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A *Bombyx mori* Transcription Factor, ATFC Binds Directly to the UPRE of Molecular Chaperones

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Cells respond to an accumulation of unfolded proteins in the endoplasmic reticulum (ER) by increasing transcription of genes encoding molecular chaperones and folding enzymes. The information is transmitted from the ER lumen to the nucleus by intracellular signaling pathway, called the unfolded protein response (UPR). In *Saccharomyces cerevisiae*, such induction is mediated by the cis-acting unfolded response element (UPRE) which has been thought to be recognized by Hac1p transcription factor. We cloned the ATFC gene showing similarity with Hac1p, and then examined to determine whether ATFC gene product specifically binds to UPRE by electrophoretic mobility shift assays. ATFC gene product displayed appreciable binding to ^{32}P-labelled UPRE. Therefore, we concluded that ATFC represents a major component of the putative transcription factor responsible for the UPR leading to the induction of ER-localized stress proteins.

Key words: ATFC, *Bombyx mori*, bZIP, ER, Hac1p, Molecular chaperone, UPR, UPRE

Introduction

One of the most important functions of the endoplasmic reticulum (ER) is to provide an environment that facilitates the proper folding and assembly of newly synthesized secretory proteins and plasma membrane proteins. Therefore the ER contains numerous ER chaperones, ER foldases and ER degradases that assist in ER quality control (Gething and Sambrook, 1992). If the unfolded proteins in the ER accumulate, transcriptional induction pathway, termed the unfolded protein response (UPR) is activated. Primary targets of the UPR are molecular chaperones and folding enzymes localized in the ER; induction of these proteins augments the capacity of the protein folding system, leading to homeostasis of the ER. In addition, some of the proteins involved in ER-associated degradation (ERAD) system to clear misfolded proteins from the ER are up-regulated by the UPR. Accumulating evidence indicates that the UPR is of fundamental importance in the quality control of proteins in the ER, under not only ER stress, but also normal growth conditions (Kaufman, 1999; Mori, 2000; Urano *et al.*, 2000a; Patil and Walter, 2001).

A majority of the UPR signaling pathway in *Saccharomyces cerevisiae* has been unraveled (Kaufman, 1999; Mori, 2000; Urano *et al.*, 2000a; Patil and Walter, 2001); the three gene products, Ire1p, Rlg1p and Hac1p, play essential roles in the yeast UPR. Ire1p has domain structures ideal to sense the presence of unfolded proteins and transduce the signal across the ER membrane. Ire1p is a type I transmembrane protein, the N-terminal half of which is located in the ER lumen; the protein kinase and endoribonuclease domains are situated on the cytoplasmic side. As a result of ER stress-induced oligomerization and autophosphorylation, the activated Ire1p specifically cleaves HAC1 precursor mRNA to remove an intron of 252 nucleotides (Chapman *et al.*, 1998). The cleaved 5' and 3' halves of mature HAC1 mRNA are ligated by Rlg1p (tRNA ligase). HAC1 mRNA encodes the basic leucine zipper (bZIP)-type transcription factor Hac1p, which binds to the unfolded protein response element

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(UPRE) in the promoters of ER chaperones and transcriptionally up-regulates their expression (More *et al.*, 1996).

In mammals, there were many attemption to isolated Ire1p, Rlg1p and Hac1p homologs, too. As a result, mammalian cells were found to express two Ire1p homologs designated as IRE1α and IRE1β; both are type I transmembrane proteins in the ER with their cytoplasmic regions carrying protein kinase and endoribonuclease domains (Tirasophon *et al.*, 1998; Wang *et al.*, 1998; Niwa *et al.*, 1999; Iwawaki *et al.*, 2001). Purified cytoplasmic regions of both IRE1α and IRE1β can cleave HAC1 precursor mRNA at the same sites as yeast Ire1p (Tirasophon *et al.*, 1998; Niwa *et al.*, 1999). These results support the importance of Ire1p homologs in the mammalian UPR and also strongly suggest the presence of a Hac1p-like transcription factor(s) in mammalian cells, expression and/or activity of which are regulated by an unconventional mRNA splicing system. However, no such protein has yet been discovered (Yoshida *et al.*, 2001).

