AMY-1, a c-Myc-binding Protein, Is Localized in the Mitochondria of Sperm by Association with S-AKAP84, an Anchor Protein of cAMP-dependent Protein Kinase*

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We have reported that a novel c-Myc-binding protein, AMY-1 (associate of Myc-1), stimulated the transcription activity of c-Myc. To access 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 HeLa cDNA library, and a clone encoding cAMP-dependent protein kinase anchor protein 149 (AKAP149), was obtained. AMY-1 was found to bind in vitro and in vivo to the regulatory subunit II binding region of AKAP149 and S-AKAP84, a splicing variant of AKAP149 expressed in the testis. AMY-1 was expressed postmeiotically in the testis, as S-AKAP84 was expressed. Furthermore, S-AKAP84 and regulatory subunit II, a regulatory subunit of cAMP-dependent protein kinase, made a ternary complex in cells, and AMY-1 was localized in the mitochondria of HeLa and sperm in association with AKAP149 and S-AKAP84, respectively. These results suggest that AMY-1 plays a role in spermatogenesis.

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‡ The abbreviations used are: PKA, cAMP-dependent protein kinase; RII, regulatory subunit II of A-kinase; GST, glutathione S-transferase; AMCA, aminomethyl coumarin acetic acid; GALAD, GAL4 activation domain.
nonspecific IgG (G). The proteins in the precipitates were separated in a 12.5% polyacrylamide gel and blotted with an anti-HA antibody (12CA5).

For IP with an anti-FLAG antibody (transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (530–AK253RIIbd) 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) or nonspecific IgG (G). The proteins in the precipitates were separated in a 12.5% polyacrylamide gel and blotted with an anti-HA antibody (12CA5).

One-fiftieth volumes of the extract used for the binding reaction were applied to the same gel (lane 5). C, AMY-1 and S-AKAP84 (AK253–530 and AK253–533RRIibd) 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) or nonspecific IgG (G). The proteins in the precipitates were separated in a 12.5% polyacrylamide gel and blotted with an anti-HA antibody (12CA5).

RESULTS AND DISCUSSION

Identification of AKAP149/S-AKAP84 as AMY-1-binding Proteins and Determination of the AMY-1 Binding Region—To screen cDNAs encoding AMY-1-associating proteins, a full-size AMY-1 starting from amino acid number 1 was fused to the LexA-DNA binding domain and introduced into S. cerevisiae L40 cells. A human HeLa 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 5.6 × 10⁹ transformant cells, 79 colonies were His- and β-galactosidase-positive, and four of the 79 positive colonies were identified as AKAP149, a PKA anchor protein, after determination of their nucleotide sequences. Since the longest clone contained amino acids 253–903 of AKAP149, the full-size AKAP149 cDNA was kindly provided by C. Hanski. S-AKAP84 is a splicing variant of AKAP149 and contains the same amino acids spanning 1–530 as does AKAP149 (3). Both proteins are composed of at least three domains (an anchor region, a leucine zipper-like (LZ) region, and an RII-binding (RIIbd) region), and AKAP149 additionally contains a KH domain (Fig. 1A). To determine the AMY-1-binding region of AKAP149 or S-AKAP84, various deletion constructs fused to the GALAD were used for the two-hybrid assay with AMY-1 as bait (Fig. 1A). The wild-types of both AKAP149 and S-AKAP84 bound to AMY-1, and the
C-terminal half-fragment spanning amino acids 531–903 of AKAP149 did not bind to AMY-1, suggesting that the same amino acids present in AKAP149 and S-AKAP84 contribute to the AMY-1 binding activity. The fragment spanning amino acids 253–530 was found to be sufficient for AMY-1 binding. Furthermore, deletion of the RII binding region (AK253–530RIIbd(143,420),(338,435) but not L2 (AK253–530LZ) from this fragment abolished AMY-1 binding activity, indicating that AMY-1 binds to the RII binding region both in AKAP149 and S-AKAP84. An in vitro binding assay was then performed by using a 35S-labeled AMY-1-binding fragment of S-AKAP84 synthesized in vitro with GST-AMY-1 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. 1B).

As in the case of binding in yeast, the fragment spanning amino acids 253–530 of S-AKAP84 bound to GST-AMY-1, while the fragment in which the RII binding region had been deleted did not have binding activity toward GST-AMY-1 (Fig. 1B, lanes 1 and 2), and no bindings of two proteins to GST alone were observed (Fig. 1B, lanes 3 and 4). To observe the complex formation of S-AKAP84 with AMY-1 in vivo, expression vectors for FLAG-tagged S-AKAP84 and its deletion fragments together with HA-tagged AMY-1 were transfected into human 293T cells. Forty-eight h after transfection, the cell extract was prepared, and the proteins in the extract were first immunoprecipitated with the anti-FLAG antibody or nonspecific IgG. The precipitates were immunoblotted against the anti-HA antibody (Fig. 1C). The anti-FLAG antibody did precipitate FLAG-S-AKAP84 (data not shown). AMY-1-HA, on the other hand, was detected in the immunoprecipitate from wild-type S-AKAP84-transfected cells with the anti-HA antibody but not with IgG (Fig. 1C, lanes 1 and 2, respectively). Furthermore, the fragment spanning amino acids 253–530, but not that in which the RII-binding region had been deleted, of S-AKAP84 was again found to bind to AMY-1 (Fig. 1C, lanes 3 and 5, respectively). These results indicate that AMY-1 is associated with the RII-binding region of S-AKAP84 in ectopic expressed 293T cells.

