A Role for Centrin 3 in Centrosome Reproduction

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Abstract. Centrosome reproduction by duplication is essential for the bipolarity of cell division, but the molecular basis of this process is still unknown. Mutations in Saccharomyces cerevisiae CDC31 gene prevent the duplication of the spindle pole body (SPB). The product of this gene belongs to the calmodulin super-family and is concentrated at the half bridge of the SPB. We present a functional analysis of HsCEN3, a human centrin gene closely related to the CDC31 gene. Transient overexpression of wild-type or mutant forms of HsCen3p in human cells demonstrates that centriole localization depends on a functional fourth EF-hand, but does not produce mitotic phenotype. However, injection of recombinant HsCen3p or of RNA encoding HsCen3p in one blastomere of two-cell stage X enopus laevis embryos resulted in undercleavage and inhibition of centrosome duplication. Furthermore, HsCEN3 does not complement mutations or deletion of CDC31 in S. cerevisiae, but specifically blocks SPB duplication, indicating that the human protein acts as a dominant negative mutant of CDC31. Several lines of evidence indicate that HsCen3p acts by titrating Cdc31p-binding protein(s).

Our results demonstrate that, in spite of the large differences in centrosome structure among widely divergent species, the centrosome pathway of reproduction is conserved.

Key words: centrosome • duplication • Ca2+-binding protein • yeast • X enopus laevis

Introduction

Although centrosome structure is strikingly different between yeasts and animal cells, proteins involved in the centrosome-associated microtubule nucleating activity are conserved (Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998). Given the important role of centrosome reproduction for cell division, one may conjecture that the corresponding molecular mechanisms also were conserved. The genes involved in animal centrosome duplication are unknown. However, several genes were identified in the spindle pole body (SPB)1 duplication pathway in budding yeast (Byers, 1981; Rose and Fink, 1987; Winy et al., 1991, 1993; Sun et al., 1992; Biggins and Rose, 1994; Donaldson and Kilmartin, 1996; McDonald and Byers, 1997), and the precise SPB duplication cycle has been described recently (Adams and Kilmartin, 1999).

The best example of a gene implicated in an early step of SPB duplication is the budding yeast CDC31, an essential gene in which mutations prevent the initiation of SPB duplication (Baum et al., 1986; Spang et al., 1993). The product of CDC31 shows homology to calmodulin, which is characterized by the presence of four potential Ca2+-binding sites, called EF-hands (Baum et al., 1986). It is localized to the half bridge of the SPB, on which the satellite forms when the SPB duplicates (Spang et al., 1993). Cdc31p localization depends on Kar1p (Biggins and Rose, 1994), which is also localized to the half bridge of the SPB (Spang et al., 1993) and is required for both SPB duplication and karyogamy (Conde and Fink, 1976; Rose and Fink, 1987). Kar1p contains a hydrophobic tail that probably anchors it in the nuclear envelope and which is necessary for its function (Vallen et al., 1992). A direct interaction between Kar1p and Cdc31p has...
been described (Biggins and Rose, 1994). KAR1 also has been shown to be in genetic interaction with DSK2 (for dominant suppressor of KAR1; Vallen et al., 1994), a gene encoding a ubiquitin-like protein (Biggins et al., 1996). Dsk2p is also involved in SPB duplication since its overexpression or the expression of the allele dsk2-1 prevents SPB duplication (Vallen et al., 1994). However, DSK2 is not an essential gene, as are CDC31 and KAR1. Dsk2p is homologous to another ubiquitin-like protein, Rrad23p, and the double deletion, Δdsk2 Δradd23, inhibits SPB duplication (Biggins et al., 1996). Neither Dsk2p nor Rrad23p were localized to the SPB, and their role in centrosome duplication is unclear.

From these data, one could infer that mammalian members of the centrin family, which are structurally related to Cdc31p, are candidates for a function in centrosome duplication. However, the green algae, Chlamydomonas reinhardtii, centrin has been shown to be required for the proper segregation of the flagellar apparatus during cell division, rather than for the duplication of the basal bodies (Kuchka and Jarvik, 1982; Wright et al., 1985; Taillon et al., 1992). In this organism, centrin is localized in the lumen of the basal bodies and forms contractile fibers connecting the basal bodies and the nucleus (Huang et al., 1988a,b; Salisbury et al., 1988). The mutation, vf2, in the centrin gene prevents the formation of the nucleus–basal body connection and the segregation of the basal bodies (Tailon et al., 1992). Moreover, centrin from C. reinhardtii is unable to complement cdc31 mutants in yeast. However, basal body–associated centrin is still detected in the vf2 mutant making it likely that C. reinhardtii contains an additional centrin gene implicated in basal body duplication.

