Interactional Cloning of the 84-kDa Major Outer Dense Fiber Protein Odf84

LEUCINE ZIPPERS MEDIATE ASSOCIATIONS OF Odf84 AND Odf27

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The tails of spermatids contain unique cytoskeletal structures, not present in unicellular flagella and cilia, that may play an important but yet undefined role in sperm integrity, motility, and durability (1–6). These structures, the outer dense fibers (ODF),1 which surround the axoneme, and the fibrous sheath (FS), which surrounds the ODF, contain multiple proteins and are highly insoluble (1, 4, 7–13). The ODF and FS proteins appear to be produced in elongating spermatids (14) and are cross-linked via disulfide bonds (7, 9, 15). Previous studies had shown that ODF morphogenesis starts at the proximal portion of the axoneme and proceeds distally, whereas FS morphogenesis begins at the distal end of the developing sperm tails and proceeds proximally (14, 16, 17). ODF, which are present in the sperm tail midpiece and principal piece, are not homogeneous but contain a thin cortex around a central medulla (4, 11).

The polypeptide composition of ODF has been investigated for a number of species (11–13, 18, 19). Rat ODF contain six major proteins (84, 71, 40, 27, 20, and 14 kDa) as well as a number of less abundant peptides (11–13). Many of these proteins are phosphorylated on serine residues, but a role for this in tail function or in ODF morphogenesis has not been established (12, 20). Antibodies raised against these peptides provided a detailed description of their synthesis in elongating spermatids and also indicated that several ODF and FS proteins appear to be immunologically related (13, 14, 21).

The study of mammalian sperm tail outer dense fibers (ODF), a structure of unknown function, is hampered by the insoluble nature of ODF proteins and the availability of only one cloned component, Odf27. We report here the first use of the Odf27 leucine zipper as bait in a yeast two-hybrid screen to isolate a novel testis-specific protein whose interaction with Odf27 depends critically on the Odf27 leucine zipper. We find that the novel gene, 111-450, encodes a product that localizes to ODF as determined by fluorescence microscopy and immunoelectron microscopy and that the gene 111-450 product is identical to the major ODF protein, Odf84. Interestingly, Odf84 contains two C-terminal leucine zippers, and we demonstrate that all leucine residues in the upstream leucine zipper are required for interaction with Odf27, demonstrating the strategic validity of our approach. The use of the yeast screening approach to isolate leucine zipper containing proteins should be useful in other systems, and our findings have implications for ODF structural models.

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ODF Sperm Proteins Interact via Leucine Zippers

By immunoelectron microscopy we found that this gene, 111-450, encodes a sperm tail-specific protein that localizes with Odf27 to outer dense fibers. Gene 111-450 encodes the 84-kDa ODF protein, which harbors two leucine ZIPs. We propose a model where leucine ZIPs are an essential motif used in specific interactions of cytoskeletal sperm-specific proteins.

EXPERIMENTAL PROCEDURES

Library Construction

Testicular Yeast cDNA Expression Library—Total RNA was isolated from testes of adult Harlan Sprague Dawley rats using the acid guanidinium thiocyanate/phenol/chloroform method as described previously (38). Poly(A)+ RNA was prepared using oligo(dT)cellulose lacking superase (39). cDNA synthesis was performed using the RiboClone cDNA synthesis system (Promega). cDNA was synthesized using 2 μg of the C-T17 primer (5′-AAGCCGCGGGCCGCTGACAT17 3′), which harbors NotI and SalI restriction sites, and 6 μg of poly(A)+ RNA, EcoRI adapters were added, and cDNA fragments greater than 400 base pairs were isolated and inserted into the EcoRI and SalI site of pGAD424. The ligated DNA was transformed into Escherichia coli DH5α. Transformed colonies were scraped from the plates and pGAD/cDNA plasmid DNA was prepared using standard techniques.

Testicular agt11 cDNA Library—Double-stranded cDNA prepared as described above was digested with NotI, and fragments greater than 400 base pairs were purified and ligated to agt11 EcoRI-NotI arms (Promega). Ligated DNA was in vitro packaged (Promega). The library contained approximately 1 × 108 recombinants and was amplified as described (39).

