COOH-Terminal Sequence Motifs Target the T Cell Protein Tyrosine Phosphatase to the ER and Nucleus

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Abstract. The noncatalytic domain of the human T cell protein tyrosine phosphatase (TCPTP) is alternatively spliced to generate a 45-kD form, $p45^{TC}$, and a 48-kD form, $p48^{TC}$ (Champion-Arnaud et al., 1991; Mosinger et al., 1992). This manuscript concerns structural motifs in the noncatalytic segment of the enzyme responsible for targeting the two forms to different subcellular compartments. Endogenous and transiently expressed $p48^{TC}$ associates with the ER, as determined by sucrose gradient fractionation and indirect immunofluorescence, respectively. By contrast, $p45^{TC}$ localizes in the nucleus even though upon cell lysis it is not retained and fractionates with markers for soluble enzymes. Using fusion proteins consisting of β-galactosidase and COOH-terminal fragments of $p48^{TC}$, two motifs necessary for ER retention within a 70–residue targeting segment have been identified. These include the terminal 19 hydrophobic residues which comprise a potential membrane-spanning segment and residues 346-358 which encompass a cluster of basic amino acids that may represent another type of ER retention motif. The sequence RKRKR, which immediately precedes the splice junction, functions as a nuclear localization signal for $p45^{TC}$.

PROTEIN tyrosine phosphatases (PTPs)[1] exist as both intracellular and receptor-linked enzymes. All are related through a conserved catalytic domain which retains an essential cysteinyl residue surrounded by the PTP consensus sequence (I/V)HCXAGXXR(S/T)G. Intracellular PTPs possess segments outside their conserved catalytic domain that often bear distinct structural characteristics. SH2 (src homology 2) domains, PEST sequences, and sequences homologous to the retinaldehyde-binding protein or to cytoskeletal proteins that include band 4.1, ezrin, and talin have been described. These noncatalytic regions appear to have both a regulatory and localization function (Fischer et al., 1991; Charbonneau and Tonks, 1992). Many PTPs display broad and overlapping substrates specificities in vitro. Consequently, restricting the subcellular localization of the intracellular PTPs may play an important role in determining which substrates they may act upon.

The T cell protein tyrosine phosphatase (TCPTP) cDNA was initially obtained from a human peripheral T cell library and found to encode a ubiquitous 48-kD protein, $p48^{TC}$ (Cool et al., 1989). It has a COOH-terminal noncatalytic segment that is largely hydrophilic except for the last 19 residues which are extremely hydrophobic. The enzyme associates with the particulate fraction in cell extracts and can be solublized by limited trypsinolysis; this results in an increase in activity of up to 20-fold as measured in vitro with the artificial substrate RCML (reduced, carboxylamidomethylated and maleylated lysozyme). A 37-kD form, generated by introducing a stop codon after the catalytic domain, is predominantly soluble indicating that the COOH terminus is instrumental in partitioning the enzyme between the soluble and the particulate fractions (Cool et al., 1990). This segment also influences the in vitro specificity of the enzyme toward two artificial protein substrates (Zander et al., 1991).

Subsequent attempts to clone TCPTP yielded an additional cDNA which encodes a 45-kD form, $p45^{TC}$ (Champion-Arnaud et al., 1991; Swarup et al., 1991; Mosinger et al., 1992). Isolation of the gene confirmed that TCPTP undergoes an alternative splicing event near the carboxyl terminus (Champion-Arnaud et al., 1991). Splicing dramatically alters the character of the COOH terminus by removing the hydrophobic tail of $p48^{TC}$; this process implies that the two forms have distinct in vivo sites of action.

Nothing is known about the regulation or cellular function of either form of TCPTP. Therefore, as an initial attempt to address these issues, the role of the noncatalytic region was investigated. $p48^{TC}$ and $p45^{TC}$ were transiently overexpressed and their subcellular localization was determined by indirect immunofluorescence. The data indicate that alternative splicing leads to a differential localization of the two variants. Examination of several mutant forms

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Abbreviations used in this paper: NLS, nuclear localization signal; PDI, protein disulphide–isomerase; PTP, protein tyrosine phosphatase; TCPTP, T cell protein tyrosine phosphatase.

of the COOH-terminal segment defined the structural motifs that specify these subcellular distributions.

Materials and Methods

Cell Culture

COS-1, Rat 2, human embryonic kidney 293, and CHO cells were maintained in DMEM (Biowhitaker, Walkersville, MD; JRH Biosciences, Lenexa, KS) with 10% FCS (GIBCO-BRL, Gaithersburg, MD) and penicillin-streptomycin (Biowhitaker). CHO cell media was supplemented also with 0.2 mM proline and 1 μM methotrexate (Sigma, St. Louis, MO). NIH 3T3 cells were maintained in DMEM , 10% CS (GIBCO-BRL) and penicillin-streptomycin. THP-1, Jurkat, and HL-60 cells were grown in RPMI 1640 (Biowhitaker, JRH Biosciences) supplemented with 10% FCS, 2 mM glutamine and penicillin-streptomycin. Hep G2 cell growth media was F12 (Biowhitaker), 10% FCS and penicillin-streptomycin. All cells were kept in 5% CO_2 at 37°C.

Antibody Preparation

Polyclonal antibody 1910H was directed against the peptide sequence CK-RPRLTDT coupled to keyhole limpet hemocyanin (Calbiochem, San Diego, CA) with sulfo-MBS (Pierce, Rockford, IL). Polyclonal antibodies 2200C and 6228 were directed against a 37-kD form of TCPTP obtained from a baculovirus expression system and purified from Sf9 cells as described (Zander et al., 1991). Antibodies were generated in New Zealand white female rabbits by subcutaneous injection after standard procedures. For purification of the antibodies by affinity chromatography, the peptide was coupled to Affigel 102 (BioRad, Richmond, CA) with sulfo-MBS, and the 37-kD form of TCPTP was coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ) as per the manufacturer's instructions. Rabbit antiserum was passed 5–10 times through the appropriate affinity matrix. The column was washed extensively with 10 mM Hepes, pH 7.4 containing 50 mM NaCl before elution with 5 M $MgCl_2$. After dialysis, the eluted antibody was concentrated by ultrafiltration using an XM-50 membrane (Amicon Inc., Beverly, MA).

Preparation of Extracts of Total Cellular Protein

Nonadherent cells were harvested by centrifugation at 5,000 rpm for 5 min at 4°C, washed with PBS and resuspended in 10 vol of extraction buffer consisting of 25 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.002% PMSF, 1 mM benzamidine, 4 μg/ml leupeptin, and 50 KI U/ml aprotinin. Adherent cells were removed from the plate by trypsinization, washed with PBS and resuspended in extraction buffer. The extracts were incubated on ice for 10 min and centrifuged at 12,000 *g* for 10 min at 4°C.

Immunoprecipitation and Western Blotting

TCPTP was immunoprecipitated with 4 μg affinity-purified antibody 2200C, 2.5 μl Protein A Sepharose (Sigma) and 7.5 μl Sepharose CL-6B (Pharmacia) overnight at 4°C. The beads were washed twice with buffer without SDS and once with either water or PBS before resuspension in SDS-PAGE sample buffer.

For Western blotting, proteins were transferred onto nitrocellulose (Schleicher and Schuell, Keene, NH). Blocking of the nitrocellulose membrane and antibody incubations were carried out in 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% BSA (Fraction V, Sigma) and 0.5% Tween-20; washes were performed in the same buffer without BSA. The primary antibody against TCPTP was 1 μg/ml mAb CF4 (Oncogene Science, Uniondale, NY), and the secondary antibody was sheep anti–mouse IgG conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL) at a 1:10,000 dilution. Immunoreactive proteins were visualized with the ECL Western blotting detection system (Amersham) on Hyperfilm-MP (Amersham).

Gradient Fractionation

THP-1 cells (10^8) were harvested by centrifugation for 5 min at 4,000 *g* at 4°C, washed with PBS and resuspended in 2 ml hypotonic buffer consisting of 5 mM Hepes-KOH, pH 6.8, 1 mM EDTA, and protease inhibitors as indicated above. Cells were swelled on ice; sucrose was added to a final concentration of 250 mM, and the cells were disrupted by 20 strokes of a type A pestle in a Dounce homogenizer. Nuclei were removed by centrifugation for 2 min at 800 *g*. Supernatants were layered over a discontinuous sucrose gradient and centrifuged as described (Bole et al., 1986). 13 1-ml fractions were collected from the bottom of the gradient. Immunoprecipitation of TCPTP was performed as described above using 300 μl of each fraction. PTP1B and docking protein were detected by Western blotting using 10 μl of each fraction. mAb FG6, which detects PTP1B, was obtained from Oncogene Science. Docking protein mAb 12B4 was a kind gift from Dr. David Meyer (UCLA).

