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Targeted Disruption of the Cation-Dependent or Cation-Independent Mannose 6-Phosphate Receptor Does Not Decrease the Content of Acid Glycosidases in the Acrosome

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ABSTRACT: The acrosome is a unique organelle containing acid hydrolases common to lysosomes as well as unique enzymes. Its ultimate exocytosis, as well as the absence of several lysosomal markers, has led to the speculation that it should be considered a secretory or zymogen vesicle rather than a specialized lysosome. The basic targeting machinery for eukaryotic lysosomal acid glycosidases are the two mannose 6-phosphate receptors. Mouse testicular germ cells are known to express both the cation-independent (CI-MPR) and cation-dependent (CD-MPR) forms of the mannose 6-phosphate receptors, but the CD-MPR is predominant. In this report, we utilized the recent targeted disruption of the CD-MPR and CI-MPR genes to determine whether these mutations affect targeting of acid glycosidases to the acrosome. Antibody to luminal fluid β-D-galactosidase was used to examine the targeting of immunoreactive product within the acrosome of permeabilized spermatozoa and testicular spermatids. No obvious changes in acrosomal immunoreactivity in either MPR homozygous mutant were observed when compared with the case of wild-type littermates. In addition, targeted disruption of either MPR did not result in decreased levels of β-D-galactosidase, α-D-mannosidase, or *N*-acetylglucosaminidase activities in spermatozoa from either MPR-homozygous mutant. These results suggest that the targeted disruption of either MPR does not result in decreased acrosomal targeting efficiency.

Key words: Acrosomal targeting, acrosomal enzymes, testicular germ cells, mouse.

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The acrosome is a unique organelle, found only in spermatids and spermatozoa, that plays an important role in fertilization (Yanagimachi, 1994). Its development occurs in 3 phases during the 14.4 days of spermiogenesis (Oakberg, 1956). In the first phase (Golgi phase), small granular vesicles (proacrosomic granules) from the trans-Golgi network coalesce into a large vesicle containing the acrosomic granule, which attaches to the nuclear envelope, forming the head cap. In the second phase (cap phase), the head cap enlarges by continuous addition of material coming from the Golgi apparatus. In the phase of spermatid elongation, the Golgi apparatus separates from the acrosomic system, marking the end of glycoprotein transport into the acrosome by the Golgi pathway (Clermont and Tang, 1985). However, late spermatids appear to possess an extra-Golgi pathway, permitting a direct protein transport from the endoplasmic reticulum (ER) to the acrosome (Tanii et al, 1992a,b). In addition, spermatids appear to possess a limited endocytic pathway to the acrosome (West and Willison, 1996). Despite recent advances in studying the nature of acrosomal biogenesis, the molecular mechanisms involved in the intracellular trafficking of acrosomal proteins are largely unknown.

The acrosome has been considered to be a lysosome-like structure because of its acidic pH and its glycosidase content, similar to that found in lysosomes (Allison and Hartree, 1970; Bellve and O'Brien, 1983; Yanagimachi, 1994). However, recent studies have questioned this identification and suggested that the acrosome should be considered analogous to a secretory granule (Noland et al, 1994; Martinez-Menarguez et al, 1996a; Foster et al, 1997). Sorting of proteins to lysosomes has been extensively studied (for review, see Kornfeld, 1992; Hille-Rehfeld, 1995; Ludwig et al, 1995). Newly synthesized lysosomal enzymes are carried from their site of synthesis, the ER, through the Golgi apparatus, where they acquire phosphomannosyl residues that serve as high-affinity ligands for binding to mannose 6-phosphate receptors (MPR). The ligand-receptor complexes are sequestered into clathrin-coated pits and bud from the trans-Golgi network in coated vesicles that deliver the ligand–receptor complexes to the endosomes, where they are dissociated by the low pH. The ligand is packaged into the lysosomes, and the receptors recycle back to the trans-Golgi

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network or the plasma membrane. Two receptors have been identified: the cation-dependent (CD-MPR) and the cation-independent MPR (CI-MPR).

Within somatic cells, the CD-MPR targets M6P glycoproteins to lysosomes, whereas the CI-MPR is a multifunctional receptor with separate binding sites for both M6P-containing ligands and the growth factor IGF-II in its extracytoplasmic domain. It binds M6P glycoproteins within the cells for lysosomal targeting and at the cell surface, where it mediates endocytosis of M6P glycoproteins (Kornfeld, 1992; Hille-Rehfeld, 1995). Targeted disruption of either CI-MPR or CD-MPR in cultured cells results in secretion of newly synthesized lysosomal hydrolases in the medium (Ludwig et al, 1993, 1994). Mice homozygous for a disrupted CD-MPR gene hypersecrete lysosomal enzymes in body fluids, but the tissue content remains in the normal range, suggesting that the secreted lysosomal enzymes may be recaptured by CI-MPR-mediated endocytosis (Ludwig et al, 1993). Because the CI-MPR also functions as the receptor for IGF-II, targeted disruption of this receptor results in perinatal death, associated with high levels of IGF-II (Wang et al, 1994; Ludwig et al, 1996), in addition to lysosomal targeting defects determined in vitro (Wang et al, 1994). In order to study the targeting defect in vivo, mice with this targeted disruption have been crossed with mice with a targeted disruption of the gene encoding IGF-II (Ludwig et al, 1996). The lethality and complex system abnormalities of CI-MPR null mice are rescued completely when bred onto an IGF-II null background, although the lysosomal targeting deficiencies remain (Ludwig et al, 1996). The creation of these IGF-II/CI-MPR null mice has allowed for the further study of the role of the CI-MPR in vivo.

