Binding of Centrins and Yeast Calmodulin to Synthetic Peptides Corresponding to Binding Sites in the Spindle Pole Body Components Kar1p and Spc110p*

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Centrins contain four potential Ca\(^{2+}\) binding sites, known as EF-hands, and have essential functions in centrosome duplication and filament contraction. Here we report that centrin from yeast, green alga, and humans bind with high affinity to a peptide of the yeast centrosomal component Kar1p. Interestingly, centrin binding was regulated by physiological relevant changes in [Ca\(^{2+}\)], and this Ca\(^{2+}\) dependence was influenced by acidic amino acids within the Kar1p peptide, which also prevented efficient binding of the related yeast calmodulin.

For accurate distribution of the chromosomes to daughter cells, the microtubule organizing center (MTOC) must be precisely duplicated to create both poles of the mitotic spindle. MTOCs from phylogenetically different organisms are quite heterogeneous in structure. Despite these structural differences, MTOCs contain conserved components, for example, γ-tubulin (1–4) and centrin (5–10), which may perform similar functions in each species. Centrins are acidic proteins containing four potential Ca\(^{2+}\) binding sites known as EF-hands (reviewed in Ref. 11). The yeast centrin, Cdc31p, has an essential function in the cell cycle-dependent duplication of the yeast MTOC, the spindle pole body (SPB). This conclusion is based on the phenotype of conditional lethal cdc31 mutants being defective in SPB duplication under restrictive growth conditions (12). Consistent with the defect in cdc31 cells is the association of Cdc31p with the cytoplasmic side of the SPB half bridge (7), which has important functions in SPB duplication (13). Genetic and biochemical evidence (15, 16) suggests that Cdc31p interacts with a component of the SPB, Kar1p. Cdc31p binds to 19 amino acids in the central part of Kar1p, and Kar1p localizes as does Cdc31p to the half bridge of the SPB (16). The similar SPB defect of kar1 and cdc31 cells reveals that both gene products are required for the same step in SPB duplication (12, 17). Cdc31p is also found in the cytoplasm of yeast cells (18). However, the role of this Cdc31p remains to be determined.

In green algae such as Chlamydomonas, centrins participate in the contractions of fibers that connect the nucleus with the basal bodies (19, 20). Centrin is a major component of these fibers, and Ca\(^{2+}\) causes shortening of the fibers, even in the absence of ATP (20). Centrin-based fiber contraction also plays an important role in flagellar excision in Chlamydomonas (21). Furthermore, green algal centrin is associated with the MTOC in its basal body in these organisms (22); however, the function of centrin at this location is not understood. Human cells contain two highly related centrin isogenes (8, 9). The two centrin may be associated with the centrioles (8) or the pericentriolar material (9) of the centrosomes.

Ca\(^{2+}\) acts as a second messenger in eukaryotic cells. At the molecular level, this implies that Ca\(^{2+}\) generates conformational changes in target proteins. Ca\(^{2+}\)-induced conformational changes have been shown for Cdc31p and centrins from the green algae Scherffelia dubia (24) and Chlamydomonas (23) and the two human centrins (24). This raises the possibility that the activity of centrin is regulated by changes in cellular [Ca\(^{2+}\)]. Ca\(^{2+}\) binding to Cdc31p (7) may function as one signal regulating SPB duplication (6, 16, 25). Ca\(^{2+}\) binding to algal centrin results in a more compact conformation that may cause the contraction of the centrin-based filaments (24).

Certainly, the best studied EF-hand protein involved in Ca\(^{2+}\) signaling is calmodulin. It is considered to be a universal Ca\(^{2+}\) receptor that interacts with over 20 proteins (26). In yeast, diverse essential functions of calmodulin were revealed by four groups of calmodulin mutants with characteristic defects in actin organization, calmodulin localization, nuclear division, or bud emergence (27). One important target of yeast calmodulin (ScCaM) is the SPB component Spc110p (28–31). ScCaM binds to a region in the cytoplasm-terminal domain of Spc110p (30, 31). This part of Spc110p is required to target ScCaM to the central plaque of the SPB (32, 33). Surprisingly, a yeast calmodulin mutant that does not bind Ca\(^{2+}\) in vitro was fully active in vivo. Therefore, it has been suggested that ScCaM fulfills its essen-
tional functions without Ca\(^{2+}\) binding (34).

In this study, we analyzed whether the binding of centrins to their targets is Ca\(^{2+}\)-regulated and how the closely related ScCaM and Cdc31p discriminate their targets. These questions were addressed by studying centrin binding to synthetic peptides corresponding to the Cdc31p and ScCaM binding sites of the SPB components Kar1p and Spc110p, respectively. We found that centrin binding to Kar1p peptide was regulated by Ca\(^{2+}\) and that this dependence was influenced by features in the centrin binding site as well as by the centrin itself. Furthermore, acidic amino acids in the centrin binding site of Kar1p prevented efficient binding of ScCaM. However, centrins bound to a ScCaM binding site in vitro but not in vivo, suggesting that mechanisms other than the structure of the target binding site prevent binding of Cdc31p to ScCaM targets in vivo. Our results suggest that centrins regulate the properties of target proteins in response to changes in [Ca\(^{2+}\)].

