

Discussion

To date, cDNAs of several kinds of α -D-mannosidase have been cloned (Bause *et al.*, 1993; Bischoff *et al.*, 1990; Camirand *et al.*, 1991; Moremen and Robbins, 1991), but little homology has been found among them. In the present study, cDNA encoding a protein homologous to the porcine epididymis-specific 135kDa MAN2B2 was cloned from the mouse testis cDNA library. It was found that 62% of the amino acids in the whole amino acid sequence deduced from the cDNA sequence were identical in boar and mouse. Especially, 80% of amino acids 29~217 of the mouse homologue (mMAN2B2) were found to be identical to the corresponding part of porcine MAN2B2. Only in this region, a low but significant homology was observed between the mMAN2B2 and other types of α -D-mannosidase, viz. 40% homology with *Dictyostelium discoideum* α -D-mannosidase (MANA,M82822) with a 121 amino acid overlap, 38% with human (D63998) and mouse (X61172) α -D-mannosidase II with a 136 amino acid overlap. The homology suggests that these conserved domains have some common function in various types of α -D-mannosidase.

We also characterized the mMAN2B2 gene. The mMAN2B2 gene was

consisted of 19 exons and 18 introns. mMAN2B2 gene was assigned to Chr5 by FISH (Ohata *et al.*, 1997). It has been shown that porcine MAN2B2 gene is located on Chr8. It is suggested that the human counterpart of the MAN2B2 gene is one of the genes located on human Chr4, because both mouse Chr5 and porcine Chr8 have extensive synteny with human Chr4.

Porcine MAN2B2 was found to be an α -D-mannosidase which was synthesized and secreted predominantly by the principal cells in the regions between the distal caput and the proximal corpus epididymis. In the present study, we found by RT-PCR analysis that the other parts of the porcine epididymis and testis also expressed the MAN2B2 mRNA though the level was extremely low.

On the other hand, it was found that in mice the testis expresses the mMAN2B2 mRNA most significantly. Furthermore, by *in situ* hybridization it was shown that the mMAN2B2 mRNA is expressed in the germ cells adjacent to the basement membranes of seminiferous tubule at stages IX~XI. The type of the germ cells in this part of the seminiferous tubule at stage IX~XI are spermatogonia and leptotene spermatocytes. It was suggested that the mMAN2B2 mRNA is expressed in spermatogonia rather than leptotene spermatocytes by the following reasons. The mMAN2B2 mRNA was detected in the seminiferous tubules at the stages from IX to VI in the next cycle, although the mRNA signal was

decreased in stages I ~VI. The type A spermatogonia in stage IX develop into the type B spermatogonia in the stages I ~VI, which are still bordered on the basement membranes of the seminiferous tubule. On the other hand, the leptotene spermatocytes develop into the early pachytene spermatocytes, which are detached from the basement membranes. Even in stages I ~VI, the mMAN2B2 mRNA was located in the germ cells adjacent to the basement membranes. These results indicate that the mMAN2B2 mRNA is specifically expressed in type A spermatogonia at stages IX ~ XI and was detected till they develop into type B spermatogonia. But the possibility that the mMAN2B2 mRNA is expressed in spermatocytes is not fully denied. It is very interesting that the mMAN2B2 mRNA is expressed when the stem cell type spermatogonia just enter into the spermatogenesis, but it is unknown whether mMAN2B2 has any role to play in the differentiation of germinal stem cells into sperm.

We found that the mMAN2B2 protein was localized into the acrosome with the progress of spermatogenesis. The signal of mMAN2B2 was first observed as a small granule, the acrosomal granule, lying near the nucleus in round spermatids at Steps 2~3. But no mMAN2B2 signal was observed either in spermatogonia, spermatocyte nor somatic cells. The mMAN2B2 signals were located in the acrosome throughout the course of the formation of acrosome, representing the specific shape of the

acrosome at each step of the spermiogenesis. So, mMAN2B2 can be used as a marker for determining the step of the development of spermatid.

