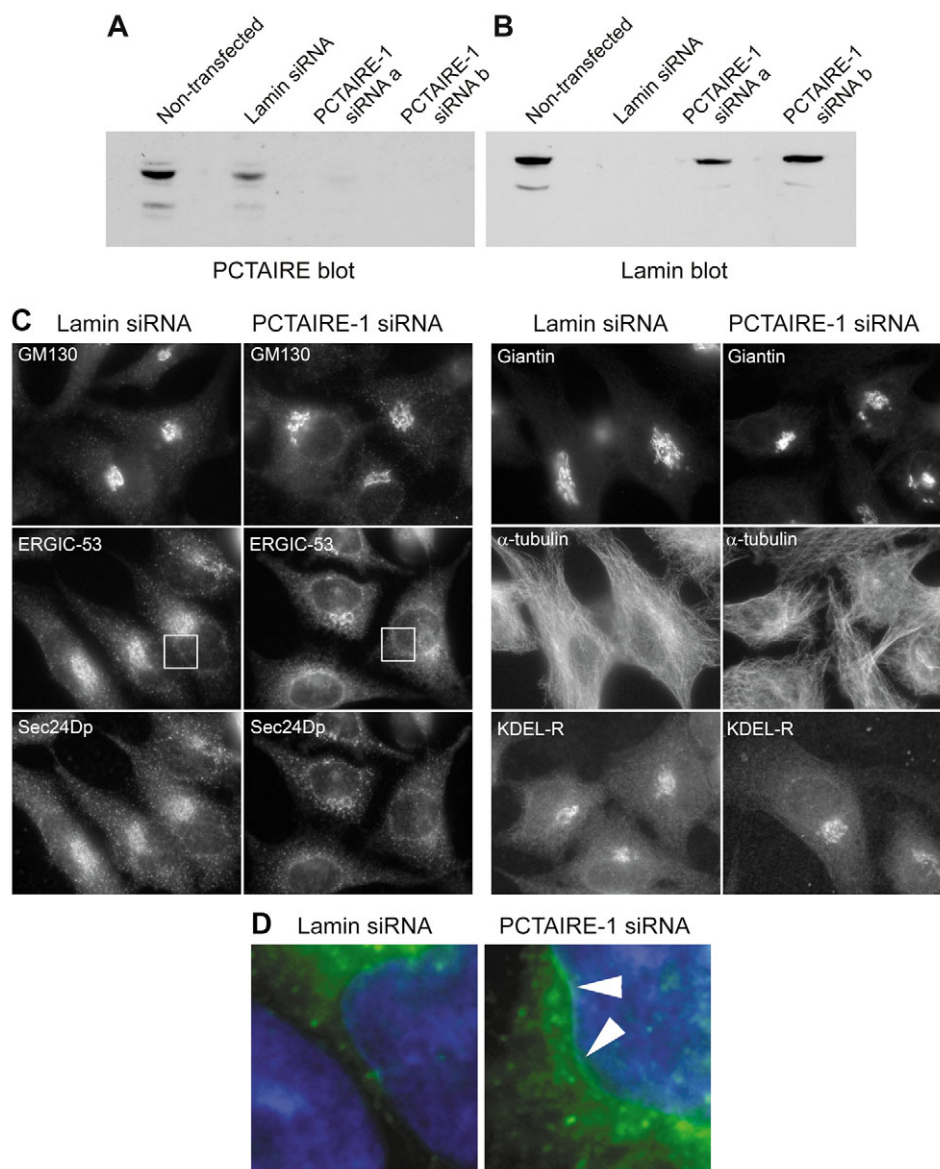


Palmer, K. J., Konkol, J. E. and Stephens, D. J. (2005). PCTAIRE protein kinases interact directly with the COPII complex and modulate secretory cargo transport. *J. Cell Sci.* **118**, 3839-3847.

In both the online and print versions of this paper, panels C and D of Fig. 4 were incorrectly labelled. The correct figure is shown below.



PCTAIRE protein kinases interact directly with the COPII complex and modulate secretory cargo transport

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Accepted 13 May 2005

Journal of Cell Science 118, 3839-3847 Published by The Company of Biologists 2005

doi:10.1242/jcs.02496

Summary

The export of secretory cargo from the endoplasmic reticulum is mediated by the COPII complex. In common with other aspects of intracellular transport, this step is regulated by protein kinase signalling. Recruitment of the COPII complex to the membrane is known to require ATP and to be blocked by the protein kinase inhibitor H-89. The identity of the specific protein kinase or kinases involved remains equivocal. Here we show that the Sec23p subunit of COPII interacts with PCTAIRE protein kinases. This interaction is shown using two-hybrid screening, direct

binding and immunoprecipitation. Inhibition of PCTAIRE kinase activity by expression of a kinase-dead mutant, or specific depletion of PCTAIRE using RNAi, leads to defects in early secretory pathway function including cargo transport, as well as vesicular-tubular transport carrier (VTC) and Golgi localization. These data show a role for PCTAIRE protein kinase function in membrane traffic through the early secretory pathway.

Key words: PCTAIRE, COPII, Protein kinase, Membrane traffic

Introduction

In mammalian cells, secretory protein transport proceeds by the accumulation of secretory cargo into ER exit sites (ERES) followed by budding of vesicular and/or tubular carriers. These then merge, probably through a combination of tethering and fusion, to form a vesicular-tubular transport carrier (VTC) (reviewed by Bonifacino and Glick, 2004; Stephens and Pepperkok, 2001). Accumulation of secretory cargo into ERES is mediated by the COPII coat complex (Barlowe, 2002; Barlowe et al., 1994). This multimeric complex is assembled onto ERES in response to GTP binding by Sar1p. This subsequently recruits the Sec23p-Sec24p heterodimeric complex and a further complex comprising of Sec13p-Sec31p. The COPII coat can directly interact with transmembrane cargo through the Sec23p-Sec24p heterodimer (Miller et al., 2003; Mossessova et al., 2003). The process of cargo export from the ER is largely constitutive but there is now increasing evidence for regulation of this process by protein phosphorylation.