Yeast Two-hybrid Screening

pGBT/NT, containing the N-terminal 145 amino acids of Odf27 fused to the GAL4 DNA binding domain, was transformed into the yeast HF7c strain as described below generating strain HF7c(bsd/NT). HF7c(bsd/NT) cells were transformed with 100 μg of the pGAD/cDNA library DNA spread on 100 9-mm-mm plates containing media. Trp-, Leu, and His, Plates were incubated for 8 days at 30°C to allow slower growing colonies to appear. His+ colonies were tested for ß-galactosidase activity using a filter assay; a filter (VWR, grade 413) was placed on transformant colonies, lifted off the agar plate, and submerged in liquid nitrogen for 10 s and thawed at room temperature to lyse cells. The mixture was vortexed for 2 min and spun for 5 min in a microcentrifuge. Five μl of the aqueous layer was used to transform 0.1 ml of competent E. coli DH5α cells. From E. coli plasmid DNAs were obtained using standard techniques as described (39).

DNA Sequence Analysis

cDNA inserts from pGAD/cDNA plasmids were subcloned into pBlueScript II KS+. Sequence analysis was performed using a cycle sequencing kit (Applied Biosystems), and samples were processed on an automated DNA sequencer (Applied Biosystems) in the DNA Sequencing Facility at the University of Calgary. Sequence data were edited and analyzed using PCGENE (Intelegenetics) programs.

Generation of Leucine Zipper Mutants

The pGAD/450 EcoRI-SalI insert was cloned in pBlueScript II KS+ and pBlueScript SK−, generating pBS-KS-450 and pBS-450, respectively (note that the orientation of the insert differs in these two vectors). Four oligonucleotides were designed that replace one of the four leucine residues in the putative leucine zipper motif of gene 111-450 by charged amino acid as indicated in Fig. 1. The oligonucleotides were as follows: mutLeu1 (5′-GACAATGGACATCTCAACATCTGCTC 3′; Leu → Gln); mutLeu2 (5′-GACAATGGACATCTCAACATCTGCTC 3′; Leu → Asn); mutLeu3 (5′-CTACAGGCTTGGTCTTGGCTAGCTGCTG3′; Leu → Asp) and mutLeu4 (5′-TTCCCGCATTCCTCCCTCCTCATAGCATTGGTTGCTT 3′; Leu → Arg). The four leucine mutants were made as follows. 1) Four separate PCR reactions were performed using the T7 primer and one of the four oligonucleotides as primers and pBS-KS-450 as template. The four PCR products were isolated. 2) These PCR products and the T7 primer were employed as primers in a second set of four PCR reactions using pBS-450 as template. The resulting PCR products were cloned in the EcoRI and SalI site of pGAD424, generating pGAD-mut1, pGAD-mut2, pGAD-mut3, and pGAD-mut4. The sequence of each mutant was analyzed. The mutants were next analyzed in yeast for interaction with pGBT/NT as described above.

In Vitro Translation and Immunoprecipitation—In vitro translations were performed using the TNT system (Promega) in the presence of [35S]Met/Cys using plasmids indicated in the text and legends as recommended by the manufacturer. For this analysis we subcloned the insert of pGAD/450 as an EcoRI-SalI fragment in a modified pBlueScript II KS+ vector, pBS-ATG; pBS-ATG was produced by insertion of one oligonucleotide into the unique XbaI site to create an ATG translation initiation codon. Inserts in the pBS-ATG vector can be efficiently translated in vitro using T7 RNA polymerase (Pharmacia Biotech Inc.) to produce RNA. The resulting plasmid was pBS-ATG-450.

Twelve μl of in vitro translation reactions and 0.4 μl of T7 Tag monoclonal antibody (Novagen) were added to 20 μl of protein A-Sepharose-Cl4B beads (Pharmacia), which had been preincubated in immunoprecipitation (IP) buffer (10% glycerol, 50 mM Heps-KOH, pH 8.0, 100 mM glutamate, 6 mM MgOAc, 0.5 mM dithiothreitol, 1 mM EDTA, 0.1% Nonidet P-40, 0.5 mg/ml bovine serum albumin) and were mixed with 200 μl of fresh IP buffer. The reactions were incubated on a Nutator for 3 h at 4°C, spun in a microcentrifuge, and washed 3 times with 1.5 ml of IP buffer. Samples were denatured and analyzed by electrophoresis on SDS-polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue, destained, and dried, and proteins were detected by autoradiography using Kodak XAR film.

