Growth-dependent subnuclear localization of a 66 kDa phosphoprotein in FER protein overexpressing cells

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Abstract **p94^{fer} and p51^{ferT} are two nuclear tyrosine kinases encoded by the FER locus in the mouse. While p94^{fer} accumulates in somatic cells, p51^{ferT} is found solely in meiotic spermatogenic cells. Ectopic expression of p94^{fer} or p51^{ferT} in CHO cells, led to tyrosine phosphorylation of cellular 66, 68 and 120 kDa proteins. A 120, 68 and 66 kDa phosphoproteins, coimmunoprecipitated with p94^{fer} and p51^{ferT} from extracts of transfected CHO cells. Subcellular fractionation analysis indicated that the 66 kDa tyrosine phosphorylated protein co-localizes with p51^{ferT} to perinuclear and nuclear fractions in actively growing cells. However, in growth arrested cells, the 66 kDa phosphoprotein was associated mainly with chromatin while its level in the other nuclear compartments was significantly reduced. The 66 kDa phosphoprotein may thus mediate the nuclear function of the FER proteins and link it to cell growth.**

Key words: FER; Tyrosine kinase; Nuclear

1. Introduction

The mouse FER locus, originally identified through its homology to v. abl [1], encodes two tyrosine kinases. One, 94 kDa in size (p94^{fer}) is present in most mammalian somatic cell lines and is evolutionarily conserved [2,3]. p94^{fer} can be detected in both cell cytoplasm and chromatin [4]. The second tyrosine kinase, termed p51^{ferT}, is encoded by a testis specific FER transcript (ferT) and exhibits a molecular mass of 51 kDa [5,6]. p51^{ferT} accumulates in spermatocytes residing in prometaphase and metaphase of the first meiotic division [6,7]. Expression of p51^{ferT} in CHO cells leads to its accumulation in the nuclei of the transfected cells [7].

p94^{fer} and p51^{ferT} share an identical SH2 sequence [8,9], and an identical kinase domain that is 70% homologous to the p92$^{fes/fps}$ kinase domain [5,10,11]. The two kinases differ, however, in their N-terminal portions. While the p51^{ferT} N-terminus is made up of 44 unique amino acids [5], that of p94^{fer} consists of 350 amino acids. It adopts a coiled-coil structure which mediates the interaction of that kinase with the catenin-like substrate pp120 [12]. Both enzymes have been partially characterized biochemically, yet their cellular functions, as well as their substrates, remain unknown. The kinase activity of the p94^{fer} cytoplasmic fraction is elevated in growth factor stimulated fibroblastic cell lines [12], thus suggesting the possible involvement of p94^{fer} in cell growth related processes. To further identify potential substrates of the FER proteins, the two tyrosine kinases were overexpressed in CHO cells, under the control of an inducible promoter.

In the present report, it is shown that ectopic expression of p94^{fer} or p51^{ferT} in somatic cell cultures leads to tyrosine phosphorylation of cellular 120, 68 and 66 kDa proteins. The subcellular distribution of the 66 kDa phosphoprotein depends on the growth state of the cells, thus supporting the linkage of the FER proteins to modulation of cell growth.

2. Materials and methods

2.1. Plasmid construction

The pHS1ferT plasmid was constructed as follows: a *Bgl*II-*Tth*III1 ferT cDNA fragment [5] was rendered blunt-ended by extension polymerization using a Klenow fragment at its *Tth*III1 end and was cloned into a *Bgl*II-*Eco*RI restricted pECE plasmid [13], whose *Eco*RI end was blunt-ended as well. That plasmid was termed pECEferT. A *Bam*HI-*Bgl*II fragment containing the ferT cDNA linked to the 3′ non-translated sequences of the early SV40 genes was excised from the pECEferT plasmid. It was then inserted in the *Bam*HI site of a pUC18 plasmid (pHS1) carrying a human metallothionein IIA promoter [14]. In the resultant pHS1ferT plasmid, the ferT cDNA was transcribed under the control of the human metallothionein promoter. The pHS1fer plasmid was constructed by replacing an *Spe*I-*Cla*I fragment in pHS1ferT with an *XBa*I-*Cla*I fragment from the mouse fer cDNA. This led to replacement of the p51^{ferT} N-terminus with the p94^{fer} N-terminus.

2.2. Cell culture and transfection

CHO cells were cultured in F-12 medium, then co-transfected with 1 μg PSV$_2$neo [15] and 10 μg pHS1ferT or pHS1fer plasmids, using the calcium phosphate precipitation technique [16]. G418-resistant clones [15] were further grown, exposed for 18 h to 100 μM $ZnCl_2$ and analyzed for accumulation of p94^{fer} or p51^{ferT}. For FER protein expression in growth arrested cells, cells were grown to confluency. The cells were left in culture for a further 24 h during which they were exposed for 18 h to 100 μM $ZnCl_2$. The accumulation of more than 90% of the cells in the G0/G1 cell cycle stage, was confirmed by exposing the cells to flow cytometry analysis.

