Targeted Disruption of Intracellular Type I Platelet Activating Factor-acetylhydrolase Catalytic Subunits Causes Severe Impairment in Spermatogenesis

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Intracellular type I platelet activating factor-acetylhydrolase is a phospholipase that consists of a dimer of two homologous catalytic subunits α1 and α2 as well as LIS1, a product of the causative gene for type I lissencephaly. LIS1 plays an important role in neuronal migration during brain development, but the in vivo function of the catalytic subunits remains unclear. In this study, we generated α1−/− and α2−/− mice by targeted disruption. α1−/− mice are indistinguishable from wild-type mice, whereas α2−/− male mice show a significant reduction in testis size. Double-mutant male mice are sterile because of severe impairment of spermatogenesis. Histological examination revealed marked degeneration at the spermatocyte stage and an increase of apoptotic cells in the seminiferous tubules. The catalytic subunits are expressed at high levels in testis as well as brain in mice. In wild-type mice, α2 is expressed in all seminiferous tubule cell types, whereas α1 is expressed only in the spermatogonia. This expression pattern parallels the finding that deletion of both subunits induces a marked loss of germ cells at an early spermatogenic stage. We also found that the LIS1 protein levels, but not the mRNA levels, were significantly reduced in both subunit-deficient mice by targeted disruption. α1−/− and double-mutant mice, suggesting that the catalytic subunits, especially α2, are a determinant of LIS1 expression level.

Platelet-activating factor (PAF)1 is a potent signaling phospholipid involved in diverse physiological events, such as inflammation and anaphylaxis (1). In addition, PAF has been implicated in the central nervous system (2, 3) and the reproductive system (4, 5). PAF is hydrolyzed to an inactive metabolite by a specific enzyme called PAF-acetylhydrolase (PAF-AH). At least three types of PAF-AH exist in mammals, namely the intracellular types I and II (6, 7) and a plasma type (8). Intracellular type I PAF-AH (PAF-AH (I)) is an oligomeric complex. It contains a dimer of two homologous catalytic subunits, α1 and α2, and a non-catalytic β subunit (6, 9–11). Interestingly, the β subunit was later found to be identical to LIS1, the product of the causative gene for type I lissencephaly (10, 12). Type I lissencephaly is a genetic brain malformation showing a smooth cerebral surface without gyri, caused by abnormal neuronal migration at early developmental stages. Mice homozygous for the Lis1 null mutation die early in embryogenesis soon after implantation (13). Heterozygous and compound heterozygous mice have expression level-dependent defects in neuronal migration (13). A series of recent studies has suggested that LIS1 interacts not only with PAF-AH (I) catalytic subunits but also with a number of proteins, including tubulin (14), cytoplasmic dynein (15, 16), and NUDE (17–20). Through interaction with these proteins, LIS1 plays important roles in microtubule-associated cellular functions such as mitotic cell division, chromosomal segregation, and neuronal migration. In contrast, the biological role of the catalytic subunits of PAF-AH (I) remains a complete enigma. Nothwang et al. (21) have described a case of functional hemizygosity of α1, possibly responsible for the resulting mental retardation, ataxia, and brain atrophy in this patient. Furthermore, Lecointe et al. (22) have proposed that deregulation of transcription of the human α2 gene is associated with the development of a certain lymphoma. Therefore, the PAF-AH (I) catalytic subunit is also likely to play an important role in some pathological conditions. The α1 and α2 catalytic subunits belong to a novel serine esterase family (9). These subunits, which show ~60% amino acid homology with each other, form homodimers and a heterodimer. Ho et al. (23) have reported the x-ray crystal structure of the α1 homodimer. The folding is unique among known lipases and phospholipases. The structure unexpectedly resembles those of the G-protein family such as p21RNase and Go. To elucidate the in vivo function of the catalytic subunits of PAF-AH (I), namely α1 and α2, we generated mice lacking either one or both of these two proteins.