RNA Analysis

RNA was isolated from the mouse organs indicated in the text as described above. Spermatozoa and spermatids were obtained by centrifugal elutriation of SD rat spermatogenic cells as described previously (30, 41), and RNA was isolated as described above. RNA samples were analyzed by Northern blotting/hybridization using Durlon-UV-75 membranes (Stratagene) and indicated random-primed probes as described (39). Membranes were washed and exposed to Kodak XAR film.

Antibody Preparation

The cDNA insert of pGAD/450 was subcloned as an EcoRI/SalI fragment into pMAL-c2, in frame with the MBP protein, generating pMBP-450. The 58-kDa MBP-450 fusion protein was induced in TB1 bacteria,
purified using amylose-agarose beads (Sigma), and used to generate polyclonal antisera in New Zealand White rabbits. Antiserum were characterized by Western blotting analyses.

Anti-ODF serum was raised in rabbits against isolated ODF fraction as described previously (13). This immune serum, which recognizes all major ODF proteins was used to affinity purify antibodies from Western blot-immobilized Od27 or Od84 proteins by a method described previously (42) and originally adapted from Ref. 43.

Isolation of Outer Dense Fibers

The isolation of rat outer dense fibers followed a previously published protocol (13) with several modifications. Briefly, epididymal spermatozoa suspended in Tris-buffered saline (TBS) were sonicated to separate heads and tails. The suspension was washed twice by centrifugation, and the final pellet was resuspended in TBS containing 80% sucrose, followed by centrifugation at 280,000 × g for 1 h in a T60 angle rotor (Beckman). The tail pellet obtained on the inside of the tube (centripetal side) was resuspended in TBS containing 40% sucrose and layered over a 60/80% sucrose gradient buffer in TBS. The discontinuous gradient was spun at 100,000 × g for 1 h on a horizontal rotor, and a virtually pure tail fraction was obtained from the 60 to 80% interface. The fraction was subsequently washed and pelleted. All steps were monitored by phase contrast and electron microscopy.

To obtain ODF the tail fractions were suspended in 10 mM Tris-HCl, pH 9.0, containing 1% SDS and 2 mM dithiothreitol and shaken at room temperature for various times, ranging from 30 to 90 min, until only the resistant ODF remained as monitored by phase contrast and electron microscopy. The ODF and solubilized tail suspension was then layered over a 35–75% sucrose gradient and centrifuged at 100,000 × g on a horizontal rotor for 30 min. The ODF band collected at the 35–75% interface was suspended in Tris-HCl and pelleted at 25,000 × g for 15 min.

Immunocytochemistry

Immunofluorescence analysis of 111-450 protein expression in frozen sections of rat testis and in isolated epididymal sperm was done as described previously (44) using polyclonal anti-111-450 antibodies and Texas Red-conjugated sheep anti-mouse IgG antibodies (Amersham Corp.). The same sections and epididymal sperm were also analyzed for expression of Od27 using anti-Od27 monoclonal antibodies and fluorescein isothiocyanate-conjugated sheep anti-mouse IgG antibodies (Boehringer Mannheim).

Immunoelectron microscopic analysis of sections through rat sperm tails was performed as described previously. Testicular sections, fixed and embedded in Locryl K4M were processed for immunogold labeling according to techniques previously used in our laboratory (13, 45).

Western Blot Analysis

ODF fractions were solubilized in 2% SDS, 5% β-mercaptoethanol sample buffer and separated by electrophoresis on 8–18% linear gradient polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose or polyvinylidene difluoride membranes in a solution of 25 mM Na2HPO4, pH 6.5, and protein bands were detected on blots by staining in 0.2% Ponceau Red in 3% trichloroacetic acid. Membrane strips were analyzed using anti-111-450 and affinity purified anti-Od84 antibodies and a secondary antibody conjugated to alkaline phosphatase (goat anti-rabbit IgG, Sigma). The phosphatase reaction was developed according to Ref. 46.

