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Protein–Protein Interactions in the Synaptonemal Complex

Madalina Tarsounas,* Ronald E. Pearlman,* Paul J. Gasser,† Min S. Park,† and Peter B. Moens*‡

*Department of Biology, York University, Toronto, Ontario, M3J 1P3 Canada and †Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

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In mammalian systems, an approximately M_r 30,000 Cor1 protein has been identified as a major component of the meiotic prophase chromosome cores, and a M_r 125,000 Syn1 protein is present between homologue cores where they are synapsed and form the synaptonemal complex (SC). Immunolocalization of these proteins during meiosis suggests possible homo- and heterotypic interactions between the two as well as possible interactions with as yet unrecognized proteins. We used the two-hybrid system in the yeast *Saccharomyces cerevisiae* to detect possible protein–protein associations. Segments of hamster Cor1 and Syn1 proteins were tested in various combinations for homo- and heterotypic interactions. In the case of Cor1, homotypic interactions involve regions capable of coiled-coil formation, observation confirmed by in vitro affinity coprecipitation experiments. The two-hybrid assay detects no interaction of Cor1 protein with central and C-terminal fragments of Syn1 protein and no homotypic interactions involving these fragments of Syn1. Hamster Cor1 and Syn1 proteins both associate with the human ubiquitin-conjugating enzyme Hsubc9 as well as with the hamster Ubc9 homologue. The interactions between SC proteins and the Ubc9 protein may be significant for SC disassembly, which coincides with the repulsion of homologs by late prophase I, and also for the termination of sister centromere cohesiveness at anaphase II.

INTRODUCTION

The synaptonemal complex (SC) assembled along the length of paired homologous chromosomes in meiotic prophase cells of most sexually reproducing organisms is a multifunctional, multicomponent organelle (Moses, 1968; Wettstein and Sotelo, 1971; von Wettstein *et al.*, 1984; Moens, 1994). Functional aspects of the SC in recombination, genetic interference, and chromosome segregation have been analyzed in budding yeast (Sym *et al.*, 1993; Sym and Roeder, 1994) and in fission yeast (Kohli and Bahler, 1994). The axial elements, protein cores formed along each pair of sister chromatids that elongate and develop into the lateral elements of the SC, seem to be required for chromosome synapsis and recombination. The SC itself is instrumental in establishing crossover interference, which inhibits or decreases the probability of crossover in regions that have already undergone exchange. Observations in *Schizosaccharomyces pombe* indicate that the absence of SCs is correlated with a lack of crossover interference (Kohli and Bahler, 1994). Similarly, in the *Saccharomyces cerevisiae zip*1 meiotic mutant, the absence of synapsis is associated with a complete elimination of crossover interference (Sym and Roeder, 1994). The SC may also be required for the conversion of crossover events into chiasmata, the cytological manifestation of crossing over, which ensure proper disjunction of homologues at meiosis I (Maguire, 1978; Engebrecht *et al.*, 1990; Rockmill and Roeder, 1990).

A molecular characterization of the SC is critical for a complete understanding of its functions. Initial SDS-PAGE separation of proteins from purified rat SCs and probing with monoclonal antibodies identified at least six proteins ranging from M_r 17,000 to 190,000 (Heyting *et al.*, 1985, 1989). The cDNAs encoding some of these proteins have been identified and characterized

‡ Corresponding author.

in rodents (Meuwissen *et al.*, 1992; Moens *et al.*, 1992; Smith and Benavente, 1992; Dobson *et al.*, 1994; Lammers *et al.*, 1994; Sage *et al.*, 1995), and a human homologue has been identified (Meuwissen *et al.*, 1997). Additional protein components associated with the SC, but not necessarily SC specific, have been detected in the SCs of various vertebrate species. These include a 65-kDa protein (Chen *et al.*, 1992), topoisomerase II (Moens and Earnshaw, 1989), testis-specific heatshock protein 70 (Allen *et al.*, 1996), and a Rad51 homologue (Ashley *et al.*, 1995; Terasawa *et al.*, 1995, Moens *et al.*, 1997).

The M_r 30/33,000 protein (Heyting *et al.*, 1987; Moens *et al.*, 1987) designated Cor1, and also referred to as Scp3, is present in the unpaired axial cores in early and late prophase I, in the lateral elements of the SC at synapsis, and it remains detectable at anaphase II in the space between the separating kinetochores (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995). The 125-kDa Syn1 (also referred to as Scp1) protein is specifically localized to the synapsed regions of the chromosomes (Heyting *et al.*, 1989; Meuwissen *et al.*, 1992; Dobson *et al.*, 1994; Liu *et al.*, 1996; Schmekel *et al.*, 1996). Localization of these antigens in the SC by immunofluorescence and electron microscopy experiments (Dobson *et al.*, 1994) leads to a prediction of possible interactions among and between these proteins. Interactions of Cor1p and Syn1p with other proteins are also likely. We have used the two-hybrid system in the budding yeast *S. cerevisiae* (Fields and Song, 1989) as well as in vitro affinity coprecipitation experiments to investigate potential interactions between known SC proteins and to detect other proteins that interact with SC proteins. The two-hybrid as well as affinity coprecipitation experiments indicate that Cor1p is involved in homotypic interactions through regions capable of coiled-coil formation. No homotypic interactions are detectable when central and carboxyl-terminal polypeptides of Syn1 are tested in the two-hybrid system. Cor1 and Syn1 proteins also interact with the ubiquitin-conjugating enzyme Ubc9, suggesting the possibility of a role for Ubc9 and a ubiquitin-mediated pathway in meiotic prophase, possibly in the termination of recombination and/or in synaptic and sister chromatid cohesion events.

