Biochimica et Biophysica Acta 1823 (2012) 2149–2156

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

ATF4 interacts with Abro1/KIAA0157 scaffold protein and participates in a cytoprotective pathway

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ARTICLE INFO

Article history:
Received 3 May 2012
Received in revised form 13 August 2012
Accepted 28 August 2012
Available online 6 September 2012

Keywords:
Activator protein-1 (AP-1) transcription factor
Activating transcription factor 4 (ATF4)
Abraxas brother 1 (Abro1/KIAA0157)
BRCC36 isopeptidase (BRISC) complex
Protein–protein interaction

ABSTRACT

Abro1 (Abraxas brother 1), also known as KIAA0157, is a scaffold protein that recruits various polypeptides to assemble the BRISC (BRCC36 isopeptide) deubiquitinating enzyme (DUB) complex. The BRISC enzyme has a Lys63-linked deubiquitinating activity and is comprised of four known subunits: MERIT40 (mediator of Rap80 interactions and targeting 40 kDa), BRE (brain and reproductive organ-expressed), BRCC36 (BRCA1/BRCA2-containing complex, subunit 3) and Abro1. We have previously shown that Abro1 has a cytoprotective role that involves the BRISC DUB complex acting on specific Lys63-linked polyubiquitinated substrates. In this report we identify three members of the AP-1 (activating protein-1) family, the ATF4, ATF5 (activating transcription factor) and JunD proteins, as specific interactors of Abro1. The function of ATF4–Abro1 interaction was investigated under normal conditions as well as under cellular stress. Abro1 is predominantly cytoplasmic, but during cellular stress it enters the nucleus and co-localizes with ATF4. Furthermore, this interaction with ATF4 is necessary and essential for the cytoprotective function of Abro1 following oxidative stress. The ability of Abro1 to specifically interact with a number of transcription factors suggests a new mechanism of regulation of the BRISC DUB complex. This regulation involves the participation of at least three known members of the AP-1 family of transcription factors.

1. Introduction

Our studies are focused on Abro1, which is the scaffold protein of the BRISC deubiquitinating enzyme (DUB) complex, and its potential regulation through specific protein–protein interactions during cellular stress. Abro1 shares 39% similarity with another protein, Abraxas, which is a scaffold protein for the BRCA1-A (breast cancer 1) DUB complex [1]. Both complexes share three subunits: MERIT40/NBA1, BRE/BRCC45 and BRCC36/BRCC3, a member of the JAMM/MPN+ (JAB1/MPN/Mov34) DUB metalloproteases [2–6]. The similarity between Abro1 and Abraxas is present at the amino-terminus and this part of the protein interacts with the common subunits. We therefore assume that the distinct function of the BRCA1-A and BRISC complexes is conferred by the unique carboxy-termini of these proteins. Abraxas' carboxy-terminus interacts with BRCA1, which targets the BRCA1-A complex to specific DNA damage foci while the function of the Abro1 carboxy-terminus domain is unknown [1,3,7–9]. Our previous studies have shown that this domain can interact with a human cardiospecific transcription factor, THAP5 (thanatos associated protein 5) [10]. THAP5 is predominantly expressed in the human heart whereas Abro1 is expressed ubiquitously, suggesting the existence of other as yet unidentified proteins that interact with the unique carboxy-terminus of Abro1 and affect its function. Furthermore, there is a disproportional number of subunits between the better characterized BRCA1-A complex consisting of six subunits whereas the BRISC complex consists of only four subunits. This is in spite of the fact that both scaffold proteins, Abro1 and Abraxas, are similar and comparable in size [2,3].

In the present study, we used the yeast two-hybrid system to isolate proteins that interact with the unique carboxy-terminus of Abro1. We screened a mouse embryonic cDNA library that provides a broad representation of various tissue specific mRNAs. We isolated JunD, ATF4 and ATF5 as specific interactors. These interactors are all distinct members of the AP-1 family of transcription factors [11]. They carry a basic-leucine zipper motif at their carboxy-terminus and our studies show this domain to interact with Abro1. We selected ATF4 for more detailed studies since this protein is expressed ubiquitously and it has previously been shown to be a stress modulator with an important function in heart disease [12,13]. Our results clearly show that Abro1 and ATF4 interact both *in vitro* as well as *in vivo* and this interaction is observed predominantly in the cell nucleus.

Abbreviations: Abro1, Abraxas brother 1; DUB, deubiquitinating enzyme; BRISC, BRCC36 isopeptidase complex; BRCA1, breast cancer 1; AP-1, activator protein 1; ATF, activating transcription factor; BRCC36, BRCA1/BRCA2-containing complex, subunit 3; BRE, brain and reproductive organ-expressed; MERIT40, mediator of Rap80 interactions and targeting 40 kDa; fosB, FBJ murine osteosarcoma viral oncogene homolog B; Maf, avian musculoaponeurotic fibrosarcoma; Ub, ubiquitin; bZIP, basic Leucine-zipper; THAP5, thanatos associated protein 5; RAP80, receptor associated protein 80; BARD1, BRCA1 associated RING domain 1; JAMM/MPN+, JAB1/MPN/Mov34 metalloenzyme

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http://dx.doi.org/10.1016/j.bbamcr.2012.08.020

Furthermore, during ER stress the protein level of ATF4 dramatically increases leading to a concurrent up-regulation of the ATF4–Abro1 complex. Our previous studies show that Abro1 provides cytoprotection against oxidative stress-induced cell death [14]. We now demonstrate that ATF4 is necessary for this function of Abro1, since reducing the ATF4 protein level significantly impairs its anti-apoptotic role. Finally, our results suggest that Abro1 can be involved in multiple specific interactions with various transcription factors. These interactions can either be tissue specific or in response to a particular stress. The potential function of these interactions could be the translocation of Abro1 scaffold protein and the BRISC complex to various subcellular compartments including the cell nucleus. This in turn potentially allows the BRISC DUB complex to act on specific local Lys63-linked polyubiquitinated substrates leading to cytoprotection.

