

# Phosphorylation Controls CLIMP-63–mediated Anchoring of the Endoplasmic Reticulum to Microtubules

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The microtubule-binding 63-kDa cytoskeleton-linking membrane protein (CLIMP-63) is an integral membrane protein that links the endoplasmic reticulum (ER) to microtubules. Here, we tested whether this interaction is regulated by phosphorylation. Metabolic labeling with  $^{32}\text{P}$  showed that CLIMP-63 is a phosphoprotein with increased phosphorylation during mitosis. CLIMP-63 of mitotic cells is unable to bind to microtubules *in vitro*. Mitotic phosphorylation can be prevented by mutation of serines 3, 17, and 19 in the cytoplasmic domain of CLIMP-63. When these residues are mutated to glutamic acid, and hence mimic mitotic phosphorylation, CLIMP-63 does no longer bind to microtubules *in vitro*. Overexpression of the phospho-mimicking mitotic form of CLIMP-63 in interphase cells leads to a collapse of the ER around the nucleus, leaving the microtubular network intact. The results suggest that CLIMP-63–mediated stable anchoring of the ER to microtubules is required to maintain the spatial distribution of the ER during interphase and that this interaction is abolished by phosphorylation of CLIMP-63 during mitosis.

## INTRODUCTION

The endoplasmic reticulum (ER) of animal cells is a continuous network of membranes connected to the nuclear envelope. In most cell types, it extends into the entire cytoplasm as interconnected tubules that form a characteristic polygonal structure characterized by three-way junctions (Terasaki *et al.*, 1986; Lee and Chen, 1988; Dailey and Bridgman, 1989). Studies in living cells showed that the ER is highly dynamic with numerous elongation, fusion, and junction sliding events occurring within the existing network (Lee and Chen, 1988). These dynamics are supported by microtubules (MTs). ER tubules often coalign with individual MTs, and the use of MT-depolymerizing drugs showed that the formation of ER tubules correlates with MT assembly (Terasaki *et al.*, 1986; Lee *et al.*, 1989; Waterman-Storer and Salmon, 1998). In migrating cells, ER tubules are pulled out by MT tip polymerization or extend toward the plus end of MTs (Waterman-Storer and Salmon, 1998), a movement known to be powered by members of the kinesin motor family (Hirokawa, 1998). Kinesins bind to cargo membranes either via transmembrane receptors that may or may not involve cytoplasmic linkers (Kamal and Goldstein, 2002) or via phospholipids (Klopfenstein *et al.*, 2002). MT minus end-directed transport of membranes, mediated by cytoplasmic dyneins,

is mainly involved in the positioning of endosomes, lysosomes, and the Golgi apparatus (Goodson *et al.*, 1997; Hirokawa, 1998). Dynein-based movement of ER membranes also has been observed in some cases but seems to occur less frequently (Allan, 1995; Lane and Allan, 1999; Wedlich-Soldner *et al.*, 2002).

The 63-kDa cytoskeleton-linking membrane protein (CLIMP-63) is a nonglycosylated type II ER membrane protein that is excluded from the nuclear envelope (Schweizer *et al.*, 1993a). Restricted localization of CLIMP-63 to the reticular part of the ER is mediated by self-association that retains the protein in the ER (Schweizer *et al.*, 1994) and limits its mobility in the membrane (Klopfenstein *et al.*, 2001). The cytoplasmic segment of CLIMP-63 binds to MTs, both *in vivo* and *in vitro*, and overexpression of the protein leads to a parallel rearrangement of ER and MTs, suggesting that the protein links ER membranes to the MT cytoskeleton (Klopfenstein *et al.*, 1998). Other nonmotor proteins linking membranes to MTs have been described, but these are soluble proteins. They include the peripheral Golgi proteins GMAP-210 (Infante *et al.*, 1999) and Hook 3 (Walenta *et al.*, 2001), both proposed to participate in Golgi architecture and anchoring to the minus end of MTs, and MT plus end-binding proteins of the cytoplasmic linker protein (CLIP) family (Galjart and Perez, 2003). Compared with these MT-binding proteins, CLIMP-63 is unique in that it 1) is a membrane protein, 2) mediates direct interaction between ER membranes and MTs via its cytoplasmic MT-binding domain, and 3) links membranes along the entire length of MTs.

In higher eukaryotes, MTs that are centrosome-nucleated during interphase undergo dramatic rearrangements into the mitotic spindle during cell division. Concomitantly, membrane traffic is inhibited (Warren, 1993). Recent fluorescence photobleaching experiments suggest that ER membranes during mitosis are continuous rather than vesiculated as previously thought (Ellenberg *et al.*, 1997; Yang *et al.*, 1997; Terasaki, 2000). In *Drosophila* and sea urchin embryos, the ER accumulates at the mitotic poles (Terasaki, 2000;

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Abbreviations used: CKII, casein kinase II; CLIMP-63, 63-kDa cytoskeleton-linking membrane protein; CLIP, cytoskeleton linking protein; ER, endoplasmic reticulum; MAP, microtubule-associated protein; MT, microtubule; pc, polyclonal antibody; PKC, protein kinase C; wt, wild-type.

Bobinnec *et al.*, 2003). If CLIMP-63 stably anchors ER membranes to MTs during interphase, it is conceivable that this interaction is abolished during mitosis. Protein binding to MTs, as studied for MT-associated proteins (MAPs), is regulated by phosphorylation that influences mainly negatively the interaction with MTs. Phosphorylation decreases MT affinity of tau proteins (Drewes *et al.*, 1995; Cho and Johnson, 2003; Hamdane *et al.*, 2003), neuronal MAP2, and ubiquitous MAP4 (Illenberger *et al.*, 1996; Ozer and Halpain, 2000; Alexa *et al.*, 2002). Inhibition of specific MAP functions, such as MT polymerization or MT nucleation-promoting activities, also have been reported (Leger *et al.*, 1997; Kitazawa *et al.*, 2000; Amano *et al.*, 2003). The phosphorylation of nonneuronal MAPs is believed to increase MT dynamics during mitosis (Masson and Kreis, 1995; Ookata *et al.*, 1995; Shiina and Tsukita, 1999). In the present study, we have investigated the role of CLIMP-63 phosphorylation in its interaction with MTs.

