Dirac-Svejstrup et al. Vol. 150, No. 3, August 7, 2000. Pages 475–487.

In the last sentence of the Abstract, the term "trans-soluble NSF attachment protein (SNAP) receptor" was mistakenly abbreviated as t-SNARE. The proper abbreviation is trans-SNARE. The last sentence in the abstract should appear as follows:

p115 phosphorylation may be required for the transition from COPI vesicle tethering to COPI vesicle docking, an event that involves the formation of trans-SNARE (trans–soluble NSF attachment protein [SNAP] receptor) complexes.

Phosphorylation of the Vesicle-tethering Protein p115 by a Casein Kinase II–like Enzyme Is Required for Golgi Reassembly from Isolated Mitotic Fragments

A. Barbara Dirac-Svejstrup,* James Shorter,§ M. Gerard Waters,‡ and Graham Warren§

*Cell Biology Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom; ‡Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544; and §Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06520-8002

Abstract. Coat protein I (COPI) transport vesicles can be tethered to Golgi membranes by a complex of fibrous, coiled-coil proteins comprising p115, Giantin and GM130. p115 has been postulated to act as a bridge, linking Giantin on the vesicle to GM130 on the Golgi membrane. Here we show that the acidic COOH terminus of p115 mediates binding to both GM130 and Giantin as well as linking the two together. Phosphorylation of serine 941 within this acidic domain enhances the binding as well as the link between them. Phosphorylation is mediated by casein kinase II (CKII) or a CKII-like kinase. Surprisingly, the highly conserved NH2-terminal head domain of p115 is not required for

the NSF (*N*-ethylmaleimide–sensitive fusion protein)–catalyzed reassembly of cisternae from mitotic Golgi fragments in a cell-free system. However, the ability of p115 to link GM130 to Giantin and the phosphorylation of p115 at serine 941 are required for NSF-catalyzed cisternal regrowth. p115 phosphorylation may be required for the transition from COPI vesicle tethering to COPI vesicle docking, an event that involves the formation of t-SNARE (trans–soluble NSF attachment protein [SNAP] receptor) complexes.

Key words: p115 • GM130 • Giantin • CKII • tethering

Introduction

The Golgins comprise a family of coiled-coil proteins that localize to the Golgi apparatus and were first identified using the sera from patients with autoimmune diseases such as Sjögren's syndrome (Chan and Fritzler, 1998). Their sequence predicts long, rod-like proteins, often with one or more flexible hinges. The NH2- and COOH-terminal ends interact either directly or indirectly with membranes and increasing evidence suggests that they act as tethering proteins, linking transport vesicles with cisternae and cisternae with each other (Linstedt et al., 1995; Nakamura et al., 1995, 1997; Sapperstein et al., 1996; Barr et al., 1998; Barr, 1999; Cao et al., 1998; Sönnichsen et al., 1998; Kim et al., 1999; Shorter and Warren, 1999). They are thought, therefore, to contribute both to the structure of the Golgi apparatus and the movement of transport vesicles through it.

The best characterized Golgins are GM130 and Giantin (Linstedt and Hauri, 1993; Nakamura et al., 1995). GM130 is mostly present on cis-Golgi membranes and is anchored at the extreme COOH terminus to GRASP65 (Nakamura

et al., 1995; Barr et al., 1998), one of at least two GRASPs implicated in the stacking of Golgi cisternae (Barr et al., 1997; Shorter et al., 1999). Giantin is a type II Golgi membrane protein (Linstedt and Hauri, 1993) and is present throughout the Golgi apparatus (Seelig et al., 1994), but appears to be most active when incorporated into COPI transport vesicles (Sönnichsen et al., 1998).

The NH2-terminal domains of both proteins interact with another coiled-coil protein, p115 (Nakamura et al., 1997; Lesa et al., 2000). This homodimeric protein has a globular head domain, a coiled-coil tail and a short acidic region at the COOH-terminal end (Waters et al., 1992; Sapperstein et al., 1995). This acidic region has been implicated in binding to both GM130 (Nelson et al., 1998) and Giantin (Linstedt et al., 2000). p115 is involved in several transport steps including ER to Golgi (Lupashin et al., 1996; Sapperstein et al., 1996; Barlowe, 1997; Alvarez et al., 1999) and transport within the Golgi stack itself (Waters et al., 1992; Seemann et al., 2000). It mediates the tethering of coat protein I (COPI)[1] vesicles

---

Address correspondence to Graham Warren, Department of Cell Biology, SHM, C441, Yale University School of Medicine, 333 Cedar St., P.O. Box 208002, New Haven, CT 06520-8002. Tel.: (203) 785-5058. Fax: (203) 785-4301. E-mail: graham.warren@yale.edu

[1]*Abbreviations used in this paper:* CKII, casein kinase II; COPI/II, coat protein I/II; GRASP65, Golgi reassembly stacking protein of 65 kD; MGF, mitotic Golgi fragments; NSF, *N*-ethylmaleimide–sensitive fusion protein; RLG, rat liver Golgi membranes; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

with Golgi membranes through its interaction with GM130 and Giantin (Sönnichsen et al., 1998; Shorter and Warren, 1999). It is thought to act as a bridge, linking GM130 on the Golgi with Giantin on the vesicle (Sönnichsen et al., 1998).

Tethering occurs before SNARE pairing (Pfeffer, 1999). The interaction of a v-SNARE on the vesicle with the cognate t-SNARE on the membrane leads to membrane fusion (Rothman, 1994). The yeast counterpart of p115, Uso1p, tethers coat protein II (COPII) transport vesicles to Golgi membranes (Cao et al., 1998). This tethering step occurs before SNARE pairing and is regulated by the small GTPase, Ypt1p, which interacts genetically with Uso1p (Sapperstein et al., 1996; Cao et al., 1998). Tethering also requires the protein complex, TRAPP (Sacher et al., 1998) and the Sec34/35p complex (VanRheenen et al., 1998, 1999; Kim et al., 1999). The yeast homologues of GM130 and Giantin have not yet been identified, but Sec34p interacts genetically with the RUD3/GRP1, a gene encoding a Golgin-160–related protein (Fritzler et al., 1993; Kim et al., 1999; VanRheenen et al., 1999). Other Golgi tethers may include Golgin-230/245/256 and Golgin-97 which have recently been shown to interact with Rab6 (Barr, 1999), another small GTPase implicated in regulating the directionality of membrane traffic and membrane dynamics within the Golgi stack (Martinez et al., 1994; Echard et al., 1998).

Tethers that bring a vesicle to a membrane with which it fuses must subsequently be dismantled to permit further rounds of tethering. The mechanism is unclear but one clue comes from recent work showing that p115 is specifically phosphorylated on a serine within the acidic tail region (Sohda et al., 1998). Phosphorylation correlates with release of p115 into the cytosol. Here we have investigated the role played by this serine in the functioning of p115. To do this we have used a system that mimics many aspects of Golgi reassembly at the end of mitosis (Rabouille et al., 1995; Shorter and Warren, 1999). Golgi disassembly during mitosis is thought to occur as part of the inheritance process (Shima et al., 1997). Mitotic Golgi fragments (MGF) prepared in vitro, will reassemble into Golgi cisternae using either one of two ATPases, NSF or p97 (Rabouille et al., 1995). The NSF pathway requires p115 as one of the components added to the incubation (Rabouille et al., 1995). Its interaction with GM130 and Giantin is essential both to reconstitute cisternae and to stack them (Shorter and Warren 1999). Using this system we have identified a crucial role for the acidic tail serine and have characterized the kinase which phosphorylates it.

Materials and Methods

Materials

All reagents were analytical grade or higher and purchased from Sigma-Aldrich, Boehringer, or BDH Chemicals Ltd., unless otherwise stated.

Antibodies

Antibodies were as follows: 4H1 mouse mAb against p115 (termed mAb 115-1; Waters et al., 1992); mouse mAb against RGS-His (QIAGEN); NN5-1 rabbit polyclonal and 2C10 mouse mAb against GM130 (Nakamura et al., 1997); rabbit polyclonal (Dr. Manfred Renz, Institute of Immunology and Molecular Genetics, Karlsruhe, Germany) and mouse

mAb (Dr. Hans-Peter Hauri, Department of Pharmacology/Neurobiology, Biozentrum, University of Basel, Switzerland) against Giantin; mouse mAbs against human CKII-α subunit and -β subunit (Calbiochem-Novabiochem).

Molecular Cloning

Site directed mutagenesis was used to construct vectors for expression of portions of bovine p115 (see Fig. 1). HTA represents the full-length unmutagenized construct. The HT construct was generated by changing the codon for D906 to a stop codon (TAG) and the H construct was made by changing the codon for V652 to a stop codon (TAG). The TA construct was generated as follows. First the R5 and G6 codons (AAG-CTT) were changed to a StuI restriction (site AGG-CCT). In a second round of mutagenesis the I651 and V652 codons (ATT-GTG) were changed to a PmlI restriction site (CAC-GTG). The plasmid was then digested with StuI and PmlI and ligated. The resulting TA construct encodes the first five amino acid residues of p115 fused with residues V652 to the COOH terminus. All constructs were subsequently cloned into pBluescriptII. TA constructs were mutagenized changing codon S941 (AGC) to either alanine (GCC) (TA [S941A]), or aspartic acid (GAC) (TA[S941D]).

GM130 pBluescriptII constructs (Nakamura et al., 1997) were used for in vitro transcription-translation reactions in the presence of ^{35}S-methionine using T3 polymerase according to the manufacturer's instructions (Promega). An amount corresponding to ~10 ng of the relevant in vitro translated protein (10 μl reticulolysate containing translation product) was used for coimmunoprecipitation reactions (see below).

Protein Expression and Purification

His$_6$-TA was generated by subcloning in the expression vector pQE9 (QIAGEN). It was expressed in *E. coli* XL1-blue, and purified according to the manufacturer's instructions.

TA, TA (S941A), and TA (S941D) were further fractionated by Superose-6 (Amersham Pharmacia Biotech) gel-filtration in 20 mM Hepes/KOH, pH 7.3, 200 mM KCl, 10 mM MgCl$_2$, 1 mM DTT, and 5% (wt/vol) glycerol. Trypsin digestion of TA mutants was performed with trypsin concentrations ranging from 0 to 10 μg/ml. Purification of rat liver p115 was performed according to Levine et al., 1996.

Synthetic Peptides

p115 peptides: the 75mer (LQNEKNKLEVDITDSKKEQDDLLVL-LADQDQKIFSLKNKLKELGHPVEEEDELES*GDQDDEDDED-EDDGKEQGHI) and 26mer (EDELES*GDQDDEDDEDEDDG-KEQGHI), were both synthesized either with or without phosphorylation of the serine marked (*) and with an NH$_2$-terminal biotin-tag. The NH$_2$-terminal GM130 peptide is characterized in Nakamura et al. (1997). The standard CKII peptide substrate (RRRDDDS*DDDDD) was synthesized with or without a phosphorylation of the marked serine (*). All peptides were provided by the Peptide Synthesis Laboratory at Imperial Cancer Research Fund (ICRF).

