Evidence for a role of transmembrane protein p25 in localization of protein tyrosine phosphatase TC48 to the ER

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Summary

T-cell protein tyrosine phosphatase gives rise to two splice isoforms: TC48, which is localized to the endoplasmic reticulum (ER) and TC45, a nuclear protein. The present study was undertaken to identify proteins that are involved in targeting TC48 to the ER. We identified two TC48-interacting proteins, p25 and p23, from a yeast two-hybrid screen. p23 and p25 are members of a family of putative cargo receptors that are important for vesicular trafficking between Golgi complex and ER. Both p23 and p25 associate with overexpressed TC48 in Cos-1 cells as determined by communoprecipitation. A significant amount of TC48 colocalized initially with ERGIC and Golgi complex markers (in addition to ER and nuclear membrane localization) and was then retrieved to the ER. Coexpression with p25 enhanced ER localization of TC48, whereas coexpression with p23 resulted in its trapping in membranous structures. Coexpression of a p25 mutant lacking the ER-localization signal KKxx resulted in enhanced Golgi localization of TC48. Forty C-terminal amino acid residues of TC48 (position 376-415) were sufficient for interaction with p23 (but not with p25) and targeted green fluorescence protein (GFP) to the Golgi complex. Targeting of GFP to the ER required 66 C-terminal amino acid residues of TC48 (position 350-415), which showed interaction with p25 and p23. We suggest that TC48 translocates to the Golgi complex along the secretory pathway, whereas its ER localization is maintained by selective retrieval enabled by interactions with p25 and p23.

Key words: Transmembrane protein p25, Tyrosine phosphatase TC48, Transmembrane protein p23, Endoplasmic reticulum

Introduction

Protein tyrosine phosphatases (PTPs) and their associated signaling pathways are crucial for the regulation of numerous cell functions including growth, mitogenesis, motility, cell-cell interactions, metabolism, signal transduction and gene expression (Tonks and Neel, 1996; Bourdeau et al., 2005). The T-cell protein tyrosine phosphatase TCPTP (encoded by PTPN2) is an intracellular protein tyrosine phosphatase, which is ubiquitously expressed (Cool et al., 1989; Mosinger et al., 1992; Kamatkar et al., 1996). There are two splice variants of TCPTP in human cells, TC48 [387 amino acids (aa)] and TC45 (415 aa), which vary at their C-terminal ends. In human and rat TC48, the last six aa of TC45 are replaced by 34 aa (mostly hydrophobic) (Lorenzen et al., 1995; Kamatkar et al., 1996). There are two splice variants of TCPTP in human cells, TC45 [387 amino acids (aa)] and TC48 (415 aa), which vary at their C-terminal ends. In human and rat TC48, the last six aa of TC45 are replaced by 34 aa (mostly hydrophobic) (Lorenzen et al., 1995; Reddy and Swarup, 1995). This difference in the C-terminus determines the different subcellular localization, substrate specificity and enzymatic properties of these two splice variants (Lorenzen et al., 1995; Kamatkar et al., 1996). TC45 localizes to the nucleus by virtue of a bipartite nuclear localization signal (NLS) present in the C-terminus (Radha et al., 1994; Tillman et al., 1994; Lorenzen et al., 1995). This C-terminal region of TC45 is also involved in binding to DNA (Radha et al., 1993; Kamatkar et al., 1996). The TC48 isoform is present in the endoplasmic reticulum (ER) and also in the nuclear membrane. The ER localization is determined by two signal sequences present in the C-terminal 70 aa of TC48 (Lorenzen et al., 1995).

Several substrates of TC45 have been identified, most of which are cytoplasmic the only exceptions are Stat proteins which are nuclear (reviewed in Bourdeau et al., 2005). However only EGF receptor in the ER is a specific substrate of TC48 whereas EGF receptor in the plasma membrane is dephosphorylated by TC45 (Tiganis et al., 1998). TC48 as well as TC45 are able to downregulate insulin-induced signaling by dephosphorylating the insulin receptor (Galic et al., 2003). Thus no substrates or regulators have been identified, which specifically interact with TC48 but not with TC45 isoform.

Although the sequences that target TC48 to the ER have been identified in the C-terminal 70-aa region, these sequences do not have any of the well-known ER targeting signals, such as KDEL, di-lysine or diarginine motifs (Lorenzen et al., 1995). Therefore, the mechanism by which TC48 is targeted to the ER is not known. In this study, we carried out yeast two-hybrid screening to identify proteins that interact with TC48 and that might be involved in determining its subcellular location. We isolated several yeast two-hybrid-positive clones encoding two members, p23 and p25, of the p24 family of putative cargo receptors (Schimmoller et al., 1995; Stennes et al., 1995). p24 family proteins are the major components of vesicles moving between ER and Golgi and show...
indicating strong dependence upon p25 to maintain their ER
and other family members also reached the plasma membrane,
cholesterol exclusion. During overexpression of p25SS, p23
inactivated by mutation to serine) is overexpressed in HeLa
which canonical KKxx motif for ER retention has been
dynamics (Emery et al., 2003). When the p25SS mutant (in
domains, thereby regulating membrane composition and
protein is enriched in COPI vesicles and its cytoplasmic
subunits of the COPI-vesicle coat and p24 proteins were found
role in retrograde transport. The C-terminal tail of some
membrane traffic at the Golgi complex, has been suggested to
have a role in retrograde transport. The C-terminal tail of some
the secretory system (Schimmoller et al., 1995; Stamnes et al.,
Belden and Barlowe, 1996; Fiedler et al., 1996; Sohn et
Dominguez et al., 1998; Bremer et al., 1999; Fullekrug et al.,
Wen and Greenwald, 1999; Kaiser, 2000; Muniz et al., 2000; Carney and Bowen, 2004).