It should be noted that M6P-independent modes of targeting are also present in somatic cells. For instance, fibroblasts from CI-MPR/CD-MPR-homozygous mutant embryos demonstrate a ''massive missorting of multiple lysosomal enzymes and accumulate undigested material in their endocytotic compartments'' (Ludwig et al, 1994). However, these cells, deficient in both M6P receptors, still transport low levels of hydrolases to lysosomes. The authors have suggested a M6P-independent pathway to explain targeting of the enzymes. This suggestion has also been used to explain the normal levels of some enzymes within the lysosomes of certain cell types of mucolipidosis II (I-cell disease) patients, a disorder that is attributed to a defective or deficient form of the phosphotransferase required for the biosynthesis of the M6P-containing recognition marker on lysosomal enzymes (von Figura and Hasilik, 1986).

The generation of CD-MPR (Köster et al, 1993; Ludwig et al, 1993) and CI-MPR (Wang et al, 1994) homozygous mutants has prompted studies to examine the role that these receptors play in targeting of acid glycosidases. Although several tissues from CD-MPR mutant mice are reported to contain a normal content of functional acid glycosidases (Ludwig et al, 1993), embryonic fibroblasts isolated from CD-MPR and CI-MPR mutant mice have shown significantly decreased levels of several lysosomal enzymes (Ludwig et al, 1993; Wang et al, 1994).

Both receptors have been found in testicular cells (O'Brien et al, 1989, 1994; Li and Jourdian, 1991). The CD-MPR predominates in germ cells and the CI-MPR in Sertoli cells, suggesting that the CD-MPR may be involved in targeting glycosidases to the acrosome (O'Brien et al, 1989, 1994). The availability of CD-MPR (Ludwig et al, 1993) and CI-MPR (Ludwig et al, 1996) homozygous mutant mice gives us the tools to determine whether either MPR is required for acrosomal biogenesis.

In this paper, we report enzyme activities of 3 acid glycosidases in spermatozoa and immunocytochemical localization of β-D-galactosidase, an acrosomal enzyme, in spermatids and spermatozoa from CD- and CI-MPR homozygous mutants and control animals. Results show that the disruption of either MPR did not decrease acid glycosidase content or localization in these haploid cells.

Materials and Methods

Materials

Male CD-MPR homozygous mutants and wild-type littermates (8–9 weeks old) have been described by Ludwig et al (1993). The genetic background of these mice is a mixture of the 129 and C57b1 mouse strains. Both of these strains were tested for enzyme levels of sperm β-D-galactosidase, and considerable difference was observed (data not shown). Therefore, we decided to obtain and compare (−/−) and (+/+) littermates to control for interstrain variability. Those used for enzyme assays were generated by backcrossing homozygous CD-MPR (−/−) mutants to C57β1/6 mice and intercrossing heterozygous F1 offspring. Because both substrains showed identical patterns of indirect immunofluorescence, those presented were obtained from matings of homozygous mutant mice. For control studies, male mice (129/Ola strain, 7–9 weeks old, and C57β1/6 strain, 7 weeks old) were purchased from Harlan UK Ltd (Oxon, United Kingdom) and from Jackson Labs (Bar Harbor, Maine), respectively.

For the CI-MPR analysis, mice were obtained from Dr T. Ludwig (Ludwig et al, 1996) that had a targeted disruption of both the IGF-II and CI-MPR genes and were used at approximately 8 weeks of age. Interestingly, in mice, the alleles for the gene for the CI-MPR (*Ifg2r*) are differentially expressed, such that disruption of the paternal and maternal alleles leads to drastically different results, a phenomenon known as imprinting (Wang et al, 1994; Ludwig et al, 1996). Mutants inheriting a disrupted paternal *Igf2r* allele are phenotypically wild type because this allele is not normally expressed in most tissues. However, mutants inheriting a disrupted maternal allele typically die perinatally or survive but are afflicted with organ defects and over-

growth because this is normally the only *Igf2r* allele expressed in most tissues (Wang et al, 1994; Ludwig et al, 1996). Therefore, to distinguish between inactivated maternal and paternal alleles, heterozygotes expressing the paternal allele (phenotypically wild type) will be denoted (+/−p), and those expressing the maternal allele (phenotypically knockout) will be denoted (+/−m).

All mice were housed under constant (12:12) lighting conditions and were fed and watered ad libitum. Animals were killed by cervical dislocation, and testis and/or epididymis were immediately retrieved and processed as described in each experiment.

Bovine serum albumin (BSA), benzamidine, and Dulbecco phosphate-buffered saline (PBS) were from Sigma (St Louis, Mo). Phosphate-buffered saline was prepared from a 10× stock solution, and the pH was adjusted as directed. Fluorescein-linked goat anti-rabbit IgG was obtained from EY Laboratories, Inc. (San Mateo, Calif). Vectashield fluorescence mounting media was from Vector Laboratories (Burlingame, Calif).

Preparation of Antiserum Against Rat Epididymal Luminal Fluid β-D-Galactosidase

A female virgin New Zealand White rabbit was immunized with purified epididymal luminal fluid β-D-galactosidase according to Tulsiani et al (1993). Blood was collected by cardiac puncture after 3–4 immunizations (on day 87) and serum prepared. Fifteen μL of immune serum immunoprecipitated 91% of β-D-galactosidase activity from luminal fluid and spermatozoa extracts containing 0.1 units of the enzyme activity. The monospecific IgG fraction (see below) was prepared from this antiserum.

Immobilization of β-D-Galactosidase and Preparation of Monospecific Anti-β-D-Galactosidase IgG

Rat cauda epididymal luminal fluid, prepared as described by Skudlarek et al (1992), was used for purification of β-D-galactosidase by using an affinity column of *p*-amino phenyl β-D-thiogalactopyranoside agarose, as described by Tulsiani et al (1995). The purified enzyme (~1.22 mg protein) was coupled to 0.3 g of cyanogen bromide–activated Sepharose 4B according to manufacturer's instructions.