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Bacterial and Yeast Transformation, and DNA Techniques**—Yeast strains are summarized in Table I. cdc31-100, cdc31-101, and cdc31-102 were obtained by in vitro mutagenesis of CDC31 and a plasmid shuffle approach as described (35). cdc31-100, cdc31-101, and cdc31-102 were integrated into their chromosomal location by a pop-in-pop-out replacement (36) to give strains UFY27, UFY28, and UFY31, respectively. cdc31-100 carries a single point mutation, causing a D144V substitution in the fourth EF-hand. cdc31-101 carries an F39I in the first EF-hand and cdc31-102, a K123E substitution affecting the helix connecting the third and fourth EF-hands. The standard medium for growth of yeast was yeast extract, peptone, and dextrose growth medium. Synthetic minimal medium and synthetic complete medium were prepared as described (38). Yeast cells were transformed using the lithium acetate method (37). DNA manipulations were performed as described (38).

**Construction of Plasmids**—Plasmids are listed in Table I. Point mutations in CDC31 affecting the four EF-hands were introduced by PCR. PCR products were subcloned into pRS425 and pRS426 (39). For the expression of cdc31 mutants under control of the T7 promoter, cdc31-1, EF, cdc31-2, EF, cdc31-3, EF, and cdc31-4, EF were amplified by PCR. The PCR products were subcloned into the expression vector pT7-7 (40). CDC31-CMD1 gene fusions were constructed by recombinant PCR. PCR products were subcloned in pRS426 and pT7-7. kar1-Δ17 was cloned from strain MS2083 by gap repair. Sequence analysis confirmed the previously reported deletion in KAR1 (14). A GST-kar1-Δ17 gene fusion was constructed as described for GST-KAR1 (16) in plasmid pGEX-3X (Pharmacia Biotech) to give plasmid pSM203.

**Protein Purification**—Centrins (pSM169, SdCEN; pSM5, CDC31; pSM248, HsCEN1; pTP6, HsCEN2), CMD1 (pSM71), CDC31-CMD1 (pSM196), or CMD1-CDC31 (pSM315) gene fusions were expressed in Escherichia coli BL21 (DE3) as described (7, 16, 41) and purified as reported (24). Briefly, after a temperature precipitation step (10 min at 80 °C), the supernatant was applied onto a DEAE-cellulose column (Sigma). Centrin- or ScCaM-containing fractions were further enriched by a phenyl-Sepharose step and finally purified using a Superdex 75 HiLoad 16/60 column on a fast protein liquid chromatography system (Pharmacia). Protein and peptide concentrations were determined according to Bradford (42) and by UV measurements.

**Purification of Cdc31p-1, EF to Cdc31p-4, EF**—Cdc31p-1, EF (pSM105), cdc31p-2, EF (pSM113), cdc31p-3, EF (pSM114), and cdc31p-4, EF (pSM93) were expressed in E. coli BL21 (DE3). Cdc31p-2, EF and Cdc31p-3, EF were purified as described for Cdc31p. For Cdc31p-1, EF and Cdc31p-4, EF cells, lysates were not subjected to temperature precipitation since
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both proteins precipitated under this condition. Instead, the lysate was directly bound onto DEAE-column and then eluted by a linear salt gradient. For Cdc31p-1.EF cells, all of the following chromatography steps were performed as described for centrins. Cdc31p-4.EF-containing fractions were further purified using hydroxyapatite (Bio-Rad Laboratories). The protein was eluted by a gradient from 45 to 300 mM KPi buffer, pH 6.8. Cdc31p-4.EF-containing fractions were further enriched by a MonoQ HR 5/5 column (Pharmacia). Finally, the protein was applied onto a Superdex 75 HiLoad 16/60 column.

Peptide Synthesis—Peptides were synthesized by the Peptide Synthesis Facility at the Max-Planck Institute. The identity of the peptide was confirmed by mass spectroscopy.

Tryptophan Fluorescence Spectroscopy—Fluorescence measurements were performed at 20 °C using a Perkin-Elmer LS 50B fluorescence spectrometer. The wavelength for excitation was 295 nm. The emission spectra of the Kar1p or Spc110p peptides showed a maximum at 350 nm. Increasing amounts of purified protein (0.5-μl steps) were added to a 1-cm square quartz cuvette containing 1 μl of a 1 μM solution of the peptide in Ca²⁺ buffers (2-10 mM calcium-EGTA in 10 mM MOPS, and 100 mM KCl, pH 7.2; Molecular Probes) with 38, 100, 225, 692, or 1350 nM free [Ca²⁺]. Binding of ScCaM or centrins to Spc110p peptide resulted in a blue shift of the fluorescence emission maximum by about 15 nm (ScCaM) or 5 nm (centrins) accompanied by a 4-fold (ScCaM) or 2-fold (centrins) increase in fluorescence intensity. The dissociation constants (Kₐ) of the reactions were determined based on the observed changes in fluorescence at 320 nm (Kar1p peptides) or 330 nm (Spc110p peptide). Experimental data were fitted using the following equation (protein) = [proteinfree] × [B/F]max × [proteinfree] + [proteinfree]. F is the measured fluorescence intensity changes at each point, and Fmax is the maximal fluorescence intensity change. B is [peptide] × (number of binding sites). Nonlinear regression curve fittings using PSI-Plot version 4.02 program (Poly Software International) was performed to fit the experimental data. Mean values were taken of two or more titrations using different batches of purified protein.

