Cell Cycle-dependent Nuclear Accumulation of the p94fer Tyrosine Kinase Is Regulated by Its NH₂ Terminus and Is Affected by Kinase Domain Integrity and ATP Binding

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Abstract

p94fer and p51ferT are two tyrosine kinases that are encoded by differentially spliced transcripts of the FER locus in the mouse. The two tyrosine kinases share identical SH2 and kinase domains but differ in their NH₂-terminal amino acid sequence. Unlike p94fer, the presence of which has been demonstrated in most mammalian cell lines analyzed, the expression of p51ferT is restricted to meiotic cells. Here, we show that the two related tyrosine kinases also differ in their subcellular localization profiles. Although p51ferT accumulates constitutively in the cell nucleus, p94fer is cytoplasmic in quiescent cells and enters the nucleus concomitantly with the onset of S phase.

The nuclear translocation of the FER proteins is driven by a nuclear localization signal (NLS), which is located within the kinase domain of these enzymes. The functioning of that NLS depends on the integrity of the kinase domain but was not affected by inactivation of the kinase activity. The NH₂ terminus of p94fer dicted the cell cycle-dependent functioning of the NLS of FER kinase. This process was governed by coiled-coil forming sequences that are present in the NH₂ terminus of the kinase. The regulatory effect of the p94fer NH₂-terminal sequences was not affected by kinase activity but was perturbed by mutations in the kinase domain ATP binding site.

Ectopic expression of the constitutively nuclear p51ferT in CHO cells interfered with S-phase progression in these cells. This was not seen in p94fer-overexpressing cells. The FER tyrosine kinases seem, thus, to be regulated by novel mechanisms that direct their different subcellular distribution profiles and may, consequently, control their cellular functioning.

Introduction

Nonreceptor mammalian 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 (1–3), membrane-associated kinases that regulate cytoskeletal-mediated signal transduction pathways (4, 5), and tyrosine kinases that can be detected both in the cytoplasm and nucleus of cells. The last group contains c-Src- and c-Abl-related tyrosine kinases (6–8).

p94fer is an evolutionarily conserved (9, 10) nonreceptor tyrosine kinase that is encoded by the FER locus in human (11), mouse (12), rat (13), and Drosophila (110). The presence of p94fer has been documented in most mammalian cell lines analyzed (11, 13), but it was not detected in pre-B, pre-T, and T cells (14). The subcellular localization pattern of p94fer is, however, less conclusively defined, and in some cell lines, it was found to reside in both the cytoplasm and nucleus of cells (15, 16). In the cytoplasm, p94fer associates with cell-cell adhesion molecules (17, 18), and its activity is induced in growth factor-stimulated cells (16).

A truncated form of p94fer, termed p51ferT, is encoded by a testis-specific FER transcript. This tyrosine kinase was shown to accumulate in the nucleus of meiotic pachytene spermatocytes (12, 19, 20).

p51ferT and p94fer differ in their NH₂ termini, but they do share common SH2 and kinase domains (Fig. 1A; Refs. 11 and 12). The FER kinase domain (11, 13) is 70 and 50% homologous to the kinase domains of two other nonreceptor tyrosine kinases, c-Fes (21–23) and c-Abl (24), respectively. Both c-fes (23) and c-Abl (24) were shown to reside in the cytoplasm and nucleus of mammalian cells.

Several other nonreceptor tyrosine kinases were found to reside in the cell nucleus. These include Wee1 and some c-Src-related kinases that were shown to accumulate in the nucleus of certain cell types (6, 8, 25). Two receptor tyrosine kinases, the fibroblast growth factor receptor (26) and the p185neu proto-oncogene (27), were also shown to reside in the nuclei of mammalian cells. The nuclear accumulation of most of these kinases was not linked, however, to defined stages in the cell cycle, nor have the regulatory elements that drive the nuclear translocation of these enzymes been characterized.

The basic element that drives the nuclear accumulation of proteins in eukaryotic cells is the NLS (28). This positively charged element mediates the interaction of the nuclear transporting factor importin-α with the protein to be translocated (29, 30). The function of the NLS was shown to be constitutive in some proteins and tightly regulated in others (31). The nuclear accumulation of a cellular protein could be
regulated, however, not only by an import process to the nucleus but also by an active export mechanism that is mediated by leucine-rich NESs (32, 33).

To further the understanding of the cellular role of p94fer and, thereby, extend the knowledge of the functions of other nuclear tyrosine kinases, a detailed analysis of the subcellular distribution pattern of this enzyme was carried out. The subcellular localization of p94fer was determined in both primary fibroblasts and fibroblastic cell lines.

In parallel, a functional assay was applied to identify elements that direct the regulated subcellular distribution of the FER proteins. This revealed unique features that imply the involvement of novel mechanisms in the regulation of the subcellular localization of mammalian tyrosine kinases.

Results

Heterogeneous Subcellular Distribution Profile of p94fer in Growing Primary Fibroblasts. To characterize the subcellular distribution profiles of p94fer in different mammalian cell lines, we determined the subcellular localization of p94fer in primary mouse fibroblasts. p94fer and p51ferT were linked at their NH2-terminal ends to a single HA epitope and were transiently expressed in primary mouse fibroblasts, under the control of the CMV promoter. Cells were then fixed 48 h posttransfection. Indirect immunocytochemistry was carried out by using antibodies against HA, and stained cells were visualized using confocal microscopy. The usage of highly specific α-HA monoclonal antibody offered us an efficient and reliable follow-up tool for detection of the expressed FER proteins. p51ferT was shown previously to accumulate in the nucleus of spermatogenic cells (20). Similarly, ectopic expression of p51ferT in primary mouse fibroblasts, under the control of the CMV promoter. Cells were then fixed 48 h posttransfection. Indirect immunocytochemistry was carried out by using antibodies against HA, and stained cells were visualized using confocal microscopy. The usage of highly specific α-HA monoclonal antibody offered us an efficient and reliable follow-up tool for detection of the expressed FER proteins. p51ferT was shown previously to accumulate in the nucleus of spermatogenic cells (20). Similarly, ectopic expression of p51ferT in growing primary fibroblasts confirmed the preferred accumulation of p51ferT in the cell nucleus (Fig. 1Bc). No staining was seen in nontransfected cells (Fig. 1Bd), thus proving the specificity of the obtained signals. However, cells expressing exogenous p94fer showed a heterogeneous subcellular distribution profile. Although p94fer was mainly cytoplasmic in ~60% of the transfected cells, in about 23% of the cells, it was equally distributed between the nucleus and the cytoplasm (Table 1 and Fig. 1Ba). The remaining 17% of the cells exhibited exclusive or preferential nuclear accumulation of p94fer (Table 1 and Fig. 1Bb). The obvious interpretation of these results is that, unlike p51ferT, the subcellular localization pattern of p94fer varies according to the cell cycle state of growth in a given population of actively growing primary cells.

