Nuclear Localization of the PEP Protein Tyrosine Phosphatase

EDWIN FLORES, GARBIÑE ROY, DHEERESH PATEL, ANDREY SHAW, AND MATTHEW L. THOMAS*

*Howard Hughes Medical Institute, Departments of Pathology and Molecular Microbiology,
Washington University School of Medicine, St. Louis, Missouri 63110*

Received 12 January 1994/Returned for modification 22 February 1994/Accepted 1 April 1994

PEP is an intracellular protein tyrosine phosphatase expressed primarily by cells of hematopoietic origin that can be divided structurally into a catalytic domain and a large carboxy-terminal domain. The carboxy-terminal domain is enriched in proline, glutamic acid, serine, and threonine residues (PEST sequences) and contains a nonperfect tandem repeat sequence enriched in proline residues and a carboxy terminus enriched in basic amino acids. Here we show that PEP is diffusely expressed in lymphoid tissues, consistent with expression by many different cell types. Analysis of the PEP protein identifies a nuclear localization sequence within the extreme carboxy terminus. Transfer of 18 amino acids from the carboxy terminus of PEP to β -galactosidase conferred nuclear localization, indicating that this sequence was sufficient for nuclear localization. Proteins enriched in PEST sequences are often rapidly degraded. However, pulse-chase analysis indicates that PEP has a half-life of greater than 5 h.

The protein tyrosine phosphatases (PTPases) are a large family of enzymes important in regulating differentiation and cellular activation and can be divided into intracellular and transmembrane forms (1, 7, 34). Although a large number of novel PTPases have been identified, the functions of most are unknown.

We have identified a PTPase, PEP (PEST-enriched phosphatase), expressed primarily in hematopoietic tissues that contains sequences enriched in proline, glutamic acid, serine, and threonine residues (PEST sequences) (16). The presence of PEST sequences in proteins has been correlated with rapid protein degradation (23). PEST sequences are found in many nuclear proteins such as c-Myc and c-Fos, raising the possibility that PEP may be a nuclear PTPase. Indeed, PEP has several putative nuclear localization sequences (16).

A role for tyrosine phosphorylation in regulating nuclear events is suggested by the presence of nuclear protein tyrosine kinases, such as FER and the protein product of the proto-oncogene *c-abl* (11, 32). Furthermore, some transcriptional activating proteins are tyrosine phosphorylated. Ligand binding of alpha, beta, or gamma interferons with their receptors results in the activation of a protein tyrosine kinase and the phosphorylation of transcriptional activating proteins (8, 26, 27, 33). The tyrosine phosphorylation of the transcriptional activating proteins correlates with nuclear translocation, DNA binding, and transcriptional activation (13, 15, 25, 28, 29). This suggests that there may be a nuclear PTPase important in the dephosphorylation of these proteins, and recently a nuclear PTPase that may function to dephosphorylate interferon-induced transcription factors has been reported (4).

There are relatively few known PTPases that have been shown to localize to the nucleus. The cell cycle-regulating threonine and tyrosine phosphatase encoded by the *cdc25* gene localizes to the nucleus and serves to illustrate the importance of nuclear tyrosine dephosphorylation (19). PAC-1, a PTPase with sequence similarity to the dual specificity phosphatase that regulates mitogen-activated protein kinase, MPK-1, is

induced after activation of T cells with the mitogenic lectin phytohemagglutinin and localizes to the nucleus (24). Finally, a *Drosophila melanogaster* PTPase of unknown function, dPTP61F, exists in either of two isoforms that are derived by alternative splicing (17). One isoform is located in the nucleus, while the other isoform is found in the cytoplasm.

Here we further characterize the PEP PTPase. PEP was found to be expressed diffusely in lymphoid tissues, and despite the presence of PEST sequences, PEP is not rapidly degraded. Transient transfection of PEP cDNA results in nuclear localization of the protein, and PEP contains a bona fide nuclear localization sequence at the extreme carboxy terminus. These data suggest that PEP serves a function in regulating nuclear tyrosine phosphorylation.

