p94fer and p51ferT are two tyrosine kinases that share identical SH2 and kinase domains but differ in their N-terminal regions. To further explore the cellular functions of these two highly related tyrosine kinases, their subcellular distribution profiles and in vivo phosphorylation activity were followed using double immunofluorescence assay. When combined with immunoprecipitation analysis, this assay showed that p94fer can lead to the tyrosine phosphorylation and activation of Stat3 but not of Stat1 or Stat2. Native p94fer exerted this activity when residing in the cytoplasm. However, modified forms of p94fer, which are constitutively nuclear, could also lead to the phosphorylation of Stat3. Endogenous Stat3 and p94fer co-immunoprecipitated with each other, thus proving the interaction of these two proteins in vivo. Unlike p94fer, p51ferT did not induce the phosphorylation of Stat3 but led to the phosphorylation of other nuclear proteins. Replacing the unique 43-amino acid-long N-terminal tail of p51ferT with a parallel segment from the N-terminal tail of p94fer did not change the subcellular localization of p51ferT but enabled it to activate Stat3. Thus the different N-terminal sequences of p94fer and p51ferT can affect their ability to induce phosphorylation of Stat3 and most probably direct their different cellular functions.

Non-receptor tyrosine kinases are localized in various subcellular compartments, where they exert specific functions. These include receptor-associated kinases that mediate signals for cell growth and differentiation (1, 2), membrane-associated kinases that regulate cytoskeletal-mediated signal transduction pathways (3, 4), and other tyrosine kinases that can be detected in both the cytoplasm and the nucleus of cells. The last group contains c-Src- and c-Abl-related tyrosine kinases (5–7) and the Fes/FER family of non-receptor tyrosine kinases (8–10). The Fes/FER family includes c-fes and the FER tyrosine kinases (8–10) and oligomerization (20) of this molecule. In p51ferT, the N-terminal 412 aa of p94fer are replaced via differential splicing, with a novel 43-aa-long N-terminal tail (17).

The FER kinases also differ in their tissue distribution profiles. Although the presence of p94fer was documented in most mammalian cell lines analyzed (11, 12), except for pre-B, pre-T, and T cells (21), p51ferT was shown to accumulate solely in meiotic pachytene spermatocytes (18, 22). The two FER enzymes not only accumulate in different tissues, but they also exhibit different subcellular distribution patterns. p94fer is mainly cytoplasmic though it enters the nucleus upon transition of cells from G1 to the S phase (10). In the cytoplasm, p94fer associates with cell-cell adhesion molecules (23, 24), and its activity is induced in growth factor-stimulated cells (19). Moreover, p94fer was shown to associate with activated epidermal growth factor and platelet-derived growth factor receptors in fibroblasts (19) and with the FceRI receptor in mast cells (25). Thus p94fer is linked to growth promoting processes. Unlike p94fer, the meiotic FER tyrosine kinase p51ferT has not been detected in the cytoplasm of cells that express it, and it accumulates constitutively in the cell nucleus (10, 22). The functional implications of these differences between p94fer and p51ferT have not been explored. Downstream effectors of these enzymes have not been identified, and their cellular roles in somatic or meiotic cells are not well understood. To further understand the cellular role of these tyrosine kinases, and to learn whether they can phosphorylate similar repertoire of cellular substrates, a panel of FER variants was subjected to double immunofluorescence assay, in addition to immunoprecipitation and Western blot analysis. These assays allowed the characterization of the subcellular distribution and in vivo phosphorylation activity of the FER kinases. This approach revealed that the N-terminal regions of the two FER kinases direct their different substrate specificity, and it implied that p94fer could serve as a novel activator of Stat3. 

EXPERIMENTAL PROCEDURES

Expression Vectors— The construction of the expression plasmids that were used in this study has been previously described (10). Native p51ferT fused to a single influenza HA epitope at its NH2-terminal tail, was expressed from the pCDNA3 fer vector under the control of the cytomegalovirus promoter. HA-p94fer devoid of a functional NLS (see Fig. 1, fer KR652/3NQ and Ref. 10) was also expressed from the pCDNA3 fer vector under the control of the cytomegalovirus promoter. The HA-tagged p94fer variants, ferΔ1–299, ferΔ1–315, ferΔ1–376, and

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for Δ1–427, were expressed from the PECE vector under the control of the SV40 early promoter. HA-p51ferT was also expressed from the PECE vector (see Fig. 1 and Ref. 10).