In human, three centrin genes have been described, named HSCEN1, HSCEN2, and HSCEN3 (Lee and Huang, 1993; Errabolu et al., 1994; Middendorp et al., 1997; the symbols in the human genome database are CETN1, CETN2, and CETN3). The products of these genes are localized in the distal lumen of the centrioles and in the procentriole bud (Paolletti et al., 1996). A nalysis of HSCEN2 revealed a possible function in cell cleavage since injection of recombinant Hscen2p in two-cell stage Xenopus laevis embryos induced undercleavage, leading to large blastomeres containing a variable number of microtubule asters (Paolletti et al., 1996). Sequence comparison revealed that Hscen3p shares more similarity with Cdc31p than the two other human centrin proteins, Hscen1p and Hscen2p (Middendorp et al., 1997), strongly suggesting the existence of two divergent subfamilies of centrin (see Fig. 1).

We have undertaken a functional analysis of Hscen3p to test a potential role in centrosome duplication. In human cultured cells, we have demonstrated that centriolar targeting of Hscen3p requires a functional fourth EF-hand. Injection of recombinant wild-type Hscen3p or of RNA coding for a mutant form of Hscen3p in two-cell stage Xenopus embryos induces undercleavage, with blastomeres containing only one or two microtubule asters. Finally, Hscen3p is able to block cell growth by impairing SPB duplication in Saccharomyces cerevisiae. CDC31 can overcome this block in a dose-dependent manner. We have shown that Hscen3p binds the Cdc31p-binding protein Kar1p, but this interaction is not sufficient to explain the effect of Hscen3p.

Materials and Methods

Cloning of cDNA of Human Centrins in pCB6

To allow detection and localization of the overexpressed protein, cDNA coding for Hscen1p or Hscen3p was cloned in the mammalian expression vector pCB6 (Brewer and Roth, 1991), in fusion with either a VSVG epitope in the NH₂-terminus region or a six histidines tag in the COOH-terminus region. Hscen2p or Hscen3p were amplified by PCR to introduce EcoRI and XbaI restriction sites, respectively, at the 5’ and 3’ ends of the cDNA. The PCR products were double digested by EcoRI and XbaI and ligated in the EcoRI/XbaI-digested pBS-KS vector containing the cDNA coding for the 15 amino acids of the VSVG protein recognized by the mAb, PSD4 (Soldati and Perriard, 1991). The cDNA encoding the fusion between VSVG and Hscen1p or Hscen3p was excised by a KpnI/BamHI double digestion and inserted in the mammalian expression vector pCB6 under the control of CMV promoter. A histidine tag was introduced by a PCR also inserting restriction sites to enable cloning in pCB6. The mutants Hscen3p-D147,149,151A, where the three aspartates in position 147, 149, and 151 were replaced by alanines and the mutant Hscen3p-P99A, where the proline in position 99 was replaced by an alanine, were generated by PCR mutagenesis and cloned in pCB6 in fusion, respectively, with a VSVG or a six histidines tag.

Transfection of HeLa Cells

Exponential growing HeLa cells were transfected by electroporation. 5 × 10⁷ HeLa cells were detached by trypsin, washed, and resuspended in 200 μl of medium containing 10% FCS and 15 mM HEPES, pH 7.5. 40 μg of plasmid and 2 μg of carrier DNA (salmon sperm DNA) were diluted in 50 μl of 210 mM NaCl solution and mixed to the cell suspension in a 4-mm electroporation cuvette. Cells were submitted to an electric pulse of 290 V, 960 mF, and an unlimited resistance in an electroporator (BioRad). Cells were then washed in 5 ml of medium containing 10% FCS and 15 mM HEPES, pH 7.5, and seeded either on collagen-coated coverslips for immunofluorescence analysis or on a petri dish for Western blot analysis. Immunofluorescence of HeLa cells was performed as described by Paolletti et al. (1996).