MATERIALS AND METHODS

In situ hybridization analysis of PEP. Tissues from adult animals were removed and fixed by immersion in 4% paraformaldehyde–0.1 M Na₂HPO₄, pH 7.4. Following fixation, tissues were equilibrated overnight in 30% sucrose at 4°C and embedded in OCT medium (Miles Laboratories). Six-micrometer cryostat sections were mounted on RNase-free precoated glass slides. Sense and antisense single-stranded [³⁵S]RNA probes (380 bp) encoding the PTPase domain (residues 112 to 232) were synthesized by *in vitro* transcription to a specific activity of 7×10^8 cpm/ μ g by following the manufacturer's instructions (Promega) and using either the T3 or the T7 promoter. Tissue sections were incubated with the radiolabeled probes for 16 h at 55°C in a solution of 10 mM Tris (pH 7.5), 300 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 10% dextran sulfate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 500 μ g of yeast tRNA per ml, and 2×10^6 to 5×10^6 cpm of [³⁵S]cRNA per slide. Slides were washed in a solution of 50% formamide, 0.3 M NaCl, 0.1 M dithiothreitol, and 30 mM sodium citrate at 65°C for 30 min, incubated with 50 μ g of RNase A per ml at 37°C for 30 min, washed again in the formamide buffer, coated with NTB2 photographic emulsion (Kodak), and exposed for 10 to 15 days at 4°C. After being developed with D19 developer, sections were counter stained with hematoxylin-eosin. For all antisense hybridizations, duplicate histological sections were examined by hybridization with a sense probe. These sections exhibited greatly reduced levels

* Corresponding author. Mailing address: Department of Pathology, Box 8118, Washington University Medical School, St. Louis, MO 63110. Phone: (314) 362-8722. Fax: (314) 362-8888. Electronic mail address: mthomas@telesphere.wustl.edu.

FIG. 1. In situ hybridization of PEP expression in lymphoid tissues. (A) Bright-field photomicrograph of hematoxylin-eosin-stained thymus section. (B) Dark-field photomicrograph of a section adjacent to that in panel A hybridized with an antisense [^{35}S]cRNA PEP probe (nucleotides 449 to 801). (C) Bright-field photomicrograph of hematoxylin-eosin-stained spleen section. (D) Dark-field photomicrograph of a section adjacent to that in panel C hybridized with an antisense [^{35}S]cRNA PEP probe (nucleotides 449 to 801). m, medulla; c, cortex; f, follicle. Bar, 0.3 mm.

of hybridization and displayed no specific topological distribution.

Nuclear localization of PEP in HeLa cells. The full-length cDNA for PEP was appended at the 3' end with sequences encoding the c-Myc epitope tag SMEQKLISEEDLN by PCR (6). The modified PEP cDNA was subcloned in Bluescript SK (Stratagene) behind the T7 promoter and verified by sequence analysis. The PEP 5' untranslated region was deleted and replaced by a consensus translational initiation site (GCCAC CATGG) (14).

Deletion constructs used to determine nuclear localization sequences were generated by PCR under the conditions of 94°C for 15 s, 52°C for 15 s, and 72°C for 30 s to 1 min for 30 cycles with a GeneAmp 9600 temperature cycler (Perkin-Elmer Cetus). Deletion or mutation of the sequence encoding amino acids 446 to 451 was made by inverse PCR with Vent polymerase (New England Biolabs). PEP Δ 446-451, PEP R/K446-451T, PEP 1-306, PEP 330-802, PEP 330-802 Δ 446-451, PEP 330-802 R/K446-451T, PEP 470-802, PEP 520-730, PEP 730-802, PEP 1-730, PEP 1-520, and PEP 330-788 (see Fig. 2) were constructed by PCR with a 5' oligonucleotide containing an *Eco*RI site adjacent to the coding sequences complementary to PEP and a 3'-end oligonucleotide containing a *Bam*HI site following the antisense coding sequences. The PCR-generated cDNAs were cloned directly into a Bluescript SK vector containing the c-myc-derived epitope tag. All

constructs used were analyzed for expression and correct size by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

HeLa cells (5×10^5) were plated in six-well plates on sterile coverslips in Dulbecco's modified Eagle medium (DMEM) (GIBCO/BRL) with 10% bovine calf serum 12 h prior to infection. Cells were washed twice with serum-free media and infected with recombinant vaccinia virus encoding the T7 RNA polymerase (VTF-7) at a multiplicity of infection of 10 in a volume of 0.5 ml of DMEM at 37°C for 1 h (9). Following infection, cells were transfected with plasmid DNA (5 μg) by using LipofectAce (15 μl) (GIBCO/BRL) in DMEM (31). Six hours posttransfection, cells were fixed for 20 min in 2% paraformaldehyde-phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100-PBS, and washed three times with PBS containing 10 mM glycine. Coverslips were incubated with anti-c-Myc monoclonal antibody 1-9E10 (ATCC CRL 1729 [6]) (1:1000 in PBS) at room temperature for 30 min. After being washed three times with PBS, coverslips were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (United States Biochemical) (1:100 in PBS) and incubated for 20 min. Coverslips were washed three times and then inverted onto 15 μl of a buffer containing 10 mM Tris (pH 8.8), 0.1 M propyl gallate (Sigma), and 90% glycerol. Immunofluorescence microscopy was performed with an inverted Nikon fluorescence microscope, and photographs