2.3. Preparation of cell protein extracts

For total cell protein extracts, cells were lysed using triple detergent lysis buffer as described [17]. Subcellular fractionation extracts were carried out essentially according to Peraness et al. [18] with the following modifications. Pellets of 2×10^7 cells were lysed with 1 ml HEPES buffer [10 mM HEPES (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM PMSF, 0.1 mM Na_3VO_4, 0.1% Nonidet P40 and 10 μg/ml each of Pefabloc SC, leupeptin, aprotinin (Boehringer Mannheim) and benzamidine (Sigma)]. Nuclei were spun down and cytoplasmic supernatants were removed and stored. Perinuclear proteins were extracted from nuclei which were suspended in a buffer containing 10 mM HEPES (pH 7.4), 0.25M sucrose, 2 mM $MgCl_2$, 0.1% Triton X-114, 0.1 mM PMSF, 0.1 mM Na_3VO_4 and 5 μg/ml each of Pefabloc SC, leupeptin, aprotinin and benzamidine. Nucleoplasmic proteins were extracted by washing the nuclei with a buffer containing 10 mM HEPES (pH 7.4), 0.25 M sucrose, 0.5% Nonidet P-40, 0.1 mM Na_3VO_4, 0.1 mM PMSF and 10 μg/ml each of Pefabloc SC, leupeptin, aprotinin and benzamidine. After extracting the nuclei with the same buffer containing 1% Nonidet P-40 and digesting the DNA with micrococcal nuclease, chromatin-associated proteins were extracted with a high salt buffer (3.5 M NaCl, 1% Nonidet P-40, 10 mM EDTA, 0.1 mM Na_3VO_4, 0.1 mM

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PMSF). The nuclear matrix was solubilized by boiling in 1% SDS. The total protein amounts, obtained in the different fractions were as follows: CY1, first cytoplasmic wash, about 2 mg; PN, perinuclear fraction, about 1.5 mg; NP1, nucleoplasmic fraction 1, about 1.25 mg; CH, chromatin fraction, about 1 mg; NM, the nuclear matrix fraction, 0.8 mg. The similar total protein amounts in the prepared fractions enabled us to analyze similar samples (30 μg) from each fraction. These samples therefore represent similar numbers of cells in each fraction.

2.4. Western blot analysis

Cellular protein extracts (30 μg) were resolved in SDS-polyacrylamide (9%) gels. Proteins were electroblotted onto a nitrocellulose membrane and incubated with a monoclonal anti-phosphotyrosine (clone: PT-66) antibody (Biomakor cat. no. 6093) at a 1:10 000 dilution, with C_1 antibody at a 1:5000 dilution or with eIF-4A antibodies at 1:500 dilution. The bands were visualized with the ECL Western blotting (RPN 2106) analysis system (Amersham International).

2.5. Immunoprecipitation and in vitro kinase assays

Extracts for immunoprecipitations and in vitro kinase assays were prepared as follows: Cells were lysed with lysis buffer (10 mM HEPES pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-114, 0.1 mM Na_3VO_4 and 10 μg/ml each of Pefabloc SC, leupeptin, aprotinin and benzamidine), the extracts were spun down and supernatants were brought to 20% glycerol. For immunoprecipitation and kinase assay, 200 μg of protein extracts were resuspended in HNTG buffer (×2) and precipitated with a 1:50 dilution of C_1 or N_1 antibody at 4°C for 3 h. Immunocomplexes were incubated with protein A-Sepharose (Sigma) at 4°C for 2 h. The antibody-protein Sepharose conjugates were washed twice with HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and once with MHNG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$ and 5 mM $MnCl_2$). The conjugates were resuspended in 50 μl MHNG buffer containing 50 mM HEPES pH 7.5 and 3 μl [γ-^{32}P]ATP (3000 Ci/mmol) was added for 30 min at room temperature.

Samples were resolved in SDS-polyacrylamide (9%) gels, electroblotted onto Immobilon (PVDF) membrane and treated for 2 h at 55°C with 1 M KOH as described before [19]. Membranes were exposed to Fuji medical X-ray film.

For Western blot analysis of immunoprecipitated proteins, immunoprecipitates were resolved in 9% SDS-PAGE after two HNTG washes (see above).

3. Results and discussion

3.1. Regulation of cellular protein tyrosine phosphorylation by p94^{fer} and p51^{ferT}

The mouse p94^{fer} and p51^{ferT} cDNAs were linked to the human metallothionein IIA promoter [14] to construct the pHS1fer and pHS1ferT plasmids, respectively. These were stably introduced into CHO cells. Western blot analysis of lysates of clonal transfected cell lines showed that both proteins were identified with antibodies (C_1) raised against a synthetic peptide derived from the last common 15 C-terminal amino acids of p94^{fer} and p51^{ferT} [5]. Fig. 1 demonstrates that both clones expressing p94^{fer} (Fig. 1, lanes 1–4) and those expressing p51^{ferT} (Fig. 1, lanes 5–8) accumulated the exogenous enzymes in a $ZnCl_2$-dependent manner. A similar expression pattern was observed in several other clones which were analyzed (data not shown). No significant level of endogenous p94^{fer} could be detected in non-transfected cells or in uninduced transfected cells (Fig. 1A). This may reflect either low cellular levels of endogenous p94^{fer}, or a lack of cross-reac-