Monospecific IgG was prepared from rabbit anti-β-D-galactosidase serum. Briefly, the antiserum was centrifuged at 130 000 g for 30 minutes at 4°C, and the clear supernatant was brought to loading buffer condition (buffer A). All other steps were carried out at 0–4°C. The sample was applied to the immobilized β-D-galactosidase column at a flow rate of 1–2 mL/h. The column was then washed with the above buffer until the column fractions showed negligible absorbance at 280 nm. The bound IgG was eluted with 7 M urea in buffer A, concentrated in an Amicon unit as above, and stored frozen at −80°C until used. The monospecific antibody, when used in immunoblotting protocols, cross-reacted with all molecular forms of β-D-galactosidase present in epididymal luminal fluid.

Preparation of Preabsorbed (Negative Control) IgG

A 50-fold excess of purified β-D-galactosidase was used to complex γ-globulin from the monospecific anti-β-D-galactosidase IgG preparation. The mixture was kept at 4°C, and the precipitated complex was removed by centrifugation at 100 000 g for 30 minutes. The supernatant was recovered, and the protein concentration was adjusted as desired.

Preparation of Spermatozoa

The cauda epididymidis was dissected free of fat, minced in PBS containing 0.1% BSA and 0.025 M benzamidine, and shaken for 15–20 minutes at room temperature. The minced tissue was passed through 16 layers of cheesecloth and centrifuged at 400 × *g* for 10 minutes at room temperature. The supernatant was removed by aspiration and saved. The pelleted spermatozoa were resuspended in approximately 0.5 mL PBS, counted with a hemacytometer, and either frozen at −80°C for enzymology or processed immediately for indirect immunofluorescence.

Preparation of Mixed Testicular Germ Cells

Testes from homozygous mutant and wild-type founder strain (129/Ola, and C57B1/6) mice were perfused gently through the testicular artery with PBS, pH 7.5; the tunica albuginea was removed; and the tissue was shaken for 15–20 minutes at 33°C in PBS, pH 7.5, containing 40 mg/mL collagenase, with occasional pipetting with a plastic transfer pipette. The suspension from this digestion, containing germ cells, Leydig cells, and other interstitial cells was spun at 400 × *g* for 10 minutes and resuspended in PBS, pH 7.5, containing 0.1% BSA. The cell suspension was washed 1 more time, as above, and then processed for indirect immunofluorescence.

Indirect Immunofluorescence

Spermatozoa or mixed testicular germ cells were centrifuged at 400 × *g* for 10 minutes at room temperature and resuspended in approximately 0.5 mL PBS (1% BSA). The sperm suspension was placed onto poly-L-lysine–coated coverslips (100 μL each) and allowed to attach for 15 minutes in a humid, sealed container. Alternatively, a sperm suspension was fixed in 4% paraformaldehyde on ice, spun at 400 × *g* for 5 minutes, resuspended in PBS containing 1% BSA, and allowed to attach to coverslips for 40 minutes. All further incubations also occurred in this container. Some coverslips were then placed in methanol at −20°C for 15 minutes for permeabilization. These were then washed twice in PBS containing 0.1% BSA for 3 minutes each, and all coverslips were incubated with primary antibody (monospecific anti-β-D-galactosidase IgG), preabsorbed IgG, or preimmune IgG at a concentration of 7 μg protein/mL for 60 minutes. These were washed as above and incubated with secondary antibody (fluorescein-linked goat anti-rabbit IgG) at a 1:100 dilution in the dark for 60 minutes. After washing as above, the coverslips were mounted with Vectashield fluorescence mounting media and viewed with a Zeiss Axiophot microscope equipped for epifluorescence.

Enzyme Assay

Samples were assayed for glycosidase activities using 5 mM of the respective *p*-nitrophenyl-substrate in a standard incubation mixture (0.5 mL total volume) containing 0.2% Triton X-100. For measuring β-D-galactosidase activity, 10 mM citrate buffer (pH 3.5) was used. For measuring *N*-acetylglucosaminidase activity, 10 mM acetate buffer (pH 4.5) was used. For measuring

α-D-mannosidase activity, 10 mM acetate buffer (pH 4.4) was used. The assay mixture was incubated for 60 minutes at 37°C and was stopped by the addition of an alkaline buffer adjusted to pH 10.7 (Tulsiani et al, 1977). The release of *p*-nitrophenol was measured by reading the spectroscopic absorbance at 400 nm. One unit of enzyme activity catalyzes the release of 1 μM *p*-nitrophenol per hour.

Results

Immunolocalization of β-D-Galactosidase in Mouse Germ Cells and Epididymal Spermatozoa

Figure 1 demonstrates the typical intracellular staining pattern seen in >90% of testicular spermatids and epididymal spermatozoa from wild-type mice. The immunolocalization studies carried out in both parental strains (129/Ola, C57B1/6; Figure 1b) as well as in CD-MPR (results not shown) and CI-MPR wild-type littermates (Figure 3b) showed similar immunoreactive patterns and intensities. Cauda epididymal spermatozoa stained in a prominent thin crescent shape, corresponding to the anterior portion of the sperm head (Figure 1a and b). This pattern did not occur when the primary IgG was preabsorbed with 50-fold excess of purified β-D-galactosidase (Figure 1c and d), nor with preimmune IgG or when the methanol treatment (permeabilization) step was omitted (data not shown). Spermatids from mixed–germ cell preparations also demonstrated a crescent-shaped immunostain corresponding to late Golgi, cap, and acrosome phase spermatids (Figure 1e and f). Elongated spermatids and testicular spermatozoa also fluoresced in a specifically acrosomal pattern (data not shown). All negative controls (preimmune or preabsorbed IgG, no permeabilization) resulted in the absence of acrosomal fluorescence (data not shown).