2. Materials and methods

2.1. Yeast two-hybrid screen

The EGY 48 yeast strain with the LexA-β galactosidase reporter construct (PSH 18–34) was used to perform a yeast two-hybrid screen on a mouse embryonic cDNA library as previously described [10,15,16]. The bait used was the unique carboxy-terminus sequence of the Abro1 protein (amino acids 199–415) cloned in the pGilda vector (Clontech) and expressed as a LexA fusion protein. Several interacting proteins were identified in this screen, including a full-length cDNA for JunD and partial cDNAs for ATF4 and ATF5 (a complete list of the interactors found is summarized in Table 1). The full-length cDNAs for ATF4 and ATF5 were isolated from the mouse embryonic cDNA library using the following specific primers: mouse ATF4 full length: Fw 5′-GCTTCGAATTCATGACCGAGATGAGCTTCCTG-3′, Rw 5′-CACCGCTCG AGTTACGGAACTCTCTTCTTCCCCC-3′; and mouse ATF5 full length: Fw 5′-GCTTCGAATTCATGTCACTCCTGGCGACCC-3′, Rw 5′-CACCGCTCGAGC TAGGTGCTGCGGGTCCTC-3′. For the bZIP constructs of ATF4, ATF5, JunD, fosB and Maf the following primers with *EcoRI* and *XhoI* restriction sites were used: mATF4 aa280: Fw 5′-GCTCGAATTCGACC CACCTGGAGTTAGTTTG-3′; mATF5 aa195: Fw 5′-GCTTCGAATTCCCC GCCCGCCCAGCCCCTTATC-3′; mJunD aa241: Fw 5′-GCTTCGAATTCCCG GACGTGCCGAGCTTCGGC-3′; mfosB aa121: Fw 5′-CCGGAATTCGGT GGGCCTTCAACCAGCA C-3′, Rw 5′-CTGCTCGAGTTATGGCAAATCTCTCA CCTCGC-3′; and mMaf aa286: Fw 5′-CCGGAATTCATGTCGGTGCGCGA GCTGAAC-3′, Rw 5′-CTGCTCGAGCTACCGTTTTCTCGGAAGCCGTTG-3′. Primers were designed with an EcoRI site at the 5′ end and XhoI site at the 3′ end. PCR products were digested and cloned in frame into the prey pJG4-5 vector as previously described [15]. The presence and stability of the recombinant proteins in yeast cells was monitored by Western blot analysis using LexA-antibodies (for baits) or HA-antibodies (for preys).

2.2. Cell culture

HEK293T cells were grown in DMEM supplemented with 10% fetal calf serum (Atlanta Biologicals), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37 °C and 5% CO_2.

Table 1
A list of Abro1$_{199–415}$ interactors isolated in our yeast two-hybrid screen using a mouse embryonic cDNA library.

Abro1$_{199–415}$ interactors	Accession number	Number of independent clones
ATF4	NM_009716.2	8
JunD	NM_010592.4	4
BRCC36	NM_001018055	4
ATF5	NM_030693.2	2
UbC	NM_019639	1

2.3. Interaction between Abro1 and ATF4 proteins in HEK293T cells

HEK293T cells were plated in 100 mm dishes and transfected with 8.0 μg of either EGFP-Abro1$_{1–415}$ plasmid or pEGFP-C1 empty vector (Clontech) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Fourteen hours after transfection, half of the cells were treated with 20 μg/ml of tunicamycin for 10 h and the other half were used as control (untreated). Cell lysates were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% NP-40, 0.25% sodium deoxycholate) containing the protease-inhibitor cocktail (Roche). The lysates were cleared by centrifugation for 10 min at 10,000 *g*, and total lysates were collected and subjected to immunoprecipitation. Approximately 200 μg of total protein cell lysate was pre-cleared by mixing with protein G-Agarose beads (Roche) for 1 h followed by incubation with ATF4 (Santa Cruz Biotechnology) or Abro1 (Bethyl) polyclonal antibodies for 2 h at 4 °C. Protein G-Agarose beads were then added and allowed to bind overnight at 4 °C. Immunoprecipitates were collected by brief centrifugation for 3 min at 800 *g* and washed three times with 500 μl RIPA buffer. After the final wash, 25 μl of RIPA buffer and 25 μl of 2× SDS-PAGE sample buffer were added. All procedures were performed at 4 °C. Samples were boiled for 5 min and resolved by SDS-PAGE. Proteins were then electro-transferred onto a PVDF membrane and probed with either a mouse monoclonal GFP (Santa Cruz Biotechnology) or a rabbit ATF4 antibody followed by a goat anti-mouse or Rabbit TrueBlot (eBiosciences) HRP-conjugated secondary-antibodies. The immunocomplex was visualized using a SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.4. Sub-cellular localization of Abro1 and ATF4 proteins

To investigate the sub-cellular localization of Abro1 protein in the presence or absence of ATF4, the cDNA encoding the full length Abro1 protein was cloned into pEGFP-C1 vector (Clontech) and the cDNA for the full length ATF4 protein was also cloned into the mRFP-C1 vector (Clontech). HEK293T cells were grown on glass cover slips to 70% confluence and they were then transfected with 1 μg of EGFP-Abro1 and mRFP-ATF4 constructs using Lipofectamine 2000 Transfection reagent. Half of the transfected cells were treated with 20 μg/ml tunicamycin for 10 h and the other half used as control. Both control and treated cells were washed and fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100 and incubated in room temperature with DAPI (Molecular Probes) in order to stain the nucleus. The cover slips were then washed and placed on microscope slides using Fluoromount-G as the mounting solution. Slides were observed using a Leica TCS SP5 II confocal laser-scanning microscope (Leica). The expression and stability of the GFP-Abro1 and RFP-ATF4 proteins was verified by Western blot analysis using ATF4 or Abro1 specific antibodies. For subcellular fractionation, HEK293 cells were grown to 80% confluence and they were either left untreated (control), treated with 20 μg/ml tunicamycin or 0.2 mM H_2O_2 for 10 h. Cells were then collected and nuclear/cytoplasmic fractionation was carried out using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (THERMO Scientific) according to the manufacturer's protocol. A total of 40 μg of protein from each corresponding fraction was resolved by SDS-PAGE and analyzed by Western blotting using ATF4, Abro1, β-actin and Histone H1 specific antibodies.

