Phage display biopanning identifies the translation initiation and elongation factors (IF1 α -3 and eIF-3) as components of Hsp70–peptide complexes in breast tumour cells

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Abstract The heat shock protein, HSP70, is over-expressed in many tumours and acts at the crossroads of key intracellular processes in its role as a molecular chaperone. HSP70 associates with a vast array of peptides, some of which are antigenic and can mount adaptive immune responses against the tumour from which they are derived. The pool of peptides associated with HSP70 represents a unique barcode of protein metabolism in tumour cells. With a view to identifying unique protein targets that may be developed as tumour biomarkers, we used purified HSP70 and its associated peptide pool (HSP70–peptide complexes, HSP70-PCs) from different human breast tumour cell lines as targets for phage display biopanning. Our results show that HSP70-PCs from each cell line interact with unique sets of peptides within the phage display library. One of the peptides, termed IST, enriched in the biopanning process, was used in a ‘pull-down’ assay to identify the original protein from which the HSP70-associated peptides may have been derived. The eukaryotic translation initiation factor 3 (eIF-3), a member of the elongation factor EF1 α family, and the HSP GRP78, were pulled down by the IST peptide. All of these proteins are known to be up-

regulated in cancer cells. Immunohistochemical staining of tumour tissue microarrays showed that the peptide co-localised with HSP70 in breast tumour tissue. The data indicate that the reservoir of peptides associated with HSP70 can act as a unique indicator of cellular protein activity and a novel source of potential tumour biomarkers.

Keywords HSP70-peptide complexes · Phage display biopanning · Interacting proteins · Biomarkers

Introduction

Heat shock proteins (HSPs) represent a family of proteins in the cell that are characterised by their ability to respond to various forms of stress, both chemical and environmental, including heat. HSPs play many diverse roles within the cell not only as molecular chaperones, assisting in protein folding, unfolding, transport, and localisation, but also participating in targeted proteasomal degradation of error-containing and/or misfolded proteins and antigen sequestration and presentation (Evans et al. 2010; Galluzzi et al. 2009; Jegu et al. 2011). HSPs also interact with a number of client proteins either protecting them from proteasomal degradation or maintaining them in an inactive state (Jegu et al. 2011). A number of steroid receptors and key effectors of the apoptotic pathway belong to this latter category of proteins. The interaction of HSPs with client proteins appears to assist in tumour growth and survival by inhibiting many anti-apoptotic pathways (Lanneau et al. 2008). Generally, HSPs are up-regulated in many tumour cells, thought to be the result of an adaptive stress response to adverse physiological and environmental conditions within the tumour microenvironment (Li et al. 2009; Lu et al. 2010; Thanner et al. 2003).

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Heat shock protein 70 (HSP70) is a highly conserved family of proteins that play essential cellular roles as protein chaperones during normal cellular growth and following exposure to environmental stresses. The C-terminal substrate-binding domain (SBD) of HSP70 interacts with a vast array of substrates including newly synthesised proteins, exposed regions of partially unfolded proteins or protein degradation products (Evans et al. 2010). A subset of HSP70 proteins stably sequester certain peptides and are referred to as HSP-peptide complexes (HSP-PCs). Since the peptides are derived from the milieu of cellular proteins, such complexes can be considered to represent the metabolic activity of the cell. HSP70 also possesses ATPase activity and interacts with ATP through its N-terminal nucleotide-binding domain. ATP hydrolysis to ADP increases the affinity of peptide substrates for the SBD.

It is generally agreed that the up-regulation of HSP70 contributes to tumour growth and survival; however, when present extracellularly, HSP70 is also capable of mounting potent innate and adaptive immune responses against the tumour cells from which it originated (Jego et al. 2011; Multhoff 2009; Srivastava et al. 2009). HSP70 and its associated peptide complexes (HSP70-PC) are taken up by antigen-presenting cells (APCs) via specific receptors. The associated peptides are represented by MHC class I molecules to elicit a CD8⁺ response against the tumour (Srivastava et al. 2009). It is solely the associated peptides that are responsible for this T cell response as HSPs stripped of peptides are ineffective in eliciting any adaptive immune responses. HSPs can also directly activate T lymphocytes through Toll-like receptor 2 on APCs (Osterloh et al. 2004; Zanin-Zhorov et al. 2003) and can activate the cytolytic and migratory activities of natural killer (NK) cells (Chen and Cao 2010; Multhoff 2009). Thus, HSP70 may play contradictory roles in tumorigenesis, promoting tumour survival through the regulation of apoptosis within the cell whilst aiding in tumour elimination by immune responses when present in the extracellular milieu.