Protein phosphorylation modulates many membrane traffic processes throughout the cell including inhibition of traffic and fragmentation of the Golgi apparatus during mitosis (Lowe et al., 2000; Lowe et al., 1998), inhibition of ER export through cdc2-dependent disassembly of ERES (Farmaki et al., 1999; Kano et al., 2004), modulation of secretory cargo transport from the trans-Golgi network (TGN) by protein kinase D (Prigozhina and Waterman-Storer, 2004; Van Lint et al., 2002; Yeaman et al., 2004) and regulation of endocytosis and many other pathways by CDK5 (Smith and Tsai, 2002). Evidence also exists for a role of protein phosphorylation in the regulation of bidirectional transport between the ER and Golgi (Aridor and Balch, 2000; Cabrera et al., 2003; Jamora et al.,

1999; Lee and Linstedt, 2000; Muniz et al., 1996; Scott et al., 2003; Vaughan et al., 2002). Importantly, the budding of COPII vesicles has been shown to require ATP as well as GTP (Balch et al., 1984; Barlowe, 1997). Furthermore, the relatively non-selective protein kinase inhibitor H-89 has been shown to block several membrane trafficking steps in and around the Golgi (Lee and Linstedt, 2000) including the recruitment of Sar1p to the ER membrane (Aridor and Balch, 2000). H-89 shows some selectivity towards PKA and causes multiple defects in ER-to-Golgi and Golgi-to-ER trafficking (Aridor and Balch, 2000; Lee and Linstedt, 2000), however, data are conflicting as to whether PKA is (Muniz et al., 1996), or is not (Lee and Linstedt, 2000) directly involved. In the case of retrograde transport from the Golgi to the ER, proper functioning of the KDEL receptor in the retrieval of ER-resident proteins has been shown to require phosphorylation of the receptor at its C-terminus (Cabrera et al., 2003). Export of some proteins from the ER has been shown to require phosphorylation in order to recruit 14-3-3 proteins that act in masking retrieval sequences (O'Kelly et al., 2002). Specifically, PKA and PKC-dependent phosphorylation of the NR1 NMDA receptor controls its export from the ER. Phosphorylation mediates release of these receptors from the ER to allow regulated transport to synapses (Scott et al., 2003). In this context, it is intriguing that both Sec23p and Sec24p were isolated in a proteomic screen for 14-3-3 binding proteins (Rubio et al., 2004). Thus a possible network of protein-protein interactions is present at ERES to regulate the export of cargo from the ER in a phosphorylation-dependent manner.

PCTAIRE kinases are a relatively uncharacterized branch of the cyclin-dependent kinase (CDK) family. They are expressed

widely, but are found predominantly in terminally differentiated cells (Besset et al., 1999), notably those that have a large population of stable microtubules (Graeser et al., 2002). They are also highly expressed in transformed cell lines (Graeser et al., 2002). Three isoforms exist in higher organisms (Okuda et al., 1992), each of which is characterized by the presence of a serine to cysteine mutation in the consensus motif for cyclin binding (PCTAIRE). They differ from most CDK family members by having large N-terminal domains in addition to the kinase domain. Each isoform shows high homology over the protein kinase domain with less homology in N- and C-terminal extensions. They have not been found to bind to any known cyclin (Graeser et al., 2002) but do associate with other cellular factors that regulate PCTAIRE activity (Charrasse et al., 1999; Graeser et al., 2002). Conflicting data exist as to whether PCTAIRE kinases are (Charrasse et al., 1999) or are not (Graeser et al., 2002) regulated in a cell cycle-dependent manner. Large-scale array-based screening of cell cycle regulated genes showed no change in PCTAIRE expression (Whitfield et al., 2002), although this does not rule out regulation of its activity. The best characterized of the PCTAIRE family is PCTAIRE-1, which has been shown to be regulated by protein kinase A (PKA) and was found to have a role in neurite outgrowth (Graeser et al., 2002). PKA phosphorylation reduces the kinase activity of PCTAIRE-1 and appears to be a significant point of regulation of the PCTAIRE kinases (Graeser et al., 2002). Interestingly, PCTAIRE-1 also binds to the p35 regulatory subunit of the CDK5 kinase; furthermore, its activity is increased by CDK5-dependent phosphorylation (Cheng et al., 2002).

A number of effectors, regulators and modulators of other vesicle budding processes have been identified in recent years: perhaps the best example being clathrin-dependent internalization at the plasma membrane (Mousavi et al., 2004). In contrast, few regulatory molecules have been implicated in COPII-mediated budding from the ER. In order to identify effectors and regulators of COPII function, we screened a yeast two-hybrid library with human Sec23A. One positive clone encoded a fragment of the protein kinase PCTAIRE-3 (PCTK3). Here we show that Sec23Ap interacts with PCTAIRE-3 as well as the closely related isoform PCTAIRE-1 (PCTK1). Sec23p and PCTAIRE can be co-immunoprecipitated from mammalian cell lysates. Expression of mutant forms of PCTAIRE kinase, or specific depletion using RNAi, causes gross changes to the organization and function of the early secretory pathway providing evidence for a role for this family of protein kinases in the regulation of COPII function and consequently ER-to-Golgi membrane traffic.

Materials and Methods

All chemical reagents were purchased from Sigma (Poole, UK) and all molecular biology reagents were from Roche (Lewes, UK) unless otherwise stated.

Two-hybrid screening

Two-hybrid screening was performed using the Matchmaker III system (Clontech/BD Biosciences, Oxford, UK). An adult human brain two-hybrid library, generated by both poly A and random priming, was screened using full-length human Sec23Ap as bait as previously described (Watson et al., 2005). Putative positive clones were isolated

and the interaction verified by re-transformation. Clones that activated all four reporter genes in the system were identified by DNA sequencing (MWG Biotech, Milton Keynes, UK). Positive clones were further analysed using yeast transformation and mating protocols to determine any interaction with other COPII subunits or lamin as a negative control. Additional assays were performed using CDK2 as a negative control. A cDNA (obtained from the MRC Gene Service, Hinxton, UK) encoding amino acids 1-101 of human CDK2 (terminating at the equivalent position to the PCTAIRE-3 clone isolated from screening) was amplified by PCR and cloned in-frame with the Gal4 activation domain of pGAD-T7 (Clontech) using standard procedures.

Generation of recombinant fusion proteins

Recombinant FLAG-Sec23Ap was expressed in insect cells using a baculovirus expression plasmid kindly provided by Peter Espenshade (Johns Hopkins University, Baltimore, MD) and Sf9 cells grown in ExCell 420 medium (JRH Biosciences, Andover, UK). FLAG-Sec23Ap was affinity-purified using anti-FLAG M2 antibody immobilized on Sepharose beads. Full-length cDNAs encoding PCTAIRE-1 and PCTAIRE-3 were obtained from the MRC Gene Service (Image clone numbers 4762081 and 3855304 respectively). These were amplified by PCR and cloned in to pGEX4T3 (Amersham Biosciences, Little Chalfont, UK) and pEGFP-C1 (Clontech/BD Biosciences, Oxford, UK). GST fusion proteins of PCTAIRE-1, and mutants thereof, were expressed in BL21DE3 *E. coli* using recommended protocols (Amersham Biosciences) and purified using glutathione-Sepharose.

Direct binding assay

Lysates (in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, complete protease inhibitors) from Sf9 cells expressing FLAG-Sec23Ap or from non-transfected Sf9 cells were incubated with anti-FLAG sepharose for 2 hours at 4°C. After washing three times for 5 minutes in lysis buffer, these beads were incubated with 5 µg GST, GST-PCTAIRE-1, GST-PCTAIRE-1(K194R) or GST-PCTAIRE-1(S153A) for 2 hours at 4°C. Complexes were then washed (three times for 5 minutes) in PBS supplemented with protease inhibitors) and eluted in SDS-PAGE sample buffer. Proteins were then separated by SDS-PAGE and immunoblotted using an anti-GST antibody (Amersham Biosciences).