**Cells and Transfections—** COS1 and CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. COS1 cells (5.0 × 10⁴) were transfected with 7 μg of DNA mixed with 30 μl of LipofectAMINE (Life Technologies, Inc.) in 100-mm dishes. CHO cells (7.5 × 10⁴) were transfected with 5 μg of DNA mixed with 20 μl of LipofectAMINE PLUS reagent (Life Technologies, Inc.) and 30 μl of LipofectAMINE in 100-mm dishes. During transfection the cells were grown in Opti-MEM medium (Life Technologies, Inc.), Two or three passages were done for each sample. The murine myoblast C2C12 cells (26) and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 15 (C2C12) or 10% (NIH3T3) fetal calf serum.

**Western Blot Analysis—** Whole cell proteins were extracted in a lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 2 mM Na₂VO₄ and were stored on ice for 1 h. The proteins were cleared by centrifugation at 17,000 × g for 30 min at 4 °C. The amount of protein was determined by Bradford analysis. For Western blot analysis, 25 μg of protein from each sample was resolved by 9% SDS-PAGE. Electroblotted proteins were detected using monoclonal anti-αHA (Babco), a phosphotyrosine (PT) monomonal antibody (PT-66, Sigma), p94fer—specific aFe/C2 antibodies (27), and αStat1, αStat2, and αStat3 monoclonal antibodies (Transduction Laboratories).

**Immunoprecipitation—** Extracted proteins (750–1000 μg) were incubated overnight at 4 °C with 1:350 diluted 4G10 monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.), 1:25 dilution of αStat3 monoclonal antibody, or 1:125 polyclonal αFER antibody. Antigen-antibody complexes were precipitated with protein A/G-Sepharose for 1 h at 4 °C and were washed four times with HNTG buffer, which contained 10% glycerol, 0.1% Triton X-100, 20 μM Hepes, pH 7.5. The first two washes were carried out with HNTG buffer containing 150 μM NaCl, and the third wash was done with HNTG containing 80 μM NaCl. The last wash was done with HNTG buffer lacking NaCl. Precipitated proteins were then resolved by SDS-PAGE, blotted onto nitrocellulose membranes, and were then reacted with monoclonal αHA (Babco), αFe/C2, or monoclonal αStat1, αStat2, and αStat3 antibodies. Co-immunoprecipitation of p94fer and Stat3 was carried out as follows: cell lysates (0.8–1 mg of protein) were incubated with 1:125 diluted pre-serum or αFER antibody (directed against the SH2 domain of p94fer) for 4 h at 4 °C. The reactions were then transferred for overnight incubation at 15 °C to avoid nonspecific association of cellular proteins. Antigen-antibody complexes were precipitated as described above.

**Immunohistochemical Analysis—** COS1 (3 × 10⁴) cells or CHO (3.5 × 10⁵) cells were seeded in eight-well chamber slides with well areas of 1 cm² and were then transfected with 125 ng of DNA mixed with 0.5 μl of LipofectAMINE PLUS reagent and 1.5 μl of LipofectAMINE, in a total volume of 125 μl. Cells were fixed 40 h post-transfection using 4% paraformaldehyde and were subsequently treated with 0.5% Triton X-100 for 30 min. Blocking was carried out with 6% skim milk, 3% bovine serum albumin, and 0.2% Tween 20 in 100 mM fetal calf serum. Cells were then exposed overnight at 4 °C to the following antibodies: 1:500 diluted monoclonal αHA and 1:500 diluted polyclonal αPT (Transduction Laboratories) and 1:500 diluted αPTStat5 or αPTStat1 (New England Biolabs) antibodies. Reacting antibodies were visualized with fluorescein isothiocyanate-conjugated donkey anti-mouse and Lyssase—Rhodamine-conjugated donkey anti-rabbit antibodies (Jackson Laboratories) using a Bio-Rad MRC 1024 upright confocal microscope with a krypton-argon ion laser. Confocal microscope image analysis was performed using Bio-Rad software, and figures were compiled using the Laser Sharp 3.0 software package.

**Preparation of Nuclear Extracts—** Nuclear extracts were prepared essentially as described before (28). Cells were washed with phosphate-buffered saline, spun down, and resuspended in buffer containing the following: 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/μl Pefabloc, 0.5 μg/μl aprotinin, 0.5 μg/μl leupeptin, and 1 μg/μl benzamidine. Cells were collected by scraping, homogenized in ice to prevent swelling and were frozen in liquid nitrogen. Nuclei were spun down by centrifugation at 10,000 × g for 15 s and suspended in high salt extraction buffer containing the following: 10 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM dithiothreitol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/μl Pefabloc, 0.5 μg/μl aprotinin, 0.5 μg/μl leupeptin, and 1 μg/μl benzamidine. Nuclear proteins were extracted by rotating the nuclei for 30 min in the extraction buffer at 4 °C, followed by centrifugation at 20,000 × g for 15 min. Supernatants were collected, divided into aliquots, and stored at −70 °C.