Previously, we selected 40 clones that differentially expressed when Bm5 cells treated tunicamycin and produced expressed sequence tags (ESTs). Among these clones, we have isolated ATFC gene (GenBank acc. No. AF325210) showing similarity with yeast HAC1 mRNA (Goo *et al.*, 2001). bZIP domain in amino acid sequences of ATFC shared homology with several transcription factors, yeast Hac1p, human CREB and mouse ATF. Also, this ATFC up-regulated by accumulation of unfolded proteins in the ER through the treatment of ER stress drugs (Goo *et al.*, 2003). However, there is as yet no evidence regarding whether ATFC is directly binds to the UPRE in the promoters of ER chaperones and transcriptionally up-regulates their expression (Goo *et al.*, 2003).

Thus, We describe here the identification of a transcription factor, ATFC that regulates the UPR by binding to the UPRE only when the signaling pathway is activated. The data in this study cover the first set of results, showing that ATFC has a major role in the insect UPR.

Materials and Methods

Construction of Expression vector for ATFC and Western blot analysis

The cDNA encoding ATFC was amplified using a sense primer (5'-CGGGATCCCGGAAATGTCGTGTCGTGCTATGGTGTCG-3'; underline indicates the initial codon) and an antisense primer (5'-CCGCTCGAGCGGTTCCTTGATTAGACCCTTC-3'; underline indicates the stop codon that was modified from the original TAA). The PCR products were ligated once to a TA cloning vector, pGEM-T (Promega). The pGEM-ATFC was digested with *Bam*HI/*Xho*I and subcloned into the baculovirus expression transfer vector (Novagen), pBAC-1. After incubation for 15 min, a mixture pBAC1-ATFC/BacVector-3000 Triple Cut Virus DNA (Novagen) with Eufectin (Novagen) was inoculated at 27°C for 5 hrs at the Sf-9 cell line. The infected cells were collected after 3 days and digested with lysis buffer (6.25 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% DTT). Electrophoresis was performed in 12.5% polyacrylamide gels. Protein transfer to a PVDF membrane was carried out in a semi-dry system from Bio-Rad. The PVDF membrane was quenched for 30 min in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 containing 2% non-fat dry milk (TBS-milk). Immunological detection was done by first incubating the PVDF membrane for 1 hr at room temperature with the primary antiserum in TBS containing 0.05% Tween 20. Subsequently, the PVDF membrane was incubated for 1 hr with peroxidase-conjugated secondary immunoglobulins. The PVDF membrane was washed extensively between each step with TBS-milk.

Purification of the ATFC-His_6 fusion protein

To facilitate the isolation of recombinant *B. mori* ATFC proteins, the C-terminal of ATFC was His_6-tagged. The histidine tagged ATFC fusion proteins were purified by IMAC using a Ni^{2+}-immobilized resin (HisTrap chelating column; Pharmacia Biotech). The column was equilibrated with start phosphate buffer (20 mM phosphate, 0.5 M NaCl, 10 mM Imidazole, pH 7.4), and the homogenized/clarified sample was applied. Several fractions (0.5 mL) were collected as a flow through. The column was washed with start phosphate buffer, and sample fractions were collected as a wash sample. The fusion proteins were eluted by elution phosphate buffer of different imidazole concentration (50, 100, 200, 300, 400, and 500 mM). Fractions were monitored at A_{280} using the corresponding elution buffer as a blank and also detected by fluorescence intensity using a fluorescence spectrometer. Most bound proteins were eluted at imidazole concentration between 100 and 200 mM.

Electrophoretic mobility shift assay

Both 5'-GGAACTGGACAGCGTGTCGAAA-3' and 3'-CCTTGACCTGTCGCACAGCTTT-5' of double-stranded, synthetic oligonucleotides containing UPRE were radiolabelled using the Klenow fragment of DNA polymeraseI and [α-^{32}P] dATP (3000 Ci/mmpl), and were purified by electropforesis through an 8% polyacrylamide gel. The binding buffer consisted of 20mM HEPS, pH 7.9, 50mM KCL, 10 mM $MgCl_2$, 0.25 mM EDTA, 0.5 mM DTT, 2% Ficoll and 5% glycerol. Binding reactions were initiated by the addition of purified ATFC-His_6 fusion protein into a binding

buffer containing 0.25 ~ 0.3 ng of a radiolabelled probe (8,000 ~ 10,000 c. p. m), 1 μg of poly (dI-dC) : poly (dI-dC) (Pharmacia), and 1 μg of denatured salmon sperm DNA. Fivefold more ATFC-His_6 fusion protein was used because it tended to aggregate and remained largely t the top of gel. After incubation for 10 min at 4°C in a final volume of 20 μl, samples were loaded onto a non-denaturing 5% polyacrylamide gel (the acrylamide: bisacrylamide ratio was 30 : 0.8) containing 0.5% Ficoll. Gels were pre-run for 2 ~ 3 hrs and samples were electrophoresed for 3 hrs at 150 V at 4°C in 0.5 × TBE (Sambrook *et al.*, 1989). The gels were then dried and exposed to X-ray film.