2.5. Regulation of Abro1 and ATF4 proteins by tunicamycin

HEK293T cells were plated in 60 mm dishes and when they reached 90% confluence they were treated with various concentrations of tunicamycin (0, 2.5, 5, 10, 20 and 50 μg/ml) for 10 h. Total cell lysates were prepared as described above and 40 μg of protein was resolved by SDS-PAGE and analyzed by Western blotting using ATF4 or Abro1 specific antibodies.

2.6. Inhibiting ATF4 protein level and Annexin V assay

HEK293T cells grown in 35 mm dishes were co-transfected with 2.0 μg pEGFP-C1 empty vector or pEGFP-Abro1 plasmid together with siRNA specific for ATF4 (siATF4) siGENOME SMARTpool or scrambled siRNA (siCON) (Thermo Scientific). Some of the transfected cells were treated with 0.2 mM of H_2O_2 for 10 h. The percentage of apoptotic cells in the transfected population was estimated by staining with phycoerythrin-conjugated Annexin V at room temperature for 15 min in 1× binding buffer followed by analysis on a FACSCalibur flow cytometer [10,14]. Cells were also used to prepare cell lysates for Western blot analysis using ATF4 and Abro1 specific antibodies.

3. Results

3.1. Isolation and characterization of Abro1 specific interactors

We employed a yeast two-hybrid system to isolate LexA-Abro1$_{199–415}$ interactors. We used a mouse embryonic cDNA library in order to screen as many diverse proteins as possible, including any tissue-specific interactors. Furthermore, using a cDNA library constructed from primary cells avoids a potential problem often seen in cDNA libraries prepared from transformed cell lines which often have deregulated expression of genes involved in cell growth as well as cell death. The bait in this screen was the LexA-Abro1$_{199–415}$, which represents the coil–coil domain as well as the unique carboxy-terminus of Abro1 protein that is completely different from its homologue, Abraxas (Fig. 1A). The screen was performed as previously described [10,15,16]. Approximately one million independent yeast colonies were screened, one hundred interactors were isolated and after further analysis several LexA-Abro1$_{199–415}$ specific interactors were identified (Table 1). One of the specific interactors was a cDNA encoding the full length JunD protein, while two other cDNAs represented partial polypeptides of ATF4 (amino acids 21–349) and ATF5 (amino acids 20–283). All three interactors are members of the AP-1 family of transcription factors [11]. Other interactors included Ubiquitin C (UbC) and BRCC36. BRCC36 is a known interactor of Abro1 and its isolation provides independent proof that the yeast two-hybrid screening was successfully performed. Using specific primers and rapid amplification of cDNA ends we were able to isolate the full-length cDNAs of ATF4 and ATF5, which were then cloned back into the pJG4-5 vector and their interaction with LexA-Abro1$_{199–415}$ was verified. As with the partial polypeptides, full-length ATF4 and ATF5 proteins were able to interact with LexA-Abro1$_{199–415}$ in yeast

