Association of the T-cell Protein Tyrosine Phosphatase with Nuclear Import Factor p97*

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Alternative splicing of the T-cell protein tyrosine phosphatase (TCPTP) transcript generates two forms of the enzyme that differ at their extreme C termini: a 48-kDa endoplasmic reticulum-associated form and a 45-kDa nuclear form. By affinity chromatography, using GST-TCPTP fusion proteins, we have isolated three cytoplasmic proteins of 120, 116, and 97 kDa that interact with TCPTP. The p120 protein associated with residues 377–415 from the C terminus of the 48-kDa form of TCPTP, whereas the recognition site for p97 and p116 was mapped to residues 350–381 encompassing the TCPTP nuclear localization sequence (NLS). The TCPTP NLS was shown to be bipartite, requiring basic residues 350–358 (basic cluster I) and 377–381 (basic cluster II), the sites of interaction with p97 and p116, for efficient nuclear translocation. The interaction between p97, p116, and the TCPTP NLS appeared unique in that these proteins did not form a stable interaction with the classical NLS of SV40 large T antigen or the standard bipartite NLS of nucleoplasmin. Sequence analysis of p97 identified it as the nuclear import factor p97 (importin-β), which is an essential component of the nuclear import machinery. In assays in vitro in permeabilized cells, p97 was necessary but not sufficient for optimal nuclear import of TCPTP. We found that TCPTP co-immunoprecipitated with the nuclear import factor p97 from cell lysates and that purified recombinant p97 and TCPTP interacted directly in vitro. These results indicate selectivity in the binding of p97 and p116 to the TCPTP NLS and suggest that p97 may mediate events that are distinct from the classical nuclear import process. Moreover, these results demonstrate that the C-terminal segment of TCPTP contains docking sites for interaction with proteins that may function to target the enzyme to defined intracellular locations and in the process regulate TCPTP function.

Protein tyrosine phosphorylation is an essential element in the control of fundamental cellular signaling events involved in proliferation and differentiation. The level of cellular tyrosine phosphorylation is determined by the activity of both protein tyrosine kinases and protein tyrosine phosphatases (PTPs)

1 The abbreviations used are: PTP, protein tyrosine phosphatase; TCPTP, T-cell PTP; ER, endoplasmic reticulum; NLS, nuclear localization sequence; PCR, polymerase chain reaction; GST, glutathione S-transferase; DTt, dithiothreitol; HPLC, high pressure liquid chromatography; IBB, importin-β binding; BSA, bovine serum albumin; NPL, nucleoplasmin; T-Ag, large T antigen.

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Association of TCPTP with p97/Importin-β

Plasmid Constructs—pGEX-KG constructs encodes human TCPTP truncation mutants were generated by PCR using the cloned pFu DNA polymerase (Stratagene, La Jolla, CA). Oligonucleotides incorporated a BamHI site immediately 5′ to the initiation codon and an EcoRI site immediately 3′ to the termination codon. The 5′ oligonucleotide encoding truncated mutants of the full-length TCPTP (residues 1–415) was 5′-GGCTCGCCAGCTGCTGCCACCAAWTGCGCCAGGGAG-3′, whereas that used for generating truncated mutants of the TCPTP C-terminal segment (residues 318–415) was 5′-GAATGGGGG-GATCCATAGGCTAGAAGAAGAAGAAAATGGC-3′. Oligonucleotides were used for generating TCPTP truncation mutants included an in-frame stop codon prior to the EcoRI site (TC-(1–415), 5′-GACAGAAAGTGT-GTACCAGATTCTATTTAAGAAAGAAG-3′; TC-(1–381), 5′-3′CCCTACTGTTGCCTTTTTTCTTTCATCGATGAGCGGTCGGTG-3′), where XhoI sites within the TCPTP C terminus.

Fischer (University of Washington, Seattle, WA) (7). The pSVL constructs encoding GST alone (pET-GST) or a GST fusion of the 48-kDa form of TCPTP (pET-GST-TCPTP48) were kind gifts from Drs. H. Cha-}

MATERIALS AND METHODS


g, 0.5% (w/v) Triton X-100 and 0.4 ml of each supernatant (12,000 g) was subsequently centrifuged at 1000 g strokes), and an equal volume of buffer B was added. This cell lysate was then centrifuged at 1000 g (20 strokes) to yield the Triton X-100 lysate. The other half of the cell suspension was washed with 0.1 ml of IgG agarose (Qiagen, Hilden, Germany). Histidine-tagged importin-α was a kind gift from Dr. Colin Dingwall.

Cell Culture and Metabolic Labeling of Cells—HeLa, NIH 3T3, WI 38, and COS1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) at 37 °C and 5% CO2. For metabolic labeling, cells were cultured on 10-cm plates to ~70% confluency, rinsed with methionine-free Dulbecco’s modified Eagle’s medium, and subsequently starved in methionine-free Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum for 1 h. Cells were incubated with 0.5 mCi/10-cm plate of L-[35S]methionine Express Protein Labeling Mix (DuPont) for 8 h. Labeled cells were washed three times with ice-cold phosphate-buffered saline and harvested in phosphate-buffered saline (100 mM sodium chloride, 10 mM sodium phosphate, pH 7.4). Harvested cells were frozen with liquid nitrogen and either stored at ~70 °C or lysed and used immediately.