**Electromobility Gel Shift Assay—** A double-stranded synthetic fragment that carries the Stat3 binding site (29) was labeled with [α-32P]SCTP by fill in reaction using Klenow fragment and was then purified on 12% acrylamide gel (30). 10–μg nuclear protein extracts were incubated at 25 °C for 15 min with 400 ng of poly(dI·dC) and 0.1 ng of labeled probe in the following binding buffer: 4 mM Tris-HCl, pH 8.0, 40 mM NaCl, 1 mM MgCl₂, and 5% glycerol in total volume of 25 μl. The assay mixture was then separated on 4% acrylamide Tris-borate EDTA gel at 100 V for 2–3 h. The gel was dried and exposed overnight on Kodak film (X-OMAT AR).

**RESULTS**

**In Situ Staining of the Tyrosine Phosphorylation Induced by p94fer—** To follow the subcellular distribution of the p94fer kinase and the tyrosine phosphorylation that it induces, we utilized double immunofluorescence staining. This assay allows one to follow the subcellular distribution and phosphorylation activity of a kinase at the single cell level, thus enabling the dissection of a mixed population of cells. A native HA-tagged p94fer protein (see Ref. 10 and Fig. 1) was transiently expressed in COS1 cells. Immunohistochemical analysis using monoclonal αHA antibody revealed the presence of the ectopically expressed p94fer in the cytoplasm of most transfected cells (Fig. 2A and B). To test whether the subcellular localization of the enzyme parallels the distribution of its induced tyrosine phosphorylation, the transfected cells were also co-stained with αPT antibodies. A prominent αPT signal high above the background was obtained in HA-p94fer-expressing cells (Fig. 2C). The induced phosphotyrosine staining was dependent on the kinase activity of p94fer, because an inactive HA-p94fer mutant (Fig. 1, ferΔ1–315) did not induce tyrosine phosphorylation in transfected cells (Fig. 2C). The subcellular distribution profile of the phosphotyrosine staining varied, however, among different transfected cells and could be seen in two typical patterns. In the majority of the cells, both HA-p94fer and the induced phosphorylation signal were located in the cytoplasm (Fig. 2A). However, in around 30% of more than 200 αPT-positive cells, while p94fer was localized in the cytoplasm, the induced phospho-
phorylation signal was mainly in the nucleus (Fig. 2B). We could never see clear cytoplasmic and nuclear phosphorylation signals combined in the same cell. Moreover, one could clearly see that native p94fer led to the accumulation of cytoplasmic or nuclear phosphorylation signals when the kinase was expressed in the cytoplasm (Fig. 3, A and B). About 30% of the transfected cells did not show an obvious induced tyrosine phosphorylation signal. A similar heterogeneous subcellular distribution profile of phosphotyrosine staining was also seen in CHO cells that transiently expressed the HA-p94fer protein (data not shown).

To test whether the p94fer kinase activity requires cytoplasmic localization, the cellular phosphorylation activity of three HA-tagged modified forms of p94fer were compared. These included fer KR652/3NQ, which bears a mutated NLS (see Ref. 10 and Fig. 1). Mutating the monopartite NLS of p94fer excludes the protein from the nucleus without compromising its kinase activity (Fig. 3, A and B). Another cytoplasmic variant of p94fer that was tested was ferΔ1–315, which lacks the first, and part of the second, N-terminal coiled-coil domains of the enzyme (see Ref. 10 and Fig. 1). The in situ phosphorylation profiles of these cytoplasmic forms of fer were compared with the phosphorylation profile of ferΔ1–299. Deleting the first N-terminal coiled-coil domain of p94fer in ferΔ1–299 (see Ref. 10 and Fig. 1, ferΔ1–299) leads to its nuclear accumulation without affecting its kinase activity (Fig. 3D). The three kinases were transiently expressed in COS1 cells. Unlike the native p94fer, the accumulation of the NLS negative FER variant (KR652/3NQ) was mainly cytoplasmic, and no preferential accumulation of this protein in the nucleus of any transfected cell could be found. However, as seen with the native p94fer, transient expression of this variant led to the accumulation of cytoplasmic phosphotyrosine in some transfected cells and to nuclear accumulation of phosphotyrosine in others (Fig. 3, A and B). Transiently expressed ferΔ1–315 also accumulated in the cytoplasm. This resulted in a perinuclear accumulation of a phosphotyrosine signal in some cells (Fig. 3C). The constitutively nuclear FER variant ferΔ1–299 exhibited exclusive nuclear accumulation and induced nuclear phosphorylation signal in the transfected cells (Fig. 3D). It therefore appears that both modified forms of p94fer expressed in the cytoplasm or in the nucleus can elicit the accumulation of tyrosine phosphorylation in the cell nucleus. This profile differs from the activity exhibited by the native p94fer, which induces phosphorylation only when residing in the cytoplasm.