Overlays

Overlays were performed using 0.1 mg/ml biotin-75mer peptide (Nakamura et al., 1997).

Light Scattering

Light scattering of TA, TA (S941A), TA (S941D), and 75mer peptide was performed in 20 mM Hepes/KOH, pH 7.3, 50 mM KCl, 10 mM MgCl$_2$, and 0.1 mM DTT using a miniDAWN machine following the manufacturer's instructions. Molecular weight was calculated using ASTRA software (Wyatt Technology Corporation).

Golgi Membrane Extracts

Purified rat liver Golgi membranes (RLG; Hui et al., 1998) were washed with 1 M KCl in sucrose buffer (0.2 M sucrose, 50 mM potassium phosphate, pH 6.7, and 5 mM MgCl$_2$) with added protease inhibitors (Nakamura et al., 1995) or 0.1 M sodium carbonate, pH 11. Membranes were rewashed with sucrose buffer and pellets extracted with 20 mM Hepes/KOH, pH 7.3, 200 mM KCl, 1 mM DTT, 1 mM EDTA, 1% (wt/vol) Triton X-100, 10 mM MgCl$_2$, and protease inhibitors for 1 h on ice. Extracts were then diluted with one vol of 20 mM Hepes/KOH, pH 7.3, to yield

Triton X-100 buffer (20 mM Hepes/KOH, pH 7.3, 100 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 0.5% [wt/vol] Triton X-100, and 5 mM MgCl$_2$), and clarified by centrifugation at 20,000 g for 10 min at 4°C. 1 mg starting RLG was extracted and diluted into 1 ml Triton X-100 buffer.

Generation of Peptide and TA Beads

NH$_2$-terminally biotinylated synthetic peptides were coupled to neutravidin beads (Pierce) in Triton X-100 buffer (Nakamura et al., 1997). His$_6$-TAs were bound to magnetic Ni-NTA agarose beads (QIAGEN) in 20 mM Hepes/KOH, pH 7.3, 200 mM KCl, 10 mM MgCl$_2$, 1 mM DTT, and 5% glycerol for 2 h at 4°C. Beads were subsequently washed into Triton X-100 buffer. Binding of proteins from salt-washed RLG extracts to peptide/TA beads was carried out by incubation in Triton X-100 buffer for 1 h at 4°C (Nakamura et al., 1997).

Immunoprecipitations

These were carried out using extracts of RLG (100 μg/reaction), which had been washed in either 1 M KCl or 0.1 M carbonate, pH 11. In some experiments, in vitro–translated and ^{35}S-labeled proteins (∼10 ng p115 [HTA, HT, H, TA], GM130, or Δ-N-GM130) were preincubated with the membrane extracts. In others, purified rat liver p115, TA, TA (S941A), TA (S941D) and p115 -26mer and -75mer peptides were added. Preincubations were performed in Triton X-100 buffer for 1 h at 4°C. In experiments involving CKII (0.2 μunits, recombinant human CKII; Calbiochem-Novabiochem), reactions were subsequently incubated at 30°C for 10 min in the presence of 10 μM GTP and 5 μCi γ-[^{32}P]GTP. Reactions were incubated with polyclonal anti-Giantin, anti-p115, or anti-GM130 antibodies (3 μl of the appropriate antiserum and 20 μl of packed protein A beads; Amersham Pharmacia Biotech) for 2 h at 4°C. Beads were washed with Triton X-100 buffer, and proteins eluted in SDS sample buffer. Samples were fractionated by SDS-PAGE, immunoprecipitated and coimmunoprecipitated proteins were detected by Western blotting using mAbs, or in experiments where ^{35}S-labeled proteins were added by exposure to a PhosphorImager. PhosphorImager quantitation was carried out using Imagequant. In all experiments, signals were corrected for immunoprecipitation efficiencies by detecting the protein the antibody was directed against using mAbs. Western blot signals were quantitated using NIH-image.

In Vitro Protein Phosphorylation

CKII-, RLG- and MGF-mediated phosphorylation of p115 was carried out by incubation of 0.2 μunits of recombinant human CKII (Calbiochem-Novabiochem), 10 μg RLG, or 10 μg MGF with either p115, TA, or TA (S941A) in the presence of either 10 μM GTP, and 5 μCi γ-[^{32}P]GTP, or 10 μM ATP and 5 μCi γ-[^{32}P]ATP (unless otherwise stated) in phosphorylation buffer (20 mM Hepes/KOH, pH 7.3, 50 mM KCl, 10 mM MgCl$_2$, 0.1 mM DTT, and 0.2 M sucrose) with added protease inhibitors (Nakamura et al., 1995). Reactions were carried out for 10 min at 30°C unless otherwise stated. In some experiments reactions contained a 100× molar excess (over the final p115 concentration in the reaction) of either the phosphorylated or nonphosphorylated CKII standard substrate. In other experiments reactions contained either staurosporine, DRB, chrysin, roscovitine, KT5720, H-89, or calphostin C at the concentrations indicated in the text or figure legends (all kinase inhibitors were from Calbiochem-Novabiochem, except chrysin, which was from Sigma-Aldrich); or, 1 μl anti–CKII-α or 1 μl anti–CKII-β antibodies. CKII-mediated phosphorylation reactions were terminated by addition of SDS-PAGE sample buffer. RLG-mediated phosphorylation reactions were terminated by addition of KCl to a final concentration of 1 M on ice, releasing p115/TA from RLG. The RLG were removed by centrifugation. SDS-PAGE sample buffer was added directly to resulting samples containing p115. Samples containing TA were incubated with Ni beads to recover His-tagged proteins. Phosphorylated p115/TA proteins were visualized by Coomassie staining followed by exposure to a PhosphorImager.

RLG-mediated phosphorylation of a standard CKII peptide substrate was carried out in the presence of 35 μM GTP and 10 μCi γ-[^{32}P]GTP at 30°C. Reactions were dotted onto phosphocellulose filters, washed in 100 mM phosphoric acid followed by 100% ethanol, dried, and phosphorylation levels quantitated using a scintillation counter.

Golgi Reassembly Reactions

MGF were generated as described (Shorter and Warren, 1999), and were incubated at 0.75–1 mg/ml for 60 min at 37°C with NSF (100 ng/μl),

α-SNAP (25 ng/μl), γ-SNAP (25 ng/μl), an ATP regeneration system, and either 0–260 nM rat liver p115, TA (or TA mutants), 75mer, or 26mer. The final reaction volume was 20 μl. In some experiments, the MGF were preincubated for 15 min on ice before reassembly with a 100× molar excess of CKII substrate or the same substrate phosphorylated on the serine residue. In other experiments, reassembly was conducted in the presence of either 100 μM staurosporine, DRB, chrysin, or roscovitine, or 1 μl anti–CKII-α or 1 μl anti–CKII-β antibodies. Reactions were fixed and processed for EM, and the amount of cisternal regrowth determined (Rabouille et al., 1995).

Results

p115 Binds to GM130 and Giantin via Its COOH-terminal Acidic Domain

To understand how p115 acts to tether membranes and vesicles we investigated the role played by phosphorylation of p115. First, however, we tested whether the COOH-terminal region of p115 that contains the serine known to be phosphorylated in vivo (Sohda et al., 1998) binds directly to GM130 and Giantin.