FIG. 2. Schematic diagram of PEP constructs indicating corresponding subcellular localization. The amino acid residue numbers corresponding to sequence from full-length PEP, mutations, and/or deletions are shown to the left. The site of the internal deletion is indicated with a $\wedge$. The different domains of PEP are indicated by shading and/or labels above the schematic diagram. The c-Myc-derived epitope tag is shown by the cross-hatch at the carboxy terminus. The potential nuclear localization sequence containing residues KRTK at position 448 to 451 is indicated above the schematic. Constructs with arginine or lysines mutated to threonines are indicated to the left and above the schematic. Subcellular localization is indicated by a + in the appropriate column.

were taken with 400 ASA Ektachrome film. Confocal microscopy was performed with a Zeiss Autophot microscope with a Bio-Rad MRC-300 argon laser.

Subcellular localization of PEP- β -galactosidase chimeric proteins. The mammalian transient-expression vector pCH110 encoding β -galactosidase (Pharmacia) was used to express chimeric proteins containing the nuclear localization signal of PEP in HeLa or COS-7 cells. PCR-generated fragments or complementary oligonucleotides encoding the indicated residues were cloned in frame into the unique *Kpn*I site of the β -galactosidase gene of pCH110 (see Fig. 6D). As a positive control for nuclear localization, the sequence encoding the simian virus 40 large T antigen, PPKKKRKVEDPV (10), was cloned into pCH110 by using two oligonucleotides with *Kpn*I complementary ends.

HeLa or COS-7 cells (5×10^5) were transfected with plasmid DNA (10 μ g) by using calcium phosphate. Alternatively, the cells were transfected with LipofectAmine (GIBCO/BRL). Briefly, 1 μ g of plasmid DNA was mixed with 100 μ l of Opti-MEM (GIBCO/BRL) containing 6 μ l of LipofectAmine, gently mixed, and allowed to stand 15 to 45 min at room temperature. The solution was diluted with 1 ml of Opti-MEM and gently added to prewashed cells in serum-free, antibiotic-

free Opti-MEM. Cells were incubated overnight at 37°C, 1 ml of growth medium containing 20% serum was added, and cells were grown again overnight. Cells were fixed 48 h later with 2% paraformaldehyde in PBS, pH 6.8, for 5 min. β -Galactosidase localization was detected in cells by incubating at 37°C for 4 h in a solution of 0.1% 5-bromo-4-chloro-3-indolyl- β -galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 5 mM $MgCl_2$.

Pulse-chase analysis of PEP. 70Z/3 pre-B cells, S194 cells, and S194 cells stably transfected with PEP cDNA containing the 3' encoded, c-myc-derived epitope tag were washed twice with cold Hanks' balanced salt solution, resuspended in prewarmed methionine- and cysteine-free DMEM (GIBCO/BRL), and incubated for 1.5 h at 37°C. Cells (6×10^7 to 12×10^7) were pulsed with 900 μ Ci of [^{35} S]methionine/cysteine for 20 min at 37°C, washed in medium, and resuspended in prewarmed medium with serum. At indicated times, 10^6 cells were removed, pelleted in a microcentrifuge at 4°C, and lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. The supernatants were precleared by incubation with 40 μ l of Pansorbin (Calbiochem) for 10 min on ice and then

FIG. 3. Nuclear localization of PEP. Indirect immunofluorescence staining of HeLa cells expressing either full-length PEP epitope tagged with a *c-myc*-derived sequence (magnification, $\times 63$) (A) or expressing Fyn modified with the same epitope tag (magnification, $\times 40$) (B). HeLa cells were infected with vaccinia virus containing the T7 RNA polymerase gene. Cells transfected with cDNAs behind the T7 promoter were fixed and stained 6 h posttransfection. The subcellular localization of the expressed proteins was detected by indirect immunofluorescence with anti-*c-myc* monoclonal antibody 9E10 and FITC-conjugated goat anti-mouse antibodies.

centrifugation at 10,000 rpm at 4°C for 10 min. For immunoprecipitation from the 70Z/3 cells, 2 μ l of affinity-purified anti-PEP antibodies or preimmune serum was added to lysates and tumbled with protein A-Sepharose (Sigma). For immunoprecipitation from the transfected and untransfected S194 cells, 1 liter of anti-*c-Myc* monoclonal antibody ascites was added, and the suspension was tumbled for 1 h at 4°C. Thirty microliters of a 50% slurry of protein G-Sepharose was added, and the mixture was rotated for 30 min. The protein G- and protein A-Sepharose complexes were washed twice with RIPA buffer, twice with RIPA buffer containing 0.5 M NaCl, and twice with RIPA buffer before being boiled in SDS-PAGE sample buffer. The proteins were resolved on SDS-8% polyacrylamide gels.