There are many evidences, which suggest a role of p24
proteins in retrograde transport from the Golgi to the ER. This
role in retrograde transport might involve their function in
COPI-vesicle (vesicles known to be involved in retrograde
transport) formation, COPI-vesicle-structure maintenance or
their role as coat-receptor, cargo-receptor, adaptor or transport
factor (Fiedler et al., 1996; Sohn et al., 1996; Bremer et al.,
1999). Coatomer, a polypeptide complex involved in
membrane traffic at the Golgi complex, has been suggested to
have a role in retrograde transport. The C-terminal tail of some
the p24 proteins (including p23 and p25) interacts with
subunits of the COPI-vesicle coat and p24 proteins were found
to be enriched in COPI vesicles in yeast as well as in
mammalian cells (Stamnes et al., 1995; Sohn et al., 1996).
Moreover, deletion of p24 proteins in yeast results in secretion
of elevated levels of the ER-resident protein kar2p, indicating that it could not be retrieved to the ER (Elrod-Érickson and
Kaiser, 1996).

In mammalian cells, p24 family proteins have been proposed to
have a structural morphogenetic role in organization and/or
biogenesis of the Golgi complex (Denzel et al., 2000; Rojo et
al., 2000). Cells from p23-heterozygous mice show reduced
stack size and altered structure of the Golgi complex (Denzel et
al., 2000). p23-heterozygous mice also show reduced p25
and p26 protein levels, indicating the interdependence of these
proteins. Homozygous p23-knockout mice are not viable,
suggesting an essential and non-redundant role in very early
stages of development (Denzel et al., 2000). p23 is proposed to
function as a coatomer receptor in the Golgi, because the
protein is enriched in COPI vesicles and its cytoplasmic
domain binds to coatomer (Stamnes et al., 1995; Sohn et al.,
1996). p25 has been shown to form highly specialized
domains, thereby regulating membrane composition and
(dynamics (Emery et al., 2003). When the p25SS mutant (in
which canonical KKxx motif for ER retention has been
inactivated by mutation to serine) is overexpressed in HeLa
cells it exits the ER and Golgi and reaches the plasma
membrane where it helps in specialized domain formation via
cholesterol exclusion. During overexpression of p25SS, p23
and other family members also reached the plasma membrane,
indicating strong dependence upon p25 to maintain their ER
and Golgi retention in early biosynthetic pathway (Emery et
al., 2003). These findings also suggested that, p25 is the major
player among p24 family by regulating the localization of other
family members by anchoring p24 oligomers in the retrograde
pathway mediated by COPI vesicles. Formation of hetero-
oligomers of p24 family members also indicates the
interdependence of p24 proteins for their functional regulation
(Jenne et al., 2002). p25 has also been implicated in the
formation of vesicular tubular clusters and ER-cargo exit sites
(Lavoie et al., 1999).

In this study, we show that TC48 interacts physically in vivo with
the two p24 family members p23 and p25. Coexpression of
p25 and p23 affects TC48 localization; whereas p25
enhances ER localization of TC48, coexpression of p23 traps
TC48 in membranous structures. TC48 escapes the ER and
gets back recycled, although it does not contain any known
retrieval signals indicating the physiological relevance of the
interaction with p23 and p25. The C-terminal sequences of
TC48 that are required for targeting a green fluorescence
protein (GFP)-TC48 fusion protein (GFP-TC48) to the ER
and Golgi, are also involved in the interaction with p25 and p23
proteins. Our results suggest that p25 and p23 proteins play an
important and direct role in targeting TC48 to its proper
subcellular location.