CD Spectroscopy—CD measurements were done using a Jobin Yvon Mark IV CD spectrometer. Measurements using 0.1 mg/ml protein were first recorded in 10 mM MOPS, pH 7.2, 50 mM KCl, 0.5 mM EGTA, and again after the addition of up to 2 mM Ca²⁺ at 20 °C in a 1.0-mm path length cuvette. Average spectra (number of 10) from 198 to 240 nm with 0.1-nm spacing were used for the calculations. The α-helical content was calculated with the CD package of CONTIN (DP version; Ref. 43).

Complementation Tests and Depletion of Cdc31p—cdc31-1.EF, cdc31-3.EF, cdc31-4.EF, or cdc31-4.EF-containing fractions were further enriched using a MonoQ HR 5/5 column (Pharmacia). Finally, the protein was applied onto a Superdex 75 HiLoad 16/60 column.

RESULTS

Binding of Centrins to Kar1p Peptide Is Ca²⁺-dependent—

Features of target binding sites and the Ca²⁺ dependence of binding reactions are well characterized for calmodulins (26) but not for centrins. These aspects of centrins were investigated by studying their binding to a peptide corresponding to the Cdc31p binding site of Kar1p (Kar1p peptide), which hitherto is the only known centrin binding site (16). The binding of centrins to Kar1p peptide was studied using the fluorescence of the amino acid tryptophan in the Kar1p peptide (Fig. 2A). This was possible, since centrins from yeast (Cdc31p; Ref. 6), the green alga S. dubia (SdCen; Ref. 46), humans (HsCen1; Ref. 8; HsCen2, Ref. 9), and ScCaM (47) lack tryptophan. Therefore, changes in the tryptophan fluorescence spectrum of the Kar1p peptide upon the addition of centrin result from the binding of centrin to the peptide. In agreement with these considerations, an emission spectrum typical for tryptophan with a maximum at 350 nm was observed for the Kar1p peptide (Fig. 1A, curve 6), and this maximum was shifted by 20 to 30 nm toward shorter wavelengths upon the addition of centrins (Fig. 1A, compare curve 6 with curves 1-4), indicating the formation of a peptide-centrin complex. Centrin addition was also accompanied by a 2-3-fold increase in relative fluorescence intensity of the emission maximum. Furthermore, the comparable shapes of the emission spectra after addition of centrins (21-4) suggested that the tryptophan of the Kar1p peptide is in a similar environment in all four peptide-centrin complexes. In contrast to centrins, the addition of a 10-fold molar excess of ScCaM over Kar1p peptide was not accompanied by an increase in the relative fluorescence intensity (Fig. 1A, curve 5), and the shift in the fluorescence maximum was less than 10 nm.

We investigated whether the binding of centrins to the Kar1p peptide was influenced by Ca²⁺. For these studies, we used Ca²⁺ buffers that maintain a constant free [Ca²⁺] (48). Centrins were titrated to the Kar1p peptide in buffers containing free [Ca²⁺] ranging from 0.038 to 1.35 μM. The dissociation constants (Kₐ) of the binding reactions were then calculated based on the changes in fluorescence at 320 nm upon the
addition of centrin (49). At 1.35 μM free [Ca\(^{2+}\)], the \(K_d\) values of centrin binding reactions were in the nanomolar range (from 60 nM for Cdc31p to 300 nM for HsCen2), indicating high affinity binding (Fig. 1B). The stoichiometry of all reactions was close to one, which is consistent with the binding of one peptide to each centrin molecule. Most interestingly, the affinity decreased (\(K_d\) increased) 20-fold by a decrease in [Ca\(^{2+}\)] from 1.35 to 0.038 μM. This change in [Ca\(^{2+}\)] is in the physiologically relevant range (50, 51), raising the possibility that the binding of centrins to target proteins is regulated by changes in [Ca\(^{2+}\)] in vivo. Taken together, our results indicate that the Kar1p peptide represents a common centrin binding site and that the binding of centrin to Kar1p peptide is regulated by physiologically significant changes in [Ca\(^{2+}\)].

