A novel direct interaction of endoplasmic reticulum with microtubules

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This paper is dedicated to the memory of Thomas Kreis

The positioning and dynamics of organelles in eukaryotic cells critically depend on membrane–cytoskeleton interactions. Motor proteins play an important role in the directed movement of organelle membranes along microtubules, but the basic mechanism by which membranes stably interact with the microtubule cytoskeleton is largely unknown. Here we report that p63, an integral membrane protein of the reticular subdomain of the rough endoplasmic reticulum (ER), binds microtubules *in vivo* and *in vitro*. Overexpression of p63 in cell culture led to a striking rearrangement of the ER and to concomitant bundling of microtubules along the altered ER. Mutational analysis of the cytoplasmic domain of p63 revealed two determinants responsible for these changes: an ER rearrangement determinant near the N-terminus and a central microtubule-binding region. The two determinants function independently of one another as indicated by deletion experiments. A peptide corresponding to the cytoplasmic tail of p63 promoted microtubule polymerization *in vitro*. p63 is the first identified integral membrane protein that can link a membrane organelle directly to microtubules. By doing so, it may contribute to the positioning of the ER along microtubules.

Keywords: cytoskeleton/endoplasmic reticulum/linker protein/microtubules/p63

Introduction

Interactions between intracellular membranes and the microtubule cytoskeleton determine the structure and positioning of organelles and direct their dynamic movements (for reviews see Allan, 1995a; Cole and Lippincott-Schwartz, 1995; Richard and Kreis, 1995; Schroer and Gill, 1996; Vallee and Sheetz, 1996; Goodson *et al.*, 1997). Membranes can associate with microtubules either dynamically or in a stable fashion, depending on the properties of the linking molecules.

Dynamic interactions are required for membrane traffic and involve microtubule motors such as kinesin and dynein (Walker and Sheetz, 1993; Holzbaur and Vallee, 1994; Schröer and Gill, 1996; Vallee and Sheetz, 1996; Presley *et al.*, 1997; Schnapp, 1997; Hirokawa, 1998). The transport of membranes along microtubules is powered by a

complex of at least two proteins: a motor protein that binds to microtubules provides motility and a cargo protein that anchors the motor molecule to the membrane. Examples of such interactions are the plus-end motor protein kinesin and kinesin-related proteins which are thought to bind to membranes via the putative membrane receptor kinectin (Toyoshima *et al.*, 1992; Burkhardt, 1996; Vallee and Sheetz, 1996; Hirokawa, 1998) and the minus-end motor protein cytoplasmic dynein that binds to membranes via the soluble protein complex dynactin (Echeverri *et al.*, 1996; Burkhardt *et al.*, 1997), perhaps involving the ankyrin–spectrin network (Hirokawa, 1998). *In vitro* studies suggest that motor proteins also play a role in the dynamics of organelles such as tubular network formation of the endoplasmic reticulum (ER; Dabora and Sheetz, 1988; Vale and Hotani, 1988; Allan, 1995b). Tubule formation at the *trans*-Golgi (Cooper *et al.*, 1990) may also depend on motor proteins.

In contrast, more stable interactions must be responsible for the positioning and structural maintenance of organelles including the ER (Terasaki *et al.*, 1986; Lee and Chen, 1988) and the Golgi apparatus (Thyberg and Moskalewski, 1985; Cole *et al.*, 1996). Such interactions are assumed to utilize soluble non-motor microtubule-binding proteins. Cytoplasmic linker proteins (CLIPs) are a class of these proteins (Rickard and Kreis, 1996), the most prominent member of which is CLIP-170 that links microtubules via a putative membrane anchor to endosomes (Scheel and Kreis, 1991; Pierre *et al.*, 1992). A different mechanism allowing the attachment and translocation of membranes independently of classical motor proteins has been found in an experimental assay using *Xenopus* egg extracts (Waterman-Storer *et al.*, 1995). In this *in vitro* system, ER membranes became attached to the growing plus ends of microtubules by membrane–microtubule tip attachment complexes by a mechanism insensitive to inhibitors of motility produced by conventional kinesins and dyneins. The molecular mechanism mediating this interaction is unknown.