RESULTS

Isolation of an Od27 Interacting Protein—We employed the yeast two-hybrid system (47) to identify testicular proteins that strongly interact with the Od27 leucine zipper. For bait we constructed a hybrid of the Od27 N terminus fused to the GAL4 DNA-binding domain, pGBT/NT. A rat testis yeast cDNA expression library containing cDNAs fused to the GAL4 transactivation domain was constructed. pGBT/NT was introduced in yeast strain HF7c, and the resulting HF7c/bd/NT yeast strain was used to screen the cDNA fusion-expression library. Of 1 × 106 yeast transformants screened 65 clones were His "lacZ". Segregants containing only pGAD/cDNA plasmids were generated and tested further to eliminate false positives. One group of related cDNA clones were identified whose products strongly interact with the Od27 N terminus. A representative plasmid, pGAD450, contained a cDNA insert of approximately 0.45 kb and was used for further initial analyses. First, the plasmid was retransformed into the original yeast host strain. Table I shows the results of tested combinations to confirm specificity of interactions; pGAD450 could not activate the lacZ reporter gene by itself or in combination with pGBT9 or pVHA3, which encodes a hybrid p53/GAL4 DBD protein. Second, to determine if the leucine zipper motif of Od27 was responsible for the observed interaction, we analyzed the interaction between pGAD-450 and different Od27 N-terminal fragments: Od27NT (amino acids 1–145), Od27NT100 (amino acids 1–100), and Od27NT15 (amino acids 35–145) that lacks the leucine zipper. The results in Fig. 1, panel A, show that Od27 fragments containing the leucine zipper strongly interact with the novel protein. Deletion of the Od27 leucine zipper abolished this interaction. These results demonstrate that the Od27 leucine zipper mediated the interaction between Od27 and the novel protein and predicted the presence of leucine zipper(s) on this protein.

To verify by an independent method the interaction between the novel protein and Od27, we carried out in vitro transcription/translation assays and co-immunoprecipitation experiments. We used pET7/RT7NT (44) containing the Od27 N terminus linked to the S10 epitope tag, which can be specifically recognized, to express the Od27 N terminus in vitro. The cDNA fragment in pGAD-450 was cloned in pBS-ATG (see “Experimental Procedures”), which provides for a translation start site. Single and co-translation reactions were performed using these plasmids, and protein complexes were immunoprecipitated using S10-specific antibodies. Control translations contained only one plasmid. The results are shown in Fig. 2; first pBS-450 encodes a 16.5-kDa protein (lane 2) that is not recognized by the anti-S10 antibodies (lane 4). The S10-Od27 N-terminal fusion fragment is efficiently immunoprecipitated using these antibodies (lanes 5 and 6). Importantly, the 16.5-kDa protein encoded by pBS-450 stably associated in vitro with the Od27 N terminus as indicated by its efficient co-immunoprecipitation (lane 8). These results demonstrate that the novel testicular gene product can associate efficiently with Od27 in vitro confirming the initial observations made in yeast.

Gene 111-450 Encodes Male Germ Cell-specific Product—For the association between Od27 and the novel gene product to be physiologically relevant, it must be expressed in spermatids, the only site of synthesis of Od27, but the possibility existed that the Od27 LZ had interacted in yeast with LZ-containing proteins that are not expressed in spermatids but rather in somatic testicular cells or other male germ cells. We therefore analyzed the RNA expression pattern of gene 111-450 in the mouse by Northern blotting assays using total tissue RNAs. The results from these assays are shown in Fig. 3, panel A, and demonstrate that gene 111-450 encodes two transcripts of 3.2 and 2.1 kb that are only detectable in mouse testis. Thus the novel gene encodes testis-specific product.