Western Blot Analysis

Samples were separated on 12% SDS-PAGE as described by Laemmli (1970) and electrophoretically transferred onto nitrocellulose and processed for immunoblotting as described by Paolletti et al. (1996). After saturation, membranes were incubated with 1/2,000 anti-Hscen1p, 1/250 anti-Hscen3p, 1/100 anti-Cdc31p rabbit sera, or 1/50 anti-Kar1p goat sera. Immune detection was carried out with anti-IgGs coupled to alkaline phosphatase (Hscen1p) or to HRP (Hscen3p), followed by enhanced chemiluminescence (ECL) detection (Nycodem A mersham, Inc.) according to the company’s instructions. For detection of anti-Cdc31p and anti-Kar1p antibodies, biotin-conjugated secondary antibodies and alkaline phosphatase-conjugated streptavidin were used (Nycodem A mersham, Inc.).
Microinjection and Immunofluorescence Experiments in Xenopus Embryos

6 histidine-tagged \( HsCEN3 \) cDNA, generated by PCR inserting NdeI and BamHI restriction sites, was cloned in the bacterial expression vector, pET3b. A recombinant His-HsCen3p was induced in the BL21 D3 E3 strain and purified on NIT A agarose (Qiagen Inc.), according to manufacturer’s instructions. High molecular weight contaminants were eliminated by chromatography on strychnosin cibacron blue (Pharmacia Biotech, Inc.) or a M (co)reduction and immunofluorescence experiments were performed as described by Paoletti et al. (1996). cDNA was cloned in pGFP-Fr/N3P (Zernicka-Goetz et al., 1996), replacing the GFP insert, and RNA were transcribed in vitro as described by Jarmolowski et al. (1994), and diluted in 10 mM Hepes before injection.

Yeast Strain Genotypes

YPH500, MATa ade2 his3 leu2 ura3 trpl1. Y M K229, MATa ade2 his3 leu2 ura3 trpl1 lys2 \\

Immunofluorescence Experiments

Cells were fixed and prepared for EM as described by Doye et al. (1994).

Electron Microscopy

Cells were fixed by resuspending a frozen pellet corresponding to 50 ml of a 10-h culture (0.7 OD 600 ) in 250 ml of buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40) containing protease inhibitors (Boehringer Mannheim Corp.). 0.4 g of glass beads were added and the samples were incubated with vigorous vortexing at 4°C for 30 min and then centrifuged 15 min at 20,000 g at 4°C. Protein G-Sepharose beads, equilibrated in PBS, were incubated with affinity-purified antibodies for 2 h at 4°C under mild agitation. Immunoglobulin-bound protein G-Sepharose beads were sedimented, washed three times in lysis buffer, and then incubated with cell extracts for 2 h at 4°C under mild agitation. Sedimented protein G-Sepharose beads were washed six times in lysis buffer and once in distilled water. The immunoprecipitates were solubilized from the Sepharose beads by incubation with the SDS-PAGE sample buffer (100°C, 5 min) and centrifugation.

Results

HsCen3p Targeting to Centrioles in Human Cells

We first attempted to test HsCen3p function in human cultured cells. We undertook transient overexpression experiments of wild-type \( HsCEN3 \) or mutants of \( HsCEN3 \) in HE.L.a cells or diploid skin fibroblasts. We chose two mutants that were expected to be defective for HsCen3p function: the first, HsCen3p-P99A, affected a proline conserved among the Cdc31p-related centrins and located between the second and the third EF-hand; the second, HsCen3p-D147,149,151A, inactivated the fourth and most conserved EF-hand (Middendorf et al., 1997). The overexpressed proteins were fused either with 16 amino acids of the VSVG protein cloned at the NH2 terminus and recognized by the mAb bPSD4, or with a 6 histidines epitope cloned at the COOH terminus. Transient overexpression of wild-type HsCen3p did not produce any obvious phenotype, such as centrosome duplication defect or abnormal mitosis. We observed that the protein was able to exchange with the centriolar endogenous HsCen3p (Fig. 2 A’), suggesting that these tags did not impair the protein function. However, when compared with overexpression of HsCen1p, we observed that localization of HsCen3p to the centrosome was slower than the localization of HsCen1p (Fig. 2 A’), suggesting either that the half-life of HsCen3p is longer than the one of HsCen1p or that the centriolar targeting of the two proteins is differently regulated. The localization of the HsCen3p-P99A mutant is less efficient than that of the wild-type protein. The HsCen3p-D147,149,151A mutant was totally unable to exchange with the centriole-associated endogenous protein (Fig. 2 A’). This suggested that centriolar localization depends upon the presence of a functional fourth EF-hand. We checked by Western blot experiments that the mutant protein was expressed (Fig. 2 B) and observed that, despite the lack of centriolar localization, part of this mutant protein was associated with a Triton X-100 insoluble fraction. However, overexpression of either of the two mutant proteins did not arrest cell growth, possibly due to the presence of a sufficient amount of wild-type protein.