## MATERIALS AND METHODS

### DNA Constructs

Phospho-mimicking mutants were constructed by standard overlapping polymerase chain reaction (PCR) techniques. For single mutants, complementary primers carrying the desired mutation were coupled to upstream (matching vector sequence, 5'-gag ctc gga tcc act agt aac-3') or downstream (matching CLIMP-63 coding sequence, 5'-cca cat gga tcc cat ccg ag-3') primers in PCR by using pcDNA3-p63 plasmid (Klopfenstein *et al.*, 1998) as a template. Amplified overlapping DNA fragments were mixed in a 1:1 ratio and used as template in a second PCR with external primers. The resulting PCR product was digested with *Bam*HI and used to replace the corresponding restriction fragment into pcDNA3-p63 initial plasmid. Double, triple, and quadruple phospho-mimicking mutants were constructed by sequentially adding point mutations by using the same strategy described above.  $\Delta$ CLIMP-63 was constructed by PCR amplification in two sequential steps. Amino acids 2–28 were deleted in pcDNA3-p63 by replacing the *Eco*RI/*Not*I fragment representing amino acids 1–118 with the correspondingly digested PCR fragment encoding the start methionine followed by amino acids 29–118 (upstream oligo, 5'-gaa ttc atg gcg gat gac gtc ggc aag aag-3'; downstream oligo, 5'-agc ggc cgc cgc cac cag ggc gag g-3'). Deletion of amino acids 62–101 was created by replacing the *Eco*RI/*Not*I fragment in pcDNA3-p63 with a PCR fragment corresponding to amino acids 1–61 fused to 102–118 (upstream oligo, 5'-gaa ttc atg ccc tcg gcc aaa caa ag-3'; downstream oligo, 5'-agc ggc cgc cgc cac cag ggc gag gta gaa gag aaa gtt gag cgc cct ggc gag cct ggc gtt ctg cgg gtc ctg ctg c-3'). Next, the *Sgr*AI restriction site encompassing the corresponding amino acids 37–39 was used to combine the two deletion regions into a single construct: *Sgr*AI/*Not*I DNA fragment from the  $\Delta$ (62–101) construct was exchanged with the corresponding fragment into the  $\Delta$ (2–28) construct, giving rise to  $\Delta$ CLIMP-63. For CLIMP-63\* constructs, the C-terminal part of CLIMP-63 was PCR amplified using a primer that introduces a stop codon in place of N<sub>538</sub> (5'-ctt ctc tag act aac tga agc 3') and a downstream primer matching pcDNA3 vector sequence (5'-ccc tct aga tgc atg ctc 3'). Resulting DNA fragment was digested with *Xba*I and exchanged with its counterpart in the adequate plasmids to create the short versions of CLIMP-63,  $\Delta$ CLIMP-63 and all triple and quadruple phospho-mimicking mutants.

### Cell Culture and Transfection

COS cells were maintained in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1  $\mu$ g/ml fungizone. HeLa cells were cultured identically, except that the medium was supplemented with nonessential amino acids. COS cells were transiently transfected using the DEAE-dextran method (Cullen, 1987). HeLa cells were transfected using FuGene 6 reagent (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. To obtain stable clones, cells were diluted into 96-wells plates (100 cells/well) 24 h posttransfection and selected in the presence of 0.7 mg/ml Geneticin (G418).

### Microtubule-binding Assays

Forty-eight hours posttransfection, transfected or nontransfected COS cells of a 60-mm dish were washed twice with ice-cold phosphate-buffered saline (PBS), scraped, and collected by centrifugation for 10 min at 750  $\times$  g at 4°C. Cell pellets were solubilized for 1 h at 4°C in 1 ml of PNT buffer (20 mM PIPES, 30 mM Tris, 100 mM NaCl, 1 mM EGTA, 1.25 mM EDTA, pH 7.0) containing 1% Triton X-100, protease inhibitors, and 100 mM iodoacetamide, and centrifuged for 1 h at 20,000  $\times$  g at 4°C. For MT binding, 50  $\mu$ g of taxol-stabilized MTs (Brugg and Matus, 1991) was resuspended in 100  $\mu$ l of

cleared lysate and incubated for 30 min at 37°C. Samples were then centrifuged for 10 min at 230,000  $\times$  g at 25°C, and supernatant and pellet were analyzed by SDS-PAGE and Western blotting for the presence of tubulin and CLIMP-63. Mitotic cells were enriched by 18-h incubation with 100 nM nocodazole.

### Immunofluorescence Microscopy

COS cells were plated on Lab-Tek multiwell glass slides (Nalge Nunc, Naperville, IL) the day before transfection. After 48 h, the cells were fixed for 30 min with 3% paraformaldehyde in PBS, permeabilized with 0.1% saponin in PBS, and processed for immunofluorescence microscopy. Cells were imaged in Mowiol mounting medium with a 63 $\times$  objective (1.32 numerical aperture) by using a Leica TCS NT confocal laser scanning microscope. Bleed-through in double-labeling experiments was avoided by sequential image acquisition and tested in single-labeling experiments.

### Antibodies

The following primary antibodies were used: mouse monoclonal antibody (mAb) G1/296 (an IgG2a) (Schweizer *et al.*, 1993a), mouse mAb A1/59 (an IgG1), a rabbit polyclonal antibody against CLIMP-63, mouse mAb 1A2 (an IgG3) against  $\alpha$ -tubulin (kindly provided by Karl Matter, University College London, United Kingdom), mouse mAb A1/182 (an IgG2a) against BAP31 (Klumperman *et al.*, 1998), and rabbit IgG against phosphorylated serine 10 of histone 3 (Upstate Biotechnology, Lake Placid, NY). mAb A1/59 was produced by the hybridoma technique (Schweizer *et al.*, 1988) by using a membrane fraction of Vero cells as an antigen (Schweizer *et al.*, 1991) and was found to cross-react with human CLIMP-63. Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG2a, Alexa Fluor 568 goat anti-mouse IgG1, Alexa Fluor 568 goat anti-mouse IgG3, Alexa Fluor 568 goat anti-rabbit (Molecular Probes, Eugene, OR), and horseradish peroxidase-coupled goat anti-mouse and goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).

### Analysis of Mitotic Phosphorylation

HeLa cells of a 10-cm dish were incubated for 1 h in phosphate-free medium and labeled for 3 h at 37°C with [<sup>32</sup>P]orthophosphate (carrier free in HCl solution; Amersham Biosciences, Piscataway, NJ) (500  $\mu$ Ci/dish). Cells were then washed twice with ice-cold PBS and harvested by scraping followed by centrifugation for 10 min at 750  $\times$  g at 4°C. Cell pellets were solubilized for 1 h on ice in 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, containing 1% Triton X-100 and protease inhibitors as well as phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO), and centrifuged for 1 h at 100,000  $\times$  g at 4°C. Endogenous CLIMP-63 and CLIMP-63 short constructs were affinity purified from the supernatant on a column of mAb G1/296 coupled to protein A-Sepharose CL-4B (Amersham Biosciences). After a 2-h incubation at 4°C, the resin was washed 12 times with lysis buffer, proteins were eluted in Laemmli buffer, and separated by 8% SDS-PAGE followed by autoradiography. For enrichment in mitotic cells, 100 nM nocodazole was added 18 h before starvation. Starvation and labeling steps were carried out in the presence of the drug, and mitotic cells were harvested by rinsing the plate with medium. Aliquots of the 100,000  $\times$  g supernatants were trichloroacetic acid precipitated and used to determine total radioactivity by scintillation counting. Phosphorylation levels of CLIMP-63 constructs were quantified by measuring signal intensity on a STORM 840 PhosphorImager (Amersham Biosciences) by using the Image-Quant software. Values were normalized referring to total incorporation of radioactivity. Nocodazole-induced mitotic enrichment was monitored by Western blotting of gels run with acid extracts of interphase and mitotic cells and probed with an antibody against phosphorylated histone 3.

