IMMUNOLOCALIZATION OF THE KINESIN RELATED PROTEIN ($KRP_{85/95}$) IN THE MIDPIECE AND FLAGELLUM OF SEA URCHIN (STRONGYLOCENTROTUS DROEBACHIENSIS) AND SAND DOLLAR (ECHINARACHNIUS PARMA) SPERM.

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Kinesin and its relatives comprise a large superfamily of homologous, ATP-dependent microtubule motor proteins which are thought to play important roles in microtubule-mediated vesicular/organellar transport and/or in mitotic spindle establishment and dynamics (for recent reviews see Skoufias and Scholey, Curr. Op. Cell Biol. 5: 95-104, 1993; Saunders, Trends Cell Biol. 3: 432-437, 1993). The best characterized kinesin-like protein in sea urchins is $KRP_{(85/95)}$, a plus end directed, heterotrimeric, kinesin related microtubule motor protein recently purified from sea urchin eggs (Cole et al., J. Cell Sci. 101: 291-301, 1992; Cole et al., Nature 366: 268-270, 1993). The $KRP_{(85/95)}$ complex consists of an uncharacterized 115 kDa subunit, and the 85 kDa and 95 kDa motor subunits. One or both of these latter subunits show sequence homology with the kinesin-like proteins encoded by the mouse KIF3a gene, the Drosophila KLP4 gene and the Chlamydomonas FLA10 gene. Using subunit-specific anti-$KRP_{(85/95)}$ monoclonal antibodies provided by our collaborators Drs. Jonathan Scholey and Douglas Cole (University of California at Davis), we have determined that $KRP_{(85/95)}$ is present on membranous vesicle-like structures in the mitotic spindle of sea urchin embryos (Henson et al., Dev. Biol. 171:182-194, 1995), suggesting that $KRP_{(85/95)}$ may play a role in vesicle transport during mitosis.

Interestingly, recent studies have suggested that kinesin-like proteins may be important within flagella, a highly ordered microtubule-based structure previously thought of as the exclusive domain of the minus-end directed microtubule motor flagellar dynein. The Chlamydomonas FLA10 gene product is a kinesin-like protein, with homology to $KRP_{(85/95)}$, which appears to be involved in flagellar assembly and maintenance (Walther et al., J. Cell Biol. 126: 175-188, 1994). In addition the Chlamydomonas kinesin-like protein Klp1 localizes to one of the central pair microtubules in the axoneme of the flagellum (Bernstein et al., J. Cell Biol. 125: 1313-1326, 1994). The results of these recent studies prompted us to examine the expression and localization of $KRP_{(85/95)}$ in sea urchin and sand dollar sperm.

Sperm were shed from locally collected S. droebachiensis sea urchins and E. parma sand dollars by intracoelomic injection of 0.5 M KCl. For immunoblotting, pellets of whole sperm cells were either directly lysed in SDS sample buffer, or the cells were dounce and ultrasonically homogenized and then diluted in sample buffer. Some samples were treated with DNase in order to break up DNA and lower the viscosity of the samples. In addition, gel samples were generated of sperm tails obtained by forcing sperm suspensions through a 25 gage needle several times prior to differential centrifugation. All gel samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose. The nitrocellulose filters were then probed with either a mixture of anti-$KRP_{(85/95)}$ monoclonal antibodies (K2.2, K2.3, K2.5; see Cole et al., 1993. loc cit.) or with an affinity purified anti-115 kDa polyclonal antiserum, followed by treatment with the appropriate alkaline phosphatase conjugated secondary antibodies. For immunofluorescent staining, sperm cells adhered to poly-lysine coated coverslips were fixed with -20° C methanol plus 40 mM EGTA, incubated with either individual or mixes of anti-$KRP_{(85/95)}$ antibodies (at a concentration of 100 µg/ml each) or the 115 kDa antiserum, and then incubated in fluorophore conjugated secondary antibodies. In double labeling experiments sperm microtubules were labeled using either an anti-sea urchin tubulin polyclonal antiserum or an anti-alpha tubulin monoclonal. Other labeling experiments utilized a commercial anti-flagellar dynein intermediate chain monoclonal antibody (Sigma Chemical Co.), an anti-centrosome monoclonal antibody raised

against Drosophila intermediate filaments (graciously provided by Dr. Calvin Simerly, University of Wisconsin at Madison; see Schatten et al., Proc. Natl. Acad. Sci. USA 84: 8488-8492, 1987), the Hoechst dye 33258 to label nuclei, and the mitochondria-specific vital dye rhodamine 123. Fluorescent specimens were viewed on a Nikon Optiphot II epifluorescence microscope using a 60X (NA 1.4) planapo phase contrast objective lens, and 35 mm photographs were taken using Kodak TriX ASA 400 film.

Immunoblotting results indicated that sperm samples probed with the anti-$KRP_{(85/95)}$ antibodies contained immunoreactive proteins which comigrated with the 85 kDa and the 115 kDa subunits of control sea urchin egg samples (see Figure 1). These immunoreactive species were present in samples of whole sperm and isolated sperm tails.