Assays of Marker Enzymes

NADPH-cytochrome c reductase was assayed with 75 μl of each fraction as described except that 190 μM NADPH was used in the reaction (Williams and Kamin, 1962). Na^+,K^+-ATPase was determined from 10 μl of each fraction according to Schimmel et al. (1973) except that the reaction volume was 200 μl and the final ouabain and NaN_3 concentrations were 2.5 and 1 mM, respectively. The reaction was stopped with 200 μl of 10% trichloroacetic acid. Galactosyl transferase activity (Pesonen et al., 1984) was measured for 60 min at 37°C with 50 μl of each fraction in a reaction volume of 100 μl. The final reaction mixture contained 25 mM Hepes, pH 7.5, 20 mM $MnCl_2$, 1 mM DTT, 1 mM ATP, 0.05 mM UDP-galactose, 8.5 mg/ml ovomucoid, 0.2% Triton X-100 and 1 μCi UDP-[H^3]galactose. The reaction was stopped by precipitating 75 μl on Whatman 3MM filters (Whatman, Clifton, NJ) with 10% TCA. Lactate dehydrogenase activity was measured with 10 μl of each fraction according to Worthington (1988).

Mutagenesis

Sequential deletion mutants of $p48^{TC}$ were constructed using the Mutagene M13 In Vitro Mutagenesis Kit (Biorad) according to the manufacturer's instructions. The mutated cDNAs were inserted into the EcoRI site of pBluescript SK+ (Stratagene, La Jolla, CA) for subsequent manipulations.

The cDNA encoding $p45^{TC}$ was obtained by modification of the $p48^{TC}$ cDNA. The COOH-terminal XbaI–HindIII fragment was ligated into the XbaI and HindIII sites of pBluescript SK+. The nucleotides coding for the sequence PRLTDT and a stop codon were inserted at position 1203 of the $p48^{TC}$ cDNA using a PCR strategy. The oligonucleotides (Howard Hughes Medical Institute facility, University of Washington, Seattle, WA) used to prime the reaction were 5′-AACGAAAAAGAAAAAGGC-CAAGATTGACAGACACCTAATGGTTATAATGGCACC-3′ and 5′-CATTCTCATTTAGCCTC-3′. The reaction conditions were 100 ng template, 10 pmol of each oligonucleotide, 500 μM of each dNTP, 2.5 mM $MgCl_2$, 1X PCR buffer (Promega, Madison, WI) and 5 U Taq polymerase. Thermocycling conditions were denaturation for 5 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 44°C and 1 min extension at 72°C followed by a final 5 min extension at 72°C. The XbaI–HindIII fragment was excised from the 3.2-kb PCR product and reinserted into the full-length cDNA. All PCR amplified DNA sequences were confirmed by sequencing. Deletion of the sequences RKRKR, 346-349, 350-358, and 359-365 and the triple point mutation R350Q;K351Q; R352Q were achieved in a similar manner.

Construction of Expression Vectors

For transient expression of full-length $p45^{TC}$ and $p48^{TC}$, the ThaI/SspI fragment of their respective cDNAs was inserted into the SmaI site of the pSVL vector (Pharmacia). To produce the β-galactosidase fusion vectors, the various COOH-terminal segments of $p48^{TC}$ were amplified by PCR using oligonucleotides complementary to the 5′ and 3′ ends of the desired fragment. The oligonucleotides had EcoRI sites at their 5′ end to facilitate cloning into the expression vector. Reaction conditions were as described above except that 2.4 μg of linearized template was used. Thermocycling conditions were 94°C for 2 min followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 38°C, 30 s extension at 72°C followed by a final 5-min extension at 72°C. The fragment obtained was inserted into the EcoRI site at position 3629 of the pSV-β-Galactosidase vector (Promega). This vector was modified in that the second EcoRI site at position 6743 had been destroyed.

Transient Expression and Indirect Immunofluorescence

COS-1 cells were seeded on gelatin-coated coverslips and allowed to at-

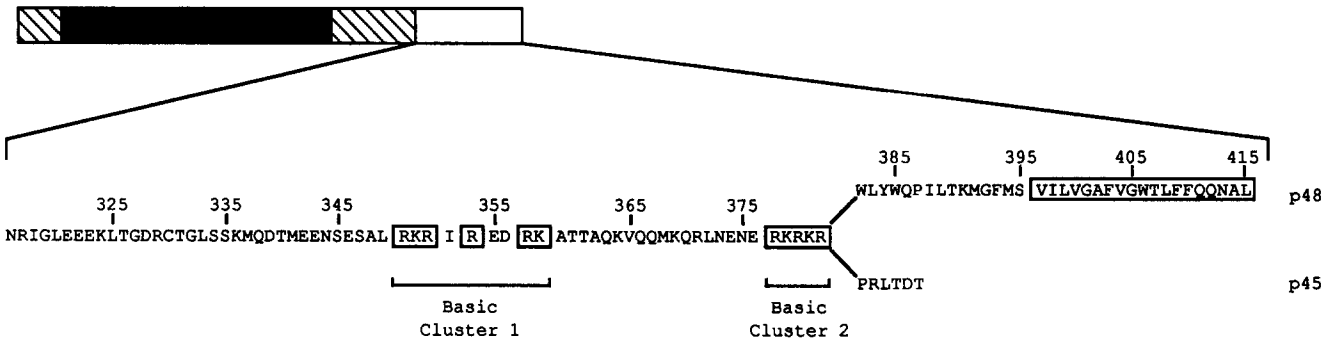

Figure 1. Schematic representation of TCPTP. The catalytic region is shown in black; the diagonal lines represent intervening sequence; the noncatalytic region is the open area. The sequence of the noncatalytic domain (residues 316-415) is shown with the site of alternative splicing, and the COOH-termini of $p45^{TC}$ and $p48^{TC}$ are indicated. Polybasic segments and the hydrophobic tail are boxed.

tach for 24 h before transfection by calcium phosphate precipitation essentially as described (Chen and Okayama, 1987). After 64 h, the cells were washed twice with PBS and fixed either with methanol for 2 min at −20°C or with 3.2% formaldehyde (methanol free, Polysciences, Warrington, PA) in PBS for 15 min at room temperature. Formaldehyde-fixed cells were washed twice in 150 mM glycine in PBS and permeabilized for 6 min in 0.2% Triton X-100 in PBS. Subsequent immunostaining procedures were identical regardless of the fixation technique. Primary and secondary antibodies were diluted in 5% goat serum in PBS and applied for 1 h at room temperature. Unbound antibody was removed by washing three times for 10 min in PBS. The primary antibodies were: (*a*) an anti–β-galactosidase mAb (Promega) at a 1:15,000 dilution; (*b*) affinity purified 6228 at a 1:1,000 dilution; or (*c*) anti-protein disulphide-isomerase mAb RL90 (a generous gift from Dr. Charlotte Kaetzel, Case Western Reserve University) at a 1:1,000 dilution; (*d*) rabbit antiserum against the EAGE peptide of β-COP (a kind gift from Dr. Richard Klausner, NIH) at a 1:500 dilution; rabbit antiserum against yeast hsp60 (a kind gift of Dr. Richard Hallberg, Syracuse University) at a 1:100 dilution. Rhodamine or fluorescein-conjugated goat anti–mouse and goat anti–rabbit secondary antibodies were from TAGO (Burlingame, CA). Mounting media contained 10.5% Airvol, 21% glycerol, 2.5% 1,4-diazobicyclo-[2,2,2]-octane (DABCO) and 50 mM Tris-HCl, pH 8.5. Immunofluorescence was visualized on a Nikon Diaphot inverted microscope, and photomicrographs were taken with a Nikon N2000 camera on Kodak Tmax 400 film.

