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Control of p62 binding to TGN38/41 by phosphorylation

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Abstract TGN38/41 cycles between the *trans*-Golgi network (TGN) and plasma membrane, traversing three sorting compartments: the TGN, plasma membrane and early endosome. The targeting signals responsible for this complex itinerary reside in a short cytoplasmic domain of 33 amino acid residues. We show that phosphorylation of the cytoplasmic domain of TGN38 prevents binding of p62 — a cytoplasmic protein essential for exocytic vesicle formation. Thus the cycle of TGN38/41 traffic, and by implication the pathway of exocytosis, could be controlled by phosphorylation of the TGN38 cytoplasmic domain.

Key words: Exocytosis; Membrane traffic; Targeting signal; Trans Golgi network; TGN38 phosphorylation

1. Introduction

TGN38/41 is a type I membrane glycoprotein predominantly located in the TGN [1]. Recent studies have shown that the cytoplasmic domain of TGN38/41 interacts with a cytosolic, autophosphorylating kinase called p62 in an essential step in exocytic vesicle formation [2,3]. A peptide corresponding to the last 12 amino acid residues of TGN38 (CKASDYQRLNLKL) was able to prevent exocytic vesicle formation [2], implying that p62 interacts principally with this sequence.

TGN38/41 becomes incorporated into the nascent exocytic vesicle and travels to the cell surface from which it is recycled back to the TGN [4–7]. Two targeting signals have been described for TGN38, a retention signal within the transmembrane region [8], and a tyrosine tetrapeptide motif (YQRL) located in the cytoplasmic domain [6,7,9]. The tyrosine and leucine residues of the YQRL moitif have been shown to be most important for internalisation of TGN38/41, suggesting that this signal is similar to that used for internalisation of recycling receptors [10,11]. Thus the signals for traffic of TGN38/41 in both directions are contained within the same 12 residue sequence motif.

We show here that p62 binds to the unphosphorylated form of the cytoplasmic domain of TGN38 and directly interacts with serine 24 (numbering from the start of the cytoplasmic domain), adjacent to the tyrosine internalisation motif. Furthermore, phosphorylation of the cytoplasmic domain at serine 15 or threonine 18 by PKC inhibits binding of p62. Thus the membrane traffic of TGN38/41 could be controlled by cycles of phosphorylation and dephosphorylation determining entry into the exocytic and endocytic pathways.

2. Material and methods

2.1. DNA cloning and protein preparation

The cDNA encoding residues 5–33 of the cytoplasmic domain of TGN38 (amino acid sequence in single letter code: IIAFALE-GKRS[15]KVT[18]RRPKAS[24]DYQRLNLKL, numbers identify possible phosphorylation sites) was amplified by polymerase chain reaction using primers that inserted a *Bam*HI site and two codons encoding glycine residues on the 5′ side of the sequence in the same reading frame as glutathione *S*-transferase (GST) and an *Eco*RI site downstream of the stop codon of TGN38. The amplified fragment was cloned into pGEX-2T cut with *Bam*HI and *Eco*RI and expressed in JPA 101 cells. The fusion protein containing the S24A mutation was generated by the same strategy as the wild-type TGN38 tail fusion protein except that a primer was used having the codon for serine changed to alanine. The GST fusion proteins were expressed and purified as described [12].

2.2. Phosphorylation

Each 100 μl reaction contained 50 μg of purified fusion protein and 30 μCi [^{32}P]orthophosphate. The reaction was started with the addition of kinase to the reaction mixture for 5 min at 37°C. Reaction buffers for the purified kinases were as recommended by the supplier (PKA/PKC: Boehringer Mannheim, CKII: Promega). For Fig. 1, lane 5, reaction buffer for PKC was used. The reaction was stopped by cooling and addition of glutathione-agarose beads in buffer A (20 mM HEPES, 300 mM KCl, 50 mM EDTA and 20% (v/v) glycerol) containing 0.1 mM pefablock, 5 μg/ml aprotinin, 1 mM benzamidine, 50 mM NaF and 2 mM sodium vanadate. After 15 min binding at room temperature, the beads were washed 3 times in buffer A and then the fusion proteins eluted in PAGE sample buffer containing 4% SDS. Fifty percent of the reaction mix was separated on a 12% polyacrylamide gel which was then dried and analysed on a phosphorimage analyser (Berthold/Fuji). Images were quantitated as PSL and corrected for equal protein loading (in arbitrary units) from the scanned Coomassie Brilliant Blue stained gels.