MATERIALS AND METHODS

Strains and Microbiological Methods

Plasmid constructions were transformed into *Escherichia coli* DH5αF′ (Life Technologies, Burlington, Ontario, Canada) either by electroporation using the BRL Cell-Porator and Voltage Booster following protocols supplied by the manufacturer or into freshly prepared cells made competent by $CaCl_2$ treatment (Sambrook *et al.*, 1989). Bacteria were grown on YT plates or liquid medium with the addition of antibiotics as required (Sambrook *et al.*, 1989). *S. cerevisiae* strain HF7c (Feilotter *et al.*, 1994; *MAT***a** *ura*3–52 *his*3–200 *lys*2–801 *ade*2–101 *trp*1–901 *leu*2–3, 112 *gal*4–542, *gal*80–538, *LYS*2::*GAL*1$_{UAS}$-*GAL*1$_{TATA}$-*HIS*3 *URA*3::*GAL*4$_{17\ mers(x3)}$-*CYC*1$_{TATA}$-*lacZ*) was used in the two-hybrid assays. Yeast were grown in YEPD or synthetic defined minimal medium (Sherman *et al.*, 1986) plus required supplements.

Plasmids and Plasmid Constructions

The *GAL*4 DNA-binding domain fusions were constructed in the pGBT9 (Amp^r, *TRP*1) vector (Bartel *et al.*, 1993). N-terminal fusions with the *GAL*4 activation domain were generated in pGAD424 (Amp^r, *LEU*2; Clontech Laboratories, Palo Alto, CA). Both pGBT9 and pGAD424 contain the *ADH*1 promoter, a polylinker, stop codons, and a transcription terminator. pGAD-GH (Hannon *et al.*, 1993), the activation domain vector in which the HeLa cell cDNA library was produced (kindly supplied by Dr. D. Beech, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), is similar to pGAD424 but carries a larger *ADH*1 promoter fragment.

A hamster testis cDNA library was constructed in the activation domain vector pACT2 (Clontech Laboratories) using the λ ZAPII cDNA synthesis kit (Stratagene, La Jolla, CA). mRNA was extracted and purified from the testis of a 26-d-old hamster with magnetic beads (Novagen, Madison, WI) following the manufacturer's instructions. The yield was 0.22 μg mRNA/mg of tissue. The cDNA produced (0.5 μg cDNA/μg of mRNA) was directionally cloned into the *Eco*RI–*Xho*I restriction sites of pACT2 and electroporated into *E. coli* DH5αF′. Restriction analysis of the cDNA from 24 independent bacterial transformants indicated that 85% of transformants contained inserts with an average size of 1.2 kb.

Hamster *COR*1 and *SYN*1 cDNA fragments used in the construction of plasmid substrates for the two-hybrid analyses are shown in Figure 1 and 2. The full-length *COR*1 cDNA was a polymerase chain reaction (PCR) product with terminal *Eco*RI and *Bam*HI restriction sites. A C-terminal (*COR*1^A) and two N-terminal (*COR*1^B and *COR*1^C) deletion derivatives of *COR*1 were generated as follows: an approximately 370-bp *Eco*RI–*Hin*dIII fragment from a full-length *COR*1 construct in pUC19 was subcloned into the corresponding sites of pET29a (Novagen). This was further subcloned as an *Eco*RI–*Xho*I fragment into the *Eco*RI–*Sal*I sites of pGBT9 and pGAD424 to generate *COR*1^A. The *COR*1^B and *COR*1^C fragments were PCR amplified using the primers CORINT1, CORINT2, and CORSTOP indicated in Figure 1 and Table 1, and cloned into the *Eco*RI–*Bam*HI sites of pGBT9 and pGAD424. The *COR*1^D fragment was PCR amplified using the primers CORINT1 and CORDSTOP indicated in Figure 1 and Table 1, and cloned into the *Eco*RI–*Bam*HI sites of pGAD424.

*SYN*1^B encoding the putative leucine zipper domain of Syn1p, and the overlapping upstream fragment *SYN*1^F (Moens *et al.*, 1992; Dobson *et al.*, 1994) were excised from their phagemid vectors as *Eco*RI–*Xho*I fragments and cloned into *Eco*RI–*Sal*I restriction sites of both pGBT9 and pGAD424. *SYN*1^G was generated by PCR amplification using SK and SYNSTOP primers (Table 1) from a cDNA clone containing the C-terminal coding and 3′-untranslated sequences of hamster *SYN*1 (Dobson *et al.*, 1994). The amplified fragment was cloned into the *Eco*RI–*Bam*HI restriction sites of pGBT9 and pGAD424.

PCR amplifications were done using a Perkin-Elmer-Cetus 9600 thermal cycler in 100-μl reactions containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM of each primer, and 10–40 ng (~10^9 template copies) of DNA. Incubation at 94°C, during which 2.5 units of Taq DNA polymerase (Sangon, Toronto, Canada) was added, was followed by 35 cycles of 30 s at 94°C, 1 min at 47°C, and 1 min at 72°C.

The DNA sequence of the 5′ fusion junction of each construct was determined before transformation into yeast to ensure that an in-frame fusion was generated. Sequences of all sequencing primers used are presented in Table 1. For pGAD424 and pACT2, the MATCHMAKER 5′ and 3′ insert screening amplimers (Clontech Laboratories) were used; for pGAD-GH, the SK primer (Stratagene) and the universal M13-20 primer were used; and for pGBT9, a

specific primer (GBT5'PR) was designed. Oligonucleotides were purchased from Dalton Chemical Co. (Toronto, Ontario, Canada).