Both Cytoplasmic and Nuclear Forms of FER Induce Tyrosine Phosphorylation of Stat3—To further identify the cellular substrates of p94fer, whole cell proteins from HA-p94fer-transfected CHO cells were immunoprecipitated with αPT antibodies. Immunoprecipitates were resolved by SDS-PAGE and then probed with αPT antibodies by Western blot analysis. Several proteins were found to be highly tyrosine-phosphorylated in transfected but not in untransfected cells. The sizes of these proteins clustered between 75 and 95 kDa and between 97 and 110 kDa, respectively (Fig. 4, upper panel). Similar results were obtained when HA-p94fer was transiently expressed in COS1 cells (data not shown). Based on the migration distances of the phosphorylated proteins seen in SDS-PAGE, the overlapping accumulation of pHA-p94fer, and the in situ phosphotyrosine signals in the cytoplasm, one would predict the presence of phosphorylated HA-p94fer in the 97–110-kDa cluster of cytosine-phosphorylated bands (Fig. 4, upper panel). Indeed reacting the αPT precipitates with αHA antibody in a Western blot revealed the immunoprecipitation of a phosphorylated HA-p94fer form that migrated slightly above the 97-kDa marker (Fig. 4, bottom panel, lane 1).
The accumulation of nuclear phosphotyrosine signals in cells overexpressing cytoplasmatic forms of FER suggests the transduction of the phosphorylation activity of FER from the cytoplasm to the nucleus. Possible downstream effectors in this process could be the signal transducers and activators of transcription, which were shown to be phosphorylated by several non-receptor tyrosine kinases (31, 32). These proteins are tyrosine-phosphorylated in the cytoplasm and are then translocated to the cell nucleus (33). The molecular mass of these proteins varies between 84 and 113 kDa (33), a size range that coincides with the size of some of the phosphorylated proteins seen in Fig. 4 (upper panel).

To test the possible link between p94fer and signal transducers and activators of transcription, phosphotyrosine precipitates from COS1 cells transfected with various FER variants were exposed to a Stat1, a Stat2, a Stat3 (Fig. 5, A and C), and a HA antibodies (Fig. 5, top panel). The phosphorylation of Stat3 (Fig. 5, lane 1), but not that of Stat1 and Stat2 (Fig. 5C), was specifically induced by the native HA-p94fer enzyme. Both cytoplasmic (ferΔ1–315 and ferKR653/3NQ) and nuclear (ferΔ1–299) modified forms of p94fer also induced tyrosine phosphorylation of Stat3 (Fig. 5, upper panel). The phosphorylation was dependent on the kinase activity of the fer enzymes, because an inactive mutated form of p94fer (ferΔ685–756, Fig. 5, lane 5) could not induce the tyrosine phosphorylation of Stat3. Similarly, native or nuclear forms of FER overexpressed in CHO cells were able to induce the tyrosine phosphorylation of Stat3 (data not shown). To verify the specificity of tyrosine phosphorylation of Stat3 in p94fer-overexpressing cells, Stat3 was directly immunoprecipitated utilizing a Stat3 antibodies, and precipitates were reacted with aPT.

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Fer Tyrosine Kinase Induces Phosphorylation of Stat3