GST-TCPTP Precipitations from Metabolically Labeled Cells—Where indicated, [35S]Met-labeled HeLa cells (1 × 10^6) were lysed in 0.8 ml of lysis buffer containing Triton X-100 (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% (w/v) Triton X-100, 150 mM NaCl, 5 mM iodoacetic acid) plus protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotonin, 1 μg/ml pepstatin A, 1 μg/ml benzamidine, and 2 μM phenylmethylsulfonyl fluoride). Lysates were precleared with 0.1 ml of IgG agarose (The Enzyme Center, Malden, MA) for 1 h at 4 °C. DTI was added to 0.5 mM, and the supernatants (12,000 g, 5 min, 4 °C) were incubated with glutathione-Sepharose coupled to 7.5 μg of GST, GST-1B-(1–435), GST-1B-(1–321), GST-1B-(290–435), GST-1B-(1–415), or GST-1B-(1–317) for 4 °C. 0.5 μg of GST fusion protein was standardized for the apperent full-length protein. Sepharose beads were washed with lysis buffer, and precipitates were analyzed by SDS-PAGE and autoradiography.

In all other cases [35S]Met-labeled HeLa cells (1 × 10^6) were lysed in 0.8 ml of hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 5 mM iodoacetic acid) plus protease inhibitors. Cells were allowed to swell for 20 min at 4 °C and then homogenized with a Dounce homogenizer. DTI was added to 0.5 mM, and the lysates were centrifuged at 70,000 g for 4 °C for 20 min. Supernatants were made to 150 mM NaCl and 0.5% (w/v) Triton X-100 and 0.4 ml of each supernatant was gently agitated for 3 h at 4 °C with 2.5 μl of glutathione-Sepharose containing approximately 3 μg of immobilized GST-TCPTP fusion protein. GST-TCPTP fusion proteins were standardized for the apparent full-length protein. Sepharose beads were then collected by centrifugation and subsequently mixed with 100 mM NaCl and 0.5% Triton X-100. Precipitates were resolved by SDS-PAGE and visualized following autoradiography.

Cellular Fractionation Studies—[35S]Met-labeled HeLa cells (2 × 10^6) were lysed in 1.2 ml of buffer A (20 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 2 mM EGTA, 5 mM iodoacetic acid, 5 μg/ml leupeptin, 5 μg/ml aprotonin, 1 μg/ml pepstatin A, 1 μg/ml benzamidine, and 2 mM phenylmethylsulfonyl fluoride) and allowed to swell for 20 min at 4 °C. The volume of one-half of the cell suspension was made to 1.2 ml with buffer B (100 mM Tris-HCl, pH 7.6, 0.5 mM sucrose, 10 mM EDTA, 5 mM iodoacetic acid, 5 μg/ml leupeptin, 5 μg/ml aprotonin, 1 μg/ml pepstatin A, 1 μg/ml benzamidine, and 2 mM phenylmethylsulfonyl fluoride). Triton X-100 was added to a final concentration of 1% (w/v), and the lysate was homogenized with a Dounce homogenizer (20 strokes) to yield the Triton X-100 lysate. The other half of the cell suspension was also homogenized with a Dounce homogenizer (20 strokes), and an equal volume of buffer B was added. This cell lysate was subsequently centrifuged at 1000 × g, 4 °C for 5 min. The pellet was resuspended in 1.2 ml of a 1:1 mixture of buffers A and B, Triton X-100 was added to 0.5% (w/v), and the lysate was termed the nuclear fraction. The supernatant was centrifuged at 70,000 g for 30 min, and the subsequent supernatant was termed the high speed supernatant. The pellet was resuspended in 1.2 ml of a 1:1 mixture of buffers A and B, and Triton X-100 was added to 1.0% (w/v). The resuspended pellet was incubated on ice for 60 min and centrifuged at 70,000 × g for 10 min, and the supernatant was termed the membrane fraction. 250 μl of each fraction was mixed with 2.5 μl of glutathione-Sepharose con-
taining ~2 μg of immobilized GST or GST-TCPTP fusion protein, and the mixture was agitated for 3 h at 4 °C. Sepharose beads were then collected by centrifugation and washed six times with buffer C (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% (w/v) Triton X-100, 150 mM NaCl, 2 mM DTT, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride). Precipitates were resolved by SDS-PAGE and visualized following autoradiography.