## RESULTS

### A Triple Phospho-mimicking Mutant and a Mutant Impaired in Microtubule Binding of CLIMP-63 Induce ER Clustering

In pilot experiments using metabolic labeling with [<sup>32</sup>P]orthophosphate, we found that the cytoplasmic domain of CLIMP-63 becomes phosphorylated in HeLa cells during mitosis (our unpublished data; Figure 6). This finding raised the possibility that CLIMP-63 may be phosphorylated at the onset of mitosis for subsequent detachment of ER membranes from the MT cytoskeleton. As shown in Figure 1, the cytosolic tail of CLIMP-63 contains four consensus sites for phosphorylation, three of which (Ser3, Ser19, and Ser101) are protein kinase C (PKC) sites, whereas Ser17 is a casein kinase II (CKII) site.

To test a potential role of the putative phosphorylation sites, several phospho-mimicking mutants of CLIMP-63

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1                               36
MPSAKQGRGSKGGHGAASPSEKGAHPSGGADDVAKKP
M-----ADDVAKKP

37                               72
PFAPQQPPPPPPAPHPQQHPQQHPQONQAHGKGGHRGG
PFAPQQPPPPPPAPHPQQHPQQHPQN-----

73                               106
GGGGGKSSSSSSASAAAAAAAASSSASCSRRLGR
-----RRLGR

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**Figure 1.** Cytosolic tail sequences of CLIMP-63 (top lane) and  $\Delta$ CLIMP-63 that is impaired in MT binding (bottom lane). Consensus phosphorylation sites for PKC and CKII are indicated in bold and underlined, respectively.

were made in which the serines were replaced by glutamic acid, either individually or in combination (Table 1). The mutants were transiently expressed in COS cells and analyzed by immunofluorescence confocal laser scanning microscopy. As a negative control for MT binding, we used  $\Delta$ CLIMP-63, a construct lacking cytoplasmic amino acids 2–28 and 62–101 (Figure 1).  $\Delta$ CLIMP-63 was constructed in a separate study aiming at the identification of the minimal CLIMP-63 MT-binding domain (Klopfenstein and Hauri, unpublished data; also see *Results*). It is unable to bind to MTs *in vitro* and was chosen for the current study because of its convenient expression *in vivo*, because more extensively deleted constructs proved to be unstable.

The immunofluorescence pattern of CLIMP-63 was described previously (Schweizer *et al.*, 1993a,b; Klopfenstein *et al.*, 1998). Endogenous CLIMP-63 has a discrete punctate pattern typical for the ER, except that the protein is excluded from the nuclear envelope. By contrast, in CLIMP-63-overexpressing cells, antibodies against CLIMP-63 label an ex-

tensively tubulated network. Additional experiments showed this pattern to be the result of alignment of ER membranes along bundled MTs. The ER rearrangement induced by CLIMP-63 can be seen in ~40% of the transfected cells (Klopfenstein *et al.*, 1998).

In the present study, we found that the MT-binding-impaired  $\Delta$ CLIMP-63 induces another type of ER remodeling. Cells transfected with  $\Delta$ CLIMP-63 indeed frequently exhibit clustering of their ER around the nucleus, from which a poorly reticulated network extends toward the cell periphery (Figure 2, A and G). Double labeling experiments with an antibody against BAP31, a major integral membrane protein of the ER (Klumperman *et al.*, 1998), confirmed that this pattern reflects an overall redistribution of the ER (Figure 2B). Additional immunofluorescence experiments showed that another ER marker, ERp72, colocalizes with BAP31 and CLIMP-63 and that the same phenotype can be induced in transiently transfected HeLa cells (our unpublished data). Clustered ER was observed in 30–40% of the expressing COS cells. We then analyzed the CLIMP-63 phospho-mimicking mutants by using the same assay. Strikingly, the quadruple mutant CLIMP-63 (S3E S17E S19E S101E) and the triple mutant CLIMP-63 (S3E S17E S19E) also were able to cluster the ER in a similar way to  $\Delta$ CLIMP-63 (in 20–30% of the cells), although the reorganization of the ER was somewhat less drastic (exemplified for the triple mutant in Figure 2, D–F). ER clustering was not observed for any other triple, double, or single phospho-mimicking mutant (Table 1), suggesting that the specific combination of the three point mutations S3E, S17E, and S19E was required for generating ER clustering. Western blot analysis confirmed that all mutant proteins were intact and expressed at similar levels (our unpublished data).

To further characterize this new phenotype, we costained cells expressing  $\Delta$ CLIMP-63, CLIMP-63 (S3E S17E S19E S101E), or CLIMP-63 (S3E S17E S19E) for tubulin. In all cases, cells having clustered ER exhibited a MT pattern that is typical for unperturbed interphase cells (Figure 2, G–I; our unpublished data). Thus, ER clustering is not a result of an altered MT cytoskeleton. We also expressed a serine-to-alanine control mutant, CLIMP-63 (S3A S17A S19A), in which the crucial serines were replaced by alanines, thereby preventing phosphorylation. This mutant was expected to behave like wild-type (wt) CLIMP-63. Indeed, this mutant colocalized with and bundled MTs (Figure 2, J–L). The change included the entire ER, as confirmed by additional double-labeling experiments with antibodies against BAP31 and CLIMP-63 or BAP31 and tubulin (our unpublished data). The results demonstrate that CLIMP-63 (S3A S17A S19A) behaves like wt CLIMP-63 (Klopfenstein *et al.*, 1998). It is important to note the difference in the MT pattern induced by the triple phospho-mimicking mutant and the triple alanine mutation. In the former case, the MT pattern remains unchanged, whereas in the latter case the MT pattern changes in parallel with the ER (Figure 2, compare I and L). We conclude that the ER clustering induced by CLIMP-63 (S3E S17E S19E S101E) and CLIMP-63 (S3E S17E S19E) is specific to phospho-mimicking mutations.