Results
Identification of p23 and p25 as TC48-interacting proteins
To identify TC48-interacting proteins, we screened a human
placental cDNA library using a Gal4-based yeast two-hybrid
system. Full-length TC48 was used as bait. Several positive
clones were obtained under various selection criteria (their
ability to grow on adenine- or histidine-deficient plates and
also by their ability to turn blue in the presence of Bgalactosidase substrate). One of the proteins identified by this
method was transmembrane protein p23, a p24 family member
also known as Tmp 21-1. The cDNA clone obtained during
screening, codes for an almost full-length p23 protein (aa 26-
219) (Blum et al., 1996; Blum et al., 1999; Rojo et al., 1997;
Rojo et al., 2000). Another protein of this family, p25, was also
identified in this screen (Dominguez et al., 1998; Emery et al.,
2000; Emery et al., 2003). The cDNA clone obtained for p25,
codes for aa 142-214, suggesting, therefore, that the C-terminal
73 aa of this protein are sufficient to interact with TC48. The
protein expressed by the control plasmid pGBK7-7 (containing
the Gal4-DNA-binding domain) did not show any interaction
with p23 or p25 (Fig. 1A). The interaction of these proteins
with TC48 was very specific because they did not show any
interaction with TC45, a splice variant of TC48 that differs
only in a short C-terminal segment (Fig. 1A,C). This
experiment also showed that C-terminal sequences of TC48
(which are not present in TC45) are necessary for interaction
with p23 and p25. A C-terminal segment of TC48 coding for
C-terminal 66 aa interacted with p23 and p25 in the yeast two-
hybrid system (Fig. 1B). TC48 did not interact with SV40 T
antigen or p53 (data not shown), further showing that the
interaction with p23 and p25 is specific. The C-terminal 66 aa
of TC48 did not show any interaction with the protein
expressed by the control plasmid (Fig. 1B).

We determined the domain requirement of p23 and p25 for
interaction with TC48 by making deletion constructs (Fig. 2A).
Truncated p23 lacking the lumenal domain but containing C-terminal sequences (aa 140 to 219) was able to interact with TC48 in yeast (Fig. 2A,B). A smaller construct lacking the coiled-coil region and containing aa 185 to 219, did not show any significant interaction with TC48 (Fig. 2B). Similarly, the p25 clone obtained during screening, containing aa 142-214 (p25CC), was able to interact with TC48, but a smaller construct coding for aa 181 to 214 of p25 was not (Fig. 2B). These results suggest that, the C-terminal sequence of p25 and p23 (encompassing the coiled-coil, transmembrane and cytoplasmic domains) is sufficient for any interaction with TC48 and, the lumenal domain is not required for this interaction. Requirement of coiled-coil domain (which is involved in dimerization) indicates that p23 and p25 interact as dimers with TC48.

Next, we examined the interaction of TC48 with p23 and p25 in mammalian cells. The cDNAs expressing full-length GFP-TC48 and myc-tagged full-length human p23 or p25 were coexpressed in Cos-1 cells (Emery et al., 2000). After 36 hours of transfection cell lysates were prepared and immunoprecipitation was carried out with anti-myc monoclonal antibody or with a control antibody. TC48 was detected in the immunoprecipitate with anti-myc antibody but not in the immunoprecipitate with control antibody (Fig. 3A,B). This result showed that TC48 can interact with p23 and p25 in mammalian cells. Endogenous p23 and p25 also interacted with exogenous TC48, as determined by coimmunoprecipitation of GFP-TC48 from GFP-TC48-transfected Cos-1 cells with antibodies that recognize native p23 and p25 proteins (Fig. 3C).

TC48 localizes to Golgi and ER

Human TTC48 has been shown to localize to the ER (Lorenzen et al., 1995). Rat TC48 (also known as PTP-S4) has been shown to localize to nuclear membranes in addition to localizing to the ER (Kamatkar et al., 1996). In these studies, the localization of TC48 was seen after 48-64 hours of transfection with their respective expression vectors. We observed that, after 12 hours of transfection GFP-TC48 was localized in most of the cells to a Golgi-like dense structure near the nucleus (Fig. 4A). The GFP-TC48 plasmid expressed full-length TC48 fused to the C-terminus of GFP. Staining of GFP-TC48-expressing cells with the known Golgi marker protein giantin showed that TC48 colocalized with this protein (determined by confocal microscopy; Fig. 4A). Proteins reaching the Golgi complex pass through the ER-Golgi intermediate compartment (ERGIC) and it is known that ERGIC vesicles fuse with each other to form the cis-Golgi complex (Hauri et al., 2000; Ben-Tekaya et al., 2005). We stained GFP-TC48-expressing cells with antibody against
ERGIC-53 (a known ERGIC marker protein) and found that ERGIC-53 colocalized with GFP-TC48 (Fig. 4B). The localization of GFP-TC48 in the Golgi and ERGIC was not due to the presence of the GFP tag because HA-tagged TC48 also showed a similar subcellular localization (data not shown). The Golgi localization of TC48 was observed even in those cells which expressed very low levels of this protein, therefore, suggesting that the Golgi localization is not due to an overflow of excessive amounts of overexpressed protein.

At 15°C, the ER-to-Golgi transport of the anterograde transport marker protein VSVG is blocked in the ERGIC. HA-tagged TC48 and VSVG-GFP expression plasmids were co-transfected in Cos-1 cells, and after 15 hours at 40°C were incubated for a further 3 hours at 15°C. VSVG-GFP showed a punctate staining pattern (typical of ERGIC) in most of the cells (Fig. 4C) as expected. Some TC48 colocalized in these VSVG-GFP-rich structures (Fig. 4C), suggesting that a fraction of TC48 accumulates in the ERGIC at 15°C.