Isolation and Sequencing of TCPTP-interacting Proteins—For preparative isolation of TCPTP-binding proteins, 2.5 × 10⁹ HeLa cells were resuspended in 40 ml of hypotonic lysis buffer, incubated on ice for 20 min, and homogenized with a Dounce homogenizer. All subsequent procedures were at 4 °C. The lysate was centrifuged at 100,000 × g for 30 min, and the resulting supernatant was brought to 150 mM NaCl, 500 ng of p97 was immunoprecipitated from supernatants with 20 μg of either the monoclonal anti-TCPTP CF4 (generously provided by Dr. David Hill, Calbiochem Oncogene Research Products, Cambridge, MA) or the anti-p97 mAb3E9 (11), respectively, for 4 °C. Immune complexes were collected on Protein A-Sepharose CL-4B (Pharmacia), washed with lysis buffer containing 200 mM NaCl, resolved by SDS-PAGE, and transferred onto Immobilon (Millipore, Bedford, MA). Immunoblots were probed with either anti-TCPTP CF4 antibody or anti-p97 mAb3E9 antibody at 1 μg/ml.

Precipitation of Purified p97 and Importin-α by GST-TCPTP—Approximately 5 μg of either GST, GST-TCPTP deletion mutants, or GST-TC-(318–415) were immobilized on 5 μl of glutathione-Sepharose and agitated at 4 °C for periods ranging from 4 h to overnight as indicated with either 5 or 10 μg of purified p97 or p97-(1–547) or with 10 or 20 μg of purified importin-α in 0.5 ml of binding buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 1 mM DTT). Precipitates were washed five times with the same buffer and analyzed by SDS-PAGE and Coomassie Blue staining.

Transient Transfections and Indirect Immunofluorescence—COS1 cells were seeded onto glass coverslips at 2.5–3 × 10⁶/cm² plate 24 h prior to transfection. Cells were transfected (10 μg of plasmid DNA) by the calcium phosphate precipitation method and processed for immunofluorescence at 36–48 h post-transfection as described previously (7). Affinity-purified polyclonal anti-TCPTP antibody 6228 (obtained from J. A. Lorenzen and E. H. Fischer, University of Washington, Seattle, WA) was applied at 0.8 μg/ml for 1 h.

NLS Binding Assay—[35S]Met-labeled HeLa cells from 4 × 10⁶/cm² dishes were resuspended in 2 ml of hypotonic lysis buffer containing protease inhibitors, homogenized with a Dounce homogenizer, and lysate was clarified at 70,000 × g, 4 °C for 20 min. NaCl was added to the supernatant to a concentration of 150 mM and concentrated 10-fold using a Centricon 10 microconcentrator (Amicon, Beverly, MA) previously blocked with 5 mg/ml BSA in lysis buffer. Concentrated supernatant was agitated at 4 °C for 1 h with 100 μg of biotinylated BSA-SV40 NLS (CGGGPNNKKKVRV) conjugate or glutathione-Sepharose coupled to 10 μg of either GST-TC-(318–381), or GST-NPL-(88–200). 50 μl of streptavidin-agarose (Sigma) was then added to the biotinylated conjugates, and the incubation of all samples continued for a further 1 h. Precipitates were collected by centrifugation, washed with lysis buffer containing 150 mM NaCl, resolved by SDS-PAGE, and analyzed by autoradiography. GST-fusion protein GST-TC-(318–349) lacking the TCPTP NLS and biotinylated BSA-mutant SV40 NLS (CGGGPKKVR) conjugate or glutathione-Sepharose coupled to 10 μg of either GST-TC-(318–381), or GST-NPL-(88–200). 50 μl of streptavidin-agarose (Sigma) was then added to the biotinylated conjugates, and the incubation of all samples continued for a further 1 h. Precipitates were collected by centrifugation, washed with lysis buffer containing 150 mM NaCl, resolved by SDS-PAGE, and analyzed by autoradiography. GST-fusion protein GST-TC-(318–349) lacking the TCPTP NLS and biotinylated BSA-mutant SV40 NLS (CGGGPKKVR) conjugate or glutathione-Sepharose coupled to 10 μg of either GST-TC-(318–381), or GST-NPL-(88–200). 50 μl of streptavidin-agarose (Sigma) was then added to the biotinylated conjugates, and the incubation of all samples continued for a further 1 h. Precipitates were collected by centrifugation, washed with lysis buffer containing 150 mM NaCl, resolved by SDS-PAGE, and analyzed by autoradiography.