DNA Sequencing

Nucleotide sequences were determined using the dideoxy chain terminator method (Sanger *et al.*, 1977). Sequence analysis was performed at the Core Molecular Biology Facility (York University) using Taq cycle sequencing reactions with dye-labeled dideoxy terminators analyzed with an ABI/Perkin-Elmer-Cetus 373A instrument.

Transformation and cDNA Library Screening

Transformation of *S. cerevisiae* used the one-step protocol as described by Chen *et al.* (1992). Transformation of the HeLa cell and hamster testis cDNA libraries into HF7c containing the full-length *COR*1 cDNA in pGBT9 followed a protocol developed by Ito *et al.* (1983) and modified by Schiestl and Gietz (1989), Hill *et al.* (1991), and Gietz *et al.* (1992) to increase the transformation efficiency. For the HeLa cell library, transformation efficiency was estimated from the number of Leu^+ Trp^+ transformants from a small sample of the transformation mixture to be 1.5×10^4 transformants per μg of supercoiled DNA. One hundred micrograms of the primary unamplified hamster testis cDNA library yielded a transformation frequency of 1.0×10^4 transformants per μg of supercoiled DNA, estimated as described above.

Transformants from the library screens were selected for a Trp^+, Leu^+, His^+ phenotype and were analyzed for β-galactosidase activity using a filter assay (Miller, 1972). Colonies were transferred onto nitrocellulose filters, which were frozen in liquid nitrogen and incubated at 30°C for minutes to overnight on filter paper soaked with 1.5 ml of Z-buffer (16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot 7H_2O$, 2.7 ml β-mercaptoethanol, all per liter, final pH, 7.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

The *GAL*4 activation domain and DNA-binding domain plasmids were isolated from His^+ $LacZ^+$ yeast by growth for at least 20 generations in synthetic defined minimal medium containing adenine, lysine, histidine, and either tryptophan or leucine. Total yeast DNA was extracted from Trp^-, Leu^+, Trp^+, or Leu^- colonies and transformed into *E. coli* DH5αF′ by electroporation. Plasmid DNA was isolated from 1.5-ml cultures of transformants by the alkaline lysis method (Birnboim and Doly, 1979; Sambrook *et al.*, 1989), and the plasmids were analyzed by restriction endonuclease mapping and DNA sequencing. The isolated plasmids were also retransformed into HF7c in appropriate combinations to verify phenotypes.

Southern Blotting

Southern blot analysis was performed on *GAL*4 activation domain plasmid DNA recovered from Trp^+, Leu^+, His^+, $LacZ^+$ colonies obtained from screening each of the two cDNA libraries. Equal amounts of plasmid DNA digested with restriction enzymes *Eco*RI and *Xho*I were loaded in each lane. DNA was electrophoresed through 0.8% agarose gels, transferred to GeneScreen+ nylon membranes (DuPont New England Nuclear, Boston, MA) and hybridized to a probe labeled with [^{32}P]dATP by random priming (Sambrook *et al.*, 1989).

Colony Hybridization

Total DNA was extracted from yeast transformants obtained in the cDNA library screens, which had subsequently been cured of the DNA-binding domain plasmid, and electroporated into *E. coli*. Bacterial transformants containing the activation domain plasmid were spotted onto a YT plate containing ampicillin (50 μg/ml), grown at 37°C overnight, and transferred to a GeneScreen+ nylon membrane (DuPont New England Nuclear). The filter was treated and probed according to the method of Grunstein and Hogness as described in Sambrook *et al.* (1989).

Expression and Purification of the Cor1 Protein and Its Deletion Derivatives

The coding region of *COR*1 (Dobson *et al.*, 1994) was cloned as a *Bam*HI–*Xho*I fragment into pGEXT (Pharmacia) and as a *Nco*I–*Xho*I fragment into pET32b (Novagen) expression vectors to produce in-frame glutathione *S*-transferase (GST) and thioredoxin fusion proteins, respectively. The cDNAs encoding the N- and C-terminal *COR*1 deletion derivatives were obtained by PCR amplification (see Table 1 for primer sequences) from a template containing full-length *COR*1 cDNA. These were subcloned as *Nco*I–*Xho*I fragments into pET32b, producing in-frame soluble fusions with thioredoxin (LaVallie *et al.*, 1993). *E. coli* BL21(DE3) (Studier *et al.*, 1990) harboring the pGEXT or pET32b constructs was grown to OD_{600} = 0.6 in Celltone medium (Martek) supplemented with ampicillin to 100 μg/ml. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and the culture was incubated overnight at 16°C. Bacteria were harvested from 1-l cultures by centrifugation and resuspended in 50-ml lysis buffer (0.1 M Tris-HCl, pH 8.0, 2 mM EDTA, 0.3 mg/ml lysozyme). After a 10-min incubation, the soluble fraction containing the fusion proteins was collected by centrifugation at 100,000 × *g* for 1 h. Protease inhibitors (phenylmethanesulfonate, 1 mM; pepstatin, chymostatin, leupeptin, 10 mg/ml) were added to the lysate. The various Cor1 fusion proteins were purified from the crude soluble extracts by glutathione (Smith and Johnson, 1988) or metal chelate affinity chromatography.

Affinity Purification Analysis

Purified GST-Cor1 fusion protein (20 μg) was incubated separately with each of the full-length or mutant Cor1-thioredoxin fusion proteins (500 μg) for 1 h at 4°C with gentle rocking. Glutathione-agarose beads were added, and incubation with gentle rocking was continued for 2 h to overnight. The beads were washed five times with wash buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.1% Triton X-100) and resuspended in 50 μl 2× SDS sample buffer (Laemmli, 1970). Samples were heated at 100°C for 5 min and analyzed by SDS-PAGE (Laemmli, 1970).