Since HSP70 is over-expressed in many tumours, harbours a large repertoire of antigenic as well as non-antigenic peptides and acts at the crossroads of many key intracellular processes, it represents a unique barcode of protein metabolism in a cancer cell. Using this rationale, we have used purified HSP70-PCs from different human breast cell lines as targets for biopanning random peptide phage display libraries to identify peptide ligands that recognise protein(s)/peptides associated with HSP70. Such ligands have the potential for development as tumour-specific biomarkers. Using this approach, we identified unique sets of phage-displayed peptides which interact with high affinity to HSP70-PC from MDA-MB-231 and MCF-7 breast cancer cell lines. Detailed 'pull-down' analysis using one of the biopanned HSP70-PC-binding peptides, referred to here as 'IST', revealed that the 78-kDa HSP, glucose-regulated protein 78 (GRP78), eukary-

otic translation initiation and elongation factors eukaryotic translation initiation factor 3 (eIF-3) and EF1A respectively are enriched in HSP70-PC from MDA-MB-231 cells. Immunohistochemical staining of tumour tissue microarrays shows that the IST peptide shows a similar staining pattern as anti-HSP70 monoclonal antibody in breast tumour tissue. Taken together, the data presented here indicate that peptides associated with HSP70 can act as a unique indicator of cellular protein activity and a novel source of potential tumour biomarkers. The random peptide phage display library biopanning methodology can be exploited to identify and characterise such peptides.

Experimental

Cell lines and media

Seed cultures of metastatic adenocarcinoma cell lines MDA-MB-231 and MCF-7 breast were obtained from Prof. David Boucher-Hayes (Department of Surgery, Beaumont Hospital, Beaumont Road, Dublin 9, Ireland) and propagated in RPMI-1640 supplemented with 2 mM glutamine and 10% foetal calf serum as previously described (Arnaiz et al. 2006). The immortalised non-tumourigenic breast epithelial cells, MCF12A, purchased from ATTC-LGC (Teddington, UK) was grown in RPMI-1640 supplemented with 2 mM glutamine, 10% foetal calf serum, 20 ng/mL human epidermal growth factor, 0.01 mg/mL human insulin and 500 ng/mL hydrocortisone. An antibiotic/antimycotic solution (Sigma Chemical Co., Poole, UK) was added during growth.

HSP70-peptide complex purification and characterisation

Approximately 10⁸ MDA-MB-231, MCF-7 or MCF-12A cells were lysed using CellLytic M reagent (Sigma Chemical Co.) following the manufacturer's instructions. The soluble protein supernatants were then adjusted to 20 mM Tris-acetate, pH 7.5, 20 mM NaCl, 3 mM MgCl₂, 5 mM DTT, 0.5 mM phenyl methyl sulphonyl fluoride and 1× complete, EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Approximately 12 mg of the cell extract was applied to an ADP agarose column (Sigma Chemical Co.) as previously described (Peng et al. 1997). The bound proteins were eluted with 3 mM ADP and then applied to an Amicon Ultra-15 centrifugal filter unit (Millipore, Ireland B.V.) with a cutoff of 10 kDa and centrifuged at 4,000 rpm at 4°C to concentrate the sample.

The ADP agarose eluate was further purified by anti-HSP70 antibody (brm-22; H1547, Sigma Chemical Co.) affinity chromatography. Protein A/G (100 µL, Ultralink; Pierce Thermo Scientific Co., Rockford, IL) was incubated with 20 µg anti-HSP70 overnight and any unbound antibody

removed by extensive washing with PBS. The antibody was cross-linked to protein A/G by incubation with 5% formaldehyde for 30 min at room temperature. The matrix was washed extensively with 1× TBS (Tris-buffered saline) followed by a quick incubation with 200 µL of 0.2 M glycine (pH 2.2) to remove any antibody that has not been cross-linked. Following equilibration in TBS, the cross-linked matrix was incubated with approximately 10 µg of protein from the ADP agarose eluate in TBS for 2 h at 4°C. The sample was spun gently to pellet the protein A/G agarose and the supernatant retained as the unbound protein fraction. The agarose was washed extensively with 40 column volumes of TBS. Bound proteins were eluted by boiling the protein A/G agarose pellet in the SDS protein sample buffer (Laemmli 1970).

