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Identification and characterization of interactions between abscisic acid and human heat shock protein 70 family members

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Olesya A. Kharenko[1], Devin Polichuk[1], Ken M. Nelson[1], Suzanne R. Abrams[1,2] and Michele C. Loewen[1,3,*]

[1]Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9; [2]Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5C9, Canada and [3]Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E5, Canada

*Michele Loewen, Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan S7N 0W9, Canada. Tel: +1 306 975 6824, Fax: +1 306 975 4839, email: michele.loewen@nrc.ca

Abscisic acid (ABA) is a stress-inducible plant hormone comprising an inevitable component of the human diet. Recently, stress-induced accumulation of autocrine ABA was shown in humans, as well as ABA-mediated modulation of a number of disease-associated systems. Now, the application of a chemical proteomics approach to gain further insight into ABA mechanisms of action in mammalian cells is reported. An ABA mimetic photoaffinity probe was applied to intact mammalian insulinoma and embryonic cells, leading to the identification of heat shock protein 70 (HSP70) family members, (including GRP78 and HSP70-2) as putative human ABA-binding proteins. *In vitro* characterization of the ABA–HSP70 interactions yielded K_ds in the 20–60 μM range, which decreased several fold in the presence of co-chaperone. However, ABA was found to have only variable- and co-chaperone-independent effects on the ATPase activity of these proteins. The potential implications of these ABA–HSP70 interactions are discussed with respect to the intracellular protein folding and extracellular receptor-like activities of these stress-inducible proteins. While mechanistic and functional relevance remain enigmatic, we conclude that ABA can bind to human HSP70 family members with physiologically relevant affinities and in a co-chaperone-dependent manner.

Keywords: abscisic acid/glucose-regulated protein 78/Heat shock protein 70/protein–ligand interaction/chemical proteomics.

Abbreviations: AB, abscisic acid; GRP78, glucose-regulated protein 78 kilodaltons; HSP70, heat shock protein 70; HEK293, human embryonic kidney 293; OG, *n*-octyl-β-D-glucopyranoside; RIN-m, rat insulinoma cells; SPR, surface plasmon resonance.

Abscisic acid (ABA) is a plant hormone known to regulate many physiological roles in development as well as biotic and abiotic stress responses (*1*). *In planta* ABA is recognized intracellularly by a family of START domain receptors and a Mg^{2+} chelatase subunit and extracellularly by G-protein coupled receptor type G-proteins; while ABA transport has been shown to be mediated by both ABC and adenine nucleotide type translocators (*2–4*). However, recent research has shown that the effects of ABA reach beyond the plant kingdom. ABA is synthesized in fungi (*5*), controls stress responses in marine sponges (*6*), regeneration in hydra (*7*), and mediates Ca^{2+} signalling in the parasite *Toxoplasma gondii* (*8*).

ABA has also been shown to elicit functional effects in mammalian model systems (*9*, *10*). This includes reports of accumulation of ABA in rat and pig brains (*11*), as well as indications of roles as an anti-inflammatory modulator in Type II diabetes, IBD and atherosclerosis in mice (*9*, *12–14*). These anti-inflammatory actions of ABA appear to involve modulation of nuclear peroxisome proliferator-activated receptor γ (PPARγ)-dependent mechanisms (*13*, *15*, *16*). In the case of the anti-diabetic effect, ABA's activation of PPARγ has been shown to be linked to cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) related signalling (*12*). In contrast, a separate series of reports have shown that ABA acts as a pro-inflammatory molecule activating granulocytes and monocytes, as well as modulating vascular smooth muscle and microglial cell functionalities (*17–19*). Further applications of ABA to insulinoma cells and pancreatic islets led to increases in insulin secretion (*20*, *21*). In these latter examples the mechanisms seem consistent with those in the live animal models, including a cAMP/PKA/Ca^{2+}-signalling system, but rather linked to a pertussis-sensitive receptor/G protein complex. Most recently, the lanthionine synthase C-like protein (LANCL2), has been shown to be cell-surface localized, bind ABA, and be essential for pertussis-sensitive ABA signalling in human cells (*20*, *22*). Finally, linkages between ABA, Ca^{2+} signalling, PPARγ and immune responses have led to the proposal that ABA could modulate cancer (*9*).

ABA is an inevitable component of the human diet present in virtually all plant-derived food ingested on a daily basis and as yet has no known toxic effects making it an attractive target for biomedical applications. At the same time recent evidence supports the possibility that mammalian cells actually produce and secrete ABA. For example: ABA levels have been shown to increase in human plasma after hyperglycaemia and stimulate glucose consumption in adipocytes and myoblasts (*23*). Similarly, autocrine ABA was been shown

to mediate UV-B-induced inflammatory responses and play a role in macrophage activation (*24*–*26*). However, while these recent findings represent important new knowledge about the roles and mechanisms of ABA in mammalian systems, the inconsistencies between proposed pro- and anti- inflammatory effects highlight gaps in our understanding that continue to undermine efforts to develop ABA as a therapeutic.