Production of anti-Hsubc9 Antibodies and Western Blot Analysis

A DNA fragment corresponding to the open reading frame of human Ubc9 (Kovalenko *et al.*, 1996) was amplified by PCR using the UBC9-F, UBC9-R pair of primers (Table 1) and cloned into the *Nco*I–*Xho*I sites of pET28b (Novagen). The Hsubc9 protein was expressed as described above and purified using a nichel chelate column according to the manufacturer's protocol (Novagen). Mouse polyclonal sera were developed by immunizing BALB/c mice with the recombinant Hsubc9 protein, and the immunoglobulin G fraction was purified using a Sepharose-protein A column (Pharmacia Biotech, Quebec, Canada).

The pGEXT/*COR*1 and pET28/*Hsubc*9 constructs were cotransformed into BL21(DE3) electrocompetent bacteria. The two fusion proteins expressed as above were coprecipitated with glutathione-agarose beads, electroblotted to nitrocellulose membranes, and detected with a mixture of anti-Hsubc9/anti-Cor1 (Dobson *et al.*, 1994) antibodies, followed by the electrochemiluminescence procedure (Amersham, Oakville, Ontario, Canada).

RESULTS

Coiled-Coil Regions Are Important in Cor–Cor Homotypic Interactions

We used the *GAL*4 two-hybrid system (Fields and Song, 1989) to identify protein–protein interactions

involving the Cor1 protein. We fused the coding region of hamster *COR1* cDNA to the N-terminus of the *GAL4* DNA-binding domain in the vector pGBT9, and also to the N-terminus of the *GAL4* activation domain in the vector pGAD424. Interactions were assessed by cotransformation into *S. cerevisiae* HF7c. Activation of the *HIS3* and *lacZ* reporter genes (Table 2) indicates homotypic interactions of the Cor1 protein. We have further investigated this interaction by identifying the regions of Cor1p required for the interaction to occur. There are four regions in the Cor1 protein capable of forming coiled-coil interactions (Figure 1), as predicted by the COILS version 2.1 algorithm (Berger *et al.*, 1995). To asses which of these regions are important for the homotypic interactions observed, we generated one C-terminal ($Cor1^A$) and two N-terminal ($Cor1^B$ and $Cor1^C$) deletion derivatives, and an internal ($Cor1^D$) fragment of the Cor1 protein (Figure 1). *GAL4* activation domain and DNA-binding domain fusion plasmids were transformed into *S. cerevisiae* HF7c in the combinations shown in Table 2 and cotransformants (Trp^+, Leu^+) were selected and screened for activation of the *HIS3* and *lacZ* reporter genes. Interaction of $Cor1^B$ with Cor1, of $Cor1^B$ with itself, of $Cor1^D$ with Cor1 and with $Cor1^B$, and the lack of interactions involving $Cor1^A$ or $Cor1^C$, identifies $Cor1^D$ as the minimal region containing sequences required for Cor1 homotypic interactions. $Cor1^A$ does not contain any potential coiled-coil forming region, whereas $Cor1^D$ harbors two such domains (Figure 1). Regions capable of coiled-coil formation, therefore, seem critical for Cor1p homotypic interactions to occur. None of the pGBT9 or pGAD424 constructs activated the reporter genes when transformed alone into the yeast host strain. To confirm that the interactions described in Table 2 result from both the DNA-activation domain and the DNA-binding domain fusion plasmids being present in a yeast cell, we have rescued the two plasmids from Trp^+, Leu^+, His^+, $LacZ^+$ yeast colonies. In all cases, restriction enzyme analysis demonstrates recovery of the plasmids initially introduced into yeast.

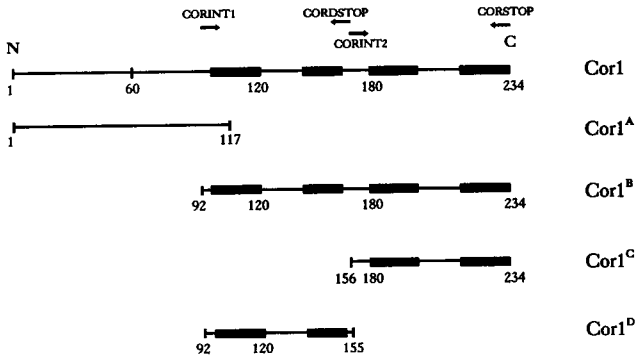

Figure 1. Schematic diagram of the full-length hamster Cor1 protein (234 amino acids), as well as the N- and C-terminal deletion derivatives used in the two-hybrid analysis. Numbers correspond to the amino acid sequence as presented in Dobson *et al.* (1994). The homologous rat protein is predicted to be 257 amino acids long (Lammers *et al.*, 1994). $COR1^A$ was produced as a restriction fragment and $COR1^B$, $COR1^C$ and $COR1^D$ by PCR amplification using primers designated above the diagram. Primer sequences are presented in Table 1. The shaded boxes mark the potential coiled-coil forming domains.

Further evidence supporting the existence of Cor1 homotypic interaction was obtained from a two-hybrid screen of a hamster testis cDNA library using a full-length *COR1* cDNA/pGBT9 construct as bait. This screen yielded 1×10^6 independent transformants, of which 48 were His^+ and $LacZ^+$. The activation domain plasmids (Leu^+) were transformed into *E. coli* and analyzed by colony filter hybridization with a *COR1* probe. Activation domain plasmids from 36 of the 48 yeast transformants expressing the reporter genes hybridized with the *COR1* probe. cDNA from four of these plasmids was sequenced. All four were partial cDNAs encoding Cor1 polypeptides fused at amino acid 93 to the *GAL4* activation domain of pACT2 and extending past the translation stop codon to a poly(A) tract.

