AAT-1, a Novel Testis-specific AMY-1-binding Protein, Forms a Quaternary Complex with AMY-1, A-kinase Anchor Protein 84, and a Regulatory Subunit of cAMP-dependent Protein Kinase and Is Phosphorylated by Its Kinase*

Hiroshi Yukitake‡, Makoto Furusawa‡§, Takahiro Taira‡§, Sanae M. M. Iuchi-Ariga§¶, and Hiroyoshi Ariga‡§¶

From the ‡Graduate School of Pharmaceutical Sciences, §College of Medical Technology, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan, and ¶CREST, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012; Japan

AMY-1 has been identified by us as a c-Myc-binding protein and was found to stimulate c-Myc transcription activity. AMY-1 was also found to be associated with protein kinase A anchor protein 84/149 (S-AKAP84/AKAP149) in the mitochondria in somatic cells and sperm, suggesting that it plays a role in spermatogenesis. To determine the molecular function of AMY-1, a two-hybrid screening of cDNAs encoding AMY-1-binding proteins was carried out with AMY-1 as a bait using a human testis cDNA library, and a clone encoding a novel protein, AAT-1, was obtained. Three isoforms of AAT-1, AAT-1α, -β, and -γ, were found to be derived from an alternative splicing of the transcripts of the aat-1 gene, which was mapped at human chromosome 13q21. AAT-1 was found to be specifically expressed in the testis during the course of spermatogenesis and also to be present in the epididymis and mature sperm, as was AMY-1. AAT-1α was found to bind to and be colocalized in mitochondria with AMY-1 in human HeLa and mouse GC-1 cells. Furthermore, AAT-1α was found to bind to the N-terminal half of S-AKAP84/AKAP149 in a quaternary complex with AMY-1 and a regulatory subunit (RII) of cAMP-dependent kinase (PKA), in which AAT-1α was associated with RII via S-AKAP84/AKAP149, in rat testis and HeLa cells. It was then found that AAT-1α weakly stimulated a phosphorylation activity of PKA and also that AAT-1α itself was phosphorylated by PKA in vivo and in vitro. These results suggest that both AAT-1 and AMY-1 play roles in spermatogenesis.

We have reported that AMY-1 (associate of Myc-1) bound to Myc box II in the N-proximal region of c-Myc, a transcriptional activation region of c-Myc (1), and stimulated E-box-dependent transcription activity of c-Myc (1). Although two mRNAs, AMY-1S and AMY-1L, which are derived from the alternative usage of poly(A) adenylation signals, encode the same protein, AMY-1, AMY-1S and AMY-1L mRNAs are strongly expressed in the testis and ubiquitously in all the tissues, respectively (1). AMY-1 was found to be a stimulating factor for the initial step of erythrocyte differentiation of human K562 cells, suggesting that AMY-1 is a trigger of differentiation of K562 cells into erythrocyte cells and that AMY-1 has a function independent of or different from that of c-Myc (2). Furthermore, we have found that AMY-1 binds to AKAP149 and its splicing variant, S-AKAP84, which is expressed in the testis and is an anchor protein of cAMP-dependent protein kinase (PKA) (3). AKAP is a protein that translocates PKA to the specific sites where the individual PKA works, and more than 10 AKAPs have so far been identified (4–6). Of the identified AKAPs, S-AKAP84 and AKAP149 have been found to anchor PKA to the mitochondria to phosphorylate the target proteins in the testis and other cells, respectively. Although some target proteins to be phosphorylated by PKA in the mitochondria have been identified, the physiological significance of their phosphorylation is not clear. It has been reported that tyrosine phosphorylation of proteins plays a key role in the acquisition of fertilization activity and that tyrosine phosphorylation is stimulated by dibutyric cAMP, 8-bromo-cAMP, or inhibitors of phosphodiesterase during fertilization (7–11). It has also been reported that an inhibitor of PKA inhibits fertilization and that an inhibitor of serine/threonine-protein phosphatase stimulates both tyrosine phosphorylation and fertilization, suggesting that PKA plays a crucial role in tyrosine-phosphorylation-mediated fertilization (8, 12, 13).

AMY-1 was found to bind in vitro and in vivo to the RII binding region of AKAP149 and S-AKAP84. AMY-1 was also found to be expressed post-meiotically in the testis, as was S-AKAP84. Furthermore, S-AKAP84 and RII, a regulatory subunit of cAMP-dependent protein kinase, were found to make a ternary complex in cells, and AMY-1 was shown to be localized in the mitochondria of HeLa and sperm in association with AKAP149 and S-AKAP84, respectively, suggesting that the function of AMY-1 is related to spermatogenesis. The molecular mechanism of AMY-1 function, however, remains to be clarified.

In this study, to elucidate the functions of AMY-1, a two-hybrid screening of cDNAs encoding AMY-1-binding proteins were performed as follows: 

1. This work was supported by grants-in-aid from the Ministry of Education, Science, Culture and Sport of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication, June 21, 2002, and in revised form, September 6, 2002
Published, JBC Papers in Press, September 9, 2002, DOI 10.1074/jbc.M206201200

45480 This paper is available on line at http://www.jbc.org

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
was carried out, and a cDNA encoding a novel protein named AAT-1 was obtained. It was found that AAT-1 was specifically expressed in the testis and located in the mitochondria. Furthermore, it was found that AAT-1 made a quaternary complex in cells with AMY-1, S-AKAP84/AKAP149, and RII and that AAT-1 was phosphorylated by PKA, suggesting that the function of AAT-1 is related to spermatogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human NEC8 and human NEC14 were purchased from the Japanese Collection of Research Bioresources, and mouse TM3, mouse TM4, and mouse GC-1 cells were purchased from American Type Culture Collection (ATCC). All of the cells used in this study were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum.

**Plasmids**—For pGlex-AMY-1, AMY-1 cDNA starting from the first ATG was inserted in-frame into the EcoRI-XhoI sites of pGlex, a modified version of the LexA-derived bait vector for yeast two-hybrid screening (14). For pcDNA3-FLAG-AAT-1a and pcDNA3-T7-AAT-1a, AAT-1a cDNA starting from the first ATG was inserted in-frame into the EcoRI-XhoI sites of pcDNA3-FLAG and pcDNA3-T7, respectively. Plasmids of various kinds of AMY-1, S-AKAP84, and RII have been described previously (3).