Figure 2 demonstrates the intracellular fluorescence pattern corresponding to the CD-MPR (−/−) mutation. Both cauda epididymal spermatozoa (panels a and b in Figure 2) and testicular spermatids (panels c and d in Figure 2) show the thin acrosomal crescent pattern seen in Figure 1. The same immunoreactive pattern was seen in spermatozoa from CI-MPR (−/−) mice (Figure 3) and spermatids (not shown). In all cases, negative controls, including preimmune, preabsorbed IgG or no permeabilization, resulted in the absence of acrosomal fluorescence (data not shown).

Enzyme Activities in Mouse Spermatozoa

To determine whether the enzyme levels were altered in spermatozoa from these mice, acid glycosidase activities were assayed. Three hydrolytic enzymes common to acrosomes and lysosomes were chosen. Figure 4 illustrates the levels of 3 acid hydrolases in spermatozoa from CD-MPR homozygous mutant and wild-type littermates. This figure demonstrates that the enzyme levels differed between the 2 groups to a variable extent, but the differences in all 3 enzymes tested were significantly different between homozygous mutant and wild-type mice when tested parametrically (*t*-test) or nonparametrically (Mann-Whitney test). In the legend to Figure 4, the results of the more conservative Mann-Whitney test are provided. Interestingly, in each case, the enzyme levels per 10^6 spermatozoa were greater in the CD-MPR (−/−) mice than in wild-type littermates. A 17% increase in β-D-galactosidase, a 63% increase in *N*-acetylglucosaminidase, and a 51% increase in α-D-mannosidase was seen in homozygous mutant males relative to controls.

Figure 5 illustrates the levels of the same 3 acid hydrolases in spermatozoa from CI-MPR homozygous mutant and wild-type mice. This figure demonstrates that CI-MPR homozygous mutant and wild-type mice yielded no significant differences in spermatozoa enzyme levels between (+/+), (+/−m), (+/−p), and (−/−) genotypes. Because of the genomic imprinting of the *Igf2r* gene (O'Brien et al, 1994), groups were pooled for statistical analysis according to phenotype (wild-type: +/+ and +/−p; knockout: +/−m and −/−).

Discussion

This study was undertaken to determine whether the targeted disruption of MPRs would lead to discernible changes in the acrosomal targeting of β-D-galactosidase in the testis. In order to assess acrosomal targeting, a qualitative analysis of β-D-galactosidase was performed with testicular germ cells and epididymal spermatozoa, as well as a quantitative analysis of β-D-galactosidase activity in epididymal spermatozoa. These studies demonstrated that disruption of the CD-MPR or the CI-MPR alone does not prevent the acrosomal localization of β-D-galactosidase. In addition, sperm enzyme levels of 3 acid hydrolases, β-D-galactosidase, α-D-mannosidase, and *N*-acetylglucosaminidase, were not decreased when compared with normal after MPR target disruption.

In the testis, both MPRs are present in germ cells and Sertoli cells, but in vitro, the germ cells (pachytene spermatocytes and spermatids) synthesize predominantly the CD-MPR and only low levels of CI-MPR, whereas the Sertoli cells synthesize predominantly the CI-MPR and little CD-MPR (O'Brien et al, 1989, 1994). This led these authors to suggest that the CD-MPR was more likely to function in acrosomal targeting. The fact that the levels of 3 hydrolases studied (β-D-galactosidase, α-D-mannosidase, and *N*-acetylglucosaminidase) are not decreased in CD-MPR homozygous mutant mice suggests that these