The self-association of Cor1 observed in vivo was confirmed in in vitro experiments. We expressed full-length and mutant Cor1 as soluble fusions with GST and thioredoxin, respectively (Figure 3A). The fusion proteins were mixed and subjected to affinity purification with glutathione-agarose beads. The results of SDS-PAGE analysis indicate a homotypic interaction with full-length Cor1p (Figure 3B, lane 1). When the purified full-length GST-Cor1 fusion protein was mixed in separate reactions with each of the thioredoxin-Cor1 full-length and deletion fusions (Figure 3B), only full-length Cor1 (lane 1), $Cor1^B$ (lane 3), and $Cor1^D$ (lane 5) interact with full-length Cor1p. No interaction is observed in this in vitro assay with $Cor1^A$ (lane 3) or $Cor1^C$ (lane 5), or when GST alone is used along with each of the thioredoxin fusions described (data not shown). This observation supports the in vivo results and confirms the region required for homotypic interaction as the 63 amino acid $Cor1^D$ fragment (Figure 3C).

The Ubiquitin-conjugating Enzyme Ubc9 Interacts with Cor1p in the Two-Hybrid Assay

Proteins produced in somatic cells, as well as proteins expressed only in meiotic cells, are present in the SC. To identify proteins that might interact with a well characterized SC protein, we screened both a HeLa cell cDNA library and a hamster testis cDNA library in *GAL4* activation domain plasmids in the two-hybrid assay with the full-length Cor1 protein *GAL4* DNA-binding domain construct in pGBT9. Of 2.5×10^6 independent transformants from a screen of a cDNA library from exponen-

tially growing HeLa cells, 140 Trp^+, Leu^+, His^+ transformants were obtained, and 130 of these were also $LacZ^+$. Of 12 Trp^+, Leu^+, His^+, $LacZ^+$ transformants initially analyzed, sequence analysis and Southern blotting of the *GAL4* activation domain plasmids rescued from these colonies indicated that six of these cDNAs encoded the human ubiquitin-conjugating enzyme Hsubc9. Subsequently, the Leu^+ activation domain plasmids were rescued from an additional 48 Trp^+, Leu^+, His^+, $LacZ^+$ yeast colonies. These colonies were hybridized with a radioactively labeled probe derived from the *Hsubc9* cDNA. Twenty-one of the 48 *E. coli* colonies hybridized to the *Hsubc9* probe. Of the 60 HeLa cell cDNAs from colonies identified in the two-hybrid screen as interacting with Cor1 protein and subsequently characterized by DNA sequence analysis, Southern blotting, or colony hybridization, 44% express the Hsubc9 protein. The remaining plasmids isolated from this screen have not as yet been characterized.

Data demonstrating the specificity of the interaction between Cor1p and Hsubc9p are presented in Table 3. A pGAD-GH plasmid expressing *Hsubc9* cDNA was transformed into yeast containing the pGBT9 vector with no insert and with pGBT9 carrying the cDNAs for *SNF*I or lamin from *S. cerevisiae*. No expression of the *HIS3* or *lacZ* reporter genes was detected in any of these experiments.

Colony hybridization with *Hsubc9* probe of *E. coli* containing the activation domain plasmid rescued from 48 His^+ $lacZ^+$ transformants from the two-hybrid screen with hamster testis cDNA and *COR1*/pGBT9 bait identified four hybridizing colonies. The cDNA inserts from these colonies were sequenced and identified as hamster Ubc9 homologues (accession number AF004814) based on 96% sequence identity over 450 bp with the mouse Ubc9 cDNA sequence (accession number X82538).

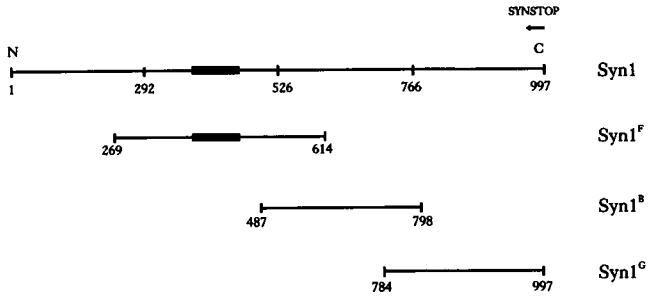

Figure 2. Schematic diagram of the full-length Syn1 protein (997 amino acids based on the murine sequence; Sage *et al.*, 1995) and the three fragments used in two-hybrid analyses. $Syn1^B$, $Syn1^F$ and $Syn1^G$ are encoded by overlapping cDNA clones, the isolation and characterization of which has been described (Dobson *et al.*, 1994). The shaded box identifies the potential leucine zipper motif.

To determine whether there is a direct physical interaction between Cor1 and Hsubc9, we produced recombinant Hsubc9, expressed in *E. coli* as a soluble fusion with a $(His)_6$ tag. The protein was purified to almost complete homogeneity using a nichel chelate affinity column (Figure 4A, lane 2) and gave a positive reaction when probed with the antiHsubc9 polyclonal antibody (Figure 4B, lane 2). Similarily, the GST-Cor1 fusion was purified using glutathione-agarose beads (Figure 4A, lane 1) and tested in a immune reaction with a mouse anti-Cor1 polyclonal antibody (Dobson *et al.*, 1994; Figure 4B, lane 1). The *E. coli* BL21(DE3) strain was cotransformed with the pGEXT/*COR1* and

Table 1. Sequences of primers[a]