**Cloning of AMY-1-binding Proteins by a Two-hybrid System**—S-cerevisiae cerevisiae L40 cells containing the lacZ gene driven by the GAL1 promoter were transformed first with pGlex-AMY-1, which did not activate lacZ transcription by itself. The transformant cells were subsequently transformed with human testis MATCHMAKER cDNA (Clontech), a cDNA library expressing the GAL4 activation domain (GALAD) fused to the cDNAs from the human testis. Approximately 5.0 × 10⁸ colonies were screened for lacZ expression, and the results indicated the association of a GALAD-fused protein with LexA DNA-binding domain-fused AMY-1. The plasmid DNAs in the lacZ-positive colonies were extracted by the procedure described in the protocols from Clontech. Nucleotide sequences of the plasmids derived from positive colonies were determined by using a Li-Cor Long Reader 4200 autosequencer.

**In Vitro Binding Assay**—35S-Labeled S-AKAP84 or RII was synthesized in vitro using the reticulocyte lysate of the Tn5 translation/translation-coupled system (Promega). Labeled proteins were mixed with GST or GST-AAT-1a expressed in and prepared from Escherichia coli at 4 °C for 1 h in a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 0.05% bovine serum albumin, and 0.1% Nonidet P-40. After washing with the same buffer, the bound proteins were separated in a 10% polyacrylamide gel containing SDS and visualized by fluorography.

**In Vivo Binding Assay**—Ten μg of pcDNA3-FLAG-AAT-1a together with 10 μg of pEF-AMY-1-HA was transfected into human 293T cells (60-mm-diameter dish by the microinjection method) using the electroporation technique (15). Forty-eight h after transfection, the whole cell extract was prepared by the procedure described previously (1). Approximately 2 mg of the 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Sigma) or with nonspecific mouse IgG under the same conditions as those of the in vitro binding assay as described above. After washing with the same buffer, the precipitates were separated in a 15% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a rabbit anti-HA antibody (Y11, Santa Cruz) or with the mouse anti-FLAG antibody.

**Indirect Immunofluorescence**—Human HeLa cells were fixed with a solution containing 4% paraformaldehyde and reacted with a rabbit anti-AMY-1 polyclonal antibody synthesized against the peptide corresponding to amino acid numbers 343–370, a rabbit anti-AAT-1a antibody synthesized against the peptide corresponding to amino acid numbers 38–51, or non-immune antisera. The cells were then reacted with a FITC-conjugated anti-rabbit IgG and observed under a fluorescence microscope. At the same time, mitochondria in the cells were stained with MitoTracker Red CM-H2Xros (Molecular Probes). At the same time, mitochondria in the cells were stained with MitoTracker Red CM-H2Xros (Molecular Probes).

**Determination of the Transcriptional Initiation Site in the Human AAT-1 Gene**— Primer extension analysis was carried out using 27-mer oligonucleotide complementary to the human AAT-1 gene, the nucleotide sequence of which is 5'-CAGGGCGTCCTTCCACTGCGCGCCG-3'. A primer labeled at the 5' end with IRdye800 was mixed with total RNA from S.cerevisiae cerevisiae (Clontech) and subjected to the primer extension reaction as described previously (16). The reaction products were separated on an 8% polyacrylamide, 5% urea sequencing gel along with a sequence ladder using the same primer as that used for the dideoxy sequencing of a 5'-genomic clone.

**RT-PCR Analysis**—Total testis RNAs were prepared by the acid guanidium thiocyanate-phenoI-chloroform method, and cDNA was synthesized using the oligo(dT) primer and BcaBEST polymerase (Takara Co., Ltd.). The first strand of cDNA products was amplified with specific primers for the first 5 min at 94 °C and then for 30 cycles of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C. The nucleotide sequences of the sense and antisense primers were aat-1 sense (5'-GGCTATGGCCTGACCCCTGGG-3') and aat-1 antisense (5'-GCTCAGACGTGTCTCAT-3'). The other nucleotide sequences of the sense and antisense primers for c-myc, c-kit, acrosin, pronamine-1, and β-actin were described previously (3). The amplified products were separated on a 2% agarose gel and stained with ethidium bromide.

**In Situ Hybridization**—The nucleotide sequences of the sense and antisense primers for aat-1 were 5'-CCAAGCAGCGG-3' and 5'-GGTCAGACGTGTCTCAT-3', respectively. PCR products were obtained from 10-week-old male mice of the ICR strain, and sections 8 μm in thickness were prepared by digestion of the testis with a cryostat (CM3000, Leica). The sections were mounted on poly-L-lysine-coating slides; fixed with a solution containing 4% paraformaldehyde, 0.1 M phosphate buffer (pH 7.4), 3% NaCl, and 0.3% sodium citrate; treated with 3 μg/ml proteinase K; dehydrated; and hybridized with 32P-labeled oligonucleotide probes for 24 h at room temperature. After the sections for the aat-1 and aat-1 genes had been autoradiographed for 60 and 21 days, respectively, they were visualized under a microscope (IX70, Olympus).

**Immunohistochemistry**—Sections of testis were obtained from 10-week-old male mice of the ICR strain were fixed in 4% paraformaldehyde and then embedded in paraffin. All of the procedures for immunostaining of the sections using anti-AAT-1a or AMY-1 antibodies have been described previously (17).

