INTRACELLULAR IMMUNOLOCALIZATION OF MOUSE AND RAT SP42 AND THE DEVELOPMENTAL PATTERN OF PROTEIN EXPRESSION IN MALE GERM CELLS

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The highly specialized structure of mammalian spermatozoa is the result of a complex process of molecular and structural cell organization which takes place mainly during the haploid phase of germ cell differentiation termed spermiogenesis. Spectacular shape changes concern both the nucleus and the cytoplasm with the remodeling of structures already present and the formation of novel structures; for both of these events synthesis of new proteins is required. It follows that the biochemical and molecular study devoted to the sperm's constitutive parts and proteins is a fundamental requisite for elucidating the biogenesis of the spermatozoon and its role in fertilization. Previous work from this laboratory has led to the identification and purification of a protein tyrosine kinase from ejaculated boar spermatozoa, termed *sp42*. More recently, with the development of specific antibodies directed to boar *sp42* we have demonstrated that the tyrosine kinase is a male germ cell-specific gene product with a presence extended to all the mammalian sperm cells studied, i.e., man, mouse and rat (Berruti and Borgonovo, 1996) and bull (our unpublished observations). Since protein tyrosine phosphorylation is an essential aspect of numerous signal transduction pathways, including cell growth and differentiation, studies devoted to a more detailed characterization of *sp42*, the first and so far unique cytoplasmic tyrosine kinase to have been found in mammalian spermatozoa, can represent an useful tool to better understand functions that are unique to germ cells. Here we report the developmental pattern of expression of *sp42* in mouse and rat testis and the immunocytochemical localization of the enzyme, that by in vitro kinase assays performed on the immunoprecipitated protein is resulted to be able to phosphorylate the synthetic substrate poly(Glu,Tyr), in intact and acrosome-reacted mouse and rat spermatozoa.

Testicular homogenates from newborn, prepuberal, puberal, and adult male mice and rats were obtained by direct homogenization of decapsulated testis in homogenization buffer by a Polytron homogenizer. Protein samples were subjected to SDS-PAGE followed by Western immunoblotting with *sp42* antibodies (Berruti and Borgonovo, 1996). Detergent protein extracts from mouse and rat epididymal spermatozoa were subjected to immunoprecipitation with *sp42* antibodies and the packed pellets of immune complexes were assayed for poly(Glu,Tyr)-phosphorylating activity. The incorporation of ^{32}P into the substrate was estimated as described by Berruti and Martegani (1989). Rat and mouse epididymal spermatozoa were experimentally induced to the acrosome reaction as described by Walensky and Snyder (1995). The acrosome-reacted spermatozoa, the acrosome reaction-released membranes and the acrosome reaction-soluble content were analyzed by SDS-PAGE and Western immunoblotting with *sp42* -antibodies to determine the sperm compartment where *sp42* localizes. Indirect immunofluorescence (IIF) analyses were carried out on smears of intact and acrosome-reacted epididymal mouse and rat spermatozoa, firstly incubated with *sp42* primary antibodies and then with fluoresceinated, anti-rabbit secondary antibodies.

Fig. 1 shows the developmental pattern of *sp42* expression in mouse testis: the specific immunosignal is detectable only at day 35 post-partum, with a maximum of intensity at day 90. A comparable pattern of expression was yielded by the rat *sp42* homologue. In vitro kinase assays carried out on the *sp42*-immunoprecipitated protein revealed a high rate of ^{32}P incorporation into poly-(Glu,Tyr). Western blots probed with *sp42*-antibodies of the three sperm's constitutive parts obtained after induction of the acrosome reaction showed that the protein is located into the acrosome-reacted spermatozoa, i.e., although *sp42* is an intracellular tyrosine kinase, it is not solubilized by the Ca^{2+}/A23187 sperm treatment. This last result is confirmed by the immunolocalization studies. As shown in Fig. 2, IIF analysis of mouse (a) and rat (b) intact spermatozoa localizes *sp42* in the sperm head and, at a much lower extent, in the sperm tail. The sperm head is compartimentalized in three major subregions: the acrosome, the perinuclear theca (PT), and the nucleus. As the *sp42* immunostaining illustrated in Fig. 2 seems to indicate, *sp42* is apparently localized at the level of PT. When acrosome-reacted mouse (Fig. 3 a) and rat (Fig. 3 b) spermatozoa are immunolabelled with *sp42* antibodies, a clear and intense immunosignal is elicited at the level of the sperm head by the structure that corresponds to the PT, which is the major

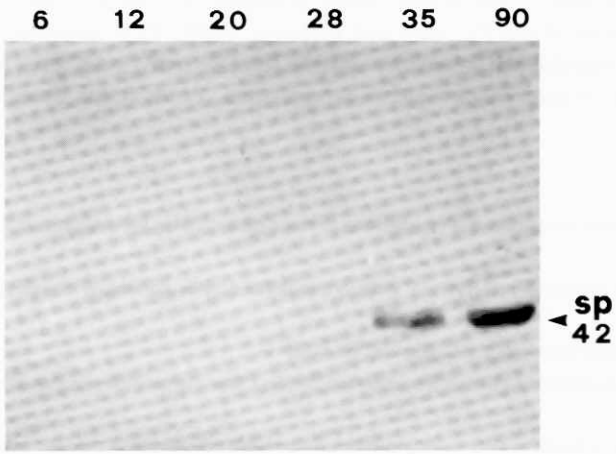

Figure 1 Developmental pattern of sp42 in mouse testis. Numbers indicate days post-partum.