Fig. 5. p94fer induces the phosphorylation of Stat3, but not of Stat1 or Stat2, in COS1 cells. A, tyrosine-phosphorylated proteins from COS1 cells overexpressing HA-p94fer (lane 1), HA-ferΔ1–299 (lane 2), HA-ferΔ1–315 (lane 3), HA-fer-NLS (lane 4), non-active HA-ferΔ685–756 (lane 5), and from non-transfected cells (lane 6) were immunoprecipitated with αPT antibody. The precipitates were resolved by SDS-PAGE and then reacted with αStat antibody in Western blot analysis (upper panel). The corresponding whole cell lysates were also reacted with Stat3 antibody in Western blot analysis (lower panel). Arrows on the left indicate the migration distances of the tyrosine-phosphorylated Stat3 (p,Stat3) and non-phosphorylated Stat3. B, the same immunoprecipitates as in A (upper panel) and the corresponding whole cell lysates as in A (lower panel) were resolved in SDS-PAGE and then reacted with αHA antibody by Western blot analysis. C, whole cell lysates from COS1 cells overexpressing HA-p94fer (lanes 1, 3, 5, 7, and 9) and from non-transfected cells (lanes 2, 4, 6, 8, and 10) were immunoprecipitated with αPT antibody (lanes 1, 2, 4, 5, 6, 9, and 10) or were left untreated (lanes 3, 4, 7, and 8). All samples were resolved in SDS-PAGE and reacted with αStat1 (upper panel), αStat2 (middle panel), or αHA (lower panel) antibody, by Western blot analysis. Ab, precipitated antibodies; IP, immunoprecipitations; IB, immunoblotting; WCL, whole cell lysates; delHAfer, tagged modified forms of p94fer; p,delHAfer, tyrosine-phosphorylated modified forms of HA-p94fer.

The tyrosine phosphorylation level of precipitated Stat3 was increased in COS1 cells overexpressing HA-p94fer and HA-ferΔ1–299. Stat3 was immunoprecipitated from COS1 cells overexpressing HA-p94fer (lane 1), HA-ferΔ1–299 (lane 2), and from non-transfected cells (lane 3). It was then resolved by SDS-PAGE and reacted with αStat3 antibody (upper panel) or with αPT (lower panel) in a Western blot analysis. Arrows on the left indicate the migration distances of the tyrosine-phosphorylated Stat3 (p,Stat3) and non-phosphorylated Stat3. IP, immunoprecipitations; IB, immunoblotting.

Fig. 6. The tyrosine phosphorylation level of precipitated Stat3 is increased in COS1 cells overexpressing HA-p94fer and HA-ferΔ1–299. Stat3 was immunoprecipitated from COS1 cells overexpressing HA-p94fer (lane 1), HA-ferΔ1–299 (lane 2), and from non-transfected cells (lane 3). It was then resolved by SDS-PAGE and reacted with αStat3 antibody (upper panel) or with αPT (lower panel) in a Western blot analysis. Arrows on the left indicate the migration distances of the tyrosine-phosphorylated Stat3 (p,Stat3) and non-phosphorylated Stat3. IP, immunoprecipitations; IB, immunoblotting.

pressing HA-p94fer—After identifying Stat3 as a downstream effector of p94fer, we turned to check to what extent does the tyrosine phosphorylation signal induced by p94fer in transfected COS1 cells reflect the tyrosine phosphorylation of Stat3. COS1 cells were transiently transfected with the HA-p94fer expression vector and were then co-stained with αHA and αpStat3 antibodies. The αpStat3 specifically detects Stat3, which became phosphorylated on Tyr-705. Tyrosine-phosphorylated Stat3 was detected in 70% of more than 200 HA-p94fer-expressing cells, which were scored (Fig. 7). This signal was prominent in the cytoplasm or in the nucleus of the HA-p94fer-expressing cells (Fig. 7, A and B). αpStat1 antibodies, on the other hand, gave only a faint signal, which was barely detected under the experimental conditions used in this work (Fig. 7, C and D). Thus, both cytoplasmic and nuclear phosphorysine signals elicited in HA-p94fer-expressing cells (Fig. 2, A and B) could reflect the accumulation of tyrosine-phosphorylated Stat3, and Stat3 is therefore activated in most cells expressing the exogenic p94fer. Tyrosine-phosphorylated Stat3 was not detected in cells expressing the inactive p94fer mutant, ferΔ685–756 (data not shown). These experiments were repeated in HeLa cells and in the murine myogenic cell line, C2C12 (26), and gave similar results (data not shown). Thus, p94fer can activate Stat3 in various cell types.

Induction of Stat3 DNA Binding Activity in FER Overexpressing Cells—Tyrosine phosphorylation of Stat3 lead to their dimerization via reciprocal SH2-phosphotyrosine interaction. The signal transducers and activators of transcription dimers then enter the nucleus and bind specific DNA elements involved in activation of specific gene transcription (34). To test whether ectopic expression of an FER kinase leads to the activation of Stat3, the DNA binding activity of Stat3 was analyzed in COS1 cells expressing or non-expressing an ectopic FER kinase. Overexpression of ferΔ1–299 induced the DNA binding activity of Stat3 to a synthetic Stat3 binding site (Fig. 8, lane 4). This binding, which was not induced by an inactive p94fer mutant (Fig. 8, lanes 6 and 7), was specific and could be competed by an unlabeled probe (Fig. 8, lane 5). Thus, a FER kinase can induce the activation of Stat3 (33, 34).