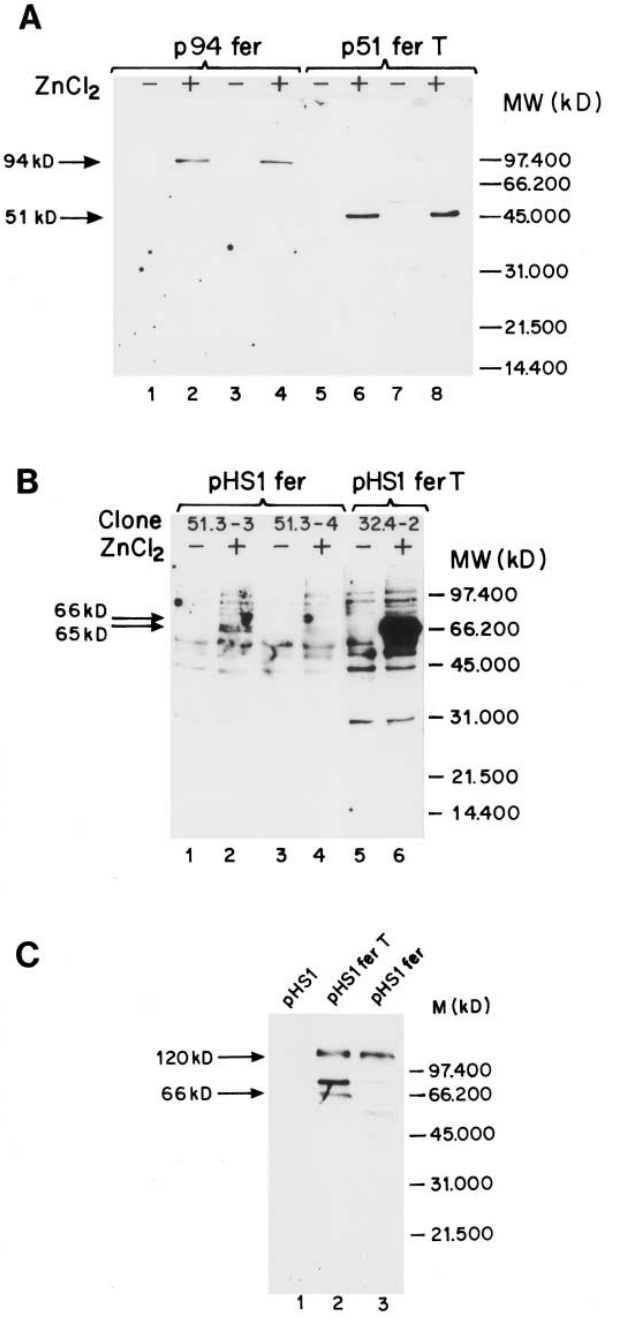

Fig. 1. (A) Exogenous expression of p94^{fer} and p51^{ferT} in transfected CHO cells. 30 μg of whole cell protein extracts prepared from clones 51.3-3 (1,2) and 51.3-4(3,4) which were transfected with pHS1fer (p94^{fer}), or clones 32.4-2(5,6) and 32.4-3(7,8) transfected with pHS1ferT (p51^{ferT}), before (−) and after (+) 100 μM $ZnCl_2$ treatment, were fractionated in a 9% SDS-PAGE. Proteins were electroblotted to a nitrocellulose membrane and incubated with C1 antibodies. Arrows on the left indicate migration distances of p94^{fer} and p51^{ferT}. Migration distances of known molecular weight markers are given on the right. (B) Phosphorylation state of proteins, in extracts untreated with vanadate from pHS1fer and pHS1ferT cells. 30 μg whole cell protein extracts from clones transfected with pHS1fer (51.3-3, 51.3.3-4) or pHS1ferT (32.4-2), before (−) and after (+) 100 μM $ZnCl_2$ treatment, were Western blotted and reacted with monoclonal anti-phosphotyrosine antibodies. Arrows on the left indicate migration distances of 66 and 65 kDa phosphoproteins. Migration distances of known molecular weight markers are given on the right. (C) Phosphorylation state of proteins, in extracts treated with vanadate from pHS1fer and pHS1 transfected cells. Protein extracts described in A were fractionated in a 9% SDS-PAGE, Western blotted and reacted with monoclonal anti-phosphotyrosine antibodies. Arrows indicate migration distances of 66 and 120 kDa phosphoproteins.