**RESULTS**

**Identification of AAT-1 as an AMY-1-binding Protein**—To screen cDNAs encoding AMY-1-associating proteins, a full-size AMY-1 starting from an amino acid number 1 was fused to the LexA-DNA-binding domain and introduced into S. cerevisiae L40 cells. A human testis cDNA library cloned in pGADGH was then introduced into the transformant yeast cells, and the colonies resistant to a His marker followed by β-galactosidase expression were selected. Among a total of 5.0 × 10⁶ transformant colonies, 37 colonies were His- and β-galactosidase-positive, and 10 of the 37 positive colonies were identified as specific clones. Two of the 10 clones were identified as c-myc, whereas 3 clones correspond to S-AKAP84, which were also cloned in the former screening using human the HeLa cDNA library (3). Two other clones were also identified as a AKAP74/RSP3 (accession no. AF353618) and a testis-specific AKAP (accession no. XM-088671), and the sequences of cDNAs of these AKAPs have recently been deposited in the database. One of the three novel clones, which was found to be specifically expressed in the testis, was termed MAP-1 (18). In the present study, we characterized one of those novel clones, no. 1, named AAT-1 (AMY-1-associating protein expressed in testis-1).

The isolated AAT-1 cDNA contained 1,266 nucleotides, encoding 98 amino acids with a poly(A) stretch at the 3'-terminal end and with the perfectly matched Kozak consensus sequence (19) at the 5'-terminal end (Fig. 1A). After an expressed sequence tag data base search, two other types of cDNA containing the same nucleotide sequences as that of the central region of the cloned AAT-1 cDNA were found and obtained from Incyte Genomics Inc. These clones contained 460 and 533 nucleotides, both of which encode the C-terminal 31 amino acids of AAT-1 with different 5' - and 3'-untranslated region from those of clones cDNA, were found to be derived from an alternative splicing in the same gene (Fig. 1C). The former of the original isolate was named AAT-1a, and the two splicing forms were named AAT-1β and AAT-1γ (Fig. 1B). Because there was an in-frame stop codon upstream of the first methionine of the AAT-1β and AAT-1γ cDNA obtained, it was thought that
Fig. 1. Characterization of AAT-1 cDNA and genomic DNA. A, nucleotide and amino acid sequences of AAT-1α, -β, and -γ are shown. The plausible initiation and stop codons are boxed. B, structures of AAT-1α, AAT-1β and AAT-1γ. Gray boxes indicate regions of AAT-1α, AAT-1β, and AAT-1γ that contain the same amino acids, and the open box in AAT-1α represents the region containing unique amino acids. C, primer extension analysis. Total RNA from the human testis was hybridized with a 5'-labeled 25-mer oligonucleotide corresponding to the cDNA sequence of 16–40
these cDNAs encode full-size AAT-1p and AAT-1y. Furthermore, a mouse homolog of human AAT-1a cDNA was cloned by RT-PCR using mouse testis RNA as a template, and the amino acid sequence of mouse AAT-1a was found to be identical to that of human AAT-1a. There were no unique structural motifs in any of the isoforms of AAT-1.

An NCBI genomic DNA data base search revealed that the human aat-1 gene was mapped at chromosome 3q13.33–3q21.1. To determine the transcriptional initiation site (CAP site) of the aat-1 gene on the sequences of chromosome 3q13.33–3q21.1, primer extension analysis was carried out with a labeled oligonucleotide corresponding to the cDNA sequence of 16–40 using total RNA from human testis as a template. As a result, an adenine located 17 nucleotides upstream of the first nucleotide of the cloned AAT-1a cDNA was identified as the CAP site, and this A has since been fixed at +1 in the human aat-1 gene (Fig. 1C). The exon-intron structure of the human aat-1 gene, consisting of 8 exons spanning ~22 kb, was then determined (Fig. 1D). It was found that AAT-1a were transcribed from exons 1, 2, 4, and 6–8; AAT-1b were from exons 5–7; and AAT-1y were from exons 3, 4, and 7 (Fig. 1D).

Each protein-coding exon of human aat-1, for instance, consisted of nucleotides 65–162, and exons 7 and 8 contained the same nucleotides (Fig. 1E). The reason for and the significance of this duplication of the gene are not known. Furthermore, the nucleotide sequences of the boundaries between exons and introns followed the GT-AG rule (20) (Fig. 1E).

Expression of AAT-1 in Various Tissues and Cells—The expression of AAT-1 mRNA was examined by Northern blot analysis in various human and mouse tissues using a probe of 1,266-base AAT-1 cDNA. In human tissues, two major mRNAs of 0.6 and 1.42 kb, which may correspond to those of AAT-1a and AAT-1b or γ, respectively, and three minor mRNAs of 1.8, 2.5, and 4.5 kb were expressed specifically or strongly in the testis, the pattern of which were similar to those of AMY-1S except for in the placenta (Fig. 2a, i). In mouse tissues, one major mRNA of 2.5 kb and two minor mRNAs of 0.46 and 1.15 kb were also expressed specifically or strongly in the testis (Fig. 2a, ii). Because it is difficult to detect AAT-1γ and AAT-γ in Western blotting because of their very small sizes, we focused on analyses of AAT-1s in this study.

A rabbit polyclonal antibody against the peptide spanning amino acid numbers 38–52 of AAT-1α, which is not present in AAT-1β and γ, was prepared. In Western blot analysis using this anti-AAT-1 antibody, protein of ~10.8 kDa was mainly detected with some minor bands in the mouse testis, whereas actin was ubiquitously expressed (Fig. 2B, i). When an excess amount of the peptide used for immunization in rabbits was added to the reaction with the antibody, the detected signal of the 10.8-kDa protein disappeared (data not shown), indicating that the 10.8-kDa protein was endogenous AAT-1α. Western blot analyses using the anti-AAT-1α antibody were further carried out on the extracts from spermatogenesis-related tissues and cultured cells (Fig. 2B, ii and iii, respectively). AAT-1a was detected in all of the spermatogenesis-related tissues examined, including the testis, the epithelium of cauda and corpus epididymis, and sperm from human, mouse or rat (Fig. 2B, ii). Expression of AAT-1a was also detected in all of the cultured cells examined, including cell lines of human prostate cancer NEC18 and NEC14, mouse Leydig TM3, mouse Sertoli TM4, SV40-transformed mouse early spermatocyte GC-1, mouse embryonal testicular teratoma F9, human chronic myelogenous leukemia K562, human cervical carcinoma HeLa, adenovirus 5-transformed-human embryonal kidney 293T, and mouse cells from NIH Swiss embryo NIH3T3 (Fig. 2B, iii).