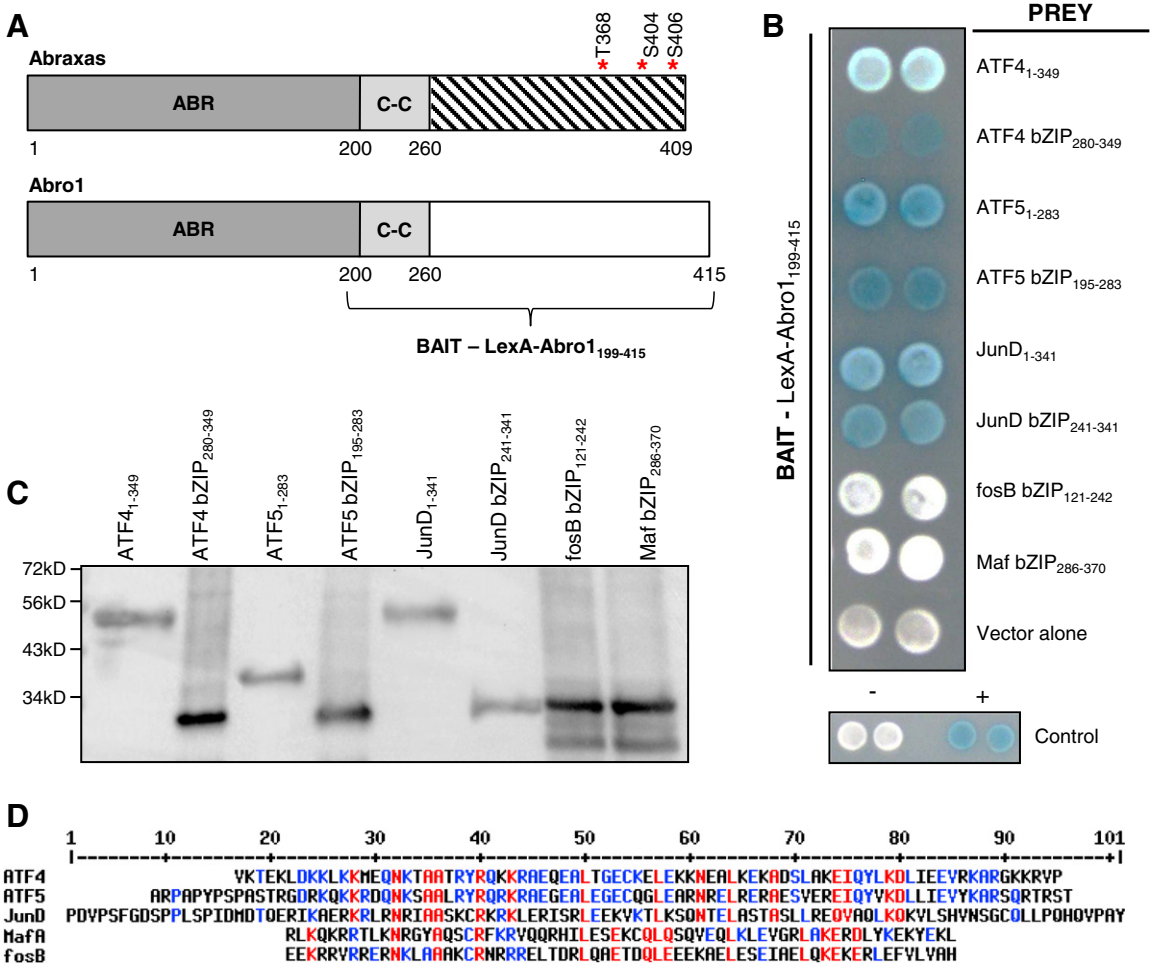

Fig. 1. ATF4, ATF5 and JunD interact with LexA-Abro1$_{199–415}$ using their bZIP domain. (A) A schematic representation of the Abraxas and Abro1 proteins showing their similar ABR and coil–coil domains as well as their unique carboxy-termini. Abraxas contains three phosphorylation sites (denoted by the *) that regulate its interaction with BRCA1–BARD1 which are absent in Abro1 [1]. The unique carboxy-terminus of Abro1 (amino acids 199–415) fused to LexA was used as the bait in the yeast two-hybrid screen. (B) Blue yeast colonies result from a positive protein–protein interaction between the bait and prey. (C) Single yeast colonies were grown overnight, induced with galactose/raffinose media the next day for 4 h and then used in a Western blot analysis to verify the expression and stability of the various recombinant proteins in yeast. (D) Alignment of the amino acid sequences of the AP-1 family members used in our experiments to illustrate the similarity in their bZIP domains. [MultAlin software (http://multalin.toulouse.inra.fr/multalin)].

(Fig. 1B). Fig. 1C shows that all constructs used in the experiments expressed the recombinant proteins in yeast.

ATF4, ATF5 and JunD are the only members of the large AP-1 family of proteins that were isolated in our screen [11]. They are distinct members but they all carry a similar bZIP domain at their carboxy-terminus (Fig. 1D). To investigate the specificity of the interaction between these three proteins and LexA-Abro1$_{199–415}$, as well as to investigate if the bZIP domain is involved in this specific interaction, we chose two other members of the AP-1 family as controls, namely fosB and Maf [17]. ATF4, ATF5 and JunD share 59% similarity at the bZIP domain, whereas fosB and Maf are only 33% similar (Fig. 1D) [18]. The bZIP domain of ATF4, ATF5 and JunD clearly interacted with the unique carboxy-terminus of Abro1 equally well or even stronger than the full-length proteins (Fig. 1B). As predicted there was no detectable interaction between LexA-Abro1$_{199–415}$, and the bZIP domain of either fosB or Maf (Fig. 1B).

3.2. Interaction of Abro1 with ATF4 in mammalian cells during stress

We focused our studies on ATF4 because this protein, like Abro1, is expressed ubiquitously, it is known to be involved in generalized stress response and participates in cardiovascular disease [19]. To investigate whether Abro1 can interact with ATF4 *in vivo*, in the presence or absence of cellular stress, HEK293T cells were transfected with a construct encoding full-length Abro1 fused to GFP (GFP-Abro1) or empty pEGFP-C1 vector. Tunicamycin was used to induce ER stress and 24 h after transfection total cell lysates were collected and a GFP antibody was used to precipitate the GFP-Abro1 protein. The presence of any ATF4 protein in the complex was monitored by Western blot analysis using an ATF4-specific polyclonal antibody. Fig. 2A shows that endogenous ATF4 interacts with GFP-Abro1 in HEK293T cells under normal conditions as well as during ER stress. After tunicamycin treatment, another form of the ATF4 polypeptide with a higher molecular weight also appears to associate with Abro1 [20]. In addition, we performed the reverse experiment using the ATF4 antibody to precipitate endogenous ATF4 and any associated GFP-Abro1 protein. Fig. 2B clearly shows that endogenous ATF4 interacts with the GFP-Abro1 protein. There was not any detectable interaction when GFP protein was expressed alone (Fig. 2A and B).

3.3. Subcellular localization of Abro1 and ATF4 proteins during stress

Abro1 is predominantly a cytoplasmic protein [3,4] while ATF4 is found both in the nucleus and the cytoplasm [21]. To better understand the biological role of the ATF4–Abro1 interaction, HEK293T cells were co-transfected with GFP-Abro1 (amino acids 1–415) and mRFP-ATF4 (amino acids 1–349). Using a Leica TCS SP5 II confocal laser-scanning microscope we observed that in the absence of stress, GFP-Abro1 was predominantly in the cytoplasm whereas mRFP-ATF4 was in the cell nucleus (Fig. 2C, top panel). When transfected cells were treated with tunicamycin, some of the GFP-Abro1 protein translocated to the nucleus where it co-localized with the mRFP-ATF4 protein (Fig. 2C, bottom panel). In cells co-transfected with GFP-Abro1 and empty mRFP, the GFP-Abro1 protein remained in the cytoplasm after tunicamycin treatment (results not shown). To quantify these results, a total of 300 co-transfected cells were counted between 15 fields of view for each control or following tunicamycin treatment. The average of co-transfected cells with GFP-Abro1 in the cytoplasm was 60%, which decreased to 28% in cells treated with tunicamycin. Cells with GFP-Abro1 in the nucleus increased from 40% in the control to 72% in tunicamycin treated cells.