Co-immunoprecipitation of Sec23Ap and PCTAIRE

HeLa cells (ATCC, CCL-2), A549 (ATCC, CCL-185), MDCK (ATCC, CCL-34) or 293 cells (ATCC, CRL-1573) were grown in DMEM containing 10% foetal calf serum (Invitrogen, Glasgow, UK). 10 cm dishes of near-confluent cells were washed twice in cold 1 × PBS and lysed in 1 ml chilled lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and complete protease inhibitors). Whole cell lysates were incubated on ice for 15 minutes and subsequently cleared at 20,000 g for 20 minutes at 4°C. The supernatant fractions were collected and incubated with 1 µg of the relevant antibody for 2 hours at 4°C with gentle rotation. The immune complexes were then incubated with 20 µl protein G-Sepharose beads (Amersham Biosciences) (pre-washed and suspended in 100 µl cold lysis buffer) for a further hour at 4°C with gentle rotation. The samples were washed three times in 1 ml cold lysis buffer (centrifuged at 500 g for 1 minute at 4°C between washes) and eluted in 20 µl SDS-PAGE sample buffer. Immunoprecipitated proteins were then detected by immunoblotting using anti-Sec23Ap (see below) or anti-PCTAIRE-1/3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence and microscopy

Images were acquired using a TILL Photonics wide-field fluorescence

imaging system or Leica TCS SP2 AOBs as described in (Stephens, 2003). Images were processed for immunofluorescence by fixing in methanol at -20°C for 4 minutes, washing in PBS, blocking in PBS with 3% BSA followed by sequential incubation in primary and secondary antibodies at appropriate dilutions. All washes were performed in PBS with 3% BSA. Highly cross-adsorbed secondary antibodies conjugated to Alexa 488, 568 or 647 were obtained from Molecular Probes (Paisley, UK). Primary antibodies were sourced as follows: PCTAIRE-1, PCTAIRE-2 and PCTAIRE-3 were obtained from Santa Cruz Biotechnology, anti-giantin from Covance (Cambridge Bioscience, Cambridge, UK), anti-ERGIC-53 was a kind gift from Hans-Peter Hauri (Biozentrum, Basel, CH), anti-VG was a kind gift from Kai Simons (MPI-CBG, Dresden, Germany), anti- α -tubulin (clone DM1A) was from Sigma (Poole, UK), anti-GM130 from BD Biosciences Pharmingen (Cowley, UK), anti-membrin and anti-KDEL-receptor were from Stressgen (Victoria, Canada). Antibodies directed against human Sec23Ap and Sec24Dp were generated in rabbits using synthetic peptides MTTYLEFIQQNEERDGVRC and MSQQGYVATPPYSQPQPGC respectively. Peptides were coupled to keyhole limpet haemocyanin. Peptides were synthesized by Graham Bloomberg (University of Bristol, UK) and antibodies were raised by Clonestar Biotech (Brno, Czech Republic). Antibodies were affinity-purified using Sulfolink (Pierce, Rockford, IL) according to the manufacturer's instructions.

Transport assay

Transport assays were performed essentially as described (Stephens et al., 2000). Briefly, HeLa cells were transfected with YFP-PCTAIRE-1 (wild type or mutants) and ts045-G-CFP using Fugene6 (Roche, Lewes, UK). After incubation at 39.5°C for 16 hours, cells were shifted to 32°C for 60 or 90 minutes and subsequently fixed in 4% paraformaldehyde in PBS. After quenching with 30 mM glycine in PBS, cells were processed for immunofluorescence using anti-VG to quantify the amount of ts045-G-CFP delivered to the plasma membrane. Fluorescence at the cell surface and total cell fluorescence was measured and transport expressed as a ratio of amount transported/total amount expressed. The data from three experiments are presented along with the error (ANOVA) calculated using Microsoft Excel.

RNA interference

Small interfering RNA (siRNA) duplexes directed against PCTAIRE-1 were synthesized by MWG Biotech using the WG online design tool. Two 21 bp duplexes were synthesized with the following sequences: 5'-GAU CUC CAC UGA GGA CAU CTT-3' and 5'-CGA GGA GUU CAA GAC AUA CTT-3'.

As a control, a 19 bp siRNA duplex directed against lamin A was used: 5'-CUG GAC UUC CAG AAG AAC A-3'. siRNA duplexes were transfected into HeLa cells seeded at 50% confluence on to live cell dishes using calcium phosphate transfection (Chen and Okayama, 1988). After incubation for 16 hours at 37°C in a 3% CO_2 atmosphere, cells were incubated at 5% $\text{CO}_2/37^{\circ}\text{C}$ for a further 56 hours (72 hours total). A time course of depletion (not shown) revealed 72 hours post-transfection to provide the most effective knockdown of PCTAIRE-1 expression. Cells were then either lysed for immunoblotting or processed for immunofluorescence. For immunoblotting, cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100. Protein concentrations were determined using the bichonic acid method (Pierce) and 10 μg were separated by SDS-PAGE (4-12% gradient gels, Invitrogen) before transferring to nitrocellulose membrane. Blots were probed with anti-PCTAIRE-1 (sc-274, Santa Cruz Biotechnology) at a dilution of 1:500 or anti-lamin A/C (Cell Signaling Technology/NEB, Hitchin, UK) at 1:1000, followed by goat anti-mouse peroxidase at 1:25,000 (Jackson ImmunoResearch/Strattech Scientific, Soham, UK) and enhanced chemiluminescence

(Amersham Biosciences). Cells were processed for immunofluorescence as described above.

Protein kinase assays

GST-PCTAIRE fusion proteins (1 μg) were assayed for kinase activity by incubating immobilized GST-PCTAIRE with rat brain lysate for 30 minutes, which is required for the activation of PCTAIRE kinase activity (Graeser et al., 2002). After washing, kinase activity was assayed in 10 μl kinase buffer (50 mM Tris-HCl, pH 7.4, 15 mM MgCl_2 , 1 mM DTT, 1 mg/ml myelin basic protein (MBP), 20 μM ATP) containing 1 μCi [γ - ^{32}P]ATP for 30 minutes at 30°C . The phosphorylated proteins were subsequently separated by SDS-PAGE and visualized by autoradiography.

Results

We screened a human brain cDNA library using the two-hybrid method to identify proteins that interact with Sec23Ap. One clone was isolated encoding the central region (amino acids 89-240) of the PCTAIRE-3 protein kinase (Fig. 1A). This clone overlapped with the N-terminal regulatory and catalytic domains of the kinase, which was detected using all four reporters in the two-hybrid system (α -galactosidase, β -galactosidase, growth in the absence of histidine and in the absence of adenine). Specificity of the interaction was shown by the absence of detectable interaction with lamin, or with another COPII protein, Sec13p (Fig. 1A). A weaker interaction was also detectable with Sec24Dp. This might be due to the high degree of structural homology of Sec23p and Sec24p (Bi et al., 2002), or it could reflect an interaction with both components of this heterodimeric complex. An interaction with Sec31Ap was detected using some, but not all of the reporters, and was therefore considered to be a false positive.