Co-immunoprecipitation Assay—HeLa cells from 2 × 10⁶/cm² plates (70% confluent) were resuspended in 0.8 ml of lysis buffer containing 200 mM NaCl, 5 mM iodoacetamide, and protease inhibitors and agitated at 30 °C for 20 min. Lysates were then diluted with 1 volume of the same buffer without NaCl and precleared with 0.1 ml of IgG Sorb for 1 h at 4 °C. Precleared lysates were centrifuged (12,000 × g, 5 min, 4 °C), and TCPTP or p97 was immunoprecipitated from supernatants with 20 μg of either the monoclonal anti-TCPTP CF4 (generously provided by Dr. David Hill, Calbiohem Oncogene Research Products, Cambridge, MA) or the anti-p97 mAb3E9 (11), respectively, for 5 h at 4 °C. Immune complexes were collected on Protein A-Sepharose CL-4B (Pharmacia), washed with lysis buffer containing 200 mM NaCl, resolved by SDS-PAGE and transferred onto Immobilon (Millipore, Bedford, MA). Immunoblots were probed with either anti-TCPTP CF4 antibody or anti-p97 mAb3E9 antibody at 1 μg/ml.
was conjugated to allophycocyanin at a ratio of 5 mol/mol as described previously (15). Recombinant 45-kDa TCPTP, expressed in E. coli using the pET21 expression vector (Novagen, Madison, WI) and purified to homogeneity by ion exchange followed by gel filtration chromatography, was a kind gift from Drs. H. Charbonneau and Luning Hao (Purdue University, West Lafayette, IN). Import experiments were performed in a 50-µl volume containing purified transport factors (400 nM importin-α, 400 nM p97, 1 µM Ran-GDP, and 1 µM RanBP1) and either 1 µg of the SV40 NLS-allophycocyanin substrate or 2 µg of the recombinant 45-kDa TCPTP as described previously (15).

RESULTS

Interaction of p97, p116, and p120 with the TCPTP C Terminus—TCPTP and PTP1B are closely related nontransmembrane PTPs. Structurally they both comprise a catalytic domain linked at its C-terminal end to a noncatalytic, targeting segment (Fig. 1). To identify proteins that interact with the C terminus, a series of GST-fusion proteins with C-terminal truncations in the 48-kDa TCPTP were generated and used to further the interactions of p97, p116, and p120 with the TCPTP C terminus, a series of GST-fusion proteins with C-terminal truncations in the 48-kDa TCPTP were generated and used to precipitate interacting proteins from [35S]Met-labeled HeLa lysates of [35S]Met-labeled HeLa cells. Cellular fractionation of [35S]Met-labeled HeLa cells and subsequent precipitation with the GST-TCPTP fusion proteins illustrated that p97, p116, and p120 were present predominantly in a high speed (70,000 × g) supernatant (Fig. 3), indicating that they are primarily cytoplasmic.

Overlapping and Distinct Binding Sites of p97, p116, and p120 within the TCPTP C Terminus—The extreme C-terminal hydrophobic residues of the 48-kDa form of TCPTP are essential for targeting to the ER, whereas residues 350–381 encompass an NLS essential for the nuclear import of the 45-kDa form (Fig. 1A) (7, 10). As such, association of the TCPTP-interacting proteins with residues within the TCPTP NLS or the hydrophobic C terminus would be consistent with a role in targeting TCPTP to the ER or to the nucleus. To characterize further the interactions of p97, p116, and p120 with the TCPTP C terminus, a series of GST-fusion proteins with C-terminal truncations in the 48-kDa TCPTP were generated and used to precipitate interacting proteins from [35S]Met-labeled HeLa lysates. In this case, lysates were prepared under hypotonic conditions, and supernatants were subsequently brought to 0.5% (w/v) Triton X-100 and 150 mM NaCl to minimize nonspecific interactions. A GST fusion protein of the full-length enzyme, GST-TC-(1–415), precipitated p97, p116, and p120 as well as p120 (Fig. 4, A and B). Truncation of the full-length enzyme to generate a mutant lacking the 34 C-terminal residues, GST-TC-(1–415), resulted in loss of association with p120 but maintained the ability to bind to p116 and, albeit more weakly, to p97 (Fig. 4A). Upon truncation of an additional 32 C-terminal residues, all binding to these proteins was lost (Fig. 4A). Similar results were obtained with lysates of NIH 3T3, WI38, and proteins for two reasons. First, their primary site of interaction appeared to be in the C-terminal segment of TCPTP, and second, they appeared to be specific for the C terminus of TCPTP and not PTP1B. Cellular fractionation of [35S]Met-labeled HeLa cells and subsequent precipitation with the GST-TCPTP fusion proteins illustrated that p97, p116, and p120 were present predominantly in a high speed (70,000 × g) supernatant (Fig. 3), indicating that they are primarily cytoplasmic.
Jurkat cells (data not shown), indicating that p97, p116, and p120 are not unique to HeLa cells but are present both in transformed and nontransformed cell lines. These results indicate that the C-terminal hydrophobic tail (residues 382–415) of the 48-kDa form of TCPTP is necessary for the interaction with p120, whereas p97 and p116 interact with residues between 350 and 381.

Two clusters of basic amino acids (residues 350–358 and 377–381) can be delineated within the p97 and p116 binding segment. Previous studies have indicated that these basic clusters are important for nuclear import (7, 10). To characterize further the interaction between TCPTP and p97 and p116, GST-TCPTP mutants lacking residues 350–352 or 350–358 (basic cluster I) or 377–381 (basic cluster II) were generated, and their ability to precipitate proteins from [35S]Met-labeled HeLa lysates was investigated. We found that deletion of residues 350–352 (Fig. 4B) or 350–358 (data not shown) did not alter the association of p97 or p120 but did result in significant loss of p116 binding. In contrast, deletion of residues 377–381 (basic cluster II) resulted in loss of p97, p116, and p120 binding.