Protein samples were separated on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes as previously described (Sambrook et al. 2001). The membranes were incubated with human anti-HSP70 mouse monoclonal antibody (Sigma Chemical Co.; 1:5,000 in blocking buffer) for 2 h at room temperature. Membranes were washed in TBS-T (Tween, 0.05%) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse-IgG antibody (Sigma Chemical Co.; 1:20,000 in blocking buffer) for 2 h. Following extensive washing, antibody binding was detected using a luminol-based chemiluminescent peroxidase substrate (Sigma Chemical Co.).

Mass spectrophotometry analysis of proteins

Proteins separated by SDS-PAGE were stained with Coomassie Brilliant Blue. The stained bands were digested in situ using the Trypsin Profile IGD Kit (Sigma Chemical Co.) as per manufacturer's instructions. Reduction and alkylation of the separated proteins was performed in-gel using the ProteoPrep Reduction and Alkylation Kit (Sigma Chemical Co.). The trypsin-digested peptides were concentrated and resuspended in 0.1% (v/v) trifluoroacetic acid. Mass spectrophotometry analysis was carried out using an Applied Biosystems 4800 Plus MALDI-TOF/TOF (Mass Spectrometry Resource, Conway Institute of Biomolecular and Biomedical Research, University College Dublin). Proteins were identified from the Swiss-Prot database using the search tool Mascot. A protein score of ≥ 78 and a confidence interval of $\geq 99\%$ were used as the criteria for identifying significant hits ($p \leq 0.05$).

Phage display biopanning

First-round biopanning

The protein fraction purified by ADP agarose chromatography (10 µg) as described above was incubated in 100 µL of 0.1 M NaHCO₃, pH 8.6, on a Maxisorp 96-well plate (Nunc

Thermo Scientific Inc., Rochester, NY) overnight at 4°C. The wells were then washed with TBS-T and blocked in 1% (w/v) casein in TBS overnight at 4°C. The PhD-C7C (New England Biolabs, Herts, UK) phage library (2×10^{11} pfu) was added to the target wells in blocking solution and incubated for 1 h at room temperature. The wells were washed with TBS-T and the bound phages were eluted with 100 µL of 0.2 M glycine buffer, pH 2.2, and immediately neutralised with 20 µL 1 M Tris-HCl, pH 9.1. The eluted phage library was amplified as instructed by the manufacturer.

Second round of biopanning

One quarter of the total amplified phage stock from round 1 above (approximately 10^{10} pfu) was first pre-absorbed onto recombinant HSP70 (HSPA1A ORF expressed in and purified from *Escherichia coli* extract; 100 µg/mL, ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) bound to anti-HSP70-Protein A/G Ultralink matrix in 100 µL TBS for 30 min at room temperature to exclude phages binding to just HSP70. The unbound phage fraction was incubated for 1 h at room temperature with approximately 2 µg of the ADP agarose eluate, also bound to the anti-HSP70 matrix as above. The blocking buffer was TBS containing bovine serum albumin (BSA, 5 mg/mL). The unbound phages were removed and the agarose was washed with 100 column volumes of TBS-T. Once again, any phages binding only to HSP70 and not to its associated peptides were removed by incubating the column with recombinant human HSP70 (100 µg/mL) in 100 µL TBS for 30 min at room temperature. The remaining bound phage were eluted with 0.2 M glycine, pH 2.2, and neutralised as above. The eluted phages were amplified as above.

Third-round biopanning was carried out in the same manner as round 1, except that the amplified phage were pre-adsorbed in a microtitre dish coated with BSA (Sigma Chemical Co.) in order to remove any nonspecific plastic- and/or BSA-binding phages prior to incubation with wells coated with the ADP agarose eluate fraction. A further two rounds of biopanning were carried out under conditions similar to round 2, except that (a) the blocking reagent was casein and (b) the HSP70-PC-binding phages were eluted in the fifth round with 3 mM ATP (Peng et al. 1997) and were not amplified.