We next investigated the RNA expression of gene 111-450 in isolated round spermatids and pachytene spermatocytes by Northern blot analysis (testis and liver RNAs were included as positive and negative controls). The results shown in Fig. 3, panel B, indicated that 111-450 gene transcripts are expressed
Sequence Analyses of 111-450 cDNAs—The yeast results predicted that the testis-specific cDNA in pGAD-450 harbors a leucine zipper motif. We initially determined the nucleotide sequence of the pGAD-450 insert, which was compared with entries in the GenBank data base. The partial pGAD-450 cDNA sequence appeared to be homologous to two expressed sequence tags, mouse MMTEST128 that is expressed in spermatocytes and spermatids (49) and EST111685 isolated from PC12 cells that were induced to differentiate to neurons by nerve growth factor (50), and to the unpublished GenBank entry RN1414 that was isolated from a spermatocyte phage cDNA library. The testicular expression patterns reported for the MMTEST128 fragment was thus identical to that of gene 111-450. We next screened a rat testis directional phage library using the insert of clone pGAD-450 and isolated 111-450 cDNAs harboring the complete open reading frame.

Fig. 4 shows the predicted protein sequence of the open reading frame encoded by 111-450 cDNAs. This latter protein sequence is shown in a comparison with the predicted RN1414 protein sequence. Our 111-450 protein sequence is similar but not identical to the predicted RN1414 protein sequence. Fig. 4 indicates the unique 111-450 amino acid residues 1–36, several conserved amino acid changes (indicated by dots) as well as a region only present in RN1414 (RN1414 residues 180–202). The molecular weight for the predicted 111-450 protein is 72,000. Importantly, the 111-450 protein contains two putative C-terminal leucine zippers (leucine residues are bold and underlined in Fig. 4), only one of which (the upstream one) was present in the original pGAD-450 cDNA clone isolated in the yeast two-hybrid screen (as indicated in Fig. 4).

Protein Interactions between Odf27 and the Gene 111-450 Product Is Mediated by the Upstream 111-450 Leucine Zipper—To investigate the involvement of the putative leucine zippers in the interaction of Odf27 with gene 111-450 product and to analyze the specificity of these interactions, the following experiments were carried out. The interaction of gene 111-450 product with itself was tested and compared with its interaction with Odf27. The result is shown in Fig. 1, panel B. Gene 111-450 protein clearly cannot associate with itself (area 2) but strongly interacts with Odf27 (area 1). We then mutated each of the four leucine residues in the upstream leucine zipper of 111-450 protein that was present in the pGAD-450 insert (see “Experimental Procedures”). The mutant 111-450 proteins were tested for interaction with Odf27 in yeast. Fig. 1, panel C, shows that wild type 111-450 interacts with Odf27 (area wt) as expected. Mutation of any of the leucine residues abolished this association completely (areas 1–4). Thus, each of the four leucine residues of the upstream leucine zipper of 111-450 protein as well as the Odf27 leucine zipper (Fig. 1A) are critically involved in the interaction between 111-450 and Odf27 proteins.

111-450 Protein Localizes to the ODF—To analyze the 111-450 protein expression pattern in male germ cells, we raised polyclonal antibodies against an MBP-450 fusion protein that were used in immunofluorescence analysis of frozen sections of rat testis. The results are shown in Fig. 5, panels A–C. Protein 111-450 staining was only detectable in tails of elongating spermatids and spermatids, and their level increases in round spermatids, a pattern resembling that of the testis-specific phosphoglycerate kinase 2 gene (PGK2) (48). In conclusion, the novel gene 111-450 is transcribed in pachytene spermatocytes and spermatids.
spermatids (panel C). No other cells within seminiferous tubules produced 111-450 protein, including pachytene spermatocytes that do transcribe the 111-450 gene. The 111-450 protein expression pattern was compared with that of Odf27 and found to be essentially identical (panel B). The localization of 111-450 protein was also investigated by immunofluorescence analysis of isolated epididymal spermatozoa (Fig. 5, panels D–F). Staining of spermatozoa using the same antibodies demonstrated that both 111-450 protein (panel F) and Odf27 (panel E) localize to sperm tails. This result suggested that 111-450 protein could be a component of either ODF or FS (note that Odf27 is only present in ODF).