To verify that the results are not due to the overexpression of these two GFP/RFP-fusion polypeptides, we investigated the potential translocation and co-localization of endogenous Abro1 and ATF4 proteins. For this experiment, untransfected cells were treated with tunicamycin or H_2O_2 to induce cellular stress. The cytoplasmic and nuclear fractions were isolated and analyzed by SDS-PAGE followed by Western blot. Fig. 2E shows that there was a small amount of Abro1 protein in the cell nucleus of control untreated cells. This level of nuclear Abro1 protein increased in cells treated with tunicamycin or H_2O_2. ATF4 was almost exclusively present in the cell nucleus; there was an increase in the ATF4 after oxidative stress but it was not as significant as the one following tunicamycin treatment. Furthermore, ATF4 protein had slightly different mobility in cells treated with H_2O_2 versus tunicamycin. These results suggest the possibility that different mechanisms are involved in regulating ATF4 in cells exposed to H_2O_2 and in cells treated with tunicamycin.

3.4. Regulation of ATF4 and Abro1 protein levels by tunicamycin

Translocation of Abro1 to the nucleus seems to occurs when ATF4 protein is overexpressed and at the same time cellular stress is induced. Therefore we investigated whether the endogenous level of ATF4 or Abro1 is regulated in cells treated with various concentrations of tunicamycin. Fig. 3A shows a progressive and significant increase in the ATF4 protein level in cells treated with various concentrations of tunicamycin. In contrast to ATF4, Abro1 protein level remained the same and was not regulated by tunicamycin (Fig. 3B).

3.5. Abro1 induced cytoprotection requires the presence of ATF4 protein

Abro1 has been previously shown to have a cytoprotective role following oxidative stress [14]. To investigate if ATF4 is involved in this process we transfected cells with GFP-Abro1 in the presence of ATF4 siRNA or scrambled control siRNA. Under normal conditions the basal level of apoptosis in cells transfected with GFP-vector alone was 12.5% versus 4% in cells transfected with GFP-Abro1 (Fig. 4A). When the cells were treated with H_2O_2, 71% of the cells transfected with GFP-vector alone were apoptotic compared to 44% of cells transfected with GFP-Abro1. Inhibiting the ATF4 protein level using siATF4 significantly reduced the Abro1 cytoprotective ability and the number of apoptotic cells increased from 44% to 64% (Fig. 4A). These results clearly demonstrate that ATF4 is necessary and essential for the cytoprotective function of Abro1 following oxidative stress. Fig. 4B shows the ATF4 protein levels under the various conditions as well as the effect of siATF4. Furthermore, induction of apoptosis by H_2O_2 in HEK293T cells does not affect the endogenous protein levels of Abro1 (Fig. 4C) and ATF4 (results not shown).

4. Discussion

Ubiquitination is a post-translational modification discovered in the 1980s that has gained a lot of attention for its biological significance and its potential role in the development of human disease (for a recent review see [22]). The modification is through the addition of ubiquitin, a small 76 amino-acid protein of about 8.5 kDa in size. There are a total of eight different polyubiquitin linkages, and depending on the lysine used on the ubiquitin for attachment, the number of ubiquitins in a chain or type of chain made, it can bestow a different fate to the substrate protein [23]. Ubiquitination is involved in diverse processes such as protein degradation, cell-cycle progression, receptor transport, immune-response and viral infection [24,25]. The most common and better-understood form of polyubiquitination involves the Lys48-linked chain. Substrates that are Lys48-linked are known to be targeted for degradation by the 26S proteasome [23]. Another form of ubiquitination is Lys63-linked, which has a non-proteolytic role but regulates a protein's function, its subcellular localization or protein–protein interaction in response to cytoplasmic or nuclear cues [26]. Ubiquitination can be reversed by deubiquitination, which is mediated by DUBs. There are over 100 putative DUBs identified in mammalian cells, subdivided into five-distinct families [27,28]. As with ubiquitination, deubiquitination is also highly regulated, it has