#### **CLIMP-63\* Constructs Behave Like Their Full-Length Counterparts**

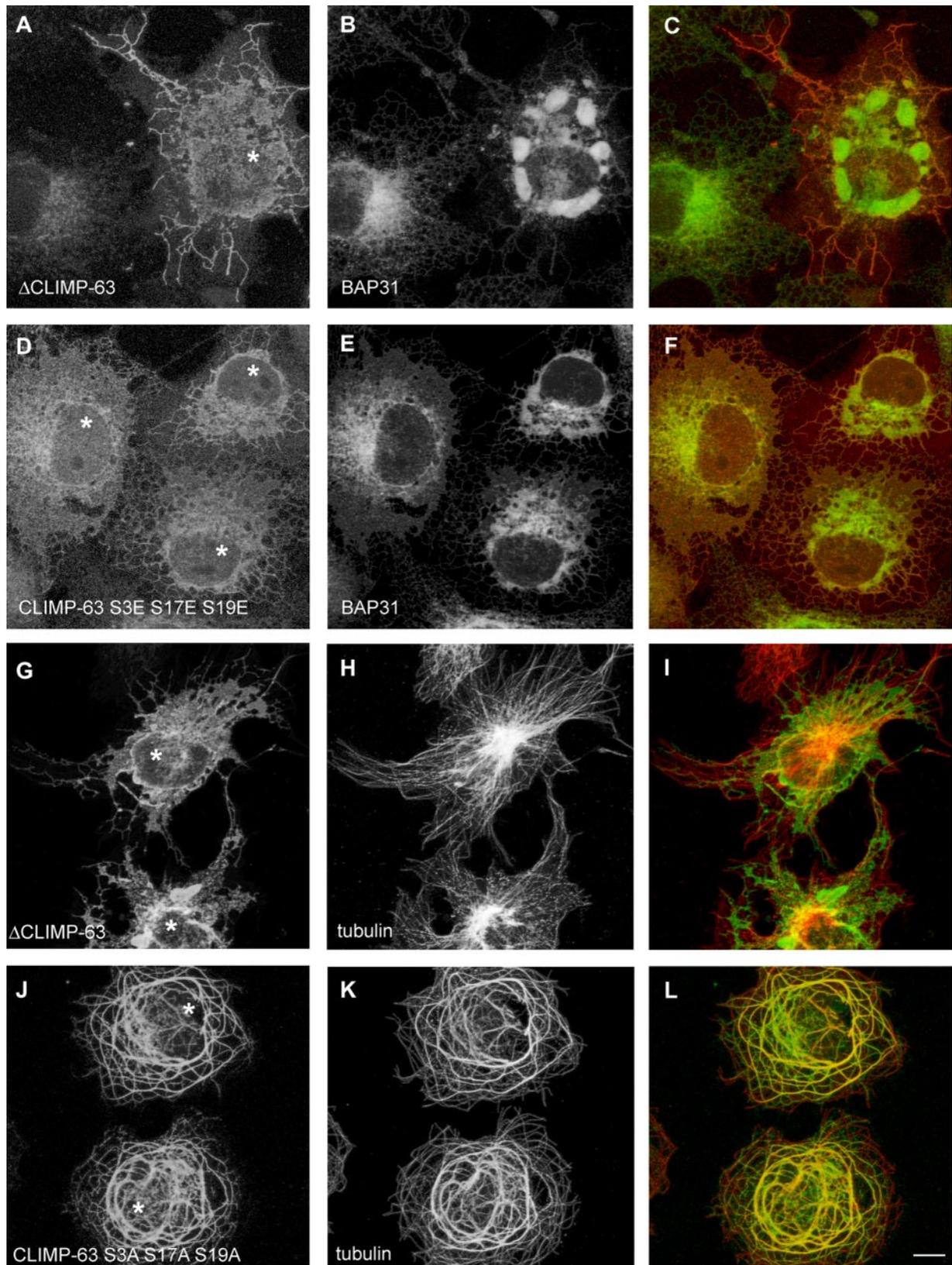
To distinguish CLIMP-63 mutants from the endogenous protein in biochemical experiments, we slightly shortened them. By using convenient restriction sites in pcDNA3-p63 constructs, we created CLIMP-63 proteins lacking the C-terminal residues 538–602. These mutants are specified with an asterisk (\*). To test whether the small luminal deletion

**Table 1.** Features of CLIMP-63 phospho-mimicking mutants

	ER clustering	MT cosedimentation
	full-length proteins	shortened proteins
WT	–	+
S3E	–	nd
S17E	–	nd
S19E	–	nd
S101E	–	nd
S3E S17E	–	nd
S3E S19E	nd	nd
S3E S101E	–	nd
S17E S19E	–	nd
S17E S101E	–	nd
S19 S101E	nd	nd
S3E S17E S19E	+ <sup>a</sup>	–
S3E S17E S101E	–	++
S3E S19E S101E	–	+-
S17E S19E S101E	–	+-
S3E S17E S19E S101E	+ <sup>a</sup>	–
$\Delta$ CLIMP-63	++ <sup>a</sup>	–

+, *in vitro* cosedimentation of the mutant with MTs, and ability and efficiency to reorganize the ER *in vivo*. Nd, not determined.

<sup>a</sup> Corresponding shortened forms also cluster the ER.



**Figure 2.** Overexpressed  $\Delta$ CLIMP-63 and CLIMP-63 (S3E S17E S19E) induce ER clustering. COS cells were transfected with  $\Delta$ CLIMP-63 (A–C and G–I), CLIMP-63 (S3E S17E S19E) (D–F), or CLIMP-63 (S3A S17A S19A) (J–L). Forty-eight hours posttransfection, the cells were fixed, permeabilized, stained for CLIMP-63 (A, D, G, and J), BAP31 (B and E), or tubulin (H and K), and analyzed by immunofluorescence confocal laser scanning microscopy. (C, F, I and L) Merged images. Transfected cells are indicated by an asterisk. Bar, 10  $\mu$ M.

had an effect on the properties of the mutants, we expressed the CLIMP-63\* constructs in COS cells and localized them by immunofluorescence microscopy by using three different antibodies against CLIMP-63. mAb G1/296, an IgG2A, (Schweizer *et al.*, 1993a) was used in single-labeling experiments where it clearly identified all transfected CLIMP-63\* constructs (our unpublished data). A rabbit antibody and mAb A1/59 (an IgG1) against CLIMP-63 could be used in double-labeling experiments with a mAb against BAP31 (an IgG2A). Figure 3 shows that the shortened proteins induced the expected phenotypes: tubular rearrangement of the ER induced by CLIMP-63\* (Figure 3, G–I) versus ER clustering induced by  $\Delta$ CLIMP-63\* (Figure 3, A–C). CLIMP-63\* (S3E S17E S19E) and CLIMP-63\* (S3E S17E S19E S101E) also maintained their ability to cluster the ER very much like their full-length counterparts (our unpublished data). CLIMP-63\* constructs did not mislocalize to the cell surface or the nuclear membrane (our unpublished data), unlike more extensively deleted CLIMP-63 constructs (Schweizer *et al.*, 1994; Klopfenstein *et al.*, 2001).