Five additional rounds of biopannings were carried out as described for round 2 above, but with the bound phages eluted with 3 mM ATP.

Single-strand DNA was extracted from individual phage plaques after rounds 4, 5, 6 and 10. PCR was performed to verify the presence of inserts in the phage DNA using the primers 5'TGGTTGTTGTCATTGTCGGCG3' and 5'GCCCTCATAGTTAGCGTAACG3'. The insert region was sequenced (GATC Biotech AG, Germany) using the

primer 5-CCCTCATAGTTAGCGTAACG-3. Generally, between 12 and 24 phage clones were sequenced from each round.

Preparation of 'recogniser peptide' affinity matrix and the 'pull-down' assay

Short peptides, deciphered from the inserted DNA in the pIII protein from the phage present in the final round eluate, referred to as 'recogniser peptides', were synthesised by Peptide 2.0 Inc. (Chantilly, VA, USA) or GL Biochem Ltd. (Beijing, People's Republic of China). In addition to the 7-mer unique amino acid sequence corresponding to the insert DNA, the synthetic peptides contained the flanking cysteine residues and a C-terminal triglycine linker with or without a biotin tag.

Approximately 1 mg of the non-biotinylated synthetic peptides (10 mg/mL in 0.1 M phosphate buffer, pH 7.4) was linked via their carboxyl group to amino-coated paramagnetic beads (Kisker Biotech GmbH & Co., Steinfurt, Germany) using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (Sigma Chemical Co.). The unreacted material was inactivated and removed by washing in TBS. The peptide-linked magnetic beads were recovered using magnetic separation and resuspended in PBS at a concentration of 1 mg/mL. As a negative control, an aliquot of beads was processed similarly in the absence of any synthetic peptides and resuspended at the same density in PBS.

Approximately 100 µg of peptide-linked or negative control beads was blocked in 1% (w/v) casein in TBS for 2 h. Following washes with TBS, the beads were incubated for 1 h with 2 mg of soluble protein extract from MDA-MB-231 breast tumour cells. The beads and any peptide-bound proteins were separated from unbound proteins through magnetisation. The bead-bound fraction was washed in TBS, then in TBS containing 0.5 M NaCl and finally in TBS alone. Beads were resuspended in 30 µL of SDS sample buffer (Laemmli 1970) and boiled prior to analysis by SDS-PAGE as before. The gel was stained with Coomassie Brilliant Blue and the selected protein bands trypsinised in situ; mass spectrometry analysis was performed as described above.

Immunofluorescence and immunohistochemical analysis

Approximately 10⁴ MDA-MB-231 or MCF12A cells were seeded onto eight-chamber glass slides (Nunc ThermoFisher, Inc., Denmark) and grown to 50–90% confluency. Cells were washed three times in PBS, fixed with 4% (w/v) formaldehyde in PBS, washed again and blocked overnight at 4°C in 1% (w/v) casein in PBS. The next day, cells were washed in PBS and incubated with biotinylated peptides

(at concentrations indicated in the figure legends) in casein blocking buffer for 2 h. Cells were washed three times in PBS and incubated with ExtrAvidin-Cy3 (Sigma Chemical Co.) at 1:100 in blocking buffer for 2 h. Cells were washed, de-chambered and mounted in aqueous mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 1 µg/mL. Slides were examined using a Nikon Eclipse E400 microscope.

Patients' tissue sections were obtained under ethical guidelines approved by The Beaumont Hospital Research Ethics Committee. All experiments were carried out in compliance with the Helsinki Declaration (<http://www.wma.net/e/policy/b3.htm>). The tissue sections underwent antigen retrieval using Bond Epitope Retrieval Solution 2 Leica Microsystems, IL, USA) prior to incubation with the biotinylated peptides (200 µg/mL) or anti HSP70 antibody (1:500 dilution). Bound peptide was detected using an HRP-conjugated anti-biotin antibody (Leica Microsystems). Antibody binding was detected using the Bond Polymer Refine Detection Kit (Leica Microsystems) and visualized using 3',3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Tissues were counterstained with haematoxylin.