RT-PCR analysis of mRNAs was then carried out to determine the timing of expression of AAT-1 and AMY-1 during spermatogenesis using total RNAs extracted from mice testis at various days after birth (Fig. 3A). Specific primers for the amplification of mRNAs of c-kit, acrosin, and protamine-1 were used to identify the times at which the expressions of spermatogonia, spermatocytes, and spermatids started (21–23), respectively. The results showed that expression of AAT-1 began in 1-week-old mice and gradually decreased after the expression of spermatid, during which time AMY-1 was expressed. On the other hand, c-myc was found to be expressed from the time of expression of spermatogonia.

To determine in which types of cells in the testis AAT-1α and AMY-1 were expressed, in situ hybridization and immunohistochemical analyses were carried out using sections of mouse testis (Fig. 3, B and C, respectively). The results of in situ hybridization using antisense oligonucleotide probes corresponding to the nucleotide sequences of AAT-1α or AMY-1 were the same as or similar to those of immunohistochemistry using the anti-AAT-1α or AMY-1 antibody; AAT-1 was observed in cells that appeared in periods during progression from spermatogonia to spermatids (Fig. 3, B (a) and C (i)), whereas AMY-1 was observed in cells after the appearance of spermatids (Fig. 3, B (c) and C (b)). Weak expression of AAT-1α was also observed in Leydig cells (Fig. 3, B (a) and C (ii)), and no cells reacted with sense oligonucleotides of AAT-1 or AMY-1 or with preimmune serum (Fig. 3, B (b and d) and C (j and l)). These results indicate that both AAT-1 and AMY-1 are expressed in spermatogenesis-related cells in the testis.

Binding of AAT-1 to AMY-1—To determine the AAT-1a-binding region of AMY-1, three deletion constructs fused to the GALAD were used for the two-hybrid assay with AMY-1α as a bait (Fig. 4A). Both the wild type and the N-terminal fragment spanning amino acids 1–33 of AAT-1 α bound to AMY-1, and the central and the C-terminal half fragment spanning amino acids 34–66 and 67–98, respectively, of AAT-1α did not bind to AMY-1, suggesting that the N-terminal 33 amino acids present in AAT-1α contribute to the AMY-1 binding activity. As suggested by the above results, AAT-1β and AAT-1γ that correspond to the C-terminal half fragment of AAT-1α spanning amino acids 67–98 were found not to bind to AMY-1 (data not shown). An in vitro binding assay was then performed by using a 35S-labeled AAT-1α synthesized in vitro with GST-AMY-1 or GST expressed in and prepared from E. coli. After GST-AMY-1 or GST trapped in glutathione-Sepharose 4B resin had been mixed with labeled proteins, the bound proteins were separated on gel and visualized by fluorography (Fig. 4B). As in the case of binding in yeast, the full-sized AAT-1α bound to GST-AMY-1, whereas no bindings of AAT-1α to GST alone was observed (Fig. 4B, lanes 3 and 2, respectively).

To observe the complex formation of AAT-1α with AMY-1 in vivo, expression vectors for FLAG-tagged AAT-1α together with HA-tagged AMY-1 were transfected into human 293T cells. Forty-eight h after transfection, cell extract was prepared and the proteins in the extract were first immunoprecipitated in A. The primer was extended by reverse transcriptase, and the product was separated on a denatured polyacrylamide gel (lane R). Sequencing of a 5′-genomic clone with this primer was used for calibration (lanes A, G, C, and T). The CAP site of transcription is marked by an arrow, and the corresponding nucleotide is indicated by a bent arrow. D, physical maps of the human aat-1 gene are shown. Exons are represented by squares, in which the regions coding for proteins are represented by black boxes, and the numbers above the boxes representing cDNA indicate the exon numbers for the aat-1 gene transcripts. E, sequences of the exon-intron junctions of the human aat-1 gene. The intronic sequence is given in base pairs. Enclosed amino acids are indicated below the first base of the corresponding codon, using the single-letter code.
with an anti-FLAG antibody or nonspecific IgG. The anti-FLAG antibody did precipitate FLAG-AAT-1α (Fig. 4C, lower panel). AMY-1-HA, on the other hand, was detected in the immunoprecipitate from FLAG-tagged AAT-1α-transfected cells with an anti-HA antibody but not with IgG (Fig. 4C, upper panel, lanes 3 and 2, respectively). These results indicate that AMY-1 is associated with AAT-1α in ectopically expressed 293T cells.

To determine the cellular localization of AAT-1α, human HeLa cells were transfected with expression vectors for FLAG-tagged AAT-1α alone or together with HA-tagged AMY-1. Forty-eight h after transfection, HeLa cells were stained with
anti-FLAG and anti-HA antibodies, and the proteins were detected by FITC- and rhodamine-conjugated second antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 4D). In HeLa cells transfected with FLAG-AAT-1α alone, AAT-1α, like AMY-1, was found to be localized in the cytoplasm (Fig. 4D(i)). In HeLa cells cotransfected with FLAG-
**Fig. 4. Association and colocalization of AAT-1α with AMY-1.** A, the wild type or three deletion mutants of AAT-1α were fused to GALAD and used for yeast two-hybrid assays in L40 cells pretransformed with LexBD-AMY-1 or a vector (PGLex). After incubation on filters, the β-galactosidase activity was assayed. B, GST or GST-AMY-1 was expressed in *E. coli* BL21(DE3) and applied to glutathione-Sepharose 4B. ³⁵S-AAT-1α synthesized in vitro in a coupled transcription/translation system was then applied to the column. The labeled proteins that had bound to the column were separated in a gel and visualized by fluorography. One-fiftieth volumes of the labeled AAT-1α used for the binding reaction were applied to the same gel (lane 1). C, AAT-1α and AMY-1 were tagged with either FLAG or HA, and their expression vectors were introduced into human 293T cells. Two days after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody (F) (M2, Sigma) or nonspecific IgG (G). The proteins in the precipitates were separated in a 15% polyacrylamide gel and blotted with an anti-HA antibody (Y11, Santa Cruz) or the anti-FLAG antibody. One-fiftieth volumes of the extract used for the binding reaction were applied to the same gel (input, lane 1). D, an expression vector for FLAG-AAT-1α was transfected with or without AMY-1-HA to human HeLa cells by the calcium phosphate precipitation technique (i and ii, respectively). Two days after transfection, the cells were fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma) or an anti-HA polyclonal antibody (Y11, Santa Cruz), and visualized with a FITC-conjugated
AAT-1 is a Phosphorylated Target of PKA