Results and Discussion

Previously, we isolated 40 clones that differentially expressed when Bm5 cell lines treated with tunicamycin using a differential screening. Among these 40 clones, we have isolated a clone showing similarity with yeast HAC1 (Goo *et al.*, 2002). It was named ATFC (activating transcription factor of chaperones), and the sequence data of the ATFC was submitted to Genbank under the accession number AF325210). Although a cDNA encoding the ATFC shows a high sequence variation compare with known transcription factors, yeast HAC1, human CREB and mouse ATF, the ATFC protein has well conserved basic leucine zipper (bZIP) motif and up-regulated by accumulation of unfolded proteins in the ER stress drugs, which is similar to the other transcription factors. However, there was by then no evidence regarding whether ATFC is directly binds to the UPRE in the promoters of ER chaperones and transcriptionally up-regulates their expression (Goo *et al.*, 2003). In order to confirm whether or not the ATFC is directly binds to the UPRE in the promoters of ER chaperones, as do other transcription factor, the cDNA corresponding to the ATFC protein was expressed in Sf9 cells as a C-terminal polyhistidin fusion protein.

To produce ATFC recombinant proteins, baculovirus expression transfer vector (pBAC1-ATFC) was constructed (Fig. 1). The constructed pBAC1-ATFC and BacVector-3000 Triple Cut Virus DNA was co-transfected into Sf9 cells, then recombinant baculovirus, vAc-ATFC was selected by plaque assay. To analysis whether the ATFC gene was introduced in wild type AcNPV viral genome, vAc-ATFC viral DNA was extracted from Sf9 cell, which harvested 72 hrs after vAc-ATFC infection. The extracted vAc-ATFC viral DNA used as template for PCR screening with sense and antisence primer used in construction of the baculovirus expression transfer vector, pBAC1-ATFC.

As shown in Fig. 2, the PCR product of 740 bp size is correctly amplified in band when vAc-ATFC viral DNA as template used for PCR screening (lane 2), whereas no band estimated ATFC appeared when AcNVP viral DNA as template used for PCR screening (lane 1). The result suggests that the polyhedrin gene of AcNVP be replaced correctly with the *B. mori* ATFC gene. To confirm whether or not the cDNA encoding ATFC translates correctly *in vivo* by ATFC, the recombinant baculovirus (vAc-ATFC) was translated in the culture insect Sf-9 cell line.

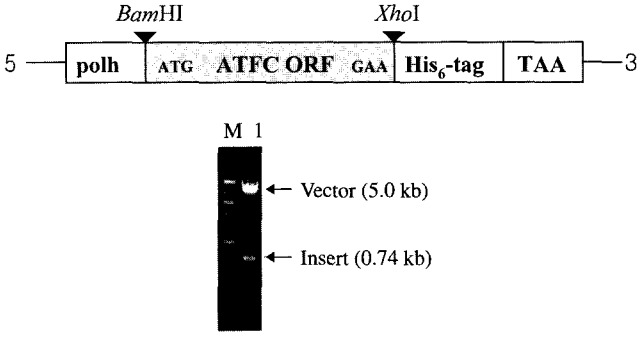

Fig. 1. Cloning of the open reading frame of the ATFC gene. M, 1 kb ladder DNA maker; lane 1, ATFC gene inserted in pBAC-1 baculovirus transfer vector digested with *Bam*HI/*Xho* I, and sample was analyzed on 1.0% agarose gel.

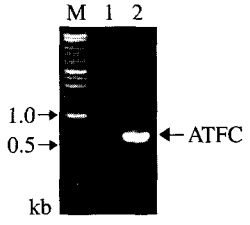

Fig. 2. PCR screening of the recombinant baculovirus, vAc-ATFC. PCR was performed with sense and antisense primer used in construction of the baculovirus transfer vector. M, 1 kb ladder DNA maker; lane 1, wild type baculovirus (AcNVPV) viral DNA as template used for PCR screening; lane 2, vAc-ATFC viral DNA as template used for PCR screening.