Cell Cycle-regulated Nuclear Accumulation of the p94fer Tyrosine Kinase. To check whether the heterogeneous subcellular distribution profile of p94fer in fibroblasts (Fig. 1B) is linked to cell cycle progression, we extended the analysis of the subcellular localization of p94fer to several fibroblastic cell lines. This offered us efficient tools for local-

Table 1 Subcellular distribution of p94fer in actively growing fibroblastic cells

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<th>Actively growing (%)</th>
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<td></td>
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<td>Primary fibroblasts</td>
<td>60</td>
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<tr>
<td>COS1</td>
<td>30</td>
</tr>
<tr>
<td>BHK21</td>
<td>40</td>
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<td>CHO</td>
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Fig. 1. A, schematic description of the p94fer and p51ferT proteins. Boxed c in p94fer, CC-forming region; SL, kinase domain small lobe; LL, kinase domain large lobe. Stippled box in p51ferT, the unique NH2-terminal sequences of the enzyme. B, subcellular distribution of the FER proteins in mouse primary fibroblasts. Subconfluent actively growing mouse primary fibroblasts were transiently transfected with HA-p94fer (a and b) or HA-p51ferT (c) expression vectors. Exogenously expressed proteins were detected with mouse monoclonal α-HA antibody which were then reacted with FITC – conjugated donkey antimouse antibodies. d, antibody incubated with nontransfected cells. Photographs represent stacked confocal laser sections taken 1 μm apart. Scale bar, 20 μm.
izing p94fer in growth-arrested and actively growing fibroblastic cells. COS1, BHK21, and CHO cells were seeded in numbers that allowed them to reach tight intercellular contact within 24 h. The cells were transiently transfected with the p53fer and p94fer expression plasmids in which the expression of the enzymes was driven by the SV40 early promoter (COS1 cells) or CMV promoter (BHK21 and CHO cells). Under these conditions, the cells significantly slowed down their division rate after 40 h, as confirmed by the observation of low numbers of BrdUrd-incorporating nuclei, compared to subconfluent transfected cells (data not shown). Inhibition of the growth rate of the cells, led to cytoplasmic accumulation of p94fer in all transfected COS1 cells. Thus, p94fer was excluded from the nucleus of the quiescent cells or was present at very low levels in this compartment. Unlike p94fer, the accumulation of p53fer in the transfected and growth-arrested COS1 (Fig. 3B), BHK21, and CHO cells was restricted to the cell nucleus (data not shown).

To check whether actively growing fibroblastic cell lines exhibit heterogeneous subcellular distribution profile of p94fer, we seeded subconfluent cultures of COS1, BHK21, and CHO cells and then transiently transfected the cells with the p53fer and p94fer expression plasmids. Cells were fixed, and subcellular localizations of the FER proteins were determined with α-HA monoclonal antibody. Actively growing COS1 cells exhibited heterogeneous subcellular distribution profile of the exogenous p94fer (Table 1 and Fig. 2Ag). Although 30% of the transfected cells exhibited cytoplasmic accumulation of p94fer (Fig. 2A, a and b) accumulation of p94fer (Fig. 2A, c and d), in the other 40%, it was present at similar levels in both cytoplasm and nucleus (Table 1). The remaining 30% of the transfected population exhibited preferential accumulation (>60% in the nucleus) in the cell nucleus (Table 1 and Fig. 2A, c and d). Around 40% of the transfected and actively growing BHK21 cells exhibited cytoplasmic accumulation of p94fer, and ~48% of the cells showed similar staining in the cytoplasm and in the nucleus (Table 1). The remaining 12% of the cells, p94fer
The nuclear accumulation of the FER proteins depends on kinase domain integrity. A, wild-type fer-intact p94*fer (a), wild-type ferT-intact p51*ferT (b), ferT.Δ1-58 (c), ferT.Δ594-634 (d), and ferT.Δ446-453 (f), all linked to a HA epitope, were exogenously expressed in actively growing COS1 cells. Cells were fixed and stained with α-HA monoclonal antibody (Babco). Photographs represent stacked confocal laser sections taken 1 μm apart. Scale bar, 20 μm. B, summary of p94*fer (fer) and p51*ferT (ferT) constructs whose subcellular distribution profiles were determined in growth arrested confluent (conf.) and nonconfluent (nonconf.) COS1 cells. The symbols within the schemes of the tested constructs (structure) are as in Fig. 1A. Enzymatically active (+) and nonactive (−) constructs which were tested in an autophosphorylation assay (kinase act.) are marked. The subcellular distribution profiles are as follows: −, 0–5% of the molecules were found in the nucleus; −+/+, 5–20%; +, 20–40%; ++, 40–60%; ++++, 60–80%; +++++, 80–95%; and ++++++, 95–100%. These values were obtained from at least three independent experiments in which at least 50 transiently transfected cells were obtained for each construct. The expressing cells were then examined by eye for the relative intensity of staining.
accumulated preferentially in the cell nucleus (>60% in the nucleus; Table 1). Growing CHO cells also exhibited a heterogeneous subcellular distribution profile. About 40% of the transiently transfected cells exhibited preferential cytoplasmic accumulation of p94fer, and ~37% showed similar staining in the cytoplasm and in the nucleus (Table 1). In the remaining 23% of the transfected CHO cells, p94fer was detected mainly in the cell nucleus (Table 1).

As was found in growth-arrested cells, the accumulation of p51ferT in the actively growing COS1 (Fig. 2A, e, f, and h), BHK21 (data not shown), and CHO (data not shown) cells was restricted to the cell nucleus. The subcellular distribution profile of p94fer seems, therefore, to be cell cycle dependent, and it differs in growth-arrested and actively growing cells. The subcellular localization of the meiotic p51ferT kinase is not affected, however, by cell growth, and it accumulates constitutively in the cell nucleus.

**Nuclear Accumulation of p94fer Coincides with Onset of S Phase.** To determine at which stages of the cell cycle p94fer is translocated to the cell nucleus, we adopted two approaches. In the first, cells were grown in the presence of aphidicolin. This drug inhibits the activity of DNA polymerase α and consequently inhibits S-phase progression, thus arresting the cells at the G1-S transition stage (34). The cells were transiently transfected with the p94fer expression plasmids and were then exposed to the drug for the last 20 h before fixation. Treating the three cell lines with aphidicolin significantly enriched the percentage of cells that exhibited preferential accumulation of p94fer in the nucleus. In CHO cells, this treatment led to the preferential accumulation of p94fer in the nuclei of 82% of the transfected cells (Table 2), whereas only 23% of the untreated cells exhibited that distribution profile (Table 2). p94fer was present preferentially in the cytoplasm of only 3% of the transfected and treated cells, compared to 40% of the nontreated cells that exhibited cytoplasmic accumulation of p94fer (Table 2). Similar results were obtained with transfected and aphidicolin-treated BHK21 and COS1 cells (data not shown). Releasing the CHO cells from aphidicolin treatment for 3 h, a procedure that allowed the entrance of 80–90% of the cells into S phase (data not shown), did not significantly change the subcellular distribution profiles of p94fer in the treated cells (Table 2), thus suggesting that the accumulation of p94fer in the nucleus persists throughout S phase progression. The prominent effect of aphidicolin treatment on the percentage of transfected cells that exhibited preferential nuclear accumulation of p94fer, suggested that the enzyme is translocated to the nucleus concomitantly with the progression of cells through G1 and toward the onset of S phase.

To determine at which of the two stages of the cell cycle p94fer reaches its maximal nuclear level, we adopted a second approach. Actively growing COS1 cells were transfected with the p94fer expression plasmids. Forty h after transfection, the cells were labeled with BrdUrd for 2 h and then double-stained with labeled α-HA and α-BrdUrd monoclonal antibodies. All of the cells that exhibited preferential cytoplasmic accumulation of p94fer did not show incorporation of BrdUrd (Fig. 2B). Preferential nuclear staining of p94fer correlated, however, with incorporation of BrdUrd in nuclei of

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**Table 2.** Effect of aphidicolin treatment on the subcellular distribution profile of p94fer

<table>
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<th>Cell treatment</th>
<th>Cytoplasmic (%)</th>
<th>Equal (%)</th>
<th>Nuclear (%)</th>
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<td>23</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>3</td>
<td>15</td>
<td>82</td>
</tr>
<tr>
<td>Aphidicolin + 3 h</td>
<td>5</td>
<td>15</td>
<td>80</td>
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* Cytoplasmic, cells that harbored 0–40% of the p94fer molecules in the cytoplasm; Equal, cells that harbored 40–60% of the p94fer molecules in the cytoplasm or in the nucleus; Nuclear, cells that harbored 60–100% of the p94fer molecules in the cell nucleus.