AAT-1a and AMY-1-HA, AAT-1a was found to be still localized in the cytoplasm and colocalized with AMY-1-HA, as shown by the yellow color (Fig. 4D(ii)). To examine the cellular localization of AAT-1a in a physiological condition, human HeLa cells were stained with an anti-AAT-1a antibody (Fig. 4E). As in the case of the ectopically transfected cells, AAT-1a was found to be localized in the cytoplasm, and no staining of cells was observed by the anti-AAT-1a antibody preabsorbed with the corresponding peptide used as an immunogen (Fig. 4E, i, indicating that the anti-AAT-1a antibody specifically stained AAT-1a in cells. Mitochondria were also stained with MitoTracker, which gives a red color (Fig. 4E, ii). In HeLa cells, AAT-1a (green), like AMY-1, and mitochondria (red) were co-localized as shown by the yellow color (Fig. 4E, ii, Merge), and these proteins were colocalized as shown in Fig. 7B. The same localization pattern of AAT-1a was also observed in mouse GC-1 cells, in which few AAT-1a proteins were localized in the nucleus as indicated by the staining by DAPI (Fig. 4E, iii). The mouse sperm extracted from epididymis was then stained with the anti-AAT-1a, and the proteins were detected by FITC-conjugated second antibody. Mitochondria were also stained with MitoTracker, which gives a red color (Fig. 4F). In sperm, AAT-1a was localized in the neck of the sperm, which was identified as mitochondria by MitoTracker staining, and these two were colocalized as shown by the yellow color (Fig. 4F, Merge). These results indicate that AAT-1a is a bona fide AMY-1-binding protein.

**Ternary Complex of AAT-1a, AMY-1, and S-AKAP84**—Because we have reported that AMY-1 binds to S-AKAP84 or AKAP149 (3), it is possible that AAT-1a is in a complex with S-AKAP84 or AKAP149. S-AKAP84 is a splicing variant of AKAP149 (3), it is possible that AAT-1a binds to S-AKAP84 or AKAP149, on the other hand, were detected in the immunoprecipitates with the anti-AAT-1a serum but not with preimmune serum. The precipitates were immunoblotted against the anti-AKAP149 antibody that recognizes both S-AKAP84 and AKAP149 (Fig. 5B, ii). The anti-AAT-1a serum did precipitate AAT-1a in two different groups of rats (Fig. 5B, ii, lower panel). Although the different amounts of S-AKAP84 and AKAP149 were found to be present in two lots of rats, S-AKAP84 and AKAP149, on the other hand, were detected in the immunoprecipitate with the anti-AAT-1a serum but not with preimmune serum (Fig. 5B, ii, lanes 3 and 2, respectively). These results clearly indicate that AAT-1a binds to S-AKAP84 and AKAP149 in cells.

Because AAT-1a and AMY-1 bind to different regions of S-AKAP84, it is possible that these three proteins make a ternary complex or that proteins competitively bind to each other. To assess this possibility, *in vitro* binding assays were performed by using 35S-labeled T7-tagged AAT-1a and nonlabeled FLAG-tagged S-AKAP84, both of which were synthesized in *vitro*, with various amounts of GST-AMY-1 (Fig. 5C). Various combinations of proteins were first mixed and then immunoprecipitated with an anti-FLAG antibody, and proteins precipitated were blotted with the anti-FLAG antibody or an anti-GST antibody or by fluorography to detect FLAG-S-AKAP84, GST-AMY-1, and T7-AAT-1a, respectively (Fig. 5C). First, binding of S-AKAP84 to either GST-AMY-1 or T7-AAT-1a was confirmed (Fig. 5C, lanes 2 and 4, respectively). When the amount of GST-AMY-1 in a reaction mixture containing T7-AAT-1a, FLAG-S-AKAP84, and GST-AMY-1 was increased, it was observed that a constant amount of T7-AAT-1a was precipitated (Fig. 5C, lanes 5–8), suggesting that AMY-1 and AAT-1a do not competitively bind to S-AKAP84 but, rather, that they both make a ternary complex with S-AKAP84. This latter possibility was further supported by the finding that these three proteins, AMY-1-HA, endogenously expressed AAT-1a and S-AKAP149, were colocalized in HeLa cells into which AMY-1-HA alone had been transfected (see Fig. 7B, i).

**Ternary Complex of AAT-1a, S-AKAP84, and RII of PKA**—Because S-AKAP84 binds either to RII or AAT-1a, it is possible that these three proteins make a ternary complex. To explore this possibility, co-immunoprecipitation assays were performed by transfection of expression vectors for FLAG-AAT-1a and RIIβ-β into 293T cells, and those for FLAG-S-AKAP84 and RIIβ-β were also transfected as positive controls (Fig. 6A). The proteins in cell extracts were immunoprecipitated with an anti-FLAG antibody, and the precipitated proteins were blotted with the anti-FLAG antibody to detect FLAG-AAT-1a or with an anti-HA antibody to detect RIIβ-β. The Flag-AAT-1a was first confirmed to be precipitated with the anti-FLAG antibody but not with IgG (Fig. 6A, lanes 4 and 5 in the lower panel). The results then showed that Flag-AAT-1a was coprecipitated with anti-mouse antibody or an rhodamine-conjugated anti-rabbit antibody (α-FLAG and α-HA, respectively). Both figures were merged (ii, Merge). E, i, to see the specificity of the proteins that were stained with an anti-AAT-1a antisera, the anti-AAT-1a antisera was first absorbed with 1 μg of peptide used for immunization for 2 h at 2 °C. HeLa cells were fixed, reacted with an anti-AAT-1a antisera or the preabsorbed anti-AAT-1a antiserum, and visualized with a FITC-conjugated anti-rabbit antibody. ii, HeLa cells were similarly treated and stained with the anti-AAT-1a antibody as described in i, and the same slides were also stained with MitoTracker Red CM-H2Xros (Mitochondria). These figures were merged (Merge). iii, GC-1 cells were similarly treated and stained with the anti-AAT-1a antibody as described for HeLa cells, and the same slides were also stained with DAPI. F, the mouse sperm extracted from epididymis was stained as described in panel E (ii). These figures were merged (Merge). The sperm was visualized under a phase-construct microscope (Phase).
RIIβ-HA but that the amount of the precipitated RIIb-HA was less than that in cells cotransfected with FLAG-S-AKAP84 and RIIβ-HA (Fig. 6A, lanes 5 and 3, respectively), suggesting that Flag-AAT-1α is associated with RIIβ-HA via endogenously expressed AKAP149 in 293T cells. To address this possibility, in vitro binding assays, in which mixtures containing various combinations of 35S-labeled S-AKAP84, 35S-labeled RIIβ, GST, and GST-AAT-1α were applied to a glutathione-Sepharose col-