**TC48 moves between Golgi and ER**

During the localization studies of TC48 we made an interesting observation. Upon overexpression, TC48 initially showed predominant localization to the Golgi complex in most of the cells. However, at later time points (24-48 hours after transfection) it showed progressively increased levels in ER. Cos-1 cells were transfected with GFP-TC48 and cells were fixed 12, 24 or 48 hours after transfection. These cells were stained with antibody against the Golgi marker giantin. At 12 hours after transfection the majority of the cells showed TC48 predominantly in the Golgi complex. However, the number of cells showing a predominant Golgi localization decreased progressively with time. Representative areas of transfected cells showing localization of TC48 are shown in Fig. 4A. Colocalization with the Golgi marker giantin by confocal
microscopy confirmed that TC48 was indeed present in the Golgi. These observations suggest that TC48 moves from Golgi to ER. We further used the protein synthesis inhibitor cycloheximide to examine its effect on localization of TC48. Cos-1 cells were transfected with GFP-TC48 for 12 hours, incubated with cycloheximide for 6 hours and then fixed. Fixed cells were stained with giantin and calnexin (an ER marker) antibodies. The cycloheximide-treated cells showed predominant ER localization of TC48, as shown by increased colocalization with calnexin (Fig. 5A, lower panel), with little staining in the Golgi (Fig. 5A, upper panel). Cycloheximide treatment did not have any visible effect upon localization of giantin. These results suggest that, after its synthesis, TC48 moves from the ER to the Golgi and then back to the ER, possibly by a process of selective retrieval.

Microtubule-dependent transport of vesicles occurs between ER and Golgi compartments. Nocodazole treatment of cells is known to inhibit microtubule-dependent transport processes, leading to disintegration of the Golgi complex. The effect of nocodazole on TC48-expressing cells was examined after 12 hours of transfection. Nocodazole treatment for 2 hours resulted in the accumulation of GFP-TC48 in many vesicle-like structures throughout the cell (Fig. 5B) and also in tubular remnants of the Golgi complex, as seen by colocalization with giantin. We also checked the effect of another Golgi-perturbing drug brefeldin A (BFA) upon localization of TC48. BFA blocks budding and formation of transport vesicles by inhibiting Arf1 and leads to redistribution of Golgi enzymes to ER, resulting in the disassembly of Golgi stacks (Klausner et al., 1992). BFA treatment for 30 minutes disturbed the Golgi localization of TC48, indicating that the Golgi pool of TC48 is present in BFA-sensitive Golgi compartments (Fig. 5B).

Since TC48 interacts with p25 and p23, we examined the possibility of their colocalization in the cell. GFP-TC48 expressed in Cos-1 cells showed partial colocalization with endogenous p23 in the Golgi after 24 hours of transfection (Fig. 6A). Endogenous p25 also showed partial colocalization with GFP-TC48 in the Golgi (Fig. 6D).

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**Fig. 4.** Time course of TC48 localization in Cos-1 cells. (A) Cos-1 cells transfected with GFP-TC48 were fixed at 12 hours, 24 hours and 48 hours after transfection and stained with anti-giantin mouse monoclonal antibody (red) to stain the Golgi complex, and observed by confocal microscopy. Yellow colour generated in the merged images indicates colocalization of TC48 and Golgi-localized giantin. Notice the progressive increase of GFP-TC48 localization in ER-like reticular structures with increasing time. (B) Cos-1 cells overexpressing GFP-TC48 were fixed after 12 hours of transfection and stained with mouse monoclonal anti-ERGIC-53 antibody. Cy3-conjugated secondary antibodies were used to visualize ERGIC (red). Yellow colour generated in the merged image indicates colocalization of TC48 and ERGIC-localized ERGIC-53. (C) Cos-1 cells were transfected with plasmids expressing VSVG-GFP and HA-TC48. After 15 hours at 40°C, the cells were shifted to 15°C for 3 hours. Cells were fixed and stained with HA-tag antibody (red) and analyzed by confocal microscopy. Bars, 10 μm.
p23 and p25 affect TC48 localization

The p24 family members p23 and p25 are believed to be involved in biosynthetic transport of proteins. Since TC48 interacted with p23 and p25 we examined the localization of TC48 upon coexpression of p23 and p25. For this purpose Cos-1 cells were transfected with GFP-TC48 and myc-p23, which expresses mature p23 protein tagged with c-myc. This construct also expresses a signal peptide that precedes the myc-tag (Rojo et al., 2000). Myc-p23 was localized to membranous structures in majority of the cells (Fig. 6B) as shown by others (Rojo et al., 2000). TC48 showed colocalization with p23 in these perinuclear membranous structures as shown by the yellow colour in the merged image (Fig. 6B). This is probably not an artifact of overexpression because previously it has been shown that overexpression of this myc-p23 protein results in the formation of similar membranous structures which selectively relocalize endogenous p23 to these structures without altering the distribution of other Golgi proteins or other members of p24 family (Rojo et al., 2000). This result indicated that p23 interacts with TC48 in vivo. A mature form of rat p23 tagged with GFP at the N-terminus has been shown to localize to the cis Golgi network and the intermediate compartment (Blum et al., 1999). This GFP-p23 construct does not contain the signal peptide. GFP-p23 was co-transfected with rat TC48 in Cos-1 cells and after 24 hours the cells were stained with G11 monoclonal antibody, which recognizes TC48. Expression of GFP-p23 did not appear to affect localization of TC48, which showed colocalization with GFP-p23 (Fig. 6C).