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**Fig. 4.** Effects of kinase domain mutations on the autophosphorylation activity of p94fer. A, wild-type fer (Lane 1), fer KR652/3NQ (Lane 2), and ferG571A–823 (Lane 3) were linked to a HA epitope, overexpressed transiently in COS1 cells, and immunoprecipitated with α-HA antibody. The precipitated proteins were exposed to Western blot analysis using monoclonal α-HA antibody (top) or monoclonal α-phosphotyrosine (bottom). The marked band (bottom) represents an in vivo autophosphorylated p94fer. B, the same analysis as in A was carried out with: wild-type fer (Lane 1), fer Y715F (Lane 2), fer G571R (Lane 3), fer G571A (Lane 4), fer K592R (Lane 5), and fer K592N (Lane 6).
transfected cells (Fig. 2B, c and d). These results strongly suggest that the maximal nuclear accumulation levels of p94\textsuperscript{fer} coincides with the onset and progression of S phase. However, as is indicated by the aphidicolin experiments, the transllocation process of p94\textsuperscript{fer} seems to initiate during the progression of the G\textsubscript{1} phase.

**Kinase Domain Integrity Affects the Nuclear Accumulation of the FER Tyrosine Kinases.** To identify functional elements that direct the different behavior of the FER kinases, various deletions and mutations were introduced in the FER cDNAs, and the effects of these modifications on the subcellular distribution profile of the FER proteins were analyzed. p51\textsuperscript{fer\textsuperscript{T}} carries a 43-aa NH\textsubscript{2}-terminal tail, which is absent from p94\textsuperscript{fer} (Fig. 1A; Ref. (12)) and which could contribute to its constitutive nuclear accumulation. Removal of that region did not affect the nuclear accumulation of p51\textsuperscript{fer\textsuperscript{T}} (Fig. 3, Ac and B, fer\textsubscript{T}1\textsubscript{A}1–58). This suggested that a common NLS directs the nuclear accumulation of the two FER proteins.

To narrow down the segment that carries the FER NLS, we then introduced serial deletions in the common kinase domain (Fig. 1A) of p51\textsuperscript{fer\textsuperscript{T}} and p94\textsuperscript{fer}. Removal of the last 147 aa of either p51\textsuperscript{fer\textsuperscript{T}} or p94\textsuperscript{fer} (Fig. 3B, fer\textsubscript{T}307–453 and fer\textsubscript{A}677–823, respectively) interfered with the nuclear translocation of the FER proteins and led to the reainment of the truncated kinases in the cytoplasm (Fig. 3B). Deletion of the last 66 COOH-terminal aa gave the same results (Fig. 3, Ad and B, fer\textsubscript{T}388–453 and fer\textsubscript{A}758–823). Surprisingly, the same effect was obtained when the extreme 8 COOH-terminial aa were removed from both p51\textsuperscript{fer\textsuperscript{T}} and p94\textsuperscript{fer} (Fig. 3, Af and B, fer\textsubscript{T}446–453 and fer\textsubscript{A}816–823, respectively). This raised the possibility of the existence of a NLS at the COOH terminus of p51\textsuperscript{fer\textsuperscript{T}} and p94\textsuperscript{fer}. Examination of the sequence of the last 8 aa (TVIKKMIT) of the FER enzymes did not reveal any potential NLS (28) but, because a cluster of basic aa is a common feature in NLS (28), we decided to mutate the two adjacent lysines in the middle of that segment. Replacing these two lysines with glutamines did not change the con-served acidic residue, even though it completely abolished the autophosphorylation activity of p94\textsuperscript{fer} (Fig. 5, Bb, fer\textsubscript{T} D315E and fer\textsubscript{A} D885E, respectively). This proved the decoupling between kinase activity and nuclear accumulation of the FER proteins. Thus, kinase domain integrity rather than kinase activity plays a role in nuclear transllocation of the FER proteins.

**Mutations in ATP Binding Site Affect the Subcellular Distribution of p94\textsuperscript{fer} but not of p51\textsuperscript{fer\textsuperscript{T}}.** Another loss of function mutation was introduced in the first glycine of the conserved G\textsuperscript{51/571}XGXXGXXV of the FER protein ATP binding site (underlined aa represents Gly-201 in p51\textsuperscript{fer\textsuperscript{T}} and Gly-571 in p94\textsuperscript{fer}). This glycine, which was shown to play a key role in ATP binding and is assumed to provide space for the ribose of ATP (38), was substituted with arginine in the two FER proteins (Fig. 5B, fer\textsubscript{G}571\textsubscript{R} and fer\textsubscript{T} G201\textsubscript{R}; Ref. (39)). Most surprisingly, this mutation, which completely abolished the autophosphorylation activity of p94\textsuperscript{fer} (Fig. 4B, Lane 3), drove constitutive accumulation of p94\textsuperscript{fer} and p51\textsuperscript{fer\textsuperscript{T}} in the cell nucleus (Fig. 5, Aa and B). Thus, although it changed the regulated translocation of p94\textsuperscript{fer} to the nucleus, the ATP binding mutation did not increase the nuclear accumulation of p51\textsuperscript{fer\textsuperscript{T}}. Besides supporting the decoupling between kinase activity and nuclear accumulation of the FER proteins, the ATP binding site mutation raised new aspects concerning the regulated nuclear entrance of p94\textsuperscript{fer}. For a better understanding of this effect, we introduced additional mutations in the ATP binding site of the FER proteins. Another mutation in the first glycine (Fig. 5B, fer\textsubscript{G}571\textsubscript{A}; this represents a change from Gly-571 to Ala), which exists naturally in the ninaC serine/threonine kinase (40), led to 80% reduction

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**Fig. 5.** Effect of ATP binding and kinase activity on the regulated subcellular distribution of p94\textsuperscript{fer} and p51\textsuperscript{fer\textsuperscript{T}}. A, fer\textsubscript{G}571\textsubscript{R} (a), fer\textsubscript{G}576\textsubscript{V} (b), fer\textsubscript{T} D885E (c) and fer\textsubscript{T} Y715F (d), all linked to a HA epitope were transiently expressed in actively growing COS1 cells. Cells were then fixed and stained with \textalpha ;HA monoclonal antibody. Photographs represent stacked confocal sections. Scale bar, 20 \textmu m. B, schematic summary of the p94\textsuperscript{fer} (fer) and p51\textsuperscript{fer\textsuperscript{T}} (fer\textsuperscript{T}) constructs, which were tested in growth-arrested confluent (conf.) and nonconfluent (nonconf.) COS1 cells. The different mutants are listed (kinase), and the mutated sites are described in parentheses. (ATP \textsuperscript{m}) mutation in ATP binding site; (cat. \textsuperscript{m}) mutation in a catalytic residue; (phos. \textsuperscript{m}) mutation in an autophosphorylation site. The expected effects on enzymatic functions are described. ATP \textsuperscript{b}, binding of ATP: --, abolished; +/−, impaired; +, normal binding. Cat. \textsuperscript{A}, autophosphorylation activity: −, loss of catalytic activity; +/−, impaired; +, normal autophosphorylation activity; n.d., not determined. Signs that describe the nuclear accumulation of the various mutants are as in Fig. 3B, and the values were determined according to the described procedure.
in the kinase activity of p94fer (Fig. 4, Lane 4). This mutation only moderately affected the regulated distribution of p94fer (Fig. 5B, fer G571A). Replacing the second glycine of the sequence G201/571XGXXGXXV with alanine (Fig. 5B, fer G573A; Gly-573 to Ala) gave the same moderate effect (Fig. 5B). Another mutation, in the third highly conserved glycine (fer G576V; Gly-576 to Val; Ref. (41)) caused a stronger effect on the nuclear accumulation of p94fer and rendered it less dependent on cell cycle progression (Fig. 5, Ab and B, fer G576V). Replacing Val-578, which is functionally important in ATP binding (42), with threonine showed only moderate effects on the nuclear entry profile of p94fer (Fig. 5B, fer V578T). A similar mutation reduced the kinase activity of erbB by ~80% (42). However, replacing Val-578 in p94fer with aspartic acid drove a constitutive and deregulated cytoplasmic accumulation of p94fer. The corresponding mutation did not affect the constitutive nuclear accumulation of p51ferT (Fig. 5B, fer V578D and ferT V208D, respectively). The ATP binding site of the FER enzymes seems therefore to affect the regulated subcellular distribution of p94fer. It does not directly affect, however, the functioning of the FER NLS.