Previously a chemical proteomics approach was successfully applied to plant tissues, using a unique ABA mimetic photoaffinity probe, to identify putative ABA-binding proteins (*3*, *27*, *28*). Now toward gaining further insight in the breadth of functionality and mechanism of ABA action in mammalian systems, this same approach and probe has been applied to two independent mammalian cell lines. This led to the identification and characterization of members of the heat shock protein 70 (HSP70) family of chaperones as ABA-binding proteins. While, the functional and mechanistic significance of an interaction between ABA and HSP70 proteins in mammalian cells remains enigmatic, the potential biological relevance of these findings is discussed.

Materials and Methods

Materials

All materials were from Sigma-Aldrich (Sigma-Aldrich, Oakville, Ontario, Canada) unless otherwise indicated. HiTrap streptavidin column, Streptavidin-HRP conjugate, ECL biotinylated protein markers and ECL plus Western Blotting Detection Reagents were obtained from Amersham Biosciences, Baie d'Urfe, Quebec. Recombinant His-tagged human GRP78 and HSP70-2 proteins with ATPase activity were purchased from StressMarq (Biosciences Inc., Victoria, Canada). Recombinant, full-length recombinant human DnaJ protein was purchased from Abcam (Abcam Inc., MA, USA) (+)-ABA was prepared as described previously (*29*). PBI 686 was synthesized according to Nyangulu *et al*. (*27*, *30*).

Cell culture

The rat insulinoma line RIN-m (ATCC# CRL-2057) was cultured in RPMI 1,640 medium according to the ATCC Product Information Sheet, supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium biocarbonate, 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. HEK293-6E (*31*) cells were propagated in F17 (Invitrogen) containing 0.1% pluronic F-68 with 100 U/ml penicillin, 100 μg/ml streptomycin.

Chemical proteomic identification of putative human ABA-binding proteins from RIN-m and HEK293-6E cells

Five 225 cm^2 T-flasks confluent with RIN-m (ATCC# CRL-2057) cells were harvested and re-suspended in 2 ml of phosphate-buffered saline pH 7.4 (PBS) containing 10 μM of PBI-686. This mixture was transferred to a Pyrex tube and incubated on ice for 45 min and irradiated with UV for a subsequent 30 min. The sample was washed 2× with PBS and lysed with 2 ml of 1% *n*-octyl-β-D-glucopyranoside (OG) in PBS on ice for 45 min. The lysate was centrifuged at 4,000 × *g*. Tagged proteins in the resulting supernatant were enriched by affinity chromatography using a HiTrap streptavidin column (1–3 ml matrix volume) and AKTATM Explorer FPLC system. The solvent system was adapted from a previous publication describing purification of biotinylated membrane proteins with minor modifications (*32*). The column was equilibrated with 10 column volumes of running buffer (1% OG in PBS). The protein solution was injected at a flow rate of 0.2–0.5 ml/min, washed with the 10 column volumes of running buffer, 10 column volumes of PBS with 500 mM NaCl and 1% OG, 10 column volumes of 50 mM Tris–HCl (pH 7.5) and finally eluted with a gradient of 8 M Guanidine–HCl at pH 2 (1 ml/min, 30 ml total). Collected fractions were desalted with a PD-10 desalting column into PBS buffer containing 1% OG, and concentrated using an AmiconTM Ultrafree centrifugal filters (Millipore, Carrigtwohill, Ireland).

Three 75 cm^2 T-flasks confluent with HEK293-6E cells were re-suspended in 2 ml of PBS containing 10 μM of PBI-686 and transferred to a 12 ml Pyrex glass tube. The mixture was incubated on ice for 45 min and irradiated with UV for 30 min. The sample was washed 2× with PBS and then lysed with 2 ml of 1% OG in PBS on ice for 45 min. The lysate was centrifuged at 4,000 × *g* and the supernatant was collected. Tagged proteins were enriched by affinity chromatography using a HiTrap streptavidin column (1–3 ml matrix volume) and AKTATM Explorer FPLC system as described above.

Mass spectroscopy analysis of streptavidin sepharose purified samples

SDS–PAGE gels were stained using FOCUS-FAST silver-stain kit (Genotech, St Louis, MO, USA) according to the manufacturer protocol. Protein bands were excised and placed in a 96-well microtitre plate (Sigma, Milwaukee, WI, USA). The resulting gel pieces were automatically de-stained, reduced with DTT, alkylated with iodoacetamide, and digested with porcine trypsin (sequencing grade, Promega, Madison, WI, USA) using a MassPREP protein digest station and recommended procedures (Waters, Manchester, UK). Peptides from tryptic digestion were analysed using a capLC ternary HPLC system (Waters, Milford, MA, USA) coupled to a Q-ToF Ultima Global (Waters, Manchester, UK). The method used for separation of the peptide digest samples and subsequent analysis using LC–MS/MS and Data Dependent Acquisition (DDA) has been described previously (*33*). The LC–MS/MS data were processed using ProteinLynx software (Waters) and searched against databases using MASCOT Daemon and Mascot MS/MS ion search performed on a MASCOT server hosted by IBS-NRC (Ottawa, Canada).