Full sequencing of the clone identified in the two-hybrid screen showed that it was flanked by predicted introns. BLAST searching of the human genome database showed that the sequence of our two-hybrid clone coded for an additional nine amino acids at the 5' end that arise from the predicted intron sequence. Detailed analysis showed predicted donor and acceptor splice sites within this region suggesting that this might arise from alternative splicing of the PCTAIRE-3 mRNA (Fig. 1B). Subsequent to this identification, the existence of a splice form (PCTAIRE-3b) was shown in which this flanking sequence forms part of an extension to exon 3 (Herskovits and Davies, 2004). The additional 3' sequence contained within the PCTAIRE-3 two-hybrid clone identified here, extends exon 8 by 110 amino acids prior to an in-frame stop codon. It is possible that this represents a truncated form of PCTAIRE-3 that is normally expressed but no such species has been detected by immunoblotting with PCTAIRE-3 antibodies. Thus, it is probable that this represents an incompletely spliced message that was incorporated into the cDNA library. Following removal of these intron-derived flanking sequences we were able to confirm that the interaction with Sec23Ap occurred with the core PCTAIRE-3 sequence (PCTAIRE-3a) (Herskovits and Davies, 2004; Okuda et al., 1992), present in all isoforms examined to date (Fig. 1C). Thus, the central regions of both PCTAIRE-3a and -3b (Herskovits and Davies, 2004) interact with Sec23Ap. We have been unable to detect any two-hybrid interaction between Sec23Ap or Sec24Dp and full-length PCTAIRE-3.

As the PCTAIRE kinases are highly homologous, we also tested whether Sec23Ap could interact with the same central region of PCTAIRE-1, the most widely expressed and best characterized family member (Graeser et al., 2002; Okuda et al., 1992; Sladeczek et al., 1997). A specific interaction was seen between Sec23Ap and the region of PCTAIRE-1 corresponding to that of PCTAIRE-3 identified in the initial screen (exons 4-8). Again, an interaction with Sec24Dp was also detectable (Fig. 1C). The specificity of this interaction is shown in Fig. 1D in which the PCTAIRE kinase CDK2 is shown not to interact with any of the COPII components. Expression of Gal4-CDK2 fusion protein was confirmed by

immunoblotting (not shown). As with PCTAIRE-3, we were unable to detect a two-hybrid interaction with full-length PCTAIRE-1.

The specificity of interaction between Sec23Ap and PCTAIRE-1 was subsequently analysed using direct binding assays (Fig. 2). Purified recombinant FLAG-Sec23Ap was incubated with immobilized GST (lane 1) or GST-PCTAIRE-1 (lane 2). Binding was measured by immunoblotting for the FLAG epitope on Sec23Ap. Fig. 2 shows that hSec23Ap can be specifically captured by immobilized GST-PCTAIRE-1 (lane 2). Some non-specific binding of Sec23Ap to immobilized GST is observed (lane 1) but this was always significantly less than the amount of binding to GST-PCTAIRE-1. We also used site-directed mutagenesis to introduce mutations into PCTAIRE-1 that have been shown to increase (S153A), or abolish (K194R), protein kinase activity (Graeser et al., 2002). The expected increase or decrease in protein kinase activity (Graeser et al., 2002) was confirmed using *in vitro* phosphorylation assays (data not shown). These mutations did not have any effect on the binding of hSec23Ap to PCTAIRE-1 (Fig. 2, lanes 3 and 4). It should be noted that these interactions are only weakly detectable above background and could be suggestive of additional factors modulating the interaction in intact cells. We were unable to produce sufficient quantities of PCTAIRE-3 for these experiments as it is extremely susceptible to proteolysis and rapidly degraded during protein expression in *E. coli*. Using these recombinant proteins and with further assays using immunisolated COPII proteins, we were unable to detect any phosphorylation of COPII by PCTAIRE-1, nor any modulation of PCTAIRE-1 activity (measured using myelin basic protein) (Graeser et al., 2002) on incubation in the presence of Sec23Ap (data not shown).

Consistent with other work (Graeser et al., 2002), expression was detectable in a number of transformed cell lines.

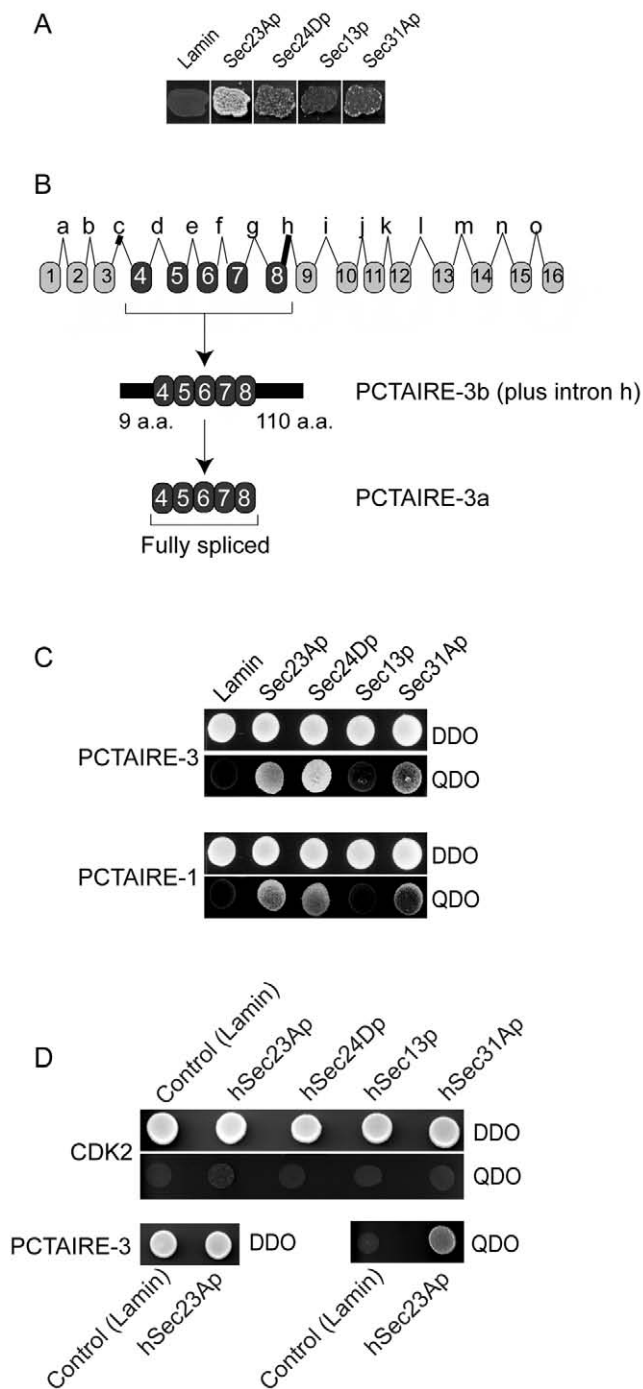


Fig. 1. Interaction of Sec23Ap with PCTAIRE protein kinases. Yeast two-hybrid screening of a human brain cDNA library with full-length Sec23Ap identified a clone encoding apart of PCTAIRE-3b.