A lysine residue positioned 21 aa COOH-terminally to the Gly-571 has also been implicated in binding of ATP, although its main function has been linked to transfer of γ-phosphate by coordinating two phosphate oxygens of ATP (43–45). Replacement of that lysine with Arg or Asn (Fig. 5B, fer K592R and fer K592N, respectively) impaired the autophosphorylation activity of p94fer (Fig. 4B, Lanes 5 and 6, respectively) and enhanced, though not in a drastic way, the deregulated nuclear accumulation of p94fer. Overall, these results suggest a good correlation between impairment of ATP binding and deregulated subcellular accumulation of p94fer. This effect, however, was not seen on the constitutive nuclear accumulation of p51ferT.

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**Table:**

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<td>KK448/50Q</td>
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**Fig. 6.** NLS in p94fer and p51ferT. A, fer KR652/3NQ (a), fer G571R-KR652/3NQ (b), ferT KR282/3NQ (c), and ferT KK185/61Q (d) were transiently expressed in COS1 cells. Cells were then fixed and stained with α-HA monoclonal antibody. Photographs represent stacked confocal sections. Scale bar, 20 μm. B, schematic summary of p94fer (fer) and p51ferT (ferT) mutants, which were tested in these experiments. Mutations in putative (putative NLS) monopartite (mono p.) or bipartite (bi p.) NLS are marked. Signs describing the nuclear accumulation of the different mutants are as in Fig. 3B, and the values were determined according to the described procedure.
The autophosphorylation site in the kinase domain activation loop was shown to modulate, in addition to substrate entrance, ATP binding in the insulin receptor (46). This does not occur in the fibroblast growth factor receptor (45). We, therefore, analyzed the role of the p94fer autophosphorylation site on the nuclear accumulation of that kinase. Tyr-715 is the major autophosphorylation site in p94fer (Fig. 4B, Lane 2). Replacing this residue with phenylalanine, which is supposed to mimic the unphosphorylated state of Tyr-715 (Fig. 5B, fer Y715F), however, did not affect the nuclear entrance of either p94fer (Fig. 5, Ad and B, fer Y715F) or p51ferT (Fig. 5B, ferT Y345F). Changing Tyr-715 or Tyr-345 to glutamic acid, in p94fer and p51ferT (fer Y715E and ferT Y345E, respectively), which is supposed to mimic the autophosphorylation state of p94fer, failed to affect the nuclear accumulation of the FER proteins (Fig. 5B). Thus, binding of ATP rather than phosphorylation activity affects the cell cycle-regulated subcellular distribution of p94fer.

A Monopartite NLS Resides in the Kinase Domain of the FER Proteins. The fact that both kinase domain integrity and ATP binding affected the nuclear accumulation of the FER proteins implied the presence of a NLS within this domain. Inspection of the kinase domain as well as the entire aa sequence of p51ferT and p94fer revealed the presence of two potential NLSs in both p51ferT and p94fer. One of these elements is a putative monopartite NLS (28), which extends from Arg-651 to Lys-660 in p94fer and from Arg-281 to Lys-290 in p51ferT, resides in the beginning of the kinase domain large lobe of the enzymes (35). This sequence, R_81/865KRRKDELLKL_990/660, is highly conserved in the mouse, rat and human FER proteins (12, 15). It bears four adjacent positive aa residues (underlined), and it possesses 60% homology to the M2 nuclear localization sequence of the avian c-myc protein (15, 47). To test the role of that sequence in the mobilization of p94fer and p51ferT to the cell nucleus, Lys-652 and Arg-653 (28) were changed to asparagine and glutamine, respectively (Fig. 6B, fer KR652/3NQ). The same mutations have been introduced in the corresponding aa residues in p51ferT (Lys-282 and Arg-283, ferT KR282/3NQ). These two positively charged aa are not conserved among protein kinases (36) and, therefore, need not necessarily affect the structure and activity of the kinase domain. In fact, FER proteins that carry these double mutations preserved their tyrosine autophosphorylation activity (Fig. 4A, Lane 2). Replacement of Lys-652 and Arg-653 in p94fer (fer-KR652/3NQ) resulted in permanent exclusion of the protein from the nucleus and its constitutive accumulation in the cytoplasm (Fig. 6, Aa and B, fer-KR652/3NQ). Similar results were obtained upon introduction of these mutations in the constitutively nuclear mutant of p94fer, which lacks ATP binding activity (fer G571R changed to fer G571R-KR652/3NQ; Fig. 6, Ab and B, fer G571R-KR652/3NQ). Inserting the corresponding mutations in p51ferT (ferT KR282/3NQ) interfered with the nuclear translocation of the kinase and caused accumulation of 60% of the molecules in the cytoplasm (Fig. 6, Ac and B, ferT KR282/3NQ). These experiments strongly suggest the involvement of this positively charged aa cluster in driving the nuclear import of p51ferT and p94fer. The presence of 40% of the mutated p51ferT molecules in the cell nucleus (Fig. 6, Ac and B, ferT-KR282/3NQ), could suggest the presence of additional nuclear localization elements in p51ferT. To check whether the residual nuclear accumulation of p51ferT resulted from the presence of a weak NLS within the unique NH2-terminus of p51ferT, the monopartite NLS was mutated in a truncated p51ferT, which already lacked the first 58 NH2-terminal aa. This double modified molecule exhibited the same nuclear accumulation profile as the entire p51ferT that carried a mutated monopartite NLS (Fig. 6B, ferT31–58/KR282/3NQ and ferT KR282/3NQ), thus suggesting the absence of an NH2-terminal NLS, in p51ferT, which could functionally cooperate with the kinase domain monopartite NLS.

Another potential NLS resembling the structure of a bipartite NLS (28) is located between the SH2 and kinase domain of the FER proteins (Fig. 1A). This element is composed of two basic aa residues followed by an interval of ten aa which precede a cluster of three basic of five aa residues (12, 48). It is composed of the sequence K_171/174 RSGVVLLNPDPKDDKKW (the first aa is numbered according to its location in p51ferT and p94fer, respectively, where the underlined aa are predicted to play a role in nuclear importing activity (48). However, replacing Lys-185 and Lys-186 with isoleucine and glutamine, respectively, did not affect the subcellular distribution of p51ferT (Fig. 6B, fer T KK185/186IQ). These mutations did not exert any effect when cointroduced with the monopartite NLS mutations described above (Fig. 6B, ferT KK185/186IQ-KR282/3NQ). These results strongly suggest the existence of one major NLS element in the kinase domain of p51ferT and p94fer. The residual nuclear accumulation of p51ferT carrying a nonfunctional NLS results most probably from a non-NLS-dependent diffusional entrance of this mutated but compact molecule into the cell nucleus.

