Identification and Characterization of a Sphere Organelle Protein

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Abstract. Sphere organelles are nuclear structures in amphibian oocytes that are easily visible by light microscopy. These structures are up to 10 μm in diameter and have been described morphologically for decades, yet their function remains obscure. The present study defines a protein component of the sphere organelle, named SPH-1, which is recognized by a mAb raised against purified Xenopus laevis oocyte nucleoplasm. SPH-1 is an 80-kD protein which is localized specifically to spheres and is undetectable elsewhere on lampbrush chromosomes or in nucleoli. We show using confocal microscopy that SPH-1 is localized to the cortex of sphere organelles. Furthermore, we have isolated a cDNA that can encode SPH-1. When epitope-tagged forms of SPH-1 are expressed in X. laevis oocytes the protein specifically localizes to spheres, demonstrating that the cloned cDNA encodes the sphere antigen. Comparison of the predicted amino acid sequence with sequence databases shows SPH-1 is related to p80-coilin, a protein associated with coiled bodies; coiled bodies are nuclear structures found in plant and animal cells. The sphere-specific mAb stains X. laevis tissue culture cells in a punctate nuclear pattern, showing that spheres or sphere antigens are present in somatic cells as well as germ cells and suggesting a general and essential function for spheres in all nuclei.

The nuclei of amphibian oocytes contain several large, morphologically distinct structures, including lampbrush chromosomes, nucleoli and "spheres." Sphere organelles were first identified as "knobs" attached to lampbrush chromosomes of the newt Notophthalmus viridescens (Gall, 1954; reviewed in Callan, 1986). The term organelle is used to describe spheres because they are distinct structures and larger than some whole cells, yet they are not membrane bound. Structures with similar morphology have since been observed on lampbrush chromosomes of many different amphibian species and in oocyte nuclei of several insects, suggesting that spheres may be common to all animal oocyte nuclei where they perform an unknown yet essential function (Gall and Callan, 1989). In amphibians some spheres appear attached to specific loci on lampbrush chromosomes while others are free in the nucleoplasm. In Xenopus laevis there are three chromosomal sphere loci, located on chromosomes VIII, IX, and XVI (Callan et al., 1987), as well as 40 to 60 free spheres (Fig. 1). Free and attached spheres are morphologically indistinguishable. They contain a central electron dense core and a surrounding cortex that is less dense (Kezer et al., 1980). Larger spheres have often been observed with 2 to 3 μm hemispherical masses on their surfaces. These hemispherical masses, which are morphologically distinct from spheres, were recently named "B snurposomes" when antibody staining experiments showed they contain a variety of proteins and small nuclear RNAs involved in pre-mRNA splicing (Wu et al., 1991). Similar studies have also shown that spheres can be stained with two antibodies that bind snRNPs and have, as a result, been referred to as "C snurposomes" (Gall and Callan, 1989; Wu et al., 1991). These common splicing components are associated with nearly all active sites of transcription on lampbrush chromosomes (Gall and Callan, 1989; Wu et al., 1991).

We report here the generation of a mAb that binds exclusively to spheres. We have used this antibody to isolate a cDNA which encodes a sphere-specific protein, SPH-1. The primary amino acid sequence of this sphere protein is related to p80-coilin; p80-coilin is localized to a functionally undefined structure called the "coiled body" found in the nuclei of many different plant and animal cells (Andrade et al., 1991). The similarity between SPH-1 and p80-coilin suggests that spheres may be related to coiled bodies.

Materials and Methods

Isolation of Oocyte Nuclei

Ovary (30 g) was removed from albino frogs and minced with scissors into pieces containing ~30 mature oocytes. The ovary was then divided into two T-150 tissue culture flasks each containing 0.1% collagenase w/v/15 grams ovary in 50 ml of Barth's medium (10 mM Hepes, pH 7.4, 88 mM NaCl, 1 mM KCl, 2.3 mM NaHCO3, 0.82 mM MgSO4, 0.66 mM NaNO3, 0.41 mM CaCl2), and rotated at 75 rpm for 90-120 min at room temperature. Oocytes were sieved from the collagenase using 100 μm nitex. The large oocytes were separated from the smaller oocytes by sequential sieving on 100 μm nitex. The oocytes were then washed twice in 5 ml of Barth's medium, and finally resuspended in 2 ml of 100 μM CaCl2 and 5 mM MgCl2.
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The percentage recovery of nuclei from oocytes was determined to be at least 80% and the contents containing 50 ml of buffer (10 mM Hepes, pH 7.4, 5 mM MgCl2, 60 mM KCl, and hypotonically swollen for 60-70 min. Several hundred oocytes were separated from the cytoplasmic contents. Nuclei were then transferred to a circular motion so that "one edge" was always in contact with the ice-containing dish. Some practice was required to know how vigorously to swirl the dish because yolk obscures the ability to see the nuclei. Once the nuclei are in the center of the dish they are removed to a second dish and reswirled; the swirling was repeated five to six times until the nuclei were separated from the cytoplasmic contents. Nuclei were then transferred to a new dish. The nuclear envelopes adhered to the clean plastic and the dish was turned quickly to create a shear force sufficient to separate the congealed nucleoplasm from the nuclear envelopes. The percentage recovery of nuclei from oocytes was determined to be at least 80% and the contents of as many as 25,000 nuclei have been recovered in one day.