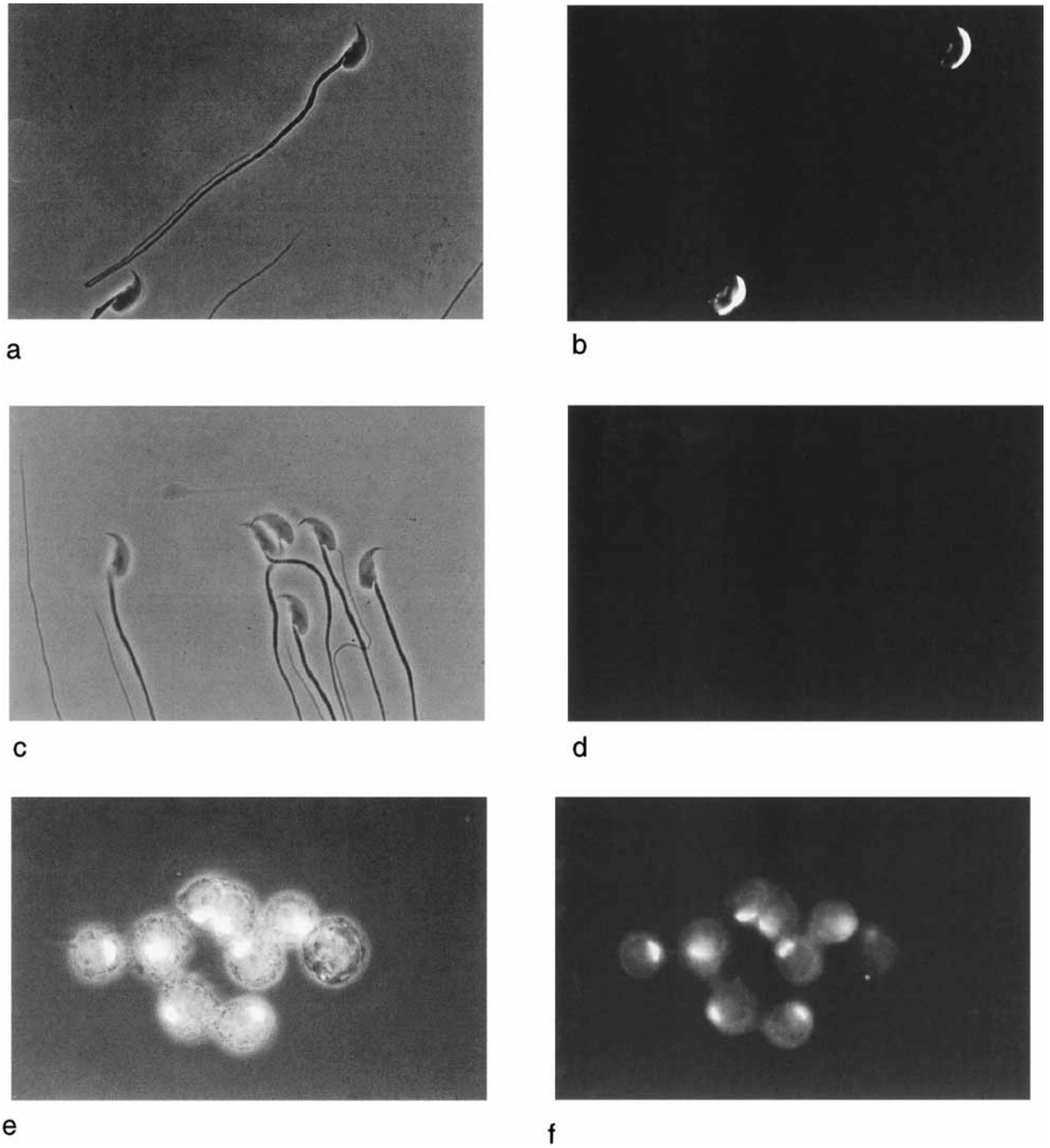

Figure 1. Indirect immunofluorescence photomicrographs of methanol-permeabilized cauda epididymal spermatozoa **(a–d)** and round spermatids **(e–f)**, from the 129/Ola mouse strain. Each matched set of pictures represents typical fluorescence patterns (625×). **(a, b)** Monospecific anti–β-D-galactosidase Ig: **(a)** phase contrast; **(b)** fluorescence. **(c, d)** Monospecific anti–β-D-galactosidase IgG preabsorbed with 50-fold–excess, affinity-purified β-D-galactosidase: **(c)** phase contrast; **(d)** fluorescence. **(e, f)** Monospecific anti–β-D-galactosidase IgG; **(e)** phase contrast and fluorescence; **(f)** fluorescence.

enzymes, and possibly other acrosomal constituents, are targeted independently of the CD-MPR.

The other obvious candidate for mediating acrosomal targeting is the CI-MPR. Murine testicular germ cells do normally express the *Igf2r* gene (encoding the CI-MPR), although at low levels compared with the case of Sertoli cells (O'Brien et al, 1994). Not only is the amount of the MPRs different, but the location of the receptors is different in germ cells and Sertoli cells. In Sertoli cells, the CI-MPR is present on intracellular membranes, whereas