Fig. 5. Ternary complex of AAT-1α, AMY-1, and S-AKAP84. A, GST or GST-AAT-1α was expressed in E. coli BL21(DE3) and applied to glutathione-Sepharose 4B. 35S-Labeled wild type or various deletion mutants of S-AKAP84 synthesized in vitro in a coupled transcription/translation system were then applied to the column. The labeled proteins that had bound to the column were separated in a gel and visualized by fluorography. One-fiftieth volumes of the labeled S-AKAP84 used for the binding reaction were applied to the same gel (I, lanes 1, 4, 7, 10, and 13). Structures of the various deletion mutants of S-AKAP84 are schematically shown in the right panel. B, i, expression vectors for FLAG-AAT-1α and T7-S-AKAP84 were transfected into 293T cells. Forty-eight h after transfection, the cell extracts were prepared, the proteins were immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (M2, Sigma), and the precipitates were blotted with an anti-T7 monoclonal antibody (Novagen) or the anti-FLAG antibody. ii, the proteins in the extract from rat testes were first immunoprecipitated (IP) with an anti-AAT-1α antiserum (A) or preimmune serum (P). The proteins in the precipitates were separated in a 15% polyacrylamide gel and blotted with an anti-AKAP149 antibody (clone 6, Transduction Laboratories) or the anti-AAT-1α antibody. One-fiftieth volumes of the extract used for the binding reaction were applied to the same gel (input, lane 1). C, GST-AMY-1, GST, FLAG-S-AKAP84, and 35S-T7-AAT-1α were mixed in various combinations as shown in the figure. The proteins in the mixture were immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (M2, Sigma) or nonspecific IgG, and the precipitates were blotted with an anti-T7 monoclonal antibody (Novagen), an anti-GST antibody (Santa Cruz), or the anti-FLAG antibody. 35S-T7-AAT-1α was visualized by fluorography.
The precipitates were blotted with an anti-HA polyclonal antibody or the anti-FLAG antibody. 35S-T7-AAT-1 with an anti-FLAG monoclonal antibody or nonspecific IgG (Fig. 6B, lane 6). These results clearly indicate that RIIβ is associated with AMY-1 via S-AKAP84.

To determine whether or not these proteins mutually or competitively bind to each other, the same binding assay as that for which the results are shown in Fig. 5C was carried out using GST-RIIβ instead of GST-AMY-1 (Fig. 6C). As in the case of a complex of AMY-1, RIIβ, and S-AKAP84, the amounts of both FLAG-S-AKAP84 and T7-AAT-1α were found not to change regardless of the amount of GST-RIIβ, suggesting that these three proteins are in the same complex. Furthermore, these proteins were colocalized in HeLa cells (see Fig. 7B, ii). Quaternary Complex of AMY-1, AAT-1α, S-AKAP84, and RII of PKA—Because we showed in this study and in a previous study (3) that there are three ternary complexes (among AAT-1α, AMY-1, and S-AKAP84; among AAT-1α, S-AKAP84, and RII; and among AMY-1, S-AKAP84, and RII) in which RII is associated with AMY-1 via S-AKAP84, it is possible that these four proteins are associated together. To explore this possibility, in vitro binding assays, in which mixtures containing various combinations of 35S-labeled S-AKAP84, 35S-labeled RIIβ, 35S-labeled AAT-1α, GST, and GST-AMY-1 were applied to a glutathione-Sepharose column and pulled down, were carried out (Fig. 7A). Although GST-AMY-1, but not GST, pulled down S-AKAP84 and AAT-1α, it did not pull down RIIβ in the mixtures containing the two respective proteins, as described in the previous section (Fig. 7A, lanes 4, 6, 7, and 5, respectively). When GST-AMY-1 was mixed with three kinds of combination of two proteins from S-AKAP84, RIIβ, and AAT-1, RIIβ was found to be precipitated only in the presence of S-AKAP84 (Fig. 7A, lanes 8–10). Finally, when all four proteins were mixed together, all of the proteins were precipitated (Fig. 7A, lane 11), indicating that these four proteins make a quaternary complex, in which S-AKAP84 bridges RIIβ to AAT-1α and AMY-1 (Fig. 7C). This conclusion was supported by the finding of colocalization of these four proteins in HeLa cells in which ectopically expressed AMY-1-1HA was colocalized with both endogenously expressed AAT-1α and S-AKAP149 or in which endogenously expressed AAT-1α, AKAP149, and RII were colocalized (Fig. 7B, i and ii, respectively).