Surface plasmon resonance

Surface plasmon resonance (SPR) was performed using a Biacore X instrument (Biacore, Uppsala, Sweden) at the Saskatchewan Structural Sciences Centre/University of Saskatchewan. NTA sensor chips (GE Healthcare) were activated and regenerated as per the manufacturer's protocols. All buffers were degassed prior to use. For immobilization, 50 μl of a 20 μg/ml solution of human His-tagged GRP78 or HSP70-2 protein solubilized in SPR buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 50 μM EDTA, 0.005% Tween 20) was injected with a flow rate of 2 μl/min until saturation of the signal was achieved. Following a 20 μl wash at a flow rate of 20 μl/min to establish a stable base line followed by a 20 μl delay, indicated concentrations of (+)-ABA in SPR buffer were injected. The data analysis was performed using BIAevaluation software (Biacore, Uppsala, Sweden). For the binding experiments in the presence of DnaJ (Abcam), GRP78 or HSP70-2 (20 μg/ml) was pre-incubated with DnaJ (20 μg/ml) for 15–30 min prior to the immobilization on a chip surface. The second cell of the instrument was used as a control cell throughout the experiments. Regeneration of the chip was performed using 350 mM EDTA, pH 8.3.

Radiolabelled ligand binding. An amount of 1 μM His-tagged GRP78 was mixed with [^{3}H]-(+/−)-ABA (Amersham Biosciences, GE Healthcare) at a range of increasing concentrations in a reaction buffer comprised of 50 mM HEPES, 150 mM NaCl and 2 mM $MgCl_2$, pH 7.5. The 50 μl reactions were incubated on ice for 2 h and then applied to GFC-filters (Whatcom, GE Healthcare) using a vacuum manifold. The filters were repeatedly washed with ice-cold reaction buffer and then transferred into the scintillation vials, which were vortexed and counted for radiation the following day. Background, non-specific, binding values were obtained by carrying out the same experiment but in the additional presence of 1,000-fold molar excess of un-labelled (+)-ABA. These background values were subtracted from the experimental values and the data subjected to first-order kinetic analysis using Enz-Fitter v2.0.18.0 (Biosoft, Cambridge, UK).

Colorimetric ATP-hydrolysis activity assay

The ATP-hydrolysis activity of GRP78 and HSP70-2 were assessed be determining the amount of free phosphate liberated using the colorimetric malachite-green assay kit (BioAssay Systems,

Hayward, CA, USA) according to the manufacturer's specifications. The assay reactions in the absence of DnaJ were performed in 70 µl reaction volumes containing 1 µM (+)-ABA, 100 µM ATP and were initiated by addition of 1 µM of recombinant full length His-tagged GRP78 or HSP70-2; a corresponding amount of a reaction buffer was used as a negative control. The reaction buffer contained 50 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, pH 7.5. The reaction mixture was incubated for 3 h at 37°C and the amount of liberated phosphate determined. Quantification was by comparison to a phosphate standard calibration curve. The ATP hydrolysis reactions in the presence of DnaJ were performed as follows: the assays were performed in 350 µl reaction volume containing 1 µM DnaJ, 1 µM (+) ABA, 100 µM ATP and were initiated by addition of 1.0 µM of recombinant full-length His-tagged GRP78 or HSP70-2. The reaction mixture was incubated for 3 h at 37°C and then aliquoted into five samples of 70 µl each which were each assayed for the amount of liberated phosphate as described above.

Results

Identification of putative mammalian ABA-binding proteins

To identify putative mammalian ABA-binding proteins, we applied a chemical proteomics approach using an ABA mimetic probe, PBI686, ((Fig. 1) described in detail elsewhere (*3*, *27*)) to RIN-m insulinoma rat cells based on a previous report proposing as many as ~6,000 cell surface ABA-binding sites per cell (*21*). Although the possibility of PBI686 translocation across the cell membrane cannot be strictly excluded, interactions with cell surface-binding proteins in particular were encouraged by incubation of PBI686 with intact RINm cells. Following UV irradiation and cell lysis covalently tagged PBI686-interacting proteins were enriched by streptavidin affinity chromatography. Active fractions were pooled, concentrated and analysed by SDS–PAGE and far western blot using streptavidin–HRP antibody. Visualization of enriched fractions of purified PBI686-interacting proteins on a far western blot revealed a single prominent band at ~80 kDa (Fig. 2A).

The corresponding region of the silver stained gel was excised and subjected to tryptic digest and analysed by an LC–MS/MS-Q-TOF coupled Mascot MS/MS ion search. This region was found to contain predominantly peptides homologous to *Rattus norvegicus* heat shock protein 5 (HSP5) (Supplementary Fig. S1A; NCBI Accession AAH62017, GI:38303969) with an expected MW of 72.5 kDa. The probability score was 510, with 16 matched peptides. Rat HSP5 shares closest homology to the human 78 kDa glucose-regulated protein (GRP78) also known as the immunoglobulin heavy chain-binding protein (BiP).