**NH2-Terminal Structures Direct the Cell Cycle-regulated Nuclear Accumulation of p94fer.** Most of the p51ferT aa sequence (410 of 453 aa) is included in p94fer (Fig. 1A), but the subcellular distribution profiles of these two kinases are different during cell cycle progression (Figs. 2 and 3). This implies the presence of specific sequences or domains in p94fer, which regulate the functioning of its NLS during mammalian cell cycle. p94fer carries a 412-aa NH2-terminal tail, which replaces the 43-aa NH2 terminus of p51ferT (Fig. 1A). Removal of 376 NH2-terminal aa residues from p94fer drove the constitutive translocation of this enzyme to the cell nucleus of quiescent and actively growing cells (Fig. 7, Aa and B, fer T 1–376), thus reconstructing the subcellular distribution profile of p51ferT (Fig. 3Ab). To delineate the p94fer NH2-terminal sequences that restrict the nuclear accumulation of p94fer at defined stages along the cell cycle, sequential deletions have been introduced in the kinase NH2-terminal tail. Removal of 147 aa residues did not change the preferential accumulation of p94fer in the cytoplasm of quiescent cells, and it allowed its translocation to the nuclei of G1-S cells (Fig. 7, Ae and B, ferT 1–147). Similar results were obtained upon deletion of an additional 31 aa from p94fer (Fig. 7B, ferT 1–178). Shortening the NH2 terminus of p94fer by 299 aa drove the deregulated constitutive accumulation of the truncated p94fer in nuclei of both quiescent and actively growing cells (Fig. 7, Ac and B, ferT 1–299). Removal of an additional 16 aa to a size of 315 aa led to accumulation of only 40% of the
truncated kinase in the nucleus of quiescent cells. In actively growing cells, however, this modified kinase showed higher levels of nuclear accumulation than those seen with intact p94fer (Fig. 7B, ferΔ1–376). The same results were obtained when 328 NH2-terminal aa were removed from p94fer (Fig. 7B, ferΔ1–328). Because ferΔ1–376 is constitutively nuclear in quiescent and growing cells and ferΔ1–328 is mainly cytoplasmic in quiescent cells (Fig. 7B), it may emerge that sequences residing between Ala-328 and Lys-376 play a major role in cytoplasmic retention of p94fer in growth-

\[ \text{fer} \Delta 1-376, \text{fer} \Delta 1-328, \text{fer} \Delta 1-299, \text{fer} \Delta 330-376, \text{fer} \Delta 1-147, \text{fer} \Delta 1-178, \text{fer} \Delta 178 \]

\[ \text{CC-forming regions; SL, the small lobe of the kinase domain; LL, large lobe of the kinase domain; NLS, nuclear localization sequence.} \]

Because ferΔ1–376 is constitutively nuclear in quiescent and growing cells and ferΔ1–328 is mainly cytoplasmic in quiescent cells (Fig. 7B), it may emerge that sequences residing between Ala-328 and Lys-376 play a major role in cytoplasmic retention of p94fer in growth-

\[ \text{Fig. 7. Effects of NH2-terminal deletions on the subcellular distribution of p94fer. A, ferΔ1–376 (a), ferΔ1–328 (b), ferΔ1–299 (c), ferΔ330–376 (d), ferΔ1–147 (e), and fer \Delta 1–178 (f) were transiently expressed in actively growing COS1 cells. Cells were fixed and stained with \( \alpha \)-HA monoclonal antibody. Scale bar, 20 \( \mu \)m. B, schematic summary of the p94fer (fer) deletions which were tested in these experiments. Signs describing the nuclear accumulation of the different constructs are as in Fig. 3B, and the values were determined according to the described procedure. C, a schematic description of the key functional and regulatory elements in p94fer. C, CC-forming regions; SL, the small lobe of the kinase domain; LL, large lobe of the kinase domain; NLS, nuclear localization sequence. The NH2-terminal elements that modulate the NLS activity are shown. The linker between CC regions I and II (negative effect) cooperates with CC regions II and III (positive effect) to impose cell cycle regulation on the FER NLS (for more details, see text).} \]
tested clones (Fig. 8). Endogenous p94fer could be detected in nontransfected cells.

Specific NH2-terminal sequences, that exert NLS or NES functions, have been identified in these proteins. The first 376 NH2-terminal aa (Fig. 7B, ferA330–421) or when 186 aa were deleted between Val-330 and Tyr-515 (Fig. 7B, ferA330–515). Interestingly, removal of 252 aa extending from Glu-124 to Ala-375 gave the same results (Fig. 7B, ferA124–375). All these last four internal deletions in p94fer, which all removed the 47 aa, residing between Ala-328 and Lys-376, all led to constitutive cytoplasmic accumulation of the truncated enzymes. This effect could reflect the presence of an NLS within these removed 47 aa. However, this interpretation is at odds with the constitutive nuclear accumulation of a truncated p94fer that lacked all of the first 376 NH2-terminal aa (Fig. 7B, ferA1–376). It seems, therefore, that it is NH2-terminal structures, rather than specific NH2-terminal sequences, that exert NLS or NES functions and regulate the cell cycle dependent subcellular distribution of p94fer.

Nuclear Accumulation of p51ferT Interferes with S-Phase Progression in CHO Cells. The presence of both p94fer and p51ferT in the nuclei of S-phase cells could imply similar effects of these enzymes on the regulation of S onset or progression. On the other hand, their different subcellular distribution profiles during cell cycle progression and NH2-terminal structures may direct different cellular roles of the two enzymes. To discriminate between these two possibilities, the p94fer and p51ferT kinases were ectopically expressed in CHO cells, and their effects on cell cycle progression were followed. To enable the monitoring of growth-promoting as well as growth-interfering effects, the two enzymes were expressed under the control of an inducible promoter. The mouse p94fer and p51ferT cDNAs were linked to the human metallothionein IIA promoter to construct the pHS1fer and pHS1ferT plasmids (49). These were stably introduced into CHO cells. Western blot analysis of lysates prepared from isolated clones was carried out with antibodies (C1) raised against a synthetic peptide derived from the last common 15 COOH-terminal aa of p94fer and p51ferT (12, 49). The accumulation of the ectopic p94fer and p51ferT proteins was ZnCl2 dependent and reached similar levels in the transfected clones (Fig. 8A, Lanes 2 and 3). No significant level of endogenous p94fer could be detected in nontransfected cells (Fig. 8A, Lane 1). This may reflect either low cellular levels of the endogenous p94fer or lack of cross-reactivity between the endogenous hamster p94fer and the antibodies used in this work. To check the effects of the FER kinases on the cell cycle of CHO cells, transfected clones expressing exogenous p94fer or p51ferT were exposed to ZnCl2 for different periods of time and were then subjected to flow cytometry analysis. Exposing p51ferT-expressing cells to ZnCl2 for 48 h significantly increased the percentage of cells residing in S phase (Fig. 8B, pHS1ferT). Although the cell population harboring the neo-resistance plasmid only (Fig. 8B, pHS1) contained 31% S-phase cells (Table 3), of the p51ferT-expressing cells, 65% resided in S phase (Fig. 8B, pHS1ferT, and Table 3). Such a prominent effect was not obtained upon exposure of ectopic p94fer-expressing cells to ZnCl2, and their cell cycle profiles did not differ significantly from that of pHS1 cells (Fig. 8B, pHS1fer, and Table 3). The cell cycle profiles of noninduced pHS1, pHSferT, and pHSfer cells were similar (Table 3) and did not differ from the profile of nontransfected CHO cells (data not shown). The increase in number of pHS1ferT cells residing in S phase is, thus, ZnCl2 dependent. Measurement of the three clones duplication time indicated elongated cell cycle of p51ferT-expressing cells as compared with the pHS1 (neomycin-resistant clone) and ectopic p94fer-expressing cells. The cell cycle of p51ferT-expressing cells...
was elongated by 30% as reflected by the reduced number of serum-starved synchronized cells that underwent cell division at 24 and 48 h after serum stimulation (data not shown). This suggests that the increase in percentage of S-phase cells in p51ferT-expressing clone reflects elongation of S phase rather than shortening of the G1 phase by p51ferT.