Here we describe a novel type of membrane–microtubule interaction that is mediated directly by an integral membrane protein, p63. p63 is a 63 kDa non-glycosylated type II integral ER membrane protein with an extracytoplasmic domain of 474 amino acids and an N-terminal cytoplasmic domain of 106 amino acids (Schweizer *et al.*, 1993a,b, 1994, 1995). The protein is palmitoylated reversibly during mitosis and under conditions that interfere with intracellular protein transport (Mundy and Warren, 1992; Schweizer *et al.*, 1993a; Mundy, 1995). Moreover, it is up-regulated in the liver after cardiogenic shock resuscitation (Buchman *et al.*, 1990; Schweizer *et al.*, 1993b), but the function of p63 is unknown. We report that the overexpression of p63 rearranges the ER and bundles microtubules along the altered ER membranes.

From *in vivo* and *in vitro* experiments, we conclude that p63 can bind directly to microtubules via its cytoplasmic domain and that the cytoplasmic domain is sufficient for this novel type of membrane-microtubule interaction.

Results

Overexpression of p63 rearranges the ER and microtubules

By immunofluorescence confocal laser scanning microscopy (CLSM), endogenous p63 of non-transfected COS cells is localized in the cytoplasm in a punctate/reticular pattern that is typical for the ER (Figure 1A), with the notable exception that the protein is largely excluded from the nuclear membrane as has been observed previously (Schweizer *et al.*, 1993a, 1995). If the cell borders were outlined by labeling the cell surface for the α -subunit of Na,K-ATPase, it was apparent that the ER classically extended to the periphery of the cell (Figure 1B and C).

Overexpression of p63 in cell culture can lead to a rearrangement of the ER network into an unusual tubular pattern (Schweizer *et al.*, 1993b, 1995). Figure 1D shows this phenomenon by CLSM. The punctate/reticular pattern of p63 changed to characteristic p63-positive tubules surrounding the nucleus upon overexpression of wild-type p63. That this change indeed reflects redistribution of the ER and not just p63 was confirmed by immunofluorescence double-labeling experiments with antibodies against total ER, and against the ER marker proteins ERP72 and BAP31. These ER markers perfectly co-localized with the p63-positive reticular pattern in non-transfected cells as well as with the p63-positive tubules induced by transfecting the cells with wild-type p63 cDNA (not shown). Under overexpressing conditions, p63 was not mislocalized to either the cell surface (Schweizer *et al.*, 1994) or the outer nuclear envelope, as shown by double-labeling for the nuclear pore complex protein p62 (Figure 1H and I), and thus the induced changes in p63 localization concern the ER only.

To determine whether these changes in ER morphology involve the cytoskeleton, we stained the cells for actin, vimentin or tubulin. The patterns of actin and vimentin were unaffected by the overexpression of wild-type p63 (not shown), indicating that microfilaments and intermediate filaments were not affected by the rearrangement of the ER. To test whether microtubules were involved, COS cells overexpressing wild-type p63 were double-stained for p63 and tubulin and analyzed by CLSM. Unlike the microfilaments and intermediate filaments, the microtubules underwent a dramatic change upon overexpression of p63 (Figure 1E). They no longer radiated into the peripheral cytoplasm, appeared thickened and, most notably, aligned with the p63-positive tubular structures (Figure 1F). At least 40% of transfected COS cells consistently showed this parallel change of ER and microtubules. The same phenomenon could be observed in CHO cells stably transfected with wild-type p63 cDNA (not shown). These observations suggest that p63 may mediate an interaction of ER membranes with microtubules *in vivo* that either becomes apparent or is induced by overexpression of p63.

To test whether p63 can bind to microtubules *in vitro*, detergent cell extracts of COS cells overexpressing p63

were incubated with taxol-stabilized microtubules of pig brain, and co-sedimentation of p63 with microtubules was analyzed by SDS-PAGE and Western blotting (Figure 2A). A considerable fraction of p63 (typically ~40–60%) was indeed found in the pellet fraction and this sedimentation was dependent on the presence of microtubules. Because these experiments cannot discriminate between a direct and an indirect interaction of p63 with microtubules, we also performed experiments with monoclonal antibody (mAb) affinity-purified p63 instead of detergent extracts. Figure 2A shows that purified p63 isolated from transfected cells binds to microtubules to a similar extent as p63 from detergent extracts. Likewise, endogenous p63 purified from non-transfected cells was equally efficient in binding to microtubules *in vitro*. Although the affinity-purified p63 fraction used for the binding assay did not reveal any major contaminating bands on silver-stained SDS gels (not shown), our data at this point leave open the possibility that p63 binds to microtubules indirectly rather than directly (but see below).