2.3. Cytosol fractionation

A cytosol fraction was made from rat liver homogenate by sequential centrifugation at 10,000 × *g* for 10 min and 100,000 × *g* for 60 min. The supernatant from the second centrifugation step was enriched in p62 by ion exchange chromatography on DEAE-Sepharose eluted by stepwise (0.05 M) increases in NaCl concentration. A fraction eluted in 0.15 M NaCl was found to contain the majority of the p62 by Western blotting. This material was concentrated and further purified by gel filtration on a 1.6 × 50 cm S-200 sephacryl column. p62 comprised approximately 5% of the enriched cytosol proteins as judged by Coomassie staining of SDS gels.

2.4. Immune depletion

Fifty microlitre samples of p62-enriched cytosol containing 110 μg of protein were incubated with 10 μl of pre-immune serum or anti-p62 antiserum overnight at 4°C. The IgG was then removed from the cytosol fraction by incubation at 4°C for 2 h with 50 μl of a 1:1 slurry of protein A-Sepharose. Protein A-Sepharose was removed by centrifugation at 14,000 × *g* for 2 min.

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Abbreviations: CKII, casein kinase II; DTSST, 3,3′-dithiobis-(succinimidylpropionate); GST, gluthaione *S*-transferase; MPR, mannos 6-phosphate receptor; NRK, normal rat kidney; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PSL, photostimulatable luminescence; TGN, *trans*-Golgi network.

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2.5. p62 binding and cross linking

GST–TGN38 fusion protein was phosphorylated with various kinases as described in Fig. 1 and then purified on glutathione-agarose beads. After 3 washes in buffer A, 1 ml of fresh rat liver cytosol (100,000 × *g* for 60 min supernatant) was added for 2 h at room temperature. DTSSP in 5 mM sodium citrate buffer, pH 5, was added to a final concentration of 0.4 mM and incubated for a further 30 min at room temperature. The remaining cross-linker was quenched in 50 mM Tris, pH 7.5, for 15 min on ice and the agarose beads then washed 3 times in 6 M urea, 20 mM Tris, pH 7.5, containing protease and phosphatase inhibitors. The disulphide cross-link was cleaved in PAGE sample buffer containing 50 mM DTT and the proteins separated on a 12% SDS gel. p62 was detected by Western blot and enhanced chemiluminescence detection (Amersham).

3. Results and discussion

We have studied the phosphorylation of the TGN38 cytoplasmic domain expressed as a fusion protein with bacterial glutathione *S*-transferase (GST–TGN38). PKC phosphorylated the GST–TGN38 fusion protein more efficiently than PKA and CKII was without effect (Fig. 1). Rat liver cytosol, and cAMP-stimulated Golgi membranes (sources of PKC and PKA in vivo) could also phosphorylate the fusion protein (data not shown). There was no phosphorylation of GST alone by any kinase tested, or of GST–TGN38 in the absence of added kinase (Fig. 1, lanes 4 and 5). It is perhaps not surprising that PKC was the most effective kinase as both serine residues (S15, S24) and the threonine residue (T18) in the TGN38 cytoplasmic domain fall within consensus sequences for phosphorylation by this enzyme [13].

We next explored the possibility that p62, which has been shown to autophosphorylate [4], was able to phosphorylate the TGN38 cytoplasmic domain in the GST fusion protein. To our surprise we found that depleting a cytosol fraction with anti-p62 antiserum (Fig. 2, lanes 1 and 2) actually increased the amount of GST–TGN38 phosphorylation, suggesting that p62 inhibited the phosphorylation reaction (Fig. 2, lanes 3 and 4). A possible explanation of this result is that p62 binds at, or close to, the site of phosphorylation on the TGN38 cytoplasmic domain, thus masking it from kinases in the cytosol fraction. An extension of this line of reasoning is that phosphorylation of the cytoplasmic domain of TGN38 might prevent p62 binding. We tested this hypothesis by incubating phosphorylated and non-phosphorylated GST–TGN38 fusion protein with a fresh cytosol fraction and then analysing the amount of p62 bound by affinity purification on agarose beads and then Western blotting. Fig. 3 shows that p62 binding was strongly inhibited by PKC phosphorylation of the GST–TGN38 fusion protein (inhibition was 74 ± 7%, mean ± SEM). In the assay shown in Fig. 3A we cross-linked the proteins interacting with GST–TGN38 to allow more stringent washing conditions without loss of signal. Similar experiments without cross-linking and with 0.5 M salt washes gave a similar result, but with a lower signal-to-noise ratio. Interestingly, when PKA was used as kinase, binding of p62 was not disrupted, suggesting that PKA phosphorylates different residues compared with PKC. As expected, CKII, which does not phosphorylate the fusion protein (Fig. 1), did not prevent p62 binding (Fig. 3A).