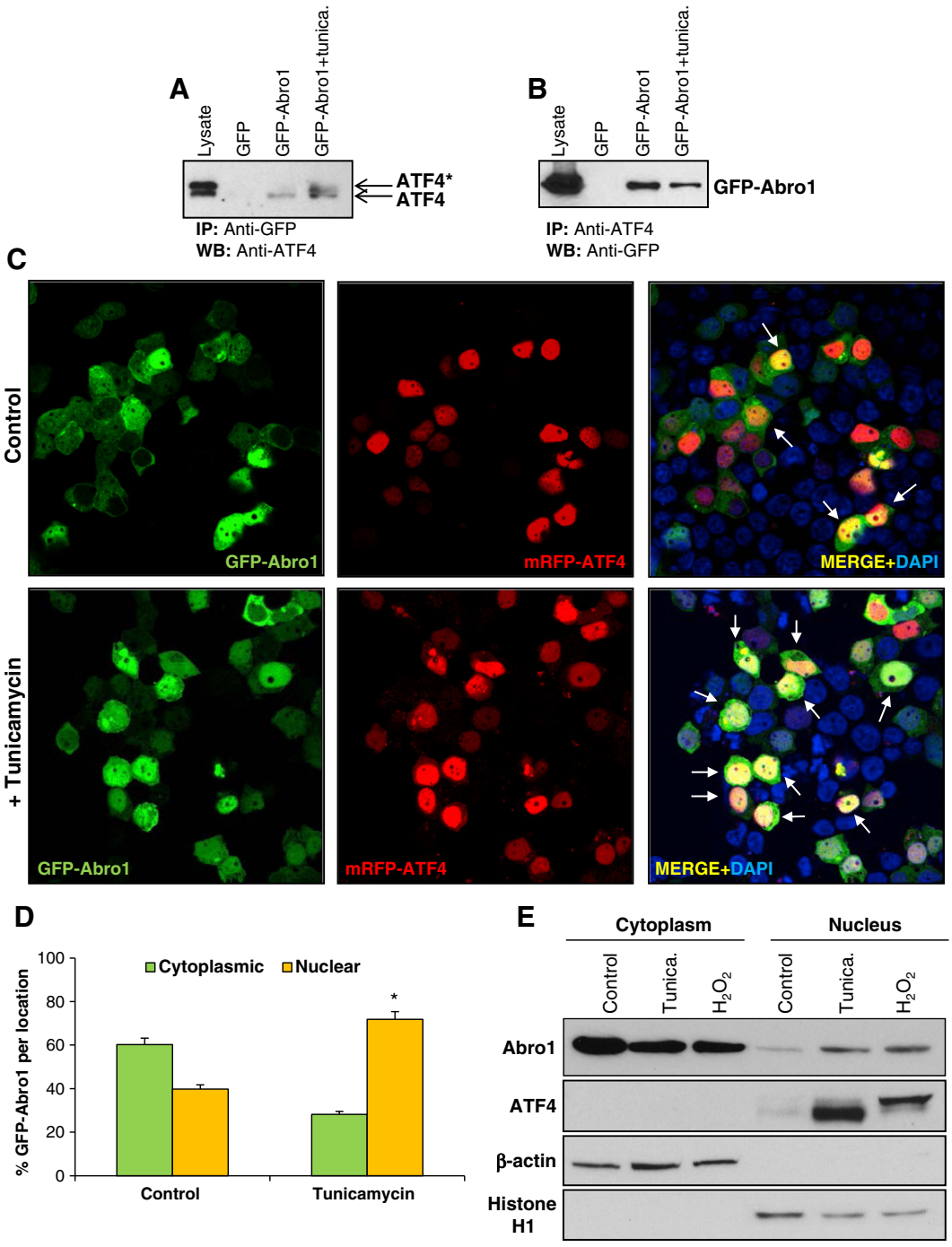

Fig. 2. Interaction and subcellular localization of Abro1 and ATF4 in mammalian cells. (A) HEK293T cells were plated in duplicate and transfected with either GFP-vector or GFP-Abro1. Twenty-four hours after transfection, one of the plates was treated with tunicamycin and the other plate was used as control. GFP-specific antibody was used to precipitate GFP or GFP-Abro1. The immuno-complex was resolved on SDS-PAGE followed by Western blot analysis and the presence of ATF4 in the precipitated complex was detected using ATF4 specific antibodies. (B) The reverse experiment was also performed where ATF4 antibodies were used to precipitate the endogenous protein complex and GFP antibodies were then used to identify any GFP-Abro1 protein bound to ATF4. (C) HEK293T cells were plated in duplicate on glass-cover slips and co-transfected with GFP-Abro1 and mRFP-ATF4. Half of the transfected cells were treated with tunicamycin and the other half used as control. Cells were stained with DAPI nuclear staining and visualized using a confocal microscope system. White arrows indicate cells that show co-localization of GFP-Abro1 with mRFP-ATF4 in the cell nucleus. (D) Three-hundred cells were counted in total between 15 different fields of view. Co-transfected cells were categorized as having GFP-Abro1 in the cytoplasm or nucleus. A percentage of the total counted cells is represented here. (E) HEK293T cells were grown and either left untreated for control, treated with tunicamycin or H_2O_2. Cells were then collected and nuclear/cytoplasmic fractionation was carried out, followed by analysis through SDS-PAGE and Western blot using ATF4 or Abro1 specific antibodies. β-actin and Histone H1 antibodies were used as cytoplasmic and nuclear markers respectively. *, $p<0.05$ versus cytoplasmic control.

been implicated in many cellular functions and its deregulation can lead to many human diseases [25,27,28].

We have recently uncovered an important role for Lys63-ubiquitination as a cytoprotective mechanism in the heart [14]. This mechanism is mediated by the BRISC DUB complex, which contains a member of the JAMM/MPN+ family of DUBs, BRCC36. Another complex that employs this JAMM/MPN+DUB is BRCA1-A. The BRCA1-A complex has been extensively studied and is found in the nucleus

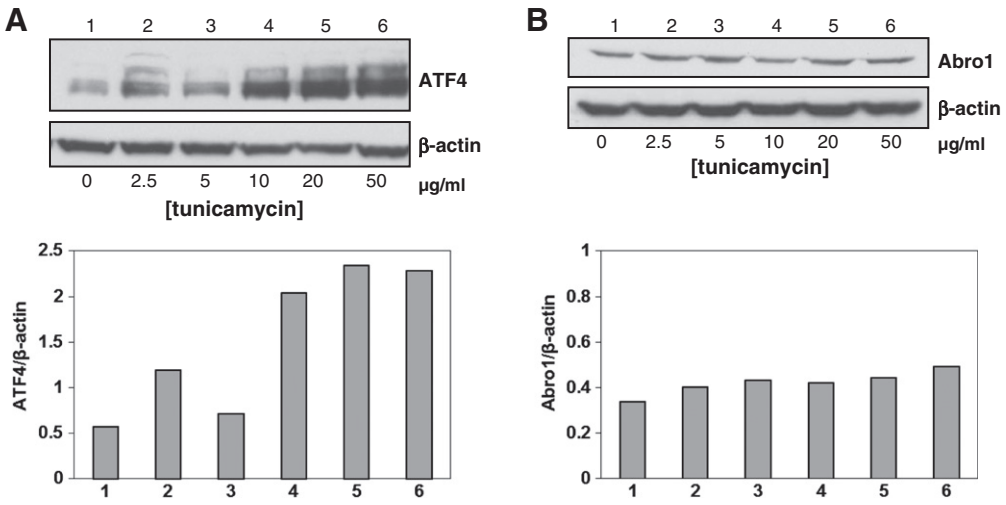

Fig. 3. Regulation of ATF4 and Abro1 protein levels by tunicamycin. HEK293T cells were treated with various concentrations of tunicamycin. Total cell lysates were collected and analyzed by SDS-PAGE and Western blotting using ATF4 (A) or Abro1 (B) specific antibodies. β-actin was used to verify equal loading of proteins in each lane. Lower panels show ATF4/β-actin or Abro1/β-actin ratio calculated after densitometry analysis.