Monoclonal Antibody Isolation and Characterization

Several mice (RF1/Da, Jackson Laboratories, Bar Harbor, MN) were each immunized with ~10000 nuclei over a 6 mo period. Polyclonal antisera were tested by indirect immunofluorescent staining of chromosome preparations (Roth and Gall, 1987; Callan et al., 1987). Hybridoma cell lines were generated by fusing the spleen cells of one of these mice to Fox-NY cells (Taggert and Samloff, 1983), and the supernatants were used to test for indirect immunofluorescent staining of spheres. Hybridoma cell lines that produced sphere binding antibodies were isolated as mAbs producing cell lines by limiting dilution cloning (Harlow and Lane, 1988). Immunoblotting and indirect immunofluorescent staining were done according to previously published methods (Roth and Gall, 1987; Roth et al. 1991). X. laevis K2 tissue culture cells were grown according to McStay and Reeder (1990). For immunoblotting, the cells were washed once in X-PBS (68 mM NaCl, 1.3 mM KCl, 4.0 mM Na2HPO4, 0.7 mM KH2PO4, 0.35 mM CaCl2, 0.25 mM MgCl2) and resuspended in SDS-PAGE sample buffer.

Identification and Characterization of SPH-1 cDNAs

A X. laevis young ovary polyA+ cDNA expression library made in the vector Lambda Zap (Stratagene Corp., Burlingame, CA) was screened using mAbH1 (Short et al., 1988). Two independent classes of inserts (700 and 1,900 bp) were isolated from the expression library and subcloned into Bluescript plasmids (Stratagene Corp.). Sequencing of the cDNAs revealed an open reading frame that could encode a 43-kD protein and that the 700-bp insert is identical to an internal region of the 1,900-bp clone. Because the 1,900-bp insert appeared to be lacking the 5' end of the open reading frame (ORF), the 700 bp cDNA was used as a hybridization probe to screen a lambda gt10 library of X. laevis ovar y cDNAs to obtain a full-length cDNA (Rebagliati et al., 1985; Maniatis et al., 1982). 15 positive phage were isolated from ~1.2 x 106 phage screened. The largest insert, referred to here as cSPH-1, is 2.3 kb. cDNA clones were sequenced on both strands according to published methods (Henikoff, 1987). Sequence databases were searched using BLAST (Altschul et al., 1990) at the National Center for Biotechnology Information and BLOCKS (Henikoff and Henikoff, 1991).
Figure 3. Indirect immunofluorescent staining of a *Xenopus laevis* lampbrush chromosome preparation with mAbH1. A and B are phase contrast and fluorescent micrographs, respectively, of the same preparation after staining with the mAbH1. Staining is specific for spheres; the fluorescent images of spheres are somewhat larger than the phase contrast images because of the intensity of fluorescence. Bar, 18 µm.

Expression and Detection of SPH-1 Protein

Epitope tagged SPH-1 protein was expressed in *X. laevis* oocytes according to previously published methods with minor modifications. The reiterated hexamer epitope tag, MT6 (Roth et al., 1991), was fused to the cSPH-1 sequence encoding either the second methionine (amino acid 25; construct A) or to amino acid 111 (construct B) as follows. The lambda cDNA insert was cloned into a bluescript plasmid (Strategene Corp.) containing the MT6 sequence with the tag ORF preceding, and in the same direction as, the SPH-1 ORF. The A construct was then generated by fusing the tag ORF to the cSPH-1 ORF by site directed mutagenesis using a 31 base oligonucleotide. Construct B was made by cloning the original 1,900-bp Lambda Zap eDNA into the MT6 Bluescript plasmid; the 1,900-bp insert encodes the SPH-1 protein from amino acid 111 to the termination codon after amino acid 536. Capped runoff in vitro synthesized transcripts encoding fusion proteins were prepared (Roth and Gall, 1989), and injected into *Xenopus* oocytes according to methods described by Gurdon et al. (1971). Immunoblotting of pools of 8–20 hand isolated oocyte nuclei 48 h after injection of RNA was done according to Roth et al. (1991). *Xenopus* lampbrush chromosomes were prepared according to Callan et al. (1987); indirect immunofluorescent staining was done according to Roth and Gall (1987). mAb9E10 was isolated by Evan et al. (1985).