As noted above, the amount of wild-type homologous protein was not critical in transient transfection experiments suggesting that, just like in yeast (Geier et al., 1996;
see also Fig. 4), the amount of homologous protein is not critical. However, we consistently failed in establishing cell lines stably overexpressing HsCen3p, whereas we could easily do so with HsCEN1 and HsCEN2. This observation suggested that increase in the dosage of HsCen3p might have a long-term effect, deleterious only after one or two centrosome duplication cycles. This possibility has been addressed in the early developing Xenopus embryo, in which several rapid rounds of division take place.

Dose-dependent HsCen3p Inhibition of Centrosome Duplication in the Xenopus Early Embryo

We investigated HsCen3p function in a heterologous animal system undergoing a simple cell division cycle, the Xenopus embryo, which has already been used to study HsCen2p function (Paoletti et al., 1996). Recombinant histidine-tagged HsCen3p (Hs-HsCen3p) was microinjected in one blastomere of two-cell stage embryos. Embryos were observed before mid-blastula transition (7 h after fertilization at 22°C) and after gastrulation (22 h after fertilization). 72% of the embryos injected with 12 mg/ml Hs-HsCen3p presented undercleavage, whereas only 2% of PBS-injected embryos presented a cleavage delay (Table I; Fig. 3 A). 22 h after fertilization, 25% of the Hs-HsCen3p-injected embryos had exogastrulated and 69% had lysed, compared with 23% and 2% for the control embryos, respectively. These effects were concentration-dependent as shown in Table I. As a control, we injected heat- or protease-treated His-HsCen3p. As other centrins (Paoletti et al., 1996), His-HsCen3p is resistant to heat treatment (95°C for 1 h). Injection of 12 mg/ml heat-treated His-HsCen3p resulted in under-segmentation (Table I; Fig. 3 A, 3). The effect was, however, weaker than with the nonheated protein, as judged by embryo lysis at 22 h, suggesting partial heat denaturation. Protease-treated His-HsCen3p had no significant effect (Table I; Fig. 3 A, 4).

Anti-α-tubulin staining demonstrated that most of the under-segmented blastomeres (91%) contained two large microtubule asters (Fig. 3 B and C; Table II). The size of the center of these asters was comparable to that of control blastomeres, making it unlikely that they contain numerous duplicated, but unseparated, centrosomes. A few blastomeres contained a single aster (5%) and 4% presented three or four asters. These observations, in marked contrast with the effect of HsCen2p injection (Fig. 3 D), strongly suggest that Hs-HsCen3p inhibits cleavage by impairing centrosome duplication or inhibits both cleavage and centrosome duplication independently.

We also tested whether the fourth EF-hand mutant, which failed to localize to the centrosome (see above), could also disturb centrosome duplication. As it is not possible to perform anticentrosome immunofluorescence in
whole *Xenopus* embryos, we microinjected the nucleus of *Xenopus* somatic cells with the plasmid coding for this mutant form. We checked that the fourth EF-hand mutant was unable to localize to the centrosome in *Xenopus*, whereas the wild-type form did (data not shown). As we were not able to concentrate the mutant protein at 12 mg/ml due to its insolubility, we injected the in vitro transcribed RNA. As a control, we injected RNA encoding wild-type *HsCEN3* or GFP, all at 0.6 mg/ml. Injection of wild-type *HsCEN3* RNA confirmed results obtained with HsCen3p (Table I). The RNA encoding the mutated HsCen3p induced under-segmentation, demonstrating a stronger effect than wild-type *HsCEN3* RNA. Injection of GFP RNA, or of Hepes buffer, had a minimal effect (Table I).

Anti–α-tubulin staining showed that most of the RNA-injected blastomeres contained two asters when observed 11 h after fertilization (Table II). Compared with protein-injected embryos, blastomeres with either one aster, or three or more asters were more numerous. This variation is probably dose-dependent. It is noteworthy that, in the case of RNA injections, pre-MBT development was analyzed 11 h after fertilization to allow RNA translation.