To determine the effect of coexpression of p25 on TC48 localization, cells were transfected with GFP-TC48 and myc-p25, fixed and stained after 12 hours of transfection. Most of the cells expressing p25 and TC48 showed predominant localization of TC48 in the ER (Fig. 6E), whereas cells transfected with TC48 alone showed predominant Golgi localization (Fig. 4). Overexpressed p25 has been shown to be present predominantly in the ER and nuclear membrane (Dominguez et al., 1998; Emery et al., 2000). TC48 showed strong colocalization with p25 as seen by the yellow colour in the merged image (Fig. 6E). These results suggest that TC48 interacts with p25 in vivo and p25 is involved in targeting TC48 to ER.

The p25 protein has an ER-retrieval di-lysine
motif (KKxx) at its C-terminus. Inactivation of this di-lysine motif results in targeting of overexpressed mutant protein p25SS to Golgi and plasma membrane (Emery et al., 2003). We examined the effect of p25SS on subcellular localization of GFP-TC48. Coexpression of p25SS resulted in enhanced localization of GFP-TC48 to the Golgi (Fig. 6F) where it colocalized with p25SS. Most of the p25SS was present in the Golgi region, although some amount of p25SS was seen at the plasma membrane after 12 hours of transfection. After 24 hours of transfection the amount of p25SS increased in the plasma membrane but we could not detect GFP-TC48 in the plasma membrane. Inability of p25SS to target GFP-TC48 to ER and enhanced localization of TC48 in the Golgi suggest that the di-lysine motif (of p25) is essential for p25 to target TC48 to ER.

**Different regions of TC48 interact with p23 and p25**

Since p23 and p25 coexpression resulted in targeting of TC48 to distinct subcellular locations we reasoned that p23 and p25 might interact with different regions of TC48 within the C-terminal 66-aa domain. Therefore, a smaller construct of TC48 was made in which the C-terminal 40 aa (C-40) of TC48 were fused with the Gal4-DNA-binding domain. This protein interacted with p23 quite well in the yeast two-hybrid system (Fig. 7A). By contrast, the p25 protein showed insignificant interaction with the C-terminal 40 aa of TC48. The C-40, C-66 and full-length TC48-Gal4 DNA-binding-domain fusion constructs did not show any interaction with the protein expressed by the control plasmid (Fig. 7A). The interaction of p25 and p23 with GFP-C66 and GFP-C40, respectively, was confirmed in mammalian cells by coimmunoprecipitation experiments (Fig. 7B).

**Interaction with p25 is essential for the targeting of TC48 to the ER**

The interaction of p23 and p25 with different regions of TC48 within the C-terminal 66-aa domain suggested that these interactions are playing a role in transporting and/or targeting this protein to specific subcellular locations. Therefore, we made vectors expressing GFP fusion proteins with C-terminal sequences of TC48. Cos-1 cells were transfected with these expression plasmids and after 12, 24 and 48 hours of transfection, the cells were examined for subcellular location of these proteins. GFP-C66 protein (C-terminal 66 aa of TC48 fused with C-terminus of GFP) showed significant Golgi localization after 12 hours and then showed progressive increase in localization to the ER as observed with full-length TC48. The GFP-C40 protein showed predominantly Golgi localization after 12, 24 or 48 hours of transfection. A representative field is shown at 24 hours of transfection for GFP-C40.
Discussion

In general, ER-localization of ER-resident proteins occurs by two different mechanisms: (1) failure of the proteins to be recognized by ER-export machinery and (2) selective retrieval of proteins from post-ER compartments such as ERGIC or cis-Golgi though some proteins seem to exit the ER by a nonselective process (bulk flow) (Wieland et al., 1987; Harter and Wieland, 1996). The protein tyrosine phosphatase TC48 has been shown by Lorenzen et al. to localize in the ER through ER retention signals located in the C-terminal 70 aa; they studied the localization of overexpressed TC48 after 64 hours of transfection in Cos-1 cells (Lorenzen et al., 1995). In the present study, we observed that TC48 shows significant localization to Golgi complex and ERGIC after 12-24 hours of transfection, as determined by colocalization with giantin and ERGIC-53. There was progressive increase in ER localization with time and predominant ER localization was shown after 48 hours of transfection. Accumulation of TC48 was seen in ERGIC at 15°C. These results suggest that, after synthesis, the TC48 protein moves from ER to ERGIC and Golgi complex through the biosynthetic protein transport machinery and then it is retrieved into ER. This suggestion is supported by the observation that, upon treatment with cycloheximide, TC48 is largely depleted from the Golgi. Association of TC48 with the Golgi complex is also supported by the observation that GFP-C40 localizes primarily to the Golgi showing the presence of ER export signal as well as a Golgi localization signal in the C-terminal 40 aa of TC48.