(A) Interactions were reconfirmed by transformation and shown to occur specifically with Sec23Ap. A weak association was also seen with Sec24Dp but not with lamin or Sec13p. The weakly detectable interaction with Sec31Ap in plate growth assays was not seen in complementary colorimetric assays and therefore was deemed a false positive. (B) The two-hybrid clone encoding PCTAIRE-3 includes nine amino acids from predicted intron c and 110 amino acids from predicted intron h. Using PCR, we generated a 'fully-spliced' cDNA including exons 4-8, which is equivalent to the central region of the predominant isoform of PCTAIRE-3, PCTAIRE-3a (Herskovits and Davies, 2004; Okuda et al., 1992). The amino acids encoded by predicted intron h are believed to arise from incomplete splicing as they include a stop codon after 61 amino acids. The nine amino acids included in this clone would encode a known splice form, PCTAIRE-3b (Herskovits and Davies, 2004). (C) Further yeast two-hybrid assays showed that Sec23Ap and Sec24Dp also interact with PCTAIRE-3a and PCTAIRE-1. DDO, double dropout medium; QDO, quadruple dropout medium. (D) Specificity of the two-hybrid interaction was determined using the PCTAIRE kinase CDK2. Co-transformants were assessed for interaction between CDK2 and lamin, Sec23Ap, Sec24Dp, Sec13p and Sec31p (upper panels). No interactions were seen. Concomitant transformation of yeast with Sec23Ap and the PCTAIRE-3 clone isolated from the library screen was used as a positive control (lower panels).

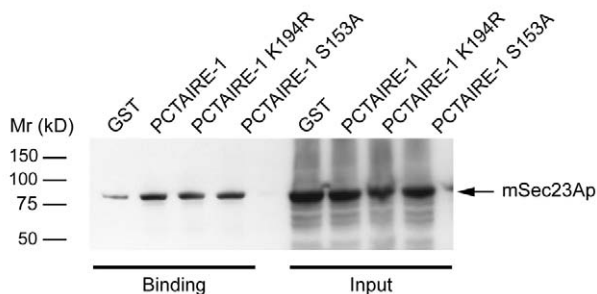


Fig. 2. PCTAIRE-1 and Sec23Ap interact directly in vitro. Purified recombinant FLAG-Sec23Ap was incubated with immobilized GST-fusion proteins as follows: GST, GST-PCTAIRE-1, GST-PCTAIRE-1(K194R) or GST-PCTAIRE-1(S153A). Beads were washed and bound protein eluted with sample buffer containing SDS, separated by SDS-PAGE and immunoblotted with an anti-FLAG antibody. Right-hand blot contains 5% of the amount of FLAG-Sec23Ap included in the binding assays (input). Position of molecular mass markers in kDa is indicated on the left.

PCTAIRE-1 was expressed in human cervical epithelial carcinoma (HeLa) cells and human embryonic kidney 293 (HEK-293) cells with lower levels in Madin-Darby canine kidney (MDCK) cells and trace levels of expression in A549 lung epithelial cells (Fig. 3A). The presence of multiple bands probably reflects the presence of multiple splice forms (Herskovits and Davies, 2004) or post-translational modification of PCTAIRE-1 (Graeser et al., 2002). In contrast, low-level expression of PCTAIRE-2 was detected in each of these four cell lines. PCTAIRE-3 was expressed at the highest level in MDCK cells with lower levels in HeLa and HEK-293 cells. Consequently, we decided to use HeLa for all subsequent analyses of PCTAIRE-1 and MDCK for analyses of PCTAIRE-3. We were able to co-immunoprecipitate hSec23Ap with PCTAIRE-3 from MDCK cell lysates (Fig. 3B, lanes 1-3), as well as with PCTAIRE-1 from HeLa cell lysates (Fig. 3B, lanes 4-6). Together, these data suggest that Sec23Ap binds directly

to PCTAIRE protein kinases under physiological conditions. As with the two-hybrid assays, specificity is confirmed by the absence of co-immunoprecipitation of Sec23Ap with antibodies directed against CDK1; furthermore, in the reciprocal experiment, antibodies directed against either Sec23Ap or PCTAIRE-1 fail to immunoprecipitate CDK1. We obtained identical results using an antibody specific to CDK2 (data not shown). Despite identifying an interaction between Sec31Ap and the truncated PCTAIRE-3 clone in two hybrid-assays, we have been unable to specifically co-immunoprecipitate these two proteins (data not shown).

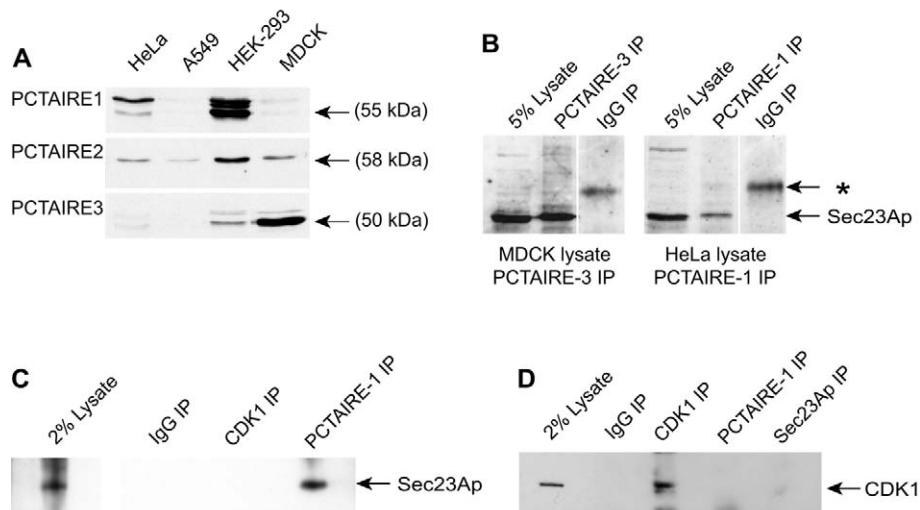
We then examined the effect of RNAi-mediated depletion of PCTAIRE-1 on the secretory pathway. Two different siRNA duplexes were designed to target the PCTAIRE-1 coding sequence. These were shown to be effective in depletion of the PCTAIRE-1 protein level by immunoblotting (Fig. 4A). Lamin was used as a control for these experiments (Fig. 4B). Immunofluorescence localization of markers of the secretory pathway in cells depleted for lamin or PCTAIRE-1 (Fig. 4C) showed that Golgi localization and positioning, microtubule architecture and the localization of the KDEL-receptor were all unperturbed. RNAi depletion of PCTAIRE-1 caused dispersion of ERGIC-53 from a more punctate localization in lamin siRNA transfected cells to a more ER-like pattern in PCTAIRE-1 siRNA-transfected cells (Fig. 4C,D). An indistinguishable phenotype was seen with two independent siRNA duplexes targeting PCTAIRE-1 (not shown). The localization of Sec24Dp in siRNA-transfected cells was also found to be more dispersed in these cells, although consistent, this is in fact a very mild phenotype.