Results

Expression of Endogenous TCPTP

The cDNAs encoding TCPTP predict proteins with molecular masses of 48 and 45 kD (Fig. 1). To determine the relative expression levels of the two species, Triton X-100 extracts were prepared from eleven cell lines and immunoprecipitated with affinity purified polyclonal Ab 2200C (Fig. 2 *A*). This antibody was raised against the catalytic domain of TCPTP common to both forms. Monoclonal Ab CF4, which recognizes an unknown COOH-terminal epitope, was used for Western blotting. Five cell lines, including CEM, Jurkat, COS-1, THP-1 and HL-60, expressed the 48-kD form in higher amounts, although the relative proportions of the two varied. $p45^{TC}$ could be seen in HL-60 cells upon prolonged autoradiograph exposure. Unexpectedly, these cells exhibited a slower migration of the $p45^{TC}/p48^{TC}$ doublet as well as a third, faster migrating band. Human embryonic kidney 293 and IMR 32 cells contained the two forms in equal quantities, while Hep G2 cells contained slightly more $p45^{TC}$ than $p48^{TC}$. Identical results were obtained when cellular extracts were prepared with 1% SDS rather than Triton X-100.

Attempts to immunoprecipitate the TCPTP doublet from NIH 3T3, Rat 2, or CHO cells (three rodent cell lines) were unsuccessful with Ab 2200C raised against the human enzyme. This was surprising because the catalytic domain is highly conserved between these species. However, Ab 1910H, which recognizes the sequence KRPRLTDT at the carboxyl terminus of the 45-kD form, readily immunoprecipitated $p45^{TC}$ from these three cell lines (Fig. 2 *B*). Since Ab 2200C did not recognize either form of TCPTP in these cells, it has not been possible to ascertain whether or not $p48^{TC}$ is expressed in these cell lines.

Immunofluorescence Localization of Overexpressed TCPTP

Endogenous levels of TCPTP are insufficient to definitively determine the localization of the enzyme by indirect immunofluorescence since no significant staining could be detected with affinity purified polyclonal Ab 6228. Therefore, $p45^{TC}$ and $p48^{TC}$ were transiently overexpressed in

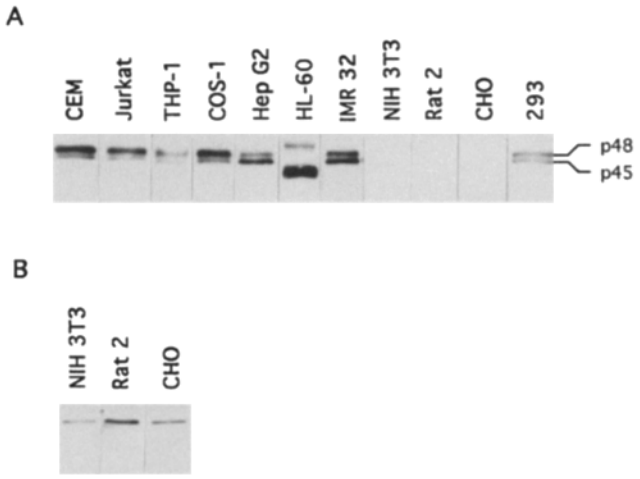

Figure 2. Relative expression of TCPTP isoforms. (*A*) $p48^{TC}$ and $p45^{TC}$ were immunoprecipitated with Ab 2200C. (*B*) $p45^{TC}$ was immunoprecipitated with Ab 1910H. mAb CF4 was used for detection in Western blot analyses (*A* and *B*). The amount of protein used for each cell line was: *CEM*, 50 μg; *Jurkat*, 50 μg; *THP-1*, 100 μg; *COS-1*, 50 μg; *Hep G2*, 100 μg; *HL-60*, 500 μg; *IMR 32*, 250 μg; *NIH 3T3*, 1 mg; *Rat 2*, 1 mg; *CHO*, 1 mg; *293*, 250 μg.

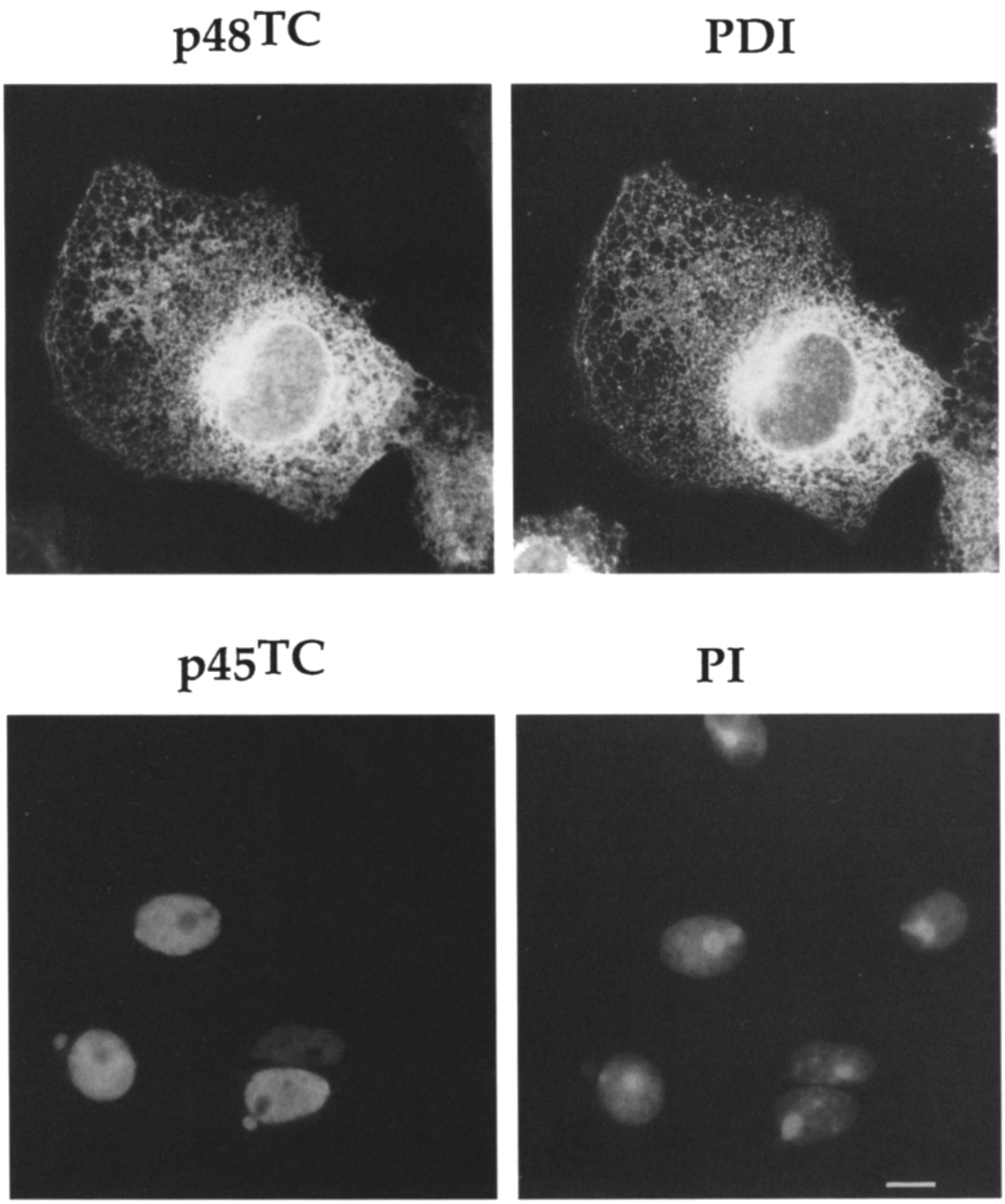

Figure 3. Localization of $p48^{TC}$ and $p45^{TC}$ by transient overexpression in COS-1 cells. Cells overexpressing $p48^{TC}$ were coimmunostained for endogenous protein disulphide-isomerase (*PDI*) to confirm ER localization. Cells overexpressing $p45^{TC}$ were colabeled with propidium iodide (*PI*) to demonstrate a nuclear distribution. Polyclonal Ab 6228 was used to immunostain $p48^{TC}$ and $p45^{TC}$, and the PDI antibody was RL90. Bar, 10 μm.

COS-1 cells to generate protein levels suitable for immunostaining.

When overexpressed, $p48^{TC}$ localizes with a reticular network that is characteristic of the ER. This was confirmed by showing that $p48^{TC}$ coimmunostains with the ER marker protein-disulphide isomerase (PDI), stained with mAb RL 90 (Kaetzel et al., 1987). By contrast, transient overexpression of $p45^{TC}$ revealed a nuclear distribution with staining excluded from the nucleoli (Fig. 3).