These results indicate that within the TCPTP NLS, the p97 binding site includes residues in basic cluster II but not I, whereas the p116 binding site includes residues in both basic clusters. The absolute dependence on an intact basic cluster II for p97 binding suggests that the decreased association of p97 with the GST-TC-(1–381) protein relative to GST-TC-(1–415) in Fig. 4A probably results from the proteolysis of its C-terminal basic residues \(^{277}{\text{RRRR}}\) rather than the loss of a binding site in the hydrophobic C terminus. Consistent with this interpretation, we find that a GST fusion protein of the TCPTP NLS is sufficient to precipitate p97 and p116 from cell lysates (Fig. 5).

In addition, p120 binding requires an intact basic cluster II, and as such the p120 binding site can be extended to include basic cluster II (residues 377–381) as well as the hydrophobic C terminus (residues 382–415). However, basic cluster II alone is not sufficient for p120 association, since truncation of the C-terminal hydrophobic residues 382–415 led to a complete loss of p120 binding (Fig. 4A).

**A Comparison of Proteins That Interact with TCPTP and Those That Bind to the NLS Motifs of SV40 Large T Antigen and Nucleoplasmin**—The clusters of basic amino acids in the C-terminal segment of TCPTP that constitute the sites of interaction with p97 and p116 have been shown to be important for nuclear import of the phosphatase (7, 10). Therefore, we compared the abilities of the classical NLS of SV40 large T antigen (T-Ag), the standard bipartite NLS of nucleoplasmin, and the NLS of TCPTP to precipitate p97 and p116 from [35S]Met-labeled HeLa cell lysates. These experiments were performed at a variety of concentrations of cell lysate and under various detergent conditions. In all conditions tested, we found that although the TCPTP NLS (residues 350–381) precipitated p97 and p116, the SV40 T-Ag and nucleoplasmin NLS sequences did not (Fig. 5). These results highlight differences between protein-protein interactions involving the TCPTP NLS and the classical NLS sequences of the SV40 T-Ag and nucleoplasmin, raising the question of whether there are unique aspects to the mechanism of nuclear import of the phosphatase.

**Isolation and Characterization of TCPTP-interacting Proteins**—To characterize further the TCPTP-interacting proteins, preparative precipitations were undertaken using the C-terminal segment GST-TC-(318–415) immobilized on glutathione-Sepharose beads. Precipitates were analyzed by SDS-PAGE, and TCPTP-interacting proteins were visualized by Coomassie Blue staining. Two major proteins bound specifically to the TCPTP C-terminal domain (Fig. 6, inset). These migrated at molecular weights corresponding to 97,000 (p97) and 120,000 (p120) as expected from the results of the analytical scale experiments performed with lysates of [35S]Met-labeled cells (Figs. 2 and 4). The 116-kDa protein was also detected but at
lower amounts than p97 and p120. The p97 and p120 bands were excised and digested in situ with Achromobacter protease I, and digests were subsequently resolved by chromatography on reverse phase HPLC. The HPLC profiles for p97 (Fig. 6) and p120 (data not shown) showed that these proteins were unrelated and that p97 was not a proteolytic product of p120. To date, the sequences of eight individual peptides from p120 do not match any proteins in the data base. In contrast, the sequences of two peptides from p97 (Fig. 6) were identical to sequences in the nuclear import factor p97, otherwise known as karyopherin-β or importin-β (11). The nuclear import factor p97 is an essential factor in the nuclear import of NLS-containing proteins (14). It localizes both to the cytoplasm and to the nuclear envelope (11), consistent with our cellular fractionation experiments. Since the identity and function of p116 and p120 are unknown, we have concentrated on characterizing the association of TCPTP with p97.

Interactions of TCPTP with p97 in Vitro and in Vivo—Using the NLS of the SV40 T-Ag as a model substrate, it has been demonstrated that the 54/56-kDa NLS receptor (importin-α) can bind directly to the NLS, whereas p97 does not bind directly but enhances 54/56-kDa NLS receptor binding (16–19). In light of this finding, we investigated whether p97 interacted directly with TCPTP. We expressed p97 as a histidine-tagged fusion protein and purified it on nickel-agarose. Purified recombinant p97 was incubated subsequently with various immobilized GST-TCPTP fusion proteins. We utilized the 48-kDa form of TCPTP in these binding studies in vitro, because the GST-45-kDa TCPTP is readily proteolyzed during its purification with disruption of the p97 binding site (data not shown). The extent to which p97 associated with the TCPTP truncation mutants was examined by SDS-PAGE and Coomassie Blue staining (Fig. 7A). p97 was shown to bind efficiently to the full-length 48-kDa TCPTP as well as to deletion mutants lacking residues 350–352 or residues 350–358 (basic cluster I) but not to a mutant lacking residues 377–381 (basic cluster II) (Fig. 6A). The data illustrate that p97 can interact directly with TCPTP independently of other proteins and that residues 377–381 are necessary for this interaction. These data are consistent with the observed association between mutant TCPTP molecules and proteins from lysates of [35S]Met-labeled HeLa cells (Fig. 4).