We then sought to identify whether the kinase activity of PCTAIRE-1 has a role in the regulation of ER export and transport to the Golgi. We transiently expressed wild-type GFP-tagged PCTAIRE-1, 'active' mutants S119A, S153A and S319E and a 'kinase-dead' (K194R) mutant (Graeser et al., 2002) in HeLa cells and examined their effect on the distribution of early secretory pathway components by indirect immunofluorescence microscopy. Although expression of the

Fig. 3. PCTAIRE kinases are expressed in many cell lines and can be co-immunoprecipitated with Sec23Ap.

(A) Analysis of PCTAIRE expression in cell lysates from four different cell lines: HeLa (cervical epithelia), A549 (lung epithelia), HEK-293 (embryonic kidney), MDCK (Madin-Darby canine kidney). (B) Sec23Ap can be co-immunoprecipitated with PCTAIRE-3 from MDCK cell lysates (lanes 1-3) and PCTAIRE-1 from HeLa cell lysates (lanes 4-6). Immunoprecipitates were washed extensively, separated by SDS-PAGE followed by immunoblotting with anti-Sec23p. Lanes 1 and 4, 5% input lysate; lanes 2 and 5, immunoprecipitation with anti-PCTAIRE-3 (lane 2) or anti-PCTAIRE-1 (lane 5); lanes 3 and 6, control immunoprecipitation using rabbit IgG.

* , non-specific band at ~100 kDa. (C) Specificity of co-immunoprecipitation was confirmed using antibodies directed against CDK1. Unlike anti-PCTAIRE-1, neither mouse IgG nor anti-CDK1 was able to immunoprecipitate Sec23Ap from HeLa cell lysates. (D) In the converse experiments, neither anti-Sec23Ap nor anti-PCTAIRE-1 were able to immunoprecipitate CDK1.



wild type or active (S153A and S153A/S119A) mutants of PCTAIRE-1 had no gross effects on the localization of the Golgi (giantin localization) or the ER-Golgi intermediate compartment (ERGIC-53 localization), expression of kinase-dead PCTAIRE-1 resulted in fragmentation of the Golgi apparatus and a redistribution of ERGIC-53 to a more peripheral localization (Fig. 5A). In addition, membrin, a SNARE protein that cycles between the ER and the Golgi (Hay et al., 1997), was also found to be disrupted in cells expressing the kinase-dead mutant PCTAIRE-1 (K194R), showing a more diffuse localization throughout the cell consistent with ER localization (Fig. 5B, asterisk). Similarly, Sec24Dp, a marker

for COPII-coated ERES showed a more diffuse pattern of localization throughout these cells (Fig. 5B, note loss of juxtannuclear accumulation of Sec24Dp). In contrast, the distribution of microtubules was unchanged compared to that in control cells (data not shown).

These data suggest that PCTAIRE kinases might regulate export of secretory protein trafficking from the ER to the Golgi. In order to measure this in a quantitative manner, we exploited the temperature-sensitive vesicular stomatitis glycoprotein (ts045-G) in transport assays in HeLa cells (Kreis, 1986; Presley et al., 1997). HeLa cells were co-transfected with plasmids encoding ts045-G-YFP (Keller and Simons, 1997)