where it interacts with histones and DNA to assist with double-stranded DNA damage repair [1,2,9]. Unlike the BRCA1-A complex, very little is known about the normal function of the BRISC complex or its role in cellular stress. Both complexes rely on similar scaffold proteins, Abro1 and Abraxas, to recruit the various polypeptides that make up the complex. The similarity of these two scaffold proteins is restricted to the amino-terminus of the proteins, whereas their carboxy-terminus is quite distinct. The BRCA1-A complex consists of the following polypeptides: RAP80, BRE, MERIT40, Abraxas, BRCC36 as well as the BRCA1–BARD1 heterodimer. The BRISC complex is made up of four subunit polypeptides: BRE, MERIT40, BRCC36 and Abro1. Three of these subunits (BRE, BRCC36 and MERIT40) are common in both complexes

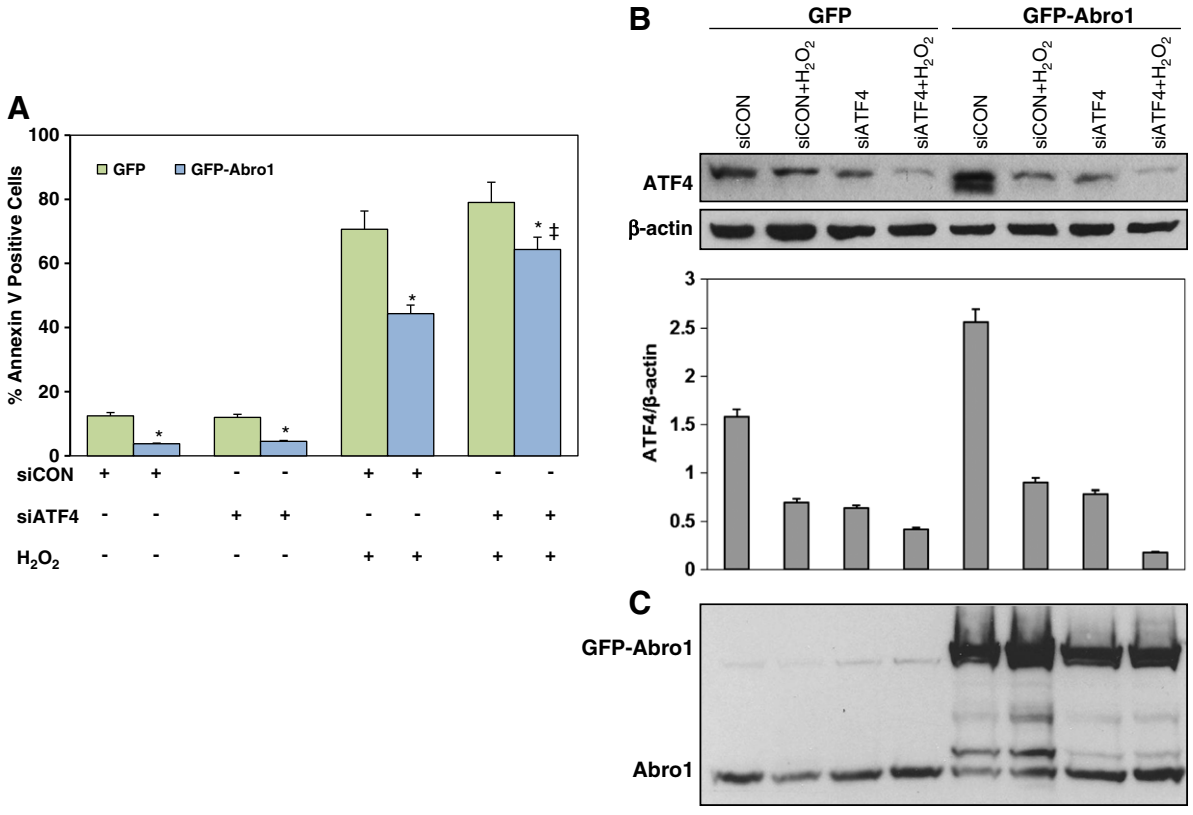

Fig. 4. ATF4 is required for Abro1-mediated cytoprotection during oxidative stress. HEK293T cells were co-transfected in duplicate with GFP-Abro1 or GFP-vector alone as well as with control siRNA (siCON) or ATF specific siRNA (siATF4). Half of the cells were treated with H_2O_2 to induce oxidative stress and cell death. (A) The percentage of apoptotic cells in the population was monitored by Annexin V staining and FACS. (B) Western blot analysis of the endogenous ATF4 protein level; bottom panel shows ATF4/β-actin ratio calculated after densitometry analysis. β-actin was used to verify equal loading of proteins in each lane. Results shown are means ± S.D. of three independent experiments. (C) Western blot analysis for the endogenous level of Abro1 as well as GFP-Abro1 proteins in the samples used for (A). *, $p<0.05$ versus GFP-vector; ‡, $p<0.05$ versus GFP-Abro1 siCON + H_2O_2.

and interact with the similar amino-terminus domain of Abro1 and Abraxas. The unique carboxy-terminus of Abraxas binds to RAP80 and the BRCA1–BARD1 heterodimer, where the interaction is regulated by phosphorylation [1,3]. RAP80 allows the complex to interact with ubiquitinated histones in DNA damage foci, while BRCA1–BARD1 interaction helps localize the complex [8,29]. Abro1 has its own unique carboxy-terminus and our recent studies have identified this domain to interact with the human zinc finger protein THAP5 [10]. THAP5 expression is highly restricted to the heart and brain, and furthermore it has no mouse or rat homologue [10]. Therefore, we assumed that, besides THAP5, there might be other protein(s) that could interact with Abro1 to modulate its activity or its subcellular localization. We used a yeast two-hybrid screen to isolate and characterize Abro1 specific interactors from a mouse embryonic cDNA library. From hundreds of potential interactors, we narrowed down our search to only a few true protein–protein interactors. These included the JunD, ATF4 and ATF5 proteins that are members of the AP-1 family of transcription factors as well as the BRCC36, a known component of the BRISC complex. JunD, ATF4 and ATF5 proteins are known to form homo- and heterodimers that bind to specific DNA sites to regulate the transcription of target genes [11].