Truncated forms of p115 (Fig. 1) were tested for their ability to bind to GM130 and Giantin. We generated DNA constructs encoding full-length p115 (HTA) and p115 lacking either the acidic domain (HT), the coiled-coil tail and acidic domain (H), or the globular head domain (TA). Serine 941, the bovine equivalent of the human serine 942 that is phosphorylated in p115 in vivo, is located within the acidic domain (A; Sohda et al., 1998). The DNA was transcribed and translated in vitro in the presence of ^{35}S-methionine. The translated proteins were mixed with detergent extracts of rat liver Golgi (RLG) membranes containing both Giantin and GM130, but from which >95% of the endogenous p115 had been removed by washing with 1 M KCl. Giantin or GM130 were then immunoprecipitated and coimmunoprecipitation of the different p115 forms monitored (Fig. 2 A). The same p115 binding pattern was obtained for both GM130 and Gian-
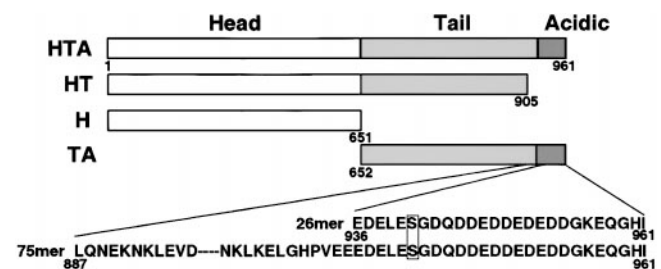
Figure 1. Schematic illustration of p115 constructs. p115 constructs were generated using the bovine cDNA. The full-length p115 sequence (HTA) encodes a globular head domain (boxed in white), a coiled-coil tail domain (boxed in light gray) and a short acidic region (boxed in dark gray). DNA encoding truncated forms of p115 was as follows: HT encodes the head region and most of the tail region (amino acids 1–905). H encodes the head domain alone (amino acids 1–651), while TA encodes the tail and acidic domains (amino acids 652–961). Two COOH-terminal peptides were also synthesized: A 75mer comprising a short stretch of coiled coil sequence and the acidic domain (amino acids 887–961), and a 26mer comprising the acidic domain alone (amino acids 936–961). Serine 941 in the bovine sequence (boxed) is equivalent to Serine 942 in the human sequence, which is known to be phosphorylated in vivo (Sohda et al., 1998).
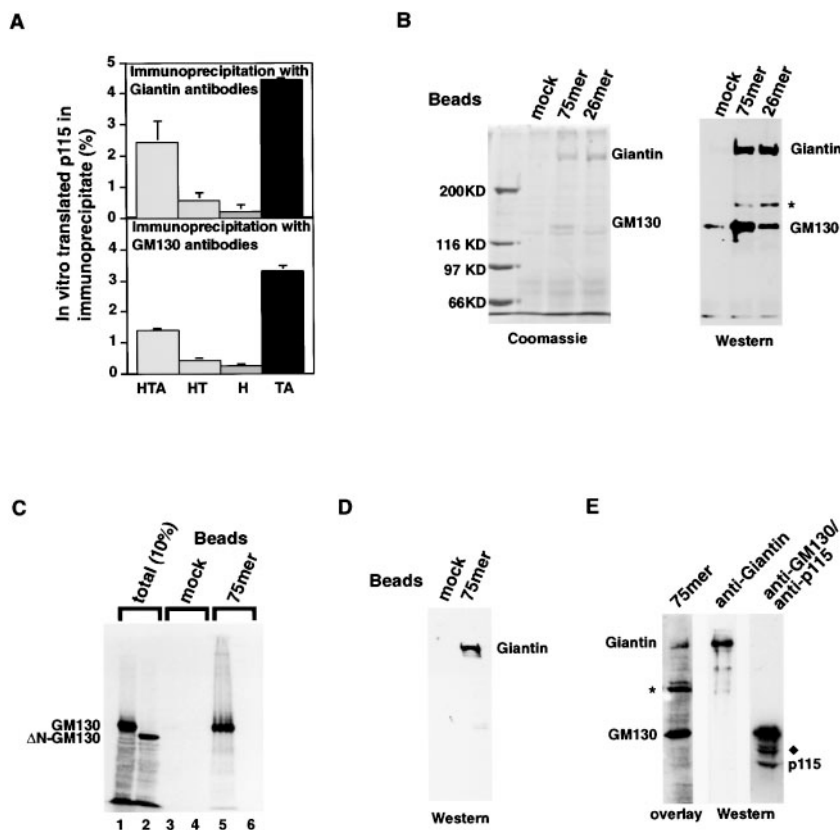
Figure 2. The COOH-terminal acidic region of p115 binds to GM130 and Giantin. (A) Coupled in vitro transcription-translation reactions were performed using plasmids encoding HTA, HT, H, and TA. Each translate was mixed with salt washed RLG extracted in Triton X-100 buffer. Either Giantin or GM130 was immunoprecipitated using polyclonal antibodies and coimmunoprecipitation of the different forms of p115 analyzed by SDS-PAGE followed by PhosphorImager visualization and quantitation. Values represent means $\pm$ SEM (n = 3–5). (B) Salt washed RLG (200 μg) were solubilized in Triton X-100 buffer and incubated with either mock, 26mer, or 75mer beads. Proteins bound were separated by SDS-PAGE and visualized by Coomassie staining or Western blotting using antibodies to Giantin and GM130. (C) ^{35}S-GM130 (lane 1) and [35]S–ΔN-GM130 (lane 2) were generated by transcription and translation in vitro. After incubation with either mock beads (GM130: lane 3, ΔN-GM130: lane 4) or 75mer peptide beads (GM130: lane 5, ΔN-GM130: lane 6) the bound proteins were visualized by SDS-PAGE followed by exposure to a PhosphorImager (lanes 3–6) as was 10% of the in vitro translated proteins added to the beads (lanes 1 and 2). (D) RLG membranes (200 μg) were washed with carbonate buffer to remove p115 and GM130 before extraction in Triton X-100 buffer. Extracts were incubated with mock or 75mer peptide beads and bound proteins were separated by SDS-PAGE and visualized by Western blotting using anti–Giantin antibodies. (E) RLG proteins (10 μg) were separated by SDS-PAGE and transferred to nitrocellulose. Blots were either overlaid with biotin 75mer followed by streptavidin-HRP and ECL to detect the bound peptide (left) or probed with antibodies directed against Giantin (middle) or GM130 and p115 (right). (*Giantin fragment; ◆ GM130 fragments.)

tin, and showed that only those forms of p115 containing the acidic domain (A) coimmunoprecipitated. HTA and TA bound very well, whereas HT and H only gave signals similar to those obtained with nonimmune sera (data not shown). Furthermore, TA bound about twice as well as HTA suggesting that the globular head domain may regulate the binding.

A direct role for the acidic domain of p115 in binding to GM130 and Giantin was obtained using two COOH-terminal peptides containing serine 941 (see Fig. 1): a 75mer representing a short stretch of the coiled coil region in addition to the acidic region; and a 26mer representing only the acidic region of p115. The peptides were NH₂-terminally biotinylated and coupled to neutravidin beads. The beads were then incubated with detergent extracts of salt washed RLG and binding of GM130 and Giantin monitored. Both peptides selectively extracted GM130 and Giantin when compared with mock beads (Fig. 2 B).

To test whether the acidic domain of p115 could bind Giantin and GM130 separately we used sources of GM130 and Giantin that contained only one of the two proteins. ^{35}S-labeled GM130 generated by in vitro transcription and translation was used as a source of GM130. 75mer and 26mer (data not shown) peptide beads, but not mock beads, bound in vitro translated GM130 (Fig. 2 C). As a control, an NH₂-terminal truncated form of GM130 (ΔN-

GM130) lacking the p115 binding site (Nakamura et al., 1997) was used. Neither mock nor 75mer peptide beads bound this form of GM130 (Fig. 2 C).

To generate a Giantin source containing no GM130, RLG were washed in carbonate buffer, pH 11, which removes more than 98% of the GM130 as well as the p115 (Nakamura et al., 1995). The membranes were then extracted in Triton X-100 buffer and incubated with either mock beads or 75mer peptide beads. The 75mer beads, but not the mock beads, bound Giantin (Fig. 2 D).

Further evidence for a direct interaction of the acidic domain of p115 with Giantin and GM130 came from overlays using the biotin-75mer peptide (Fig. 2 E). RLG proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, incubated with biotin-75mer and the bound peptide visualized using streptavidin-HRP and ECL. The 75mer bound to Giantin and GM130, but not to p115. Similar results were obtained using the 26mer peptide (data not shown). We conclude that Giantin and GM130 can bind directly and independently to the acidic domain of p115.

The Acidic Domain of p115 Is Not Sufficient for Reassembly of Golgi Cisternae

The forms of p115 that could bind Giantin and GM130 were next tested for their ability to regrow cisternae from
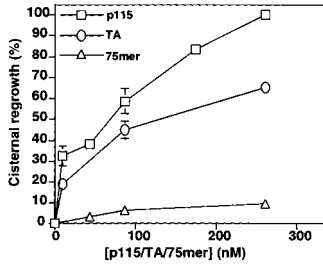
Figure 3. The acidic domain of p115 alone is not sufficient to stimulate cisternal regrowth. MGF were incubated with NSF, α-SNAP, and γ-SNAP and increasing concentrations of either p115 (squares), TA (ovals), or the 75mer peptide (triangles) for 60 min at 37°C. Cisternal regrowth was measured by quantitative EM. The results are presented as the mean of three experiments ± SEM.

MGF. p115 lacking the head domain (TA) was generated in *E. coli* and purified using an NH$_2$-terminal His$_6$tag. We compared the ability of TA and the short peptides to stimulate cisternal reassembly with that of purified rat liver p115 (Fig. 3).

Purified RLG were incubated with mitotic cytosol to generate MGF (Rabouille et al., 1995). Recovery of MGF (Shorter and Warren, 1999) was followed by incubation with NSF, α-, and γ-SNAP and increasing concentrations of either purified rat liver p115, TA, or the short COOH-terminal peptides. Cisternal regrowth was monitored by quantitative EM (Rabouille et al., 1995).

As shown in previous work, p115 stimulated reassembly of cisternae in a concentration-dependent manner (Shorter and Warren, 1999; Fig. 3). Even without the head domain TA was almost as efficient, reaching ∼70% of the maximum cisternal reassembly obtained using p115. The 75mer peptide and the 26mer peptide (data not shown) could not reassemble Golgi cisternae, despite their ability to bind to GM130 and Giantin (Fig. 2 B). Gel-filtration (data not shown) and light scattering methods (Table I) showed that the peptides were monomeric, whereas TA was dimeric, as is p115 (Waters et al., 1992; Sapperstein et al., 1995). This suggests the peptides might not function in the assay because they cannot form dimers.

Serine 941 in the Acidic Domain of p115 Is Essential for Reassembly of Golgi Cisternae

To determine whether phosphorylation of serine 941 was required for cisternal reassembly we mutated serine 941. So far it has not been possible to express full-length p115 in *E. coli*. So, since TA was almost as active as full-length p115, we chose to mutate TA instead. We generated an

Table I. Light Scattering Analysis of TA Constructs and the 75mer Peptide

	Mol wt (calculated)	Mol wt (measured by light-scattering)	Predicted oligomerization state
	g/mol	*g/mol*	
TA	3.80×10^4	$(7.42 \pm 0.06) \times 10^4$	Dimer
TA (S941A)	3.80×10^4	$(7.13 \pm 0.04) \times 10^4$	Dimer
TA (S941D)	3.80×10^4	$(7.75 \pm 0.50) \times 10^4$	Dimer
75mer	8.62×10^3	$(8.37 \pm 2.90) \times 10^3$	Monomer

Light-scattering measurements of TA (2.5 mg/ml), TA (S941A) (1.71 mg/ml), TA (S941D) (1.6 mg/ml), and the 75mer peptide (1 mg/ml) were carried out using a miniDAWN machine. Errors represent the standard deviation of the mean molecular weight.

alanine mutant (TA [S941A]) to prevent phosphorylation at this site and an aspartic acid mutant (TA [S941D]) since aspartic acid can mimic a phosphorylated serine residue (Huang and Erikson, 1994). All three constructs were expressed in *E. coli*, purified, and characterized so as to ensure that the mutations had no effect on the physical or biochemical properties of the protein. Gel filtration showed that all three had the same molecular weight (data not shown) and light scattering confirmed that all three were dimers (Table I). They all had the same sensitivity to trypsin (data not shown) and they each selectively removed GM130 and Giantin from detergent-solubilized Golgi extracts (Fig. 4 A). They were all similarly efficient in linking GM130 to Giantin, as shown below in Fig. 9 B, GM130 was coimmunoprecipitated with Giantin in a similar dose-dependent manner for all three of the TA constructs.