We subsequently applied this same chemical proteomics strategy with an identical experimental work flow to an unrelated cell line, human embryonic kidney 293-6E cells (HEK293-6E; (*31*)). Significantly, far-western blot of affinity enriched PBI686-interacting proteins using streptavidin-HRP antibody again revealed a single band at ~70 kDa, similar to what was observed for the RIN-m cell experiments (Fig. 2B). The LC/MS/MS-Q-TOF coupled Mascot MS/MS ion search analysis of the corresponding region of the silver stained gel identified heat shock protein HSP70-2 [Homo sapiens] (NCBI Accession AAD21815, GI:4529892) identified as an ABA-interacting protein

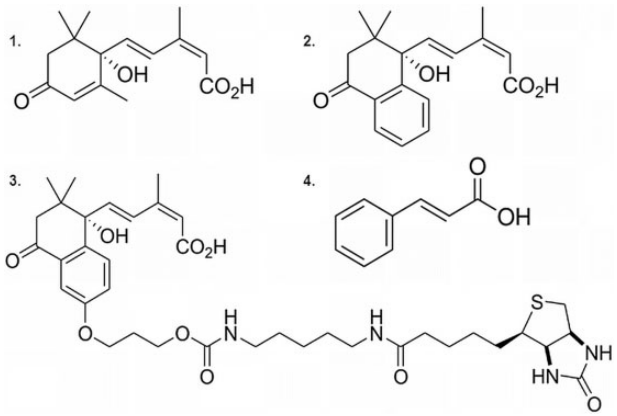

Fig. 1 Chemical structures. (**1**) (+)-(S)-ABA, (**2**) (+)-PBI410, (+)-tetralone. (**3**) (+)-PBI686 photoactive, bioactive ABA-mimetic biotynylated probe used to pull out putative ABA-binding proteins, (**4**) *trans*-CA.

with a probability score of 1293 and 32 matched peptides (Supplementary Fig. S1B). HSP70-2 belongs to the same HSP70 family as GRP78 sharing ~60% amino acid identity (*34*). Thus, from two independent cell lines, we identified two homologous proteins, belonging to the same HSP70 protein family, as putative ABA-binding proteins.

ABA binds to GRP78 and HSP70-2 in vitro

In light of the chemical proteomics results, stress-induced nature of both ABA and HSP70s and the documented linkages between GRP78 and disease conditions shown to be modulated by ABA, further characterization of the ABA–HSP70 interaction was initiated. To test binding affinities, a biosensor system was applied to measure SPR arising from the interaction. A His-tagged version of human GRP78 protein was immobilized onto the surface of a Ni(II)-activated NTA chip (*35*). Following optimization of conditions, the binding of ATP (a known natural substrate of GRP78) and salicylic acid (SA; a plant hormone previously shown to bind to GRP78 (*36*)) were assessed to test reliability of the method. A concentration dependent plot of ATP binding to immobilized GRP78, fitted to a steady-state affinity model, yielded an equilibrium dissociation constant (K_d) of 38 µM (Supplementary Fig. S2), which fit within the broad range of nanomolar to micromolar values reported for ATP binding to HSP70s (*37*). Evaluation of the binding of SA to GRP78 yielded a K_d of 62 µM (Supplementary Fig. S3) which correlated nicely with the K_d of 56 µM established previously using a radiolabel-binding assay (*36*). Following demonstration of the reliability of the SPR technique for GRP78 analysis, ABA-binding affinities were determined. Binding of (+)-ABA to GRP78 was found to saturate at ~30 µM (+)-ABA, and when fitted to a steady-state affinity model, the concentration dependence plot yielded a K_d of 22 µM (Fig. 3A and B). Binding of ABA to human HSP70-2 was determined to occur with a K_d of 59 µM (Fig. 3C and D). These values both fall within the range of other known