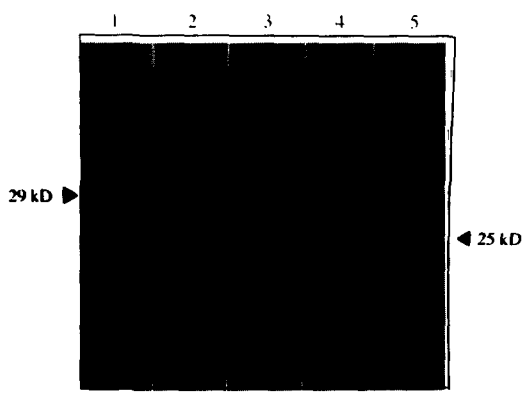

Fig. 1. PKC and PKA can phosphorylate the cytoplasmic domain of TGN38 in vitro. Fifty micrograms of purified GST–TGN38 fusion protein were incubated with purified kinases at 37°C for 5 min as follows: lane 1 = 0.05 mU (Casein) PKC; lane 2 = 0.4 units (Kemptide) PKA; lane 3 = 4 units of casein kinase II; lane 4 = GST alone; lane 5 = no kinase added.

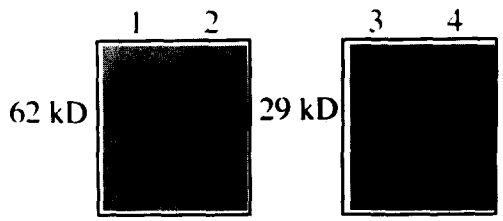

Fig. 2. p62 inhibits TGN38 phosphorylation in vitro. A rat liver cytosol fraction was treated with a pre-immune serum (lanes 1 and 3) was or immunodepleted with anti-p62 serum (lanes 2 and 4) and tested for phosphorylation activity as described in Fig. 1 (using a general phosphorylation buffer: 2 mM $MnCl_2$, 10 mM $MgCl_2$, 50 mM Tris, pH 7.5, 1 mM $CaCl_2$, 25 μM ATP and 30 μCi [^{32}P]orthophosphate). Lanes 1 and 2 show a Western blot using anti-p62 antibody showing that depletion of p62 was effective (the lower band in lane 2 has the mobility of the antibody IgG heavy chain used for immunodepletion). Lanes 3 and 4 show the [^{32}P]orthophosphate incorporated into GST–TGN38 fusion protein.

The YQRL motif in TGN38 is preceded by a serine residue (SDYQRL) that has been implicated [9] as being important in determining the correct intracellular location of TGN38. We therefore made a fusion protein between GST and the TGN38 cytoplasmic domain in which this serine was changed to alanine (GST–S24A), expecting that this mutant might bind p62 regardless of phosphorylation. Surprisingly, the S24A fusion protein exhibited much reduced p62 binding compared to wild-type (Fig. 3B, compare lanes 1 and 2), suggesting that p62 interacts directly with this residue. PKC was able to phosphorylate the S24A mutant as well as the wild-type fusion protein (13396 ± 1960 compared with 14572 ± 1450 mean PSL/h/unit protein ± S.E.M.), indicating that this serine is not a target for phosphorylation by PKC. Furthermore, PKC phosphorylation of S24A abolished the residual binding of p62 (Fig. 3B, lane 4) suggesting that either, or both, S15 or T18 are the principal targets for PKC phosphorylation and control p62 binding. The S24A mutant was phosphorylated by PKA to a lower degree than wild-type GST–TGN38 (1208 ± 188 compared with 2062 ± 305 mean PSL/h/unit protein ± S.E.M.), but this phosphorylation did not abolish the residual p62 binding that was apparent when using PKC (Fig. 3B, compare lanes 6 and 4). This confirmed the result in Fig. 3A.