Results

Identification and Characterization of a Sphere-specific Monoclonal Antibody

To begin a biochemical characterization of spheres, we developed a method for isolating large numbers of oocyte nuclei (Fig. 2; see Materials and Methods). We immunized mice with *X. laevis* oocyte nucleoplasm that had been purified away from cytoplasm and nuclear envelopes (Fig. 2, inset). Immunostaining of lampbrush chromosomes with the resulting polyclonal antisera indicated high titers of antibodies against spheres as well as nucleoli, chromosomes, and B snurposomes (data not shown). The spleens of all of these mice were fused to a mouse myeloma cell line and the resulting hybridoma cell lines tested for production of antibodies that bind to spheres by indirect immunofluorescent staining of lampbrush chromosome preparations. Of a total of 384 cell lines tested, four produce antibodies that preferentially bind to spheres. After subcloning, four independent hybridoma cell lines were recovered which produce antibodies that bind spheres. While three of the antibodies show some staining of other nuclear structures, one of the antibodies, mAbH1, shows selective binding to spheres with no detectable staining of other nuclear structures (Fig. 3).

Electron microscopic observations of sections through spheres have shown that spheres have morphologically distinct domains including an electron dense core and a cortex that is less dense (Kezer et al., 1980). Confocal microscopy of spheres stained with mAbH1 shows that the antigen is concentrated in the sphere's outer cortex with negligible staining in the inner core (Fig. 4, a and b). Conversely, the inner core is preferentially stained by mAbD7 (Fig. 4, c and d); this antibody binds a conserved epitope on a class of alternative pre-mRNA splicing factors called SR proteins (Tuma and Maniatis, 1990). The spatial segregation of specific antigens within the sphere is consistent with the view that the core and cortex represent distinct functional domains.

We were also interested to determine whether spheres are present in somatic cells. We stained *X. laevis* K2 tissue culture cells with mAbH1 and found that it stains nuclei in a punctate pattern (Fig. 5). Although some of the nuclei show a few larger aggregations of staining, most show a fine granular pattern. This observation indicates the mAbH1 antigen is expressed in somatic cells. If spheres are present in somatic
Characterization of the mAbH1 Antigen, SPH-1

To determine the molecular nature of the mAbH1 antigen, oocyte nuclei (germinal vesicles) were extracted in sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose, the oocyte nuclear proteins were probed with mAbH1 and a single protein of 80 kD was detected (Fig. 6, lane 1). We refer to this 80-kD protein as SPH-1. In addition, mAbH1 binds a band of similar mobility in extracts of Xenopus K2 tissue culture cells (Fig. 6, lane 2).

To isolate a full-length cDNA for this 80-kD protein, two Xenopus ovary cDNA libraries were screened. Initially, we screened ~1 × 10^9 phage from an expression library with mAbH1 and isolated several overlapping, partial cDNAs of 700 and 1,900 bp. Using the 700-bp expression clone as a probe, we screened a lambda gt10 library and identified a 2.3-kb cDNA. This cDNA is referred to as cSPH-1. The nucleotide sequence of cSPH-1 reveals an open reading frame that can encode a 536 amino acid protein (Fig. 7).

To demonstrate that cSPH-1 encodes the sphere-specific protein recognized by mAbH1, expression and localization studies were done in X. laevis oocytes. To distinguish between endogenous SPH-1 and protein expressed from the cDNA, a sequence encoding a repeated epitope derived from the chicken myc proto-oncogene was fused to the 5' end of cSPH-1 (Munro and Pelham, 1984; Roth et al., 1991). This allows detection of myc “tagged” SPH-1 fusion protein in Xenopus oocytes by mAb9E10, an antibody which specifically binds the myc epitope (Evan et al., 1985). Two

