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

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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 $K_{D}$s 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.

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., Cambridge, MA, USA) (+)-ABA was prepared as described previously (29). PBI 686 was synthesized according to Nyangul 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 bicarbonate, 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 10 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 cm² 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 then lysed with 2 ml of 1% OG in PBS on ice for 45 min. The lysate was centrifuged at 4,000 x 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 [3H](+/-)-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 MgCl2, 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 v.2.18.0 (Biosoft, Cambridge, UK).

Colorimetric ATP-hydrolysis activity assay

The ATP-hydrolysis activity of GRP78 and HSP70-2 were assessed by determining the amount of free phosphate liberated using the colorimetric malachite-green assay kit (BioAssay Systems,
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 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 (Kd) 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 Kd of 62 μM (Supplementary Fig. S3) which correlated nicely with the Kd 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 Kd of 22 μM (Fig. 3A and B). Binding of ABA to human HSP70-2 was determined to occur with a Kd of 59 μM (Fig. 3C and D). These values both fall within the range of other known
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
[3H]-(+/-)-ABA binding to rubisco revealing a $K_d$ of
60 $\mu$M, 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 [3H]-(-)-ABA portion of the [3H]-(+/-)-ABA
having lower affinity than the [3H]-(+)-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-bind-
ing 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 corres-
ponding concentration dependence plot yielded a $K_d$
of 7 $\mu$M (Fig. 5A and B). This highlights a 3.5-fold in-
crease 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 $\mu$M 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
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 or 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. 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 [3H]-(±)-ABA-binding assay. A representative plot of the interaction of increasing concentrations of [3H]-(±)-ABA with recombinant His-tagged GRP78. First-order kinetic analysis of the data yields as $K_d$ of 60 μM.
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 modal 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 GRP78 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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Abscisic acid binds to mammalian heat shock 70 proteins

(R)-Abscisic acid from plant cell cultures supplied with racemic ABA. Phytochem. 28, 2885–2889