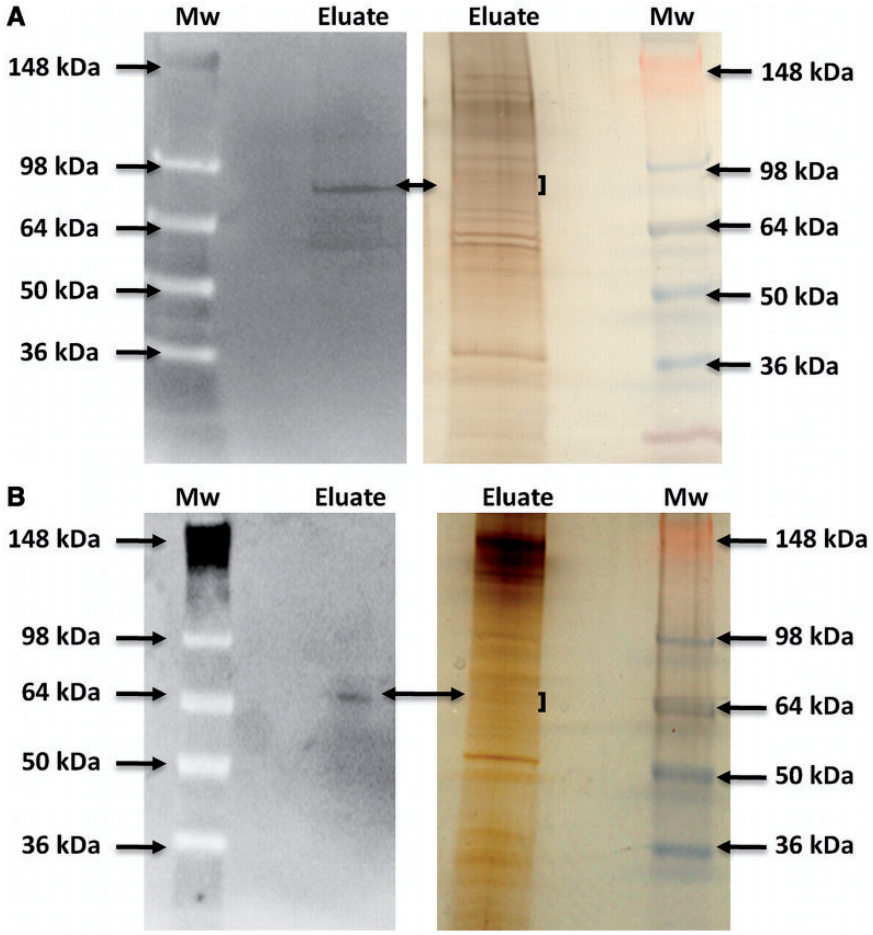

Fig. 2 Streptavidin-affinity-enriched PBI686-tagged protein extracts from RINm and HEK293 cells. Total protein extracts, photocross-linked with PBI686, were enriched using streptavidin sepharose affinity chromatography and analysed using far-western blot with a streptavidin–HRP conjugate (left-panels) and silver-staining (right panels). Band regions that were excised are indicated with the symbol '] '. (**A**) Extracts from RINm cells. (**B**) Extracts from HEK293-6E cells.

pharmacological targets of HSP70s (*38*). A structurally reminiscent, but functionally unrelated molecule to ABA, *trans*-cinnamic acid (CA; Fig. 1) was assessed for binding to GRP78. CA did not produce any noticeable SPR signal, highlighting the specific nature of the ABA interaction with GRP78 (Supplementary Fig. S4).

The GRP78–ABA interaction was corroborated by [^{3}H]-(+/−)-ABA binding to rubisco revealing a K_d of 60 μM, 3-fold higher, but within the same general range as the K_d determined by SPR (Fig. 4). The observed difference between the SPR and radiolabel derived values for ABA could be representative of the [^{3}H]-(−)-ABA portion of the [^{3}H]-(+/−)-ABA having lower affinity than the [^{3}H]-(+)-ABA. However this remains to be tested. Together these data suggest strong selective binding interactions between ABA and both GRP78 and HSP70-2.

Co-chaperone DnaJ stimulates ABA-binding to GRP78 and HSP70-2

HSP70 chaperone activity is based on a dynamic cycle of ATP-hydrolysis associated with a nucleotide-binding domain, modulating protein substrate binding into the substrate-binding domain (*38*). The cycle is generally stimulated by interaction with a family of co-chaperones, termed HSP40s or DnaJs, where the HSP70's affinity for protein substrate can be increased by ~10-fold (*39*). To further investigate the biological relevance of the ABA–GRP78 interaction we tested whether the presence of a human DnaJ protein had any effect on ABA binding. Following pre-incubation of GRP78 with human DnaJ, the protein mixture was immobilized on a biosensor chip. Analysis of overlaid sensogram plots arising from the application of increased concentrations of (+)-ABA and the corresponding concentration dependence plot yielded a K_d of 7 μM (Fig. 5A and B). This highlights a 3.5-fold increase in ABA affinity for GRP78 in the presence of DnaJ, lending weight to the possibility of biological relevance of the ABA–GRP78 interaction. Similarly, analysis of the binding of ABA to HSP70-2 in the presence of DnaJ yielded a K_d of 2 μM indicating a 30-fold increase in binding affinity due to the presence of the biologically relevant partner (Fig. 5C and D).

ABA differentially modulates the ATP-hydrolysis activity of GRP78 and HSP70-2

Molecules that compete with ATP or protein substrate for binding affect the overall dynamic functional cycle

