known and is difficult to address experimentally. Our findings contrast with previous results for Fps, in which a marked reduction in Fps activity was observed when Fps was immunoprecipitated with inactive Fps proteins (8). Also, a recent study suggested a dominant negative effect of overexpressed FerK65QR on the activity of endogenous Fer (13). However, in both studies a molar excess of the inactive Fer or Fps proteins were required for this effect, whereas in our experiments the amounts of FerD743R or Fer-N were closer to equimolar with wild type Fer (Fig. 3, lane 5, and Fig. 6, lanes 8–13). In contrast, dominant negative mutations were identified in the Kit PTK that produce a marked reduction in pigmenta
tion but does not greatly affect accessibility of the active site (53). We have preliminary evidence that mutation of tyrosine 715 in Fer reduces autophosphorylation (53). We are currently attempting to identify all of the autophosphorylated residues in Fer, which will be of utmost importance in delineating binding sites for substrates and/or effectors of Fer.

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