The Endogenous P94fer Kinase Associates with Stat3 in NIH3T3 and C2C12 Cells—To substantiate the interaction of p94fer and Stat3 in vivo, the association of these two proteins was analyzed in two unrelated cell types. These were the fibroblastic NIH3T3 cell line and the murine myogenic C2C12 cell line (26). The endogenous p94fer protein was immunoprecipitated from whole cell extracts, and the presence of Stat3 was tested in the obtained precipitates. Stat3 co-immunoprecipitated with p94fer in extracts prepared from the two analyzed cell lines. Polyclonal αFER antibodies directed against the SH2
domain of p94fer specifically immunoprecipitated the FER enzyme and Stat3, which co-immunoprecipitated with it but failed to co-immunoprecipitate Stat1 (Fig. 9). These results clearly demonstrate the specific interaction of p94fer and Stat3 in vivo and strongly implies that p94fer is an activator of Stat3.

In Situ Staining of the p51ferT Phosphorylation Activity—Our observation that both cytoplasmic and nuclear forms of p94fer can lead to the tyrosine phosphorylation of Stat3 prompted us to study the ability of the nuclear meiotic form of FER, the p51ferT kinase, to induce a similar phosphorylation profile. Transiently transfected COS1 cells expressing an HA-tagged p51ferT were immunostained with a PT and a HA antibodies. As expected, HA-p51ferT accumulated in the nucleus of all expressing cells (Fig. 10A). Staining the transfected cells with the PT antibody revealed the induction of tyrosine phosphorylation signal by p51ferT, which was confined to the cell nucleus (Fig. 10A). This profile was similar to the one seen in cells expressing the nuclear truncated form of the somatic p94fer (Fig. 3D, ferΔ1–299). Western blot analysis showed that p51ferT was able to induce phosphorylation of proteins in two main bands, around 80 and around 105 kDa (data not shown).

p51ferT Does Not Induce the Phosphorylation of Stat3—To check whether the in situ nuclear phosphorylation signal seen in HA-p51ferT-expressing cells (Fig. 10A) reflects, at least in part, increased phosphorylation of Stat3, immunoprecipitation and Western blotting were performed. Whole cell proteins were immunoprecipitated from HA-p51ferT-expressing cells using αPT antibodies. As expected, HA-p51ferT accumulated in the nucleus of all expressing cells (Fig. 11B). Staining the transfected cells with the αPT antibody revealed the induction of tyrosine phosphorylation signal by p51ferT, which was confined to the cell nucleus (Fig. 11B). This profile was similar to the one seen in cells expressing the nuclear truncated form of the somatic p94fer (Fig. 3D, ferΔ1–299). Western blot analysis showed that p51ferT was able to induce phosphorylation of proteins in two main bands, around 80 and around 105 kDa (data not shown).

FIG. 7. In situ immunofluorescence of tyrosine-phosphorylated Stat3 in COS1 cells overexpressing HA-p94fer (A and B). Cells were double-stained with monoclonal αHA antibody, which detects the ectopic HA-p94fer protein (green) and polyclonal anti-phospho Stat3 antibodies (red). A, HA-p94fer localization (a); induced cytoplasmic tyrosine-phosphorylated Stat3 (b); the merged images (c). B, HA-p94fer localization (a); induced accumulation of tyrosine-phosphorylated Stat3 in the cell nucleus (b); the merged images (c). C and D, double staining with monoclonal αHA antibody (green) and polyclonal anti-phospho Stat1 antibodies (red). HA-p94fer localization (a); induced tyrosine-phosphorylated Stat1 (b); the merged images (c). These photographs represent stacked confocal laser sections taken 1 μm apart. Experiments were repeated more then 10 times, and more than 100 cells were scored in each experiment. Representative photographs are shown. Scale bar, 50 μm.

FIG. 8. FER tyrosine kinase activates the DNA binding activity of Stat3. Nuclear extracts from non-transfected COS1 cells (lanes 2 and 3), COS1 cells overexpressing HA-ferΔ1–299 (lanes 4 and 5), and cells overexpressing the non-active HA-ferD685–756 mutant (lanes 6 and 7) were incubated with a 32P-labeled probe in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of 175 ng of unlabeled competitive fragment, which was used as a probe in that assay. Lane 1, free probe.
or Stat2 (data not shown). In addition, HA-p51ferT was not autophosphorylated in the transfected cells, because it was not precipitated by the αPT antibodies (Fig. 11B, upper panel, lane 2). Thus, the in situ nuclear tyrosine phosphorylation signal seen in p51ferT-expressing cells (Fig. 10A) most likely reflects the phosphorylation of a nuclear protein(s) that differs from Stat1, Stat2, Stat3, and p51ferT.