Fractionation of Endogenous TCPTP

THP-1 cells were chosen for the fractionation of endogenous TCPTP on a discontinuous sucrose gradient (Fig. 4). COS-1 cells were not used for these experiments because

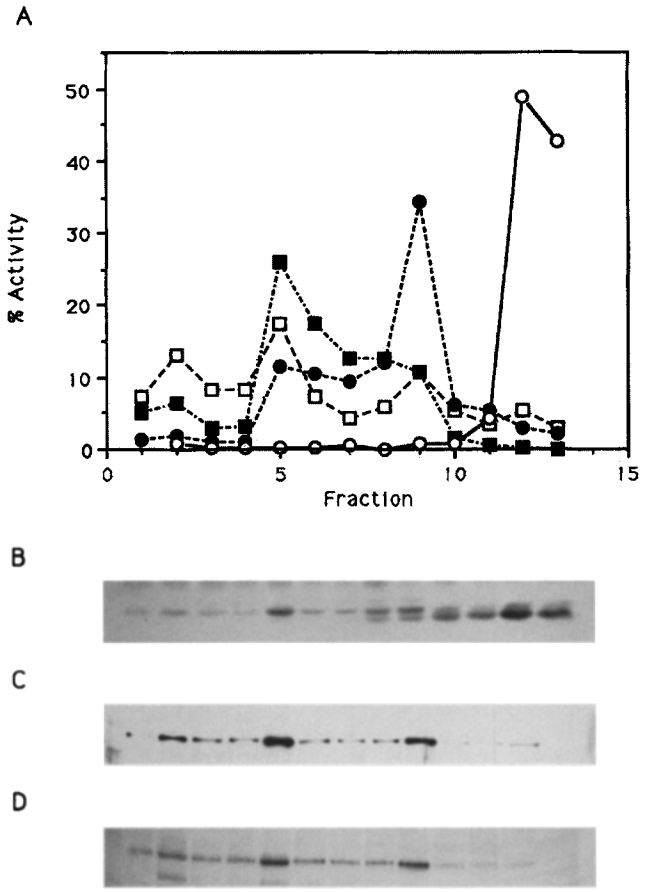

Figure 4. Discontinuous sucrose density gradient fractionation of endogenous TCPTP in THP-1 cells. (*A*) Marker enzyme distribution: *open squares*, ER (NADPH-cytochrome c reductase); *closed squares*, plasma membrane (Na^+,K^+-ATPase); *open circles*, soluble (lactate dehydrogenase); *closed circles*, Golgi (galactosyl transferase). The values represent the percent of total marker enzyme activity recovered. (*B*) Immunoprecipitation and Western analysis of $p48^{TC}$ and $p45^{TC}$. (*C* and *D*) Western analysis of docking protein and PTP1B, respectively. $p48^{TC}$ fractionates with ER-associated proteins while $p45^{TC}$ appears to be soluble.

suitable resolution of the ER, Golgi, and plasma membranes could not be obtained. Using immunoprecipitation with Ab 2200C followed by Western analysis with mAb CF4, $p48^{TC}$ was detected in all fractions, with levels peaking in fractions 2, 5, and 9. This pattern followed that of the ER marker NADPH-cytochrome c reductase, and differed from that exhibited by the Golgi marker galactosyl transferase and the plasma membrane marker Na^+,K^+-ATPase. Furthermore, fractions were immunoblotted for PTP1B, a second ER-associated protein tyrosine phosphatase, and docking protein, a 73-kD integral membrane protein of the rough ER (Hortsch et al., 1985). The distribution of these markers corresponded to that of $p48^{TC}$ and NADPH-cytochrome c reductase. Earlier fractionation using a 20% percoll density gradient clearly indicated that $p48^{TC}$ was not associated with lysosomes or mitochondria. Even though $p45^{TC}$ resides in the nucleus, upon cell lysis it fractionates as a soluble protein as indicated by the marker lactate dehydrogenase. Immunoblotting of the nuclear pellet fraction confirmed that none of the 45-kD material remained with the nuclei (data not shown).

Based on the immunoblot of THP-1 cell extract, the level of $p45^{TC}$ detected in the sucrose gradient fractions was disproportionately high relative to the amount of $p48^{TC}$ recovered. It seemed possible that much of the 48-kD protein had been removed with the nuclei by low speed centrifugation since the ER is contiguous with the nuclear envelope. However, immunoblotting showed that less than 5% of $p48^{TC}$ was present in the nuclear pellet fraction (data not shown). It is not known why the majority of the 48-kD protein escapes detection after density gradient centrifugation but may be due to proteolysis of the COOH terminus during fractionation and the concomitant removal of the epitope for mAb CF4.

Minimal ER Targeting Sequence

Given the hydrophobic nature of the carboxyl terminus of $p48^{TC}$, it was of interest to determine the minimum sequence required for ER targeting. Therefore, chimeric proteins were constructed in which the terminal 16 residues of β-galactosidase were replaced by fragments consisting of the last 20 (a.a. 396-415), 25 (a.a. 391-415), 30 (a.a. 386-415), 40 (a.a. 376-415), 70 (a.a. 346-415), or 100 (a.a. 316-415) residues of $p48^{TC}$. The 100–residue segment (see Fig. 1) was considered as a positive control because residue 316 occurs approximately at the boundary that separates the catalytic from the noncatalytic domain. Moreover, deletion of an 11-kD segment had been shown previously to affect the distribution of the enzyme, converting the particulate $p48^{TC}$ into a soluble material (Cool et al., 1990).

The chimeric protein, β-galTC316-415, was transiently expressed in COS-1 cells, and its localization was determined by indirect immunofluorescence using an anti–β-galactosidase monoclonal antibody. As shown in Fig. 5, unmodified β-galactosidase appeared as a diffuse cytoplasmic protein. In contrast, β-galTC316-415 presented a reticular profile characteristic of the ER. The noncatalytic segment of $p48^{TC}$ was capable, therefore, of redirecting the localization of β-galactosidase from the cytoplasm to the ER. Nontransfected cells exhibited no immunostaining with the β-galactosidase mAb (data not shown).

Frangioni et al. (1992) found that PTP1B, a close homologue of TCPTP within the catalytic segment, localizes to the ER through its carboxyl terminal hydrophobic segment. Therefore, it was unexpected that the hydrophobic tail of $p48^{TC}$ was insufficient for ER targeting since β-galTC396-415 distributed throughout the cytoplasm and nucleus. A segment of 25 residues (β-galTC391-415) was slightly less abundant in the nucleus but in addition displayed intense immunostaining in the perinuclear region. Further lengthening of the COOH-terminal fragment to 30 (β-galTC386-415) and 40 (β-galTC376-415) residues progressively redistributed the fusion proteins from the cytoplasm and nucleus to a structure(s) in the region of the Golgi apparatus. Extension of the fragment to 70 residues (β-galTC346-415) resulted in immunostaining of the ER suggesting that a second ER targeting motif may lie between residues 346 and 375 (Fig. 5).