Role of p25 in targeting of TC48 to ER

The transmembrane proteins p25 and p23 were found to interact with TC48 in yeast as well as in mammalian cells. Co localization experiments suggest that TC48 interacts with p25 and p23 in vivo. This interaction was specific and mediated by C-terminal sequences of TC48. The p25 and p23 proteins (and other p24 family proteins) have been reported to play a role in protein transport in early biosynthetic pathways (Kaiser, 2000; Nickel et al., 2002). For p25 protein two different roles have been suggested. p25 has been shown to control the fidelity of membrane transport by maintaining cholesterol-poor membrane in the Golgi complex. p25 protein has the intrinsic capacity to form highly specialized domains that control membrane composition and dynamics. In addition, p25 protein also plays a crucial role in the retention of members of p24 family in early biosynthetic membranes, presumably by anchoring them in the COP-I recycling pathway (Emery et al., 2003).

Based on the results presented here, we suggest that p25 protein plays an important role in targeting TC48 to the ER by direct binding. This suggestion is supported by the following observations: (1) p25 interacts with the same C-terminal sequence of TC48 that is sufficient for targeting to the ER; (2) coexpression of p25 enhances ER localization of TC48; (3) coexpression of a p25 mutant that is defective in the ER-retrieval di-lysine motif is unable to enhance ER localization of TC48. Instead, Golgi localization of TC48 is increased in the presence of this mutant p25. Additional support is provided by the observation that the C-terminal 40 aa of TC48, which are not sufficient for interaction with p25, do not target GFP to the ER. Instead, the GFP-C40 protein ends up in the Golgi.
p25 targets TC48 phosphatase to ER

Possible role of p23 in biosynthetic transport of TC48

Although the interaction of TC48 with p25 protein appears to play an important role in targeting TC48 to the ER, the significance of interaction with p23 is not clear. Endogenous p23 protein is present in the cis-Golgi network in high amount (Sohn et al., 1996; Rojo et al., 1997; Barr et al., 2001). Therefore, one possibility is that the interaction with p23 plays a role in transporting or targeting TC48 to the Golgi. Some support for this suggestion is provided by the observation that the C-terminal 40 aa of TC48, which are sufficient for interaction with p23, are also sufficient for targeting GFP to the Golgi complex. Since p23 binds to coatomers and is enriched in COPI vesicles (which are involved in retrograde transport), p23 by itself or together with p25 is probably involved in the retrieval of TC48 from the Golgi and other post ER compartments to the ER. An indication for the role of endogenous p23 for proper subcellular targeting of TC48 is provided by the following observation. Overexpression of myc-p23 results in trapping of TC48 (and GFP-C40) in myc-p23-rich membranous structures. Previously it has been shown that, expression of myc-p23 selectively relocates endogenous p23 to the myc-p23-rich membranous structures without altering the distribution of other Golgi proteins or other members of p24 family (Rojo et al., 2000). Therefore, trapping of TC48 in p23-rich membranous structures indicates a requirement of endogenous p23 in cis Golgi for proper subcellular targeting of TC48.

Previously it has been shown that, the C-terminal hydrophobic tail and residues within the region 346-358 must act in concert to target TC48 to the ER (Lorenzen et al., 1995). The hydrophobic tail lies in the region of TC48 that interacts with p23 (our data). The second ER-targeting sequence (346-358 aa) identified by Lorenzen et al. (Lorenzen et al., 1995) overlaps with the region required for interaction with p25. Therefore, it seems likely that interactions with both p23 and p25 play a role in targeting and/or transporting of TC48 to Golgi and ER. Our results suggest that the C-terminal 66 aa of TC48 that begin at residue 350 are sufficient to target GFP to the ER, whereas Lorenzen et al. found that internal deletion of residues 346-349 in a 100-aa construct (316-415 aa) results in loss of ER retention of β-galactosidase (Lorenzen et al., 1995). This apparent discrepancy may be due to the requirement of residues 346-349 for proper folding of TC48.

It is worthwhile to mention that the fusion protein of GFP and the C-terminal

![Fig. 8. Localization of GFP-C40 and GFP-C66 fusion proteins. Cos-1 cells transfected with vectors expressing GFP-C40 (A) or GFP-C66 (B) were allowed to express exogenous cDNA for 24 hours. Cells were fixed and stained with anti-giantin mouse monoclonal antibody (red) to stain the Golgi complex, and observed by confocal microscopy. Yellow colour indicates colocalization of GFP-C40 or GFP-C66 and Golgi-localized giantin. (C) GFP-C66 and myc-p25 expression plasmids were co-transfected in Cos-1 cells; after 12 hours of transfection the cells were fixed, stained with anti-myc antibody (red) and observed by confocal microscopy. (D) GFP-C40 and myc-p23 expression plasmids were co-transfected in Cos-1 cells; after 12 hours of transfection the cells were fixed, stained with myc antibody (red) and observed by confocal microscopy. Bars, 10 μm.](image)
40 aa of TC48 shows prominent Golgi localization, indicating that the C-terminal 40 aa of TC48 contain some ER-export signals. By carefully analyzing the sequence of TC48, we found that it does contain three known ER-export motifs, a di-basic RR-motif (RRKKKR) at the start of this 40 aa sequence (Fiedler et al., 1996; Marks et al., 1997; Sevier et al., 2000; Giraudo et al., 2003). Presence of these ER-export motifs indicates the possible functional significance of TC48 localization in post ER-compartment where TC48 might regulate some Golgi-emanated signal transduction pathways. Recently, there have been reports from various laboratories regarding the role of Golgi-associated proteins in regulating various cellular functions (Donaldson and Lippincott-Schwartz, 2000; Preisinger and Barr, 2001; Alton-Bonnet et al., 2003; Alton-Bonnet et al., 2004). The Src family kinases Hck and Src are present to some extent in the Golgi complex (Carreno et al., 2000; Bard et al., 2002; Radha et al., 2004). Tyrosine phosphorylation might be involved in pathways regulated by these kinases (Bard et al., 2003), which might be affected by Golgi-localized TC48.