### Table I. Short- and Long-term Effects of Heterologous Centrin 3 in Two-Cell Stage Injected *Xenopus* Embryos

| Injected solution         | Pre-MBT development | Post-MBT development | Eggs |
|---------------------------|---------------------|---------------------|------|
|                           | N | U | L | % | N | E | L | % | n |
| Protein injection         |   |   |   |   |   |   |   |   |   |
| HsCen3p (1.5 mg/ml)       | 80 | 20 | 0 | 49 | 48 | 3 | 90 |
| HsCen3p (3 mg/ml)         | 25 | 75 | 0 | 25 | 75 | 0 | 20 |
| HsCen3p (6 mg/ml)         | 30 | 70 | 0 | 10 | 48 | 42 | 91 |
| HsCen3p (12 mg/ml)        | 28 | 72 | 0 | 6 | 25 | 69 | 77 |
| Heat-treated HsCen3p      | 31 | 69 | 0 | 10 | 77 | 13 | 97 |
| Protease-treated HsCen3p  | 95 | 5 | 0 | 58 | 28 | 14 | 56 |
| PBS                       | 98 | 2 | 0 | 75 | 23 | 2 | 133 |
| RNA injection             |   |   |   |   |   |   |   |   |   |
| WT HsCen3                 | 63 | 32 | 5 | 30 | 46 | 24 | 106 |
| Mutant HsCEN3             | 38 | 58 | 4 | 6 | 29 | 65 | 103 |
| GFP                       | 89 | 11 | 0 | 64 | 19 | 17 | 37 |
| Hepes                     | 94 | 5 | 1 | 64 | 25 | 11 | 79 |

25 ml of native or treated recombinant HsCen3p, or 25 ml of wild-type HsCEN3 (WT), mutant HsCEN3, or GFP RNA at 0.6 µg/ml or control solutions (PBS or Hepes) were injected in one blastomere of two-cell stage embryos. Short-term effects were observed 7 h (protein injection) or 11 h (RNA injection) after fertilization. The effect on blastomere cleavage was scored as normal (N), under-segmented (U), or lysis (L) of the injected half. Long-term effects were observed 22 h after fertilization. Development of the injected side of the embryo was scored as normal (N), participating in exogastrulation of the whole embryo (E), or lysed (L). Note that in the case of RNA injections, pre-MBT development was analyzed 11 h after fertilization to allow RNA translation.

### Table II. Number of Asters per Blastomere in the Injected Half of Embryos

| Injection                     | Number of asters/blastomere |
|-------------------------------|-----------------------------|
|                              | 1 | 2 | 3 | 4 | 5 or more | n |
| HsCen3p                      | 5 | 91 | 1 | 3 | 0 | 103 |
| WT HsCEN3 RNA                | 10 | 78 | 3 | 3.5 | 5.5 | 111 |
| Mutant HsCEN3 RNA            | 18 | 68.5 | 3 | 2.5 | 8 | 126 |

7 h (protein injection) or 11 h (RNA injection) after fertilization, embryos were fixed and labeled with monoclonal anti-α-tubulin. For each condition, the number of asters per large blastomere in the injected half of the embryo was scored in two embryos from two independent experiments. One or two asters were observed in blastomeres cleaved at normal rate.
HsCen3p Inhibits SPB Duplication in Budding Yeast

Finally, another approach to support a possible involvement of HsCEN3 in centromere duplication was to test whether HsCEN3 could complement S. cerevisiae strains bearing temperature-sensitive mutations in CDC3 (cdc31-100D144V, cdc31-101F291, cdc31-102K123E) or a complete deletion of CDC3. Expression of HsCen3p did not rescue the temperature-sensitive phenotype of the mutants nor did it complement the deletion of SCDC3 (data not shown). However, overexpression of HsCen3p in a wild-type background was lethal: cells transformed with a galactose inducible 2μ or CEN (centromeric) plasmid encoding HsCen3p did not grow when expression was induced (Fig. 4). This effect was specific because overexpression of HsCen1p, HsCen2p, or Cdc31p (Fig. 4, see also Geier et al., 1996) did not inhibit growth of wild-type cells. We checked by Western blotting that HsCen1p, HsCen2p, and HsCen3p, and Cdc31p were expressed to comparable levels in the strains transformed with the 2μ plasmid (data not shown).

To further investigate the effect of HsCen3p, yeast strains bearing HsCEN3 or CDC3 on a galactose inducible plasmid were grown in liquid medium complemented with glucose (repression), raffinose (low level of expression), or raffinose + galactose (induction). Whereas cell growth was identical in glucose medium for both strains, cells expressing HsCen3p stopped growing 6 h after induction (Fig. 5 A). Moreover, cell growth of this strain in raffinose, where a very low expression of HsCen3p was detected (Fig. 5 B), decreased after a 10-h culture. This indicates that even a very low level of HsCen3p is able to impair cell proliferation. This was further confirmed by the effect of HsCEN3 cloned on a centromeric plasmid, which blocked cell proliferation (see Fig. 4). The effect of HsCen3p overexpression was not reversible: cells grown in raffinose + galactose medium for 12 h were not rescued from the block when seeded onto glucose plates.