To test whether TCPTP and p97 can associate at physiological levels of expression, the p97-specific antibody mAB3E9 and the TCPTP-specific antibody CF4 were used to test whether p97 and TCPTP co-immunoprecipitate from HeLa cell lysates. The 45-kDa form of TCPTP and low levels of the 48-kDa form were shown to co-immunoprecipitate with p97 from cell lysates (Fig. 7B). The ability of p97 to interact with low levels of the 48-kDa form of TCPTP indicates that part of the association may occur after lysis. We did not detect p97 in immunoprecipitates of TCPTP (data not shown); however, we have mapped the epitope for the TCPTP antibody CF4 to the last 38 residues of the 45-kDa form of the enzyme (data not shown), indicating...
overlap between the epitope and the p97 binding site. As such, steric hindrance due to antibody binding most likely prevented isolation of a complex with p97.

Comparison of the Interaction of TCPTP with p97/Importin-β and Importin-α—The classical mechanisms underlying nuclear translocation of proteins depend upon interaction of importin-α with the protein to be imported. Considering that we did not detect significant levels of importin-α associated with GST-TCPTP fusion proteins following incubation of the phosphatase with labeled cell lysates, we decided to compare directly the binding of importin-α with that of p97 to TCPTP using purified proteins (Fig. 8A). We observed that although p97 formed a stable complex with the TCPTP C terminus, which could be visualized by Coomassie Blue staining of SDS-PAGE gels of the precipitates, the binding of importin-α with that of p97 to TCPTP using purified proteins (Fig. 8A). We observed that although p97 formed a stable complex with the TCPTP C terminus, which could be visualized by Coomassie Blue staining of SDS-PAGE gels of the precipitates, the binding of importin-α was at best weak and at times not detectable (Fig. 8A). In addition, we observed first that TCPTP interacted with a truncated mutant of p97, p97-(1–847) (Fig. 8B), which does not bind importin-α (12) and, second, that a 40-fold molar excess of the importin-α binding (IBB) domain of importin-α (20, 21) did not prevent the association of p97 with TCPTP (Fig. 8A). These results indicate that TCPTP does not interact with p97 in the same way as importin-α and that the binding site for TCPTP is unique. Moreover, these results first demonstrate the strong association between p97 and TCPTP and, second, suggest that if importin-α functions in the nuclear import of TCPTP this must reflect a transient interaction. Moreover, since p97 formed a more stable complex with the TCPTP NLS than importin-α, it is possible that in vivo p97 may have a more direct role in nuclear transport of TCPTP than previously described.

TCPTP Nuclear Localization in Transfected COS1 Cells—The ability of p97 to interact directly with basic cluster II of the TCPTP NLS suggests that it may function to target the 45-kDa form of TCPTP to the nucleus. In overexpression studies, the 45-kDa form of TCPTP is exclusively nuclear (7, 10) and its import is independent of phosphatase activity (Fig. 9A). The contribution of basic cluster II in nuclear import however is not clear. Lorenzen et al. (7) have previously reported that residues 377–381 (basic cluster II) constitute the TCPTP NLS. In contrast, Tillmann et al. (10) have reported that residues 350–352 and 377–381 are sufficient to target TCPTP to the nucleus. Because of the discrepancies as to the exact nature of the TCPTP NLS, we have delineated further the TCPTP NLS and investigated the relationship between p97 association (residues 377–381), p116 association (residues 350–352 and 377–381), and NLS function. Truncation mutants encoding residues 1–376, 1–359, and 1–349 were expressed in COS1 cells, and their localization was investigated by indirect immunofluorescence (Fig. 9B). Although the nuclear translocation of all three truncated forms of TCPTP was impaired relative to the full-length 45-kDa protein, the localization of the 1–376 and 1–359 proteins was predominantly nuclear. In contrast, the proportion of 1–349 protein in the cytosol relative to the nucleus was higher than for either the 1–376 or 1–359 protein. The 1–349 protein appeared to be evenly distributed between the nucleus and cytoplasm (Fig. 9B), and a similar distribution was obtained for the catalytic domain (residues 1–317) of TCPTP (data not shown). These results are consistent with the ability of small proteins (<40–60 kDa) to diffuse readily in and out of the nucleus (reviewed by Dingwall and Laskey (22)). The results of this study indicate that although basic cluster I (residues 350–358) can function to a limited extent to import TCPTP, basic cluster II (residues 377–381) is also required for the efficient nuclear
translocation of the enzyme. These results suggest that the TCPTP NLS is bipartite in nature and help explain the discrepancies in previously published work (7, 10).