**The Acidic Amino Acids in the Kar1p Peptide Influence the Ca\(^{2+}\) Dependence of the Binding Reaction and Prevent Efficient ScCaM Binding**—A basic amphipathic \(\alpha\)-helix is a structural feature common to many calmodulin binding peptides (52). Analysis of the Kar1p peptide (Fig. 2A) in a helical wheel model (Fig. 2B) revealed that this feature is prevented by three acidic amino acids within the peptide. Consequently, the exchange of the acidic amino acids to alanine results in a peptide with the potential to form a basic amphipathic \(\alpha\)-helix (Fig. 2B). This implies that the negatively charged amino acids of the Kar1p peptide are important for centrin binding and that they may prevent binding of calmodulin.

We first tested whether the acidic amino acids of the Kar1p peptide have a function in the Ca\(^{2+}\)-dependent binding to Cdc31p. Therefore, the affinity of Cdc31p toward modified Kar1p peptides (Fig. 2A) with only one (Kar1-1p to Kar1-3p) or no negatively charged amino acid (Kar1-4p) was determined. At 1.35 μM [Ca\(^{2+}\)], Cdc31p bound with similar high affinity to the mutant as well as the wild-type Kar1p peptides (Fig. 2C, ■). Most interestingly, lowering the [Ca\(^{2+}\)] to 0.038 μM reduced the affinity of Cdc31p for the modified Kar1p peptides with one acidic amino acid 4–6-fold (Fig. 2C; Kar1-1p to Kar1-3p, □), whereas the high affinity binding to Kar1-4p was not affected (Fig. 2C; Kar1-4p, compare ■ with □). We conclude that the negatively charged amino acids within the Kar1p peptide are not required for high affinity Cdc31p binding but influence the Ca\(^{2+}\) dependence of the binding reaction.

Next, we investigated whether ScCaM bound to Kar1p peptide. Analysis of the fluorescence spectra upon the addition of ScCaM to Kar1p peptide (Fig. 1A) revealed that ScCaM bound at 1.35 μM [Ca\(^{2+}\)] with a 170-fold lower affinity to the Kar1p peptide in comparison to Cdc31p (Fig. 2; compare Kar1p peptide with D (ScCaM)). To test whether the acidic amino acids contribute to this ability of the Kar1p peptide to discriminate between centrins and calmodulin, we measured the binding of ScCaM to the modified Kar1p peptides (Kar1-1p to Kar1-4p). The exchange of two acidic amino acids to alanine increased the affinity for ScCaM approximately 5-fold (Fig. 2D; compare Kar1p with Kar1-1p to Kar1-3p), whereas the exchange of the three acidic amino acids increased the affinity 15-fold relative to the wild-type Kar1p peptide (Fig. 2D; compare Kar1p with Kar1-4p). However, ScCaM still bound with a 10-fold lower affinity to Kar1-4p compared to Cdc31p, suggesting that additional features than the acidic amino acids of the Kar1p peptide prevent high affinity ScCaM binding. In conclusion, the acidic amino acids of the Kar1p peptide are important for the specificity of the binding reaction by preventing high affinity binding of ScCaM.

**Centrins Bind to a ScCaM Target**—It is assumed that calmodulin and centrin evolved from a common four-domain precursor (53). How the features of the target binding sites co-evolved with the EF-hand proteins is not well understood. We identified the acidic amino acids within the Kar1p peptide as a feature preventing high affinity binding of ScCaM. We now investigated whether features in a ScCaM binding site prevent binding of centrin.

The binding of centrins to a peptide corresponding to the ScCaM binding site of the SPB component Spc110p (amino acids 897 to 917 of Spc110p; Fig. 3A) was determined. The amino acid leucine at position 10 of the Spc110p peptide was similar to ScCaM at 1.35 μM free [Ca\(^{2+}\)]. The affinity of Cdc31p, SdCen, and HsCen1 binding to the Spc110p peptide was similar to ScCaM at 1.35 μM [Ca\(^{2+}\)] but about 6-fold lower for HsCen2 (Fig. 3B). In addition, centrin-Spc110p peptide interactions were either Ca\(^{2+}\)-independent in the range examined (SdCen) or only 2–3-fold influenced by Ca\(^{2+}\) (Cdc31p, HsCen1, and HsCen2).

**CDC31 Is a Dosage-dependent Suppressor of Yeast Calmod-**
Abstract

Mutations in the EF-Hand region of Cdc31p interfere with calmodulin binding and function. Cdc31p binds to calmodulin with high affinity and links actin to kinetochore microtubules to control cell division. The EF-hand region of Cdc31p is a dosage-dependent suppressor of CMD1, which encodes a Kinesin-like protein that is required for kinetochore microtubule attachment. The mutation of the EF-hands of ScCaM revealed surprising results that ScCaM may fulfill its essential function—in vivo binding to Kar1p peptide and competing with Cdc31p for an EF-hand site on Kar1p. In contrast, the Ca2+-dependent binding of Cdc31p to Kar1p peptide was still 10-fold lower compared to Cdc31p, suggesting that EF-hands II of Cdc31p also contribute to high affinity binding.