Microtubule redistribution depends on p63's cytoplasmic domain

The bundling of microtubules along the ER *in vivo* suggested that the overexpression of p63 enhanced the interaction of the two components and did not just change the morphology of the ER. Together with the above observation that p63 can bind to microtubules *in vitro*, these findings pointed to an ER-microtubule linker function of p63. We therefore asked whether p63 carries a microtubule-binding determinant in its cytoplasmic domain. Such a determinant would have to be novel because the sequence of p63 does not reveal any of the microtubule-binding motifs known in microtubule-associated proteins. To test this assumption, we generated p63 mutants with deletions in different regions of the cytoplasmic domain and tested their effects by overexpressing them in COS cells. According to previous results (Schweizer *et al.*, 1994), amino acids 1–23 of the cytoplasmic tail of p63 are sufficient to induce the morphological changes of the ER. Are they also sufficient for microtubule binding? Deletion construct Δ 24–101 induced an ER redistribution similar to wild-type p63 but the ER tubules appeared shorter, thinner and were more concentrated near the center of the cell as shown by CLSM (Figure 3A). The ER tubules accumulated close to the nucleus (Figure 3D and E) and did not extend into the peripheral cytoplasm (compare Figure 3G–I). However, the microtubule pattern remained unchanged in cells overexpressing Δ 24–101 (Figure 3B). The microtubules neither co-aligned with p63 tubules (Figure 3C) nor did they increase in thickness. Consistent with this, the mutant protein Δ 24–101 was unable to bind to microtubules in the *in vitro* binding assay, irrespective of whether it originated from detergent extracts or affinity purification (Figure 2B). The observations that the ER can tubulate without altering the general microtubule appearance and that the Δ 24–101 construct did not bind to microtubules *in vitro* show that the cytoplasmic domain of p63 is required for microtubule binding.

The cytoplasmic domain of p63 carries two functionally distinct determinants

The results with the Δ 24–101 construct also suggest that the microtubule-binding determinant and the tubulation

Fig. 1. Overexpressed p63 co-aligns with microtubules *in vivo*. COS cells were mock transfected (A–C) or transiently transfected with p63 wild-type cDNA (D–L). After 48 h, the cells were fixed with paraformaldehyde, permeabilized with saponin, double-stained for p63 (A, D, G and J), tubulin (E), Na,K-ATPase (B and K) or the nuclear pore complex protein p62 (H), and analyzed by immunofluorescence confocal laser scanning microscopy (CLSM). Note that overexpression of p63 leads to tubulation of the ER (D, G and J) and a parallel alteration of microtubules (E). The microtubules appear thickened (E) and co-align with the ER (F). Bar, 10 μm.

Fig. 2. Binding of wild-type and mutant p63 to polymerized microtubules *in vitro*. (A) In the presence of microtubules (+MT), a substantial fraction of wild-type p63 from detergent-solubilized transfected COS cells co-sediments with microtubules and appears in the pellet (p). In the absence of microtubules (–MT), p63 stays in the supernatant (s). Affinity-purified p63 also binds to microtubules (+MT), indicating a direct interaction. (B) The Δ24-101 mutant fails to bind to microtubules irrespective of whether it originates from cell extracts or affinity purification. (C) The Δ2-21 mutant binds to microtubules like wild-type p63 (compare with A). (D) Amino acid sequence (single letter code) of the entire cytoplasmic domain of p63 (Schweizer *et al.*, 1993b).

determinant of the p63 tail are spatially different. To test this notion, the Δ2-21 p63 construct was generated in which 20 amino acids close to the N-terminus were deleted. This construct thus lacks the putative ER rearrangement determinant. Expression of Δ2-21 in COS cells resulted in a morphological pattern distinct from that observed with wild-type p63 and Δ24-101. Neither the microtubule pattern nor the reticular appearance of the ER were affected (Figure 4A and B). The ER still extended as a network throughout the entire cytoplasm, as demonstrated by the localization of p63-positive membranes relative to the nuclear membrane and to the cell border (Figure 4D–F and G–I), and co-localized significantly with microtubules (Figure 4A–C). Consistent with these morphological findings, construct Δ2-21 was found to bind to microtubules *in vitro* with an efficiency comparable with that of wild-type p63 (Figure 2C). These findings suggest that amino acids 2–21 comprise a determinant required for ER rearrangement that functions independently from the microtubule-binding determinant which is contained within amino acids 24–101. Moreover, the results show that bundling of the ER membranes is not a prerequisite for microtubule binding.