To distinguish these possibilities we examined 111-450 protein localization in sections of sperm tails by immunoelectron microscopy. The anti-450 antibodies described above were used. Cross-sections were prepared from tails of rat spermatozoa at the midpiece, the principal piece, and the endpiece. We also examined the distribution of 111-450 protein in longitudinal sections through sperm tails. The results of these experiments are shown in Fig. 6, panels A and B. The electron

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**Fig. 4.** The 111-450 product harbors two putative leucine zippers. Presented is the complete predicted 111-450 amino acid sequence in a comparison with the predicted protein described by GenBank entry RN1414. The amino acid residues that form the two putative leucine zippers are bold and underlined. Indicated are identical residues (line) and conserved amino acid changes (dots) as well as the extent of the original pGAD-450 fragment.
FIG. 5. 111-450 protein localizes to sperm tails. Panels A–C, the expression of the 111-450 protein was examined by immunofluorescence of frozen rat testis sections and compared with that of Odf27. The same sections were incubated with polyclonal antisera raised against the 111-450 protein (panel C) and monoclonal antisera raised against Odf27 (panel B), and these were detected using Texas Red-conjugated donkey anti-rabbit antibodies and fluorescein isothiocyanate-conjugated sheep anti-mouse MAb-Ig antibodies, respectively. Panel A shows a phase contrast image of the same frozen section used for the analyses shown in panels B and C. Magnification: ×25. Panels D–F, the primary and secondary antibodies described above were used to localize the 111-450 protein in epididymal spermatozoa. Panel D, phase contrast image of the slide used for the analyses shown in panels E and F. Panel E, Odf27 protein detection in sperm tails, and panel F, 111-450 protein localizes to sperm tails. Magnification: ×40.

Micrographs in panel A show that in cross-sections 111-450 protein is evenly distributed in the nine ODF (odf) in the midpiece and in the seven ODF in the principal piece. 111-450 protein is not detectable in the FS (fs), the mitochondrial sheath (m), or the axoneme (ax). These results are confirmed by the analysis of longitudinal sections (panel B), which show that 111-450 protein localizes to the ODF and is absent from the other major tail structures. We conclude that the gene 111-450 encodes an outer dense fiber protein.

Gene 111-450 Encodes the 84-kDa Major Outer Dense Fiber Protein—Initial protein characterizations and the localization of the 111-450 protein to ODF indicated the possibility that the gene 111-450 could encode Odf84, one of the major ODF proteins. To identify the gene 111-450 protein, we performed the following assays. First, we used anti-450 antiserum in a Western blotting analysis of isolated ODF proteins and compared the results with those obtained using affinity purified anti-Odf84 antibodies. The results shown in Fig. 7 indicate that both antisera recognize the same proteins of 84, 71, 40, 56, and 27 kDa, in descending order of strength (lanes 3 and 4), in spite of their completely independent generation. No ODF proteins were recognized by preimmune sera (lane 2). This result strongly suggested that gene 111-450 encodes Odf84.

In a second approach we analyzed the in vitro translation products of 111-450 cDNA by anti-450 antibodies and by affinity purified anti-Odf84 antibodies. This assay shows (Fig. 7) that a 2.1-kb 111-450 cDNA, which harbors the open reading frame shown in Fig. 4, encodes a protein with an apparent molecular weight of 82,000 on SDS-polyacrylamide gel electrophoresis (lane 6) that is recognized by both anti-450 antibodies and anti-Odf84 antibodies (lanes 9 and 12, respectively). The products of included negative controls, 55-800 and 69-1400 cDNAs (lanes 7 and 8, respectively), are not recognized by these antisera (lanes 10, 13, and 11, 14, respectively), as expected. The smaller 111-450-encoded products detected in this assay likely derive from internal translation start sites. Taken together with the immunoelectron microscopy studies we conclude that gene 111-450 encodes the major outer dense fiber protein Odf84.

FIG. 6. Immunoelectron microscopic analysis of 111-450 protein distribution in sperm tails. Electron microscopic sections of rat spermatozoa immunogold-labeled with anti-450 antibodies. Panel A, cross-sections through mid-pieces and principal piece of tail. Note that the immunogold labeling is specific to the outer dense fibers (odf). fs, fibrous sheath; ax, axoneme; m, mitochondria. Panel B, longitudinal section showing the junction of the mid-piece and principal piece of the tail. Only the outer dense fibers (odf) are labeled. fs, fibrous sheath; a, annulus; m, mitochondria; smr, submitochondrial reticulum; ax, axoneme. Magnification: ×42,000.