We then compared the activity of the mutant TAs with that of TA in cisternal reassembly (Fig. 4 B). TA (S941A) was unable to support reassembly even at high concentrations, showing that serine 941 is important for its function. Since the alanine substitution did not significantly alter the interaction of TA with Giantin and GM130, phosphorylation of TA appears to be essential for TA function in
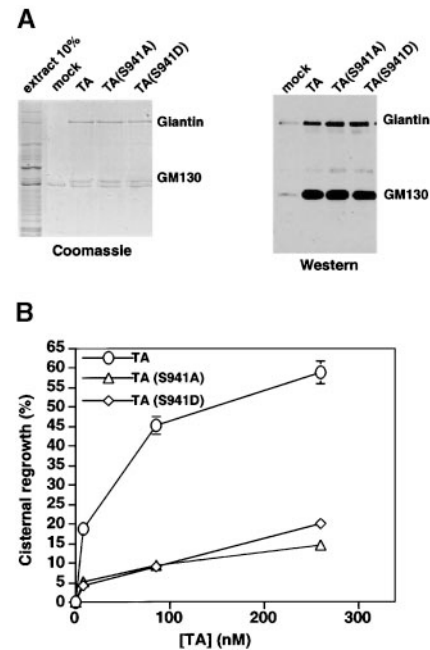
Figure 4. Cisternal regrowth requires serine 941. (A) Serine 941 in TA was substituted with either alanine (TA [S941A]) or aspartic acid (TA [S941D]) by site-directed mutagenesis. Salt-washed RLG membranes (200 μg) were extracted in Triton X-100 buffer and incubated with either Ni-magnetic beads alone or Ni-magnetic beads to which an equal amount of TA, TA (S941A) or TA (S941D) had been coupled. Proteins bound were separated by SDS-PAGE and visualized by Coomassie staining or Western blotting using antibodies to Giantin and GM130. (B) MGF were incubated with NSF, α-SNAP, and γ-SNAP and increasing concentrations of either TA (ovals), TA (S941A) (triangles) or TA (S941D) (diamonds) for 60 min at 37°C. Cisternal regrowth was measured by quantitative EM. The results are presented as the mean of three experiments ± SEM.

Golgi reassembly. TA (S941D) was also unable to stimulate reassembly (Fig. 4 B). Hence, it is likely that either the aspartic acid substitution did not mimic the phosphorylated form of TA or that a cycle of phosphorylation/dephosphorylation is required for the process by which p115 functions in stimulating membrane fusion.

p115 Forms Containing Serine 941 Can Be Phosphorylated by CKII

Having shown that serine 941 is essential for Golgi reassembly it became important to identify the responsible kinase. The kinase would have to be associated with Golgi membranes, since Golgi reassembly reactions were carried out in the absence of cytosol. This would also be in agreement with the findings of Ikehara and colleagues who reported that Golgi prepared from HeLa cells contained a kinase activity capable of phosphorylating Golgi-bound p115 (Sohda et al., 1998).

We tested whether RLG contained a kinase capable of phosphorylating purified p115 (Fig. 5 A). p115 was incubated with RLG in the presence of either γ-[^{32}P]ATP or γ-[^{32}P]GTP and phosphorylation of p115 monitored. RLG could phosphorylate p115 in vitro and the responsible kinase was able to use both ATP and GTP as a phosphate source.

Next, we wanted to know whether p115 was being phosphorylated at serine 941. We therefore compared TA and TA (S941A) as substrates for RLG mediated phosphorylation and found that only TA was phosphorylated in the presence of either ATP or GTP (Fig. 5 B). Serine 941 is clearly the target for the kinase in TA and most likely in the full-length protein since this serine was the only phosphorylatable residue in full-length p115 (Sohda et al., 1998).

CKII is the best-characterized kinase known to be able to use GTP as well as ATP as a phosphate source (Allende and Allende, 1995). So the fact that RLG-mediated phosphorylation of p115 was very efficient in the presence of GTP indicated that the kinase in question might be CKII or a CKII-like kinase. Another hint came from the amino acid sequence surrounding Serine 941, which is very acidic. CKII is known to phosphorylate serine or threonine residues flanked by acidic amino acids (Songyang et al., 1996). Furthermore, CKII has been found associated with membranes (Dittié et al., 1997), although it is mainly a soluble protein residing predominantly in the nucleus, as well as in the cytosol (Lorenz et al., 1993).

When RLG was incubated with γ-[^{32}P]GTP in the presence or absence of a CKII peptide substrate, an increase in protein phosphorylation was observed in the reactions containing the CKII peptide (Fig. 5 C). Increasing the amount of RLG used in the reaction increased the amount of CKII peptide phosphorylation showing that RLG contains CKII or a CKII like activity.

If the RLG kinase, which phosphorylates p115, is indeed CKII, pure CKII should be able to phosphorylate p115. p115 was incubated with pure recombinant human CKII protein (Calbiochem-Novabiochem) in reactions containing either ATP or GTP. Phosphorylation of p115 took place using either nucleotide (Fig. 5 D). TA was also a substrate for recombinant CKII, whereas TA (S941A) was not (Fig. 5 D). This strongly suggests that CKII phosphorylates p115 at serine 941.
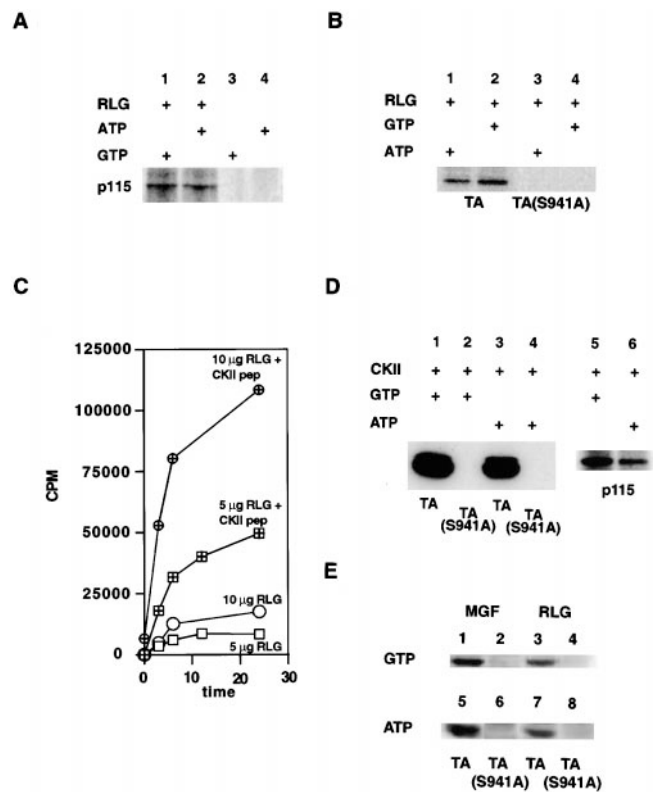
Figure 5. CKII and RLG can phosphorylate serine 941. In vitro phosphorylation of p115, TA or TA (S941A) was carried out using RLG, MGF, or recombinant human CKII in the presence of γ-[^{32}P]ATP or γ-[^{32}P]GTP. Phosphorylated substrates were visualized after SDS-PAGE and Coomassie staining by exposure to a PhosphorImager. (A) p115 (0.6 μg) was incubated in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of RLG for 10 min at 30°C. Reactions contained either γ-[^{32}P]ATP (lanes 2 and 4), or γ-[^{32}P]GTP (lanes 1 and 3). (B) 0.2 μg TA (lanes 1 and 2) or TA (S941A) (lanes 3 and 4) was incubated with RLG in the presence of either γ-[^{32}P]ATP (lanes 1 and 3), or γ-[^{32}P]GTP (lanes 2 and 4) for 10 min at 30°C. (C) RLG was incubated in the presence or absence of 10 μg CKII peptide substrate for increasing time at 30°C. Reactions contained 35 μM GTP and γ-[^{32}P]GTP. Phosphorylation activity was quantitated on phosphocellulose filters by scintillation counting. (D) Recombinant human CKII was incubated with either 5 μg TA (lanes 1 and 3), TA (S941A) (lanes 2 and 4), or 0.5 μg p115 (lanes 5 and 6) in the presence of either γ-[^{32}P]GTP (lanes 1, 2, and 5) or γ-^{32}P-ATP (lanes 3, 4, and 6). (E) MGF (lanes 1, 2, 5, and 6) or RLG (lanes 3, 4, 7, and 8) was incubated with 0.2 μg TA (lanes 1, 3, 5, and 7) or TA (S941A) (lanes 2, 4, 6, and 8) in the presence of γ-[^{32}P]GTP (lanes 1–4) or γ-[^{32}P]ATP (lanes 5–8).

Since MGF were used to reassemble cisternae we wanted to make sure that they retained the kinase activity found in the RLG from which they were generated. We compared the ability of MGF to phosphorylate TA and TA (S941A) with that of RLG (Fig. 5 E). We found that MGF did possess the kinase activity that recognized TA, but not TA (S941A).

Inhibition of CKII Inhibits Golgi Reassembly

Three separate approaches were used to implicate CKII in the reassembly of Golgi cisternae. We examined the effect

of adding either drugs known to inhibit CKII, antibodies to CKII, or CKII peptide substrates.

Four different kinase inhibitors were tested at 100 μM: staurosporine, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), chrysin and roscovitine (Fig. 6 A). Staurosporine has limited selectivity for the kinases it inhibits as it binds to the catalytic domain of several kinases that share homology within that region. It is not a potent inhibitor of CKII, but does inhibit when added in the μM range (Meggio et al., 1995). Addition of staurosporine inhibited cisternal reassembly by about 50%. DRB caused an ~50% reduction of cisternal regrowth. DRB is a relatively specific inhibitor of CKII, but it may inhibit other kinases (Shugar, 1994; Yankulov et al., 1995). In contrast, chrysin is thought to be specific for CKII (Critchfield et al., 1997) and reduced Golgi reassembly by >75%. Roscovitine, which was added as a control, is specific for cdc2 and cdk2 (De Azevedo et al., 1997) and showed no significant effect on cisternal regrowth.

To determine whether the effect of the drugs was related to phosphorylation of p115 they were added to in vitro phosphorylation reactions containing RLG and p115 (Fig. 6 B). The same pattern of results was obtained as for the Golgi reassembly reactions. Addition of staurosporine resulted in an ~70% inhibition of p115 phosphorylation and DRB inhibited to a similar extent. The most specific inhibitor, chrysin, reduced phosphorylation by ~90%. The same pattern of inhibition was seen when TA was used as a substrate for RLG mediated phosphorylation (data not shown). Several control inhibitors were also tested: roscovitine (Fig. 6 B), KT5720 (a specific inhibitor of protein kinase A), H-89 (an inhibitor of protein kinases A, C and D and casein kinase I), and calphostin C (inhibitor of protein kinases C, A, and G). In all cases, at all concentrations tested, none of these had an effect on RLG mediated phosphorylation of p115 in the presence of ATP or GTP (data not shown).

Next, we examined the effect of adding antibodies to CKII to Golgi reassembly reactions (Fig. 6 C). Antibodies raised against human CKII-α or -β subunits (Calbiochem-Novabiochem) both inhibited Golgi reassembly by ~45%. When added to the in vitro phosphorylation assay, these antibodies inhibited p115 phosphorylation by ~50% (Fig. 6 D). Again the effect on Golgi reassembly paralleled the effect on p115 phosphorylation.

If phosphorylation of p115 by CKII is required for Golgi reassembly, the addition of CKII peptide substrates should inhibit by competing with p115 for the kinase. Addition of the CKII peptide substrate at a 100-fold molar excess over added p115, resulted in a 70% reduction in cisternal regrowth (Fig. 6 E). As a control, the same peptide phosphorylated on the serine target residue had only a modest effect (Fig. 6 E).