To test whether cells were arrested at a specific stage of the cell cycle upon HsCen3p overexpression, we monitored the budding index throughout the induction. As shown in Fig. 5, C and D, cells with a large bud started to accumulate 6 h after induction, reaching a maximum value of 80% at 12 h. At this time, cells overexpressing Cdc31p were mainly unbudded, only 10% of them having a large bud (data not shown). We demonstrated by flow cytometry analysis that the large budded HsCen3p-expressing cells had a G2 DNA content (Fig. 5 E) and an increased size, compared with control cells in G2-M.

Immunofluorescence analysis of HsCen3p-induced large budded cells with anti-tubulin antibody did not show the spindle characteristic of the G2-M phase (Fig. 6 A). mAb 45D10, which recognizes the SPB component Spc110p (Out and Kilmartin, 1990), revealed only one dot (Fig. 6 B). A single dot corresponding to SPB was also observed in a yeast strain expressing HsCen3p and GFP-Spc42p (Fig. 6, C and C'). Cytoplasmic background, but no staining of the SPB, could be detected with anti-HsCen3p antibodies, whatever the fixation method used, indicating that HsCen3p was not able to localize to the SPB. These results, together with the DAPI staining showing the nucleus most often trapped into the neck (Fig. 6, A and B), strongly suggested that cells failed to complete mitosis due to unduplicated SPB or unseparated SPBs.

To discriminate between these two possibilities, cells were fixed for EM. The observation of SPB in 35 sagittally sectioned cells blocked in G2-M showed no associated satellite nor additional SPB (Fig. 6 D). This was further established by serial sectioning (five successive nuclear sections of five individual cells). In all cases, a single unduplicated SPB was observed (Fig. 6 E). The second, third, and fourth sections always showed part of the single SPB and a few microtubules radiating from the SPB, whereas the first and the fifth sections did not contain any SPB. Examination of the unduplicated SPB at higher magnification showed that their size had not been affected. Altogether, this data shows that HsCEN3 behaves as a dominant negative mutant of CDC31, inhibiting the initiation of SPB duplication.

The most divergent region between HsCen3p and Cdc31p lies in the NH2 terminus region (Middendorp et al., 1997). To test whether this region was responsible for the dominant effect of HsCen3p, the 5′ region of the two cDNA s were swapped. The cDNA encoding amino acids 1-17 of Cdc31p in fusion with amino acids 24-167 of HsCen3p still blocked cell growth, whereas the reciprocal construction (1-23HsCen3p/18-161Cdc31p) had no effect (Fig. 7 A). This demonstrated that the effect of HsCen3p was not due to the divergent NH2 terminus end.

It is possible that HsCen3p acts by titrating either Cdc31p or a protein that normally interacts with Cdc31p. If such a complex between HsCen3p and Cdc31p or a Cdc31p-binding protein exists, overexpressing Cdc31p might restore the native complex. To test this possibility, we performed cooverexpression of HsCen3p and Cdc31p. A yeast strain containing a multicopy plasmid expressing HsCen3p was transformed with a multicopy plasmid bearing CDE31 and DNA. As shown in Fig. 7 A, these cells did not grow under inducing conditions. However, when we attempted to find yeast genes able to suppress the effect of HsCen3p expression by transforming a yeast strain expressing HsCen3p on a centromeric plasmid with a vector that...
genomic library of S. cerevisiae cloned on a 2μ multicopy plasmid, we found only one gene able to rescue the phenotype, namely CDC31 (Fig. 7 A). In this condition, Western blot analysis showed that Cdc31p was expressed to a higher level than HsCen3p. This result suggested that HsCen3p might interact with either Cdc31p itself or with Cdc31p-binding protein(s) with a higher affinity than Cdc31p.