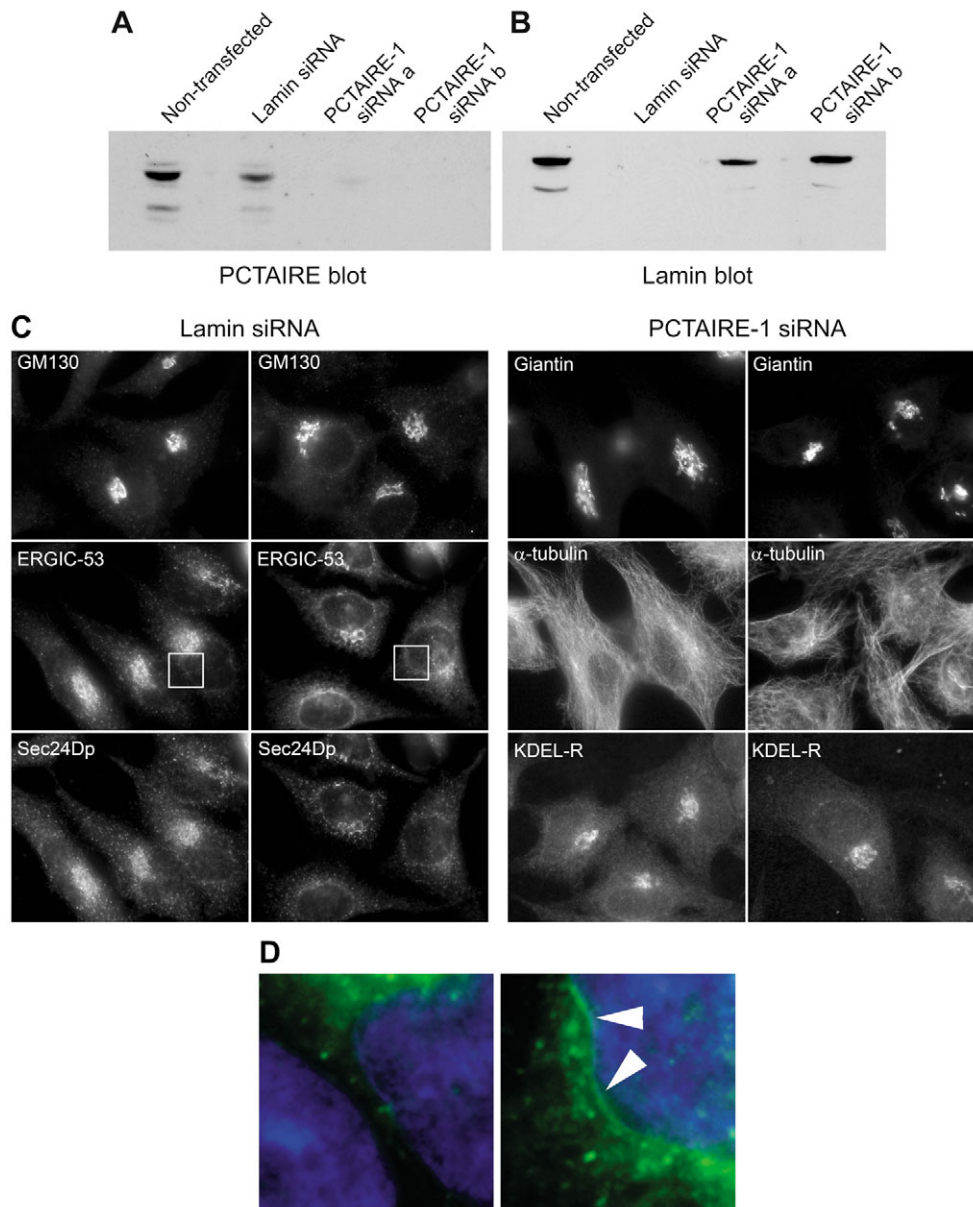


Fig. 4. Depletion of PCTAIRE-1 expression using RNAi causes disruption of morphology of the early secretory pathway. HeLa cells were transfected with siRNA duplexes targeting lamin or PCTAIRE-1. After 72 hours, cells were lysed and blotted for PCTAIRE-1 (A) or lamin (B). (C) Cells grown on coverslips, transfected with siRNA specific for PCTAIRE-1 and incubated for 72 hours. Cells were then fixed and processed for immunofluorescence with antibodies specific to GM130, ERGIC-53, Sec24Dp, giantin, α -tubulin or the KDEL-receptor. (D) Enlargement of the boxed regions of ERGIC-53 immunolabelling (green) from C show that PCTAIRE-1 siRNA results in increases ER labelling clearly shown by the nuclear envelope localization. The nucleus (DNA labelled with DAPI) is shown in blue.

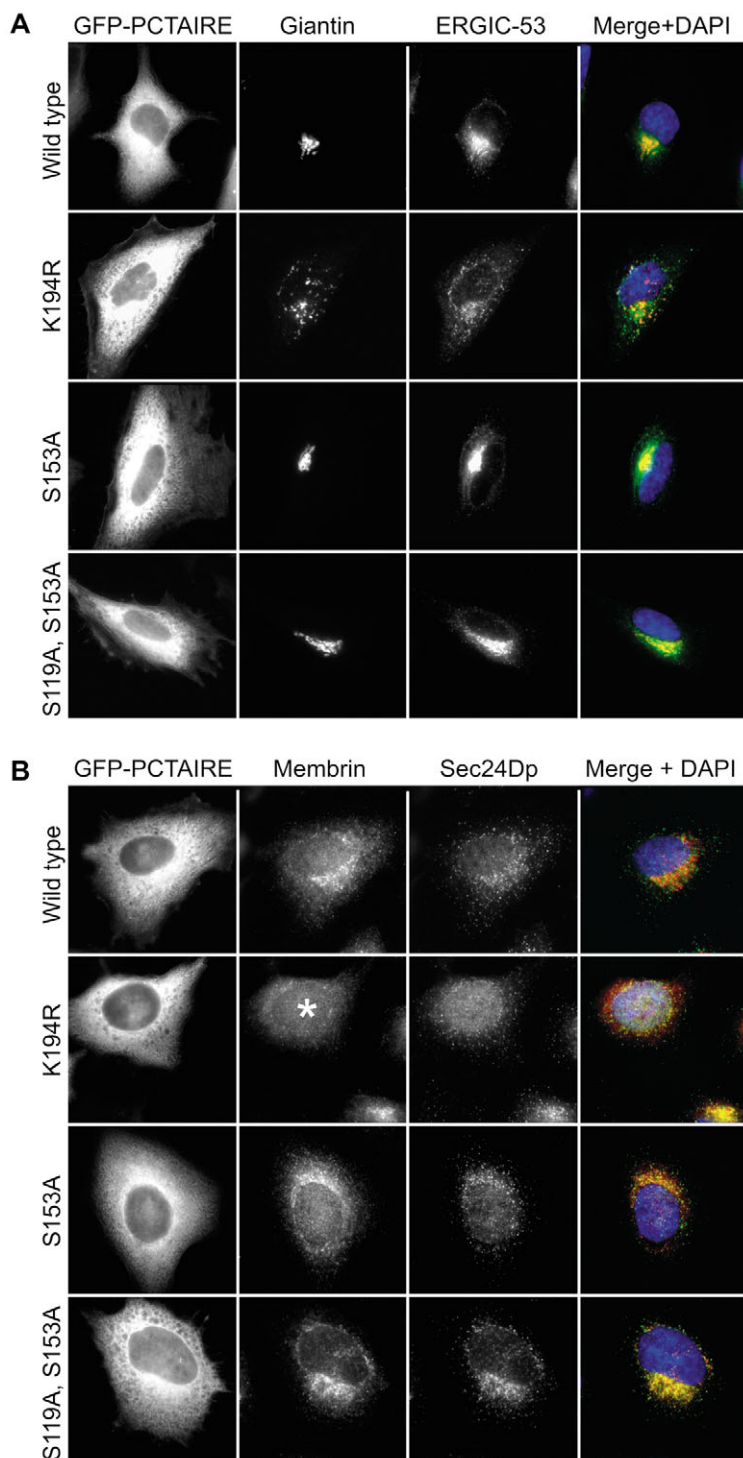


Fig. 5. Kinase-dead PCTAIRE-1 disrupts protein localization within the early secretory pathway. Cells were transfected with plasmids to express GFP-PCTAIRE-1 (wild type or point mutants as indicated). (A) The K184R ('kinase-dead') point mutation in PCTAIRE-1 results in fragmentation of the Golgi (giantin) and redistribution of ERGIC-53 to a more dispersed localization. In contrast, the wild type or 'active' mutants of PCTAIRE-3 do not cause these effects. (B) The K184R point mutation in PCTAIRE-1 results in redistribution of the ER-Golgi SNARE protein membrin to a more dispersed localization (asterisk), and a reduction in the intensity and juxtannuclear clustering of COPII-coated ERES marked with Sec24Dp. In contrast, the wild type or 'active' mutants of PCTAIRE-3 do not cause these effects.

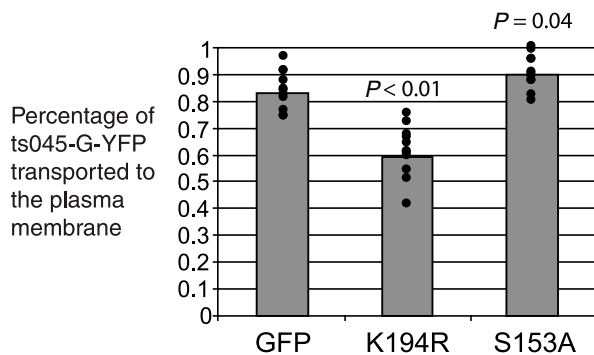


Fig. 6. Expression of kinase-dead PCTAIRE-1 causes an inhibition of secretory cargo transport. Quantitative transport assays using ts045-G-CFP was performed using cells transfected with YFP, kinase-dead mutant YFP-PCTAIRE-1(K194R), or active mutant YFP-PCTAIRE-1(S153A). Histogram of the mean amount of ts045-G-CFP transported to the plasma membrane after a 60-minute incubation at 32°C. The points indicate the scatter of values from ten cells for each experiment. Errors (ANOVA) show statistical significance for the inhibition of transport following PCTAIRE-1(K194R) expression.

and GFP-PCTAIRE-1 (wild type or mutants). Following incubation at 39.5°C to accumulate ts045-G-YFP in the ER, cells were incubated at 32°C to allow export of cargo and transport to the plasma membrane. The amount of protein transported to the plasma membrane was then measured 60 minutes after temperature shift using immunofluorescence with an antibody directed against the extracellular domain of ts045-G-YFP. The proportion of ts045-G-YFP transported to the plasma membrane in cells expressing GFP, GFP-PCTAIRE-1(K194R) or GFP-PCTAIRE-1(S153A) was determined (Fig. 6). An 18% reduction in transport was seen in cells coexpressing the kinase-dead (K194R) mutant. In contrast, cells expressing an active S153A mutant of PCTAIRE-1 showed an equivalent enhanced amount of transport of ts045-G-YFP. Together these data suggest that PCTAIRE-1 can regulate protein trafficking through the secretory pathway, at least in part at the level of ER export.