In the work described by Lorenzen et al. (7), mutation of basic residues 350–352 to glutamine (p45 TCR350Q/K351Q/R352Q) did not alter nuclear import, whereas deletion of basic residues 377–381 (p45TC ΔRKRKR) impaired import. Nevertheless, the localization of the p45TC ΔRKRKR mutant appeared to be predominantly nuclear (7). Localization similar to that for the p45TC ΔRKRKR mutant was obtained in this study for the 1–376 and 1–359 truncation mutants (Fig. 9B). Previously, it has been shown that single mutations in either basic cluster of the bipartite NLS of nucleoplasmin have little or no effect on nuclear transport, but mutations in both basic clusters of the same molecule abolish nuclear transport (23). In light of the fact that the truncations performed in this study indicate that the TCPTP NLS is bipartite (Fig. 9B), we examined the effect of incorporating the mutation of basic residues 350–352 and deletion of basic residues 377–381 into the same molecule. We found that when mutations in basic clusters I and II are com-

Fig. 9. Nuclear localization of TCPTP in transfected COS1 cells. A, overexpressed 45-kDa TCPTP (i) or inactive C216A mutant (ii). B, overexpressed TCPTP truncation mutants encoding residues 1–376 (i), 1–359 (ii), or 1–349 (iii). C, overexpressed p45R350Q/K351Q/R352Q (i), p45Δ377–381 (ii), and p45Δ377–381/R350Q/K351Q/R352Q (iii). Localization of overexpressed TCPTP protein was visualized by immunofluorescence and nuclei with Hoechst stain.
importin-
imal TCPTP import was achieved only when both p97 and
nuclear intensity. The S.D. for each condition was approximately 5–10% of the mean nuclear intensity.

| Condition   | Nuclear accumulation |
|-------------|-----------------------|
| TCPTP       |                       |
| With Ran    | 6                     |
| With Ran + α| 23                    |
| With Ran + p97 | 26               |
| With Ran + α + p97 | 35          |
| SV40 T-Ag   |                       |
| With Ran + α + p97 | 36          |

bined, TCPTP is evenly distributed between the nucleus and the cytoplasm (Fig. 9C), and its distribution is similar to that observed for the 1–349 truncation mutant (Fig. 9B). These results are consistent with the NLS of TCPTP being bipartite in nature and reinforce the results obtained with the truncation mutants. Although each basic cluster can function to a limited degree independently, both basic clusters are required for optimal nuclear localization of TCPTP. As such, the results demonstrate a direct correlation between the binding of p97 and p116 and the bipartite nature of the nuclear import of TCPTP.

Nuclear Import of TCPTP in Permeabilized Madin-Darby Bovine Kidney Cells—Of the nuclear proteins in the database, at least 50% possess a bipartite NLS similar to that found in nucleoplasmin (22). The nucleoplasmin NLS comprises two basic clusters separated by a 10-residue spacer sequence. In the bipartite NLS of TCPTP, the two basic clusters are separated by a spacer sequence of 24 residues. The observation that this spacer in nucleoplasmin can be increased in length to 22 residues without inhibition of nuclear import (23) suggests that the motif in TCPTP satisfies the structural requirements for a conventional bipartite NLS. However, the stable interaction we observe between TCPTP and p97, together with the unusually long spacer sequence, suggests the possibility that TCPTP may be unique and undergo nuclear import by an alternative mechanism. Previous studies have shown that p97 can function independently of importin-α and import a heterologous protein containing the IIB domain of importin-α (20, 21), raising the possibility that, in light of the stable association of p97 with the TCPTP NLS, such an import mechanism may apply to the phosphatase.

To test this possibility directly, import experiments were performed in digitonin-permeabilized Madin-Darby bovine kidney cells (13, 14). In these experiments, the ability of the purified nuclear import factors to translocate recombinant TCPTP to the nucleus was assessed. It has been shown that high concentrations of either p97 or importin-α alone may, to a variable extent, be sufficient to achieve import (24). We found that p97 alone could not translocate TCPTP to the nucleus to a greater extent than that achieved with importin-α alone. Optimal TCPTP import was achieved only when both p97 and importin-α were present (Table I). Similar results were obtained with the SV40 T-Ag NLS (data not shown). Therefore, these results indicate, first, that the association of TCPTP with p97 does not simply mimic the association of the importin-α IBB domain with p97 and, second, that TCPTP can be imported by a process involving both p97 and importin-α, at least in this in vitro assay system.

DISCUSSION

A knowledge of the intracellular distribution of nontrans-membrane PTPs and the factors dictating their distribution may facilitate an understanding of the regulation and function of these enzymes. In the case of TCPTP, two distinct forms of the enzyme exist: a 48-kDa ER-associated form and a 45-kDa nuclear form. These TCPTP variants are generated by alternative splicing of the TCPTP transcript, and the two enzymes differ only at the extreme C terminus in the residues immediately following the TCPTP NLS. In the case of the 48-kDa TCPTP, the NLS is followed by a segment of 34 predominantly hydrophobic residues, whereas, in the case of the 45-kDa TCPTP, the NLS is followed by the 6-residue segment PRLTDT.