Figure 5. X. laevis K2 tissue culture cells stained with mAbH1. A shows a confocal pseudo-phase contrast image of Xenopus K2 cultured cells; B is a corresponding indirect immunofluorescent image after staining with mAbH1. The nuclei are stained in a punctate pattern; the nucleoli are unstained but are clearly visible in A. The cell on the far right shows some fluorescence in the cytoplasm; this

cell nuclei, they appear to be much smaller than somatic nucleoli; in oocytes spheres and nucleoli are of comparable size.
Figure 7. Detection of epitope-tagged SPH-1 in a *Xenopus laevis* lampbrush chromosome preparation. Chromosome preparations were made from oocytes that were injected with transcripts encoding epitope-tagged SPH-1 fusion protein B. A is a phase contrast micrograph and B a fluorescent image of a chromosome preparation stained with the anti-tag antibody, mAb9E10. The fusion protein is predominantly localized to spheres. Bar, 20 μm.

such constructs were made by fusing the myc tag to the amino terminal region of SPH-1, either to the second methionine (construct A; Fig. 7, bp 301) or to an internal site, deleting the NH2-terminal region of the protein (construct B; Fig. 7, bp 553).

We expressed the fusion proteins by injecting oocytes with capped in vitro synthesized RNA transcripts from either construct A or construct B. 48 h after injection, nuclei were removed from the injected oocytes and tested for expression of the fusion protein. The A fusion protein was unstable and expressed less efficiently than the B fusion protein as detected by immunoblotting (data not shown). Expression from the B construct resulted in production of an 80–83-kD protein that was detectable with both mAbH1 and mAb9E10 (Fig. 8, lanes 1 and 3). Because the predicted size of the B fusion protein is only 58 kD (including 10 kD contributed by the epitope tag), the retarded mobility of this fusion protein is likely to reflect post-translational modification. We assume that the modification occurs on SPH-1 itself because fusion of the same tag to other proteins results in the predicted mobility shift of only 10 kD when expressed in *X. laevis* oocytes (Zahler and Roth, unpublished observations). It is likely that cSPH-1 contains the entire open reading frame encoding the sphere protein SPH-1 because fusion protein B, which contains 10 kD of tag sequence, has the same mobility as the endogenous protein despite the absence of 9.5-kD of NH2-terminal SPH-1 sequence.

We confirmed that cSPH-1 encodes the sphere protein, SPH-1, by observing the localization of the B fusion protein in oocyte nuclei. Lampbrush chromosomes were prepared from oocytes that had been injected with in vitro synthesized transcripts encoding the B fusion protein. Indirect immunofluorescent staining with the anti-tag antibody, mAb9E10, showed preferential localization of the B fusion protein to spheres (Fig. 9). Previous results show that this localization is independent of the tag because the same tag fused to a nucleolar protein results in nucleolar localization (Roth et al., 1991).

Protein sequence databases were searched with SPH-1 to identify related proteins. The COOH-terminal 185 amino acids of SPH-1 are 75% similar to a partial amino acid sequence of human p80-coilin (Fig. 10), a protein found in coiled bodies (Andrade et al., 1991). Furthermore, comparison of SPH-1 to the predicted amino acid sequence of the full-length p80-coilin reveals an additional region of similarity in the NH2 terminus spanning amino acids 1 to 125 with 28% similarity (personal communications, Takano and Chan; Wu and Gall). Coiled bodies are morphologically defined nuclear structures found in somatic cells (Hervás et al., 1980; LaFarga et al., 1983); their function is unknown. The regions of similarity shared by *Xenopus* SPH-1 and human p80-coilin suggests that spheres and coiled bodies may be related.

**Discussion**

The sphere protein we have identified, SPH-1, is the first sphere-specific macromolecule to be characterized. To date, several studies have shown that spheres contain pre-mRNA splicing components (Gall and Callan, 1989; Wu et al., 1991). The antibodies used in this work include mAbY12 which recognizes a conserved epitope on several small nuclear RNA binding proteins (Lerner et al., 1981) and mAbK121 which binds the trimethylguanosine cap structure present on the 5' ends of all the major U-snRNAs except U6 (Krainer, 1988). In addition, both of these antibodies also stain other nuclear structures, including B snurposomes and most lateral loops on lampbrush chromosomes. mAbH1 stains only spheres, indicating that the function of SPH-1 is unique to spheres. The only other known sphere-specific protein is a 120-kD protein identified in the newt *Pleurodeles waltl* (Lacroix et al., 1985); a cDNA for this protein has yet to be isolated.

