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The enigma of ATCE1, an acrosome-associated transcription factor

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Abstract

Atce1 belongs to the CREB3/LZIP subtype of the ATF/CREB transcription factor gene family. Its transcription has previously been shown to be testis-specific and within the testis to be restricted to haploid spermatids. In this study, we characterized the protein's distribution in the testis and found that it accumulates in late round and in elongating spermatids, corresponding to developmental stages considered transcriptionally silent. ATCE1 accumulation is acrosome-specific and persists up to mature epididymal cells, at which stage the protein remained associated with the inner acrosome membrane even after acrosomal reaction. No nuclear localization was evident at any spermatogenic stage. Expression of full-length ATCE1 in various cell lines revealed ER and Golgi localization whereas truncation of the C-terminus allowed entrance into the nucleus. Potent transcriptional activation activity, from kB-containing regulatory elements (but not from CRE elements as one might expect), was observed using the C-terminally truncated nuclear form of ATCE1. These results raise the question of why would a transcription factor be specifically anchored to the acrosome inner membrane? An intriguing speculation that ATCE1 might be paternally delivered to the newly formed zygote is discussed.

Keywords: Acrosome; ATCE1; Spermatid; Transcription factor; Zygotic gene activation

Introduction

An important molecular regulatory mechanism that has been shown to operate at different stages of spermatogenesis is the cAMP-dependent transcriptional regulation. Two predominant transcription factors, which take part in this pathway, are CREB (cAMP-responsive element binding protein) and CREM (cAMP-responsive element modulator). Both factors contain a basic domain/leucine zipper motif (bZIP) through which they bind a specific *cis* DNA element, designated cAMP-responsive element (CRE), present in the promoter of target genes. cAMP-dependent phosphorylation of a specific serine residue (S^{133} on CREB and S^{117} on CREM) activates these transcription factors to bind p300/CBP (CREB binding protein), a co-activator that recruits the basal transcription machinery to enable transcription initiation (Andrisani, 1999; Behr and Weinbauer, 1999). CREB plays an important role mainly in Sertoli cells (Andrisani, 1999; De et al., 1993) while CREM is more dominant in germ cells (for a review, see Don and Stelzer, 2002). As spermatocytes complete the meiotic division and enter the haploid phase, a prominent switch occurs from inhibitory to activating isoforms of CREM (De Cesare et al., 2000). An interesting feature of CREM activity in haploid round spermatids is its phosphorylation independence. It has been shown that CREMô transcription factors interact with ACT (activator of CREM in testis), which functions as a co-activator, thus bypassing the requirement for the phosphorylation-dependent binding to CBP (Fimia et al., 1999).

Atce1 was identified in a two-hybrid screen for mouse genes that might interact with TCTEX2 (Stelzer and Don, 2002) and simultaneously as *Tisp40* in a search for mouse genes that are significantly upregulated during spermiogenesis (Fujii et al., 2002). Its polypeptide contains a basic domain followed by a leucine zipper motif (bZIP domain). The six C-terminal amino acids of the basic domain constitute a putative nuclear localization signal. In addition, two glutamine-rich regions are present. The first is located at the amino terminal region of the peptide and the second is included in the leucine zipper region and extends beyond it towards the carboxy end of the peptide (Stelzer and Don, 2002). This peptide shows significant homology to a CREB subfamily whose prototype members are the

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mouse LZIP-1 and LZIP-2 genes (Burbelo et al., 1994). More specifically, it shows the most significant homology to the human Luman protein (CREB3, Lu et al., 1997; and human CREB-H, Omori et al., 2001). Tissue distribution of *Atce1* transcripts revealed that it is restricted to late haploid round spermatids and to elongating spermatids (Stelzer and Don, 2002). In addition, it was shown that an in vitro translated ATCE1 protein binds to the NF-κB enhancer rather than to the CRE element, suggesting that ATCE1 plays a role distinct from that of CREM. Here we show that the ATCE1 protein localizes specifically to the sperm acrosome and that it stays anchored to the inner acrosome membrane even after the acrosome reaction has occurred. We also show that ATCE1 can indeed transactivate transcription of a reporter gene, specifically through the NF-κB element. To our knowledge, this is the first report of a transcription factor localized to the acrosome, and we suggest that this acrosomal inner membrane localization might enable transfer of ATCE1 into the zygote.