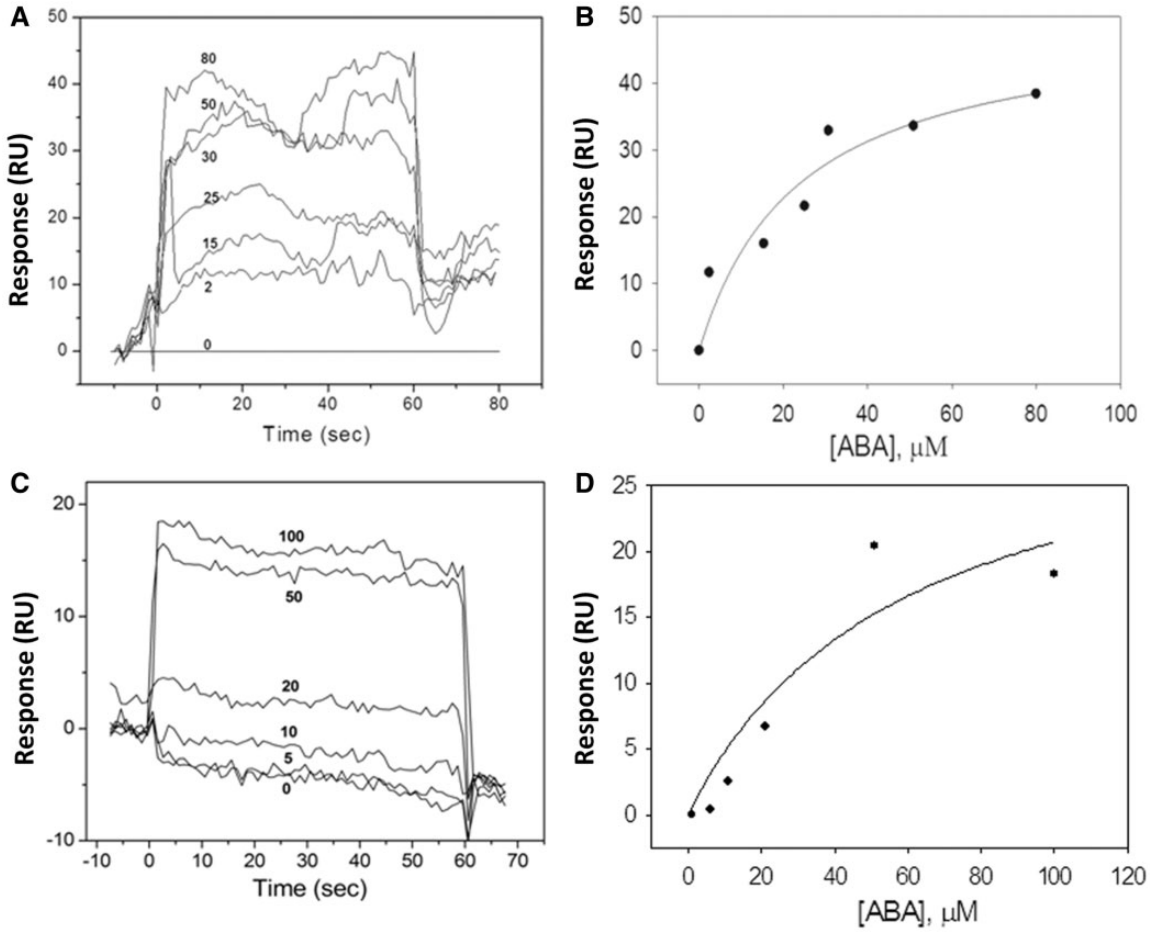

Fig. 3 ABA binds to GRP78 and HSP70-2. His-tagged GRP78 and HSP70-2 were immobilized on Ni (II)-activated NTA SPR chips, respectively, and changes in resonance observed upon application of (+)-ABA at various concentrations. Overlay plots of SPR sensograms showing binding of ABA (concentrations as indicated in micromolars) to immobilized (**A**) GRP78 and (**C**) HSP70-2. Concentrations of ABA are indicated in micromolar concentration dependence plot of ABA binding to immobilized (**B**) GRP78 and (**D**) HSP70-2, fitted to a steady-state affinity model.

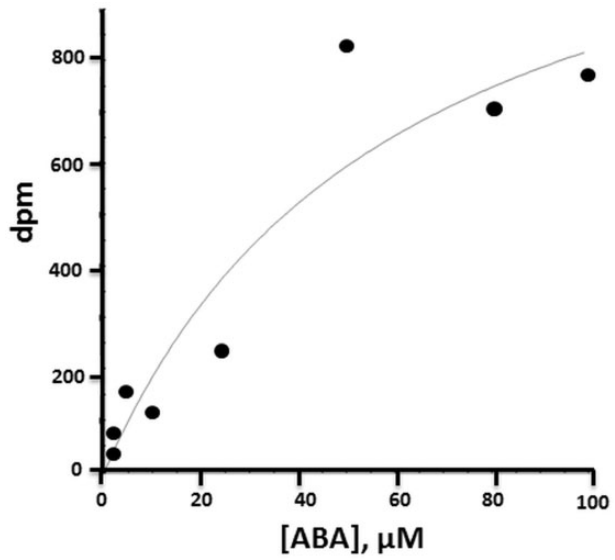

Fig. 4 ABA binds to GRP78 by radiolabel [^{3}H]-(±)-ABA-binding assay. A representative plot of the interaction of increasing concentrations of [^{3}H]-(±)-ABA with recombinant His-tagged GRP78. First-order kinetic analysis of the data yields as K_d of 60 μM.

modulating chaperone activity (*38*, *40*, *41*). To evaluate whether ABA might modulate the chaperone functionality of GRP78 and HSP70-2, heat shock protein-mediated ATP-hydrolysis was assessed in the presence and absence of (+)-ABA and DnaJ. Consistent with a previous report for HSPA6 protein, recombinant GRP78 exhibited high basal ATPase activity and DnaJ did not further stimulate it (*42*). Further comparison of the amounts of phosphate released demonstrated that the presence of 1 μM (+)-ABA had no effect on ATP-hydrolysis activity compared to untreated GRP78 regardless of the presence of absence of DnaJ (Fig. 6A). This lack of any effect of (+)-ABA on GRP78 ATP hydrolysis suggests its role may not be associated with modulation of the protein chaperone functional cycle of GRP78. In contrast to this, the presence of 1 μM (+)-ABA elicited a ~60% increase in the ATP-hydrolysis activity of HSP70-2; albeit again independent of the presence of DnaJ (Fig. 6B). Interestingly, as with GRP78, DnaJ had no effect on HSP70-2 by itself. Such differential ATPase activity within sequence-conserved HSP family members has been described previously (*42*, *43*).