Furthermore, we investigated whether structural studies with calmodulin revealed that the first two and the last EF-hands form functional units organized in two domains separated by a flexible linker region (54, 55). Based on this analysis, we constructed Cdc31p-ScCaM and ScCaM-Cdc31p chimeras.

Although cmd1–1 with pRS246 did not grow at 33 °C (Fig. 3C; sector: cmd1–1, pRS426, CDC31 on pRS426 restored growth (sector: cmd1–1, pRS426-CDC31). However, suppression of cmd1–1 was not observed by CDC31 on a low-copy, centromere-based plasmid, indicating that overexpression of CDC31 was required (data not shown). In contrast, the growth defects of cdc31-100 (sector: cdc31-100, pRS426-CMD1), cdc31-101, and cdc31-102 (data not shown) were not suppressed by CMD1. In summary, our suppression analysis is consistent with the in vitro binding studies and suggest that Cdc31p can fulfill ScCaM functions in vivo by binding to ScCaM targets.

The 3rd and 4th EF-Hands of Cdc31p Influence the Binding Specificity—Cdc31p but not ScCaM bound with high affinity to the Kar1p peptide, suggesting that features within the EF-hand proteins influence their binding specificity. These features were investigated by studying the binding of purified Cdc31p-ScCaM hybrid proteins to Kar1p peptide. Structural studies with calmodulin revealed that the first two and the last two EF-hands form functional units organized in two domains separated by a flexible linker region (54, 55). Based on this analysis, we constructed Cdc31p-ScCaM and ScCaM-Cdc31p chimeras.

Cdc31p-ScCaM carries EF-hands I and II from Cdc31p (amino acids 1 to 106) and III and IV from ScCaM (amino acids 95 to 147), whereas ScCaM-Cdc31p contains EF-hands I and II from ScCaM (amino acids 1 to 94) and III and IV from Cdc31p (amino acids 107 to 161). Although Cdc31p-ScCaM bound to the Kar1p peptide with a similar low affinity as ScCaM, ScCaM-Cdc31p interacted with intermediate affinity, dependent on [Ca2+] (Fig. 3C). Immunoblot analysis revealed that overexpression of CDC31 did not influence ScCaM levels in cmd1–1 cells (data not shown).

Prf. 3. Centrin binding to the ScCaM binding site of the SPB component Spc110p. A, modified amino acid sequence of the ScCaM binding site of Spc110p (30). The synthetic Spc110p peptide carried LI0W and C155 exchanges.

Additional Results

In the present study, we have shown that Cdc31p interacts with a ScCaM target (Spc110p) as well as a centrin target (Kar1p peptide), whereas ScCaM hardly binds to the centrin binding site. This raised the possibility that Cdc31p is partly able to perform ScCaM functions in vivo. However, it is less likely that ScCaM substitutes for Cdc31p. We tested this hypothesis in Saccharomyces cerevisiae (strain JGY44–2A; Ref. 66) and cdc31-100 cells (UY27) were transformed with plasmids pRS426, pSM56 (pRS426-CDC31), or pSM529 (pRS426-CMD1). Transformants were tested for growth at 33 °C. Immunoblot analysis revealed that overexpression of CDC31 did not influence ScCaM levels in cmd1–1 cells (data not shown).

As expected, the overexpression of CDC31 restored growth of cdc31-100, cdc31-101, and cdc31-102 (data not shown). Our results clearly demonstrate that the carboxyl-terminal half of Cdc31p contains features that contribute to the high affinity binding to Kar1p peptide and thereby to the binding specificity of this centrin.

Mutations in the 1st or 4th EF-Hand of Cdc31p Interfere with Ca2+-induced Conformational Changes and Impair Cdc31p Function—The mutation of the EF-hands of ScCaM revealed the surprising result that ScCaM may fulfill its essential functions without Ca2+ binding (34). In contrast, the Ca2+-dependent binding of Cdc31p to Kar1p peptide suggests that Cdc31p requires Ca2+ to be functional. To study the role of Ca2+ in Cdc31p functions, the first highly conserved aspartate residue of each of the four potential Ca2+ binding sites of Cdc31p was changed to alanine (Fig. 4A). It has been shown that this type of mutation severely reduces the affinity of Ca2+ for an EF-

| Protein          | 0.1 μM [Ca2+] | 1.35 μM [Ca2+] |
|------------------|---------------|---------------|
| Cdc31p           | 0.59 ± 0.13   | 0.60 ± 0.01   |
| Cdc31p-1.EF (D33A) | 0.54 ± 0.09   | 0.85 ± 0.05   |
| Cdc31p-2.EF (D69A) | 0.61 ± 0.05   | 0.59 ± 0.01   |
| Cdc31p-3.EF (D106A) | 4.75 ± 1.18   | 0.53 ± 0.05   |
| Cdc31p-4.EF (D142A) | ND           | >10           |
| ScCaM           | ND           | >10           |
| Cdc31p-ScCaM     | ND           | 5–10          |
| ScCaM-Cdc31p     | 5–10         | 0.56 ± 0.06   |

*ND, not determined. Shown is the average of three or four independent experiments.