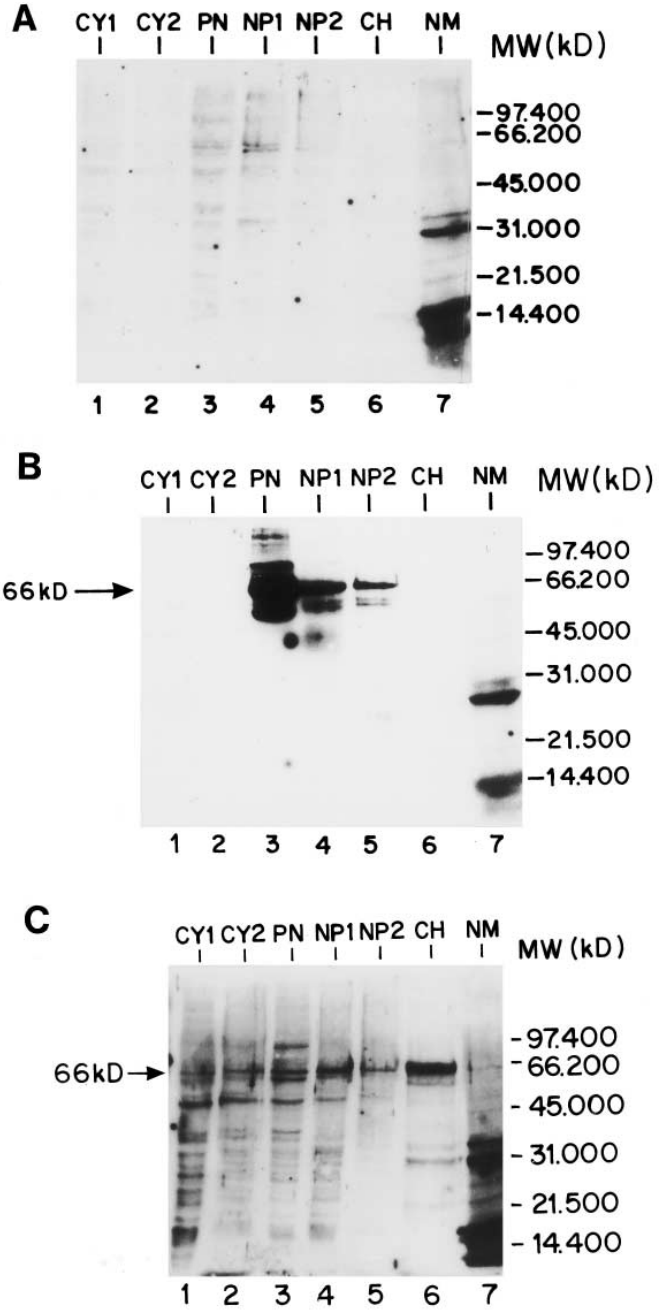

Fig. 2. Subcellular localization of the 66 kDa phosphoprotein. 30 μg proteins from subcellular fractions of non-induced (A), induced (B) actively growing and induced growth arrested (C) 32.4-2 pHS1ferT cells, were blotted and incubated with monoclonal anti-phosphotyrosine antibodies. CY1, cytoplasmic fraction; CY2, cytoplasmic wash; PN, perinuclear fraction; NP1, nuclear plasmic fraction; NP2, second nuclear plasmic wash; CH, chromatin associated proteins; NM, nuclear matrix. Arrow on the left indicates migration distance of the 66 kDa phosphotyrosine protein. Migration distances of known molecular weight markers are given on the right.

tivity between the endogenous hamster $p94^{fer}$ and the antibodies used in this work.

The effects of $p94^{fer}$ and $p51^{ferT}$ overexpression, on the tyrosine phosphorylation pattern of cellular proteins, were studied using monoclonal anti-phosphotyrosine antibodies. Whole cell protein extracts were prepared with buffer lacking vanadate, from clones transfected with $p94^{fer}$ or $p51^{ferT}$ cDNA before and after $ZnCl_2$ treatment. The proteins were separated on 9% SDS-PAGE, Western blotted and exposed to anti-phosphotyrosine antibodies (Fig. 1B). A 66 kDa protein became tyrosine phosphorylated in the clones expressing $p51^{ferT}$, upon exposing the cells to $ZnCl_2$ (Fig. 1B, lanes 5,6). No such effects could be detected in the parental non-transfected cells, in cells expressing exogenous mutant $p51^{ferT}$ deleted of its kinase domain (data not shown) or in cells transfected with the pHS1 plasmid lacking any FER cDNA (Fig. 1C, lane 1).

In clones expressing $p94^{fer}$, two proteins of similar molecular mass (~66 kDa) underwent tyrosine phosphorylation concomitantly with the accumulation of $p94^{fer}$ in these cells (Fig. 1B, lanes 1–4). These phosphotyrosyl levels were significantly lower than those of the 66 kDa band, detected in extracts prepared from clones expressing $p51^{ferT}$ (Fig. 1B, lanes

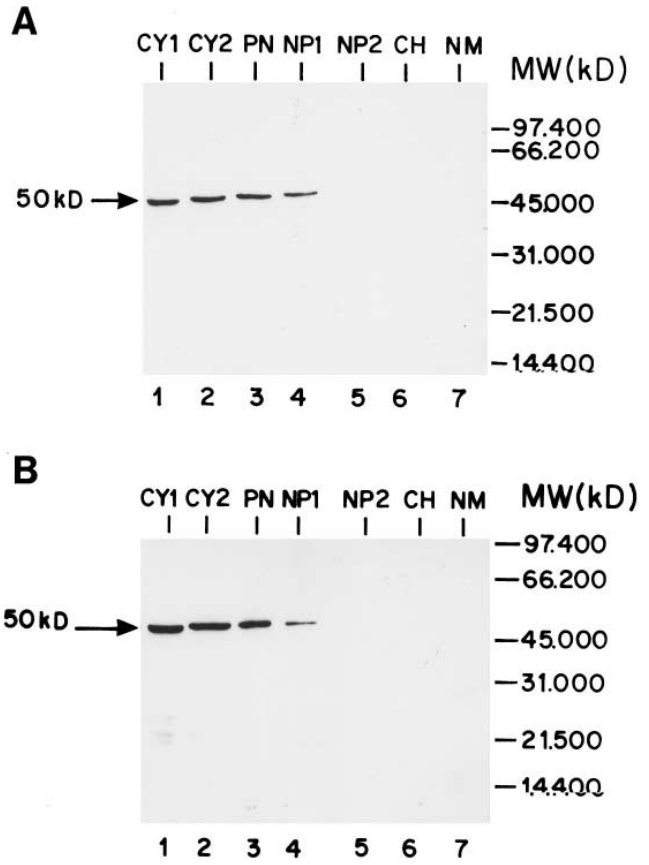

Fig. 3. Subcellular localization of eIF-4A in non-induced (A) and induced (B) 32.4-2 pHS1ferT cells. Proteins from the same fractions as described in Fig. 2 were Western blotted and reacted with eIF-4A antibodies. Arrow on the left indicates migration distance of eIF-4A.