Effect of AAT-1α on Kinase Activity of PKA—Because AAT-1α is associated with RII, a regulatory subunit of PKA, we examined whether or not AAT-1α affects the kinase activity of PKA. To examine this, 293T cells were transfected with expression vectors for FLAG-S-AKAP84 with or without T7-AAT-1α, the proteins in cell extracts were immunoprecipitated with an anti-FLAG antibody, and the kinase activity in the precipitated proteins was measured using a mixture containing [γ-32P]ATP and a synthetic peptide kemptide as a substrate (Fig. 8). It was first confirmed that both FLAG-S-AKAP84 and T7-AAT-1α were expressed in transfected cells and that both proteins were coimmunoprecipitated with the anti-FLAG antibody but not with nonspecific IgG (Fig. 8, A and B, respectively). Although it was shown that the level of kinase activity in the extract from cells transfected with both FLAG-S-AKAP84 and T7-AAT-1α was a little higher than that in the extract from cells transfected with FLAG-S-AKAP84 alone (Fig. 8C), it is not thought that this small difference in kinase activities was because of the direct effect of AAT-1α on PKA; rather, AAT-1α itself might be a substrate for PKA. When the kinase reaction was carried out without the synthetic peptide, a 5-fold higher level of kinase activity was found in the extract from cells transfected with both FLAG-S-AKAP84 and T7-AAT-1α than that in the extract from cells transfected with FLAG-S-AKAP84 alone (Fig. 8D), suggesting that AAT-1α was used as a substrate for PKA. To
confirm that AAT-1α was indeed phosphorylated with PKA in the above reaction, 293T cells were transfected with expression vectors, and the kinase reaction was carried out under the same conditions as those used in the experiment for which the results are shown in Fig. 8. The proteins in the reaction mixture were then immunoprecipitated with an anti-AAT-1 antibody, separated on the gel, and visualized by autoradiography (Fig. 9A). The results showed that, although no band was observed in cells not transfected with T7-AAT-1α, a phosphorylated band corresponding to the size of AAT-1α was immunoprecipitated with the anti-AAT-1α antibody, and this band disappeared in the kinase reaction mixture containing a PKA inhibitor, suggesting that AAT-1α was phosphorylated by PKA that was anchored by S-AKAP84. To confirm further that
AAT-1 Is a Phosphorylated Target of PKA

In this study, we identified a novel AMY-1-binding protein, AAT-1α, and its expression profiles, genes, and cellular localization were determined. The results showed that AAT-1α was specifically expressed in the testis throughout the course of spermatogenesis and was also present in mature sperm. Furthermore, histochemical analysis showed that some AAT-1α proteins are expressed in Leydig cells, which are known to be essential cells for testis development. AMY-1, on the other hand, was shown to be expressed in the testis from the period of late spermatocytes and was also present in mature sperm. These results indicate that there are two periods of expression of AAT-1. These expression patterns between AAT-1α and AMY-1 are reminiscent of those between AKAP149 and S-AKAP84, whose expression patterns are similar to those of AAT-1α and AMY-1, respectively (5, 6). Because we showed in this study that AAT-1α binds to both AKAP149 and S-AKAP84, AAT-1α might change its binding partner among AKAPs during the course of spermatogenesis, from AKAP149 to S-AKAP84, and AMY-1 might participate in the latter complex with AAT-1α and S-AKAP84. Although the functional differences between two complexes of AAT-1α, one containing AMY-1 and the other not containing AMY-1, are not known at present, it may be necessary to use the fractionated cells corresponding to specific spermatogenic stages.

AAT-1α was found to bind to the N-proximal region spanning amino acid numbers 30–253 of S-AKAP84/AKAP149, within which the amino acid sequence KGVLF, a consensus sequence for binding of protein phosphatase 1 (PP1), is present (24), and an interaction of PP1 with AKAP220, another AKAP expressed in the testis and brain, has been reported (25–27). Because it was reported that inhibitors for PKA and protein phosphatase affected fertilization (12, 13, 21, 25) and because we showed in this study that AAT-1α was phosphorylated by PKA, it is thought that the phosphorylated form of AAT-1α is dephosphorylated by PP1 or that these two proteins competitively bind to S-AKAP84/AKAP149, leading to modulation of PKA activity.

Both AMY-1 and AAT-1α were found to be associated with RII via AKAP149 or S-AKAP84 in a quarterly complex and to be localized in the mitochondria of the testis and mature sperm. Because transgenic mice harboring the human AMY-1

of late spermatocytes and was also present in mature sperm. These results indicate that there are two periods of expression of AAT-1. These expression patterns between AAT-1α and AMY-1 are reminiscent of those between AKAP149 and S-AKAP84, whose expression patterns are similar to those of AAT-1α and AMY-1, respectively (5, 6). Because we showed in this study that AAT-1α binds to both AKAP149 and S-AKAP84, AAT-1α might change its binding partner among AKAPs during the course of spermatogenesis, from AKAP149 to S-AKAP84, and AMY-1 might participate in the latter complex with AAT-1α and S-AKAP84. Although the functional differences between two complexes of AAT-1α, one containing AMY-1 and the other not containing AMY-1, are not known at present, it may be necessary to use the fractionated cells corresponding to specific spermatogenic stages.

AAT-1α was found to bind to the N-proximal region spanning amino acid numbers 30–253 of S-AKAP84/AKAP149, within which the amino acid sequence KGVLF, a consensus sequence for binding of protein phosphatase 1 (PP1), is present (24), and an interaction of PP1 with AKAP220, another AKAP expressed in the testis and brain, has been reported (25–27). Because it was reported that inhibitors for PKA and protein phosphatase affected fertilization (12, 13, 21, 25) and because we showed in this study that AAT-1α was phosphorylated by PKA, it is thought that the phosphorylated form of AAT-1α is dephosphorylated by PP1 or that these two proteins competitively bind to S-AKAP84/AKAP149, leading to modulation of PKA activity.

Both AMY-1 and AAT-1α were found to be associated with RII via AKAP149 or S-AKAP84 in a quarterly complex and to be localized in the mitochondria of the testis and mature sperm. Because transgenic mice harboring the human AMY-1
gene tend to be infertile and because mitochondria are energy-producing organelles, it is possible that AMY-1 or AAT-1α affects the PKA activity that is used to preserve the energy necessary for fertilization. In addition to S-AKAP84/AKAP149, AMY-1 has been found to bind to many AKAPs, including other testis-specific AKAPs, and to play a positive role in cell differentiation (2, 3). It is known that the formation and maturation of sperm are affected by several factors, including the ion gradient, various stresses, and phosphorylation signals (7–13, 25, 28–30). These findings further suggest that AMY-1 or AAT-1 has versatile functions in spermatogenesis, during which time AMY-1 or AAT-1 changes its respective AKAPs as binding partners to effectively modulate AKAP activity.