Primer designation	DNA sequence
CORINT1 (*Eco*RI)	5′-GGC GAA TTC ATG TAT ACC AAA GCT-3′
CORINT2 (*Eco*RI)	5′-GGC GAA TTC CAG AAG ATT TTT CAG-3′
CORDSTOP (*Bam*HI)	5′-GCG GGA TCC TTG TCG AAA AAG-3′
CORSTOP (*Bam*HI)	5′-GCG GGA TCC TCA GAA TAA CAT GGA-3′
COR1-1F (*Bam*HI)	5′-CCG CGT GGA TCC ATG GTG CCT GGT GGA AGA AAG-3′
COR1-1F (*Nco*I)	5′-AGA TAT ACC ATG GTG CCT GGT GGA AGA AAG-3′
COR1-276F (*Nco*I)	5′-AGA TAT ACC ATG GAA ATG TAT ACC AAA GCT-3′
COR1-486F (*Nco*I)	5′-AGA TAT ACC ATG GCG CAG AAG ATT TTT CAG CAG-3′
COR1-465R (*Xho*I)	5′-GAG GAG CTC GAG TTG TTG TCG AAA AAG ATT-3′
COR1-702R (*Xho*I)	5′-GAG GAG CTC GAG GAA TAA CAT GGA TTG AAG-3′
GBT5′PR	5′-GAC TGA TAT GCC TCT AAC-3′
M13-20	5′-GTA AAA CGA CGG CCA GT-3′
MATCHMAKER 5′	5′-TAC CAC TAC AAT GGA TG-3′
MATCHMAKER 3′	5′-GTT GAA GTG AAC TTG CGG GG-3′
UBC9-F (*Nco*I)	5′-AGA TAT ACC ATG TCG GGG ATC GCC CTC AGC-3′
UBC9-R (*Xho*I)	5′-GAG GAG CTC GTC ATT GTA TGG AGC AAC TTT-3′
SK PRIMER	5′-CGC TCT AGA ACT AGT GGA TC-3′
SYNSTOP (*Bam*HI)	5′-GCG GGA TCC AGC AAA TAA CTT TTC-3′

[a] Sequences of the primers used in PCR amplifications of *SYN1*, *COR1*, and *UBC9* cDNA fragments and for DNA sequence analysis.

Table 2. Results obtained in two-hybrid assays[a]

		pGBT9			
		Cor1 (234 aa)	Cor1^A (117 aa)	Cor1^B (142 aa)	Cor1^C (78 aa)
pGAD424	Cor1 (234 aa)	His$^+$LacZ$^+$	His$^-$	His$^+$LacZ$^+$	His$^-$
	Cor1^A (117 aa)	His$^-$	His$^-$	His$^-$	His$^-$
	Cor1^B (142 aa)	His$^+$LacZ$^+$	His$^-$	His$^+$LacZ$^+$	His$^-$
	Cor1^C (78 aa)	His$^-$	His$^-$	His$^-$	His$^-$
	Cor1^D (63 aa)	His$^+$LacZ$^+$	His$^-$	His$^+$LacZ$^+$	His$^-$

[a] Phenotype of reporter genes in two-hybrid assays with the *GAL4* DNA-binding domain plasmid (pGBT9) and the *GAL4* activation domain plasmid (pGAD424) containing full-length and various fragments of the hamster *COR1* cDNA.

pET28/*Hsubc9* constructs, and clones resistant to both ampicillin and kanamycin were selected, grown, and induced with isopropyl-1-thio-β-D-galactopyranoside. The total lysate was incubated with glutathione-agarose beads. Coprecipitated proteins are shown in Figure 4C, lane 1, along with the GST control after glutathione-agarose precipitation in the presence of Hsubc9-$(His)_6$ (Figure 4C, lane 2).

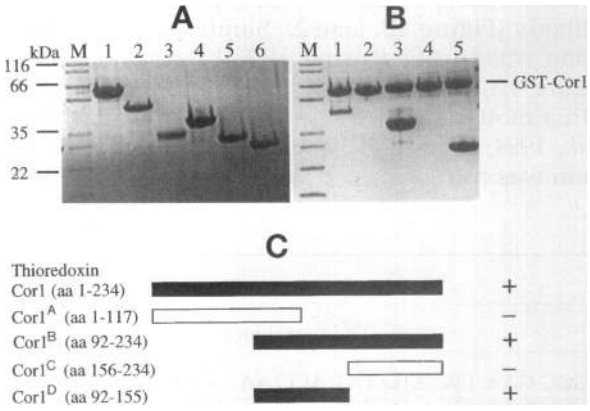

Figure 3. In vitro mapping of Cor1 domains required for homotypic interactions. (A) Coomassie blue-stained SDS-polyacrylamide gel of purified Cor1 fusion proteins. M, molecular weight markers; lane 1, GST-Cor1 (amino acids [aa] 1–234); lane 2, thioredoxin-Cor1 (aa 1–234); lane 3, thioredoxin-Cor1^A (aa 1–117); lane 4, thioredoxin-Cor1^B (aa 92–234); lane 5, thioredoxin-Cor1^C (aa 156–234); lane 6, thioredoxin-Cor1^D (aa 92–155). (B) Coomassie blue-stained SDS-polyacrylamide gel of glutathione-agarose affinity-purified proteins. The purified GST-Cor1 (aa 1–234) fusion protein was mixed with various purified thioredoxin-Cor1 fusion proteins and affinity purified with glutathione-agarose prior to electrophoresis through an SDS-polyacrylamide gel. M, molecular weight markers; lane 1, thioredoxin-Cor1 (aa 1–234); lane 2, thioredoxin-Cor1^A (aa 1–117); lane 3, thioredoxin-Cor1^B (aa 92–234); lane 4, thioredoxin-Cor1^C (aa 156–234); lane 5, thioredoxin-Cor1^D (aa 92–155). (C) Schematic representation of the interactions between thioredoxin-Cor1 deletion mutants with the full-length GST-Cor1 protein. + and − indicate interaction or lack of interaction, respectively.