Initially, the 20 hydrophobic C-terminal residues of 48-kDa TCPTP were identified as being responsible for the targeting of the protein to the particulate fraction of cell lysates (5, 6) and, more recently, have been shown to be necessary for directing the overexpressed protein to the ER (7). The fact that the 48-kDa form of TCPTP contains both the ER-targeting and NLS motifs but is found exclusively in the ER suggests that ER targeting is the dominant event. The C-terminal, hydrophobic segment of TCPTP, together with the second cluster of basic residues (377–381), is necessary for interaction with the binding protein p120. Interestingly, this interaction appeared specific in that the enzyme most closely related to TCPTP, PTP1B, which also contains a hydrophobic C-terminal segment and localizes to the ER, did not associate with p120. The ability of p120 to interact with residues in the hydrophobic C terminus of TCPTP is consistent with a role in directing the enzyme to the ER. In addition, the fact that the binding site for p120 overlaps with the second cluster of basic residues in the bipartite NLS of TCPTP suggests that it may also play a regulatory role in controlling nuclear import by controlling the accessibility of the NLS to nuclear import factors. We are currently pursuing the identity of p120 to characterize further the function of its interaction with TCPTP.

The TCPTP-associated protein p97 was identified as the nuclear import factor p97, otherwise known as importin-β or karyopherin-β, which is an essential factor in the nuclear transport of NLS-containing proteins (11, 14). Nuclear import is separated into two distinct steps. The first is NLS-dependent binding to the nuclear pore complex at the nuclear membrane, and the second is translocation into the nucleus. The binding step includes at least two cytoplasmic factors, the 54/56-kDa NLS receptor, otherwise known as importin-α or karyopherin-α, and the nuclear import factor p97. Using the SV40 T-Ag NLS as a model, numerous studies have reported that importin-α interacts directly with the NLS, whereas p97 interacts with importin-α and docks the complex to the nuclear pore complex (16–18). The complex is believed subsequently to be translocated through the pore by an energy-dependent mechanism that requires RanGTP (25–27).

In this study we report the binding of two distinct proteins, the nuclear import factor p97 and p116, to the TCPTP NLS. This interaction appears specific to the NLS of TCPTP in that in parallel binding assays in vitro we did not detect interaction of p97 or p116 with either the classical NLS of SV40 T-Ag or the standard bipartite NLS of nucleoplasmin. The association of p97 with the TCPTP NLS was direct, occurring independently of other proteins, and was also shown to occur at physiological levels of expression. Both in precipitations from [35S]Met-labeled cell lysates using GST-TCPTP constructs as an affinity support and in direct binding assays in vitro using purified GST-TCPTP NLS and importin-α, we find that importin-α binds poorly relative to the binding of p97. Furthermore, TCPTP interacts with p97 at a site on the import factor that is...
Association of TCPTP with p97/Importin-β

1. **p97** is a component of the nuclear import machinery distinct from the importin-α binding site and therefore does not simply mimic the association of p97 with the importin-α IBP domain. Thus, it appears that the NLS of TCPTP participates in a unique, stable interaction with p97. Moreover, we have provided evidence to indicate that the TCPTP NLS is bipartite in nature, and we have demonstrated that p97 and p116 interact with the two basic clusters lying at the termini of the NLS.

The nuclear import factor p97 associated with residues 377–381 (basic cluster II), whereas p116 interacted with residues 350–352 (basic cluster I) and residues within basic cluster II. This illustrates a direct correlation between the binding of p97 and p116 to the basic clusters of the TCPTP NLS and NLS function. However, the precise function of this interaction remains to be ascertained. Our results suggest that there may be unique features to the mechanism of nuclear import of TCPTP, in particular that p97 may play a more direct role in the process than previously anticipated. In this respect, it is interesting to note that p97 has been shown to function independently of importin-α and to import a protein to which has been fused the IBB domain of importin-α (20, 21). Nevertheless, at least in the context of a nuclear import assay in permeabilized Madin-Darby bovine kidney cells in vitro, we have demonstrated that although p97 is essential for the import of TCPTP, it is not sufficient. Optimal import required the presence of both p97 and importin-α. However, it is important to bear in mind that these assays in permeabilized cells, in which an excess of purified factors is present, may not reflect accurately the normal physiological condition and do not exclude the possibility of a more direct role for p97 in the transport process in vivo.

TCPTP is one of few PTPs including PTPs (28), PTP/blepharidomerase (29), and DPTP61F (30) that exist in two forms with alternative splicing and differ only at their extreme C termini. In this study, we have isolated three proteins, p97, p116, and p120, that interact with the TCPTP C terminus. p120 interacts with the C-terminal residues that dictate an ER localization for the 48-kDa TCPTP, whereas the nuclear import factor p97 and p116 interact with the basic clusters of the TCPTP bipartite NLS, which targets the 45-kDa TCPTP into the nucleus. Although p97 appears to be essential for nuclear import of TCPTP, it is not sufficient. Thus, the identification and characterization of p116 will be required to elucidate whether or not the stable interaction of p97 and p116 with TCPTP reflects a unique mechanistic aspect to the nuclear import of this phosphatase.

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