mAb A1/59 was unable to detect CLIMP-63\* proteins. Cells expressing CLIMP-63\* or  $\Delta$ CLIMP-63\* (as judged from their tubulated or clustered ER, respectively) exhibited a very weak immunofluorescence staining (Figure 3, D–F, J–L). The staining was weaker than the A1/59 staining in nontransfected cells, presumably because the nonreacting transfected constructs mask the A1/59 epitope of the endogenous CLIMP-63. The inability of A1/59 mAb to recognize CLIMP-63\* constructs was confirmed by immunoprecipitation experiments in which mAb A1/59 only immunoprecipitated endogenous CLIMP-63 (Figure 4). Obviously, CLIMP-63\* constructs lack the A1/59 epitope. In contrast, mAb G1/296 and the polyclonal anti-CLIMP-63 immunoprecipitated the shortened proteins as efficiently as full-length CLIMP-63.

Collectively, these results suggest that CLIMP-63\* constructs behave like their full-length counterparts. They were therefore considered suitable for the subsequent biochemical experiments.

#### **Endogenous CLIMP-63 of Mitotic Cells and CLIMP-63 Phospho-mimicking Mutants Fail to Bind to MTs**

Our observations that overexpression of some phospho-mimicking mutants induces the ER to collapse without altering MT cytoskeleton in the same way as a mutant impaired in MT binding suggest that phosphorylation blocks the interaction of CLIMP-63 with MTs. To directly test this notion and its relevance for mitosis, we performed *in vitro* MT-binding assays. We first studied the ability of endogenous CLIMP-63 to bind to MTs in mitotic and interphase cells. For this purpose, COS cells were either mitotically arrested by nocodazole or left untreated (interphase cells). Detergent extracts were incubated with taxol-stabilized MTs, and CLIMP-63 binding to MTs was monitored by MT cosedimentation. As shown in Figure 5, top left panels, a significant amount of interphase CLIMP-63 bound to MTs as described previously (Klopfenstein *et al.*, 1998). In contrast, CLIMP-63 extracted from mitotic cells was unable to bind to MTs.

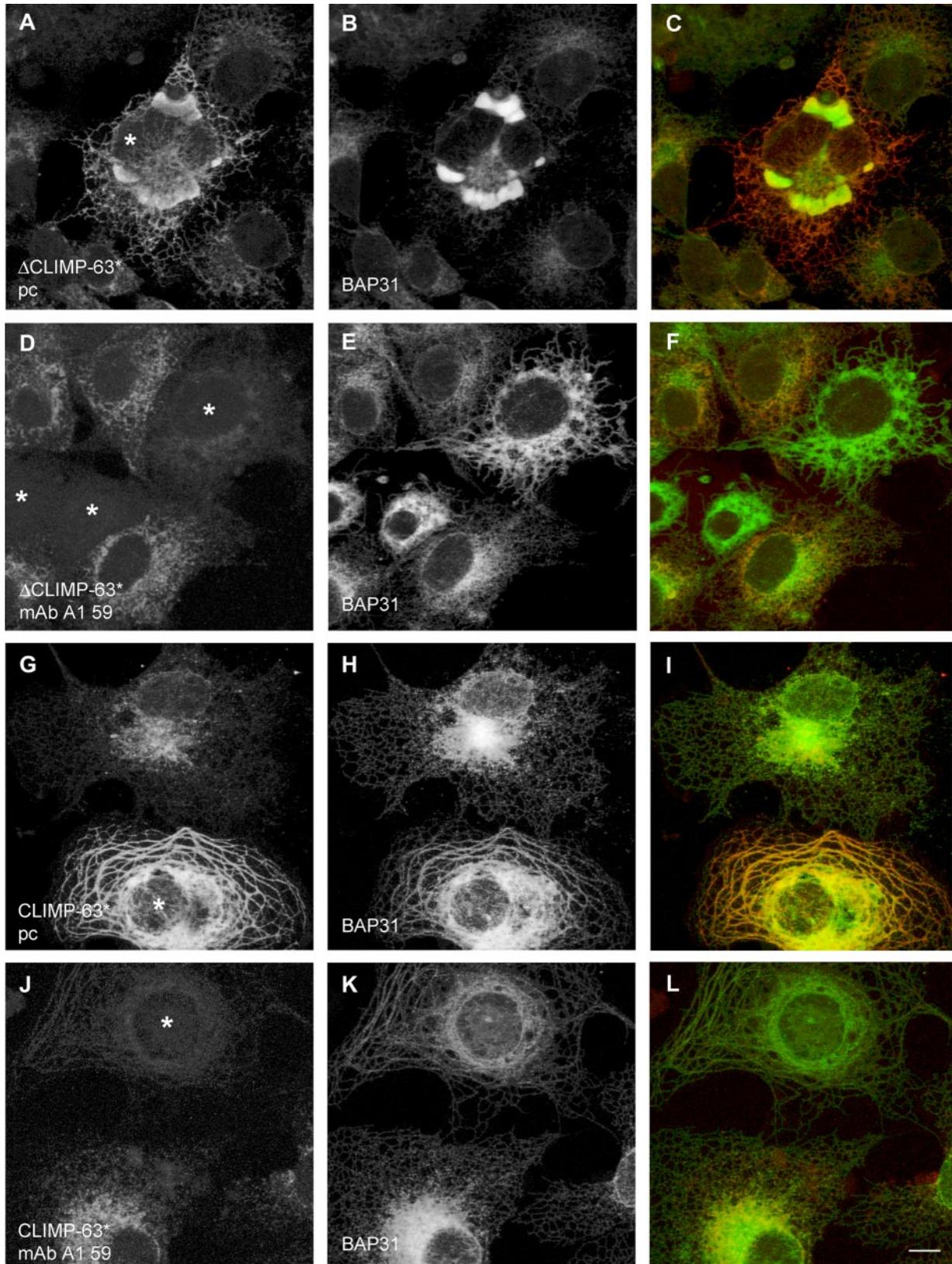
To study the effect of phosphorylation on the binding of CLIMP-63 to MTs *in vitro*, we analyzed the phospho-mimicking mutants extracted from interphase COS cells that had been transfected with the shortened forms of these mutants. CLIMP-63\* was used as a positive control and  $\Delta$ CLIMP-63\* as a negative control.  $\Delta$ CLIMP-63\* did not show any binding to MTs, identical to  $\Delta$ CLIMP-63 (our unpublished data), whereas CLIMP-63\* bound to MT with similar efficiency as