**Discussion**

Nonreceptor tyrosine kinases were shown to play a regulatory role in growth and differentiation of mammalian cells (50). Of the nonreceptor tyrosine kinases, the cellular role of the nuclear tyrosine kinases is least understood. Most of these kinases were reported to reside both in nucleus and cytoplasm of cells, yet the functional significance of the presence of these enzymes in the two subcellular compartments has not been revealed (6–8). Here, we show that the relative accumulation levels of p94fer in the cytoplasm and nucleus of cells vary along cell cycle progression in fibroblastic cells. While residing in the cytoplasm of growth-arrested fibroblasts, p94fer was translocated to the nucleus, concomitantly with the progression of cells through G1 toward the onset of S phase. The maximal nuclear accumulation levels of p94fer were seen in cells residing in S phase. The absence of p94fer from the nuclei of G0 cells suggests its removal from nuclei of late S or G2-M cells. How could p94fer get out of late S or G2/M nuclei? One possibility is that dissolution of the nuclear envelope during M enables the subcellular redistribution of p94fer.

Alternatively, the nuclear p94fer may be degraded at the onset of G2-M phases. It should be noted, however, that no obvious destabilizing motifs (51) could be detected in p94fer.

p94fer was shown to be activated in the cytoplasm of growth factor-stimulated fibroblasts (16). The activation of p94fer, thus, seems to precede its translocation to the nucleus. This sequence of events may be part of the regulated translocation of p94fer to the cell nucleus, thus ensuring the accumulation of an active kinase at the right time (S phase) and at the right place (the nucleus) during progression of the cell cycle. The cytoplasmic activation of p94fer could also imply the involvement of that kinase in modulation of cytoplasmic signal transduction pathways. Both the cytoplasmic and nuclear pools of p94fer were shown to be highly and equally active in an in vitro kinase assay (15).

The nuclear accumulation of p94fer in S-phase cells suggests a role for this kinase in regulation of the onset and/or progression of S phase. Intriguingly, the nuclear p94fer was shown to be associated with nuclear chromatin (15), thus envisaging the involvement of p94fer in chromosomal DNA-related processes.

p94fer was shown to phosphorylate the TATA element modulatory factor (TMF) (52), which can suppress the functioning of RNA polymerase II promoters (53). Additional experiments should reveal whether this activity is related to the onset or progression of S phase.

Another nuclear tyrosine kinase, c-Abl, was found to be regulated along the progression of mammalian cell cycle. Despite the fact that c-Abl resides in the cell nucleus throughout the entire cell cycle, its activity was found to be cell cycle regulated. Whereas in G0/G1 cells, c-Abl is inhibited by the retinoblastoma protein (Rb), it becomes active at the G1-S transition stage (54). Interestingly, c-Abl was also shown to shuttle between the cytoplasm and the cell nucleus under defined cellular conditions. This was dependent on the attachment or detachment of cells from constituents of the extracellular matrix (55, 56).

The nuclear entrance of the FER proteins is driven by a NLS which resides within their kinase domain, at the beginning of the kinase large lobe (35); Fig. 7C). In the murine FER proteins analyzed in this work as well as in rat FER, the NLS is composed of the sequence RKKDELKKL (12, 13). The human FER NLS is highly similar and is composed of the sequence RKKDELKKL (11). These sequences resemble the M2 NLS of c-myc, RQRINELKLSF, which was shown to function as a relatively weak nuclear translocation element (47). Despite the fact that the FER NLS contains at its beginning four basic aa residues, a feature that is common in monopartite NLS (28), it cannot be considered a typical NLS. This stems from the fact that the first basic aa residue in a typical monopartite NLS [K(R/K)X(R/K)] is lysine and not arginine (28, 57), as is found in the FER proteins (15). Yet, the two FER proteins exhibit a most prominent nuclear accumulation profile under defined cellular conditions. The p51ferT FER kinase is mainly nuclear throughout the entire cell cycle, and the p94fer kinase accumulates preferentially in the nuclei of S-phase cells. These findings suggest an efficient functioning of the FER NLS and effective translocation of these proteins to the cell nucleus. The efficient functioning of the FER NLS could result from its cooperation with other NLSs in the FER proteins. However, no additional NLS could be detected in p51ferT or p94fer. This raised the possibility that a structural element, rather than another sequence-specific element, potentiates the activity of the FER monopartite NLS. Indeed, the efficient functioning of the FER NLS is highly dependent on the integrity of the FER kinase domain within which it resides. The interaction between importin (29, 30), and the FER proteins seem, therefore, to be mediated by the FER monopartite NLS and to be stabilized by the specific tertiary structure of the kinase domain within which the NLS is embedded. Any change in the structure of the FER kinase domain drastically impaired the functioning of its NLS. Similar localization of a cryptic NLS within a kinase domain was shown for the cGMP-dependent protein kinase (58). In c-Abl, however, three NLSs were identified at the COOH-terminal half of the protein, outside of the SH2 and kinase domains (59).

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### Table 3 Percentage of cells residing in different phases of the cell cycle

<table>
<thead>
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<th>Condition</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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<td>pH51 ferT</td>
<td>56%</td>
<td>33%</td>
<td>11%</td>
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<td>33%</td>
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<td>65.2%</td>
<td>8%</td>
</tr>
<tr>
<td>pHS1 fer</td>
<td>+</td>
<td>55.3%</td>
<td>10%</td>
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NH2 Terminus-dependent Cell Cycle Regulation
Despite the common kinase domain and NLS in the two FER enzymes, they exhibit different subcellular distribution profiles. Whereas the FER NLS is constitutively active in p51ferT, it is tightly regulated in p94fer. However, we did not detect any effect of unique p51ferT sequences (Fig. 1A) on the nuclear accumulation of that enzyme. This implied the involvement of unique p94fer sequences in the cell cycle-dependent modulation of the NLS activity of this kinase. p94fer bears a 412-aa NH2-terminal tail that is absent from p51ferT (Fig. 1A) and that carries three potentially CC-forming regions (as predicted by the Pepcoil application program). These extend from aa 121 to 178 (Figs. 1A and 7C, I), from aa 301 to 342 (Figs. 1A and 7C, II), and from aa 357 to 387 (Figs. 1A and 7C, III; Ref. (16)). Removal of the entire NH2-terminal tail of p94fer (Fig. 3B, ferTΔ 1–58, which is identical to ferΔ1–427) or of 376 aa (Fig. 7B, ferΔ1–376) drove the constitutive accumulation of p94fer in the cell nucleus, a pattern that is typical for p51ferT (Fig. 3). The regulated nuclear accumulation of p94fer is dictated, therefore, by the unique NH2-terminal sequences of that kinase.