The isolated cytoplasmic domain of p63 binds to microtubules *in vivo* and *in vitro*

To test whether the cytoplasmic domain of p63 is sufficient for microtubule binding, a construct encompassing the cytoplasmic portion with an additional C-terminal c-myc tag (p63tail-myc) was expressed in COS cells. The subcellular localization of p63tail-myc showed a fine reticular/tubular pattern co-localizing with microtubules (Figure 5A–C), while no bundling of microtubules was observed. While sufficient for microtubule binding, the isolated cytoplasmic tail was unable to induce microtubule bundling, suggesting that the cytoplasmic domain needs to be anchored to the ER to promote microtubule bundling.

Unlike wild-type p63, its isolated cytoplasmic domain did not rearrange the ER. Obviously such a change requires the additional presence of other p63 domains.

To confirm further that the cytoplasmic determinant required for ER-microtubule interactions carries microtubule-binding information, a His₆-tagged peptide corresponding to amino acids 1–106 was produced in *Escherichia coli*, purified by Ni-agarose affinity chromatography, and its binding to microtubules was tested *in vitro*. Figure 6 shows that this fragment can indeed bind to microtubules while a control peptide (a His₆-tagged peptide corresponding to amino acids 39–189 of the leptin receptor) was unable to bind. Together with the *in vitro* binding experiments in which purified intact p63 was tested (Figure 2A), these data strongly suggest that the cytoplasmic domain can bind directly to microtubules *in vitro*. Obviously amino acids 1–106 in p63 comprise microtubule-binding information which is independent of the rest of the molecule.

To test whether the cytoplasmic domain of p63 can induce tubulin polymerization *in vitro*, we used a turbidity assay in which the absorbance at 350 nm was measured. The incubation of a tubulin preparation with the recombinant peptide corresponding to the cytoplasmic domain of p63 led to a concentration-dependent increase in absorbance over time, with a threshold at 10 μM peptide (Figure 7c–g). The increase in absorbance was preceded by a lag phase of ~1 min at peptide concentrations ≥10 μM. A 20 μM peptide concentration induced the same maximal turbidity as 20 μM taxol (Figure 7h) though with slower kinetics. Incubation of tubulin with buffer alone (Figure 7a) or 20 μM bovine serum albumin (BSA) (Figure 7b) did not increase the absorbance, and the p63 peptide-induced turbidity change required the presence of tubulin in the assay (not shown). These data suggest that the cytoplasmic domain of p63 is able to promote tubulin polymerization *in vitro*.

Discussion

Numerous previous studies have suggested that the ER network is connected to microtubules. This interaction is believed to determine the structure and dynamics of the ER (Terasaki *et al.*, 1986; Lee and Chen, 1988). Although *in vitro* data have provided compelling evidence that microtubule motors play a role in tubular network formation of the ER (Dabora and Sheetz, 1988; Vale and Hotani, 1988; Allan, 1995b), the precise mechanism by which this organelle maintains its characteristic structure *in vivo* is largely unknown.

Here we report a novel mode of interaction by which an integral membrane protein, p63, can link the ER and microtubules directly. That p63 binds to microtubules *in vivo* became apparent due to the remarkable ability of p63 to rearrange the ER upon its overexpression, which led to a concomitant redistribution of microtubules. The information for ER rearrangement is localized in the first 23 amino acids of p63, since the construct Δ24-101 could still induce this phenotype while the construct Δ2-21 was unable to do so. Obviously when overexpressed, the first 23 amino acids are necessary to cross-link the ER as indicated by the increased thickness of the tubular ER elements observed in this study and the previously reported

Fig. 3. The overexpressed p63 mutant $\Delta 24-101$ rearranges the ER but does not co-align with microtubules. Transient expression of $\Delta 24-101$ in COS cells can lead to rearrangement of the ER into short tubular structures (A). Microtubules in the same cell are largely unaffected (B) and there is little apparent co-localization of ER and microtubules (C). Note that the ER tubules preferentially stay near the nucleus (D–F and G–I). Immunofluorescence CLSM analysis was as in Figure 1. Bar, 10 μ m.

parallel arrangement of ER membranes seen in electron micrographs (Schweizer *et al.*, 1995). It is conceivable that the capacity of p63 to form large aggregates in the ER (Schweizer *et al.*, 1994) is related intimately to ER tubule formation, but the precise mechanism underlying this rearrangement remains to be determined. Overexpression of some invariant chain mutations in the ER can also lead to a tubular remodeling of the ER (Schutze *et al.*, 1994), but it has not been investigated whether this change involves microtubules.