RESULTS

In situ hybridization of PEP mRNA. We have previously shown by Northern (RNA) blot analysis that PEP is primarily expressed in cells of hematopoietic origin (16). A single transcript of 2.8 kb was detected in the thymus, spleen, bone marrow, and lymph nodes. Furthermore, PEP is expressed by cells of either myeloid or lymphoid lineages (16). To examine the pattern of PEP expression within tissues, in situ hybridization analysis was performed. Sense and antisense RNA probes were hybridized to a variety of mouse tissue sections. The thymus and spleen exhibited significant hybridization with the PEP antisense probe (Fig. 1). Interestingly, the antisense probe hybridized diffusely throughout the thymus and did not hybrid-

FIG. 4. The nuclear localization of PEP is directed by the carboxy terminus. Confocal microscopy of HeLa cells expressing PEP mutated by deletion of various regions was done. All PEP polypeptides are modified by the addition of a carboxy-terminal epitope tag derived from *c-myc*. (A) Cells expressing PEP 1-306 encompassing the PTPase domain of PEP; (B) cells expressing PEP 330-802 encompassing the carboxy terminus of PEP; (C) cells expressing PEP 730-802 encompassing the carboxy-terminal 72 amino acids; (D) cells expressing PEP 330-788, encompassing the carboxy-terminal domain with the last 14 amino acids deleted. The subcellular localization of the expressed proteins was detected by indirect immunofluorescence with the monoclonal antibody 9E10 and FITC-conjugated goat anti-mouse antibodies. Bar, 10 μ m.

ize more strongly to either the medulla or the cortex (Fig. 1B). Similarly, diffuse hybridization was detected in the spleen, with neither the white pulp nor the red pulp demonstrating stronger hybridization (Fig. 1D). Similar results were obtained with hybridization of lymph node tissue (data not shown). Low-level hybridization was also detected in the kidney and brain, and expression in these tissues was verified by Northern blot analysis (data not shown). This pattern of expression is consistent with PEP being expressed by many different cell types within lymphoid tissues.

Nuclear localization of PEP. Many proteins containing PEST sequences localize to the nucleus (23). PEP contains several clusters of basic residues that are potential nuclear localization sequences. To determine the intracellular localization, PEP was epitope tagged at the carboxy terminus with a short sequence derived from *c-Myc*, and the cDNA was transfected into HeLa cells. The epitope tag allowed us to monitor the subcellular localization of a series of mutant PEP proteins by using the same monoclonal antibody. A series of deletions and/or mutations was generated to identify subcellular localization sequences (Fig. 2).

To obtain high levels of transient expression, the T7 promoter was used to drive PEP expression in HeLa cells infected

with VTF-7, a recombinant vaccinia virus that expresses the T7 RNA polymerase (9, 20). As a control for appropriate intracellular localization in this transient-expression system, Fyn cDNA was epitope tagged with the same *c-myc* sequence (31). Transient expression of PEP demonstrated nuclear and perinuclear localization as detected by indirect immunofluorescence (Fig. 2 and 3A). The amount of perinuclear staining was variable between transfected cells and appeared to reflect increased expression levels. In contrast, expression of Fyn showed plasma membrane localization as well as some punctate staining, indicating some localization to intracellular organelles (Fig. 3B). Nuclear staining was not detected in any Fyn-transfected cells. Nonexpressing adjacent cells exhibit minimal staining, demonstrating specificity for the procedure and indicating that under the conditions used the monoclonal antibody, 9E10, does not recognize the endogenous *c-myc* protein.

To determine the sequences responsible for nuclear targeting, deletion mutagenesis was employed. Expression of PEP amino acids 1 to 306, which encompass the phosphatase domain, localized to the cytoplasm (Fig. 2 and 4A). However, a cDNA encoding the carboxy-terminal domain, residues 330 to 802, showed strong nuclear localization (Fig. 2 and 4B).

Furthermore, expression of the last 330 amino acids of PEP (PEP 470 to 802) also resulted in nuclear localization (Fig. 2). This indicated that the nuclear localization sequence was contained within the carboxy terminus of PEP.