**EXPERIMENTAL PROCEDURES**

Generation of α1, α2 Mutant Mice—α1, α2 genomic clones were isolated from a mouse 129/SvJ genomic library in the Lambda FIXII vector (Stratagene). Targeting vectors were constructed for replacing part of exon 2 and 3 of the α1 and α2 genes, which include a translation initiation site and catalytic motif (GXXSV) with a PGKNeoP cassette (24). A PGKDTA (diphtheria toxin A fragment) cassette was inserted at the 3′-end of the short arm for negative selection. The targeting vectors were linearized and electroporated into ES cell line RW4 (Genome
Systems), which was cultured on neomycin-resistant mouse embryonic fibroblasts. G418-resistant colonies were screened for homologous recombiantants by PCR. Candidates of homologous recombinants were verified by Southern analysis using fragments at the 3’-ends of the genes, external to the targeting vectors as probes. Chimeric mice were generated as follows. a1−/− and a2−/− female mice were immunized with each purified recombinant rat protein with Freund’s complete adjuvant (DIFCO), followed by six boosters at 2-week intervals with 20 μg of protein and established monoclonal antibody, producing hybridoma cell lines as previously described (11). Monoclonal antibody against LIS1 (clone 338, a kind gift from Dr. O. Reiner, Weizmann Institute, Rehovot, Israel) and α-tubulin (clone DM1A, Sigma) were used for a Western blot analysis. Polyclonal antibody against LIS1 (N-19, Santa Cruz Biotechnology) was used for an immunohistochemistry staining of testis cross-sections of wild-type (C57BL/6J), a1−/− (C), and a2−/− (F) adult mice was performed using specific antibodies for a1 (A–C), a2 (D–F), and LIS1 (G and H). Hematoxylin was used for counterstaining. Scale bars: A, C, D, F, and G, 100 μm; B, E, and H, 25 μm.

**Fig. 2.** Localization of PAF-AH (I) subunits in mouse testis. Immunohistochemical staining of testis cross-sections of wild-type (A, B, D, E, G, and H), a1−/− (C), and a2−/− (F) adult mice was performed using specific antibodies for a1 (A–C), a2 (D–F), and LIS1 (G and H). Hematoxylin was used for counterstaining. Scale bars: A, C, D, F, and G, 100 μm; B, E, and H, 25 μm.

**Fig. 1.** Expression pattern of PAF-AH (I) subunits in mice. Expression of a1, a2, LIS1, and α-tubulin in various mouse tissues was examined by Western blotting using specific antibodies.
Reduced testis size in mutant mice

|               | Wild-type | a1−/− | a2−/− | a1−/−/a2−/− | a1−/−/a2−/+ | a1−/+/a2−/+ | a1−/+/a2−− |
|---------------|-----------|-------|-------|-------------|------------|------------|------------|
| Testes weight (mg) | 128.0 ± 6.9 | 126.0 ± 8.3 | 76.0 ± 9.0 | 95.3 ± 6.8 | 56.2 ± 4.6 | 45.6 ± 2.2 |
| Body weight (g)    | 16.7 ± 1.6 | 17.0 ± 2.0 | 16.1 ± 0.7 | 17.6 ± 0.8 | 16.4 ± 0.7 | 15.7 ± 0.7 |

Adult testis (Fig. 1). The expression levels of a2 and LIS1 were observed to be essentially proportional to that of α1-tubulin, a component of microtubules. Because LIS1 plays an important role in microtubule dynamics (14, 16), it can be postulated that both α subunits are also involved in this process.

Immunohistochemical staining of adult mouse testes revealed that a2 and LIS1 immunoreactivity was present in all seminiferous tubule cell types (Fig. 2, D and G). Intense staining of a2 and LIS1 was observed in meiotically dividing spermatocytes and elongating spermatids. In contrast, a1 staining was restricted to the cells lining the basement compartment of seminiferous tubules (Fig. 2A). Magnification revealed that a1 was specifically localized in spermatogonia cytoplasm (Fig. 2B, arrow), whereas a2 and LIS1 were expressed in the cytoplasm of all types of spermatogenic cells and Sertoli cells (Fig. 2, E and H), suggesting that a1 is involved specifically in proliferation and/or differentiation of spermatogonia. LIS1 was also localized at meiotic spindles of spermatocytes (Fig. 2H, arrowhead) and manchetttes of elongating spermatids (Fig. 2H, arrow), both of which are specific microtubule structures. No staining of α1 or α2 was detected in the seminiferous tubules of null mutant mice (Fig. 2, C and F).

We used homologous recombination in embryonic stem cells to generate mice lacking the a1 (Pafah1b3) and a2 (Pafah1b2) genes. Parts of exon 2 and exon 3 of each gene, including the translation initiation site and the catalytic serine residue, were replaced with a neomycin-resistance gene (Fig. 3A). Targeted embryonic stem cell clones and subsequent germ line transmissions were detected by PCR and/or Southern blot analysis (Fig. 3B). Both a1−/− and a2−/− mice were born with the expected Mendelian frequencies, viable and apparently indistinguishable from their wild-type littermates. Western blot analysis of the brain and the testis homogenates showed no immunoreactive bands in either a1−/− or a2−/− mice (Fig. 3A). a1−/−/a2−/− mice were also viable and apparently indistinguishable from wild-type mice. However, a1−/−/a2−/+ mice were found to be infertile, whereas female fertility was not affected. Testes weights of 5-week-old a1−/−/a2−/+ mice were significantly (−35%) smaller than those of wild-type mice (Table I). Testes weights were not noticeably reduced in a1−/− mice, whereas they were reduced to 60% in a2−/− mice (Table I). There was no significant difference in body weight among any of the genotypic combinations (Table I).