Materials and methods

Antibody production and affinity purification

Two polyclonal antibodies were raised in rabbits against synthetic or recombinant ATCE1 peptides, both of which represent sequences from the N-terminus of ATCE1. This region shows no sequence similarity to any other member of the CREB/CREM family of transcription factors or to any other mouse peptide present in the various databases. The first antibody was raised against the short synthetic peptide ETSPGRDSGVSEDPGS (amino acids 6–21), whereas the second antibody was raised against a recombinant fusion protein consisting of the ATCE1 N-terminus (amino acids 2–103) fused in-frame, and C-terminally to GST (GST-ATCE1). The immune sera were affinity purified on a GST-ATCE1 Sepharose column, and antibody specificity was verified by ELISA (Supplementary material, Fig. S1). Subsequent experiments were conducted with both antibodies revealing identical results.

Protein extraction, immunoprecipitation and Western blotting

Proteins were extracted from the various tissues with RIPA buffer (Tris pH 7.4 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 50 mM, SDS 0.1%, Triton X-100 1%, NaDeoxycholate 1%, NaVO4 0.2 mM supplemented with anti-protease cocktail), incubated on ice for 1 h and centrifuged at 10,000×*g* for 20 min. Insoluble material was then incubated for 1 h on ice with urea buffer (100 mM DTT, 9.8 M urea and 100 mM Tris, corrected to pH 7.4) for further extraction of proteins from the insoluble material, and lysates were centrifuged at 10,000×*g* for 20 min to remove yet insoluble material. Cellular fractionation of cultured cells was basically performed according to Braiman et al. (2001). In short, cells were pelleted at 500×*g* for 10 min at 4°C, resuspended in sonication buffer (Tris HCl pH 7.4 50 mM, NaCl 150 mM, EDTA 2 mM, EGTA 1 mM, sucrose 25 mM, supplemented with anti-protease cocktail), homogenized in a Dounce glass homogenizer and centrifuged at 1100×*g* for 5 min at 4°C (pellet 1 and supernatant 1). Pellet 1 was washed twice with PBS, resuspended in buffer B (50 mM HEPES, 25% glycerole, 0.42 M NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA, supplemented with anti-protease cocktail) for 45 min (short vortex every 5 min) and centrifuged at 30,000×*g* for 45 min at 4°C to obtain pellet 2 and supernatant 2. Pellet 2 was discarded whereas supernatant 2 contained the soluble nuclear fraction. Supernatant 1 was centrifuged at 31,000×*g* for 60 min (pellet 3 and supernatant 3) and the supernatant from this centrifugation (supernatant 3) was further centrifuged at 190,000×*g* for 60 min (pellet 4 and supernatant 4) to collect the light-density microsome fraction, or the inner membrane (IM) fraction (pellet 4). Supernatant 4 contained the soluble cytoplasmic fraction. To further discriminate between peripheral or integral membrane proteins within the IM fraction, the pellet (pellet 4) was resuspended in 50 µl of sodium carbonate 0.15 M at pH 11.3 (or alternatively 0.2 M at pH 9.6) for 30 min in 4°C, according to Gibson et al. (2005) with minor modifications, and centrifuged at 100,000×*g* for 15 min at 4°C. The pellet of this centrifugation consisted of integral membrane proteins whereas the supernatant contained the non-integral peripheral proteins. All fractions were stored at minus 70°C until use. For immunoprecipitation, 1 mg total protein from each sample was brought to a final volume of 2 ml with PBS containing 0.55% Triton X-100, and incubated under gentle agitation for 2 hours at 4°C with 20 µl slurry of protein A/G PLUS-Agarose bead (Santa Cruz), to remove proteins binding non-specifically to the beads. Beads were removed by centrifugation at 20,000×*g* for 1 min and supernatant was transferred into new microcentrifuge tubes. 5 µg of affinity purified anti-ATCE1 antibody was added to each tube and incubated overnight at 4°C with agitation, followed by addition of 30 µl slurry of the protein A/G Agarose bead and incubation for 2 h at 4°C with agitation. Beads were then washed 5 times with PBS, boiled 5 min in 2% SDS containing loading buffer and resolved by SDS PAGE. For Western blotting analysis proteins were resolved by SDS PAGE and then transferred to a nitrocellulose membrane which was blocked for 1 h with 5% skim milk in PBST (PBS containing 0.05% Tween), incubated overnight with affinity purified anti-ATCE1 antibodies (diluted 1:1000 in 1% skim milk PBST), washed with PBST and incubated for 1 h with alkaline phosphatase (AP) conjugated monoclonal anti-Rabbit IgG antibody (Sigma), diluted 1:3000 in PBST. Signal was visualized by a colorimetric reaction with the AP substrate Western Blue® (Promega).