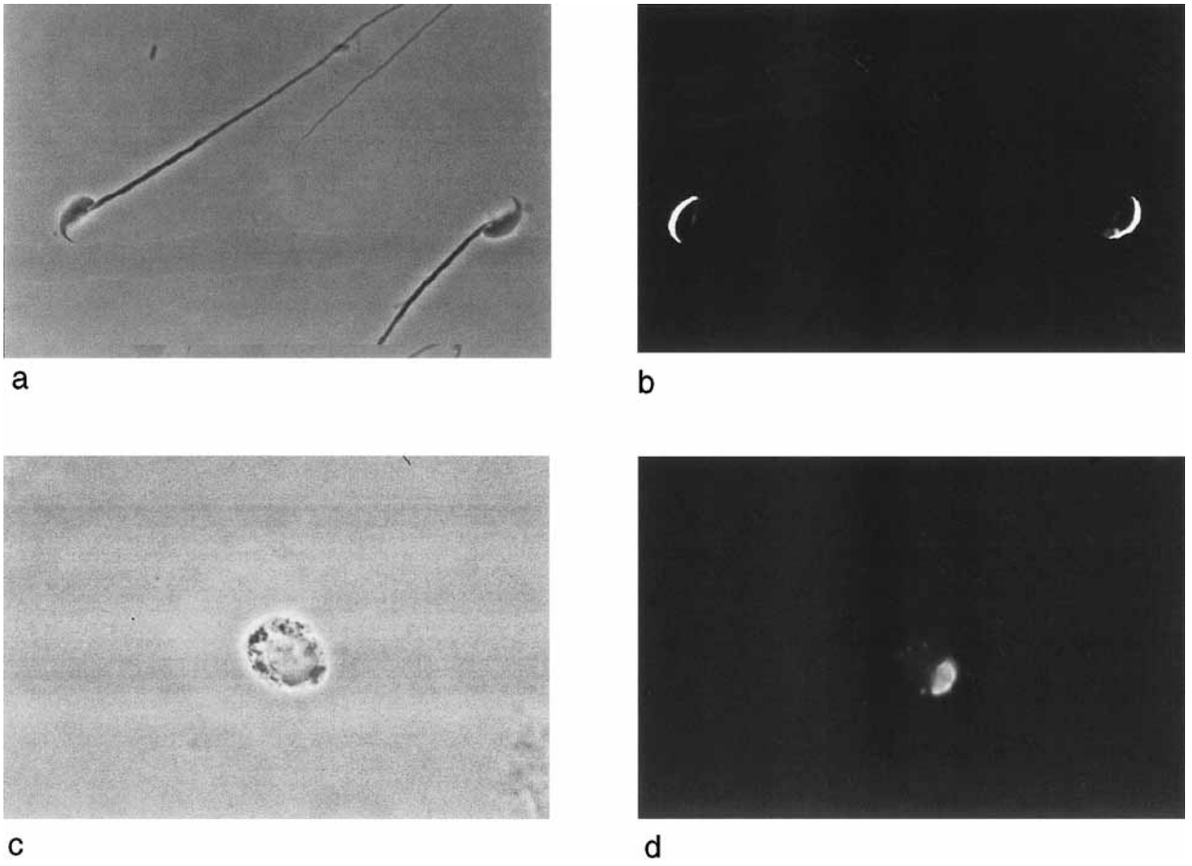

Figure 2. Indirect immunofluorescence photomicrographs of methanol-permeabilized cauda epididymal spermatozoa **(a–b)** and round spermatid **(c–d)** from the cation-dependent mannose 6-phosphate receptor (−/−) mouse strain (Ludwig et al, 1994). Each matched set of pictures represents typical fluorescence patterns (625×). **(a, b)** Monospecific anti–β-D-galactosidase IgG: **(a)** phase contrast; **(b)** fluorescence. **(c, d)** Monospecific anti–β-D-galactosidase IgG: **(c)** phase contrast; **(d)** fluorescence.

in germ cells, it is present predominantly on the cell surface (O'Brien et al, 1989), suggesting that the CI-MPR functions at the cell surface as a receptor for M6P-bearing ligands and IGF-II. This seems especially likely, given the dose-dependent increase in gene expression induced, in cultured germ cells, by IGF-II and M6P-containing ligands secreted by Sertoli cells (Tsuruta and O'Brien, 1995). These authors suggested that the function of the CI-MPR in germ cells is to mediate transmembrane signal transduction like other growth factor receptors. Whether or not the CI-MPR functions also in the endocytosis and targeting of M6P glycoproteins to the acrosome is controversial. Internalization and processing of radiolabeled M6P-ligands by spermatogenic cells has been demonstrated by O'Brien et al (1989). In vivo and in vitro studies with endocytic tracers have shown that spermatids are active in absorptive endocytosis and target tracers to endosome and lysosomes but not to the acrosome (Segretain, 1989; Segretain et al, 1992). West and Willison (1996) have confirmed that endocytic vesicles do not interact with the acrosome when spermatids are exposed in vitro to an endocytic marker. However, when the spermatids were cocultured with Sertoli cells, a small population of late spermatids transported the marker to the acrosome. This transport was inhibited by M6P, suggesting that there is a limited M6P-specific endocytosis pathway to the acrosome (West and Willison, 1996). Our study shows that spermatozoa from the CI-MPR homozygous mutant mice have normal levels of the 3 glycosidases studied, suggesting that the CI-MPR is not predominantly involved in acrosomal biogenesis.

It is possible that both the CD-MPR and the CI-MPR normally have overlapping functions in the targeting of acrosomal hydrolases and can compensate for each other when one is deficient. For example, it has been suggested that the normal concentration of hydrolases found in tissues on the CD-MPR–deficient mice could be explained by the CI-MPR–mediated endocytosis of the missorted enzymes (Ludwig et al, 1993). However, a single type of MPR is not sufficient for targeting proteins to lysosomes along the normal intracellular route (Ludwig et al, 1994; Pohlmann et al, 1995; Kasper et al, 1996; Munier-Leh-