5,6). This phenomenon can be clearly seen in clones 51.3-3 and 32.4-2 which expressed comparable levels of $p94^{fer}$ and $p51^{ferT}$, respectively, but exhibited significantly different levels of phosphotyrosine, associated with the 66 kDa protein (Fig. 1B). Inclusion of vanadate to suppress phosphatases in protein extraction buffer did not change the relative expression levels of $p94^{fer}$ and $p51^{ferT}$ (data not shown) but led to tyrosine phosphorylation of additional 68 and 120 kDa proteins (Fig. 1C). The exact migration distance of the 68 kDa phosphoprotein varied in some experiments, a fact which may reflect several potential phosphorylation levels of that protein. Unlike the 66 and 68 kDa proteins, the tyrosine phosphorylation levels of the 120 kDa protein were similar in clones expressing $p51^{ferT}$ or $p94^{fer}$ (Fig. 1C, lanes 2,3). Although $p94^{fer}$ and $p51^{ferT}$ seemed to be active in the G418-resistant clones, the proteins were not significantly autophosphorylated in that system. No obvious tyrosine phosphorylated proteins corresponding in size to either $p94^{fer}$ or $p51^{ferT}$ could be detected in the transfected $ZnCl_2$-treated clones (Fig. 1B,C).

3.2. Subcellular localization of the 66 kDa phosphoprotein

It has previously been reported that $p51^{ferT}$ and approx. 50% of the cellular $p94^{fer}$ molecules are located in the cell nucleus [4,7]. We therefore sought to characterize the subcellular localization of the 120, 66 and 68 kDa phosphoproteins and to determine whether they co-localize with $p94^{fer}$ or $p51^{ferT}$. Clones expressing $p94^{fer}$ and $p51^{ferT}$ were lysed with vanadate-containing buffer and were biochemically fractionated to subcellular constituents. Proteins extracted from each fraction were Western blotted and exposed to anti-phosphotyrosine antibodies. The 120 and 68 kDa phosphoproteins could not be reproducibly detected in the prepared fractions. This may reflect the high sensitivity of these phosphoproteins to cellular dephosphorylation activities (Fig. 1) Detectable levels of the 66 kDa phosphoprotein were obtained in fractions prepared from induced $p51^{ferT}$ expressing clones only (Fig. 2). The 66 kDa phosphoprotein was not detected in fractions prepared from $p94^{fer}$ expressing clones (data not shown). This could result from the relatively low phosphorylation levels of the 66 kDa phosphoprotein or low concentrations of the 66 kDa protein in these cells. In clones expressing $p51^{ferT}$, the 66 kDa phosphoprotein associated mainly with the cell perinuclear and nuclear cytosolic fractions (Fig. 2B). However, it was not detected in other nuclear compartments like chromatin and nuclear matrix (Fig. 2B, lanes 67). Probing the same fractions with antibodies to $p51^{ferT}$, revealed an identical subcellular distribution of $p51^{ferT}$ and the 66 kDa phosphoprotein in transfected cells (data not shown). This coincides with previous subcellular localization experiments of $p51^{ferT}$, in transfected CHO cells [7]. The presence of the 66 kDa protein in the perinuclear fraction may reflect its association with the nuclear or other cellular membranes. Alternatively, it may reflect leakage of the 66 kDa phosphoprotein from the intranuclear environment, due to detergent inclusion in the extraction procedure adopted in these experiments (see also Section 2). The subcellular localization of the 66 kDa phosphoprotein differed, however, from the distribution profile of eIF-4A, a soluble cytoplasmic translation initiation factor [20], which is equally present in the cytoplasmic and perinuclear fractions in induced and non-induced cells (Fig. 3A,B). The absence of the 66 kDa phosphoprotein from the soluble cytoplasmic fractions indicates that it is associated mainly with either the nuclear cytosol or cellular membranes. An additional 60 kDa phosphoprotein was detected in perinuclear and nuclear fractions of $p51^{ferT}$ expressing clones (Fig. 2B). This could reflect degradation or partial dephosphorylation of the 66 kDa phosphoprotein. The 66 kDa phosphoprotein was not detected in non-induced clones (Fig. 2A) or in CHO cells transfected with pHS1 plasmid lacking a FER cDNA (data not shown). The cellular levels of $p94^{fer}$ do not change along the cell division cycle of fibroblastic cells (Bern and Nir, unpublished data). The enzyme is, however, activated in growth factor stimulated fibroblasts [11]. The functioning of the FER pathway constituents may thus vary in growing and quiescent cells. The relative phosphotyrosyl levels and the subcellular distribution profile of the 66 kDa phosphoprotein were therefore analyzed in growth arrested quiescent cells. The induced production of $p51^{ferT}$ in confluent growth arrested cells led to significant reduction in the 66 kDa phosphotyrosyl levels in the perinuclear and nuclear fractions (Fig. 2C). Unlike in growing cells, however, a 66 kDa phosphoprotein was clearly detected in the chromatin fraction of the $p51^{ferT}$ expressing cells (Fig. 2C). That phosphoprotein was not detected in non-induced confluent cells (data not shown), thus linking its appearance to the activity of $p51^{ferT}$. The subnuclear localization of $p51^{ferT}$ and the subcellular distribution of eIF-4A did not change in growth arrested cells (data not shown). The different subnuclear localization of a 66 kDa phosphoprotein in growing and