Our *in vivo* and *in vitro* experiments showed that amino acids 24–101 in the cytoplasmic tail of p63 are required for linking the ER to microtubules while the first 23 amino acids conferring tubule formation were dispensable for this interaction. The interaction with microtubules appears

to be a direct one, since purified p63 as well as its isolated cytoplasmic domain can bind to microtubules. We conclude, therefore, that amino acids 24–101 comprise a microtubule-binding determinant. The *in vitro* sedimentation assays showed a consistent ~50% microtubule binding for all forms of p63 irrespective of their origin or purity, suggesting an intrinsically low affinity of this interaction.

It is important to note that the $\Delta 2-21$ construct which bound to microtubules like wild-type p63 was not mislocalized to post-ER compartments, indicating that the peptide segment comprising the microtubule-binding domain, either alone or together with the transmembrane and/or luminal domain of p63, confers retention in the ER. This retention is not mediated by microtubules, since the construct $\Delta 2-21$ also remained in the ER when micro-

Fig. 4. The overexpressed p63 mutant $\Delta 2-21$ co-aligns with microtubules but does not rearrange the ER. COS cells transiently transfected with $\Delta 2-21$ exhibit a typical reticular ER (A). Microtubules (B) in part co-localize with ER membranes (C) but remain unbundled. $\Delta 2-21$ remains excluded from the nuclear membrane and extends from the center (D–F) to the periphery of the cell (G–I). Immunofluorescence CLSM analysis was as in Figure 1. Bar, 10 μm .

tubules were depolymerized by nocodazole treatment (not shown). A previous mutagenesis study has shown that amino acids 1–23 also contain information for ER retention (Schweizer *et al.*, 1994). Obviously the retention information provided by the cytosolic tail of p63 is highly redundant.

The rearrangement of the ER does not depend strictly on the expression level of p63. Both at early (24 h) and late (72 h) times after transfection, ~40% of the transfected COS cells exhibited a tubular appearance of the ER although p63 levels increased considerably between 24 and 72 h. Moreover, a strict correlation of tubulation and fluorescence signal was not observed when cells of the same transfected cell culture dish were compared visually. It is also important to note that the expression

levels of the different p63 constructs were in the same range as compared by Western blotting (not shown), excluding the trivial possibility that the different ER phenotypes simply reflected different expression levels. That tubulation did not depend strictly on p63 expression levels may suggest that additional mechanistic factors are involved in ER rearrangement.

The region providing microtubule binding does not have any significant sequence similarities to the microtubule-binding domains of MAP2, MAP4 and tau (Lee *et al.*, 1988; Lewis *et al.*, 1988; Chapin and Bulinski, 1991) or to that of MAP1B (Noble *et al.*, 1989). In a previous study, an *in vitro* interaction of the E3/19K adenovirus membrane protein and microtubules was reported and found to depend on a motif related to microtubule-binding

Fig. 5. The cytoplasmic domain is sufficient to bind to microtubules *in vivo*. COS cells were transiently transfected with a construct encoding the cytoplasmic domain of p63 with a C-terminal myc tag, fixed with paraformaldehyde, permeabilized with saponin, double-stained with antibodies against the c-myc epitope and tubulin, and analyzed by CLSM. Note that the myc staining (A) co-localized with microtubules (B and C). Bar, 10 μ m.

Fig. 6. A His₆-tagged peptide corresponding to the cytosolic domain of p63 (amino acids 1–106) binds to purified microtubules *in vitro*. The peptide co-sediments with microtubules as detected by a horseradish peroxidase conjugate (Ni-NTA HRP) while a His₆-tagged peptide corresponding to amino acids 39–189 of the leptin receptor, used as a control, does not bind (Lep-R).