The ability of phosphorylation of the TGN38 cytoplasmic domain to prevent p62 binding suggests that phosphorylation might control TGN38/41 membrane traffic by flipping the structure of the TGN38 cytoplasmic tail between a p62 binding

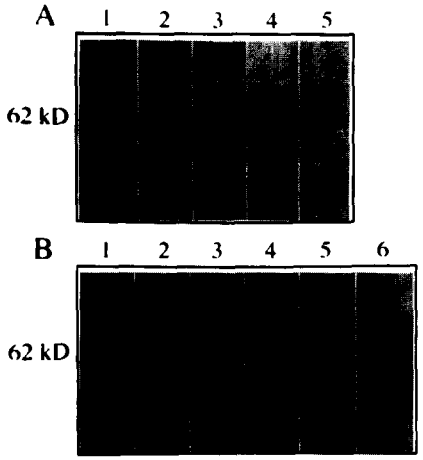

Fig. 3. p62 binding to TGN38 is prevented by PKC phosphorylation and mutation of serine 24 of the TGN38 tail. (A) Effect of phosphorylation on p62 binding. Lane 1 = unphosphorylated control; lane 2 = +PKC; lane 3 = +PKA; lane 4 = +casein kinase II; lane 5 = GST alone. (B) Effect of S24A mutation on p62 binding. Lanes 1, 3 and 5 = GST–TGN38 wild type; lanes 2, 4 and 6 = GST–S24A. Kinases added were: lanes 1 and 2, none; lanes 3 and 4, PKC; lanes 5 and 6, PKA.

structure found in the TGN (unphosphorylated) and an AP-2 binding structure found on the cell surface (phosphorylated). In support of this hypothesis it has been shown that the (unphosphorylated) TGN38 cytoplasmic tail peptide assumes a 'nascent helix' conformation in solution [14] and not a tight turn as proposed for internalisation signals [10]. Thus p62 might recognise the nascent helix form of the cytoplasmic domain, and AP-2 the phosphorylated, presumably tight turn, conformation. It will be interesting to determine the 2D NMR structure of the phosphorylated TGN38 cytoplasmic domain peptide to see if this is the case. Further evidence comes from the observation [15] that treatment of NRK cells with okadaic acid leads to a 10-fold increase in surface TGN38/41. In the presence of okadaic acid, TGN38/41 would remain phosphorylated and become locked into the surface recycling pool. Another clue to the mechanism of TGN38/41 membrane traffic is the observation that mutation of R28 of the YQRL motif to D destroys internalisation of TGN38/41 beyond the early endosome [7]. On our hypothesis this could be due to inhibition of phosphatase recognition of the cytoplasmic tail.

A parallel situation has been described for the recruitment of AP-1 adaptors onto clathrin-coated vesicles at the TGN. Here, the casein kinase II-dependent phosphorylation of serine residues on the cation-independent MPR has been shown to be important for entry of this receptor into vesicles targeted to the late endosomes [16–19]. Thus transient phosphorylation may be a general mechanism for controlling the presentation of targeting signals to the cytosolic sorting machinery, especially for proteins like TGN38/41 and the MPR which traverse more than one sorting compartment in the cell.

Although exocytosis has been regarded as a constitutive process, implying a lack of control mechanisms, evidence is accumulating to indicate that both individual steps in the pathway, and the overall rate of release of secreted protein may be subject to control mechanisms involving kinases [20–22], heterotrimeric G proteins [23–25] and calcium [26]. Our data suggest that increased phosphorylation retards the rate of exocytic vesicle formation in as much as p62 recruitment onto TGN38/41 is required for this process. Phosphorylation has also been reported to inhibit the traffic of membrane proteins from ER to Golgi [20], between endosomes [27] and from recycling endosomes to the cell surface [28]. In contrast, exocytosis of secretory proteins can be stimulated by PKC [21], perhaps reflecting the different pathways of exocytosis for membrane and secretory components [29,30]. p62-dependent vesicle formation at the TGN may therefore be the mechanism of exocytosis for membrane proteins.

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