To ascertain if β-galTC376-415 associates with the Golgi

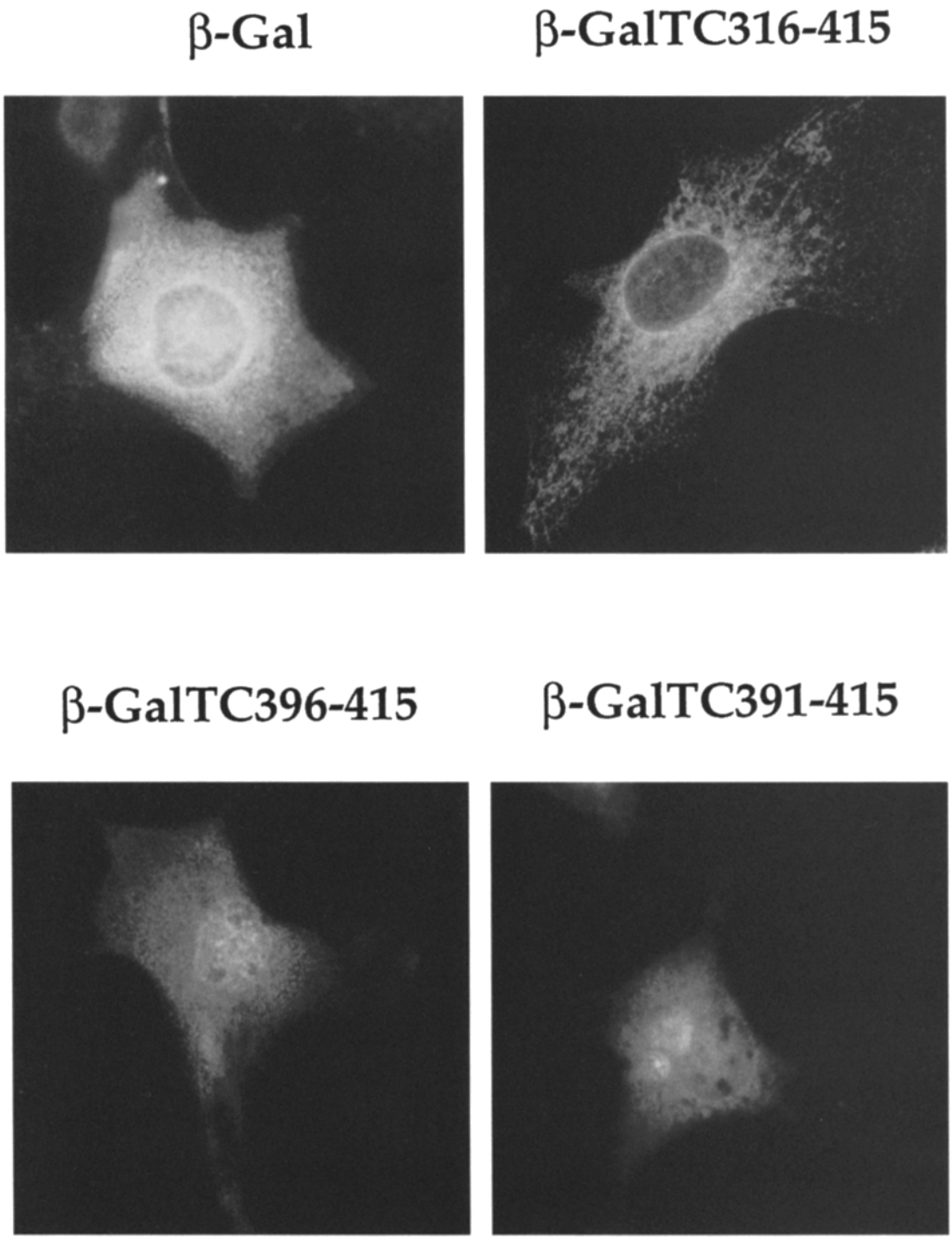

Figure 5.

complex, cells expressing this fusion protein were colabeled with antibody directed against the EAGE peptide of β-COP, a coatomer subunit of nonclathrin-coated vesicles associated with the Golgi complex and pre-Golgi compartments (Allan and Kreis, 1986; Duden et al., 1991). Costaining indicated that β-galTC376-415 did indeed associate with the Golgi (Fig. 6). This localization could be abrogated by exposure of the cells to brefeldin A, a compound which disrupts Golgi structure (data not shown) (Fujiwara et al., 1988; Doms et al., 1989; Lippincott-Schwartz et al., 1989). The fusion protein also decorated a tubular array not recognized by the β-COP antibody. Since this structure resembles mitochondria (Willingham and Pastan, 1985), cells were costained with anti-hsp 60, a highly conserved heat shock protein that is a normal component of the mitochondria (McMullin and Halberg, 1988). The fusion protein coimmunostained with hsp 60 indicating that β-galTC376-415 localized to the extensive mitochondrial network in addition to the Golgi apparatus (Fig. 6). Brefeldin A did not affect mitochondrial distribution.

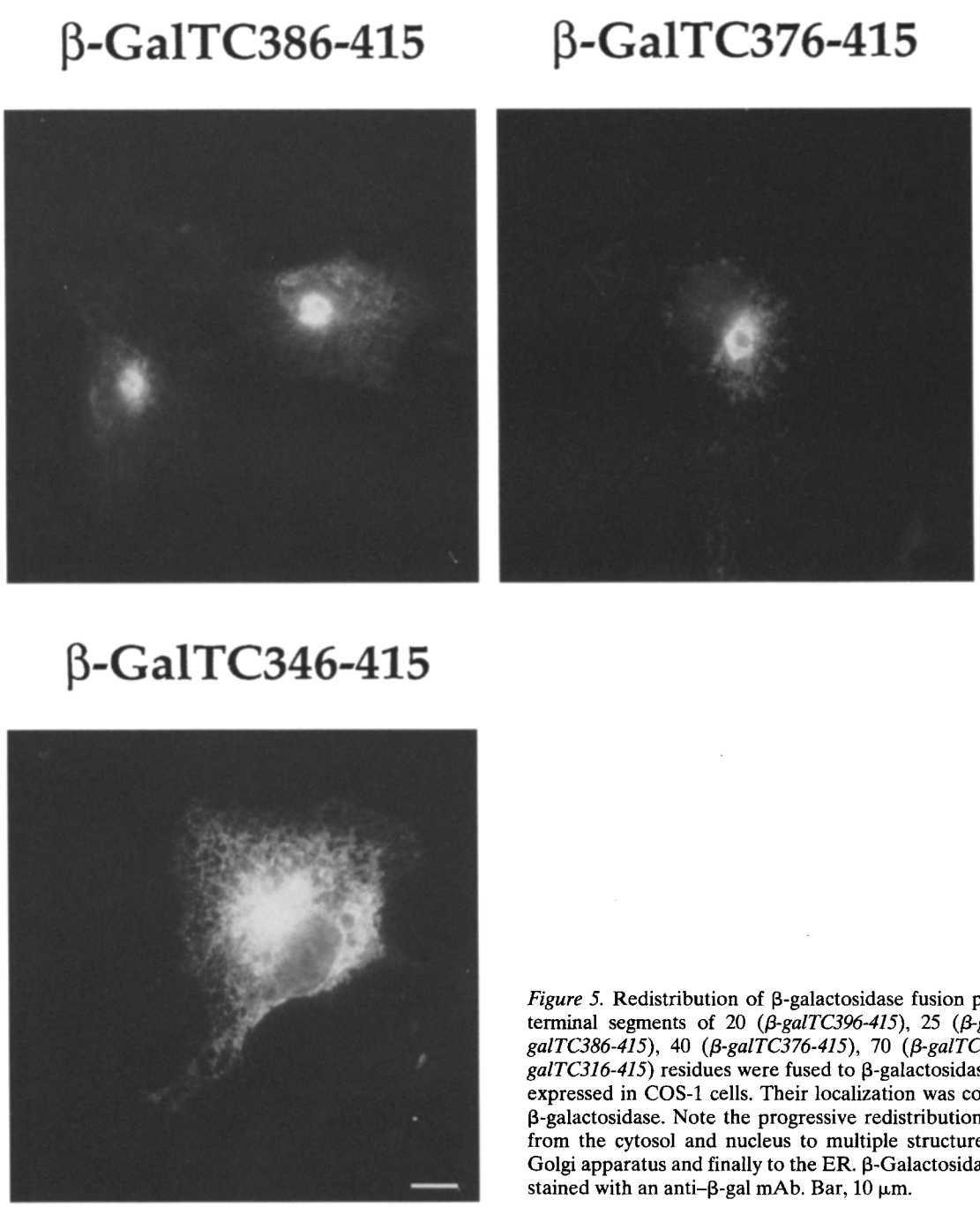

Figure 5. Redistribution of β-galactosidase fusion proteins. $p48^{TC}$ COOH-terminal segments of 20 (*β-galTC396-415*), 25 (*β-galTC391-415*), 30 (*β-galTC386-415*), 40 (*β-galTC376-415*), 70 (*β-galTC346-415*), and 100 (*β-galTC316-415*) residues were fused to β-galactosidase and transiently overexpressed in COS-1 cells. Their localization was compared to unmodified β-galactosidase. Note the progressive redistribution of the fusion proteins from the cytosol and nucleus to multiple structures in the region of the Golgi apparatus and finally to the ER. β-Galactosidase fusion proteins were stained with an anti–β-gal mAb. Bar, 10 μm.

Identification of ER Targeting Sequences

The finding that $p48^{TC}$ required a 70–residue carboxyl terminal segment for ER targeting prompted a deletion analysis to identify structural motifs responsible for this localization. A series of mutations was introduced into the region encompassing the last 100 residues (a.a. 316-415) in which blocks of 10 amino acids were successively deleted while leaving the remaining 90 residues intact. Additionally, the last 20 amino acids comprising the hydrophobic tail were removed as a single mutation. Attempts to assess the impact of the mutations on localization of the full-length phosphatase directly were unsuccessful because such deletions often resulted in proteins that either did not immunostain or stained very poorly, while others were expressed in very few cells. Since it has been difficult to express active, delocalized PTPs (Zander et al., 1993), the mutant proteins were inactivated by changing the essential cysteinyl residue to serine. This, however, did not improve the immunofluorescence. Therefore, these mutant noncatalytic segments were examined as fusion proteins with β-galactosidase.