The histology of mutant testes was examined at age 5 weeks, the time when the first wave of murine spermatogenesis is completed. The seminiferous tubules of a1−/−/a2−/+ mice showed a 50% reduction in diameter, and spermatogenic cells were dramatically decreased (Fig. 4F) when compared with wild-type mice (Fig. 4A). Spermatocytes beyond the pachytene stage and round spermatids were significantly reduced in num-
Expression in B pairment of spermatogenesis (Fig. 4 12492ogenesis fails, leading to induction of programmed cell death in PAF-AH (I), the differentiation of prehaploid stages of spermatogenesis. Our results indicate that in the absence of the catalytic subunits of both /H9251 and/or /H9251 mice, LIS1 levels in both brain and testis were reduced to about 20% of the levels in the wild type mice. Because a large portion of LIS1 forms complexes with /H9251 and/or /H9251, we examined the LIS1 protein level in a1/+/a2/−/− mice and found that the level was reduced to about 20% of the normal level in a2/−/− mice (Fig. 6C). Although the exact molecular mechanism and function of the

**Fig. 4. PAS and hematoxilin staining of various 5-week-old mutant mice testis sections.** Wild-type (A), a1/−/− (B), a2/−/− (C), a1/−/−a2/−/− (D), a1/+/−a2/−/− (E), and a1/−/−a2/−/− (F) mice were examined. Note severe reduction of spermatogenic cells, especially after pachytene spermatocyte stage in a1/+/−a2/−/− (E) and a1/−/−a2/−/− (F) mice. Elongating spermatids were almost absent in the latter. Scale bar: 100 μm

**Fig. 5. Increased apoptosis in spermatogenic cells of a1/−/−/ a2/−/− mice.** Sections of seminiferous tubules of wild-type (A) and a1/−/−a2/−/− mice (B) at 5 weeks of age are shown. Apoptotic cells are labeled by TUNEL staining. Scale bar: 100 μm

**DISCUSSION**

In this study, we demonstrated that the catalytic subunits of intracellular PAF-AH (I) are involved in murine spermatogenesis. Our study gives new insights into the in vivo function of PAF-AH (I). We showed by Western blotting that in mice the catalytic subunits a1 and a2 as well as LIS1 are present at high levels in both brain and testis. Interestingly, it was noted that PAF-AH (I) subunits exhibit expression levels proportional to those of a-tubulin, a major component of intracellular microtubules. The above findings and the fact that LIS1 is a microtubule-associated protein lead us to speculate that the catalytic subunits are also involved to a major extent in microtubule dynamics. Microtubule structures undergo dramatic rearrangements in the process of spermatogenesis. Processes involving microtubule rearranging include mitotic division of spermatogonia, meiotic division of spermatocytes, manchette formation, and flagellar axoneme assembly in spermatids. The most severe degeneration occurs in primary spermatocytes of a1/−/−a2/−/− mice, but degeneration also occurs in meiotically dividing spermatocytes (increased apoptosis) and in elongating spermatids (abnormal nuclear morphogenesis). Therefore, it can be stated that PAF-AH (I) catalytic subunits are involved in the several processes of spermatogenesis and not just in a specific stage of spermatogenesis. Although the exact molecular mechanism and function of the...
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PAF-AH (I) catalytic subunits in spermatogenesis are unclear, we found that depletion of both catalytic subunits leads to a major decrease in LIS1 protein levels, suggesting that the catalytic subunits are associated with LIS1. It is most likely that LIS1 protein levels are post-transcriptionally influenced by the catalytic subunits, because LIS1 mRNA levels are not altered in a2−/− mice. Because LIS1 levels are crucial for cortical brain development (13), we speculate that LIS1 is involved in microtubule organization of spermatogenesis and that reduced LIS1 protein levels are responsible for the testicular defects occurring in a1−/−/a2−/− mice. However, our studies also revealed that the more severe testicular degeneration in a1−/−/a2−/− mice than in a2−/− mice cannot be explained solely by the amount of reduction in the level of LIS1, because the reduction of LIS1 was not very different between a2−/− and a1−/−/a2−/− mice. Given that PAF-AH (I) closely resembles trimeric G-proteins (23), the catalytic subunits may mediate a novel intracellular signaling to LIS1 in mammals, and depletion of this signaling pathway may result in severe impairment in spermatogenesis.