Expression of cDNA in which the sequence encoding the carboxy terminus was deleted, residues 1 to 520 or 1 to 730, resulted in cytoplasmic localization (Fig. 2). This indicated that the carboxy-terminal 72 amino acids contained the nuclear localization sequence. The protein expressed by the construct containing the final 72 amino acids, residues 730 to 802, was located in the nucleus (Fig. 4C). Deletion of the last 14 amino acids from the cDNA encoding the carboxy terminus, residues 330 to 788, resulted in cytoplasmic localization (Fig. 4D). In addition to the nuclear localization sequence at the extreme carboxy terminus, a potential nuclear localization sequence exists at positions 446 to 451. However, deletion of this sequence or mutation of the basic amino acids to threonine did not affect subcellular localization (Fig. 2). This indicates that sequences within the last 14 amino acids are necessary for nuclear localization.

An additional sequence that may affect nuclear localization was found at residues 520 to 730 (Fig. 2). The carboxy-terminal domain of PEP contains a nonperfect tandem repeat sequence enriched in proline residues, as well as sequences enriched in PEST residues. Deletion of the proline-enriched repeat sequence and PEST sequences did not affect subcellular localization (Fig. 2). Interestingly, however, approximately half of the cells that expressed just residues 520 to 730 (a fragment that contained the tandem proline-enriched repeat and PEST sequences but not the carboxy terminus) demonstrated nuclear and cytoplasmic staining (Fig. 5A), while the rest exhibited strictly cytoplasmic staining (Fig. 5B). The reason for the difference in subcellular localization is not known.

Transfer of the PEP nuclear localization sequence to β -galactosidase. To demonstrate that the sequence at the carboxy terminus of PEP was sufficient for nuclear localization, β -galactosidase chimeric proteins containing different regions of PEP were expressed, and the subcellular localization was examined. The recombinant plasmids were transiently transfected into HeLa or COS-7 cells. After 48 h, cells were fixed and β -galactosidase protein was visualized with a colorimetric substrate. Expression of β -galactosidase alone resulted predominantly in cytoplasmic localization (Fig. 6A). Expression of either a β -galactosidase chimeric protein containing the carboxy terminus of PEP, residues 330 to 802, or the simian virus 40 nuclear localization sequence resulted in nuclear staining (Fig. 6B and C). Transfer of 18 amino acids, residues 782 to 799, to β -galactosidase was sufficient to confer nuclear localization (Fig. 6D). Transfer of other PEP regions enriched in basic residues, amino acids 1 to 30 or 429 to 480, did not direct nuclear localization of β -galactosidase (Fig. 6D). Thus, the sequences at the carboxy terminus of PEP were both necessary and sufficient for nuclear localization.

The PEP PEST sequences are not involved in rapid protein degradation. The presence of PEST sequences within the carboxy-terminal domain of PEP suggested that PEP is rapidly degraded (23). To examine the half-life of PEP protein, pulse-chase analysis was performed with 70Z/3 pre-B cells, a cell line that constitutively expresses PEP. Concomitantly, S194 plasmacytoma cells that had been stably transfected with PEP cDNA containing the *c-myc*-derived epitope tag were analyzed. S194 cells do not constitutively express PEP (16). Cells were methionine and cysteine starved, pulsed for 20 min with [³⁵S]methionine/cysteine, and chased with cold methionine and cysteine for various times. A specific protein of approximately 90 kDa, the predicted mass of PEP, was de-

FIG. 5. Expression of a polypeptide containing the PEST sequences and the tandem proline-enriched repeat domains results in differential localization in HeLa cells. (A) Cells expressing PEP 520-730, appended with a *c-myc*-derived epitope tag demonstrating both nuclear and cytoplasmic staining of the polypeptide repeats by confocal microscopy; (B) cells in the same culture dish as those in panel A demonstrating exclusion from the nucleus. The subcellular localization of the expressed proteins was detected by indirect immunofluorescence with the monoclonal antibody 9E10 and FITC-conjugated goat anti-mouse antibodies.

tected upon immunoprecipitation of 70Z/3 cells with anti-PEP antibodies (Fig. 7A). No appreciable loss of the labeled protein was seen after 5 h of cold chase. Similarly, immunoprecipitation of transfected or untransfected S194 cells with anti-*c-myc* monoclonal antibody specifically detected a protein from the transfected cells that demonstrated little, if any, appreciable degradation after 5 h of chase (Fig. 7B). The difference in the apparent molecular weight of the proteins from 70Z/3 and S194 cells is likely due to the presence of the epitope tag. Therefore, the PEST sequences in PEP do not, under these conditions, direct rapid degradation despite the fact that lymphoid cells express PEST-containing nuclear proteins that are rapidly degraded (23). Furthermore, the epitope tag did not appear to interfere with the half-life of PEP.