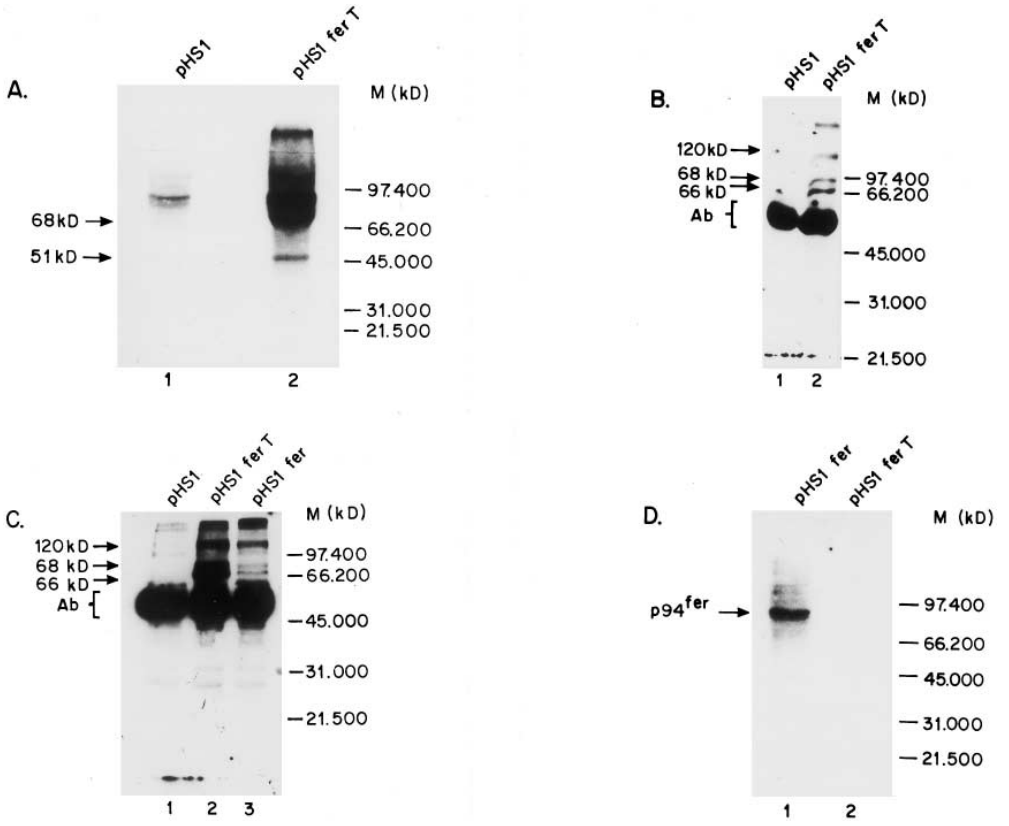

Fig. 4. Coimmunoprecipitation of cellular phosphoproteins with $p94^{fer}$ and $p51^{ferT}$. (A) Whole cell proteins from neo resistant CHO cells (PHS1) and CHO cells expressing $p51^{ferT}$ (PHS1ferT) were immunoprecipitated with N1 antibodies. Precipitated proteins were exposed to in vitro kinase assay in the presence of [γ-^{32}P]ATP. Phosphorylated proteins were resolved on 9% SDS-PAGE, transferred to PVDF (immobilon) membrane which was then treated for 2 h at 55°C with 1 M KOH. Membranes were exposed to Fuji X-ray medical film. Arrows indicate migration distances of $p51^{ferT}$ and its putative 68 kDa substrate. (B) Whole cell proteins from PHS1 (lane 1) and mouse $p51^{ferT}$ expressing cells (lane 2), were immunoprecipitated with N1 antibodies. Precipitated proteins were Western blotted and reacted with monoclonal anti-phosphotyrosine antibodies. Arrows indicate migration distances of the 66, 68 and 120 kDa phosphoproteins. Ab denotes precipitating antibodies which react with the peroxidase conjugated protein A. (C) Whole cell proteins from PHS1 (lane 1), mouse $p51^{ferT}$ expressing cells (lane 2), and mouse $p94^{fer}$ expressing cells (lane 3) were immunoprecipitated with C1 antibodies. Precipitated proteins were Western blotted and reacted with monoclonal anti-phosphotyrosine antibodies. Arrows denote calculated molecular weights of precipitated proteins. Ab denotes the precipitating antibodies which react with the peroxidase conjugated protein A. (D) Whole cell proteins from CHO cells expressing the mouse $p94^{fer}$ (lane 1) or $p51^{ferT}$ (lane 2) were immunoprecipitated with antiserum E. Precipitated antibodies were exposed to in vitro kinase assay in the presence of [γ-^{32}P]ATP. Phosphorylated products were resolved on 9% SDS-PAGE and transferred to PVDF membrane. The membrane was treated with 1 M KOH, at 55°C for 2 h, dried and exposed to Fuji medical X-ray film. Arrow on the left indicates migration distance of the mouse $p94^{fer}$.