CLIMP-63 (Figure 5, top right). The ER clustering mutants CLIMP-63\* (S3E S17E S19E S101E) and CLIMP-63\* (S3E S17E S19E) also were unable to bind to MTs (Figure 5, bottom). The three other phospho-mimicking triple mutants were affected to various extents in their MT binding (Figure 5, bottom; and summarized in Table 1). In the conditions we used to visualize overexpressed mutants, endogenous CLIMP-63 is sometimes barely visible. However, when detected, it often seems to bind less efficiently to MTs in transfected cells. Although 40–60% of the protein routinely bind to MT *in vitro* (Klopfenstein *et al.*, 1998) (Figure 5), this percentage is lower in cells that express phospho-mimicking mutants that are severely affected for MT binding. In contrast, binding of endogenous CLIMP-63 is unaltered in cells overexpressing CLIMP-63\* and  $\Delta$ CLIMP-63\*. When probed by coimmunoprecipitation experiments under conditions used for MT-binding assay, shortened CLIMP-63 phospho-mimicking mutants, but not  $\Delta$ CLIMP-63\*, oligomerize with endogenous CLIMP-63 (our unpublished data). Therefore, the binding of endogenous CLIMP-63 is negatively affected when cells coexpress a mutant that retains full oligomerization capacity and binds less efficiently to MTs. These results further argue that phospho-mimicking mutants are indeed impaired for MT binding.

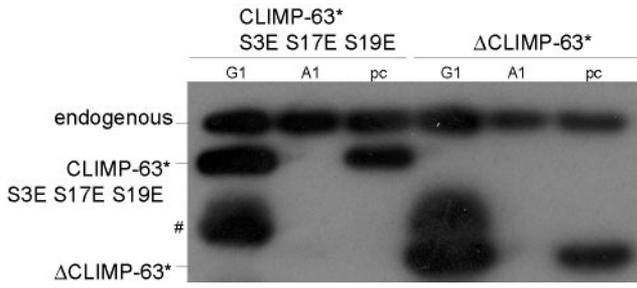
In conclusion, endogenous CLIMP-63 from mitotic cells is unable to bind to MT *in vitro*, and the mimicking of phosphorylation on the serines 3, 17, and 19 is sufficient to prevent CLIMP-63 from MT binding.

#### **CLIMP-63 S3E S17E S19E Is Not Phosphorylated during Mitosis**

To analyze the phosphorylation state of CLIMP-63 during the cell cycle, HeLa cell lines were established that stably express CLIMP-63\*, CLIMP-63\* (S3E S17E S19E), or  $\Delta$ CLIMP-63\*. Several clones were obtained for each construct, only in some of which few cells exhibited tubulated or clustered ER (see *Discussion*). Instead, the ER in these cell lines retained a reticular appearance as judged by immunostaining for CLIMP-63. The clones were treated with nocodazole to arrest cells in mitosis or left nonsynchronized, and then metabolically labeled with [<sup>32</sup>P]orthophosphate followed by immunoprecipitation with mAb G1/296. These experiments showed that endogenous CLIMP-63 was <sup>32</sup>P labeled to some extent in interphase cells, but that its level of phosphorylation increased two- to threefold during mitosis (Figure 6A, top band in all three panels). CLIMP-63\* exhibited a similar increased mitotic signal, indicating that the C-terminal deletion does not interfere with CLIMP-63's ability to undergo mitotic phosphorylation (Figure 6A, top). In contrast, CLIMP-63\* (S3E S17E S19E) did not exhibit increased phosphorylation during mitosis, although it was phosphorylated to some extent during interphase (Figure 6A, bottom). This result indicates that CLIMP-63\* (S3E S17E S19E) has lost its mitosis-responsive phosphorylation sites. As a negative control, we used the cytosolic deleted construct  $\Delta$ CLIMP-63\* that does not contain any of the phosphorylation sites of interest. This protein was phosphorylated neither in interphase nor at mitosis (Figure 6A, middle). To verify the mitotic state of the nocodazole-treated cells, we analyzed the phosphorylation state of histone 3, which is specifically phosphorylated at mitosis, by using a phosphospecific antibody against serine 10. Figure 6B shows such an analysis performed with cells stably expressing CLIMP-63\* (S3E S17E S19E). The mitotic phosphorylation of histone 3 was apparent in nocodazole-treated cells but undetectable in untreated cells, validating our procedure to isolate mitotic cells.



**Figure 3.** Immunofluorescence analysis of CLIMP-63\* constructs. COS cells were transfected with  $\Delta$ CLIMP-63\* (A–F) or CLIMP-63\* (G–L) and double stained for BAP31 (B, E, H, and K) and CLIMP-63, by using rabbit pc antibody (A and G) or mAb A1/59 (D and J). Transfected cells are indicated by an asterisk. Bar, 10  $\mu$ M.



**Figure 4.** Analysis of CLIMP-63\* (S3E S17E S19E) and ΔCLIMP-63\* by immunoprecipitation. The mutants were expressed in COS cells, immunoprecipitated with mAb G1/296 (G1), mAb A1/59 (A1), or rabbit pc (pc), and analyzed by SDS-PAGE and Western blotting by using mAb G1/296. #, antibody heavy chain.