Discussion

We have identified a direct interaction between the COPII machinery and a ubiquitously expressed protein kinase family, PCTAIRE. Furthermore, we have shown that manipulation of PCTAIRE kinase activity modulates membrane trafficking through the early secretory pathway. These findings are of particular interest for a number of reasons. First, there is considerable evidence for protein kinase regulation of membrane traffic at the ER-Golgi interface. The relatively non-selective kinase inhibitor H-89 has been shown to modulate transport between the ER and Golgi by affecting the formation and function of COPII-coated ERES (Lee and Linstedt, 2000). Specifically, it blocks recruitment of Sar1p to the ER membrane (Aridor and

Balch, 2000). In addition, protein kinase A has been shown to regulate a number of steps in bidirectional transport between the ER and Golgi, including transport from the ER to the Golgi (Lee and Linstedt, 2000; Muniz et al., 1996), and the recycling of the KDEL-receptor from the early Golgi (Cabrera et al., 2003). Intriguingly, PCTAIRE kinases are themselves subject to regulation by protein kinase A (Graeser et al., 2002). There is also considerable evidence for the regulation by phosphorylation of motor proteins such as dynein (Vaughan et al., 2001) and its adaptor dynactin (Vaughan et al., 2002), which are known to be involved in COPII-dependent export from the ER (Watson et al., 2005) and ER-to-Golgi transport (Presley et al., 1997). There is also evidence that both the COPI and COPII complexes can be phosphorylated (Dudognon et al., 2004; Salama et al., 1997; Sheff et al., 1996), however, PCTAIRE protein kinases were not found to phosphorylate either of these complexes in vitro (data not shown). It remains possible that the role of PCTAIRE kinase activity lies in the direct phosphorylation of one or more specific cargo molecules to regulate its export from the ER.

Direct binding assays showing an interaction between PCTAIRE-1 and Sec23Ap, although reproducible, consistently showed very weak binding of the two components. This might reflect a low affinity interaction or the fact that there is some regulation of this interaction. Alternatively, some unknown component may be required to increase the affinity of binding, or even to facilitate the interaction. This would explain the more reliable co-immunoprecipitation of these two components from cell lysates in which additional factors would be present. Another possibility is that a particular phosphorylation state of PCTAIRE is required for Sec23Ap binding. Any additional factor would need to be conserved from humans to yeast, the latter of which do not possess a PCTAIRE homologue. Thus, the simplest interpretation of these data is a low affinity direct interaction. It is also possible that the large N-terminal extension of PCTAIRE-1 is in fact inhibitory with regard to Sec23Ap binding. This would also explain the lack of interaction observed in the two-hybrid system between Sec23Ap and full-length Sec23Ap. We have only been able to detect an interaction with Sec31Ap in some two-hybrid assays and not in any other binding assays tested. We cannot rule out some role for this interaction, possibly in the recruitment of PCTAIRE kinases to fully coated COPII vesicles but it seems more likely that PCTAIRE kinases do not interact with Sec31Ap.

We observed a significant effect on early secretory pathway organization following expression of PCTAIRE-1(K194R) in HeLa cells. This suggests that the localization of proteins that cycle between the ER and Golgi, such as ERGIC-53 and membrin, as well as the localization of the COPII complex itself, depends on PCTAIRE kinase activity. We do not currently have an explanation for these observations. However, we can detect no defect in secretory cargo export from the ER using the ts045-G-GFP cargo transport assay. One possibility is that through binding to COPII, PCTAIRE regulates some downstream component possibly involved in the assembly or mobility of transport-competent carriers such as VTCs. In the absence of proper regulation of this process, defective transport could result in perturbed localization of the ERGIC and VTCs.

Strong evidence also now exists for regulated export of specific cargo molecules from the ER. The decision to retain

or export NMDA receptors from the ER is controlled by phosphorylation of the ER retention motif (Scott et al., 2003; Scott et al., 2001). Furthermore, KCNK3 potassium channels have been shown to require phosphorylation on their cytosolic domains in order to be efficiently exported from the ER (O'Kelly et al., 2002). In this case, phosphorylation initiates binding to 14-3-3 β , which masks an ER retrieval motif. This mechanism was also shown to control the export of the invariant chain (O'Kelly et al., 2002). Intriguingly, PCTAIRE kinases have also been shown to bind to 14-3-3 family members (Sladeczek et al., 1997), as indeed have the COPII subunits, Sec23p and Sec24p (Rubio et al., 2004). Thus, the interaction we describe here provides a potential mechanism for integrating the requirements for COPII binding, protein phosphorylation and 14-3-3 recruitment at a single intracellular location. This notion of COPII acting as a platform for the recruitment of the necessary machinery molecules to ERES is consistent with the long-lived nature of ERES (Hammond and Glick, 2000; Stephens, 2003; Stephens et al., 2000) and the fact that COPII can recruit other machinery molecules such as SNARES (Mossessova et al., 2003), phospholipases (Pathre et al., 2003) and motor protein complexes (Watson et al., 2005).

PCTAIRE kinases have also been implicated in neurite outgrowth, possibly through modulation of the pool of stable microtubules (Graeser et al., 2002). As ERES align along microtubules and recruit dynactin for the transport of VTCs to the Golgi (Watson et al., 2005), it is possible that the PCTAIRE-COPII interaction has some role in coordinating this process. A signalling cascade based around cdc42 and atypical PKC has been described that operates in the reorientation of the Golgi apparatus during directed cell migration and neurite outgrowth (Etienne-Manneville and Hall, 2003). It is possible that PCTAIRE activity has a role in the organization of ERES in polarizing cells or in the selective export of specific cargo proteins that are required for directed cell migration and/or polarization. Clearly, the roles of this and other protein phosphorylation events in the regulation of transport through the secretory pathway require further investigation.

We are very grateful to the Medical Research Council (UK) for providing an Infrastructure Award to establish the University Of Bristol School Of Medical Sciences Cell Imaging Facility. Thanks to Pete Watson, Jon Lane, George Banting, Jeremy Tavaré and Harry Mellor for helpful advice and critical reading of the manuscript. This work was supported by a Research Career Development Award from the Medical Research Council (UK) (G120/617) to D.J.S. and an Undergraduate Research Bursary from the Nuffield Foundation to J.E.K.

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