Plasmids, constructs and mutagenesis

For constructing the GST-ATCE1 plasmid, ATCE1 N-terminus coding region was PCR amplified using the following primers: forward 5′-CCCCCggatccATACACTGCTCAGAAACATCTCCT-3′ and reverse 5′-GGGGGActcgagGGACGGCATGGCTGCAGG-3′. *Bam*HI/*Xho*I digested PCR product was cloned at the corresponding sites into pGEX-4T-1 (Pharmacia) in frame to Glutathione-S-Transferase (GST). For constructing various HA-tagged ATCE1 plasmids, PCR fragments coding for either full-length or C-terminal deleted ATCE1 were amplified using the same forward primer: 5′-CCCCCgaattcACTGCTCAGAAACATCTCCTG-3′ but different reverse primers: 5′-CCCCCctcgagCTCATCTGTATGCACCATTCC-3′ and 5′-AAAAActcgagGTCTGGGCAGCTCTGCTG-3′, respectively. *Eco*RI/*Xho*I digested PCR products were cloned at the corresponding sites into the pCMV-HA expression vector (Clontech) obtaining the pHA-ATCE1 and pHA-ATCE1-C-del vectors. The Putative Trans Membrane Domain (PTMD, amino acids 220–237) was deleted from pHA-ATCE1 using the following primers: forward 5′-CAGACCAGCACCTGTGTTTTTCAGGGTCAATCAGAAG-3′, and reverse 5′-CTTCTGATTGACCCTGAAAAACACAGGTGCTGGTCTG-3′. The N-terminal domain (amino acids 10–131) was deleted from both pHA-ATCE1 and pHA-ATCE1-C-del, using the QuickChange Site Directed Mutagenesis kit (Statagene) and the following primers: forward 5′-CACTGCTCAGAAACATCTCCTACCAAGGCAGAGGAGAGAATAC-3′, and reverse 5′-GTAT TCTCTCCTCTGCCTTGGTAGGAGATGTTTCTGAGCAGTG-3′. This gave rise to the pHA-ATCE1-N-del and pHA-ATCE1-C-del-N-del vectors.

Directional 5 tandem repeats of either NF-κB or CRE elements were created as follows: For the NF-κB repeats the following complementary oligonucleotides 5′-gatccCTCGGAGGGGAATCTCCCGGGTTACTa-3′ and 5′-gatctAGTAACCCGGGAGATTCCCCTCCGAGg-3′ (taken from the IL-2 receptor-α promoter sequence) were used, whereas for the CRE repeats we used the oligonucleotides 5′-gatccGTATGTAGTGACGTCACAAGAGAGCa-3′ and 5′-gatctGCTCTCTTGTGACGTCACTACATACg-3′ (taken from the Transition Protein 1 promoter sequence). Briefly, for each construction, 2 µg of oligos was incubated with 5 units of BamHI, 5 units BglII and 0.1 unit of T4 DNA ligase in manufacturer's ligation buffer (ROCHE) at 37°C for 60 min, after which an aliquot of 0.5 µg DNA, 2.5 units BglII, 2.5 units BamHI and 0.1 unit ligase was added every 30 min for 2 h under the same conditions. After additional 30 min at 37°C, ligase was inactivated at 70°C for 15 min. To verify that fragments ligated in the undesired orientation are digested, 10 units of both BamHI and BglII were then added for additional incubation at 37°C for 60 min. Upon phenol extraction, a 165 bp fragment was isolated from a 2.5% TBE agarose gel, purified and cloned into the *Firefly* luciferase reporter vector (pGL3, Promega) via *Bgl*II site. Composition and orientation of all constructs were verified by sequencing.