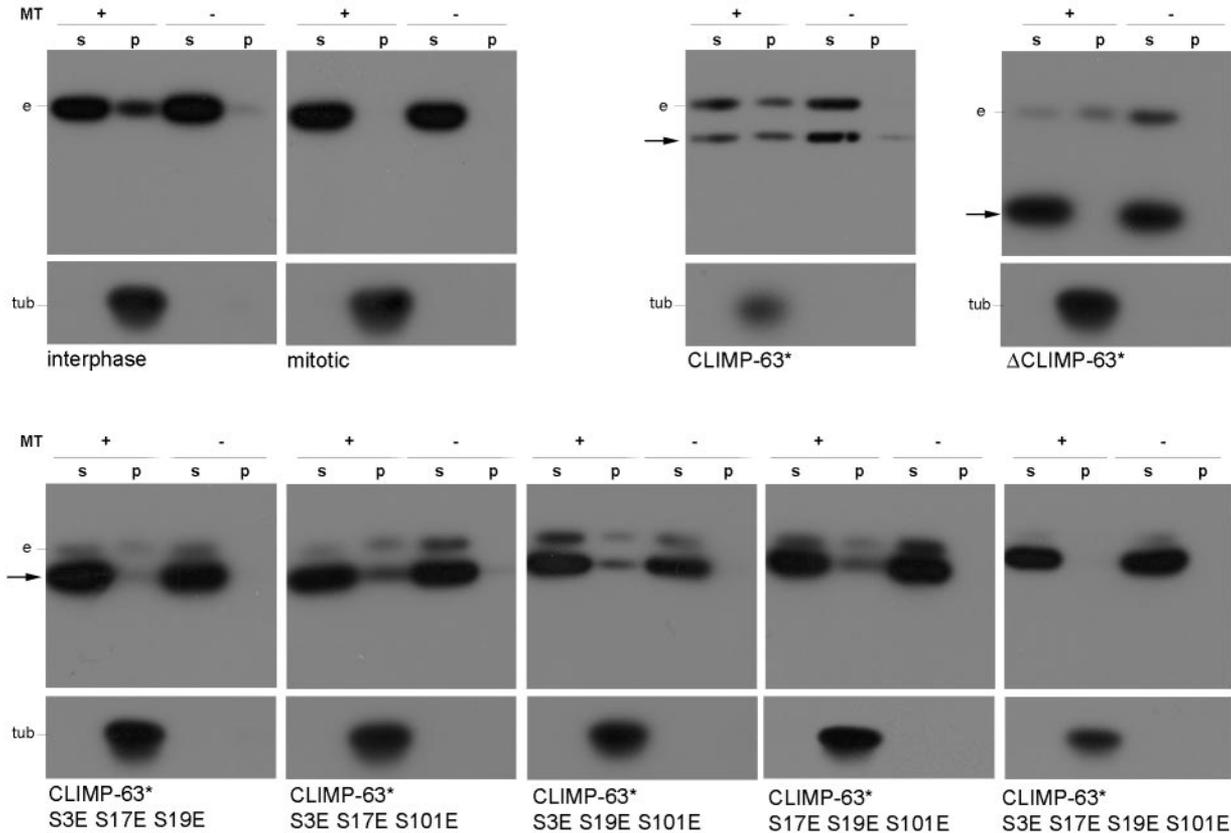
**DISCUSSION**

*Cell Cycle-dependent Phosphorylation of CLIMP-63*

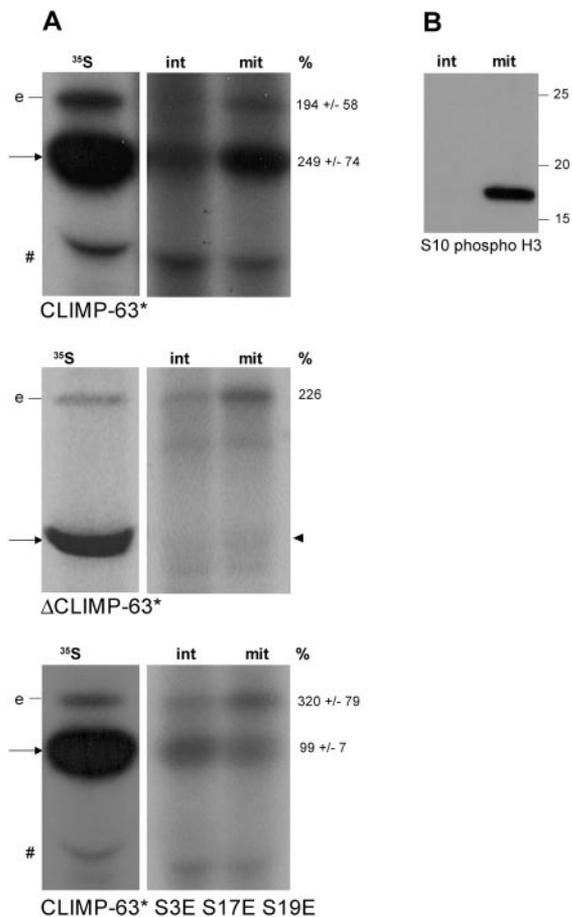
Because phosphorylation is known to regulate in many cases the interaction between MTs and their associated proteins, we studied the *in vivo* phosphorylation state of CLIMP-63. Our results show that CLIMP-63 is already phosphorylated to some extent during interphase but undergoes increased phosphorylation during mitosis. The phosphorylation signal of CLIMP-63 detected in interphase cells may originate from

some mitotic cells present in the nonsynchronized cell population. Alternatively, CLIMP-63 may be a phosphoprotein during interphase and undergo further phosphorylation during mitosis. This seems possible because, apart from the four putative sites for known kinases we have investigated, the cytosolic tail of CLIMP-63 contains another 13 serine residues. In support of CLIMP-63 phosphorylation during interphase, we were unable to detect any phosphate-labeled ΔCLIMP-63\* in interphase cells. In this construct, none of the additional serine residues is present (Figure 1). This finding is consistent with some constitutive phosphorylation on one or more of the additional serine residues during interphase.

Interphase phosphorylation may reflect local detachment of CLIMP-63 from ER membranes. While maintaining its characteristic steady-state structure, the ER undergoes constant small-scale rearrangements that are likely mediated by molecular motors (Lee and Chen, 1988; Waterman-Storer and Salmon, 1998). Several publications have shown that MAPs compete with motors for MT binding or exert a steric blocking effect on motor-based movement (Bulinski *et al.*, 1997; Ebneth *et al.*, 1998; Trinczek *et al.*, 1999). It has been proposed that MAP phosphorylation is required to facilitate MT-based transport (Lopez and Sheetz, 1995; Sato-Harada *et al.*, 1996). In a similar way, it is conceivable that a subset of CLIMP-63 is phosphorylated on additional serine residues during interphase to allow local rearrangement of ER membranes.



**Figure 5.** Binding of endogenous CLIMP-63 and CLIMP-63\* constructs to polymerized MTs *in vitro*. Endogenous CLIMP-63 of interphase or mitotic COS cells, as well as CLIMP-63\* proteins from COS transfected cells, were incubated either in the presence (+) of MTs (to test their binding) or in absence (-) of MTs (autoprecipitation control). MTs were sedimented by centrifugation, and supernatant and pellet fractions were analyzed by SDS-PAGE. Western blots were probed for CLIMP-63 (top) and tubulin (bottom). arrow, CLIMP-63\* proteins; e, endogenous CLIMP-63; p, pellet; s, supernatant; tub, tubulin.



**Figure 6.** Mitotic phosphorylation of CLIMP-63. (A) Endogenous wt CLIMP-63 and CLIMP-63\* mutants were affinity purified from  $^{35}\text{S}$ -labeled interphase cells ( $^{35}\text{S}$ ) and  $^{32}\text{P}$ -labeled interphase (int) or mitotic (mit) cells. After separation by 8% SDS-PAGE, radioactive proteins were visualized by autoradiography. Phosphorylation levels of endogenous CLIMP-63 and of CLIMP-63\* constructs were quantified. Mitotic phosphorylation is indicated at the right margin of each panel as percentage of the interphase level of phosphorylation. Values for the CLIMP-63\* and CLIMP-63\* (S3E S17E S19E) panels are means  $\pm$  SD ( $n = 3$ ). The value for the endogenous protein in the  $\Delta$ CLIMP-63\* panel is the mean of two values differing by 19%. (B) Control for mitotic phosphorylation. Acid extracts of untreated and nocodazole-treated cells expressing CLIMP-63\* (S3E S17E S19E) were separated by 12% SDS-PAGE. Western blots were probed for serine 10-phosphorylated histone 3. Molecular weights are indicated at the right margin. arrow, CLIMP-63\* proteins; arrowhead, no phosphorylation signal; #, background band; e, endogenous wt CLIMP-63.