**Cdc31p-ScCaM consists of amino acids 1–106 of Cdc31p and 95–147 of ScCaM.

**ScCaM-Cdc31p consists of amino acids 1–94 of ScCaM and 107–161 of Cdc31p.
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FIG. 4. The 1st and 4th EF-hands of Cdc31p are important for viability. A, amino acid sequence of Cdc31p showing the four potential Ca\^{2+}-binding loops. The first aspartate (encircled) of each of the four EF-hands of Cdc31p was mutated to alanine. The dotted lines in EF-hands II and III indicate that they may not bind Ca\^{2+}. In B, the four mutant cdc31 alleles were tested for functionality. Strain ESM109 (Δcdc31::HIS3 pCH1122-CDC31) was transformed with the control plasmid pRS425 or CDC31 (pSM234), cdc31-1.EF (pSM224), cdc31-2.EF (pSM225), cdc31-3.EF (pSM226), or cdc31-4.EF (pSM237) on the multicity plasmid pRS425. Transformants were tested for growth on 5-FOA plates at 30 °C. Failure to grow on 5-FOA indicates that the plasmid-encoded cdc31 is non-functional. C, expression of mutant cdc31 alleles. ESM56 contains the CDC31 gene under the control of the MET3 promoter. The addition of methionine to MET3-CDC31 cells results in Cdc31p depletion. This property of ESM56 was used to determine whether plasmid-encoded cdc31 mutants were expressed. ESM56 cells with the control plasmid pRS426 (control), pRS425 carrying CDC31 (pSM56), cdc31-1.EF (pSM230), cdc31-2.EF (pSM231), cdc31-3.EF (pSM232), or cdc31-4.EF (pSM233) were incubated in medium with (repression of the MET3 promoter) or without methionine. Cells (80 μg of total protein) were then analyzed by immunoblotting with anti-Cdc31p antibodies. After 6 h in the repressing methionine medium, most of the MET3-CDC31 was degraded (control, Met.: -/+). Therefore, the Cdc31p-signal of MET3-CDC31 cells grown in methionine-medium (Met.: +/+) corresponds to the constitutive expressed, plasmid-encoded CDC31 genes.

hand (34). To test the functionality of the mutant cdc31 alleles, we used strain ESM109, which carries a deletion in the chromosomal CDC31 (Δcdc31::HIS3) and is kept alive by a URA3-based plasmid containing CDC31. Therefore, ESM109 with a LEU2-based control plasmid (pRS425) did not form colonies on 5-FOA plates, which select against the URA3-based CDC31-plasmid (Fig. 4B, control). In contrast, ESM109 carrying a second CDC31 on a LEU2-based plasmid formed colonies on 5-FOA plates (Fig. 4B; CDC31). In this fashion we tested whether the cdc31 alleles were functional. Although cdc31-1.EF was not functional at either 23 °C (data not shown) or 30 °C (Fig. 4B; cdc31-1.EF), cdc31-2.EF and cdc31-3.EF supported growth at 30 °C (Fig. 4B) and 37 °C (data not shown). Finally, cdc31-4.EF formed colonies at 23 °C (data not shown) but not at 30 °C (Fig. 4B; cdc31-4.EF). An explanation for the non-complementation of some of the cdc31 EF-hand mutants could be the degradation of the encoded proteins. We used strain ESM56 carrying wild-type CDC31 under control of the MET3 promoter to establish that all Cdc31p proteins accumulated in similar amounts in yeast cells. Expression of MET3-CDC31 is repressed by methionine, causing a 10-fold depletion of Cdc31p (Fig. 4C, control, compare Met.: - and +) and cell cycle arrest (data not shown). Depletion of Cdc31p then allowed the detection of the mutant Cdc31p proteins expressed from the CDC31 promoter. The steady-state levels of Cdc31p-1.EF to Cdc31p-4.EF were similar or higher in comparison to Cdc31p expressed from the MET3 promoter (Fig. 4C; compare control, Met.: -/+ with cdc31-1.EF to cdc31-4.EF, Met.: +/+), suggesting that the non-functionality of Cdc31p-1.EF and the temperature-sensitive phenotype of Cdc31p-4.EF were not caused by protein instability.

We were interested whether the growth defect of cdc31 mutants resulted from a failure to respond to changes in [Ca\^{2+}]\textsuperscript{vitro}. Ca\^{2+}-induced conformational changes of ScCaM have been determined by CD measurements (56). Similarly, purified Cdc31p showed a characteristic change in its CD spectrum upon Ca\^{2+} binding corresponding to an increase in a-helical content by 12% (Fig. 5A). Most interestingly, while Cdc31p-2.EF and Cdc31p-3.EF responded to Ca\textsuperscript{2+} as wild-type (Fig. 5A), point mutations in the 1st and 4th EF-hand severely affected the Ca\textsuperscript{2+}-induced conformational change (Fig. 5B). Remarkably, the mutant Cdc31p proteins that failed to respond to Ca\textsuperscript{2+} \textsuperscript{vitro} were defective in their function in vivo, which is consistent with the notion that Ca\textsuperscript{2+}-induced conformational changes are essential for Cdc31p function.