To ensure that the peptides did not have a general inhibitory effect on Golgi reassembly, the effect of the peptides was examined in the Golgi reassembly pathway that uses p97 and p47 but does not require p115 for cisternal regrowth (Shorter and Warren, 1999). Neither the phosphor-
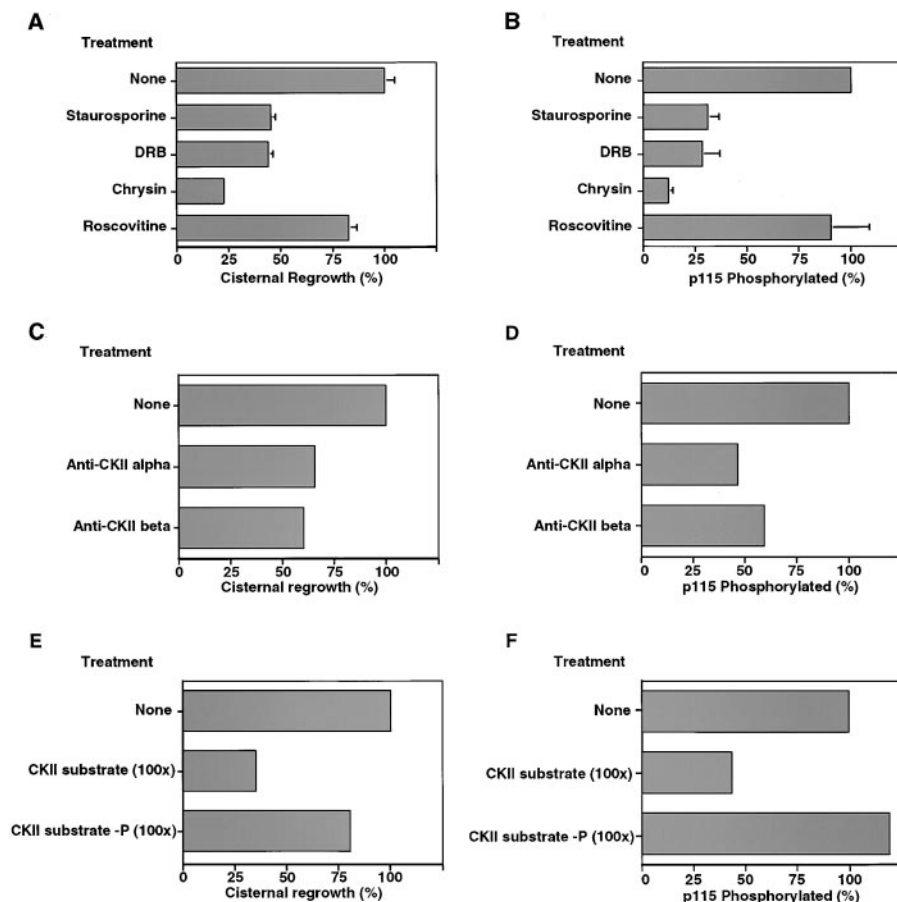
Figure 6. CKII inhibitors inhibit cisternal regrowth from MGF and phosphorylation of p115 by RLG. (A, C, and E) 10 μg MGF were incubated with NSF, α-SNAP, γ-SNAP, and maximum concentrations of p115 (261 nM). After incubation for 60 min at 37°C cisternal regrowth was measured by quantitative EM. (B, D, and F) 10 μg RLG was incubated with 0.6 μg p115 for 10 min at 30°C in the presence of γ-[³²P]GTP. Phosphorylation of p115 was monitored by SDS-PAGE and Coomassie staining followed by exposure to a PhosphorImager. (A and B) 100 μM staurosporine, DRB, chrysin, or roscovitine were present during the incubation. (C and D) MGF or RLG were pretreated with antibody directed against either the α or the β subunit of human CKII. (E and F) A CKII peptide substrate or the same substrate phosphorylated on the serine was present during incubations at a 100-fold molar excess over added p115. For all experiments, quantitations represent the means of two experiments, except where error bars are shown and values represent the means of three experiments ± SEM.

ylated nor the nonphosphorylated forms of the CKII peptide had any effect (data not shown). The inhibition was therefore specific for the NSF catalyzed Golgi reassembly pathway, which uses p115.

The effect of adding the peptides to the in vitro phosphorylation reactions was examined next (Fig. 6 F). As in the Golgi reassembly reactions only the nonphosphorylated CKII peptide substrate had a significant inhibitory effect on phosphorylation of p115 by RLG.

We conclude from these data that p115 dependent reassembly of Golgi cisternae requires the activity of CKII or a CKII like enzyme. Furthermore, it is likely that the requirement is due to its ability to phosphorylate p115, although we cannot exclude the possibility that other CKII targets are also involved in p115 mediated Golgi reassembly.

Phosphorylation of Serine 941 Enhances Binding to Giantin and GM130

Having established a role for serine 941 in the reassembly of Golgi cisternae, we then wanted to determine how phosphorylation of this residue affected the function of p115. The effect of phosphorylating the equivalent of serine 941 in the 75mer and 26mer peptides on binding to their Golgi binding partners, GM130 and Giantin, was therefore tested (Fig. 7).

The phosphorylated and nonphosphorylated 26mers were coupled to beads and incubated with RLG extracted in Triton X-100 buffer (Fig. 7 A). The membranes had been washed with 1 M KCl before extraction to remove any endogenous p115 that might have interfered with binding. Phosphorylation of the nonphosphorylated peptide was prevented by omitting ATP and GTP and also by the salt wash, which removed endogenous CKII activity (data not shown). As shown in Fig. 7A the phosphorylated form of the 26mer peptide was at least twice as efficient in binding Giantin and five times as efficient in binding GM130 than the nonphosphorylated form.

The binding was also tested in free solution by mixing the 26mer peptide with extracts of RLG. Again the RLG

was washed with salt before extraction and ATP and GTP were both omitted from the reaction. The complexes that formed were then isolated on neutravidin beads using the biotin tag on the peptide. Since the components were more dilute, the amounts of complex formed were smaller. The difference in binding of GM130 and Giantin to the phosphorylated versus the nonphosphorylated 26mer was less striking under these conditions, but the phosphorylated peptide did bind more of both proteins (Fig. 7 B). The longer 75mer peptide was also tested and the results showed a strong effect of phosphorylation of serine 941. The phosphorylated 75mer bound ~10 times more GM130 and Giantin than its nonphosphorylated counterpart (Fig. 7 B).

p115 Links GM130 to Giantin

Since phosphorylation of serine 941 increased the binding of p115 peptides to GM130 and Giantin it suggested that phosphorylation might enhance the function of p115 as a tethering protein. p115 tethers COPI vesicles to Golgi membranes and binds to Giantin on the vesicles and GM130 on the membranes (Sönnichsen et al., 1998). We suggested that p115 acts as a bridge, linking Giantin to GM130 and hence the vesicle to the membrane. There was, however, no direct evidence that such a tethering complex existed.

Therefore, we set up a system to show that p115 can link GM130 to Giantin. By carbonate washing of RLG we generated a Giantin enriched fraction that had <2% of the original levels of both p115 and GM130. This was solubilized in Triton X-100 buffer. GM130 was generated by transcription and translation in vitro and labeled with ^{35}S-methionine. Solubilized Giantin and ^{35}S-GM130 were then mixed in the presence of increasing concentrations of p115. Giantin was immunoprecipitated and the recovery of GM130 monitored. As shown in Fig. 8 A, GM130 coimmunoprecipitated with Giantin only in the presence of p115, the amount increasing linearly with increasing concentrations of p115. Formation of the complex depended on the presence of the NH[2] terminus of GM130, containing the p115 binding site (Nakamura et al., 1997). Mutant GM130 lacking this NH[2] terminus did not coimmunoprecipitate with Giantin (Fig. 8 C), neither did full-length GM130 in the presence of an NH[2]-terminal GM130 peptide (Fig. 8 B) representing the p115 binding site (Nakamura et al., 1997). The interaction of p115 with Giantin was not affected by the presence of the competing GM130 peptide or by the presence of the truncated GM130. Together these data show that p115 can link GM130 to Giantin.

Similar experiments were also carried out using the TA construct. As shown in Fig. 8 D, TA was able to link GM130 to Giantin and did so with a similar efficiency to p115 (see Fig. 8, A and D).