How does p25 help in targeting of TC48 to the ER? There are at least two possibilities: (1) p25 might be involved in anchoring TC48 to the ER or (2) it might transport TC48 to the ER from post-ER compartment. These two possible explanations are not necessarily mutually exclusive. The known properties of p25 and p23 suggest that both proteins are probably involved in transporting TC48 to its proper subcellular locations.

In summary, this article is the first report to show a physical interaction of the p24-family member p25 with a cargo protein, TC48, in vivo in mammalian cells and adds on the suggested role of p24-family members p23 and p25 in vesicular transport of proteins between Golgi and ER. The interaction with p25 plays a crucial role in the targeting of TC48 to ER and the interaction with p23 might be involved in taking TC48 through the Golgi also helping in targeting it to the ER. The interactions with TC48 provide a model to further study the details of the mechanism by which p25 and p23 transport and target a cellular protein to ER and Golgi in mammalian cells. This manuscript also sheds light on the possible role of p24-family proteins in targeting cell signaling molecules to the organelles of the early secretory pathway.

Materials and Methods

Cell culture and transfections

Cos-1 cells were grown as monolayers in a humidified atmosphere of 5% CO₂ at 37°C in Dulbecco's Minimum Essential Medium (DMEM) containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Transfections were performed on cells grown as a monolayer in either 60-mm dishes or glass coverslips using the cationic lipid DHDEAB as described (Bamerjee et al., 1999; Radha et al., 2004). Briefly, 1 μl lipid diluted in 50 μl serum-free DMEM was mixed with 1 μg DNA in 50 μl serum-free DMEM. The mixture was kept at room temperature for 30 minutes to allow complex-formation before adding it to the (60-80% confluent) cell monolayer. Cells were fed with serum 4 hours later and harvested at indicated time points after transfection. Cyclchoeximide (10 μg/ml, Sigma), nocodazole (5 μg/ml, Calbiochem) or brefeldin A (BFA, 5 μg/ml, Sigma) were added to the medium at indicated concentrations.

Antibodies

Mouse monoclonal anti-HA and anti-myc antibodies were from Roche Molecular Biochemicals and Oncogene Research Products, respectively. The mouse monoclonal G11, which recognizes rat TC45, TC48, overexpressed human TC45 and TC48 proteins, was made in our laboratory (Radha et al., 1994). Mouse monoclonals anti-ERGIC-53 and anti-giantin were kind gifts from Hans-Peter Hauri (Biocentre, Basel, Switzerland) (Schweizer et al., 1988; Linstedt and Hauri, 1993). Rabbit antibodies that recognize p23 and p25 proteins were kindly provided by Felix Z. T. Wieland (Heidelberg, Germany) and have been described before (Jenne et al., 2002). Cy-3-conjugated anti-mouse and anti-rabbit IgGs were from Amersham. Texas-Red-conjugated anti-mouse and anti-rabbit IgGs were from Vector Labs.

Expression vectors

All cloning experiments were performed according to standard procedures. Full-length TC48 was amplified from a cloning-vector clone containing full-length TC-PTP (Cool et al., 1989), with gene-specific primers containing EcoRI and SalI restriction sites at its ends. The resulting PCR product was cloned in the pMOS cloning vector (Amersham) and its sequence was verified by automated sequencing. TC48 cDNA was excised with specific restriction enzymes and cloned in the yeast two-hybrid expression vector pGBK7T (Clontech), mammalian expression vectors pcDNA 3.1 (HA) (Invitrogen) and pEGFP-C3 (Clontech). TC45 cDNA was cloned in various vectors the same way as TC48. DNA sequences coding for the C-terminal 66 aa or 40 aa of TC48 were amplified with specific primers and cloned in the yeast two-hybrid expression vector pGBK7T and also in the GFP-expression plasmid pEGFP-C2. The constructs in pEGFP-C2 produce GFP-C66 and GFP-C40, in which the C-terminal 66 aa or 40 aa of TC48 are fused at the C-terminus of GFP. The nucleotide sequence of all constructs was confirmed by automated DNA sequencing. Rat TC48 cloned in the mammalian expression vector pCB6 has been described before (Kamatkar et al., 1996). Myc-p23, myc-p25 and myc-p25SS (in which the ER-retrieval motif KKKxx is inactivated by mutation of KK to SS) expression plasmids have been described before (Emery et al., 2000; Rojo et al., 2000) and were kindly provided by Jean Graevenburg (University of Geneva, Switzerland). These plasmids express mature human p23 and p25 proteins with a myc-tag at the N-terminus of the molecule. GFP-tag is preceded by the signal peptide. The GFP-p23 expression plasmid, which expresses the mature form of rat p23 tagged with GFP at the N-terminus was kindly provided by Irene Schulz (University of Saarland, Germany) (Blum et al., 1999). This construct does not contain a signal peptide. Smaller constructs of p23 and p25 were prepared by amplifying the required region by PCR and cloning in the EcoRI and XhoI sites of pEGFP-C2. A GFP-labeled vesicular stomatitis virus G-protein (VSVG-GFP tsO45) expression plasmid was kindly provided by Jennifer Lippincott-Schwartz (NIH, Maryland, USA) and has been described before (Presley et al., 1997).