Fig. 7. Polymerization of pig brain tubulin by a recombinant peptide corresponding to the cytoplasmic tail of p63. Tubulin (1 mg/ml) was incubated with buffer (a), 20 μ M BSA (b) or different concentrations of the recombinant p63 peptide: 4 μ M (c), 8 μ M (d), 10 μ M (e), 16 μ M (f) or 20 μ M (g). Change in absorbance at 350 nm was measured at 37°C. In the absence of tubulin, 10 μ M of the p63 peptide had no effect on the absorbance (not shown). As a positive control, tubulin polymerization in the presence of 20 μ M taxol is shown in (h).

repeats present in MAP2 and tau (Dahllöf *et al.*, 1991). However, this *in vitro* finding was not validated in an *in vivo* system.

Numerous other microtubule-binding proteins are known for which the binding domain has, at least in part, been characterized; none of these, however, appears to share similarity with the microtubule-binding domain of p63. They include Stu2p/p93^{dis1} that localizes to the

spindle pole body and along spindle microtubules of yeast (Nakaseko *et al.*, 1996; Wang and Huffaker, 1997), the plus-end motor protein kinesin (Woehlke *et al.*, 1997), and the minus-end motor dynein (Gee *et al.*, 1997; Koonce, 1997). Common to all these proteins is that their microtubule-binding domain is positively charged and their binding to microtubules is sensitive to salt (Vallee, 1986), suggesting that they bind to microtubules through electrostatic interactions. The microtubule binding of p63 *in vitro* is also salt dependent, suggesting an ionic interaction, although binding was only affected by salt concentrations >200 mM NaCl (not shown). Protein binding to microtubules can also be mediated by Src homology 2 domains of signaling molecules (Itoh *et al.*, 1996). No Src homology 2 domain is present in the microtubule-binding domain of p63.

The recombinant cytoplasmic domain of p63 was found to exert a concentration-dependent stimulatory effect on tubulin polymerization *in vitro*, confirming the existence of at least one microtubule-binding domain in p63. In contrast to p63, the microtubule-binding domains of MAPs are known to contain multiple repeats of determinants that are essential for binding. However, studies with MAP2 have shown that the microtubule-related properties including tubulin polymerization *in vitro* can arise from a single 31-amino-acid repeat, although the precise mechanism by which this occurs is unknown (Ludin *et al.*, 1996). The microtubule-binding region of p63 may contain two binding sites that are capable of tethering two neighboring tubulin subunits together. Alternatively, a single binding site may modify the features of the tubulin subunit to which it binds and thereby increase its affinity for other tubulin molecules. It is possible that the expressed cytosolic domain of p63 is even capable of bundling microtubules when present in relatively high concentrations. This is indicated by the fact that the experiment with 20 μ M p63tail reached an OD similar to that of 20 μ M taxol. Bundled microtubules have much higher ODs than unbundled ones at similar polymer concentrations and, at this concentration of taxol, much of the OD is contributed normally by bundling. Additional experiments are required to confirm this suggestion.

In addition to motor proteins, organelle-microtubule interactions involve the activity of CLIPs (Richard and

Kreis, 1996), such as CLIP-170 that links endosomes to microtubules (Pierre *et al.*, 1992, 1994) and CLIP-115, a brain-specific protein that mediates the localization of dendritic lamellar bodies (De Zeeuw *et al.*, 1997). These two proteins share a double repeated microtubule-binding domain, which can also be found in various other proteins including the p150^{Glued} subunit of the dynactin complex (for a recent comparison of these microtubule-binding domains, see De Zeeuw *et al.*, 1997). Again no sequence similarity to p63 is apparent. Unlike p63, CLIPs are soluble proteins preferentially associated with microtubules. In order to promote docking of organelles to microtubules, CLIPs must interact with membrane receptors that are still unknown.

The microtubule-binding domain of p63 has a number of interesting features of potential importance for its binding to microtubules: strings of consecutive identical amino acids (i.e. prolines, glycines, serines, alanines) and a repeated tetrapeptide motif, HPQQ. Moreover, p63 is palmitoylated reversibly during mitosis and by inhibitors interfering with membrane traffic (Mundy and Warren, 1992; Schweizer *et al.*, 1993; Mundy, 1995). However, it is presently unclear which features in fact determine the binding of p63 to microtubules. Despite extensive mutagenesis of this region (unpublished), it was not possible to define a continuous minimal microtubule-binding motif. It appears that three-dimensional structural information will be required in addition to mutagenesis to define the microtubule-binding domain of p63.

