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Activation of the unfolded protein response and alternative splicing of ATF6α in HLA-B27 positive lymphocytes

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Abstract Misfolding of major histocompatibility complex (MHC) class I molecules has been implicated in the rheumatic autoimmune disease ankylosing spondylitis (AS), and has been linked to the unfolded protein response (UPR) in rodent AS models. XBP1 and ATF6α are two important transcription factors that initiate and co-ordinate the UPR. Here we show that misoxidised MHC class I heavy chains activate XBP1 processing in a similar manner to tunicamycin, with tunicamycin and dithiothreitol (DTT) inducing differential XBP1 processing. Unexpectedly, ATF6α mRNA is alternatively spliced during reducing stress in lymphocytes. This shorter ATF6α message lacks exon 7 and may have a regulatory role in the UPR.
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Keywords: Endoplasmic reticulum; Unfolded protein response; MHC; Transcription factor; Protein folding

1. Introduction

Controlled protein secretion by specialized cells like the B cell requires the co-ordinated synthesis of endoplasmic reticulum (ER) membranes, chaperones and folding factors. Terminally misfolded proteins in the ER must be removed to prevent chronic intracellular indigestion and the onset of apoptosis, and this is monitored by the unfolded protein response (UPR) [1]. The eukaryotic UPR consists of a number of specialized signaling molecules, including IRE1 [2], ATF6α [3,4] and the transcription factor XBP1 [5]. XBP1 binds upstream of a UPR target gene to initiate transcription, but only after it has been activated by unconventional mRNA processing. Activation of XBP1 mRNA is achieved by IRE1, which dimerises, autophosphorylates, and becomes active as a site-specific endo-RNase when unfolded proteins accumulate in the ER [6,7]. IRE1 is likely to bind unfolded proteins directly and is kept in the inactive (monomeric) form by BiP.

Whereas XBP1 is a cytosolic/nuclear transcription factor that is activated by IRE1, ATF6α can sense and initiate UPR gene transcription directly. ATF6α is a member of the ATF/CREB family of bZIP proteins [8] and the protein is cleaved in the Golgi by regulated intra-membrane proteolysis [9]. Stress-induced cleavage of the ATF6α precursor occurs after dissociation from BiP [10], releasing a cytosolic fragment. This liberated transcription factor can bind to consensus ERSE sequences (with NF-Y) upstream of target genes [11]. ATF6α can initiate XBP1 expression [5], and can dimerise with other transcription factors such as SRF [3], SREBP2 [12] and CREB-H [13].

Recently, ER stress has been proposed to be involved in ankylosing spondylitis (AS) [14], an arthritic condition in which most sufferers express the HLA-B27 allele of the major histocompatibility complex (MHC) class I family [15]. Although HLA-B27 homodimers at the cell surface may have altered signaling properties [16,17], HLA-B27 can also misfold in the ER and could trigger the UPR [18–20]. However, it is not clear how UPR initiation might result in disease, or how findings from animal models relate to the clinic. To investigate whether MHC class I molecules cause an UPR in humans, we have examined HeLa cells expressing misfolded HLA-B2705. We find that over expression of MHC class I heavy chains is sufficient to induce the UPR and that HLA-B27 positive lymphocytes are pre-sensitised to XBP1 splicing compared to the monocyte cell line THP1. Dithiothreitol, but not tunicamycin, induces a long lasting UPR that culminates in the alternative splicing of ATF6α in lymphocytes. Our results show that UPR induction by faulty glycosylation and UPR induction by disulfide bond reduction can be discriminated at the level of both XBP1 processing and ATF6α transcription.

2. Materials and methods

2.1. Cell lines and constructs

The human lymphoblastoid cell lines JESTHOM, HOM-2 (both HLA-B2705) and WEWAK1(HLA-B2704) were obtained from the ECACC. The human acute monocytic leukaemia cell line THP1 was a gift from J. Robinson, Newcastle University, UK. These cell lines were cultured in RPMI 1640 (Invitrogen). The human cervical carcinoma cell line HeLa was maintained in minimum essential medium. All cell lines were supplemented with 8% FCS, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM glutamax. The cDNA encoding wild-type HLA-B2705 (confirmed by DNA sequencing) and the HC10 monoclonal antibody recognising free MHC class I heavy chains were gifts from J. Neefjes, Netherlands Cancer Institute.