quiescent cells which express $p51^{ferT}$ suggests the possible involvement of the FER proteins in the modulation of nuclear processes, related to cell growth. This may also apply to meiotic cells which endogenously express $p51^{ferT}$ [7], and in which some somatic nuclear processes like DNA replication are suppressed [21]. Indeed, induced expression of $p51^{ferT}$ in CHO cells elongated their S phase and consequently slowed down the division rate of these cells (data not shown).

3.3. $p94^{fer}$ and $p51^{ferT}$ associate with tyrosine phosphorylated 66, 68 and 120 kDa proteins in growing cells

The subcellular co-localization of $p51^{ferT}$ and the 66 kDa phosphoprotein in growing cells suggests that these two proteins associate in vivo. To check this possibility, the coimmunoprecipitation of tyrosine phosphorylated cellular proteins with the FER enzymes was tested. The in vitro phosphorylation pattern of cellular proteins, coimmunoprecipitated with $p94^{fer}$ and $p51^{ferT}$, was studied as well. C_1 antibodies raised against the C-terminus of $p94^{fer}$ and $p51^{ferT}$, immunoprecipitated these enzymes but interfered with their in vitro autophosphorylation activity (data not shown). This coincides with previous results which indicated that antiserum G, raised against the C-terminus of $p94^{fer}$, did not allow autophosphorylation of the immunoprecipitated enzyme [3]. The interference of C_1 antibodies with the autophosphorylation of the FER proteins suggests the possible localization of the tyrosine autophosphorylation acceptor site to the C-terminus of these proteins. A similar localization of the autophosphorylation acceptor site was established in $p93^{c-fes}$ [22], which is closely related to the FER proteins. Two other antisera raised against $p94^{fer}$ and $p51^{ferT}$ were therefore used for the immunocomplex kinase assays. N_1 antibodies, raised against a synthetic peptide (Glu^{11}–Lys^{24}) derived from the unique N-terminus of $p51^{ferT}$ [7], were used to immunoprecipitate this enzyme from extracts of $p51^{ferT}$ expressing cells. These antibodies do not interact with $p94^{fer}$ [7]. An in vitro kinase assay carried out with the immunoprecipitated proteins led to tyrosine phosphorylation of a 51 kDa protein, and an additional protein of approx. 68 kDa. The relatively broad 68 kDa band may reflect, as mentioned above, several phosphorylation levels of that protein (Fig. 4A, lane 2). The 51 and 68 kDa bands were not detected in precipitates from pHS1 cell (harboring

the parental SVneo gene containing plasmid) extracts (Fig. 4A, lane 1) indicating that the 51 kDa protein is p51^{ferT} and the 68 kDa protein represents its putative, associated substrate.

Immunoprecipitation of p94^{fer} with antiserum E [3] and analysis of its activity in an in vitro kinase assay led to autophosphorylation of that enzyme (Fig. 4D, lane 1). No additional cellular protein with an apparent different molecular weight was phosphorylated by this enzyme in an in vitro kinase assay. This coincides with previous results obtained in in vitro kinase assays carried out with antiserum E precipitates [3]. Since antiserum E was raised against the N-terminal half of the FER protein's SH2 domain [3], it may interfere with the interaction of these enzymes and their putative substrates. The autophosphorylation activity of p51^{ferT} is much weaker than that of p94^{fer} and was barely detected in the immunokinase assay (Fig. 4D, lane 2). Longer exposure of the dried gel revealed weak autophosphorylation activity of p51^{ferT} in Fig. 4D, lane 2 (data not shown).

To verify the association of the 66 kDa phosphotyrosyl protein with p51^{ferT} in vivo, cellular proteins were immunoprecipitated with N_1 antibodies from pHS1 and p51^{ferT} expressing cells. The precipitated proteins were fractionated in SDS-PAGE, Western blotted, and reacted with monoclonal anti-phosphotyrosine antibodies. In Fig. 4B, it can be seen that 66, 68 and 120 kDa tyrosine phosphorylated proteins coimmunoprecipitated with p51^{ferT}. These proteins were not detected in immunoprecipitates from pHS1 cells. The C_1 antibodies, raised against the common C-terminus of p94^{fer} and p51^{ferT}, coimmunoprecipitated these enzymes with 66, 68 and 120 kDa tyrosine phosphorylated proteins as well (Fig. 4C). While the 120 kDa phosphotyrosyl band appeared in similar levels in p94^{fer} and p51^{ferT} precipitates (Fig. 4C, lanes 2,3), the 66 and 68 kDa phosphotyrosyl bands appeared in much higher levels in p51^{ferT} precipitates as compared with p94^{fer} precipitates. The 66 and 68 kDa bands may represent two different proteins or alternatively two phosphorylation levels of the same protein. The coimmunoprecipitation of a 66 kDa protein and p51^{ferT}, by antibodies directed toward either the N- or C-terminus of this enzyme, strongly suggests the association of p51^{ferT} and a 66 kDa tyrosine phosphorylated protein, in vivo. Stable in vivo association of the cytoplasmic src [23,24] and the nuclear c-abl [25] with their putative substrates in vivo has been shown before. This association may play a role in modulating the turnover activity of cytoplasmic and nuclear tyrosine kinases.