To discriminate between these two possibilities, we tested whether Cdc31p or Kar1p, a Cdc31p-binding protein involved in SPB duplication, were coimmunoprecipitated by anti-HsCen3p antibodies. As shown in Fig. 8, anti-HsCen3p antibodies did not immunoprecipitate Cdc31p in the yeast strain overexpressing HsCen3p, despite their capacity to weakly cross-react with Cdc31p, as observed in the strain overexpressing Cdc31p. By contrast, anti-HsCen3p antibodies immunoprecipitated Kar1p in the HsCen3p-overexpressing strain, whereas they did not in the Cdc31p-overexpressing strain. As expected, Kar1p was not immunoprecipitated in the Δkar1 YMKH229 strain.
strain overexpressing HsCen3p (see below). It is noteworthy that when we performed anti-HsCen2p immunoprecipitation in yeast strain overexpressing HsCE N2, we found that HsCen2p was able to interact in vivo with Kar1p, as previously shown in vitro (Geier et al., 1996). As HsCen2p does not impair SPB duplication, this result suggests that the specific blocking effect of HsCen3p in S. cerevisiae is dependent on interaction of HsCen3p with ScCdc31p-binding protein(s) other than Kar1p.

To investigate this possibility, we tested whether HsCen3p could inhibit growth of the Y M K 229 strain that is deleted for Kar1p and expresses cdc31-16, the product of which is able to localize to the SPB in absence of Kar1p (Vallen et al., 1994). To facilitate the phenotypic analysis, we used a Y M K 229 strain in which Spc42p is expressed in fusion with GFP. If HsCen3p acts by sequestering Kar1p only, expression of HsCen3p in a Δkar1p strain would be expected to be without effect. We observed the opposite.
result: expression of HsCen3p in this genetic background remained lethal (Fig. 7A), 80% of the cells accumulating with a large bud after a 12-h induction (data not shown). Moreover, a single dot corresponding to GFP-Spc42p was observed in this strain, indicating that the SPB failed to duplicate (Fig. 7B). This strongly suggests that HsCen3p interacts with at least another unidentified Cdc31p-binding protein involved in SPB duplication.

**Discussion**

To study the possible involvement of HsCen3p in centrosome duplication, we performed overexpression of wild-type and mutant forms of HsCen3p in human cultured cells, but did not succeed in disturbing the centrosome duplication cycle in transient transfection experiments. The overexpression of HsCen3p was found to be consistently less efficient than overexpression of HsCen2p, suggesting that either the level of HsCen3p is more tightly regulated than that of HsCen2p in human cells or that the half life of the two proteins are quite different. We observed that inactivation of the fourth EF-hand prevents centriole localization of the protein. However, part of this mutant protein can associate with Triton X-100 insoluble structures. A long-term effect of high dosage of HsCen3p was suggested by the fact that we could not establish stable cell lines expressing HsCen3p, whereas we could easily do it with the two other human centrin genes. We reasoned that a high dosage of HsCen3p could have a deleterious effect only after one or two centrosome duplication cycles, a possibility which was difficult to test in culture cells. Thus, we turned to the Xenopus embryo, which we previously used to study HsCen2p function (Paoletti et al., 1996). In this rapidly dividing system, HsCen2p and HsCen3p led to completely different effects, although undercleavage was observed in both cases: undercleaved blastomeres produced by injection of HsCen2p contained numerous asters, indicating that the centrosome duplication process itself was not prevented. Thus, HsCen2p could disturb either cytokinesis itself or any upstream event, for example, the timing of centrosome duplication or its coupling with cytokinesis. On the contrary, HsCen3p is shown in this work to impair centrosome duplication. As a consequence, cleavage is inhibited. Our results do not exclude, however, that HsCen3p also impairs events downstream of centrosome reproduction, including the cleavage process itself. This could explain that a few uncleaved blastomeres contained more than two microtubule asters. Injection of RNA encoding the mutant HsCen3p-D147,149,151A protein, which is unable to localize to the centrosome, also impairs centrosome duplication, suggesting that HsCen3p acts by interacting with a cytoplasmic Xcen3p-binding protein.

We observed that HsCEN3 could not complement S. cerevisiae strains bearing temperature-sensitive mutations in CDC31 or a complete deletion of CDC31. On the contrary, HsCen3p inhibited cell growth and SPB duplication, whereas the two other human centrin proteins were without effect. We have shown that HsCen3p interacts with Kar1p, a protein which is also involved in SPB duplication (Biggins and Rose, 1994), whereas the other known Cdc31p-binding protein, Kic1p, is involved in cell wall
pressing the HsCen3p still inhibits SPB duplication in a yeast strain expressing Cdc31p-binding proteins. This was further confirmed: HsCen3p disturbs SPB duplication by titrating other previously described in vitro (Geier et al., 1996). This suggests HsCen2p, which did not impair cell growth when overexpressed, is not coimmunoprecipitated, whereas HsCen3p and Kar1p are coimmunoprecipitated. It is noteworthy that in the conditions we have used, Cdc31p and Kar1p are not coimmunoprecipitated, whereas HsCen2p and Kar1p are.