#### Mitosis-specific Phosphorylation of CLIMP-63

Our systematic analysis of CLIMP-63 phospho-mimicking mutants revealed that CLIMP-63 (S3E S17E S19E S101E) and CLIMP-63 (S3E S17E S19E) but not other mutants can rearrange the ER in a way similar to the MT-binding-impaired  $\Delta$ CLIMP-63 and are unable to bind to MTs in vitro. Moreover, mutating serine residues 3, 17, and 19 abolishes completely the mitosis-specific phosphorylation of CLIMP-63. These results suggest that CLIMP-63 is detached from MTs upon phosphorylation of serine residues 3, 17, and 19 during mitosis.

A negative effect of protein phosphorylation on MT affinity has been studied in detail for stabilizing MAPs, but little

is known concerning cytoskeleton linkers. Although phosphorylation mediates dynamic binding to MTs in the case of CLIP-170 (Rickard and Kreis, 1991; Choi *et al.*, 2002) and CLIP-115 (Hoogenraad *et al.*, 2000), this effect has not been related to a cellular function. In the case of the dynactin subunit p150<sup>Glued</sup> that shares a common MT binding domain with CLIPs, phosphorylation-mediated release from MTs was proposed to be a required step to initiate dynein-based movement (Vaughan *et al.*, 2002). With CLIMP-63, we have now studied a membrane-anchored cytoskeleton linker protein and found that phosphorylation also is involved in detachment from MTs, supporting the notion that this post-translational modification is a general mechanism in regulating MAP/MT interaction. Three phosphorylation events are required to detach CLIMP-63 from MTs. Because the resulting modifications are clustered near the N terminus of the protein they can be expected to considerably change the charge of this domain and thereby modify the interaction of CLIMP-63 with MTs. It also is possible that a change in protein shape is involved in this process, because phosphorylation on Ser/Thr-Promotifs (S17 is followed by a proline) is believed to affect the *cis/trans* isomerization properties of the proline residue (Ramelot and Nicholson, 2001).

The serine residues we have identified in CLIMP-63 as targets for mitotic phosphorylation belong to consensus sites for PKC (S3 and S19) and CKII (S17). To our knowledge the involvement of these kinases in the cell division process is poorly documented. For example, a CKII-like kinase has been proposed to be necessary for p115-mediated Golgi reassembly of mitotic fragments (Dirac-Svejstrup *et al.*, 2000). Regarding structural MAPs, cyclin-dependent kinases and mitogen activated protein kinases are responsible for their mitotic phosphorylation (Shiina *et al.*, 1992; Ookata *et al.*, 1997; Illenberger *et al.*, 1998; Jiang *et al.*, 1998; Shiina and Tsukita, 1999; Cassimeris, 2002). In related studies, cAMP-dependent protein kinase has been proposed to actively participate in tau release from MTs during mitosis when this neuronal MAP is expressed in Chinese hamster ovary cells (Illenberger *et al.*, 1998), and it is interesting to note that the same pathway has been suggested to facilitate organelle transport when tau is expressed in COS cells (Sato-Harada *et al.*, 1996). Whether PKC and CKII mediate the mitosis-specific phosphorylation of CLIMP-63 remains to be shown. We can also not exclude that an unknown cell cycle-dependent kinase is involved, because MAP-specific kinases also exist (Drewes *et al.*, 1995; Ebnet *et al.*, 1999).

#### CLIMP-63 Binding to Microtubules and ER Morphology

The interdependence of MT and ER networks in mammalian cells has been extensively described (Terasaki *et al.*, 1986; Lee *et al.*, 1989; Cole and Lippincott-Schwartz, 1995). From a mechanistic point of view, it is known that tubules extending from the ER in living migrating cells are formed by two main processes: 1) motor-dependent membrane sliding along preexisting MTs and 2) MT growing tip attachment (Waterman-Storer and Salmon, 1998). These data support a model in which the ER network forms by membrane extension toward the plus end of MTs. The importance of such a transport also has been shown in several other studies, where kinesin-dependent inhibition of transport, either by antisense suppression of kinesin heavy chain (Feiguin *et al.*, 1994) or by overexpressing tau (Ebnet *et al.*, 1998), led to the retraction of the ER to the cell center. This phenotype is very reminiscent of what we observe when overexpressing CLIMP-63 mutants that fail to bind to MTs: ER membranes no longer extend into the cell periphery and the number of three-way junctions is considerably reduced. Our results

strongly suggest that, in addition to plus end-directed active transport, a stable anchoring of membranes to MTs also is required to establish the characteristic ER organization. We propose that molecular motors pull membranes along MTs, whereas nonmotile linkers, such as CLIMP-63, stabilize the tubulated membrane processes. Interfering with either of these processes would lead to ER retraction either due to tension (Upadhyaya and Sheetz, 2004) or unmasking of the slow retrograde flow of membrane-bound MTs that continuously occurs within the cells (Terasaki and Reese, 1994; Waterman-Storer and Salmon, 1997, 1998), leading to ER retraction. Our experiments showed that some poorly branched tubules almost always emerge from the ER concentrated in the perinuclear area. These processes may be due to the presence of endogenous wt CLIMP-63. Alternatively, they may represent tubules formed by attachment to MT growing tips or by a proposed intrinsic ER membranes property to reticulate (Dreier and Rapoport, 2000).

A collapse of the ER was observed in transiently transfected cells but was almost absent from HeLa cells stably expressing  $\Delta$ CLIMP-63\* or CLIMP-63\* (S3E S17E S19E). Likewise, the tubulated phenotype was absent in cells stably expressing CLIMP-63\*. This discrepancy is consistent with the view that a reticular characteristic of the ER is essential for cell viability.

CLIMP-63 or homologous proteins are absent from yeast, in which the ER is much simpler organized into perinuclear ER and cortical ER underlying the plasma membrane. Yeast ER mainly relies on the actin cytoskeleton for its dynamics and maintenance (Prinz *et al.*, 2000; Estrada *et al.*, 2003). In contrast, cells of higher eukaryotes with their complex reticular ER largely rely on MTs and various mechanisms to allow for membrane dynamics while maintaining the overall structure of the ER. Our study presents CLIMP-63 as a novel player in maintaining the ER as an extended polygonal network and more generally highlights the necessity for a synergistic action of motor- and nonmotor-dependent interactions between MTs and membrane in this process.

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