Our finding that SPH-1 is similar to p80-coilin is the first piece of evidence that suggests oocyte spheres and somatic cell coiled bodies may be related. This hypothesis is sup-
ported by the fact that both spheres and coiled bodies are nuclear structures and by the observation that SPH-1 is present in oocyte and somatic cell nuclei. Furthermore, coiled bodies and spheres are thought to contain snRNPs and snRNAs (Eliezeri et al., 1984; Fakan et al., 1984; Wu et al., 1991; Carmo-Fonseca et al., 1992). However, there are apparent differences between spheres and coiled bodies. For example, coiled bodies contain fibrillarin (Andrade et al., 1991) which has been detected in nucleioli (Lischwe et al., 1985) but has not been shown to be in spheres. Similarly, mAbSCS5, which stains spheres (Wu et al., 1991), does not stain coiled bodies (Carmo-Fonseca et al., 1991). Hence, the similarity of SPH-1 to p80-coilin is interesting, but further studies are necessary to determine if spheres and coiled bodies are functionally similar.

The identification of SPH-1, together with other studies, more clearly defines the structure of spheres and sheds light on how this organelle is generated. Our working hypothesis is that the formation of spheres is analogous to the formation of nucleioli. Nucleoli arise as a result of rDNA transcription and accumulation of variously assembled pre-ribosomal subunits around the site of transcription (Sheer and Benavente, 1990; Fisher et al., 1991). We propose that spheres arise by a similar mechanism; that is, DNA at the sphere loci is transcribed and a sphere-specific ribonucleoprotein complex (RNP) is formed as a result of specific proteins binding to these nascent transcripts. The accumulation of this RNP gives spheres their distinctive morphology. This model is supported by common features shared by nucleioli and spheres. Each has been mapped to distinct genetic loci on newt lambrush chromosomes (reviewed in Callan, 1986) and each has an electron dense core and a cortex that is less dense (Kezer et al., 1980; Goessens, 1984). Another feature common to spheres and nucleioli is that they both exist as chromosomally attached and extrachromosomal “free” structures in amphibian oocyte nuclei. Extrachromosomal nucleioli are formed as a result of amplification of rDNA during oogenesis, nucleating the formation of hundreds of free nucleioli (Brown and Dawid, 1968; Gall, 1968). It is possible that sphere DNA is similarly amplified, and that this amplified DNA gives rise to the free spheres. Alternatively, free spheres may arise via “shedding” of accumulated sphere RNP from the chromosomal sphere loci (Callan, 1986). If spheres do arise in the same way as nucleioli, then spheres may be sites of assembly of a distinct RNP. The identification of RNP as the nucleolar organizer played a fundamental role in understanding the function of nucleioli; we presume that identification of the sphere organizer sequence will be of equal importance for understanding sphere function.

In addition to providing a tool for the direct characterization of spheres, the work presented here provides some useful insight into the possible function of spheres. First, the observation that SPH-1 is present in somatic cells, as well as oocytes, suggests that the function carried out by spheres is not restricted to oocytes, and is therefore likely to be general in nature. Second, preferential staining of the sphere core by mAbD7, which binds a family of pre-mRNA splicing factors called SR proteins (Zahler et al., 1992), suggests that RNA processing may occur in the sphere core. Consistent with this, mAbSCS5 (Fu and Maniatis, 1990) which binds the same set of SR proteins as mAbD7 (Neugebauer and Roth, unpublished observations), also stains the core of spheres (Wu et al., 1991). Finally, the observation that the antibody mAbH1 binds the cortex of spheres preferentially suggests that the sphere protein SPH-1 does not directly interact with SR proteins and is therefore unlikely to be involved in pre-mRNA splicing. The binding of the cortex by mAbH1 does suggest, however, that spheres may be a component of an RNP produced by spheres.

We thank Judith Roon for her extensive help in screening hybridoma cell lines and for the Xenopus K2 tissue culture cell line. We would also like to thank Seako Takano and Ed Chan and Zheng’s Wu and Joe Gall for providing their unpublished sequences of the full-length p80-coilin cDNA. We thank Ali Hammami Brivanlou and Doug Melton for the use of their Xenopus ovari cDNA library. We thank Karl Neugebauer, Janet Partridge, Laurie B. Roth, and Alan Zahler for their comments on the manuscript, as well as Tim Knight and Paul Goodwin for help with the production of figures.

R. S. Tuma was supported by National Research Service Award T32 GM07270. This work was supported by grant 42786-02 from the National Institutes of General Medical Sciences to M. B. Roth.

Received for publication 21 April 1993 and in revised form 3 June 1993.

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