Yeast two-hybrid screening

The full-length TC48 protein was used as a bait. Yeast strain PJ69-4A was used for two-hybrid analysis (James et al., 1996). Expression of TC48 fused to the Gal4-DNA-binding domain (expressed by the construct in a pGBK7T vector) was checked in western blot with lysates from yeast cultures grown in Trp dropout liquid culture. The TC48 (pGBK7T) expressing PJ69-4A strain was then transformed with human placental cDNA library (Clontech). Transforms were plated on yeast dropout medium lacking Trp, Leu and Ade. A small plate (0.1%) of transformant mix was plated to determine the efficiency of transformation on the Leu and Trp dropout medium plate. The positives obtained on Trp’, Leu’ and Ade’ plates were cloned for activation of positive clones reporter plasmids containing pTrp and Leu dropout medium adjusted to pH 7.0 and β-galactosidase substrate. Plasmids were isolated from positive yeast colonies, propagated in E. coli and then sequenced. The interactions were confirmed by retransformation of positive clones with TC48 or control plasmids. Yeast culture reagents and media were obtained from Himedia and Sigma.

Indirect immunofluorescence and confocal microscopy

Indirect immunostaining of cells and microscopy was carried out essentially as described (Kamatkar et al., 1996; Radha et al., 2004). Cells grown on coverslips were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 10 minutes at room temperature and then washed thrice in PBS buffer. Fixed cells were permeabilized for 6 minutes in PBS containing 0.5% Triton X-100 (Sigma) and 0.05% Tween-20 (Sigma). Permeabilized cells were washed thrice in PBS and incubated with 2% BSA (Sigma) in PBS for 1 hour. Following this, the cells were incubated with primary antibodies diluted in blocking solution for 1 hour at room temperature. Antibodies were removed and the cells were washed with PBS three times. Cells were then incubated with secondary antibodies for 1 hour at room temperature, washed three times in PBS and finally in H₂O. Labeled cells were mounted in Vectashield mounting medium containing DAPI (Vector labs). Colocalization was determined by observing the staining patterns, with the LSM 510 Meta Confocal Microscope from Carl Zeiss. Serial optical sections in the Z-axis of the cell were collected at 0.33 μm or 0.5 μm intervals with 63× oil immersion objective lens (NA 1.4). Then, two to three middle optical sections were projected and colocalization was observed in a total thickness of 1 μm by using LSM 510 (version 3.2) software. Immunofluorescence staining and colocalization was also observed with a Zeiss LSM 510 Meta 2 microscope. The Apoptome (from Carl Zeiss Microimaging) is a new 3D imaging system for contrast-enhancement in fluorescence microscopy. It uses structured illumination to reject signals belonging to regions of the sample that are outside the best focus position.
of the microscope. Images were captured using the Axioscan (Zeiss) CCD camera and processed with Axiosvision 4.0.

Coimmunoprecipitation and western blotting

Cos-1 cells in 60-mm dishes were co-transfected with GFP-TC48 and myc-p23 or myc-p25 (pCB6). Thirty six hours later, cells were washed twice with ice-cold PBS and then lysed at 4°C for 20 minutes in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 0.1% BSA, 5 mM EDTA and protease inhibitor cocktail (Roche Biochemicals). Lysates were centrifuged at 10,600 g for 10 minutes at 4°C and the supernatant was used for immunoprecipitation using 2 μg of c-myc monoclonal antibody or 2 μg of normal mouse IgG as control antibody. Supernatants were incubated overnight with antibodies. Then, 20 μl protein A/G agarose beads (Santa Cruz) were added for another hour at 4°C. After three washes in wash buffer (20 mM Hepes pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM PMSF, protease inhibitors) and after adding 20 μl of 2× SDS-PAGE sample buffer, the beads containing immune-complexes were boiled. The samples were resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane for western blot analysis. Membranes were blocked with 5% nonfat dried milk (Santa Cruz) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and immunostained with the required primary antibodies. Membranes were then washed three times with TBST and incubated with anti-mouse secondary antibodies (1:5000; Amersham) conjugated to horseradish peroxidase. Bands were visualized with the ECL western blotting detection system (NEG Life Sciences).

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