We also identified three isoforms, AAT-1α, -β, and -γ, which were derived from an alternative splicing human aat-1 gene mapped at chromosome 3913–3921, in which exons 7 and 8 were duplicated. Both AAT-1β and AAT-γ comprise 31 amino acids encoding 3.4-kDa proteins, and their corresponding mRNAs were found to be expressed in the testis like that of AAT-1α. Direct bindings of AAT-1β and AAT-γ to AMY-1, however, were not observed, suggesting that AAT-1β and AAT-γ have different functions from that of AAT-1α.

Although the functions of AMY-1 and AAT-1α in testis development, spermatogenesis, and fertilization are not known at present, we are now establishing knockout mice for AMY and AAT-1 genes to clarify these issues.

Acknowledgments—We thank Yoko Misawa and Kiyomi Takaya for technical assistance. We also thank Masahiko Watanabe and Iuya Yoshida for help in the experiments of in situ hybridization and immunohistochemistry.

REFERENCES
1. Taira, T., Maeda, J., Onishi, T., Kitaura, H., Yoshida, S., Kato, H., Ikeda, M., Tamai, K., Iguchi-Ariga, S. M. M., and Ariga, H. (1998) Genes Cells 3, 551–567
2. Furusawa, M., Onishi, T., Taira, T., Iguchi-Ariga, S. M. M., and Ariga, H. (2000) Int. J. Oncol. 6, 339–345
3. Furusawa, M., Onishi, T., Taira, T., Iguchi-Ariga, S. M. M., and Ariga, H. (2001) J. Biol. Chem. 276, 36647–36651
4. Feliciello, A., Gottesman, M. E., and Avvedimento, E. V. (2003) J. Mol. Biol. 339, 99–114
5. Lin, R. Y., Moss, S. B., and Rubin, C. S. (1995) J. Biol. Chem. 270, 27804–27811
6. Trendelenburg, G., Hammel, M., Riecken, E. O., and Hanski, C. (1996) Biochem. Biophys. Res. Commun. 225, 313–319
7. Gianetto-Homer, H. L., Visconti, P. E., and Kopf, G. S. (1997) Biol. Reprod. 56, 707–719
8. Leyton, L., and Salin, P. (1989) Cell 57, 1123–1130
9. Nix, R. K., Ahmad, K., and Kumar, R. (1991) J. Cell Sci. 99, 157–165
10. Visconti, P. E., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995) Development 121, 1129–1137
11. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995) Development 121, 1139–1150
12. Aitken, R. J., Paterson, M., Fisher, H., Buckingham, D. W., and van Duin, M. (1995) J. Cell Sci. 108, 2017–2025
13. Leclerc, P., de Lamirande, E., and Gagnon, C. (1997) Free Radical Biol. Med. 22, 643–656
14. Ono, T., Kitaura, H., Ugai, H., Mutara, T., Yokoyama, K. K., Iguchi-Ariga, S. M. M., and Ariga, H. (2000) J. Biol. Chem. 275, 31145–31154
15. Graham, F. L., and Vander Eb, A. J. (1973) Virology 52, 456–467
16. Haigermoser, C., Fujimoto, M., Iguchi-Ariga, S. M. M., and Ariga, H. (1996) Nucleic Acids Res. 24, 3856–3857
17. Fujimoto, M., Matsumoto, K., Iguchi-Ariga, S. M. M., and Ariga, H. (2001) Genes Cells 6, 1067–1075
18. Yukitake, H., Furusawa, M., Taira, T., Iguchi-Ariga, S. M. M., and Ariga, H. (2002) Biochim. Biophys. Acta 1577, 126–132
19. Kozak, M. (1987) J. Mol. Biol. 198, 947–950
20. Breathnach, R., and Chambon, P. (1981) Annu. Rev. Biochem. 50, 456–467
21. Florke, S., Phi-van, L., Muller-Esterl, W., Scheuber, H. P., and Engel, W. (1983) Differentiation 24, 250–256
22. Mali, P., Kaipa, A., Kangasniemi, M., Toppari, J., Sandberg, M., Hecht, N. B., and Farvstinen, M. (1989) Reprod. Fertil. Dev. 1, 369–382
23. Sorrentino, V., Giorgi, S. M., Geremia, R., Besmer, P., and Rossi, P. (1991) Oncogene 6, 149–151
24. Steen, R. L., Martino, S. B., Tasken, K., and Collas, P. (2000) J. Cell Biol. 150, 1251–1261
25. Leclerc, P., de Lamirande, E., and Gagnon, C. (1996) Biol. Reprod. 55, 684–692
26. Reinton, N., Collas, P., Haugen, T. B., Skalbeg, B. S., Hansson, V., Jahnsen, T., and Tasken, K. (2000) Dev. Biol. 233, 194–204
27. Schillace, R. V., and Scott, J. D. (1999) J. Biol. Chem. 274, 1251–1261
28. Schillace, R. V., and Scott, J. D. (1999) J. Biol. Chem. 274, 319–325
29. Reinton, N., Collas, P., Haugen, T. B., Skalbeg, B. S., Hansson, V., Jahnsen, T., and Tasken, K. (2000) Dev. Biol. 233, 194–204
30. Ryan, A. K., and Rosenfeld, M. G. (1997) Genes Dev. 11, 1207–1225
AAT-1, a Novel Testis-specific AMY-1-binding Protein, Forms a Quaternary Complex with AMY-1, A-kinase Anchor Protein 84, and a Regulatory Subunit of cAMP-dependent Protein Kinase and Is Phosphorylated by Its Kinase
Hiroshi Yukitake, Makoto Furusawa, Takahiro Taira, Sanae M. M. Iguchi-Ariga and Hiroyoshi Ariga

J. Biol. Chem. 2002, 277:45480-45492. doi: 10.1074/jbc.M206201200 originally published online September 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206201200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 11 of which can be accessed free at http://www.jbc.org/content/277/47/45480.full.html#ref-list-1