As shown in Fig. 3, the cDNA encoding the *Bombyx mori* ATFC was successfully translated in ATFC (line 3 in panel A), which was also recognized by anti-His_6 antibodies (line 3 in panel B). No band estimated ATFC appeared between the normal cells and the cells infected with the wild type baculovirus (line 1 and 2 in panel A and B). In addition, to facilitate the isolation of the ATFC recombinant protein, the C-terminal of ATFC was His_6-tagged (Fig. 1). That is, the ATFC recombinant proteins were expressed from Sf9 as fusion histidin-binding protein (ATFC-His_6), purified ATFC-His_6 fusion proteins were purified by IMAC using a Ni^{2+}-immobilized resin (Fig. 3) and tested for electrophoretic mobility shift assays.

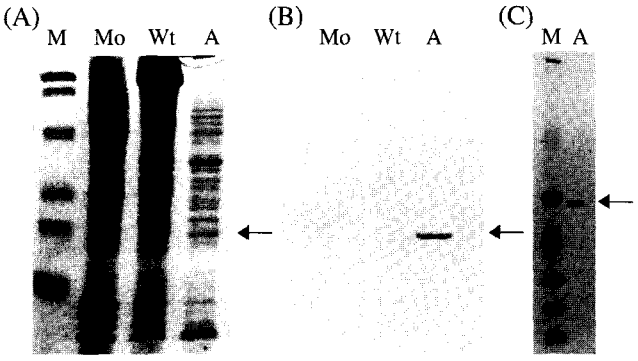

Fig. 3. SDS-PAGE (A), Western blot analysis (B) and purification of ATFC recombinant protein (C). Sf-9 cells (3.0×10^6) were infected with recombinant baculovirus (vAc-ATFC) encoding ATFC-His_6 at a total m.o.i of 5. Cells were harvested 72hrs after infection (lane 4). Western blot analysis was performed using a His_6tag antibody. The ATFC was expressed and purified from Sf-9 cells infected with vAc-ATFC as fusion protein (ATFC-His_6). Lane 2 (Mo), protein extracted from normal cellss; lane 3 (Wt), protein extracted from cells infected with wild type baculovirus; M, mid range protein size maker. Arrows indicate prepro ATFC protein.

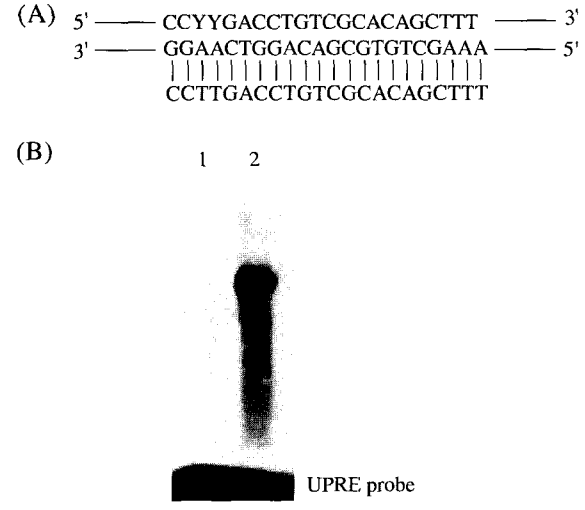

Fig. 4. Specific binding of the ATFC gene product to UPRE. Electrophoretic mobility shift assay was carried out using purified ATFC-His protein and 32P-labelled, synthetic double-stranded oligonucleotides containing 22-bp element, the UPRE as probe (A). Probe (0.3 ng) was mixed with ATFC-His fusion protein (0.5 μg) (line 2 in panel B) or no other addition (line 1 in panel B). Sample was separated by electrophoresis through a 5% nondenaturing polyacrylamide gel (B).

Electrophoretic mobility shift assays were carried out to determine whether ATFC-His_6 fusion proteins bind to the UPRE directly to activate transcription of genes encoding ER resident proteins. To this end, we incubated with ATFC-His_6 fusion proteins [α-^{32}P] labeled 22 bp oligonucleotide containing the UPRE sequence (Fig. 4a). After incubation, the mixture was fractionated on nondenaturing polyacryamide gels. ATFC-His_6 fusion proteins bound to the UPRE and retarded its migration in the gel, resulting in the formation of a new band (Fig. 4b).

These results are consistent with the conjecture that ATFC itself is the transcription factor binding the UPRE in the promoters of ER resident proteins. Therefore, we conclude that the *B. mori* ATFC represents a major component of the putative transcription factor responsible for the insect UPR leading to the induction localized stress proteins.

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