Figure 8. HsCen3p interacts with Kar1p extracts of yeast cells overexpressing HsCen3p, Cdc31p, HsCen3p in aΔkar1/cdc31-16 background, or HsCen3p were immunoprecipitated with anti-HsCen3p affinity-purified antibodies or with anti-HsCen2p antibodies. Immunoprecipitated proteins were separated on SDS-PAGE, transferred onto nitrocellulose, and revealed with anti-HsCen3p, anti-Cdc31p, or anti-Kar1p antibodies, or with anti-HsCen2p and anti-Kar1p antibodies. In cells overexpressing HsCen3p, HsCen3p and Kar1p are coimmunoprecipitated. It is noteworthy that in the conditions we have used, Cdc31p and Kar1p are not coimmunoprecipitated, whereas HsCen2p and Kar1p are.

integrity (Sullivan et al., 1998). We also found that HsCen2p, which did not impair cell growth when overexpressed in yeast, is also able to interact with Kar1p as previously described in vitro (Geier et al., 1996). This suggests that HsCen3p disturbs SPB duplication by titrating other Cdc31p-binding proteins. This was further confirmed: HsCen3p still inhibits SPB duplication in a yeast strain expressing the cdc31-16 allele that can grow in the absence of Kar1p. It is noteworthy that, in the conditions we used to demonstrate the interaction between HsCen3p and Kar1p, we could not detect an interaction between Cdc31p and Kar1p. This result suggests that Kar1p has a higher affinity for HsCen3p than for Cdc31p or, more likely, that the functional Cdc31p/Kar1p complex, which is part of the half bridge, is not soluble in the conditions we used. No SPB localization has been detected for HsCen3p, making it possible that HsCen3p sequesters Cdc31p-binding proteins in the cytoplasm. The existence of complexes between HsCen3p and Cdc31p-binding protein(s) is in favor of a functional conservation between HsCen3p and Cdc31p.

A together, our data strongly argue in favor of the existence of two functionally distinct centrin families (see Fig. 1): a first one implicated in centrosome duplication, to which Cdc31p and HsCen3p belong; and a second family that participates in other cell division events, such as centrosome segregation or cytokinesis, and which includes centrin from C. reinhardtii and HsCen2p (Paoletti et al., 1996). The single centrin gene of the budding yeast might be able to fulfill both functions. It has been shown that, in addition to SPB duplication, Cdc31p regulates the activity of Kic1p, a kinase involved in cell integrity and in cell separation (Sullivan et al., 1998).

Recently, several studies have shown that cyclin-CDKs are required for driving centrosome duplication in animals. During embryonic cell cycles, cyclin E-CDK2 activity is required for centrosome reproduction (Hinchcliffe et al., 1999; Lacey et al., 1999), whereas cyclin A-CDK2 is also required in somatic cells (Matsumoto et al., 1999; Meradji et al., 1999). A together, these data suggest that the cell cycle machinery indeed regulates centrosome reproduction, coupling it with the cell cycle. However, the centrosomal targets of cyclins-CDK2 are unknown. It is possible that these kinases transcriptionally regulate genes involved in centrosome duplication. Identification of genes coding for centrosomal proteins regulated by cyclin-CDKs will be a crucial step in understanding centrosome duplication regulation.

The intriguing possibility that γ-tubulin, a protein involved in microtubule nucleation and stability, is required for basal body assembly in Paramecium suggests that controlling microtubule dynamics might also regulate centriole/basal body duplication and probably that the regulation of centriolar microtubule assembly shares common steps with cytoplasmic microtubule nucleation (Ruiz et al., 1999). Centrin 3 is the first centriole-associated protein, actually concentrated in the distal lumen of each centriole and in the early procentriole bud, to be shown to participate in the initiation of centrosome duplication in animals. Identification of proteins interacting with Cen3p in a Ca²⁺-dependent manner should be critical for further study of the regulation of the centrosome duplication. A s HsCen3p blocks yeast SPB and frog centrosome duplication most likely by competing with Cdc31p and with Xenopus centrin for their physiological targets, the two experimental systems used in this study provide valuable tools to identify new proteins involved in SPB or centrosome duplication.

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