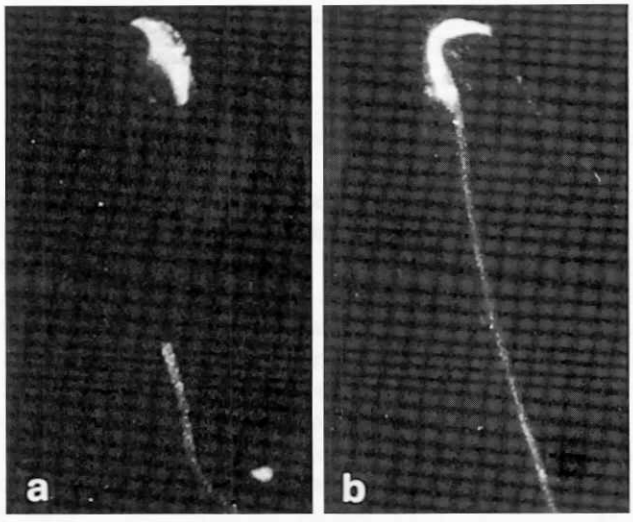

Figure 2 Localization of *sp42* by IIF in adult mouse (a) and rat (b) intact spermatozoa.

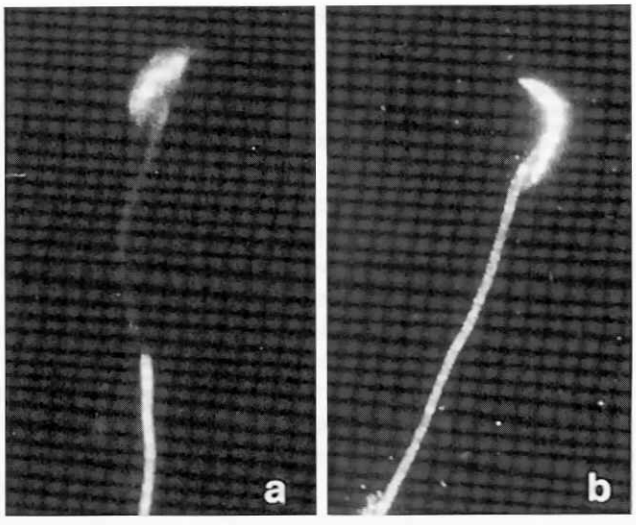

Figure 3 Localization of *sp42* by IIF in adult acrosome-reacted mouse (a) and rat (b) spermatozoa.

cytoskeletal element of the sperm head. Again, a positive, even if decisely weaker, *sp42* -staining can be seen at the level of the sperm tail, which in turn is subdivided in mid-piece, principal piece and terminal piece; it is to remember that, in contrast to cilia and flagellae, the sperm tail possesses besides to the central contractile axoneme additional massive cytoskeletal elements, i.e., the outer dense fibres (ODF) and the fibrous sheath (FS): here *sp42* seems to locate in the sperm tail.

Summarizing, our results show that the appearance-time of *sp42* in mouse and rat testis is coincident with the appearance into the seminiferous epithelium of the elongating spermatids, cellular expression of the last phase of spermiogenesis. This indicates that the protein is synthetized during the haploid phase of the male germ cell differentiation process; moreover, this finding together with the datum that *sp42* is subjected to storage being maintained in the mature spermatozoon suggests a possible involvement of the sperm tyrosine kinase in the sperm cell final development and/or fertilization. Mouse and rat homologues to boar *sp42* are here shown to be enzymatically active when immunoprecipitated from epididymal spermatozoa. Not only, but also the intracellular localization exhibited by both the mouse and rat enzyme is in agreement with a sperm cell-specific role of *sp42*. The apparent co-localization of *sp42* with distinct structural entities, which appear at the very end of spermatogenesis and represent the bulk of the mammalian sperm cytoskeleton, suggests that *sp42* may be involved in the control of sperm cytoskeleton arrangement in response to specific physiological stimuli. Intracellular tyrosine kinases, differently from the receptorial tyrosine kinases which are involved in the early response in the signal transduction pathway, act downstream mediating the intracellular transduction of the signal elicited by surface receptors without an intrinsic tyrosine kinase activity; two of the more studied intracellular tyrosine kinases, i.e., pp125 ^{FAK} and p56 ^{lck}, are known, when activated, to influence the cell cytoskeletal architecture. Recently reported molecular cloning of proteins of the PT (Oko and Morales, 1994), ODF (Morales et al., 1994) and FS (Fulcher et al., 1995) has established that these are cytoskeletal elements specific to male germ cells; thus the sperm-specific sp42 may be the tyrosine kinase involved in the control of sperm cytoskeleton organization. Work in this direction is in progress in our laboratory.

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