Results

Purification of HSP70 from breast tumour cell lines

Previous studies have shown that HSP70-PCs can be isolated by a one-step purification using ADP agarose column chromatography (Peng et al. 1997). Whole cell lysates were prepared from the breast tumour cell lines MDA-MB-231, MCF-7 and the non-tumour breast cell line MCF12A and applied to an ADP agarose affinity column. The bound proteins were eluted as previously described (Peng et al. 1997). As shown in Fig. 1a, four prominent bands of molecular weights 70, 41, 17 and 15 kDa were retained on the column. The band migrating at 70 kDa, which in fact represents a number of closely migrating proteins, was confirmed as the HSP70 family of proteins by Western blot using an anti-HSP70 antibody (Fig. 1b). To identify the co-eluting proteins, the bands were excised from the gel and digested in situ with trypsin. The peptide products were subjected to matrix-assisted desorption/ionization time-of-flight (MALDI-TOF) peptide mass fingerprinting and the proteins were identified from the Swiss-Prot database using Mascot search engine (Table 1). The closely migrating bands at 70 kDa all represented members of the HSP70 family, including the stress-induced HSP70, the constitutively expressed HSP71 and the mitochondrion-associated HSP70, GRP-75. The co-eluting band at 41 kDa was identified as actin, whilst the 17- and 15-kDa bands corresponded to nucleotide diphosphate kinase A and B, respectively. The

Fig. 1 HSP70-PC purification. **a** Lane M Molecular weight markers. Lane 1 Recombinant Human HSP70 (1 µg; inducible form HSPA1A gene product). Lanes 2–4 ADP agarose column eluate (5 µg) from MCF-7, MDA-MB-231 and MCF-12A cells, respectively. **b** Western blot of gel from (a) incubated with anti-HSP70 antibody. **c** Anti-HSP70 affinity-purified MDA-MB-231-derived HSP70-PC. The ADP agarose eluate was applied to an anti HSP70-A/G agarose column. The bound proteins were eluted by boiling in the SDS sample buffer. Lane M Molecular weight marker. Lane 2 ADP agarose column eluate (5 µg). Lane 3 Anti-HSP70/Protein A/G eluate (half of total sample). The two additional bands present in lane 3 correspond to the reduced heavy and light chains of the IgG anti-HSP70 antibody

latter three proteins are known to interact with ADP, thus explaining their retention on the ADP agarose column. Similar results were obtained in at least two independent experiments.

In order to use HSP70-PC as a target for biopanning, we needed to purify HSP70-PCs away from the co-eluting proteins. The ADP agarose-bound fraction was incubated with anti-HSP70 mouse monoclonal antibody which was cross-linked to a protein A/G agarose column. The antibody employed (Brm-22) was raised against human HSP701A (HSPA1A) and recognises the major constitutive (HSPA8 and HSPA2) and inducible (HSPA1A and HSPA1B) isoforms of HSP70 (Brocchieri et al. 2008; Tavaría et al. 1996). This ensured that at a minimum, the majority of isoforms of Hsp70 would be captured on the beads. Following the removal of unbound proteins, the bound material was eluted, separated by SDS polyacrylamide electrophoresis and stained with Coomassie Brilliant Blue (Fig. 1c). The major forms of HSP70 were retained on the anti-HSP70 column (Fig. 1c, lane 3). Furthermore, the

HSP70 proteins could be separated from the other ADP-binding proteins, which were evident in the unbound fraction (data not shown).