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Abbreviations: AS, ankylosing spondylitis; DTT, dithiothreitol; ER, endoplasmic reticulum; ERSE ER, stress response element; GAMPO, goat anti-mouse peroxidase; HLA, human leukocyte antigen; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NEM, *N*-ethylmaleimide; PMA, phorbol myristate acetate; PVDF, polyvinylidene fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; UPR, unfolded protein response

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2.2. Cell treatments

Suspension cells were grown to a density of ~2–10 × 10^5 cells ml^{-1} and resuspended with fresh medium containing 10 mM DTT or 10 μg ml^{-1} tunicamycin to induce ER stress for given times. THP1 cells were treated with 20 μg ml^{-1} LPS for 4 days to induce partial differentiation to adherent macrophages. Adherent HeLa cells were supplemented with 10 mM DTT or 10 μg ml^{-1} tunicamycin for 6 h.

2.3. Cell transfections and lysis

HeLa cells in 6 cm dishes were transfected with 1 μg DNA in the presence of 5 μl lipofectamine 2000 for 6 h in optiMEM (Invitrogen). Cells were then provided with complete medium and analysed 24 h post-transfection. Cells were lysed at 4 °C in 300 μl MNT lysis buffer (20 mM MES, 100 mM NaCl, 30 mM Tris, pH 7.4, supplemented with 20 mM *N*-ethylmaleimide (NEM), 1% Triton X-100 and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A). Post-nuclear supernatants were taken up in sample buffer in the presence (reducing) or absence (non-reducing) of 50 mM DTT and then subjected to 8% SDS–PAGE.

2.4. Western blotting

Gels were wet transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) for 2 h at 150 mA and the membranes were blocked in 8% milk in PBS–Tween for 1 h. Membranes were incubated with HC10 (1:200 tissue culture supernatant) for 1 h before being washed five times with PBS–Tween. Membranes were then incubated with goat anti-mouse peroxidase (GAMPO) secondary antibodies (DAKO) at 1:3000 for 1 h, and subsequently washed five times with PBS–Tween. Proteins were then visualised with 200 μl enhanced chemiluminescence fluid (Amersham) per membrane and exposed to film (Kodak).

2.5. Primers

The primers used for reverse transcriptase polymerase chain reaction (RT-PCR) were as follows:
ATF6α: TGATGCCTTGGGAGTCAGAC and GTGTCAGAGAACCAGAGGCT.
XBP1: GAAACTGAAAAACAGAGTAGCAGC and GCTTCCAGCTTGGCTGATG.
Actin: CCACACCTTCTACAATGAGC and ACTCCTGCTTGCTGATCCAC.

2.6. RT-PCR, XBP1 analysis and sequencing of ATF6α

Cells were lysed in TRI reagent (Sigma) and the concentration of extracted, precipitated RNAwas determined by spectrophotometry. Fifty nanograms total cell RNA was subjected to RT-PCR using the Access-Quick RT-PCR kit (Promega). XBP1 cDNA was subjected to *Pst*I digestion for 2 h at 37 °C and DNA extracted using a PCR purification kit (Qiagen). All cDNA was either analysed by 1% or 2% agarose gel at 100 mV for ~50 min before visualising by UV light. ATF6α RT-PCR products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced by the departmental sequencing centre. White-on-black images were inverted in Adobe Photoshop.