Expression of AMY-1 during Spermatogenesis—Since S-AKAP84 has been reported to be expressed in testis and to be related to spermatogenesis, Northern blot analysis of AMY-1 expression was carried out using a multiple tissue blot filter to see its expression profile (Fig. 2A). AMY-1S and S-AKAP84 mRNAs were found to be strongly expressed in the testis, while AMY-1L, a variant of alternative usage of the poly(A) signal of AMY-1S, and AKAP149, a splicing variant of S-AKAP84, were ubiquitously expressed in tissues. Reverse transcriptase-polymerase chain reaction analysis of mRNAs was then carried out to examine the timing of expression of AMY-1S and S-AKAP84 during spermatogenesis using total RNAs extracted from the mice testis at various days after birth (Fig. 2B). Specific primers for the amplification of mRNAs of c-kit, acrosin, protamine-1, and β-actin on total RNA extracted as substrates.

**Fig. 2.** Expressions of AMY-1 and AKAP149/S-AKAP84 mRNAs in mouse tissues and in the testis of various stages. A. Northern blot analyses were carried out using multiple Northern blot sheets of mouse tissues (CLONTECH) with a labeled AMY-1L cDNA (upper panel), AKAP149 cDNA (middle panel), or β-actin (lower panel) as a probe. B, total RNAs were extracted from the mice testes at various days after birth, and the expressions of AMY-1 and S-AKAP84 were analyzed by Northern blot hybridization as described in A. Reverse transcriptase-polymerase chain reaction analysis of mRNAs was then carried out to examine the timing of expression of AMY-1S and S-AKAP84 during spermatogenesis using total RNAs extracted from the mice testis at various days after birth (Fig. 2B). 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, spermatocyte, and spermatid started (8–10), respectively. The results showed that AMY-1S began to be weakly expressed in 2-week-old mice and was strongly expressed after the expression of spermatid, during which time S-AKAP84 was expressed. On the other hand, c-myc was found to be expressed from the time of spermatogenesis. These results indicate coordinate expression between AMY-1S and S-AKAP84.
HA, and cell extracts were prepared 48 h after transfection. Expression levels of introduced FLAG-S-AKAP84, RIIβ-HA, and AMY-1-HA were determined by Western blot analysis (Fig. 3B, a). The proteins in the extracts were immunoprecipitated with an anti-FLAG monoclonal antibody (M2; Sigma), and the precipitates were blotted with the anti-FLAG antibody or the anti-HA polyclonal antibody (Y-11; Santa Cruz Biotechnology) (Fig. 3B, b). The expression vectors for FLAG-S-AKAP84 and various doses of RIIβ-HA were transfected into AM416 cells. Forty-eight h after transfection, the cell extracts were prepared, and the proteins in the extracts were blotted with the anti-FLAG monoclonal antibody or the anti-HA polyclonal antibody (Fig. 3B, a). The proteins in the extracts were immunoprecipitated with the anti-FLAG monoclonal antibody, and the precipitates were blotted with the anti-HA polyclonal antibody (Fig. 3B, b). One-fiftieth volumes of the extract used for the binding reaction were applied to the same gel (Input, lane 15).

FIG. 3. Ternary complex of AMY-1, S-AKAP84, and RIIβ in cells. A, the expression vectors for FLAG-AMY-1, T7-S-AKAP84, and RIIβ-HA were transfected in various combinations into 293T cells. Forty-eight h after transfection, the cell extracts were prepared, and the proteins in the aliquots of extracts were blotted with an anti-T7 monoclonal antibody (Novagen) or an anti-HA polyclonal antibody (Y-11; Santa Cruz Biotechnology) (Fig. 3A, a). The proteins in the extracts were immunoprecipitated with an anti-FLAG monoclonal antibody (M2; Sigma), and the precipitates were blotted with the anti-FLAG antibody or the anti-HA polyclonal antibody (Y-11; Santa Cruz Biotechnology) (Fig. 3A, b). B, the expression vectors for FLAG-S-AKAP84 and various doses of RIIβ-HA were transfected into AM416 cells. Forty-eight h after transfection, the cell extracts were prepared, and the proteins in the extracts were blotted with the anti-FLAG monoclonal antibody or the anti-HA polyclonal antibody (Fig. 3B, a). The proteins in the extracts were immunoprecipitated with the anti-FLAG monoclonal antibody, and the precipitates were blotted with the anti-HA polyclonal antibody (Fig. 3B, b). One-fiftieth volumes of the extract used for the binding reaction were applied to the same gel (Input, lane 15).