Much of the sequence within the COOH-terminal region could be removed and still retain proper ER targeting (Table I). As expected, all three mutations affecting the hydrophobic tail (a.a. 396-415) abrogated localization to

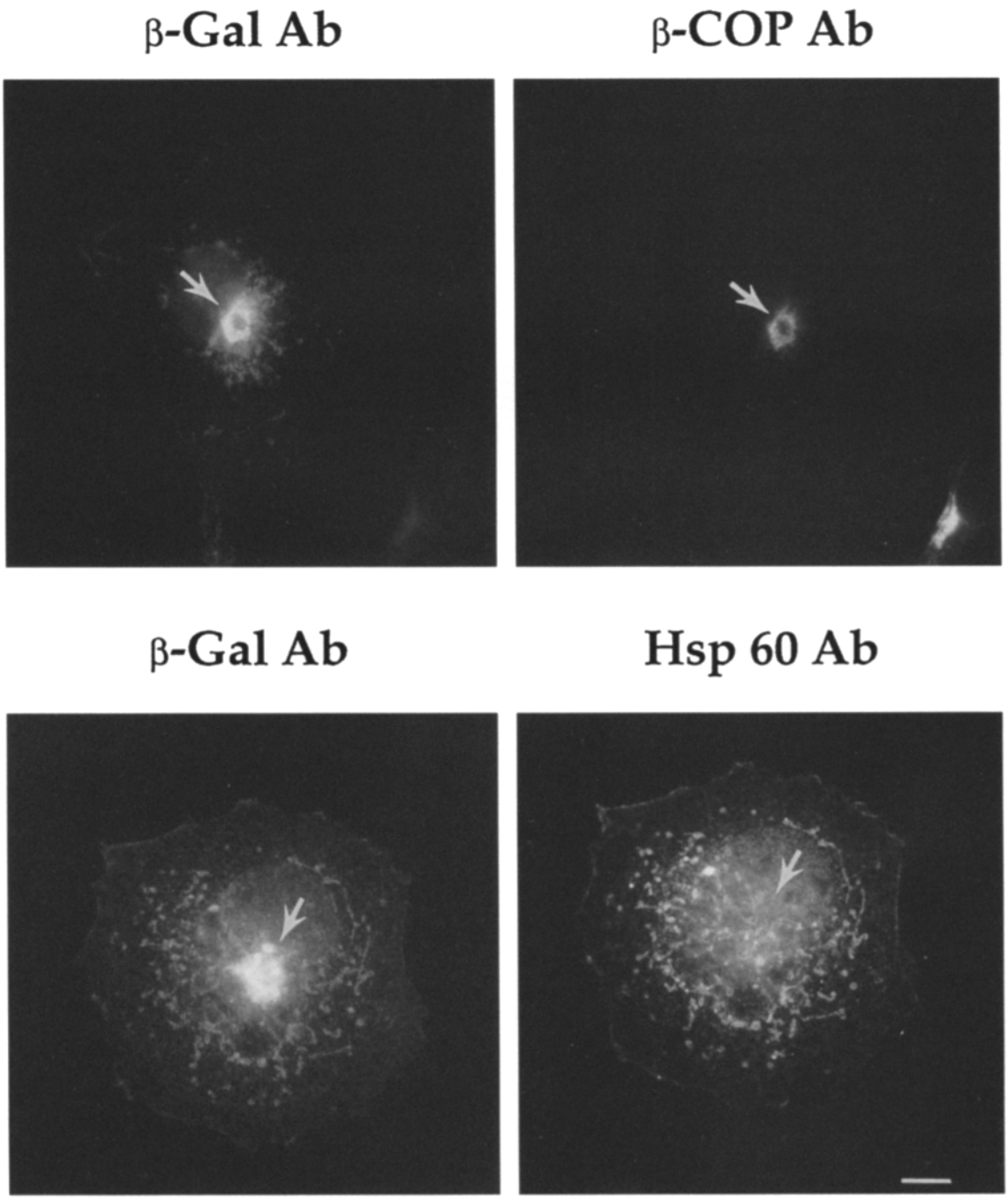

Figure 6. β-Gal376-415 localizes to the Golgi apparatus and the mitochondria. β-Galactosidase fused to the 40 terminal residues of $p48^{TC}$ distributed to a brightly staining perinuclear body and an extensive tubular array as indicated by an anti–β-gal mAb (*upper and lower left panels*). Coimmunostaining with an antibody directed against the EAGE peptide of β-COP revealed β-galTC376-415 was targeted to the Golgi apparatus (compare *upper right and left panels*). Colocalization with an anti-Hsp 60 antibody demonstrated β-gal376-415 also associated with the mitochondria (compare *lower right and left panels*). Note the absence of Golgi staining by the Hsp 60 antibody. Arrows indicate position of the Golgi apparatus. Bar, 10 μm.

the ER and resulted in both cytoplasmic and nuclear distribution. Two other deletions also interfered with ER distribution. β-galTCΔ346-355 (data not shown) and β-galTCΔ356-365 (Fig. 7) produced an immunofluorescence pattern similar to that obtained with β-galTC376-415. That is, all three localized with the Golgi apparatus and the mitochondria as indicated by costaining with β-COP and hsp 60 antibodies, respectively (data not shown). Note that the deletions Δ346-355 and Δ356-365 occur in a region critical for the ER localization of β-galTC346-415. As these results differed significantly from those reported for PTP1B (Frangioni et al., 1992), the same set of deletions was introduced into a second vector which expressed the mutations as fusion proteins with chicken muscle pyruvate kinase (Frangioni and Neel, 1993). This vector has been used for a similar targeting experiment with the hydropho-

Table I. Localization of COOH-Terminal Mutants

Mutation*	Distribution
Δ316–325	ER
Δ326–335	ER
Δ336–345	ER
Δ346–355	Golgi/Mitochondria
Δ356–365	Golgi/Mitochondria
Δ366–375	ER
Δ376–385	ER
Δ386–395	ER
Δ396–405	Cytosolic/Nuclear
Δ406–415	Cytosolic/Nuclear
Δ396–415	Cytosolic/Nuclear
Δ346–349	Golgi/Mitochondria
Δ350–358	Golgi/Mitochondria
Δ359–365	ER
R350Q:K351Q:R352Q	Golgi/Mitochondria

* Mutations were introduced into the 100-residue COOH-terminal fragment (amino acids 316–415) of the 48-kD form.

bic segment of PTP1B and, consequently, allows a direct comparison of the ER targeting motifs found in the two enzymes. Immunostaining patterns produced by the pyruvate kinase chimeras were similar to those obtained with β-galactosidase (data not shown). Therefore, we conclude that, unlike PTP1B, the ER localization of $p48^{TC}$ requires a second signal distinct from the hydrophobic tail.

The most striking characteristic of the two mutations β-galΔ346-355 and β-galΔ356-365 is that both remove a portion of polybasic region 1 (Fig. 1). To assess if this cluster participates in localizing $p48^{TC}$ to the ER, four smaller mutations were introduced between residues 346-365. These include point mutation of RKR to QQQ (β-galTCR350Q; K351Q:R352Q) and the deletion of basic segment 1 (β-galTCΔ350-358) and the flanking sequences on either side (β-galTCΔ346-349 and β-galTCΔ359-365). Table I summarizes the results obtained with these four mutations. Only β-galTCΔ359-365 associated with the ER. Mutation of either the basic cluster or the sequence ESAL (β-galTC346-349) was sufficient to produce immunostaining of the Golgi and mitochondria and implicate the first basic cluster as having a direct role in maintaining ER distribution (Fig. 7).

Nuclear Localization Signal

The nuclear localization signal (NLS) of the 45-kD form was also investigated. NLS are characterized by a high proportion of basic residues (Dingwall and Laskey, 1991). To examine the possibility that a grouping of basic amino acids at the COOH terminus may function in this capacity, several deletions and point mutations were introduced into these clusters and transiently expressed as either the native PTP or a β-galactosidase fusion protein carrying the terminal 72 residues of the $p45^{TC}$. Identical results were obtained for both constructs. Deletion of basic region 2 (RKRKR; a.a. 377-381) severely deterred movement into the nucleus as there was a dramatic increase in the level of cytosolic immunofluorescence (Fig. 8). Point mutation of each residue to glutamine revealed that within this cluster K380 and R381 were critical for efficient nuclear translocation. The K380Q mutation (Fig. 8) resulted in an immunofluorescence pattern similar to Δ377-381 while the R381Q mutation was slightly less effective in impairing entry into the nucleus, as seen by a lower level of cytosolic immunostaining (data not shown). The R377Q, K378Q, and R379Q mutations were without effect, distributing entirely within the nucleus. The enzyme never appeared to be associated with the nucleoli.