Linking of Giantin to GM130 by p115 was also shown by another means (Fig. 8 E). GM130 peptide beads were generated by coupling the biotinylated peptide representing the p115 binding site in GM130 to neutravidin beads. GM130 peptide or mock beads were then incubated in the presence or absence of excess p115. The beads were subsequently washed to remove free p115 and an excess of the Giantin enriched Golgi fraction added. Mock beads bound neither p115 nor Giantin. GM130 peptide beads with pre-
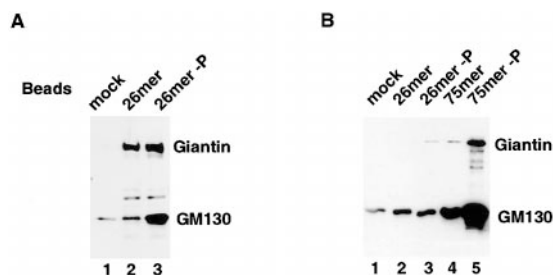
Figure 7. Phosphorylation of serine 941 enhances binding to GM130 and Giantin. (A) Triton X-100 extracts of salt washed RLG (200 µg) were incubated with either mock beads (lane 1), 26mer beads (lane 2) or beads bound to the 26mer phosphorylated at the equivalent of serine 941 (lane 3; 100 µg peptide). Bound GM130 and Giantin were separated by SDS-PAGE and visualized by Western blotting. (B) Triton X-100 extracts of salt washed RLG (40 µg) were incubated alone (lane 1) or with 4 µg of either the 26mer peptide (lane 2), the 75mer peptide (lane 4) or their phosphorylated counterparts (lane 3 and 5). The peptides were recovered on neutravidin beads and bound Giantin and GM130 were visualized as in A.
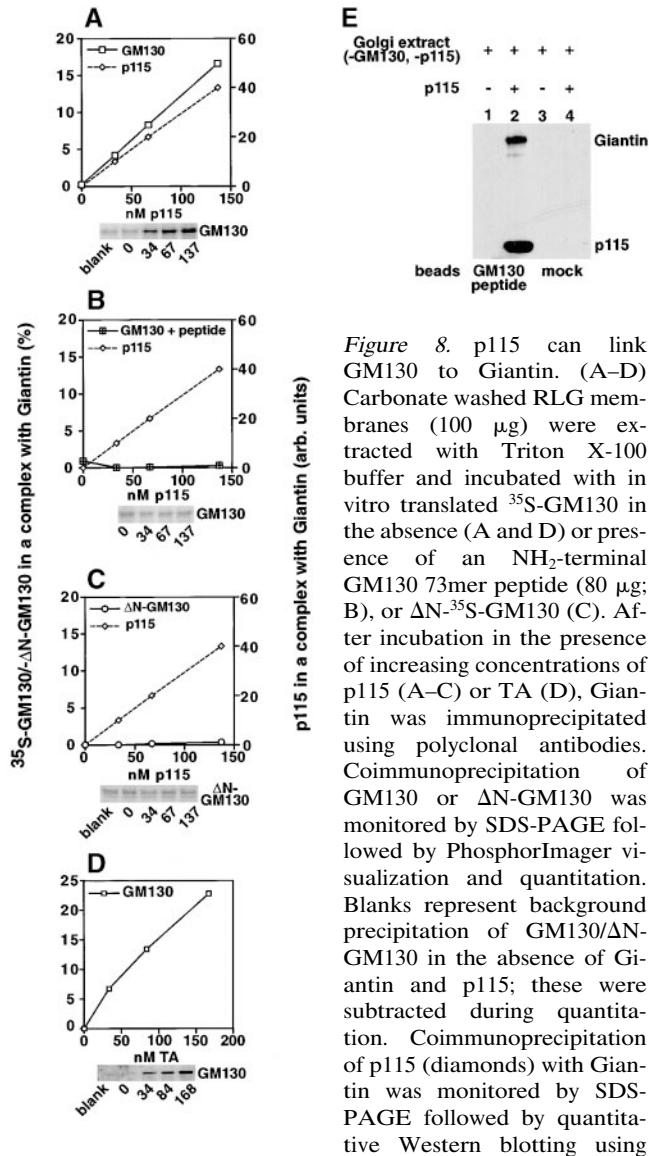
Figure 8. p115 can link GM130 to Giantin. (A–D) Carbonate washed RLG membranes (100 μg) were extracted with Triton X-100 buffer and incubated with in vitro translated ^{35}S-GM130 in the absence (A and D) or presence of an NH$_2$-terminal GM130 73mer peptide (80 μg; B), or ΔN-^{35}S-GM130 (C). After incubation in the presence of increasing concentrations of p115 (A–C) or TA (D), Giantin was immunoprecipitated using polyclonal antibodies. Coimmunoprecipitation of GM130 or ΔN-GM130 was monitored by SDS-PAGE followed by PhosphorImager visualization and quantitation. Blanks represent background precipitation of GM130/ΔN-GM130 in the absence of Giantin and p115; these were subtracted during quantitation. Coimmunoprecipitation of p115 (diamonds) with Giantin was monitored by SDS-PAGE followed by quantitative Western blotting using mAbs to p115. (E) Mock beads (lanes 3 and 4) or beads to which the GM130 peptide had been coupled (lanes 1 and 2) were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of excess p115. Free p115 was removed by washing and the beads were incubated with carbonate washed Golgi extracts (100 μg) containing Giantin, but no GM130 or p115. Bound p115 and Giantin were visualized by Western blotting.

bound p115 pulled down Giantin, whereas GM130 peptide beads with no p115 did not (Fig. 8 E). The binding of Giantin did not displace any p115 (data not shown) showing that Giantin only bound GM130 through its binding to p115. These data argue that p115 can link Giantin to GM130 by interacting with its binding site on GM130 while simultaneously interacting with Giantin. p115 can, therefore, act as a bridging molecule.

Phosphorylation of Serine 941 Enhances p115-mediated Tethering of GM130 to Giantin

We next tested whether the p115 peptides that were able to bind to GM130 and Giantin could also link them (Fig. 9 A). Since the data in Fig. 8 showed that Giantin and GM130 do

not interact in the absence of p115, we simplified the assay by using salt-washed Golgi membranes as the source of both proteins. Salt washing removes >95% of the endogenous p115, and that remaining (≤3 nM final concentration in the assay) was well below the levels of added p115 and other constructs. Coimmunoprecipitation of GM130 with Giantin was monitored by Western blotting. The percentage of GM130 brought down was lower than that seen in the previous assay because chemical rather than radiochemical amounts were being precipitated.

Using this system the TA construct of p115 was again similar to p115 in its ability to link GM130 to Giantin (Fig. 9 A). Higher levels of TA could be added because it is a recombinant protein available in larger amounts than p115. In marked contrast, the 26mer (data not shown) and 75mer (Fig. 9 A) were unable to link GM130 to Giantin even though they could bind to both proteins (Figs. 2 and 7). The same was true for the phosphorylated peptides (data not shown). This supports the earlier suggestion that dimers are needed for tethering.

To test the role of CKII-mediated phosphorylation in tethering we carried out the assay in the presence or absence of added recombinant CKII (Fig. 9 B). The TA constructs were used so as to exploit mutations at serine 941. Incubation of the reaction mix with CKII resulted in phosphorylation of TA and enhanced the ability of TA to link Giantin and GM130, the effect being most striking at low concentrations of TA. At higher concentrations mass action overcame the stimulation by phosphorylation. The tethering complex formed in the presence of TA (S941A) was not improved by the addition of CKII, showing that phosphorylation of serine 941 is solely responsible for the increased efficiency of linking in the presence of CKII. TA (S941D) did not show elevated linking as compared with TA (S941A) suggesting that it does not mimic the phosphorylated form of p115.

Together, these data argue that p115 acts as a link between Giantin and GM130 and that this link can be strengthened by phosphorylation of p115 mediated by CKII or a CKII-like enzyme.

Discussion

Using a cell-free assay that mimics Golgi reassembly, we have identified a role for phosphorylation in the vesicle docking reactions that lead to membrane fusion. The target is the vesicle tethering protein, p115, and the kinase is either CKII or a closely related enzyme. Phosphorylation strengthens the bridge formed by p115 as it links GM130 and Giantin and may work to transfer a tethered vesicle to the next step of the docking reaction.

p115 Links GM130 to Giantin

The region of GM130 required for p115 binding had previously been mapped to the first 73 NH$_2$-terminal amino acids (Nakamura et al., 1997). The Giantin domain required for p115 binding has also been mapped to the NH$_2$ terminus though it varies from 70 to 448 amino acids, depending on the laboratory (Linstedt et al., 2000; Lesa et al., 2000). Research shows that the COOH-terminal tail of p115 is required for binding to GM130 (Nelson et al., 1998), and, more recently, for binding to Giantin (Linstedt et al.,
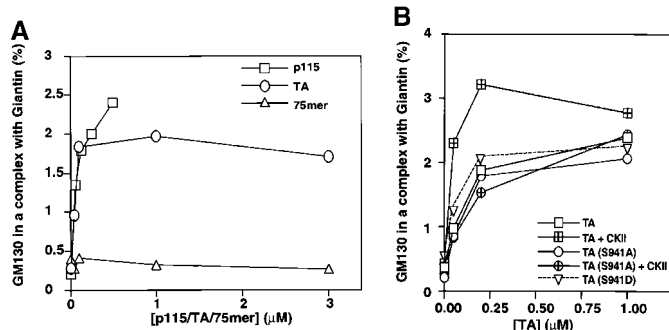
Figure 9. Phosphorylation of serine 941 enhances TA mediated tethering of GM130 to Giantin. (A) Salt washed RLG membranes (100 μg) were extracted in Triton X-100 buffer and incubated with increasing concentrations of either p115 (squares), TA (ovals), or the 75mer peptide (triangles). Giantin was immunoprecipitated using polyclonal antibodies and coimmunoprecipitation of GM130 measured by SDS-PAGE followed by quantitative Western blotting using a mAb against GM130. Values represent means of two separate experiments. (B) Salt washed RLG (100 μg) were solubilized in Triton X-100 buffer and either TA (squares), TA (S941A) (ovals) or TA (S941D) (inverted triangles) added at increasing concentrations. GTP and γ-[^{32}P]GTP was subsequently added to reactions containing TA and TA (S941A) and incubation continued for 10 min at 30°C in the presence (crossed symbols) or absence (open symbols) of recombinant human CKII (0.2 μunits; Calbiochem-Novabiochem). Subsequently, immunoprecipitation of Giantin was performed using polyclonal antibodies and coimmunoprecipitation of GM130 measured by SDS-PAGE followed by quantitative Western blotting using mAbs to GM130. Values represent means of two separate experiments.

2000). We have confirmed and extended these results, mapping the binding site to the extreme COOH-terminal acidic domain. Two peptides covering this region of p115 (a 26mer and a 75mer) both bind to GM130 and Giantin in RLG detergent extract. They also bind to each protein in the absence of the other. Hence, in vitro translated GM130 binds to these peptides but not when the NH$_2$ terminus of GM130 is missing. Carbonate treatment of RLG removes >98% of GM130 and p115 leaving Giantin, which alone can bind to these peptides. Lastly, these peptides will recognize Giantin and GM130 in overlay blots showing that there is a direct interaction between the COOH terminus of p115 and both of these proteins.

However, these synthetic peptides were unable to link GM130 to Giantin, whether they were phosphorylated or not. Tethering assays showed that GM130 could only be coprecipitated with Giantin in the presence of p115 or the TA construct lacking the globular head domain. Dose-dependent assays showed that the TA construct was ~60–70% as efficient as p115 in linking GM130 to Giantin. The lack of any linking by the synthetic peptides might reflect their oligomerization state. Gel-filtration and light scattering data show that TA, like p115, is dimeric, whereas the 26mer and the 75mer are monomeric. An attractive model is that dimerization is essential for p115-mediated linking of GM130 and Giantin, since it would explain how p115 could link GM130 to Giantin when both proteins interact with the same small region of p115. GM130 and Giantin would be linked if they each bind to one of the two acidic domains in the p115 dimer.

This suggestion is inconsistent with recent work from Linstedt et al. (2000). It showed that p115 and its constructs could bind to either GM130 or Giantin but could not link them. There could be many reasons for the difference between their results and ours but two points are worth noting. The first is that none of the reported experiments designed to detect linked complexes of GM130, p115 and Giantin used full-length GM130 and Giantin. One or the other of these two proteins was always present as an NH$_2$-terminal recombinant protein. Though the binding sites for p115 have been mapped to the NH$_2$ termini of both proteins, it is possible that other parts of these molecules might be needed to stabilize the tether.

The second reason is that p115 was limiting in those ex-

periments that measured tethering. In the one experiment we report, where we used the NH$_2$-terminal fragment of GM130 to bind Giantin, we could only detect bound Giantin when we used excess p115 to saturate the NH$_2$-terminal fragment (Fig. 8 E). Since stable tethering of vesicles to membranes is likely the consequence of multiple tethers, individual tethers might be quite weak so it is important to keep the concentration of interacting components as high as possible to observe the tethering complex.