The Unique N-terminal Sequences of p51ferT Interfere with Its Ability to Phosphorylate Stat3—The fact that truncated nuclear forms of the somatic p94fer led to the phosphorylation of Stat3 (Fig. 5A), whereas the nuclear meiotically expressed p51ferT failed to do so, raised the possibility that specific sequences in the p51ferT protein impair the ability of this meiotic enzyme to induce phosphorylation of Stat3. To test this hypothesis, the unique 43-aa N-terminal region of p51ferT (Fig. 1) was removed, together with further downstream 15 aa. The new truncated form of p51ferT (Fig. 1, ferΔ1–427) was tyrosine-phosphorylated in vivo (Fig. 11B, upper panel, lane 1 and Fig. 10C). However, the truncated p51ferT did not gain the ability to induce phosphorylation of Stat3 (Fig. 11A, top panel, lane 1). This could be because of the newly introduced truncation point in p51ferT, which is proximal to the SH2 domain (starting at aa 460 in p94fer) of the kinase (Fig. 1, ferΔ1–427).

To further understand the effects of the unique sequences in the N terminus of p51ferT, the 43 N-terminal aa of p51ferT were replaced with the parallel 36 unique aa of p94fer (Fig. 1, ferΔ1–376). In this modified enzyme, the unique N-terminal tail of p51ferT was replaced by a fragment extending from aa 376 to 412 in p94fer. This fragment is 36 aa in length, and its position in p94fer parallels the position of the unique N-terminal tail in p51ferT (Fig. 1). The replacing p94fer fragment includes the last 11 aa of the p94fer coiled-coil domain III, and it was attached to the p94fer/p51ferT divergence point (Fig. 1, ferΔ1–376). Thus the modified enzyme, ferΔ1–376, differs from the native p51ferT by its replaced N-terminal sequences. Like p51ferT, ferΔ1–376 accumulated mainly in the cell nucleus and induced a nuclear tyrosine phosphorylation signal (Fig. 10B). However, unlike p51ferT, the transient expression of ferΔ1–376 enabled prominent phosphorylation of Stat3 (Fig. 11A, upper panel, lane 3). Thus, it seems that the N-terminal tail of p51ferT plays a role in the inability of this enzyme to induce phosphorylation of Stat3. Replacement of the tail with parallel N-terminal p94fer sequences endowed the meiotic enzyme with the ability to induce Stat3 phosphorylation.

**DISCUSSION**

The FER tyrosine kinases are members of the Fes/FER family that belong to the wider group of non-receptor tyrosine kinases. However, unlike most other known tyrosine kinases,
the FER tyrosine kinases include both somatically and meiotically expressed members, whose cellular roles are not well understood. To further explore the function of these enzymes, immunofluorescent staining approaches accompanied by Western blotting and immunoprecipitation were employed. Double immunofluorescence was found to be a most valuable tool in understanding. To further explore the function of these enzymes, immunofluorescent staining approaches accompanied by Western blotting and immunoprecipitation were employed. Double immunofluorescence was found to be a most valuable tool in understanding the function of the Fes/FER tyrosine kinase to regulation of cell growth, with Stat3 as a downstream effector of p94fer. This coincides with the activation of p94fer by growth factors and its association with their receptors. The involvement of p94fer in growth-promoting processes may also explain the ability of Drosophila Fer to transform mammalian cells (16) and the association of p94fer expression with the proliferation of prostatic cancer cells (49). p94fer could thus be a novel mediator for the activation of Stat3 by growth factors like platelet-derived growth factor and epithelial growth factor (50, 51). As mentioned above, the other somatic member of the Fes/FER family, the tyrosine kinase p51ferT, is exceptional in this respect. Despite the fact that it shares the same kinase and SH2 domains with p94fer, these two highly related kinases do not elicit the same phosphorylation events in fibroblastic cells. Although overexpression of p94fer led to elevated tyrosine phosphorylation of Stat3 but not of Stat1 or Stat2, p51ferT failed to induce the phosphorylation of any of these proteins. This difference between p94fer and p51ferT did not result from the different sub-

Fig. 11. N-terminal sequences of p51ferT affect its ability to induce the phosphorylation of Stat3. A, tyrosine-phosphorylated proteins from COS1 cells overexpressing HA-ferΔ1–427 (lane 1), HA-p51ferT (lane 2), HA-ferΔ1–376 (lane 3), and from non-transfected cells (lane 4) were immunoprecipitated with PT antibody and then resolved by SDS-PAGE (upper panel) together with their corresponding whole cell extracts (WCL, lower panel). The membranes were exposed to pStat3 antibody. Arrows on the left indicate the migration distances of the tyrosine-phosphorylated Stat3 (pStat3) and non-phosphorylated Stat3. B, the immunoprecipitates from A (upper panel) and their corresponding untreated lysates (lower panel) were exposed to pHA antibody in a Western blot analysis. IP, immunoprecipitations; IB, immunoblotting; delHAfer, tagged modified forms of p94fer; delHAfer, tyrosine-phosphorylated modified forms of HA-p94fer.