From previous analyses it became apparent that the nine ODF in the midpiece and the remaining seven ODF in the principal piece each have a distinguishable, characteristic shape, contain a cortex surrounding a medulla, and are highly insoluble. Many of the major ODF proteins (as well as many of the FS proteins) are specifically produced in elongating spermatids and are thus likely encoded by testis-specific genes (14, 51). As a consequence the elongating spermatid is faced with several problems that need resolution; all required ODF proteins need to be synthesized at the appropriate time and in the appropriate amounts. Furthermore, since the proteins that constitute the mature ODF are highly insoluble, the elongating spermatid must provide an environment to synthesize and store these proteins in a solubilized state and then transport them to the assembly points of the developing ODF. In support of a possible storage, granular bodies have been observed in elongating spermatids at a time of maximal production of ODF proteins, and these bodies are near exclusive depots of ODF proteins, including Odf84, in the cytoplasm (52). At later stages that coincide with maximal ODF assembly the number of these granular bodies declines. Finally, in order to ensure sufficient stocks of ODF protein for assembly, elongating spermatids appear to produce an excess of the required proteins, which still in granular form are recycled in residual bodies (52). It is therefore reasonable to propose an important role for ODF protein interactions in at least two stages as follows: interactions that occur (a) during synthesis to store ODF proteins in a soluble state (perhaps with the aid of unidentified chaperons) and (b) during ODF assembly to determine the mature ODF structure. The possibilities to investigate these points have been poor due to the lack of cloned ODF proteins, and the...
nature of the motifs involved in ODF protein interactions cannot be addressed directly due to their insolubility.

We demonstrate here that the leucine zipper motif of the outer dense fiber protein Odf27 can be successfully used to clone a novel testis-specific gene based on an interaction screen in yeast. We identify the novel product as the major outer dense fiber protein Odf84. Odf84 harbors two leucine zipper proteins, one of which strongly and specifically interacts with Odf27.

A Leucine Zipper from a Structural Protein as Bait in the Yeast Two-hybrid System—Since only Odf27 had been cloned, we planned to isolate cDNAs of new member(s) of the family of structural testis-specific proteins to identify molecular determinants of protein interactions that play a role in ODF morphogenesis. To increase the likelihood of obtaining proteins relevant to Odf27 and its function, we decided to exploit our previous Odf27 structural predictions of a leucine zipper motif in a yeast two-hybrid system, rather than screen testis cDNA libraries by subtractive procedures. The latter approach can result in the isolation of testis-specific proteins, but they would be of unknown relevance to Odf27 biology. We thus used an Odf27 N-terminal fragment, which harbors a leucine zipper (26, 37), to isolate testicular proteins that could interact with Odf27 in a yeast two-hybrid system. The cDNA library used was made from rat total testis mRNA and as a consequence contained leucine zipper proteins derived from germ cells and somatic cells (among others the bZIP proteins Creb (53) and Crem (54)). Interestingly, we did not isolate members of the bZIP family of transcription factors in this screen but instead obtained cDNA for gene 111-450 that we showed encodes the 82-kDa product encoded by a 2.1-kb 111-450 cDNA in translation/immunoprecipitation assays. Furthermore, antibodies raised against protein 111-450 and affinity purified anti-Odf84 antibodies display identical patterns in Western blot analyses of isolated ODF proteins. Finally and importantly, the product of gene 111-450 localizes to the outer dense fibers as determined by immunoelectron microscopy. We do not yet know why other smaller ODF proteins are also recognized by anti-Odf84 antisera in in vitro translation/immunoprecipitation assays and Western blot assays: possibly, translation of one or more of these smaller proteins initiates at internal AUG codons on 111-450 mRNA. Alternatively, smaller proteins may result from processing of the initial translation product. A less likely possibility is that the antibodies raised against the 111-450 product and the affinity purified anti-Odf84 antibodies cross-react with some of the other ODF proteins.