Protein–Protein Interactions Involving Central and C-Terminal Regions of Syn1

We have examined the ability of hamster Syn1 polypeptides to engage in homotypic interactions as well as to associate with Cor1 and Hsubc9 proteins in vivo. The regions of Syn1p used to study these interactions, Syn1^B, Syn1^F, and Syn1^G, are shown in Figure 2. Syn1^F contains a potential "leucine zipper" motif, which might provide a dimerization interface of Syn1p. Syn1^G is the C-terminus of the Syn1 protein. The data indicate that none of the three fragments tested is involved in homotypic interactions. In addition, no interaction between the full-length Cor1 protein and any of the three Syn1 regions is detected. The data presented in Table 3 also indicate that Hsubc9 undergoes protein–protein interaction with Syn1^F but not with Syn1^B or Syn1^G. The N-terminus (amino acids 2–101) of the murine Syn1 homologue has been shown to undergo homotypic interaction, but no interactions were observed with the murine Cor1 homologue in similar two-hybrid assays (Liu *et al.*, 1996). Because the hamster *SYN1* cDNA lacks the fragment encoding the 269 N-terminal amino acids (Dobson *et al.*, 1994), we were not able to assess the ability of this region of the hamster protein to participate in protein–protein interactions.

DISCUSSION

Using the two-hybrid system and molecular manipulation of the *COR1* cDNA, we have shown that the Cor1 protein undergoes homotypic interactions in vivo. In addition, we have demonstrated that the potential coiled-coil forming regions of Cor1p are critical interfaces for these interactions. Potential for coiled-coil formation may, however, not be the only determinant for homotypic interactions among Cor1 polypeptides to be detected in the two-hybrid assay. This is evidenced by the observation that no interaction is detected between Cor1^C and Cor1, or between Cor1^C and Cor1^C, despite the prediction that *COR1*^{C} encodes two regions with potential coiled-coil forming capability. This lack of interaction with the Cor1^C polypeptide may, however, be explained by its small size (78 amino acids), which could prevent the folding required for interaction to be demonstrated in this assay.

Previous studies using immunofluorescence microscopy have localized the Cor1 antigen along the length of the homologous chromosome cores in early prophase I, in the axial elements of the SC at synapsis,

at discrete spots along the separating cores at metaphase I, and between the kinetochores of segregating sister chromatids at meiotic anaphase II (Dobson *et al.*, 1994). These data suggest that the Cor1 protein may be involved not only in SC assembly but also in sister chromatid cohesiveness. The evidence for in vivo Cor1 homotypic interactions offers some insight into molecular details determining the localization of Cor1p on chromosomes. The data do not, however, indicate the orientation of the Cor1p molecule with respect to the central element of the SC. Nevertheless, homotypic interactions of Cor1 are consistent with the cytological data indicating the presence of the Cor1 antigen in the form of small aggregates detached from the SC at pachytene, and also in the form of short filaments located exclusively between the sister kinetochores at anaphase II (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995).

The exact role that Cor1 homotypic interactions play in determining the structure and the function of the SC remains to be determined. Some clues to function might be obtained by comparisons with other systems. Cor1p shares some features with the previously characterized product of the *Drosophila mei-S332* gene (Kerrebrock *et al.*, 1992). Both are meiosis-specific proteins present at the centromeric region of meiotic chromosomes until anaphase II, suggesting a possible role in sister centromere cohesion during meiosis II. A region at the N-terminus of mei-S332 protein has the potential for coiled-coil formation, allowing homotypic interaction, which might be important for normal meiotic function. This suggestion is supported by the characterization of two nonfunctional *mei-S332* alleles having missense mutations in this N-terminal domain (Kerrebrock *et al.*, 1995). It is possible that analogous Cor1 homotypic interactions may be important for Cor1 function in the SC.

In a structure such as the SC, some of the structural and functional complexity could be mediated by protein–protein interaction. Studies on the organization of Syn1 protein molecules within rat SCs (Schmekel *et al.*, 1996) and immunogold localization of Syn1p in hamster SCs (Dobson *et al.*, 1994) suggest a model in which Syn1p homotypic interactions and Syn1p–Cor1p interactions might be predicted, the former within transverse filaments, the structural units between and perpendicular to the paired chromosome cores in which the synaptic proteins assemble, and the latter toward the outside of the SC. We have not observed such interactions using two-hybrid assays in *S. cerevisiae*. This is particularly striking because all of the polypeptides tested are predicted to be able to form coiled-coil structures, suggesting a potential for protein–protein interaction. It is possible that even where immunoreactive epitopes of Cor1p and Syn1p overlap, such as toward the outside of the SC (Dobson *et al.*, 1994), either the proteins do not interact directly and other proteins mediate any interaction, or conditions of our two-hybrid analysis are not appropriate to detect direct interaction. The latter seems unlikely because we have detected other heterotypic interactions involving these proteins in vivo.