Because PAF-AH (I) catalytic subunits are predominantly expressed in brain as well as in testis and because haploinsufficiency of LIS1 leads to severe brain malformation in both humans and mice (13), we expected that mice lacking the catalytic subunits would exhibit brain abnormalities. However, Nissl staining of adult a1−/−/a2−/− mice brain showed no obvious abnormalities in lamination of neurons in the cerebral cortex, hippocampus, or cerebellum (data not shown). To our surprise, in E14.5 brain of a1−/−/a2−/− mice, there was no significant reduction of LIS1 protein levels, suggesting that there is a mechanism to maintain LIS1 protein levels and the function of catalytic subunits in brain.

In a2−/− mice, both a1 and LIS1 protein levels were significantly reduced compared with wild-type mice, whereas in a1−/− mice both a2 and LIS1 levels were not changed markedly. In preliminary experiments, supernatants of mice testis or brain homogenates were subjected to DEAE-Sepharose ion exchange column chromatography. In the case of the a1−/− homogenates, a2 eluted in the same fraction as LIS1, whereas in the case of the a2−/− homogenates a1 and LIS1 eluted at the different positions. These results suggest that a2, probably the a2 homodimer, has a strong affinity for LIS1 and that the a1 homodimer has a weak or negligible affinity for LIS1 in vivo. Considering the fact that a1 mRNA levels are not changed in a2−/− mice (data not shown), the present results also suggest that the a1 protein is not stably expressed in the absence of a2 in vivo. These observations are consistent with our previous report (26) that a1/a2 heterodimers and a2 homodimers are the major PAF-AH (I) catalytic units present in vivo.

Immunostaining studies revealed that a2 is expressed in all spermatogenic cells, whereas a1 is expressed only in spermatogonia. This expression pattern parallels the finding that deletion of both subunits induces a marked loss of germ cells, even at an early spermatogenic stage. We have previously shown that a1 is specifically expressed in migrating neurons in the embryonic and postnatal stages, whereas the a2 expression level is almost constant from the fetal stage through adulthood (26, 27). As a result, the catalytic subunits change from the a1/a2 heterodimer to the a2a2 homodimer in neurons during brain development. It is likely that the same type of alteration in the catalytic dimer occurs during differentiation from spermatogonia to spermatocytes. Interestingly, it has been shown that undifferentiated spermatogonia move to specific sites within the seminiferous tubule and spread their progeny laterally along the base of the tubule (28). Transplantation experiments demonstrated that spermatogonia are capable of moving along the length of the seminiferous tubule at a rate of more than 50 μm/day (29, 30). Although the biological significance of the change in the catalytic subunit combination is not known, it is interesting to speculate that a1 mediates a common signaling pathway in migrating neurons and spermatogonia.

The cellular function of the enzyme activity and the physiological substrate of this enzyme are largely unknown. PAF has been detected in sperm from several mammalian species and has been shown to affect sperm motility and fertility (4). High-fertility spermatozoa, for example, have a substantially greater PAF content than low-fertility spermatozoa (31, 32). Exogenously added PAF increases the motility of human spermatozoa.

2H. Koizumi, N. Yamaguchi, J. Aoki, K. Inoue, and H. Arai, unpublished observations.
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zou (33). Our study gives hints at the possibility that PAF is not only involved in spermatozoal maturation and penetration but is also involved in spermatogenesis itself. On the other hand, PAF-AH(I) shows striking substrate specificity for an acetyl group but hydrolyzes other types of acetyl-containing esters in vitro (34). Studies on the tertiary structure of the catalytic dimer suggest that the substrate of this enzyme is not necessarily a lipophilic substance (23). Because PAF-AH(I) shows similarities to trimeric G-proteins (23), the PAF-AH(I)-mediated novel intracellular signaling is likely operating in mammals, with PAF or a related substance as a GTP-like switch.

When measuring cytosolic PAF-AH activity of α1–/–α2–/– mice in brain and testis, enzymatic reduction to ~65% of wild-type mice was seen in both tissues (data not shown). This phenomenon is likely because type I PAF-AH is the only affected subtype, whereas enzymatic activities of type II PAF-AH and further not yet identified PAF-AH subtypes are probably responsible for the remaining activity.

In conclusion, we found that the depletion of the PAF-AH(I) catalytic subunits induces reduction of LIS1 protein on the cellular level and severe testicular malformation on the phenotypic level. The next question to be considered is whether the catalytic activity of PAF-AH(I) is required for LIS1 protein stability and spermatogenesis. To answer these questions, we are planning to insert the catalytically inactive α1 and α2 subunit genes into our double-knockout mouse model.

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