Extending the NH2-terminal end of a truncated p94fer from aa 376 to 328 (Fig. 7B, ferΔ1–328) or to aa 315 (Fig. 7B, ferΔ1–315) reduced the tendency of the enzyme to accumulate in the cell nucleus. This did not result from the presence of an NES in NH2-terminal sequences because deletion of this region from p94fer (ferΔ330–376 and other deletions that include this region; Fig. 7B) did not drive its nuclear translocation but rather led to deregulated accumulation of p94fer in the cytoplasm (Fig. 7B). In addition to that, artificial attachment of the first 124 NH2-terminal aa of p94fer to aa 376 led to constitutive exclusion of p94fer from the cell nucleus (Fig. 7B, ferΔ124–376). These first 124 aa, however, did not play a regulatory role in the subcellular distribution of the intact p94fer (Fig. 7B, ferA 1–147). Thus, attachment of a nonredundant segment, which is 124 aa long, to a p94fer, truncated at aa 376, led to constitutive cytoplasmic accumulation of the truncated enzyme. Surprisingly, however, further extension of the NH2-terminus of the truncated p94fer from aa 376 up to the preceding aa 299, completely regained the constant nuclear accumulation profile that characterizes p51ferT and p94fer truncated at aa 376 (Fig. 7A and B). This does not seem to result from the inclusion of the NLS in the segment extending from aa 300 to 315 in p94fer, because no cluster of basic aa could be identified in that segment. These results suggest that addition of 124 aa residues beyond the NH2-terminal position 376 suffices for interfering with the nuclear entry of p94fer, unless it possesses the CC regions II and III, which reside between aa 301 and 387 (Fig. 7A, c and d; B, ferΔ1–299; and C). Thus, a NH2-terminal tail of 160 aa (124 aa + the remaining 36 unique aa residues in ferΔ1–376; Fig. 7B), which lacks CC regions II and III interferes with the functioning of the p94fer NLS, which is located in the kinase domain of the enzyme. Indeed, deletion of CC regions II and III from p94fer, which was left with a residual unique NH2-terminal 160 or 366 aa (Fig. 7B, ferΔ124–376 and ferΔ330–376, respectively), abolished the nuclear accumulation of the enzyme. Intramolecular interference of NH2-terminal sequences with kinase domain activity was demonstrated in MEKK-1 (60) and Raf-1 (61). In cGMP-dependent protein kinase, NH2-terminal sequences were shown to interfere with both the kinase activity and NLS functioning of the enzyme (58). COOH-terminal sequences were proposed to intramolecular mask the NLS of p105 nuclear factor κB (62). Intramolecular self-regulation was also seen in the c-Abl (63) and FAK (4) tyrosine kinases.

Inclusion of CC regions II and III in the unique NH2-terminal sequences of p94fer relieved their NLS interfering activity and allowed either constitutive or regulated accumulation of the enzyme in the cell nucleus (Fig. 7B, ferΔ1–299 and ferΔ1–178, respectively). Whereas the nuclear accumulation of p94fer depends on the presence of CC regions II and III, the cell cycle regulation of that process depends on sequences that reside NH2-terminally to CC regions II and III. Because p94fer deleted of the first NH2-terminal 147 or 178 aa residues (ferΔ1–147 and ferΔ1–178, respectively) exhibit a subcellular distribution profile that is similar to that of the intact enzyme (Fig. 7B), one can conclude that sequences residing between aa 179 and 299 are essential for cell cycle regulated nuclear accumulation of p94fer. This fragment links between CC region I and II (Figs. 1A and 7C). Thus, three elements seem to play a major role in the regulatory function of the p94fer NH2-terminal sequences. These are: (a) the sequences that link CC regions I and II, (b) CC region II, and (c) CC region III (Fig. 7C). CC region I does not seem to play a critical role in the regulated nuclear entrance of p94fer. It may contribute, however, as was shown for c-fes (64), to the oligomerization and autophosphorylation of p94fer.

The above described analysis suggests that it is not NH2-terminal NLS or NES elements that direct the cell cycle-regulated nuclear accumulation of p94fer but rather structural changes in CC regions II and III. CC regions II and III could be engaged in inter- (16) or intramolecular interactions. Involvement of the CC region II in mediating the oligomerization of p94fer could be stabilized by the catenin like pp120 protein, which binds to the NH2-terminus of p94fer in quiescent cells (16). This would release CC region III to intramolecularly interfere with the accessibility of the kinase domain-embedded FER NLS. This would most probably restrict also the p94fer tyrosine kinase activity. Growth factor stimulation was shown to promote tyrosine phosphorylation of pp120 and consequently drive its dissociation from p94fer (16). This may lead to preferential intramolecular interactions between CC II and III. Engagement of CC III in intramolecular structures with CC II should relieve the NLS masking by CC III, thus allowing the intramolecular self-regulation was also seen in the c-Abl (63) and FAK (4) tyrosine kinases.

Similarly, regulated transition processes from CC-mediated intramolecular interactions to intermolecular structures were presumed to occur in the heat shock-activated heat shock transcription factors HSFl and HSFl2 (65, 66).

One cannot exclude, however, the possibility that the nuclear accumulation of p94fer is regulated by additional cellular factors that bind to its NH2-terminal CC regions or by

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I. Ben-Dor, K. Orlovsky, and U. Nir, unpublished data.
phosphorylation and dephosphorylation events, which may take place under defined cellular conditions and could affect the CC structures in the kinase.

Most interestingly, the regulatory effect of the p94FER NH2-terminal elements depended on the ability of the enzyme to bind ATP. This effect was most pronouncedly observed in the G571R mutant, in which the highly conserved Gly-571 was replaced with arginine, a mutation that abolishes ATP binding ((38)), and which led to constitutive nuclear accumulation of p94FER (Fig. 5B). The cell cycle-regulated NLS-interfering effect of the NH2-terminal structures in p94FER, thus, depends, at least partially, on the ATP binding by the kinase domain. ATP/ADP may stabilize a structure of the kinase domain that is prone to interact with the NH2-terminal CC region III.

The different subcellular distribution profiles of the somatic (p94FER) and meiotic (p51FER) FER enzymes may affect their cellular activities. For example, the permanent accumulation of p51FER in the cell nucleus may allow it to interact with substrates that are not encountered by p94FER. p51FER could, therefore, exert cellular functions that are not carried out by p94FER. Indeed, ectopic expression of p51FER in fibroblastic cells interfered with the S-phase progression in these cells. This effect was much slighter in p94FER expressing cells (Fig. 8B). Thus, although the cellular role of p94FER could be linked to regulation of cell proliferation, the functioning of p51FER in meiotic cells may interfere with proliferation-related events like DNA replication (67). It was shown before that ectopic overexpression of p94FER in fibroblastic cells led to detachment of cells from the substratum and to arrest of cells at G1 (18). We did not see such a dramatic effect under the ectopic expression levels that were achieved in the CHO cells transfected with the metallothionein expression vectors. These levels were most probably lower than those achieved by Rosato et al. (18).

The two FER kinases seem thus to exert different cellular roles. This could result from their different subcellular distribution profiles. One cannot rule out, however, the possibility that the different NH2-terminal tails of p94FER and p51FER dictate their interactions with different cellular proteins, thus leading to their different cellular effects.

The FER proteins represent, therefore, two tyrosine kinase that share identical SH2 and kinase domains but are regulated by novel mechanisms that direct their different subcellular distribution profiles. This could consequently affect their cellular regulatory roles.