Mutations in 3rd and 4th EF-Hands of Cdc31p Affect Binding to Kar1p Peptide—The growth defect of cdc31 mutants may be caused by a change in their affinity toward the target pro-
tein. This should affect the Ca\(^{2+}\)-dependent binding of the mutant Cdc31p proteins to Kar1p peptide. Surprisingly, Cdc31p-1.EF bound to the Kar1p peptide with very similar affinity and Ca\(^{2+}\) dependence as Cdc31p (Table II), although it did not provide Cdc31p function. A similar binding behavior was observed for the functional Cdc31p-2.EF. In contrast, the functional Cdc31p-3.EF bound the Kar1p peptide with a 6–10-fold lower affinity compared to wild-type Cdc31p (Fig. 6; Table II). Finally, a drastic decrease in affinity by a factor of 170 was measured for Cdc31p-4.EF (Table II), indicating that Ca\(^{2+}\) binding to the 4th EF-hand is absolutely essential for high affinity Kar1p binding.

The high Affinity Cdc31p Binding Site in Kar1p Is Not Essential for Growth—We tested whether the Cdc31p-binding site in Kar1p is essential for the viability of yeast cells. The conditional lethal kar-1\(\Delta17\) encodes a mutant Kar1p with a deletion in the central part of Kar1p (14), removing one-half of the Cdc31p binding site. In agreement with this deletion, binding of \[^{35}S\]Cdc31p to filter immobilized, recombinant Kar1-\(\Delta17\)p was completely abolished (Fig. 7A; compare Kar1p-\(\Delta17\) with Kar1p). Since kar-1\(\Delta17\) cells grow at temperatures below 33 °C, we conclude that the characterized high affinity Cdc31p binding site in Kar1p is not essential for growth at lower temperatures.

It has been reported that CDC31 is a dosage-dependent suppressor of kar-1\(\Delta17\) cells (14). Since Kar1-\(\Delta17\)p does not contain the characterized Cdc31p binding site suppression by CDC31 is most likely the result of the interaction of Cdc31p with another protein. The requirements of the interaction of Cdc31p with this additional binding site may be different compared to the Kar1p peptide. In this case, binding of Cdc31p to Kar1p peptide and the ability to suppress the growth defect of kar-1\(\Delta17\) cells should not correlate. To address this possibility, the dosage-dependent suppression of kar-1\(\Delta17\) by CDC31 and the four cdc31 EF-hand mutants was investigated (Fig. 7B). As reported before (14), CDC31 suppressed the growth defect of kar-1\(\Delta17\) cells at 33 °C but not at 37 °C (Fig. 7B). An even better suppressor was cdc31-3.EF, which restored growth at 37 °C. However, cdc31-3.EF was unable to suppress a total deletion of KAR1 (data not shown). Interestingly, the functional cdc31-2.EF that binds to the Kar1p peptide with the same affinity as wild-type Cdc31p did not suppress kar-1\(\Delta17\). These results are consistent with the notion that CDC31 suppresses the growth defect of kar-1\(\Delta17\) by binding to a site with different properties than the Kar1p peptide.

**Discussion**

Centrins Are Ca\(^{2+}\)-regulated Proteins—Our results show that centrins are Ca\(^{2+}\)-regulated proteins. This conclusion is based on the finding that the four centrins from phylogenetically diverse organisms bind to the Kar1p peptide in a Ca\(^{2+}\)-dependent fashion. In addition, point mutations in the first or fourth EF-hands of Cdc31p, which prevent Ca\(^{2+}\)-induced conformational changes, severely impair the function of Cdc31p. We also determined features that influence the Ca\(^{2+}\) dependence of centrin binding. The four centrins bound with different, Ca\(^{2+}\)-dependent affinities to the Kar1p peptide, revealing that the individual structural properties of each centrin influence their binding behavior. In this respect, it is interesting that the two human centrins, despite their high identity, are distinct in their interaction with the Kar1p peptide, indicating that they bind differently to substrates *in vivo*. In addition, we identified the negatively charged amino acids of the Kar1p peptide as important modulators of Ca\(^{2+}\)-dependent centrin-binding. It is easy to imagine that their number and distribution along the centrin binding site will influence the properties of centrin binding to a target protein. Interestingly, the absence of negatively charged amino acids in the Kar1p peptide resulted in high affinity centrin-binding that was not influenced by the examined changes in Ca\(^{2+}\). The reason for this might be quite complex. The acidic amino acids in Kar1p peptide may weaken Cdc31p binding, and this is then overcome by Ca\(^{2+}\) binding to Cdc31p. Substitution of the acidic amino acids in the Kar1p peptide to alanine may allow binding to Cdc31p at lower Ca\(^{2+}\), and this interaction may subsequently increase the affinity for Ca\(^{2+}\), resulting in an apparent Ca\(^{2+}\) independence of the binding reaction. Such an influence of a target peptide on the affinity of Ca\(^{2+}\) binding sites has been reported for calmodulin (57, 58).