DISCUSSION

PEP is an intracellular PTPase expressed primarily by cells of hematopoietic origin. In situ hybridization analysis revealed that PEP was diffusely expressed in lymphoid tissues. This is consistent with PEP being expressed by a wide variety of leukocyte cell types (16). The pattern of PEP expression in lymphoid tissues is distinct from that observed for two other hematopoietic PTPases, CD45 and SHP (data not shown). PEP is not regulated during the cell cycle, and thus the pattern

FIG. 6. The PEP nuclear localization sequence is contained within residues 782 to 799. PEP- β -galactosidase fusion proteins were transiently expressed in HeLa cells. Subcellular localization was visualized with the enzymatic activity of β -galactosidase and a colorimetric substrate. (A) Cells expressing β -galactosidase; (B) cells expressing PEP- β -galactosidase chimeric protein containing the carboxy-terminal amino acids 330 to 802; (C) cells expressing simian virus 40 large T antigen nuclear localization sequence PPKKKRKVEDPV- β -galactosidase fusion protein; (D) schematic diagram indicating subcellular localization of PEP- β -galactosidase chimeric proteins. The regions of PEP added to β -galactosidase are shown. The amino acid residue numbers corresponding to the sequence from full-length PEP are shown to the left. β -GAL and SV40 NLS indicate the subcellular localization of β -galactosidase and the simian virus 40 large T nuclear localization sequence- β -galactosidase fusion protein, respectively. The different domains of PEP are indicated by shading and/or labels above the schematic diagram. Subcellular localization is indicated by a + in the appropriate column, and the cells shown in A, B, and C are representative of the results obtained for the different fusion proteins. There was some sample-to-sample variation in the intensity of staining likely due to the expression level of each fusion protein.

of expression does not reflect different stages of the cell cycle (21).

Overexpression of proteins, including the protein tyrosine kinase c-Abl, has been used to determine nuclear location (32). To determine the subcellular location of PEP, we transiently expressed the protein in HeLa cells. These experiments demonstrated that PEP is capable of nuclear localization. In contrast, expression of the protein tyrosine kinase p59^{lck} demonstrated plasma membrane localization. While it is pos-

sible that the transient-expression system does not accurately reflect PEP intracellular location, the correct location of p59^{lck} suggests that the system is capable of demonstrating proper subcellular localization of expressed proteins. This is supported by the identification of a nuclear localization sequence within PEP which when added to a cytoplasmic protein is sufficient to direct nuclear localization.

Deletion analysis of PEP and experiments with chimeric proteins suggest that the nuclear localization sequence is

FIG. 7. Pulse-chase analysis of PEP PTPase reveals that PEP is not rapidly degraded. (A) 70Z/3 pre-B cells were labeled with [^{35} S]methionine/cysteine for 20 min and cold chased for 0, 15, and 30 min and 1, 3, and 5 h. Proteins were immunoprecipitated with preimmune sera or anti-PEP antisera. (B) S194 plasmacytoma cells stably expressing PEP containing an epitope tag derived from *c-myc*, or untransfected cells were labeled and chased as in panel A. PEP was immunoprecipitated with the anti-*c-Myc* monoclonal antibody 9E10. Proteins were resolved on an SDS-8% polyacrylamide gel, and radiolabeled proteins were visualized by autoradiography. The period of cold chase is indicated above the lanes. Migrations of molecular weight markers are indicated to the left. An arrow indicates the migration of PEP.

contained minimally within amino acids 788 to 799. This sequence, SKPKGPRNPPSA, is reminiscent of other nuclear localization sequences, although it does not contain adjacent basic residues (10). A more widely distributed PTPase that is highly similar to PEP, PTPG1 (also known as P19-PTP or PTP-PEST), has recently been identified (5, 30, 35). The catalytic domains of PEP and PTPG1 have 62% identical residues, and both contain a large carboxy-terminal domain enriched in PEST sequences. The carboxy-terminal domains, however, have only 20% identical residues. Significantly, a similar sequence, GKPKGPPEPSE, is found at the extreme carboxy terminus of PTPG1 (30), suggesting that PTPG1 also localizes to the nucleus.

PEP contains a tandem nonperfect repeat sequence between residues 605 and 705. Each repeat sequence is approximately 25 amino acids and is enriched in proline residues. These polypoline repeats are similar in sequence to the minimal binding epitope identified on proteins bound by the SH3 domain of *c-Abl* (3). Additionally, it was recently shown the GRB-2 adapter protein functions in signal transduction by binding to son-of-sevenless, a GTPase-activating protein (2). This interaction is via the GRB-2 SH3 domain and four short polypoline repeats located in son-of-sevenless. We have preliminary data to suggest that this region binds to other proteins and to suggest that this region is a protein-protein interaction domain. This is of interest since expression of this portion of PEP in HeLa cells resulted in either subcellular localization to the nucleus and the cytoplasm or exclusion from the nucleus (Fig. 5A and B). The reason for the different subcellular localization is unknown but may reflect different response states of cells due to electroporation and/or viral infection or may reflect different phases of the cell cycle. Regardless, the difference in subcellular localization seen with this fragment may reflect the presence or absence of some factor that interacts with this region.