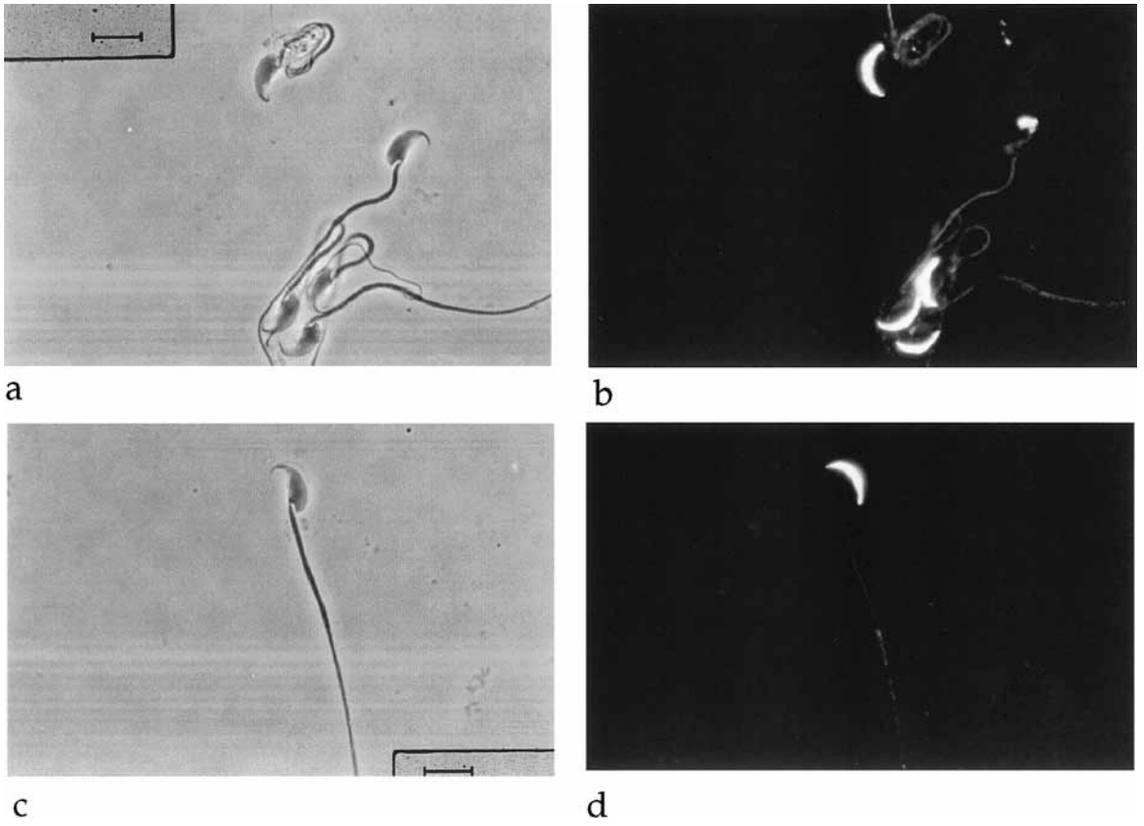

Figure 3. Indirect immunofluorescence photomicrographs of methanol-permeabilized cauda epididymal spermatozoa from the IGFII (−/−) cation-independent mannose 6-phosphate receptor (+/+; **a, b)** or (−/−; **c, d)** mouse strain. Each matched set of pictures represents typical fluorescence patterns (625×). **(a, b)** monospecific anti–β-D-galactosidase IgG: **(a)** phase contrast; **(b)** fluorescence. **(c, d)** monospecific anti–β-D-galactosidase IgG; **(e)** phase contrast; **(f)** fluorescence.

mann et al, 1996). For example, fibroblasts prepared from embryos that lack the 2 receptors exhibit a massive missorting of lysosomal enzymes, whereas those lacking either one of the MPRs are only partially impaired in sorting (Ludwig et al, 1994; Pohlmann et al, 1995). Overexpression of either MPR in these MPR-deficient fibroblasts only partially corrected lysosomal missorting (Pohlmann et al, 1995; Kasper et al, 1996; Munier-Lehmann et al, 1996). Comparison of the phosphorylated glycoproteins or the phosphorylated oligosaccharides of lysosomal enzymes secreted by fibroblasts deficient in either MPRs revealed differences, suggesting that the 2 receptors have different sets of ligands in somatic cells (Ludwig et al, 1994; Pohlmann et al, 1995; Munier-Lehmann et al, 1996). More important, in vivo, lack of the CI-MPR disrupts lysosome function to a greater extent than lack of the CD-MPR, and the pattern of serum Man-6-glycoproteins is different (Sohar et al, 1998). Finally, in vitro binding assays have shown that the ligand-binding specificities of the 2 MPRs are different and that the CD-MPR binds only a subset of the ligands bound by the CI-MPR (Sleat and Lobel, 1997). It thus appears that although the MPRs have complementary targeting functions, neither one can fully compensate for the absence of the other.

In germ cells, it is unclear whether the recently described limited-MPR endocytosis pathway to the acrosome (West and Willison, 1996) could account for the levels of hydrolases found in spermatozoa of CD-MPR–deficient mice. In murine testicular germ cells (as well as most tissues), both MPRs are expressed at quite distinct levels, leading to the speculation that they have distinct, nonoverlapping roles (O'Brien et al, 1989). The data shown in Figure 5 provide another line of evidence for nonoverlapping roles of the 2 MPRs because the levels of sperm hydrolases in this study were normal in the case of the CI-MPR disruption and statistically greater in the case of the CD-MPR disruption. The increase in sperm glycosidases in the CD-MPR homozygous mutant mice was unexpected because it has not been observed in other somatic cells and tissues. It does suggest that in germ cells, as in somatic cells, there is a disparity between the 2 MPRs because the measurable alteration in sperm hydrolase content differed for the 2 MPR disruptions. The

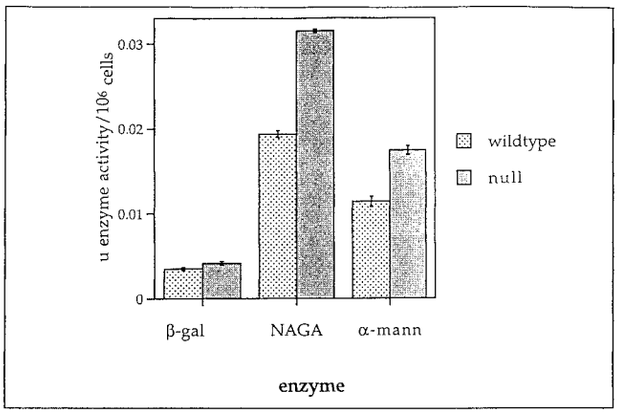

Figure 4. Comparison of spermatozoa enzyme levels in cation-dependent mannose 6-phosphate receptor (−/−) and wild-type (+/+) littermates. Spermatozoa were collected from 3 wild-type and 4 cation-dependent mannose 6-phosphate receptor (−/−) age-matched littermates, as described in "Materials and Methods." Spermatozoa were assayed in the presence of 0.2% Triton X-100. The number of spermatozoa added to the reaction mixture depended on the assay. For β-D-galactosidase, 8×10^5; for *N*-acetylglucosaminidase (NAGA), 4×10^5; and for α-D-mannosidase, 1×10^6 spermatozoa per tube were added. Assays were performed in 3 sets of triplicate for each enzyme, including enzyme blanks. The Mann-Whitney nonparametric test for small independent samples was performed by using Systat software. *P* values were as follows: for β-D-galactosidase, $<.05$; for *N*-acetylglucosaminidase (NAGA) and α-D-mannosidase, $<.005$.