3. Results

3.1. Differential activation of XBP1 by DTT and tunicamycin

Prior to analyzing the relationship between HLA-B27 and the UPR, we optimized UPR induction by stimulating the human monocyte cell line THP1 with either tunicamycin or DTT for 0, 3, 6 and 24 h. We took advantage of the fact that activation (splicing) of XBP1 results in the removal of a unique *Pst*I site from the mRNA transcript [7]. Thus the UPR is activated if a higher molecular weight (undigested) band is observed on agarose gels after incubation of the cDNA with *Pst*I. The fragments expected from this digestion are explained in Fig. 1A. In the absence of DTT or tunicamycin, XBP1 was not spliced, resulting in a *Pst*I sensitive product that was digested into two fragments of 289 and 255 nucleotides (Fig. 1B, lanes 1 and 5). Both DTT and tunicamycin induced XBP1 mRNA processing in THP1 cells (Fig. 1B, lanes 2 and 6). However, tunicamycin stimulation resulted in a resistant species that ran as a doublet (S) and persisted as a singlet after 24 h (Fig. 1B, lane 4). In contrast, DTT stimulation resulted in a single resistant species that ran at the same height as the lower band in the tunicamycin stimulated doublet (Fig. 1B, lanes 6–8). THP1 cells partially recovered from tunicamycin stimulation after 24 h, whereas in DTT stimulated cells, XBP1 remained processed after this time (Fig. 1, compare lanes 4 and 8), despite the fact that DTT is a labile reductant that likely becomes oxidized in the culture medium. Lower concentrations of DTT also triggered the appearance of a single XBP1 band (not shown). These results suggest that XBP1 splicing can follow more than one pathway, and that DTT and tunicamycin can activate subtly different processing or RNA ligation events upstream of XBP1.

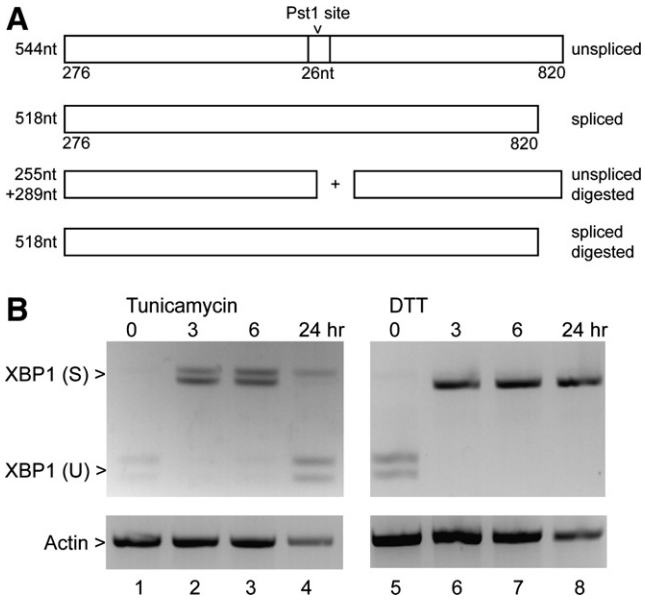

Fig. 1. Induction of XBP1 processing by DTT and tunicamycin. (A) Diagram illustrating the two expected mRNA/cDNA products of XBP1, and their digestions by *Pst*I. The unique *Pst*I site is removed when XBP1 is processed. (B) THP1 cells treated with (lanes 2–4) and without (lane 1) 10 μg ml^{-1} tunicamycin or with (lanes 6–8) and without (lane 5) 10 mM DTT were lysed in TRI reagent, and the lysates subjected to RT-PCR to detect XBP1. Resultant cDNA was digested with *Pst*I and analysed by 2% agarose gel to visualize the activated spliced (S) and unspliced (U) products. Actin was used as a control (lower panel).

3.2. Misoxidised MHC class I heavy chains induce XBP1 processing in HeLa cells

UPR activation has been linked to misfolding of HLA-B2705 heavy chains in a rat model of AS [14]. Thus we investigated whether misoxidised HLA-B2705 could activate the UPR in HeLa cells. MHC class I heavy chains require both an accessory light chain protein, β2m, and a peptide to fold properly in the ER [21] and we have previously shown that over expressed heavy chains misoxidise and form abundant disulfide linked complexes in HeLa cells [22]. HeLa cells transfected (or mock transfected) with HLA-B2705 were simultaneously lysed in the presence of the alkylating agent NEM to preserve disulfide bonds or were taken up in TRI reagent for

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