FER Tyrosine Kinase Induces Phosphorylation of Stat3

Stat3 was shown to be activated in several human tumors (37–41) and can act as an oncogene (42). The constitutive activation of Stat3 was found to be essential for cellular transformation by v-src (43). Several oncogenic tyrosine kinases including v-Abl (44), v-Fps (45), Bcr-Abl (46), and v-Eyk (47) also activate Stat3. Together these findings link the function of Stat3 to cellular proliferation. This is supported by the fact that Stat3 is required for the activation of v-src (43). However, the specific activation of Stat3 by p94fer directly links this tyrosine kinase to regulation of cell growth, with Stat3 as a downstream effector of p94fer. This coincides with the activation of p94fer by growth factors and its association with their receptors. The involvement of p94fer in growth-promoting processes may also explain the ability of Drosophila Fer to transform mammalian cells (16) and the association of p94fer expression with the proliferation of prostatic cancer cells (49). p94fer could thus be a novel mediator for the activation of Stat3 by growth factors like platelet-derived growth factor and epithelial growth factor (50, 51). As mentioned above, the other somatic member of the Fes/FER family, the tyrosine kinase p51ferT, is exceptional in this respect. Despite the fact that it shares the same kinase and SH2 domains with p94fer, these two highly related kinases do not elicit the same phosphorylation events in fibroblastic cells. Although overexpression of p94fer led to elevated tyrosine phosphorylation of Stat3 but not of Stat1 or Stat2, p51ferT failed to induce the phosphorylation of any of these proteins. This difference between p94fer and p51ferT did not result from the different sub-
cellular distribution profiles of the two kinases, because truncated forms of p94fer, which lack p51fer tail, exhibit constitutive nuclear localization profiles, did induce the increased tyrosine phosphorylation level of Stat3. p94fer and p51fer thus appear to have different substrate specificity.

We found that p51ferT increases the phosphotyrosine levels of 80- and 105-kDa proteins in COS1 cells (data not shown), which prove not to be Stat1, Stat2, or Stat3. One of these proteins could be related to the 66-kDa nuclear protein that is linked to growth-promoting processes that do not involve Stat3. It could induce growth-suppressive pathways, or it could be linked to growth-promoting processes that do not involve Stat3. The impairment imposed by p51ferT on S phase progression in transfected CHO cells (10), indicates the possible involvement of p51ferT in growth-inhibitory processes in meiotic cells.

The inability of p51ferT to phosphorylate Stat3 seems to result from an inhibitory effect that is imposed by its unique 43-aa-long N-terminal tail. This was demonstrated by showing that replacement of these 43 aa with a parallel N-terminal sequence from p94fer (Fig. 1, ferΔ1–376) restored the ability of p51ferT to phosphorylate Stat3 (Fig. 11). One can not exclude, however, the possibility that the unique tail of p51ferT does not inhibit Stat3 phosphorylation but rather lacks some positive signal that is present in the 36 N-terminal aa of ferΔ1–376 and that is essential for the interaction of the FER kinases with Stat3. Interestingly, the regulatory effect of the N-terminal tail of p51ferT is specific and does not prevent the interaction of that enzyme with other cellular substrates. It should be noted, however, that the unique N-terminal sequence also interfere with the autophosphorylation activity of p51ferT in COS1 cells (Fig. 11). This could be related to the inability of p51ferT to phosphorylate Stat3. The unique N-terminal tail of p51ferT may thus be involved in the formation of a structure that dictates the defined substrate specificity of the meiotic kinase. It should be mentioned that we did not identify any post-translational modifications that could be linked to the modulatory effect of these N-terminal 43 aa in p51fer (data not shown).

The FER kinases constitute a unique subgroup of tyrosine kinases in which two related enzymes share identical kinase and SH2 domains but are each linked to a different N-terminal tail. This directs different substrate specificity of these enzymes and most probably leads to their different cellular roles, which are linked to key regulatory processes of cell growth.

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