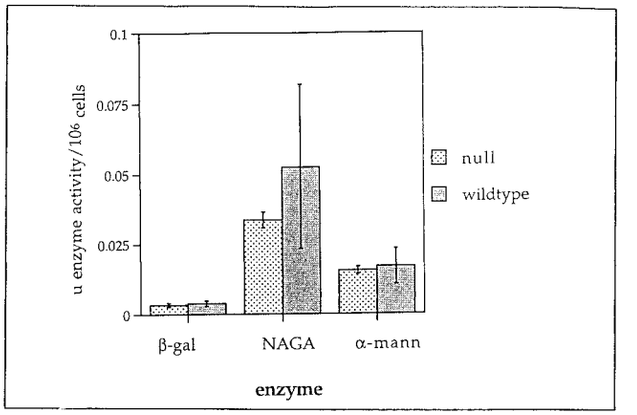

Figure 5. Comparison of sperm enzyme levels in CI-MPR homozygous mutant phenotype [pooled from (−/−) and (+/−m)] and wild-type phenotype [pooled from wild-type (+/+) and (+/−p)] littermates. Spermatozoa were collected from 2 wild-type and 2 homozygous mutant age-matched littermates as described in "Materials and Methods." Spermatozoa were assayed in the presence of 0.2% Triton X-100. The number of spermatozoa added to the reaction mixture depended on the assay. For β-D-galactosidase, 8×10^5; for *N*-acetylglucosaminidase (NAGA), 4×10^5; and for α-D-mannosidase, 1×10^6 spermatozoa per tube were added. Assays were performed in 3 sets of triplicate for each enzyme, including enzyme blanks. The Mann-Whitney nonparametric test for small independent samples was performed by using Systat software. *P* values were as follows: for β-D-galactosidase, $<.324$; for NAGA and α-D-mannosidase, $<.443$.

issue of complementarity of the MPRs in hydrolase targeting in both somatic and germ cells could be addressed by analysis of crosses between homozygous mutants for the CD- and CI-MPR. However, the double-homozygous mutant mice are severely compromised and usually die perinatally (Ludwig et al, 1996).

This study suggests that neither MPR receptor is required for the proper targeting of acrosomal hydrolases. Although the acrosome has been considered a lysosome-like structure (Allison and Hartree, 1970), it has several distinctive features. It is a large vesicle overlying the sperm nucleus with a distinct structural matrix that sequesters hydrolases into specific spatial domains (Olson et al, 1988; Peterson et al, 1992; NagDas et al, 1996). It contains components unique to the acrosome, such as acrosin or acrogranin (Meizel and Mukerji, 1975; Anakwe and Gerton, 1990), and lacks components found in lysosomes, such as the lysosomal membrane glycoproteins 1gp 120 and LAMP-1 (Martinez-Menarguez et al, 1996b). Because the acrosome undergoes exocytosis at the site of fertilization, it has been considered a regulated secretory granule (Eddy and O'Brien, 1994; Fraser, 1994; Noland et al, 1994; Foster et al, 1997). Labeling studies have shown that glycosylated molecules are incorporated into the Golgi apparatus and delivered to the developing acrosome during the early steps of spermiogenesis (Clermont and Tang, 1985). In addition, transport vesicles (clathrin and coat-protein vesicles) of the Golgi complex, which have been implicated in anterograde and retrograde protein transport in other cell types, have been identified in the Golgi complex of the spermatids (Griffiths et al, 1981; Martinez-Menarguez et al, 1996b; Thorne-Tjomsland et al, 1998). After exposure to Brefeldin A, a membrane traffic inhibitor, early rat spermatids exhibit ultrastructural acrosome alterations (Tanii et al, 1998). However, in addition to the Golgi pathway, it has been suggested that the acrosome possesses an extra Golgi pathway of protein sorting. Electron microscopy studies have shown that 1) in late spermatids, small vesicles fuse with the acrosomal membrane at a time when the Golgi complex has migrated away from the acrosome (Fawcett, 1974) and 2) an acrosomal antigen not only immunolocalizes in the acrosome and not the Golgi apparatus but does so during the terminal step of spermiogenesis, when the Golgi complex no longer contributes to the formation of the acrosome (Clermont and Tang, 1985). The fact that round spermatids from transgenic mice expressing a regulated secretory protein, human growth hormone, import the protein into the acrosome (Braun et al, 1989) strongly suggests that acrosomal biogenesis involves the secretory pathway of the spermatids, whether through a Golgi route, an extra-Golgi route, or both. Our results, taken together, agree with the speculation of a nonlysosomal origin of the acrosome.

Given the lack of a uniform model in the collective data on acrosomal biogenesis, it may be worthwhile to

consider the acrosome a hybrid between a lysosome and a secretory granule. This possibility has been suggested for a lytic granule, the specialized organelle of cytotoxic lymphocytes and natural killer cells (Griffiths and Isaaz, 1993). Like the acrosome, this acidic structure contains unique isozymes as well as lysosomal enzymes and fuses with the plasma membrane to release its contents upon appropriate extracellular stimuli. Phosphomannose labeling, as well as studies of T-cell lines from I-cell disease patients, suggests that both MPR-dependent and MPR-independent pathways to this organelle exist. Furthermore, the data suggest that the granzymes A and B correctly immunolocalize in these T-cell lines but at a diminished level, suggestive of multiple pathways for the same proteins (Burkhardt et al, 1989; Griffiths and Isaaz, 1993). These data have led to a model of flexibility in sorting secretory and lysosomal enzymes, such that a given protein may take alternate routes (MPR dependent and independent) to reach its target organelle. Given the dual nature of the acrosome, multiple targeting modes seem plausible.

In summary, our results imply that neither MPR is solely required for the acrosomal targeting of β-D-galactosidase and 2 other acrosomal hydrolases. These data suggest that either the acrosome does not utilize the biosynthetic transport pathways of lysosomal hydrolases or that the acrosome may use mechanisms involved in the targeting of secretory proteins. In somatic cells, the regulated secretory pathway has not been as clearly outlined as those directing hydrolases to lysosomes. Further studies will be required to determine whether acrosomal biogenesis relies primarily on the regulated secretory pathway, or whether the spermatid MPRs complement each other in their targeting functions.

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