Materials and Methods

Construction of p94FER and p51FER Expression Vectors. A mouse fer cDNA5 fragment extending from the first ATG to nt 586 was amplified by PCR using the forward primer AGCCCTGCAGATGGATTGGAATGGAC, which included a PstI site (underlined), and the reverse primer TTTGTCAATAGCGCTCTTGG. The amplification product was cut with PstI and EcoRI and then inserted into the corresponding sites in Bluescript KS+ (12). The cloned fragment was cut out again with BamHI and EcoRI and was ligated to a BamHI and EcoRI cut pcDNA3 vector that carried a single copy of the influenza HA epitope (YPYDVPDYA). The entire fer cDNA sequence was then completed in the pcDNA3 vector by inserting a fer EcoRI-NotI fragment that encodes the missing p94FER sequences. This plasmid was termed CMVfer, and it allowed the expression of an intact p94FER fused to a single HA epitope at its NH2-terminal end under the control of the CMV promoter. A mouse fer cDNA ((12)) fragment extending from the second translation initiation site GAT to nt 1014 was amplified using the forward primer TGCTCTGCAGATAGAGTGGAATGGTC and the reverse primer GAAGTGGTGAATATAAAGC. This amplification product replaced the NH2-terminal sequences of p94FER in pcDNAfer, thus forming the HA-p51FER expression vector pcDNAferT. The pECfer and pECferT expression plasmids were constructed by removal of the FER cDNAs from CMVfer and CMVferT and their reinsertion between the HindIII and XbaI sites. The pECfer and pECferT expression plasmids were transformed under the control of the SV40 early promoter ((68, 69)).

Point mutations in p94FER and p51FER were introduced using the "oligonucleotide directed-mutagenesis without phenotypic selection" approach (69)) with the following modifications. Mutagenized fragments were cloned in pBluescript (KS+) and the R408 M13 strain (Promega) was used as a helper phage for preparation of single-strand DNA stocks. Modified oligonucleotides were extended with T7 DNA polymerase. Modified codons are underlined. All mutations were verified by DNA sequencing.

Point mutations introduced into p94FER (fer) and p51FER (ferT) as follows. For fer-G571R and ferT-G201R, Gly-571 in fer and Gly-201 in ferT were replaced with arginine by using the oligonucleotide GAATTCTGGCGAGGGGGA in which the underlined cytosine replaced the original guanine ((12)). For fer-G571A and ferT-G201A, Gly-571 in fer and Gly-201 in ferT were replaced with alanine by using the oligonucleotide GAATTCTGGCGAGGGGGA in which the cytosine replaced the guanine. For fer-G573A and ferT-G203A, Gly-573 (Gly-203 in ferT) was replaced with alanine by using the oligonucleotide CTGGCGAGGGGA in which the underlined cytosine replaced the guanine. For fer-G575V and ferT-G206V, Gly-575 in fer and Gly-206 in ferT were replaced by valine by using the oligonucleotide GGGGACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine. For fer-G576V and ferT-G206V, Gly-576 in fer and Gly-206 in ferT were replaced by valine by using the oligonucleotide GGGGACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine. For fer-G576V and ferT-G206V, Gly-576 in fer and Gly-206 in ferT were replaced by valine by using the oligonucleotide GGGGACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine.

For fer-V578T and ferT-V208T, Val-578 in fer and Val-208 in ferT was replaced with threonine by using the oligonucleotide GAATTCTGGGAGGGCAAC in which the underlined cytosine replaced the original alanine. For fer-K592R and ferT-K222R, Lys-592 (Lys-222 in ferT) was replaced with arginine by using the oligonucleotide GCCATCTCACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine. For fer-K592R and ferT-K222R, Lys-592 (Lys-222 in ferT) was replaced with arginine by using the oligonucleotide GCCATCTCACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine.

For fer-D315E and ferT-D685E, Asp-315 (Asp-685 in ferT) was replaced with alanine (A) in ferT. For fer-D315E and ferT-D685E, Asp-315 (Asp-685 in ferT) was replaced with alanine (A) in ferT. For fer-L592R and ferT-L222R, Lys-592 (Lys-222 in ferT) was replaced with arginine by using the oligonucleotide GCCATCTCACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine. For fer-L592R and ferT-L222R, Lys-592 (Lys-222 in ferT) was replaced with arginine by using the oligonucleotide GCCATCTCACTGGTTGGAATATTAGTAAG in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the original guanine. For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.

For fer-Y345F and ferT-Y715F, Tyr-345 (Tyr-715 in ferT) was replaced with phenylalanine by using the oligonucleotide CTGTATGGTGAA in which the underlined cytosine replaced the guanine.
DNA fragment AACGCTTACACACAGTGATACATACTGATGCCTAT-TAGGGTTCTACTATTG was cloned into a Saci-DraII-cut pECeFeR.

The following deletions were introduced into p94 fer and p51 fer: ferT31–58 (ferT31–427); pECeFeR was partially cut with Murl and subsequent digestion with BarnHI, and ends were filled in and religated. ferG67–232 and ferL307–453 were constructed by cutting pECeFeR and pECeFerT with XhoI and XbaI, filling in the ends with Klenow fragment, and religation. ferA758–823 and ferA7388–453 were constructed by cutting pECeFeR and pECeFeR T with BlnI and XbaI, and the ends were filled in and religated. ferA816–823 and ferA3446–453 were constructed by cutting pECeFeR and pECeFeR T with PspI and BlnI and after being propagated in dcm E. coli strains. Ends were filled in and religated. The following deletions were introduced into p94 fer, ferA1–148 was obtained by cutting pECeFeR with Smal and EcoRI, and the ends were filled in and religated. For ferA1–178, pECeFeR was cut with XbaI and Ndel, and then the ends were filled in and religated. For ferA1–299: nt 965–1648 were amplified with the forward primer GGCTGACACAGCAGTTTGG, in which the first three underlined nt were exogenously added, and the reverse primer QAATGTTGCTCTAAGCC. The amplified fragment was cut with CiaI and was then inserted into a pECeFeR plasmid that was cut with CiaI and Smal. For ferA1–315, pECeFeR was partially cut with EcoRI and subsequently cut with Smal and self-ligated. For ferA1–330, pECeFeR was cut with CiaI and filled in with cohesive ends and religated. ferA330–376, a fragment encoding Val-330 to Lys-376 was deleted as follows: a downstream fer fragment extending from nt 1194 to 1666 was amplified with a forward primer GGAACGGCCGTTGACTGAGCACACA-AAAAAG, which harbors an EagI site at its 5′ end (underlined), and a reverse primer QAATGTTGCTCTAAGCC. The PCR product was cut with EagI and CiaI. This was reinserted into a CiaI-cut pECeFeR. A similar strategy was used for deleting a fer fragment encoding Leu-331 to Phe-421. In this case the forward primer was CACATCGGCCGTTGAGTCTATTCGT-GGG and the reverse primer used was CACATCGGCCGTTGAGTCTATTCGT-CCTCTA-TCTGTTGATGAATG. The PCR product carried an EagI site at its 5′ end (underlined), and a reverse primer QAATGTTGCTCTAAGCC. The PCR product was cut with EagI and CiaI. This was reinserted into a CiaI-cut pECeFeR. A similar strategy was used for deleting a fer fragment encoding Leu-331 to Phe-421.

Flow Cytometry Analysis. Cells (2 × 10^5) were seeded and exposed to 100 μM ZnCl2 3 h later. Cells were harvested after 48 h, spun down, and washed with PBS and resuspended in 1 ml of staining buffer containing: 0.1% BSA, 0.01% sodium azide, 50 mg/ml propidium iodide, 0.1% Triton X-100, and 2.5 mg/ml RNase for 10 min. Nuclei were spun down, resuspended in 0.5 ml PBS, and analyzed for relative DNA content by an Epics XL-MCL flow cytometer.

Acknowledgments

We thank Dr. R. S. Goldstein and Dr. F. Lebkowicz for helpful discussions; Dr. L. Rolfman and L. Varhavsky for help with the confocal microscope image analysis; U. Karo for help with the flow cytometry analysis; Dr. R. Wides for his critical remarks; and A. Goldreicht for typing this manuscript.

References