It is likely that other organellar membrane proteins will be found in the endomembrane system that can bind directly to the cytoskeleton. We propose to name these proteins CLIMPs (cytoskeleton-linking membrane proteins) and accordingly rename p63 to CLIMP-63. Another potential CLIMP is the Golgi protein giantin (Linstedt and Hauri, 1993, 1994). CLIMP-63 may stabilize the ER in its reticular appearance by directly anchoring it to microtubules and/or stabilizing microtubules. In line with such a proposed function are the observations that the ER retracts from peripheral to more central areas of the cytoplasm upon overexpression of mutant $\Delta 24-101$ which is unable to bind microtubules, and the selective localization of CLIMP-63 in the reticular part of the rough ER. Experiments with living epithelial cells have shown that only a small proportion of cells possess actively moving ER, while in most cells the ER is less dynamic (Lee and Chen, 1988). It was suggested that these movements may be limited to a particular phase of the cell cycle that requires ER proliferation. In conjunction with CLIPs and molecular motors, CLIMPs may also regulate the dynamic interaction of organelles with the cytoskeleton. CLIMP-63 may play a role in *de novo* ER network formation by anchoring tubulo-vesicular membranes to microtubules and thereby aiding motor protein-driven tubulation that ultimately will result in an ER network. Although CLIMP-63 can bind directly to microtubules *in vitro*, it is conceivable that additional proteins will be required for the regulation of this interaction *in vivo*, in particular during mitosis when the microtubules are required for spindle formation.

Materials and methods

Antibodies

The following primary antibodies were used: mouse mAb G1/296 (an IgG2a) against p63 (Schweizer *et al.*, 1994); mouse mAb 1A2 (an IgG3)

against α -tubulin (kindly provided by Thomas Kreis, University of Geneva; Kreis, 1987); mouse mAb 414 (an IgG1) against nuclear pore complex protein p62 (BAbCO, Richmond, CA, kindly provided by Birthe Fahrenkrog, Biozentrum Basel, Switzerland); and mouse mAb 9E10.2 (an IgG1) (Evan *et al.*, 1985) against the c-myc epitope. mAbs against BAP31 (an IgG1) and ERP72 (an IgG1) were produced in mice by the hybridoma technique (Hauri *et al.*, 1985) using a membrane fraction of Vero cells enriched in ERGIC-53 (Schweizer *et al.*, 1991) as an antigen. Antibody specificity was established by N-terminal sequencing of the recognized antigens (Schweizer *et al.*, 1993b). Mouse mAb N1/123 (an IgG1) against the α -subunit of Na-K-ATPase was produced by using a membrane fraction of human kidney enriched in Na-K-ATPase (Jorgensen, 1974) as an antigen and the immunization protocol of Kashgarian (1985). Secondary antibodies were: fluorescein isothiocyanate (FITC)-goat anti-mouse IgG3 (ICN, Eschwege, Germany); Texas red-goat anti-mouse IgG2a; Texas red-goat anti-mouse IgG1; and FITC-goat anti-mouse IgG1 (Southern Biotech., Birmingham, AL).

Cell culture and transfection

COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin and 1 μ g/ml fungizone at 37°C according to standard techniques. The cells were transiently transfected using the DEAE-dextran method (Cullen, 1987).

Recombinant DNAs and expression of recombinant protein

p63 cDNA was mutated using standard PCR protocols. Construct $\Delta 24-101$ has been described (Schweizer *et al.*, 1993b). Inserts were excised from the pECE vector with *EcoRI* and blunt-end ligated into the *EcoRV* site of a modified pcDNA3 vector (Invitrogen, Leers, The Netherlands) lacking the *NotI* restriction site to give the plasmids pcDNA3-p63 or pcDNA3- $\Delta 24-101$. For the construction of the $\Delta 2-21$ mutant, pcDNA3-p63 was used as template for PCR. For the construction of p63tail-myc, the DNA sequence coding for the c-myc epitope EQKLISEEDL followed by the translation termination codon TAA was introduced after amino acid position 106 of p63 by PCR. A His₆-tagged construct encompassing amino acids 1–106 of the cytoplasmic domain of p63 was obtained by PCR amplification of the corresponding DNA sequence using an upstream primer with a *SphI* and a downstream primer with a *BglII* restriction site. The resulting fragment was cloned into the *SphI*-*BglII* site of the *E. coli* expression vector pQE-70 (Qiagen, Basel, Switzerland). The recombinant His₆-tagged peptide was expressed in *E. coli* strain M15 and purified by Ni-agarose affinity chromatography.