Many nuclear proteins, including proteins expressed by hematopoietic cells, that contain PEST sequences are rapidly degraded (23). The PEST sequences in PEP suggested that PEP would have a short half-life. However, pulse-chase analysis indicated that the half-life of PEP is greater than 5 h. It is

possible that the turnover characteristics of PEP are affected by the state of cellular activation. Alternatively, the PEST sequences may be used for some other purpose. In this regard, similar sequences in nuclear proteins have been demonstrated to be sites of O-linked glycosylation (12), and a 65-kDa PTPase, of unknown sequence, has been shown both to be glycosylated and to have a nuclear localization (18). Therefore, it is possible that the PEST sequences within PEP are sites of glycosylation.

The phosphorylation of nuclear proteins plays a role in controlling a variety of responses. Mitosis is controlled in part by tyrosine dephosphorylation. The phosphatase responsible for this dephosphorylation, *cdc25*, is located primarily in the nucleus (19, 22). Transcriptional activation following stimulation by interferons leads to the tyrosine phosphorylation of the ISGF3 and GAF transcription factors (27, 33). Furthermore, two protein tyrosine kinases, *c-Abl* and *FER*, are known to localize to the nucleus and are likely to affect nuclear phosphorylation levels (11, 32). The localization of PEP to the cell nucleus has important implications for its function and is likely to be important in regulating nuclear activities such as mitosis or transcription.

ACKNOWLEDGMENTS

We thank our colleagues for helpful discussion, advice, support, and reagents, particularly J. Mullersman, C. Weaver, E. Wong, and R. J. Matthews. We also thank M. Olszowy for the SR α -EMCVneo vector; L. Samelson for the *fyn* construct containing the epitope tag; E. Brown for the use of the Nikon fluorescence microscope; K. Grindstaff, D. Shearer, and J. Cooper for help with confocal microscopy; and T. A. Woodford-Thomas for critically reading the manuscript.

This work was supported by the Howard Hughes Medical Institute. E.F. was supported by a Patricia Roberts Harris Fellowship and the Division of Biological and Biomedical Sciences, Washington University.

REFERENCES

- Charbonneau, H., and N. K. Tonks. 1992. 1002 protein phosphatases? *Annu. Rev. Cell Biol.* 8:463-493.
- Chardin, P., J. H. Camonis, N. W. Gale, L. Van Aelst, J. Schlesinger, M. H. Wigler, and D. Bar-Sagi. 1993. Human *Sos1*: a