Changes in cytosolic Ca\(^{2+}\) may regulate the affinity of centrins to target proteins, thereby influencing their biochemical
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properties. For example, the Ca\(^{2+}\)-regulated interaction of Cdc31p with Kar1p or an additional protein at the SPB may function in the cell cycle-dependent initiation of SPB duplication (13, 16). An increase in Ca\(^{2+}\) influx during the time of SPB duplication has been reported (59), which may result in elevated cytosolic Ca\(^{2+}\) levels. Centrins are also involved in the contraction of centrin-containing filaments in green algae (60, 61). Conformational changes of algal centrin induced by Ca\(^{2+}\) binding may cause the contraction of the centrin filament (24). Centrioles change their architecture in response to the removal of divalent cations (62), a process in which HsCen1 or HsCen2 may be involved.

Binding of Centrins to Target Proteins—Eukaryotic cells contain a number of highly related EF-hands proteins thought to have evolved from a common four-domain precursor protein (53). How the target binding sites of these EF-hand proteins co-evolved with the EF-hand protein to achieve specific binding is not well understood. For centrins, we have shown that the three negatively charged amino acids in the Cdc31p binding site of Kar1p prevented the binding of the related ScCaM. In contrast, the ScCaM binding site of Spc110p was less discriminating. Centrins bound in vitro with a similar affinity to the Scp110p peptide as ScCaM. Furthermore, the dosage-dependent suppression of the conditional lethal calmodulin mutant cmd1–1 by CDC31 suggests that centrins can fulfill calmodulin functions under specific conditions in vitro, probably by binding to calmodulin targets. However, the exclusive association of Cdc31p with the half bridge of the SPB (7) reveals that Cdc31p does not interact with the ScCaM binding site of Spc110p in wild-type cells, which is located near the central plaque (32, 33). Therefore, factors besides the features of the binding site determine the binding specificity of Cdc31p. The conformation of Spc110p in the SPB, Cdc31p-binding proteins or the concentration of Cdc31p relative to ScCaM at the SPB may prevent binding of Cdc31p to Spc110p. These mechanisms may then be overcome by overexpression of CDC31 and by the defect of cmd1–1.

Since ScCaM does not bind with high affinity to the Kar1p peptide but Cdc31p does, Cdc31p must contain features that allow binding. We found that a hybrid protein with the 3rd and 4th EF-hands from Cdc31p and the amino-terminal half from ScCaM behaved more like Cdc31p in its interaction with Kar1p peptide. An attractive hypothesis is that EF-hands three and four of centrins interact first with the binding site, thereby determining the binding specificity. In a second step, which requires EF-hands one and two, centrin may then fold around determining the binding specificity. In a second step, which four of centrins interact first with the binding site, thereby activating cytosolic Ca\(^{2+}\) binding to Kar1p peptide. Cdc31p-4.EF that failed to bind the ScCaM behaving more like Cdc31p in its interaction with Kar1p or an additional protein at the SPB may be involved. Either the 1st EF-hand is not required for function in the cell cycle-dependent initiation of SPB duplication, which starts in G1 phase of the cell cycle (12, 13). The interaction of Cdc31p with the SPB component Kar1p seems to be important in this process (15, 16). However, since a partial deletion of the Cdc31p binding site in Kar1p (kar1-1\(\Delta\)) allowed growth at low temperatures (14), we suggest that the characterized Cdc31p binding is not essential for the viability of yeast cells under some growth conditions. Furthermore, Cdc31p was still associated with the SPB and required for SPB duplication in kar1-1\(\Delta\) cells grown at the permissive temperature (15), indicating that Cdc31p interacts with an additional protein, Xp, at the half bridge.

What is the role of Cdc31p in SPB duplication? We propose a model in which Cdc31p regulates the activity of at least two SPB proteins, Kar1p and Xp. The consequences of Cdc31p interaction with Kar1p and Xp are not understood, but suppression of KAR1 deletion by certain mutations in CDC31 (14) suggest that the function of Kar1p can be bypassed by an altered Cdc31p. Assuming that Xp and Kar1p have overlapping functions in SPB duplication, KAR1 deletion may be suppressed by an altered regulation of Xp through the mutant Cdc31p. Such a bypass model also explains the dosage-dependent suppression of the temperature-dependent growth defect of kar1-1\(\Delta\) cells by CDC31. An increase in [Cdc31p] could allow the interaction with Xp at lower [Ca\(^{2+}\)], thereby activating Xp differently in the cell cycle. The latter would compensate for the failure of Cdc31p to regulate Kar1p-1\(\Delta\). The more efficient suppression of kar1-1\(\Delta\) by cdc31-3.EF compared to CDC31 may be the result of a higher affinity of Cdc31p-3.EF toward Xp.

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