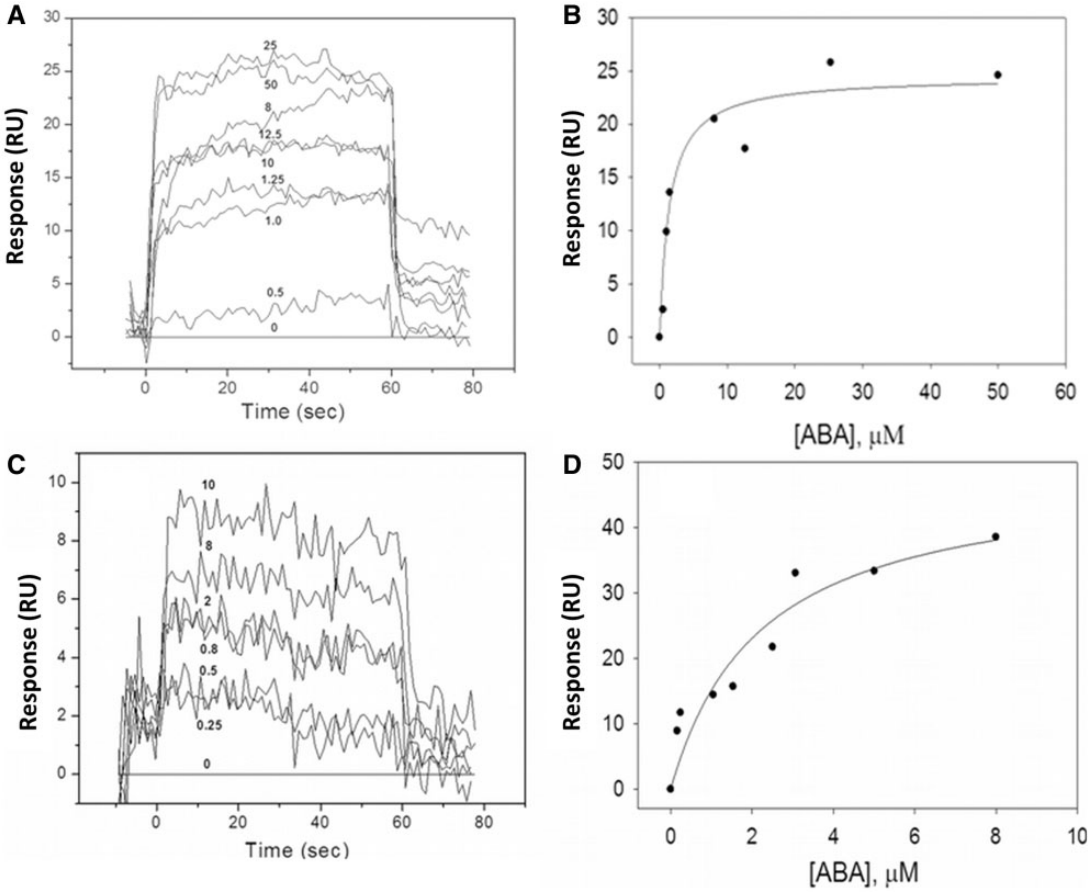

Fig. 5 DnaJ activates ABA binding to GRP78 and HSP70-2. Recombinant human His-tagged GRP78 and HSP70-2 were pre-incubated with recombinant human DnaJ, and then immobilized on Ni (II)-activated NTA SPR chips and changes in response units observed upon application of (+)-ABA at various concentrations. Overlay plots of SPR sensograms showing binding of ABA (concentrations as indicated in micromolars) to immobilized (**A**) GRP78 and (**C**) HSP70-2, in the presence of DnaJ. Concentration dependence plot of ABA binding to immobilized (**B**) GRP78 and (**D**) HSP70-2, in the presence of DnaJ, fitted to a steady-state affinity model.

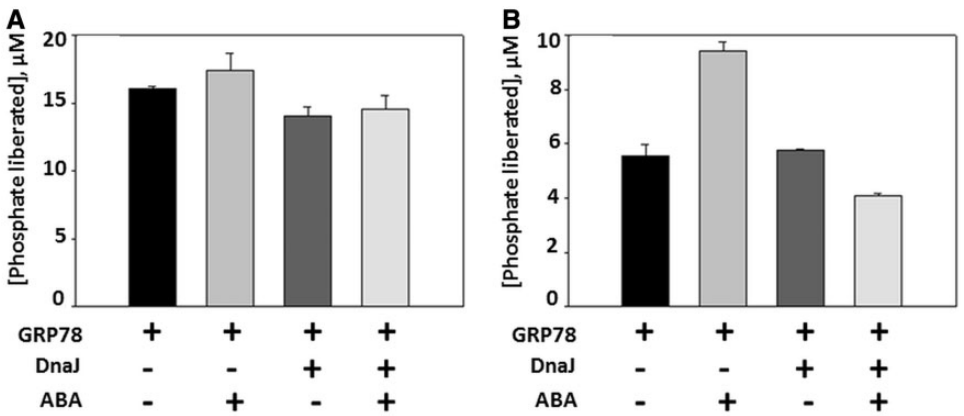

Fig. 6 ABA differentially modulates the ATP-hydrolysis activity of GRP78 and HSP70-2. The effects of ABA on the ATP hydrolysis activity of GRP78 (left panel) and HSP70-2 (right panel) were determined in the presence or absence of 1 μM DnaJ. 1 μM GRP78 (± DnaJ) was incubated ± 1 μM (+)-ABA and the liberation of free phosphate determined using the malachite green phosphate release assay. Results are the mean ± SD of $n = 30$ for GRP78 and $n = 5$ for HSP70-2.