- guanine nucleotide exchange factor for Ras that binds to GRB2. *Science* **260**:1338–1343.
3. Cicchetti, P., B. J. Mayer, G. Thiel, and D. Baltimore. 1992. Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho. *Science* **257**:803–806.
  4. David, M., P. M. Grimley, D. S. Finbloom, and A. C. Lerner. 1993. A nuclear tyrosine phosphatase downregulates interferon-induced gene expression. *Mol. Cell. Biol.* **13**:7515–7521.
  5. den Hertog, J., C. E. G. M. Pals, L. J. C. Jonk, and W. Kruijer. 1992. Differential expression of a novel murine non-receptor protein tyrosine phosphatase during differentiation of P19 embryonal carcinoma cells. *Biochem. Biophys. Res. Commun.* **184**:1241–1249.
  6. Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.* **5**:3610–3616.
  7. Fischer, E. H., H. Charbonneau, and N. K. Tonks. 1991. Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. *Science* **253**:401–406.
  8. Fu, S.-Y. 1992. A transcription factor with SH2 and SH3 domains is directly activated by an interferon α -induced cytoplasmic protein tyrosine kinase(s). *Cell* **70**:323–335.
  9. Fuerst, T. R., P. L. Earl, and B. Moss. 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. *Mol. Cell. Biol.* **7**:2538–2544.
  10. Garcia-Bustos, J., J. Heitman, and M. N. Hall. 1991. Nuclear protein localization. *Biochim. Biophys. Acta* **1071**:83–101.
  11. Hao, Q.-L., D. K. Ferris, G. White, N. Heisterkamp, and J. Groffen. 1991. Nuclear and cytoplasmic location of the FER tyrosine kinase. *Mol. Cell. Biol.* **11**:1180–1183.
  12. Hart, G. W., R. S. Haltwanger, G. D. Holt, and W. G. Kelly. 1989. Glycosylation in the nucleus and cytoplasm. *Annu. Rev. Biochem.* **58**:841–874.
  13. Khan, K. D., K. Shuai, G. Lindwall, S. E. Maher, J. E. Darnell, Jr., and A. L. M. Bothwell. 1993. Induction of the Ly-6A/E gene by interferon α/β and gamma requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds. *Proc. Natl. Acad. Sci. USA* **90**:6806–6810.
  14. Kozak, M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **115**:887–903.
  15. Lerner, A. C., M. David, G. M. Feldman, K. Igarashi, R. H. Hackett, D. S. A. Webb, S. M. Sweitzer, E. F. Petricoin III, and D. S. Finbloom. 1993. Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science* **261**:1730–1733.
  16. Matthews, R. J., D. B. Bowne, E. Flores, and M. L. Thomas. 1992. Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. *Mol. Cell. Biol.* **12**:2396–2405.
  17. McLaughlin, S., and J. E. Dixon. 1993. Alternative splicing gives rise to a nuclear protein tyrosine phosphatase in *Drosophila*. *J. Biol. Chem.* **268**:6839–6842.
  18. Meikrantz, W., D. M. Smith, M. M. Sladicka, and R. A. Schlegel. 1993. Nuclear localization of an O-glycosylated phosphotyrosine phosphatase from human cells. *J. Cell Sci.* **98**:303–307.
  19. Millar, J. B. A., J. Blevitt, L. Gerace, K. Sadhu, C. Featherstone, and P. Russell. 1991. p55^{CDC25} is a nuclear protein required for the initiation of mitosis in human cells. *Proc. Natl. Acad. Sci. USA* **88**:10500–10504.
  20. Moss, B. 1991. Vaccinia virus: a tool for research and vaccine development. *Science* **252**:1662–1667.
  21. Nargi, J. L., and T. A. Woodford-Thomas. 1993. Cloning and characterization of a mouse cdc25 phosphatase. *Immunogenetics* **39**:99–108.
  22. Nurse, P. 1992. Universal control mechanism regulating onset of M-phase. *Nature (London)* **344**:503–508.
  23. Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**:364–368.
  24. Rohan, P. J., P. Davis, C. A. Moskaluk, M. Kearns, H. Krutzsch, U. Siebenlist, and K. Kelly. 1993. PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science* **259**:1763–1766.
  25. Ruff-Jamison, S., K. Chen, and S. Cohen. 1993. Induction by EGF and interferon- γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. *Science* **261**:1733–1736.
  26. Schindler, C., K. Shuai, V. R. Prezioso, and J. E. Darnell, Jr. 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* **257**:809–813.
  27. Shuai, K., C. Schindler, V. R. Prezioso, and J. E. Darnell, Jr. 1992. Activation of transcription of IFN- γ : tyrosine phosphorylation of a 91-kD DNA binding protein. *Science* **259**:1808–1812.
  28. Shuai, K., G. R. Stark, I. M. Kerr, and J. E. Darnell. 1993. A single phosphotyrosine residue of Stat 1 required for gene activation by interferon- γ . *Science* **261**:1744–1746.
  29. Silvennoinen, O., C. Schindler, J. Schlessinger, and D. E. Levy. 1993. Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. *Science* **261**:1736–1739.
  30. Takekawa, M., F. Itoh, Y. Hinoda, Y. Arimura, M. Toyota, M. Sekiya, M. Adachi, K. Imai, and A. Yachi. 1992. Cloning and characterization of a human cDNA encoding a novel putative cytoplasmic protein-tyrosine-phosphatase. *Biochem. Biophys. Res. Commun.* **189**:1223–1230.
  31. Timson Gauen, L. K., A.-N. T. Kong, L. E. Samelson, and A. S. Shaw. 1992. p59^{lyn} tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. *Mol. Cell. Biol.* **12**:5438–5446.
  32. van Etten, R. A., P. Jackson, and D. Baltimore. 1989. The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. *Cell* **58**:669–678.
  33. Velazquez, L., M. Fellous, G. R. Stark, and S. Pellegrini. 1992. A protein tyrosine kinase in the interferon α/β signalling pathway. *Cell* **70**:313–322.
  34. Walton, K. M., and J. E. Dixon. 1993. Protein tyrosine phosphatases. *Annu. Rev. Biochem.* **62**:101–120.
  35. Yang, Q., D. Co, J. Sommercorn, and N. K. Tonks. 1993. Cloning and expression of PTP-PEST. A novel, human, nontransmembrane protein tyrosine phosphatase. *J. Biol. Chem.* **268**:6622–6628.