Both the interaction of p115 with GM130 and with Giantin is required in the NSF-catalyzed pathway of membrane fusion (Shorter and Warren, 1999), which involves p115-mediated tethering of COPI vesicles to Golgi membranes (Sönnichsen et al., 1998). When the interaction with one p115 binding partner is blocked, the other binding partner alone is insufficient to allow fusion implicating both proteins in the same fusion process (Shorter and Warren, 1999). We therefore favor the idea that p115 functions by interacting with both proteins simultaneously, acting as a bridge to link GM130 to Giantin.

Truncated forms of p115 only stimulated Golgi reassembly in vitro if they were able to link GM130 to Giantin. Thus, TA linked GM130 to Giantin and stimulated cisternal regrowth with 60–70% of the efficiency observed for full-length p115. The shorter p115 peptides (whether phosphorylated or not) did not stimulate Golgi reassembly and failed to link GM130 to Giantin. This strongly suggests that linking of GM130 to Giantin via p115 is a prerequisite for Golgi reassembly by the NSF pathway and likely represents the tethering of vesicles before membrane fusion. Intriguingly, the head domain of p115 was not required for bridging between GM130 and Giantin, or for stimulating Golgi reassembly. The head domain has been shown to facilitate targeting of p115 to the Golgi membrane in vivo (Nelson et al., 1998). Likewise, association of p115 with vesicular tubular clusters (VTCs) is also affected by deletions in the head domain, which cause p115 to localize more with ER-like structures (Nelson et al., 1998). Binding to these structures does not involve interaction with GM130 or Giantin (Nakamura et al., 1997; Lesa et al., 2000). An attractive possibility is that the head domain facilitates the transport of p115 from VTCs to the Golgi in vivo, where p115 then performs its function of linking Giantin to GM130 via its acidic domain.

Phosphorylation of Serine 941 by CKII or CKII-like Kinase

Mutations in serine 941 prevented cisternal regrowth, thereby establishing the importance of this residue in the functioning of p115. Whereas TA could stimulate the regrowth of MGF into stacked cisternae, TA (S941A) could not. This suggested that phosphorylation of p115 is required for its function in Golgi reassembly. However, replacing serine 941 with aspartic acid, which can mimic a phosphorylated serine by introducing negative charge, also prevented TA function in cisternal reassembly. Yet since the TA (S941D) did not link GM130 to Giantin better that TA or TA (S941A), while phosphorylated TA did (Fig. 9 B), and the phosphorylated COOH-terminal p115 peptides bound both GM130 and Giantin better than their nonphosphorylated counterparts (Fig. 7), it seems likely that the substitution of serine for aspartic acid failed to mimic the phosphorylated serine.

The Golgi associated kinase that phosphorylates p115 at serine 941 was identified as CKII or a closely related kinase. CKII is an essential, multi-functional enzyme found in the nucleus and cytoplasm of all eukaryotic cells (Padmanabha et al., 1990; Lorenz et al., 1993; Allende and Allende, 1995). Well over 100 substrates are known (Pinna, 1990) and these include components of the core SNARE fusion machinery (Nielander et al., 1995; Risinger and Bennett, 1999). CKII has previously been implicated in transport events (e.g., Alconada et al., 1996; Cotlin et al., 1999). However, only its function in recruitment of the clathrin coat and sorting of Golgi targeted proteins is well characterized (Mauxion et al., 1996; Dittié et al., 1997; Wan et al., 1998; Hao et al., 1999).

The identification of CKII as the kinase responsible for phosphorylating p115 at serine 941 was based of several observations. First, the sequence surrounding serine 941 resembles a CKII phosphorylation site, comprising EDELESGDQDDE. It does lack the canonical acidic residue at position +3 but other CKII substrates are known to lack this residue as well (e.g., Korner et al., 1994). Second, RLG was found to phosphorylate a generic CKII substrate. Third, RLG phosphorylated p115 and both MGF and RLG specifically phosphorylated TA at serine 941. In so doing it could use GTP as well as ATP as the phosphate source, a hallmark of CKII (Allende and Allende, 1995). Fourth, recombinant human CKII could phosphorylate p115 and specifically phosphorylated TA at serine 941. Fifth, inhibition of CKII by several different types of inhibitor prevented phosphorylation of p115 by RLG as well as Golgi reassembly by the NSF (but not the p97) pathway. Drugs, most notably the CKII-specific inhibitor, chrysin, inhibited cisternal regrowth by >75% and p115 phosphorylation by 90%. Antibodies specific for CKII inhibited both reactions by 30–50%. Competing CKII substrates also inhibited both assays. In every case, the effect of CKII inhibitors on Golgi reassembly was paralleled by a similar inhibitory effect on p115 phosphorylation, suggesting that inhibition of CKII-mediated p115 phosphorylation is responsible for the effect on Golgi reassembly. Since we were unable to obtain biochemical amounts of full-length p115 mutated at serine 941, we could not be certain that this was the only site acted upon by CKII. It

does, however, appear very likely since Sohda et al. (1998) showed that serine 941 (942 in the human sequence) is the only site that is phosphorylated on p115 in vivo. Our findings, therefore, provide very strong support for the idea that CKII or a very closely related kinase modulates p115 function by phosphorylating serine 941.

Modulation of Bridging by CKII

Two biochemical approaches gave insight into how phosphorylation might affect p115 function. First, phosphorylation of serine 941 resulted in an enhanced affinity for GM130 and Giantin. COOH-terminal peptides (26mers or 75mers) phosphorylated at serine 941 bound more GM130 and Giantin than their nonphosphorylated counterparts, both in solution and when immobilized on beads. The increase in binding ranged from 2- to 10-fold (Fig. 7). Second, CKII-mediated phosphorylation of TA increased the efficiency with which complexes between GM130 and Giantin were formed (Fig. 9 B). Importantly, TA (S941A) mediated linking of GM130 to Giantin was insensitive to the addition of CKII, showing that serine 941 was the target for phosphorylation.

Even without phosphorylation, TA and the TA mutants could still bind GM130 and Giantin (Fig. 4 A) and link GM130 to Giantin (Fig. 9 B) yet the TA mutants could not support cisternal regrowth (Fig. 4 B). This suggests that TA needs to do more than link GM130 to Giantin to rebuild cisternae and that phosphorylation plays a role in this additional function. One possibility is that it mediates the transition from vesicle tethering to trans-SNARE pairing perhaps by rearranging the tethers to bring the vesicle closer to the membrane. Such a rearrangement could explain the enhanced linking of GM130 to Giantin by phosphorylated p115 in our biochemical assay. Phosphorylated p115 could even recruit SNAREs to the sites where the vesicle now meets the membrane. Further work will be required to test this hypothesis, but it would help explain why overexpression of all ER to Golgi v-SNAREs can compensate for mutations in the yeast p115 homologue, Uso1p (Sapperstein et al., 1996). Uso1p mediates the tethering of COPII vesicles to Golgi membranes before SNARE pairing and tethering involves the small GTPase, Ypt1p (Sapperstein et al., 1996; Cao et al., 1998). Uso1p does not have the equivalent serine but there are two sites at the very beginning of the acidic domain (serines 1,759 and 1,756) that are potential CKII phosphorylation sites. It is, therefore, possible that CKII phosphorylation is a general feature of the tethering cycle.

Whatever the role played by phosphorylation it must act rapidly since Ikehara and colleagues found very little phosphorylated p115 on Golgi membranes. Almost all was found in the cytosol suggesting that phosphorylated p115 is released immediately after it has operated on the tethering complexes. During mitosis, however, there was very little phosphorylated p115 on either the membranes or in the cytosol (Sohda et al., 1998). Since mitotic phosphorylation of GM130 by Cdc2 inhibits the binding of p115 (Lowe et al., 1998) this suggests that p115 is phosphorylated only after it has bound to GM130 (and perhaps Giantin). Phosphorylation of p115 might alter the tethering complex making it more stable during the tethering phase, which

might be rapidly followed by the formation of a fusion complex, which subsequently displaces phosphorylated p115 to the cytosol. This of course leaves open the question as to whether SNAREs are recruited specifically to the linked complexes containing phosphorylated p115 and where, when and by which phosphatase the p115 is dephosphorylated. Looking for downstream fusion components and identifying the p115 phosphatase is the next important step in working out the other parts of the tethering cycle.

References

Alconada, A., U. Bauer, and B. Hoflack. 1996. A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the *trans*-Golgi network. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6096–6110.

Allende, J.E., and C.C. Allende. 1995. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J.* 9:313–323.

Alvarez, C., H. Fujita, A. Hubbard, and E. Sztul. 1999. ER to Golgi transport: requirement for p115 at a pre-Golgi VTC stage. *J. Cell Biol.* 147:1205–1221.

Barlowe, C. 1997. Coupled ER to Golgi transport reconstituted with purified cytosolic proteins. *J. Cell Biol.* 139:1097–1108.

Barr, F.A. 1999. A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. *Curr. Biol.* 9:381–384.

Barr, F.A., M. Puype, J. Vandekerckhove, and G. Warren. 1997. GRASP65, a protein involved in the stacking of Golgi cisternae. *Cell.* 91:253–262.

Barr, F.A., N. Nakamura, and G. Warren. 1998. Mapping the interaction between GRASP65 and GM130, components of a protein complex involved in the stacking of Golgi cisternae. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:3258–3268.

Cao, X.C., N. Ballew, and C. Barlowe. 1998. Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:2156–2165.

Chan, E.K.L., and M.J. Fritzler. 1998. Golgins: coiled-coil-rich proteins associated with the Golgi complex. *Eur. Biophys. J.* 1:1–10.

Cotlin, L.F., M.A. Siddiqui, F. Simpson, and J.F. Collawn. 1999. Casein kinase II activity is required for transferrin receptor endocytosis. *J. Biol. Chem.* 274:30550–30556.

Critchfield, J.W., J.E. Coligan, T.M. Folks, and S.T. Butera. 1997. Casein kinase II is a selective target of HIV-1 transcriptional inhibitors. *Proc. Natl. Acad. Sci. USA.* 94:6110–6115.

De Azevedo, W.F., S. Leclerc, L. Meijer, L. Havlicek, M. Strnad, and S.H. Kim. 1997. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. *Eur. J. Biochem.* 243:518–526.

Dittié, A.S., L. Thomas, G. Thomas, and S.A. Tooze. 1997. Interaction of furin in immature secretory granules from neuroendocrine cells with the AP-1 adaptor complex is modulated by casein kinase II phosphorylation. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:4859–4870.