Discussion

We hypothesized that by analogy with the plant kingdom, there may exist a variety of proteins in mammals to which ABA binds, and through which it regulates its plethora of reported mammalian responses (*9*, *10*). Toward testing this, two independent mammalian cell lines, RIN-m and HEK293-6E, were applied to a chemical proteomics approach based on a previously described ABA mimetic bioactive photoaffinity probe

(*3*, *27*). Significantly, two homologous proteins, GRP78 and HSP70-2 (belonging to the same family of HSP70s (*34*)) were independently identified and subsequently characterized as ABA-binding proteins. Thus ABA becomes the second stress inducible phytohormone, after SA, known to bind mammalian HSP70's (*36*).

Physiologically, GRP78 has been implicated in the modulation of a wide variety of human disease states (*44*–*46*). It is known for its roles in protein folding and degradation, Ca2+ signalling, and in the unfolded protein response of the endoplasmic reticulum (*47*). More recently it has also been shown to function as a cell-surface receptor in immune responses (*44*, *48*–*54*). Interestingly, over-expression of GRP78 in INS-1 832/13 insulinoma cells has been shown to increase insulin secretion (*55*), much as ABA has also been reported to do (*21*). Combined with recent findings that show secretion of autocrine ABA from pancreatic β cells into plasma upon hyperglycaemia (*21*, *23*), these data fit the possibility of a modulatory ABA–GRP78 interaction. Expression and secretion of both molecules is modulated by high glucose, placing both protein and ligand in the same location at the same time, where their interaction could lead to insulin secretion.

Mechanistically, while HSP70 proteins are best known for their roles as chaperones, the DnaJ stimulated increases in ABA binding to HSP70s might be consistent with recent literature describing a second, non-chaperone-based role for GRP78 and HSP70's in general. According to this model, stress-induced accumulation of the HSP70s at the cell surface is involved in mediating immune signalling and responses to disease (*44*, *48*, *50*). At least one report highlights a role for an interaction between cell-surface localized GRP78 and a DnaJ type protein, MTJ-1 (*51*). Moreover, ATP-hydrolysis does not seem to be involved in the cell-surface functionalities of GRP78, such that the lack of any ABA-mediated effect on GRP78 ATPase activity is also consistent with a role at the surface-signalling level. Ultimately, however, the findings reported here do not differentiate between intracellular or extracellular roles for ABA with respect to HSP70 binding.

Also of note is that ABA seems to affect many of the same disease states modulated by the cell-surface receptor functionalities of GRP78 (*9*, *48*). Details of the mechanism by which GRP78 mediates cell-signalling events is dependent on the cell type and thus molecules available for association (*48*). In this context, GRP78 has been shown to interact with a diverse assortment of partners including activated α2-macroglobulin, Kringle 5 and Par-4 ligands, cell-surface anchored proteins like Cripto and T-cadherin and membrane proteins such as tissue factor. These interactions modulate an equally diverse array of signalling events; for example in the case of macroglobulin binding, signals have been shown to increase cell motility, proliferation and survival through activation of P21-activated kinase-2 and LIM domain kinase, extracellular-signal-regulated kinases 1/2, p38 mitogen-activated protein kinase and phosphoinositide 3-kinase, as well as Akt and nuclear factor-κB, respectively (*52*, *53*). To date GRP78 signalling has not been linked to the ABA-modulated cAMP/PKA/CD38 pathway. However, GRP78's ability to interact with a wide variety of different co-receptors modulating different signalling pathways suggests that many as yet uncharacterized interactions could remain to be identified. Further to this, LANCL2, a plasma membrane localized lanthionine synthetase C-like protein previously shown to bind ABA, was shown to mediate ABA binding to the plasma membrane and for the activation of ABA signalling in various cells (*15*, *20*, *22*). Whether there exists any relationship between LANCL2 and GRP78 remains to be determined.

Finally, looking to plants where HSP70s are normally produced in response to stress (*56*), a recent report has demonstrated that *in planta* cross-talk between the stress induced phytohormones ABA and SA, is actually dependent on the presence HSP70s (*57*). But here to, the mechanism of the ABA–SA–HSP70 interaction remains to be characterized.

Based on the findings described herein, we conclude that ABA binds to human HSP70 proteins including GPR78 and HSP70-2. While ultimately inconclusive about the molecular mechanisms governed, and the physiological effects modulated by ABA binding to mammalian HSP70's, these findings do raise important new possibilities and provide new hypotheses for future evaluation.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest
None declared.

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