The subcellular distribution of the 66 kDa phosphoprotein in growing cells is identical to the subcellular localization of the human GAP-associated p62 protein [26]. In addition, the stable association of the 66 kDa protein with p51^{ferT} in vivo resembles the tight association of the p62 related protein (p68) with c-src in vivo [23,24]. Commercially available (Santa Cruz Biotechnology) anti- p62 antibodies were used for Western blot analysis, and failed to detect any protein in p51^{ferT} precipitates (data not shown), suggesting that the 66 kDa phosphoprotein differs from the previously cloned p62 and p68 proteins [23,24,26]. Shc antibodies (Santa Cruz Biotechnology) also failed detect any protein in the p51^{ferT} precipitates (data not shown).

The catenin-like substrate pp120 was previously shown to associate in vivo with p94^{fer} [11]. Since pp120 interacts with p94^{fer} through the unique N-terminus of the enzyme, the 120 kDa phosphoprotein associated with p51^{ferT}, seems to be of another identity. Antibodies (Santa Cruz Biotechnology) directed against other known 120 kDa tyrosine phosphorylated proteins, like ras GTPase-activating protein (GAP) and FAK, failed to detect any protein in the p51^{ferT} immunoprecipitates (data not shown). Further characterization of the presently described 66, 68 and 120 kDa phosphoproteins should offer new tools for elucidating the nuclear functions of the FER proteins.

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References

[1] Hao Q.-L., Heisterkamp N. and Groffen J. (1989) Mol. Cell. Biol. 9, 1587–1593.
[2] Letwin K., Yee S.-P. and Pawson T. (1988) Oncogene 3, 621–627.
[3] Pawson T., Letwin K., Lee T., Hao Q.-L., Heisterkamp N. and Groffen J. (1989) Mol. Cell. Biol. 9, 5722–5725.
[4] Hao Q.-L., Ferris D.K., White G., Heisterkamp N. and Groffen J. (1991) Mol. Cell. Biol. 11, 1180–1183.
[5] Fischman K., Edman J.C., Shakleford G.M., Turner J.A., Rutter W.J. and Nir U. (1990) Mol. Cell. Biol. 10, 146–153.
[6] Keshet E., Itin A., Fischman K. and Nir U. (1990) Mol. Cell. Biol. 10, 5021–5025.
[7] Hazan B., Bern O., Carmel M., Lejbkowicz F., Goldstein R.S. and Nir U. (1993) Cell Growth Differ. 4, 443–449.
[8] De Clue J.E., Sadowski I., Martin G.S. and Pawson T. (1987) Proc. Natl. Acad. Sci. USA 84, 9064–9068.
[9] Sadowski I., Stone J.C. and Pawson T. (1986) Mol. Cell. Biol. 6, 4396–4408.
[10] Roebroe A.J.M., Schalken J.A., Verbeek J.S., Van der Ouweland A.M.W., Onnekink C., Bloemers H.P.J. and Van de Ven W.J.M. (1985) EMBO J. 4, 2897–2903.
[11] Wilks A.F. and Kurban R.R. (1988) Oncogene 3, 289–294.
[12] Kim L. and Wang T.W. (1995) Mol. Cell. Biol. 15, 4553–4561.
[13] Ellis L., Clauser E., Morgan D.O., Edery M., Roth R.A. and Rutter W.J. (1986) Cell 45, 721–732.
[14] Karin M., Hoslinger A., Heguy A., Dietin T. and Cooke T. (1987) Mol. Cell. Biol. 7, 606–613.
[15] Southern P.J. and Berg P. (1982) J. Mol. Appl. Genet. 1, 327–341.
[16] Graham F. and Van der Eb A.A. (1973) Virology 52, 456–457.
[17] Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY.
[18] Peraness J., Liljestron P. and Kaariainen L. (1990) J. Virol. 64, 1888–1896.
[19] Kamps M.P. and Sefton B.M. (1989) Anal. Biochem. 176, 22–27.
[20] Lejbkowicz F., Joyer C., Darveau A., Meron S., Lemieux R. and Sonenberg N.P. (1992) Proc. Natl. Acad. Sci. USA 89, 9612–9616.
[21] Bellve A.R. (1979) in: Oxford Reviews of Reproductive Biology (Finn, C.A. ed.) pp. 159–261, Clarendon Press, Oxford.
[22] Hjermstad S.J., Briggs S.D. and Smithgall T.E. (1993) Biochemistry 32, 10519–10525.
[23] Fumagalli S., Edman J.C., Shakleford G.M., Turner J.A., Rutter W.J. and Nir U. (1994) Nature 368, 871–874.
[24] Taylor S.J. and Shalloway D. (1994) Nature 368, 867–871.
[25] Ren R., Ye Z.S. and Baltimore D. (1994) Genes Dev. 8, 783–795.
[26] Wong G., Muller O., Clark R., Coroy L., Moron M.F., Polakis P. and McCormick F. (1992) Cell 69, 551–558.