The possibility that basic cluster 2 (RKRKR) may constitute half of a bipartite NLS was also considered. Bipartite NLS consist of left and right elements separated by an intervening spacer of typically 10 or 11 residues. The upstream component is composed of two basic residues while the downstream component requires that at least 3 out of 5 amino acids be basic. While RKRKR satisfies the requirement for a downstream element, the nearest potential upstream element is basic cluster 1. Therefore, the triple point mutation R350Q;K351Q:R352Q (Fig. 8) and the two deletions Δ346-355 and Δ356-365 were introduced. However, these mutations had no observable effect as cells exhibited the same immunostain as those expressing $p45^{TC}$.

Discussion

The data presented here demonstrate that the 48-kD and 45-kD forms of TCPTP localize to the ER and nucleus, respectively. To date, the subcellular distribution of only a few other intracellular PTP's has been reported (Frangioni et al., 1992; Seki et al., 1992; Woodford-Thomas et al., 1992; Heald et al., 1993; McLaughlin and Dixon, 1993; Rohan et al., 1993). Previous studies suggest that delocalization of TCPTP can have profound effects. Expression of a 37-kD form which lacks the noncatalytic segment causes the multinucleation of BHK cells and suppresses the v-fms–induced transformation of rat-2 cells (Cool et al., 1992; Zander et al., 1993). In fact, the 37-kD form can be readily expressed only in cells that display a transformed phenotype, suggesting that expression of this truncated species could be lethal in the context of a normal cell.

Association of some proteins with the ER is often mediated by specific retention signals. Soluble ER proteins have a KDEL sequence at the carboxyl end, and various ER transmembrane proteins contain a dilysine motif at their cytoplasmic COOH terminus (Jackson et al., 1990; Munro and Pelham, 1987). Furthermore, a variation of the dilysine motif has been described in the ER chaperone calnexin (Rajagopalan et al., 1994). It is thought that proteins with these signals remain in the ER because they are continuously retrieved from post-ER compartments (Pelham, 1988; Rothman and Orci, 1992). In support of this, the KDEL receptor has been localized to the Golgi where it sorts KDEL-tagged proteins for transport back to the ER, and the dilysine grouping interacts with the coatomer assembly of Golgi-derived vesicles, suggesting that the coatomer proteins participate in the sorting mechanism (Lewis and Pelham, 1992; Cosson and Letourneur, 1994). There is also evidence for retrograde vesicular flow between the Golgi and the ER in brefeldin A–treated cells (Lippincott-Schwartz et al., 1989; Doms et al., 1989).

$p48^{TC}$ contains neither the KDEL nor the dilysine motif. Rather, the data obtained through the use of two different reporter constructs indicate that the hydrophobic tail and

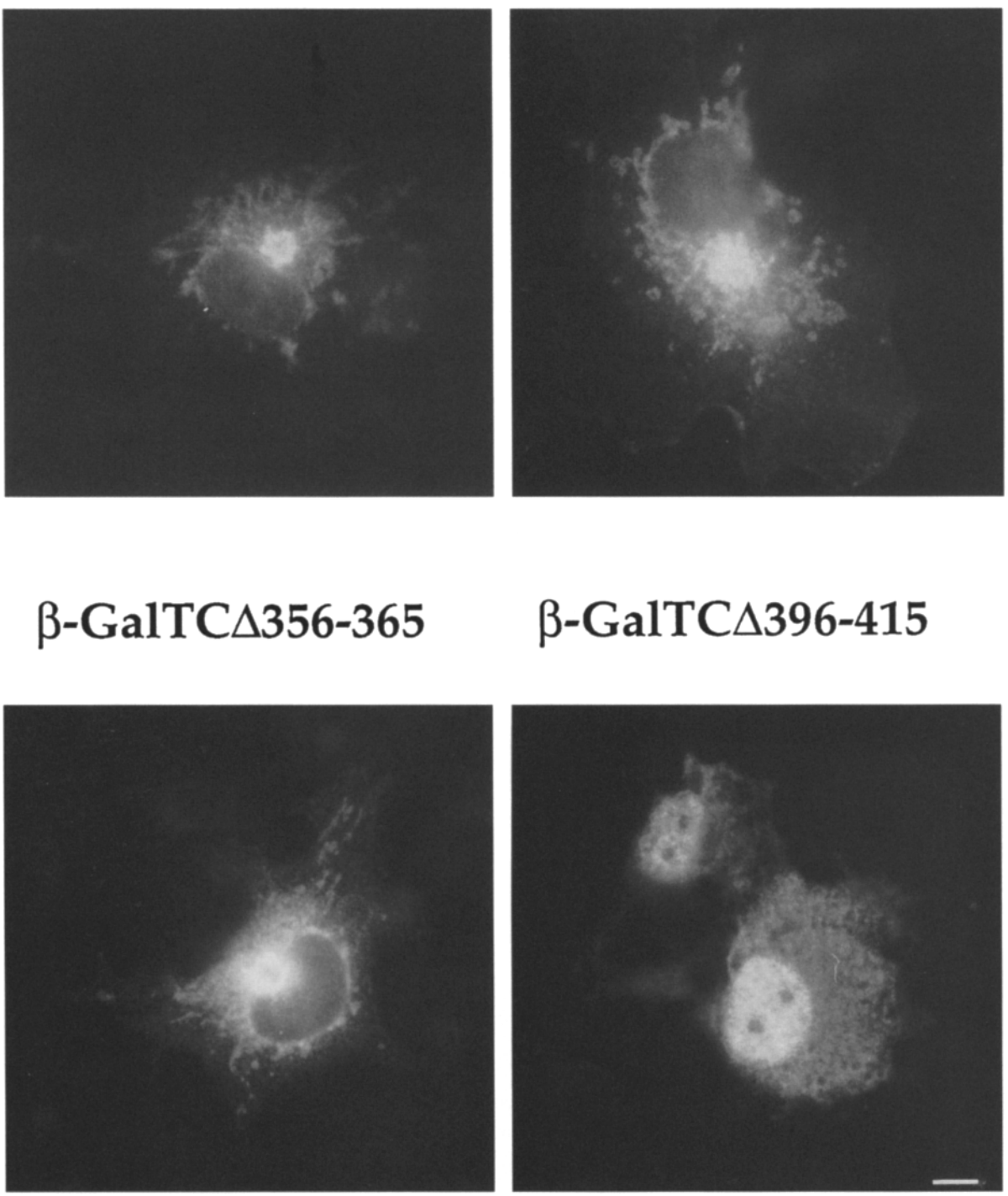

Figure 7. Mutations in the COOH terminus of $p48^{TC}$ abrogate ER localization. A series of deletions and one triple point mutation were introduced into the $p48^{TC}$ COOH-terminal segment of the β-galTC316-415 fusion protein. Several mutations resulted in a loss of ER immunostaining in COS-1 cells. Removal of the hydrophobic tail (a.a. 396-415) led to a cytosolic and nuclear distribution of the fusion protein, while those with mutations affecting residues 346-358 were found in the Golgi apparatus and the mitochondria. β-Galactosidase fusion proteins were stained with an anti–β-gal mAb. Bar, 10 μm.