In preliminary experiments we observed that in vitro translation of RNA derived from 111-450 cDNAs that are approximately 2 kb in size or larger results in the synthesis of the 82-kDa protein and that this product co-migrates on SDS-polyacrylamide gel electrophoresis with Odf84 translated from testicular polyadenylated mRNAs. The in vitro translation efficiency of 111-450 cDNAs larger than 2.1 kb (up to the near full-length 3.1 kb cDNA) rapidly drops, but the size of products synthesized remains the same, viz. 82 kDa. At present we do not know why the 111-450 gene product, which has a predicted molecular weight of 72,000, migrates in SDS-polyacrylamide gel electrophoresis conditions as an 82-kDa protein. Our preliminary translation data thus indicate that the 5’-UTR of 111-450 mRNA decreases translational efficiency in vitro and raise the possibilities of (i) translational control by the 5’-UTR (see below) and (ii) a role for the smaller testicular 2.1-kb 111-450 mRNA in the synthesis of Odf84.

Interestingly, sequence analysis of Odf84 cDNA predicted the presence of two putative leucine zipper motifs in the C-terminal region of the protein, only one of which, the upstream leucine zipper, was present in the original pGAD-450 cDNA (Fig. 4). Site-directed mutagenesis of any one of the four leucine residues in the upstream Odf84 leucine zipper abolished interaction with Odf27. Thus, Odf27 and Odf84 co-localize and strongly interact via their leucine zippers. However, they differ with respect to interacting partners; whereas Odf84 can only associate with Odf27, Odf27 can associate to a limited extent with itself (37). This finding has implications for the model of ODF protein interactions described below.

The RNA expression pattern of gene 111-450 is interesting and differs from that of Odf27. Odf84 mRNA is detectable in meiotic, pachytene spermatocytes and accumulates further in round spermatids, whereas Odf27 mRNA is exclusively synthesized in round spermatids. Odf84 protein is, however, only detectable in elongating spermatids (similar to Odf27 protein expression). Odf84 mRNA expression resembles that of PGK2, but PGK2 protein is synthesized in both spermatocytes and spermatids. This suggests that Odf84 mRNA is subject to translational repression in both spermatocytes and round spermatids. This level of gene regulation has been described before for several spermatid-specific genes (reviewed in Ref. 55), including the protamine genes mP1 and mP2 (56, 57), but not for genes transcribed in spermatocytes. Translational regulation of mP1 mRNA results from the action of cis-acting sequences in the 3’-untranslated region (UTR) (56). Sequences in the 3’-UTR can interact with proteins (58–60) one of which was recently cloned and fulfills the criteria predicted for a translational repressor protein (61). Recently, the translation regula-

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2 X. Shao and F. A. van der Hoorn, unpublished observations.
ODF Sperm Proteins Interact via Leucine Zippers

A functional role for the Odf27 PC repeats is supported by the analysis of mutations in genes belonging to the Drosophila multigene Mst(3)PCG family, which encodes seven male germ line-specific proteins (33). These proteins localize to satellite fibers in Drosophila spermatozoa, considered functional homologs of mammalian ODF (36). A homozygous deletion of four of these genes (located in cluster 84D) caused a 2-fold decrease in sperm production, and examination of spermatozoa revealed numerous malformations (35). The evolutionary conservation of the PC repeats in male germ cell-specific proteins and the similar regulation of expression and localization of Odf27 and Mst(3)PCG proteins supports an important role for these repeats in mammalian ODF. We also determined recently in a yeast two-hybrid approach and in vitro experiments that the PC repeats mediate Odf27 self-association (37). Computer analysis predicted that these repeats could form coiled coils. Taken together, these observations lend support to the first model, presented in Fig. 8, model A, which we favor: it is based on the available experimental data concerning the Odf27 leucine zipper and PCX repeat domains. Of course the presented models are subject to variations, e.g., the Odf27 gene also encodes a minor protein of 20 kDa that contains the PCX repeats but lacks the leucine zipper and would limit the branching opportunity provided by the full-length Odf27 molecule. A direct demonstration of protein interactions in isolated ODF is not possible due to the insoluble nature of these proteins; however, the availability of the novel cDNAs and our identification of the crucial dimerization domain will allow us to confirm such interactions in transgenic mice and study the consequences of disruption of these interactions on sperm tail development.

In conclusion, our data demonstrate that specific interactions between two testis-specific cytoskeletal proteins exist and that these are mediated by leucine zipper motifs.

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