Echard, A., F. Jollivet, O. Martinez, J.J. Lacapere, A. Rousselet, I. Janoueix-Lerosey, and B. Goud. 1998. Interaction of a Golgi-associated kinesin-like protein with Rab6. *Science.* 279:580–585.

Fritzler, M.J., J.C. Hamel, R.L. Ochs, and E.K. Chan. 1993. Molecular characterization of two human autoantigens: unique cDNAs encoding 95- and 160-kD proteins of a putative family in the Golgi complex. *J. Exp. Med.* 178:49–62.

Hao, W., Z. Luo, L. Zheng, K. Prasad, and E.M. Lafer. 1999. AP180 and AP-2

interact directly in a complex that cooperatively assembles clathrin. *J. Biol. Chem.* 274:22785–22794.

Huang, W., and R.L. Erikson. 1994. Constitutive activation of Mek1 by mutation of serine phosphorylation sites. *Proc. Natl. Acad. Sci. USA.* 91:8960–8963.

Hui, N., N. Nakamura, P. Slusarewicz, and G. Warren. 1998. Purification of rat liver Golgi stacks. *In* Cell Biology: A Laboratory Handbook. Vol. 2. J. Celis, editor. Academic Press, Orlando, FL. 46–55.

Kim, D.-K., M. Sacher, A. Scarpa, A.M. Quinn, and S. Ferro-Novick. 1999. High-copy suppressor analysis reveals a physical interaction between Sec34p and Sec35p, a protein implicated in vesicle docking. *Mol. Biol. Cell.* 10:3317–3329.

Korner, C., A. Herzog, B. Weber, O. Rosorius, F. Hemer, B. Schmidt, and T. Braulke. 1994. *In vitro* phosphorylation of the 46-kDa mannose 6-phosphate receptor by casein kinase II. Structural requirements for efficient phosphorylation. *J. Biol. Chem.* 269:16529–16532.

Lesa, G.M., J. Seemann, J. Shorter, J. Vandekerckhove, and G. Warren. 2000. The N-terminal domain of the Golgi protein Giantin interacts directly with the vesicle tethering protein p115. *J. Biol. Chem.* 275:2831–2836.

Levine, T.P., C. Rabouille, R.H. Kieckbusch, and G. Warren. 1996. Binding of the vesicle docking protein p115 to Golgi membranes is inhibited under mitotic conditions. *J. Biol. Chem.* 271:17304–17311.

Linstedt, A.D., and H.P. Hauri. 1993. Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350kDa. *Mol. Biol. Cell.* 4:679–693.

Linstedt, A.D., M. Foguet, M. Renz, H.P. Seelig, B.S. Glick, and H.P. Hauri. 1995. A C-terminally-anchored Golgi protein is inserted into the endoplasmic reticulum and then transported to the Golgi apparatus. *Proc. Natl. Acad. Sci. USA.* 92:5102–5105.

Linstedt, A.D., S.A. Jesch, A. Mehta, T.H. Lee, R. Garcia-Marta, D.S. Nelson and E. Sztul. 2000. Binding relationships of membrane tethering components. *J. Biol. Chem.* 275:10196–10201.

Lorenz, P., R. Pepperkok, W. Ansorge, and W. Pyerin. 1993. Cell biological studies with monoclonal and polyclonal antibodies against human casein kinase II subunit beta demonstrate participation of the kinase in mitogenic signaling. *J. Biol. Chem.* 268:2733–2739.

Lowe, M., C. Rabouille, N. Nakamura, R. Watson, M. Jackman, E. Jämsä, D. Rahman, D.J.C. Pappin, and G. Warren. 1998. Cdc2 kinase directly phosphorylates the *cis*-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. *Cell.* 94:783–793.

Lupashin, V.V., S. Hamamoto, and R.W. Schekman. 1996. Biochemical requirements for the targeting and fusion of ER-derived transport vesicles with purified yeast Golgi membranes. *J. Cell Biol.* 132:277–289.

Martinez, O., A. Schmidt, J. Salamero, B. Hoflack, M. Roa, and B. Goud. 1994. The small GTP-binding protein rab6 functions in intra-Golgi transport. *J. Cell Biol.* 127:1575–1588.

Mauxion, F., R. Le Borgne, H. Munier-Lehmann, and B. Hoflack. 1996. A casein kinase II phosphorylation site in the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor determines the high affinity interaction of the AP-1 Golgi assembly proteins with membranes. *J. Biol. Chem.* 271:2171–2178.

Meggio, F., A. Donella Deana, M. Ruzzene, A.M. Brunati, L. Cesaro, B. Guerra, T. Meyer, H. Mett, D. Fabbro, P. Furet, et al. 1995. Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur. J. Biochem.* 234:317–322.

Nakamura, N., C. Rabouille, R. Watson, T. Nilsson, N. Hui, P. Slusarewicz, T.E. Kreis, and G. Warren. 1995. Characterization of a *cis*-Golgi matrix protein, GM130. *J. Cell Biol.* 131:1715–1726.

Nakamura, N., M. Lowe, T.P. Levine, C. Rabouille, and G. Warren. 1997. The vesicle docking protein p115 binds GM130, a *cis*-Golgi matrix protein, in a mitotically regulated manner. *Cell.* 89:445–455.

Nelson, D.S., C. Alvarez, Y.S. Gao, R. Garciamata, E. Fialkowski, and E. Sztul. 1998. The membrane-transport factor TAP/p115 cycles between the Golgi and earlier secretory compartments and contains distinct domains required for its localization and function. *J. Cell Biol.* 143:319–331.

Nielander, H.B., F. Onofri, F. Valtorta, G. Schiavo, C. Montecucco, P. Greengard, and F. Benfenati. 1995. Phosphorylation of VAMP/synaptobrevin in synaptic vesicles by endogenous protein kinases. *J. Neurochem.* 65:1712–1720.

Padmanabha, R., J.L. Chen-Wu, D.E. Hanna, and C.V. Glover. 1990. Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 10:4089–4099.

Pfeffer, S.R. 1999. Transport-vesicle targeting: tethers before SNAREs. *Nat. Cell. Biol.* 1:E17–E22.

Pinna, L.A. 1990. Casein kinase 2: an 'eminence grise' in cellular regulation? *Biochim. Biophys. Acta.* 1054:267–284.

Rabouille, C., T.P. Levine, J.M. Peters, and G. Warren. 1995. An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell.* 82:905–914.

Risinger, C., and M.K. Bennett. 1999. Differential phosphorylation of syntaxin and synaptosome-associated protein of 25kDa (SNAP-25) isoforms. *J. Neurochem.* 72:614–624.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature.* 372:

55–63.

Sacher, M., Y. Jiang, J. Barrowman, A. Scarpa, J. Burston, L. Zhang, D. Schieltz, J.R. Yates, 3rd, H. Abeliovich, and S. Ferro-Novick. 1998. TRAPP, a highly conserved novel complex on the *cis*-Golgi that mediates vesicle docking and fusion. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:2494–2503.

Sapperstein, S.K., D.M. Walter, A.R. Grosvenor, J.E. Heuser, and M.G. Waters. 1995. p115 is a general vesicular transport factor related to the yeast endoplasmic reticulum to Golgi transport factor Uso1p. *Proc. Natl. Acad. Sci. USA.* 92:522–526.

Sapperstein, S.K., V.V. Lupashin, H.D. Schmitt, and M.G. Waters. 1996. Assembly of the ER to Golgi SNARE complex requires Uso1p. *J. Cell Biol.* 132:755–767.

Seelig, H.P., P. Schranz, H. Schroter, C. Wiemann, G. Griffiths, and M. Renz. 1994. Molecular genetic analyses of a 376-kilodalton Golgi complex membrane protein (Giantin). *Mol. Cell. Biol.* 14:2564–2576.

Seemann, J., E. Jamsa-Jokitalo, and G. Warren. 2000. The role of the tethering proteins, p115 and GM130, in transport through the Golgi apparatus *in vivo. Mol. Biol. Cell.* 11:635–645.

Shima, D.T., K. Haldar, R. Pepperkok, R. Watson, and G. Warren. 1997. Partitioning of the Golgi apparatus during mitosis in living HeLa cells. *J. Cell Biol.* 137:1211–1228.

Shorter, J., and G. Warren. 1999. A role for the vesicle tethering protein, p115, in the post-mitotic stacking of reassembling Golgi cisternae in a cell-free system. *J. Cell Biol.* 146:57–70.

Shorter, J., R. Watson, M.E. Giannakou, M. Clarke, G. Warren, and F.A. Barr. 1999. GRASP55, a second mammalian GRASP protein involved in the stacking of Golgi cisternae in a cell-free system. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:4949–4960.

Shugar, D. 1994. Development of inhibitors of protein kinases CKI and CKII and some related aspects, including donor and acceptor specificities and viral protein kinases. *Cell. Mol. Biol. Res.* 40:411–419.

Sohda, M., Y. Misumi, A. Yano, N. Takami, and Y. Ikehara. 1998. Phosphorylation of the vesicle docking protein p115 regulates its association with the Golgi membrane. *J. Biol. Chem.* 273:5385–5388.

Songyang, Z., K.P. Lu, Y.T. Kwon, L.H. Tsai, O. Filhol, C. Cochet, D.A. Brickey, T.R. Soderling, C. Bartleson, D.J. Graves, et al. 1996. A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol. Cell. Biol.* 16:6486–6493.

Sönnichsen, B., M. Lowe, T. Levine, E. Jamsa, B. Dirac-Svejstrup, and G. Warren. 1998. A role for Giantin in docking COPI vesicles to Golgi membranes. *J. Cell Biol.* 140:1013–1021.

VanRheenen, S.M., X. Cao, V.V. Lupashin, C. Barlowe, and M.G. Waters. 1998. Sec35p, a novel peripheral membrane protein, is required for ER to Golgi vesicle docking. *J. Cell Biol.* 141:1107–1119.

VanRheenen, S., X. Cao, V. Luphasin, C. Barlowe, and G. Waters. 1999. Sec35p, a novel peripheral membrane protein, is required for ER to Golgi vesicle docking. *J. Cell Biol.* 141:1107–1119.

Wan, L., S.S. Molloy, L. Thomas, G. Liu, Y. Xiang, S.L. Rybak, and G. Thomas. 1998. PACS-1 defines a novel gene family of cytosolic sorting proteins required for *trans*-Golgi network localization. *Cell.* 94:205–216.

Waters, M.G., D.O. Clary, and J.E. Rothman. 1992. A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack. *J. Cell Biol.* 118:1015–1026.

Yankulov, K., K. Yamashita, R. Roy, J.M. Egly, and D.L. Bentley. 1995. The transcriptional elongation inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription factor IIH-associated protein kinase. *J. Biol. Chem.* 270:23922–23925.