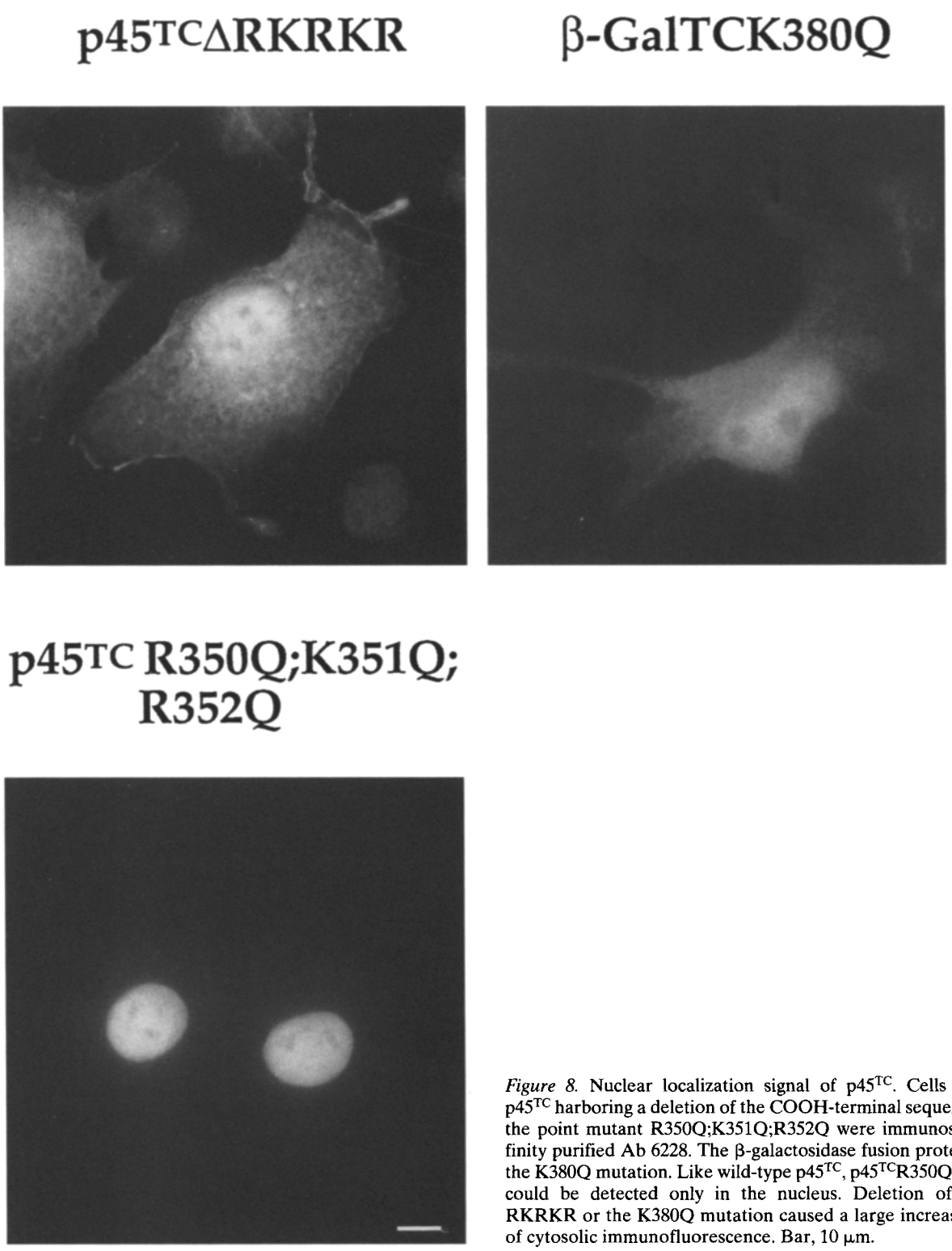

Figure 8. Nuclear localization signal of $p45^{TC}$. Cells overexpressing $p45^{TC}$ harboring a deletion of the COOH-terminal sequence RKRKR or the point mutant R350Q;K351Q;R352Q were immunostained with affinity purified Ab 6228. The β-galactosidase fusion protein is shown for the K380Q mutation. Like wild-type $p45^{TC}$, $p45^{TC}$R350Q;K351Q;R352Q could be detected only in the nucleus. Deletion of the sequence RKRKR or the K380Q mutation caused a large increase in the extent of cytosolic immunofluorescence. Bar, 10 μm.

residues within the region 346-358 must act in concert to target $p48^{TC}$ to the ER. The hydrophobic tail likely inserts into the ER membrane serving as an anchor. Without it, the phosphatase becomes both cytosolic and nuclear as seen with mutations affecting residues 396-415. As to the sequence ESALRKRIREDRK (a.a. 346-358), it may be necessary for retrieval of the enzyme from a post-ER compartment. Fusion proteins harboring mutations in this region associate with the Golgi apparatus. This distribution is consistent with the export of these proteins and their failure to be retrieved. A similar immunostain is seen with calnexin when its ER retention signal has been deleted (Rajagopalan et al., 1994). While the positioning of this sequence is unlike that of the dilysine or the calnexin ER re-

tention motif, basic residues are integral components of each and could perhaps serve an analogous function.

An alternative possibility is that the sequence ESALRKRIREDRK prevents recycling of $p48^{TC}$ by interacting with a binding protein that would facilitate permanent ER residency. In this case, the hydrophobic tail may not be sufficient to anchor the enzyme in the absence of this upstream motif. Consequently, the phosphatase associates with other intracellular membranes, namely those of the Golgi and mitochondria. These models are not necessarily mutually exclusive; the second model could explain the mitochondrial distribution even if residues 346-358 could serve as a retention motif.

The results also highlight important differences in the ER targeting mechanisms of $p48^{TC}$ and PTP1B. While these two enzymes have homologous catalytic domains, their carboxyl terminal noncatalytic segments are unrelated. The most notable difference is the absence of any COOH-terminal basic clusters in PTP1B. Although the last 35 residues of PTP1B are sufficient for ER localization (Frangioni et al., 1992), $p48^{TC}$ COOH-terminal fragments of up to 40 residues were incapable of redirecting the distribution of two different fusion proteins. Hence, these two phosphatases may localize to distinct subcompartments of the ER.

The identification of two mammalian ER phosphatases may point to new cellular roles for tyrosine phosphorylation. The ER is a highly dynamic structure that controls a multitude of cellular events including the regulation of cytoplasmic calcium and lipid metabolism, the maturation, degradation and sorting of proteins, and membrane trafficking (Rose and Doms, 1988; Klausner and Sitia, 1990; Sambrook, 1990). Additionally, the structural integrity of the ER, which is highly dependent on microtubules, must be maintained except during mitosis when secretion stops and the ER network breaks down into small vesicles (Warren et al., 1983; Featherstone et al., 1985; Terasaki et al., 1986). $p48^{TC}$ may be involved in any one of these processes. Since the catalytic domain of $p48^{TC}$ lies on the cytoplasmic side of the ER (Lorenzen, J. A., unpublished observations), the catalytic domain could be accessible to various cytosolic substrates and play a more general role in the regulation of cellular events.

Proteins destined for the nucleus typically harbor a lysine and/or arginine-rich NLS that is either contiguous, as is the case in SV40 large T antigen, or bipartite, as illustrated by nucleoplasmin (Dingwall and Laskey, 1991). Our data, obtained with two different expression vectors, indicate that basic cluster 2 (RKRKR) assists in the nuclear translocation of $p45^{TC}$. It is interesting to note that $p48^{TC}$ also contains the NLS; the fact that it does not enter the nucleus indicates the hydrophobic tail masks the NLS and acts as the dominant targeting signal. It remains undetermined as to whether the NLS is bipartite. No upstream component of the NLS could be identified as mutations in basic cluster 1 had no effect on nuclear distribution. Tillmann et al. (1994) reported on the NLS of murine $p45^{TC}$. The COOH-termini of the two PTPs are highly related with the basic clusters being strictly conserved. The authors concluded that a 25–residue segment lying immediately upstream of basic cluster 2 contains the NLS and specifically implicated the sequence RKR (a.a. 350-352) in basic region 1 as being important. This was not the case for the human enzyme; removal of this region demonstrated that it was not essential. Furthermore, these authors observed nucleolar, rather than nuclear, immunofluorescence for several of their β-galactosidase chimeric proteins. No nucleolar localization could be observed here even when a chimeric protein that had an identical portion of the noncatalytic segment was stained (data not shown).

Physiological substrates of $p45^{TC}$ are presently unknown; in fact, few tyrosyl phosphorylated nuclear proteins have been identified. These include the mitotic kinase $p34^{cdc2}$ (Gould and Nurse, 1989; Morla et al., 1989); RNAPII (Baskaran et al., 1993); the STAT proteins (Ruff-Jamison et al., 1993; Sadowski et al., 1993; Shuai et al., 1993; Silvennoinen et al., 1993; Larner et al., 1993) and a few Ser/Thr kinases such as MAP kinase (Lenormand et al., 1993), $p85^{S6K}$ (Reinhard et al., 1994), and GSK-3 (Plyte et al., 1992). Most of these affect transcription either directly or indirectly. Thus far, c-abl is the only nuclear tyrosine kinase reported (Van Etten et al., 1989). Its substrate may be RNAPII whose tyrosyl phosphorylation has been implicated in the control of cell cycle–dependent transcription of specific genes (Welch and Wang, 1993). The above possibilities suggest a role for $p45^{TC}$ in the regulation of gene expression.

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