We focused our studies on the ATF4–Abro1 interaction because this transcription factor is expressed ubiquitously (similarly to Abro1), it is known to be involved in cellular stress and to potentially play a role in the development and progression of heart disease [12,13]. The interaction of ATF4 with Abro1 was verified in yeast as well as in mammalian cells. The carboxy-terminus of ATF4 was shown to be responsible for the interaction with Abro1. This domain is known to carry the bZIP motif that is involved in protein–protein and protein–DNA interactions [30]. Furthermore, this motif is similar in JunD and ATF5 proteins that were also isolated in our screen. We verified the specificity of this interaction by using two other members of the AP-1 family (fosB and Maf) that have limited similarity at their bZIP motif with that of ATF4, ATF5 and JunD, and because of this they were unable to interact with Abro1. To investigate the biological significance of the Abro1–ATF4 interaction in normal cells and during cellular stress, cells were treated with tunicamycin (an ER stressor) and the subcellular localization of these two proteins was observed. Under normal conditions Abro1 is predominantly cytoplasmic and ATF4 is mostly nuclear but in the presence of tunicamycin Abro1 is translocated into the nucleus where it co-localized with ATF4. Since the translocation of Abro1 occurred when ATF4 was overexpressed and stress was applied, we investigated whether the endogenous ATF4 protein level might be regulated by tunicamycin. The ATF4 protein level was found to substantially increase and be directly proportional to the concentration of tunicamycin used. Abro1, on the other hand, did not respond to the tunicamycin treatment and its protein level remained unchanged. Furthermore, tunicamycin treatment not only increased the ATF4 protein level but also its post-translational modification. At higher tunicamycin concentrations various forms of ATF4 with higher molecular weight appeared. We assume that these represent polyubiquitinated forms of the polypeptide since it is known that ATF4 is ubiquitinated and degraded by the proteasome [31,32]. The biological relevance of the ATF4–Abro1 interaction was further investigated using a previously described assay where overexpression of Abro1 protects cells from apoptosis induced by oxidative stress [14]. Oxidative stress also increased the amount of nuclear Abro1 protein. In this assay, the cytoprotective effect of Abro1 was dramatically reduced when the ATF4 protein level was inhibited by siRNA. The interaction of Abro1 with ATF4, as well as ATF5 and JunD, suggests that various nuclear proteins can bind to this scaffold protein in the nucleus. These proteins could act as a "driver" for the Abro1 polypeptide in a similar manner to the BRCA1–BARD1 interaction with Abraxas to direct the BRCA1-A complex to the DNA damage foci [8]. In addition, we cannot exclude the possibility that the interaction of ATF4, ATF5 and JunD with Abro1 can also modulate the DUB activity of the BRISC complex. Based on our data, as well as previously published work, we were able to assign three discernible functional domains to the BRISC and BRCA1-A complexes: (a) the scaffold domain, represented by Abro1 and Abraxas; (b) the catalytic domain which includes the common polypeptides BRCC36, MERIT40 and BRE; and (c) the "driver" domain which in BRCA1-A includes the BRCA1–BARD1 heterodimer and RAP80, and in BRISC it can include one of the following four polypeptides: ATF4, ATF5, JunD and THAP5 (Fig. 5). The presence of several "driver" proteins that could interact with Abro1 at any time suggests that this process could be further regulated. The identity of the "driver" proteins that interact with Abro1 could be determined by the particular tissue/cell line or the type and severity of the cellular stress. In addition, we have previously shown that the translocation of Abro1 to the cell nucleus by THAP5 leads to the deubiquitination of specific substrates [14]. Our results suggest that either Abro1 translocation carries the complete BRISC complex to the nucleus or the complex assembles soon after nuclear localization of Abro1. It will be important to identify the Lys63-linked polyubiquitinated substrates of BRISC and to investigate how their deubiquitination results in cytoprotection against oxidative stress-induced cell death.

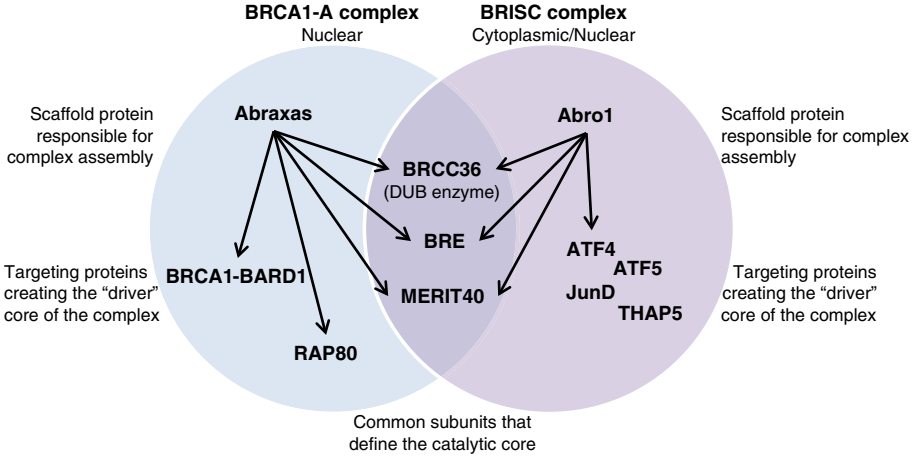

Fig. 5. Schematic diagram showing the current composition of the BRISC and BRCA1-A DUB complexes. Both BRISC and BRCA1-A complexes share three common subunits (BRCC36, BRE, MERIT40) that compose the catalytic core. BRCA1-A complex contains two unique proteins, RAP80 and the BRCA1–BARD1 heterodimer, that are used to target the complex. Our work also identified four unique proteins (THAP5, ATF4, ATF5 or JunD), three of which are members of the AP-1 family, which can be used to target the BRISC complex to the cell nucleus under conditions of cellular stress.

Acknowledgements

This work was supported by grant 2KB05 to A.S.Z. by the James and Esther King Biomedical Research Program, Florida Department of Health.

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