Affinity purification of p63

After 60 h of transfection with wild-type p63 cDNA, COS cells were harvested by scraping followed by centrifugation for 10 min at 750 g at 4°C. Cell pellets were solubilized for 1 h on ice in 100 mM NaH₂PO₄ pH 8.0, containing 1% Triton X-100 and protease inhibitors, and subsequently centrifuged for 1 h at 100 000 g. p63 protein was affinity purified from the resulting supernatant on a column of mAb G1/296 coupled to Affi gel-10. The protein was eluted with 0.05 M triethylamine pH 11.5, 0.1% Triton X-100, and the eluates were adjusted immediately to pH 7.0 with 1 M Tris base. Fractions containing the purified p63 were pooled and dialyzed against PNT buffer (20 mM PIPES, 30 mM Tris, 100 mM NaCl, 1 mM EGTA, 1.25 mM EDTA), pH 7.0, containing 0.1% Triton X-100 and 40 μ g/ml phenylmethylsulfonyl fluoride (PMSF).

Confocal laser scanning immunofluorescence microscopy

Transiently transfected COS cells were trypsinized 24 h after transfection and seeded into 8-well multi-chamber slides. After 19–20 h, the cells were fixed with paraformaldehyde, permeabilized with 0.1% saponin, and processed for immunofluorescence microscopy (Schweizer *et al.*, 1993a). The specimens were examined with a Leica confocal laser scanning microscope (0.28 μ m optical sections). Corrections of bleed-through in double-labeling experiments were done according to the recommendations of the manufacturer and were tested in single-labeling experiments.

Microtubule binding assay

Tubulin was prepared from pig brain by two temperature-induced cycles of microtubule assembly by the method of Weingarten *et al.* (1974) with slight modifications. The brain was homogenized in 1 ml of assembly buffer per gram of brain tissue [0.1 M *N*-morpholinoethanesulfonic acid (MES), 1 mM EGTA, 1 mM MgSO₄, 40 μ g/ml PMSF, pH 6.8] containing 0.5 M sucrose. Homogenization was performed with a Teflon/glass homogenizer at 4°C. The homogenate was centrifuged at 54 000 g for

60 min at 4°C in a TST70.38 rotor. The supernatant was collected, diluted 1:1 with assembly buffer containing 7 M glycerol, warmed to 37°C for 45 min, centrifuged at 100 000 g for 60 min at 26°C, and the supernatant was discarded. The pellet was resuspended in 0.3 ml of assembly buffer per gram of brain, incubated on ice for 30 min and centrifuged at 69 000 g for 20 min at 4°C. The supernatants were diluted 1:1 with assembly buffer containing 7 M glycerol, warmed at 37°C for 45 min and centrifuged for 60 min at 100 000 g at 26°C. The pellets containing the purified microtubules were resuspended in assembly buffer, frozen in liquid nitrogen and stored at -80°C.

After 52–62 h of transfection, COS cells of a 60 mm dish were washed twice with ice-cold phosphate-buffered saline (PBS), scraped into 1 ml of PBS and collected by centrifugation for 10 min at 750 g at 4°C. The cells were solubilized in 1 ml of PNT buffer, pH 7.0, containing 1% Triton X-100, 100 mM iodoacetamide and protease inhibitors. Tubulin (5 mg/ml in buffer A: 100 mM MES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8) was polymerized with 30 µM taxol in the presence of 2.5 mM GTP, 350 mM NaCl for 30 min at 37°C. For microtubule binding, 30 µg of repolymerized microtubules per assay were pelleted by centrifugation for 30 min at 20 800 g in an Eppendorf centrifuge. The microtubules were washed once with buffer A, resuspended in 80 µl of solubilized cell supernatant (or purified p63) containing 20 µM taxol and incubated for 30 min at 37°C. The sample was then centrifuged at 20 800 g, and supernatant and pellet were analyzed by SDS-PAGE and Western blotting for the presence of tubulin and p63.

In vitro polymerization

The polymerization of pig brain tubulin was monitored at 350 nm in a Uvikon 820 spectrophotometer. Tubulin at a concentration of 1 mg/ml in assembly buffer containing 1 mM GTP was mixed with various amounts of the recombinant cytoplasmic domain of p63 to a final volume of 400 µl and the increase of absorbance at 350 nm was measured at 37°C.

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