Although *COR1* is expressed exclusively in the testis (Offenberg *et al.*, 1991; Lammers *et al.*, 1994; Kara-

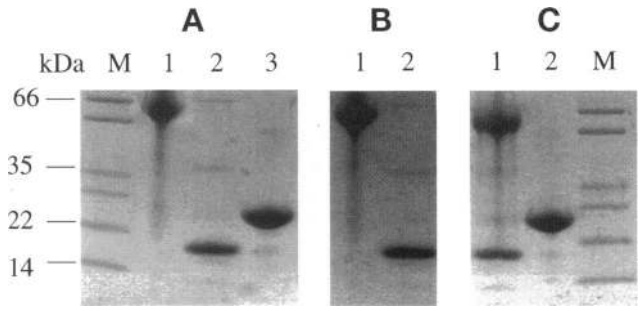

Figure 4. Coprecipitation experiments confirming a direct interaction between Cor1 and Hsubc9. (A) Coomassie blue-stained SDS-polyacrylamide gel of purified fusion proteins. M, molecular weight markers; lane 1, GST-Cor1 fusion; lane 2, Hsubc9-$(His)_6$; lane 3, GST. (B) Immunoblot of purified fusion proteins reacted with a mixture of mouse polyclonal antiHsubc9 (described here) and antiCor1 (Dobson *et al.*, 1994) antibodies. Lane 1, GST-Cor1 fusion; lane 2, Hsubc9-$(His)_6$. C, Coomassie Blue stained SDS-polyacrylamide gel of glutathione-agarose precipitated proteins. lane 1, GST-Cor1/Hsubc9-$(His)_6$ complex was collected by centrifugation after reaction with agarose-glutathione beads, and analyzed by SDS-PAGE; lane 2, GST does not form a complex with Hsubc9-$(His)_6$ treated as for lane 1; M, molecular weight markers.

Table 3. Results obtained in two-hybrid assays[a]

		pGBT9							
		Cor1	Syn1^F	Syn1^B	Syn1^G	Hsubc9	SNFII	Lamin	No insert
pGAD-GH	Hsubc9	His^+ $LacZ^+$	His^+ $LacZ^+$	His^-	His^-	His^+ $LacZ^+$	His^-	His^-	His^-

[a] Phenotype of reporter genes in two-hybrid assays with the *GAL4* activation domain plasmid (pGAD424) containing the human *UBC9* cDNA and the *GAL4* DNA-binding domain plasmid (pGBT9) containing cDNAs encoding various proteins.

iskakis, 1997) and Corlp localizes exclusively to male meiotic structures, other SC proteins have been identified that are not meiosis specific. To identify proteins that might interact with Corlp, both a HeLa and a hamster testis cDNA library were screened with *COR1* as bait in a two-hybrid assay in *S. cerevisiae* (Fields and Song, 1989). As expected, Corl homotypic interactions occurred only with testis cDNAs. Although no full-length *COR1* cDNAs were characterized from this screen, all of the cDNAs analyzed were equivalent to the $Corl^B$ fragment, confirming the in vivo and in vitro results on Corlp homotypic interactions. The human ubiquitin-conjugating enzyme Hsubc9 was identified in the screen with HeLa cell cDNA. Interaction of Corlp with Ubc9 was confirmed by the identification of a hamster Ubc9 homologue in the two-hybrid screen with hamster testis cDNA and also by in vitro coprecipitation experiments. We also demonstrated the interaction between Hsubc9 and the $Syn1^F$ fragment using the two-hybrid assay. Analysis of the Hsubc9 amino acid sequence (Berger *et al.*, 1995) does not predict a potential for coiled-coil formation, which suggests differences in mechanisms of recognition and interaction between Corlp and Ubc9 compared with those for Corlp homotypic interactions.

The ubiquitin-conjugating enzyme is part of a nonlysosomal proteolytic pathway in which ubiquitination targets proteins for degradation by a 26S proteasome complex (Ciechanover, 1994). Generally, activated ubiquitin is transferred by the ubiquitin-conjugating enzyme E2 or ubc to the substrate, which is bound to a ubiquitin-protein ligase E3. Some instances, however, have been described in which Ubc catalyzes the transfer of activated ubiquitin directly to the substrate to which the Ubc binds. The Corlp or $Syn1^F$ interaction with Hsubc9 could occur in this way. It has been proposed that the ubiquitin-proteasome pathway may represent a mechanism for cell cycle regulation, whereby degradation of proteins may induce termination of one phase and transition to the next (Ciechanover, 1994). In *Schizosaccharomyces pombe* (Funabiki *et al.*, 1996) and *Drosophila* (Stratmann and Lehner, 1996), the destruction of proteins involved in sister chromatid cohesion is required for separation of centromeres at the metaphase/anaphase transition of mitosis. A specific interaction between Hsubc9p and all three subunits of the *S. cerevisiae* centromere DNA-binding core complex CFB3 has been demonstrated (Jiang and Koltin, 1996). This is consistent with a role for the ubiquitination process in centromere function linked to chromosome segregation in mitosis. In general, ubiquitin-mediated proteolysis has been shown to be required for sister chromatid separation at anaphase (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995). This may involve a protein attaching the sister chromatids, which is destroyed by proteolysis at the anaphase transition (Holloway *et al.*, 1993). In meiosis in rodents, Corlp and Synlp are specifically localized, and their appearance and disappearance are precisely temporally controlled (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995; Schmekel *et al.*, 1996). Disappearance coincides with the transition from one meiotic phase to another. The disappearance of Syn1 starting at the end of pachytene correlates with SC disassembly and bivalent separation at diplotene, and degradation of Corlp, which is present until anaphase II, correlates with sister centromere segregation at the onset of anaphase II. Kovalenko *et al.* (1996) have recently demonstrated a Rad51 interaction with Hsubc9 using a two-hybrid screen. Rad51/RecA foci localize to chromosome cores in mammalian spermatocytes and oocytes prior to synapsis and are lost after synapsis (Moens *et al.*, 1997), suggesting the possibility of a ubiquitin-mediated, temporally specific breakdown of proteins involved in homology search at meiosis. Similarly, ubiquitination dependent on the binding of Ubc9 to SC proteins such as Cor1 and Syn1 could target these proteins for cell cycle-specific degradation. This could represent a mechanism for regulation of events in meiosis analogous to that described for mitosis (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995; Willems *et al.*, 1996).

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