FIG. 4. Colocalization of AMY-1 with S-AKAP84/AKAP149 in mitochondria. A, HeLa cells were fixed, reacted with an anti-AMY-1 polyclonal antibody and an anti-AKAP149 monoclonal antibody (clone 6; Transduction Laboratories), and visualized with a fluorescein isothiocyanate-conjugated anti-rabbit antibody and an AMCA-conjugated anti-mouse antibody. The same slides were also stained with Mitotracker Red CM-H2Xros (Mitochondria). These figures were merged (Overlay). B, the ejaculated human sperm was stained as described for A. These images were merged (Overlay). The cells were visualized under a phase-contrast microscopy (phase).

FIG. 5. Expressions of AKAP149/S-AKAP84 mRNAs in human HeLa cells and sperm. Proteins were extracted from human HeLa cells or the ejaculated human sperm with the radioimmune precipitation buffer contained 1× PBS, 1% Nonidet P-40, and 0.1% SDS. Fifty μg of proteins were separated on 7.5% polyacrylamide gel, blotted with an anti-AKAP149/S-AKAP84 antibody (Transduction Laboratories), and visualized by ECL. The same filter was reprobed with an anti-actin antibody.
Colocalization of AMY-1 with AKAP149/S-AKAP84 in the Mitochondria in HeLa and Sperm—Previous studies have shown that AMY-1 was usually located in the cytoplasm and translocated from the cytoplasm to nuclei only during the S phase of the HeLa cell upon the expression of c-Myc (1) and that S-AKAP84 was localized in the mitochondria of HeLa, elongating spermatid (4), and mature sperm (12). To determine the cellular localization of AKAP149/S-AKAP84 and AMY-1 more precisely, human HeLa cells and the ejaculated human sperm were stained with anti-AMY-1 and anti-AKAP149/S-AKAP84 antibodies, and the proteins were detected by fluorescein isothiocyanate- and AMCA-conjugated second antibodies, respectively, and then visualized under a confocal laser microscopy (Fig. 4). Mitochondria were also stained with MitoTracker, giving a red color. In HeLa cells, AMY-1 (green), AKAP149 (blue), and mitochondria (red) were co-localized as shown by the white color (Fig. 4A, Overlay). In sperm, both AMY-1 and S-AKAP84/AKAP149 were localized in the neck of the sperm, which was identified as mitochondria by MitoTracker staining, and these three were colocalized as shown by the white color (Fig. 4B, Overlay). To distinguish which AKAP, S-AKAP84 or AKAP149, is located in the ejaculated human sperm and HeLa cells, proteins extracted were blotted with the anti-AKYAP84/S-AKAP84 antibody and with an anti-actin antibody as an internal control (Fig. 5). Results clearly showed that S-AKAP84 was strongly expressed and a faint amount of AKAP149 was expressed in sperm (Fig. 5, lane 2). In HeLa cells, on the other hand, only AKAP149 was expressed (Fig. 5, lane 1). These results clearly show that AMY-1 is colocalized with AKAP149 or S-AKAP84 in the mitochondria of HeLa cells or sperm, respectively.

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 dibutylyryl cAMP, 8-bromo-cAMP, or inhibitors of phosphodiesterase during fertilization (13–17). It has also been reported that an inhibitor of PKA inhibits 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 (18–20). It has been shown that AKAP determines the place where PKA works in cells and that S-AKAP84 and AKAP149 anchor PKA to the mitochondria in sperm, and various somatic cells, respectively (3, 4). In this study, we found that AMY-1 was located in the mitochondria in sperm and HeLa cells in a complex with S-AKAP84/AKAP149 and RIIβ and that both AMY-1 and S-AKAP84 were expressed after the appearance of spermatocytes in the testis, while the expression profile of c-myc during spermatogenesis was not parallel with that of AMY-1. These results suggest that AMY-1 plays a role independent of that of c-Myc in spermatogenesis. In addition to the functions of S-AKAP84 in spermatogenesis, it has been reported that S-AKAP84/AKAP149 may be involved in apoptosis regulation by anchoring PKA to mitochondria in which the unphosphorylated active form of BAD, a protein for apoptosis, is bound to Bcl-XL, an anti-apoptosis protein, to be inactivated. After BAD is phosphorylated by PKA in mitochondria, the phosphorylated inactive form of BAD is translocated to the cytoplasm and binds to 14-3-3 protein, thereby leading to the free active form of Bcl-XL that functions as an anti-apoptosis protein (21). Transgenic mice carrying overexpressing AMY-1 are apt to become infertile (data not shown). Although the precise mechanisms of the AMY-1 function in testis are not clear at present, it is possible that AMY-1 affects the apoptosis of spermatogenesis-related proteins by modification of PKA anchored by S-AKAP84. Furthermore, since AMY-1 and RIIβ were found to bind mutually to the RII-binding region of S-AKAP84/AKAP149, which spans 22 amino acids, it is possible that both proteins bind to the same surface of the RII-binding region side by side that each protein binds to the opposite surface of the RII-binding region of S-AKAP84/AKAP149. To examine these possibilities, structural analysis of the complex containing AMY-1 is now being carried out.

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