As mentioned previously, the mMAN2B2 mRNA was detected in spermatogonia but not in spermatid where the mMAN2B2 protein was first detected by the anti mMAN2B2 antibody. Two possible explanations for the time lag between transcription and translation can be made : (1) the mMAN2B2 mRNA may be trapped in the chromatoid body which keeps the synthesized mRNA inactive till spermatid and are not detected by *in situ* hybridization (Fawcett *et al.*, 1970 ; Moussa *et al.*, 1994 ; Oko *et al.*, 1996). (2) Although mMAN2B2 has been synthesized in spermatogonia or spermatocyte, mMAN2B2 can not be detected by anti mMAN2B2 antibody until it is concentrated into the acrosomal granule in the round spermatid at steps 2~3. The exact reasons for the time lag has not been clarified yet, but it is known that the synthesis of some protein is delayed from the transcription of their mRNA in germ cells (Tsunekawa *et al.*, 1999).

The present study showed that the monospecific antibody against mMAN2B2 reduced the fertilization rate 21% from that in the absence of the antibody, whereas the normal rabbit IgG had no effect (Data not shown). This result suggests that the inhibitory effects caused by the antibody were not due to the merely steric hindrance by the immunoglobulin molecules in sperm-egg interaction but due to the specific

interaction between mMAN2B2 and the antibody. This indicates that mMAN2B2 plays an important role on fertilization in mouse as well as in boar. In this connection, it is a very interesting report that the number of sperm-bound per egg was inhibited in rat by an antibody against the sperm surface α -D-mannosidase, α -D-mannose and methyl- α -D-mannoside (Yoshida-Komiya *et al.*, 1999). It was also found that the rates of fertilization in the presence of α -D-mannose or methyl- α -D-mannoside were 74.6% or 76.6%, respectively, which were similar extent of reduction of the fertilization rate induced by the anti mMAN2B2 antibody. It has been indicated that ZP3, one of the glycoprotein components of ZP which highly contains mannose residues in its sugar chains, is the primary ligand for sperm (Mortillo and Wassarman, 1991 ; Benoff *et al.*, 1997b ; Tulsiani *et al.*, 1992 ; Shalgi *et al.*, 1986 ; Sinowatz *et al.*, 1998 ; Akatsuka *et al.*, 1998). Furthermore, those mannose residues of ZP3 were shown to act as a ligand for the sperm surface α -D-mannosidase. All these results suggest that α -D-mannosidases including MAN2B2 are one of the important molecules in sperm involved in the interaction between sperm and oocyte.

In addition to α -D-mannosidase, human and bull spermatozoa are reported to possess a mannose-binding protein (Benoff *et al.*, 1993b ; Benoff *et al.*, 1993c ; Revah *et al.*, 2000). The mannose-binding protein appears on the sperm head after the incubation under the conditions for

inducing the capacitation. Especially, in human spermatozoa, it is suggested that the mannose-binding protein migrates from sub-plasma membrane space to an integral membrane position accompanied with the loss of cholesterol from the sperm membrane and plays important roles on fertilization. In this connection, we have recently found the 16kDa cholesterol-binding protein (ChBP) in the porcine epididymal fluid, which specifically binds free cholesterol and decreases the cholesterol contents in the sperm plasma membranes (Appendix II). It was also found that ChBP was expressed in the mouse female reproductive organs such as ovary, oviduct and uterus in addition to the epididymis. The expression of ChBP in the oviduct where sperm undergo the capacitation is dependent on the estrous cycle ; high in estrus and metestrus whereas low in diestrus (unpublished data). These results indicate that ChBP is involved in the processes conferring fertilizing abilities on sperm : epididymal maturation and capacitation, through regulating the cholesterol contents in the plasma membranes.

Although the molecular mechanisms involved in the function of mMAN2B2 on fertilization have not been elucidated yet, it is likely that the mMAN2B2 is relocalized to the sperm surface where sperm interact with oocyte. In hypothesis, mMAN2B2 is relocalized to the equatorial segment of sperm surface during the acrosome reaction and mediates sperm-egg interaction and ChBP enables mMAN2B2 relocation by

reducing cholesterol contents of sperm plasma membranes. Indeed, it has already been found that in boar MAN2B2 is localized to the small area in the equatorial segment of the mature sperm (Okamura *et al.*, 1992). It is also possible that mMAN2B2 enables sperm to pass through the zona pellucida by digesting carbohydrate moieties of ZPs. Further study must be done to elucidate the exact mechanisms for the mMAN2B2 function on fertilization. Along with this line, we intend to produce mMAN2B2 knock out mouse. We have already prepared the targeting vector as shown in Fig.15. Analysis of the mMAN2B2 knock out mouse is the next step of this study.