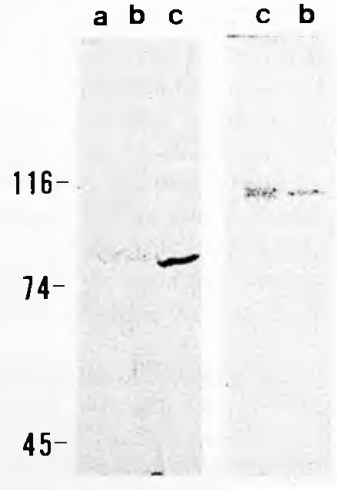

Figure 1: Immunblot of anti-$KRP_{(85/95)}$ monoclonals K2.2-2.5 (left panel) and the anti-115 kDa polyclonal (right panel) against samples from sea urchin sperm (lane a), sand dollar sperm (lane b) and sea urchin eggs (lane c). Molecular masses of standards are given in kDa on the left.

Immunofluorescent localization of $KRP_{(85/95)}$ in sperm revealed faint labeling of the flagellum and more intense staining of the midpiece (Figure 2). The midpiece is defined as the portion of the sperm lying between the nucleus and the flagellum which contains the centrosome, many mitochondria, and the majority of the sperm's cytoplasm. The midpiece-specific nature of the $KRP_{(85/95)}$ staining pattern was confirmed in cells triple labeled for $KRP_{(85/95)}$, microtubules and nuclei. In addition, the $KRP_{(85/95)}$ midpiece staining pattern localized to the same region that was labeled by either the centrosome-specific antibody, or the mitochondria-specific dye rhodamine 123.

The $KRP_{(85/95)}$ labeling of the midpiece and flagella often appeared punctate suggestive of an association with cytoplasmic vesicles. This pattern of punctate labeling was different from the more continuous, linear pattern seen in the flagella of sperm labeled with anti-flagellar dynein intermediate chain. An association between $KRP_{(85/95)}$ and membranous vesicles is also suggested by the loss of staining intensity seen in sperm extracted prefixation with Triton X-100 detergent under microtubule stabilizing conditions (see Wright et al., J. Cell Biol. 113: 817-833, 1991). Flagellar dynein labeling of sperm tails was not affected by prefixation extraction.

The results of the present study suggest that the kinesin-like heterotrimeric protein $KRP_{(85/95)}$ is associated with membranous structures in the midpiece and flagella of echinoderm sperm. It is interesting to note that a very recent meeting abstract (Kozminski et al., Mol. Biol. Cell 6: 253a, 1995) reports genetic and immunolocalization results which indicate that the $KRP_{(85/95)}$ homologue in Chlamydomonas, FLA10, is associated with the intraflagellar transport of granule-like particles. These results are consistent with our localization findings reported here. We are currently attempting to purify $KRP_{(85/95)}$ from sea urchin sperm in order to begin a biochemical characterization of the protein.

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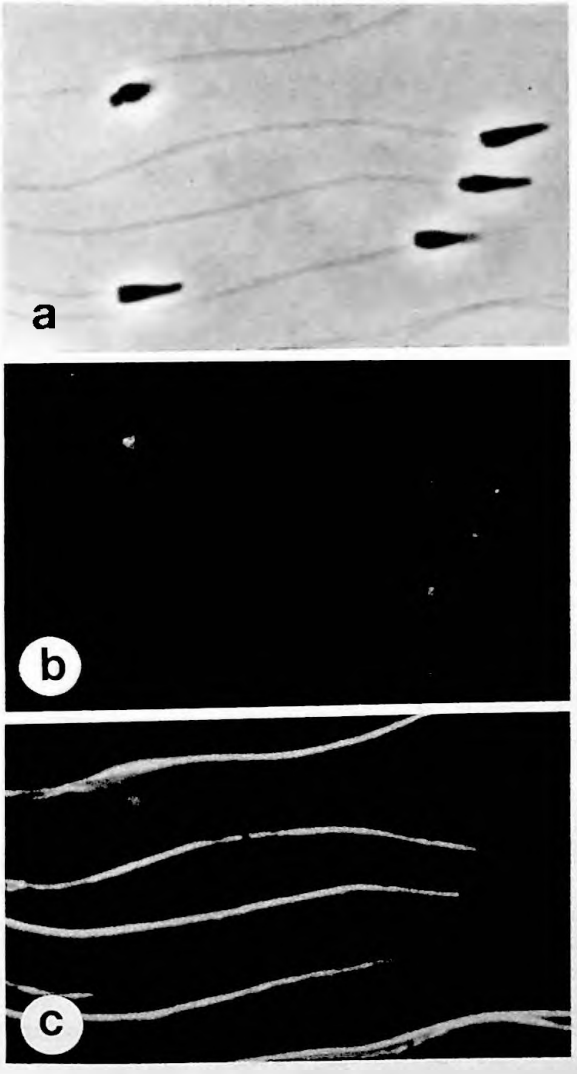

Figure 2: Immunofluorescent labeling of the $KRP_{(85/95)}$ 115 kDa subunit (panel b) and microtubules (panel c) in sand dollar sperm. Panel a shows a phase contrast image of the sperm. Note that the 115 kDa antiserum labels both the midpiece and the flagella of the sperm in a punctate manner. Magnification = 1,200X