Biopanning against tumour cell line-specific HSP70-PCs

Since the peptides associated with HSP70 represent a unique collection of cellular peptides indicative of protein metabolism in a cancer cell, we set out to design probes capable of interacting with one or more of the peptides in the pool. Such probes would be valuable tools both in diagnosis and targeted drug therapy. The physical quantity of HSP70-PCs in a cell is not sufficient enough for use in routine biochemical and/or biophysical characterization unless one resorts to prohibitively expensive, very large-scale cell culture preparations. An alternative approach to generate peptide probes that selectively bind to pure or enriched target molecules is to use phage display biopanning, which requires only relatively small quantities of target proteins. Such peptide probes can be mass-produced and modified for stability and/or affinity for any downstream applications. We used immunoaffinity-purified HSP70-PC as targets in the biopanning of commercially available phage display libraries (Deutscher 2010). The biopanning protocol incorporated various contingency measures to reduce the selection of false positives. Typically, the ADP agarose column eluate from MDA-MB-231 and MCF-7 cells, prepared as described above, was separately bound to microtitre plates and incubated with the PH.D.-C7C phage display library. This library displays random 7-mer peptides flanked by cysteine residues, fused to the minor coat protein pIII of the phage virion. The spontaneous formation of a disulphide bond between the cysteine residues results in the display of a constrained cyclic peptide. The bound phage was eluted and amplified. In the second round of biopanning, phages were incubated with immunoaffinity-purified HSP70-PCs, thus removing those phages binding to any of the contaminating proteins in the ADP agarose eluate. Phages interacting with the HSP70 moiety of the HSP70-PC were removed using a subtractive biopanning step with recombinant HSP70 before proceeding to the third round of

Table 1 MALDI-TOF peptide mass fingerprinting of ADP agarose binding proteins

Band	Identified protein	Swiss-prot name	Protein MW	CI (%)
1	Stress-70 protein, mitochondrial	GRP75	73,635	100
	Heat shock 70-kDa protein 1	HSP71	70,009	100
	Heat shock cognate 71-kDa protein	HSP7C	70,854	100
2	Actin, cytoplasmic 1	ACTB	41,709.7	100
3	Nucleoside diphosphate kinase A	NDKA	17,137.7	100
4	Nucleoside diphosphate kinase B	NDKB	17,286.9	100

CI confidence interval

biopanning with the ADP agarose column eluate. The recombinant HSP70 protein was derived from the heat-inducible gene HSP1A1; however, due to the conserved nature of HSP70 proteins, this subtractive step should remove the majority of phages recognising the major isoforms of HSP70. Up to six rounds of further biopanning was carried out with the immunoaffinity-purified HSP70-PC. The subtractive step was repeated after each of the first five rounds of biopanning, thus enriching for phages binding to the polypeptide fraction of HSP-PC. Additionally, interacting phages were selectively eluted from the column with ATP, thereby enriching for peptide-interacting phages. By carrying out up to ten rounds of consecutive biopanning, we hoped to identify peptides with the highest affinity for HSP70-PCs. Individual phage DNAs were isolated and sequenced after rounds 4, 5, 6 and 10 to evaluate the enrichment of any selected sequences. The peptide sequences identified after ten rounds of biopanning from each of the breast tumour cell lines are shown in Table 2. A single peptide, ISTHGPL, dominated the final phage pool from the MDA-MB-231 cell line, whilst four unique peptides were identified in the final phage pool from the MCF-7 biopanning, with the sequence STLNVLQ (STL) representing 50% of the phages sequenced (Table 2). The other isolated peptides in the final round from MCF-7 were TQKWPPH (TQK), APNETRS (APN) and HPLIHPD (HPL). Amino acid motifs present in the final round peptides, in particular the motifs THXPL, IXTH found in IST and SXLN from the STL peptide, were evident in peptides identified in earlier rounds of biopanning (Electronic supplementary material (ESM) Table 1). The HPL peptide was also found in phages isolated from rounds 4 and 5 (ESM Table 1). The peptide sequences enriched in the earlier rounds (4–9) were subsequently outcompeted by the peptides found in round 10, although the enrichment of specific peptides in earlier rounds indicates that they also have affinity for HSP70-PC. Thus, the biopanning approach selectively enriches for peptides with the highest affinity for the target, HSP70-PC. The peptides isolated from round 10 were designated as ‘recogniser peptides’ of HSP70-PC.

Table 2 Recogniser phage from HSP70-PCs

Peptides	Frequency (%)	Cell line
ISTHGPL	100	MDA-MB-231
TQRPDKS ^a	25	MDA-MB-231
STLNVLQ	50	MCF-7
TQKWPPH	17	MCF-7
HPLIHPD	17	MCF-7
APNETRS	17	MCF-7

^a Round 5

Interestingly, some of the newly identified recogniser peptides have partial sequence identity to two peptides (AIPNKLNVWPPH and SQELTQRPYKWH; see ESM Table 2) independently isolated from an previous biopanning scheme using the ADP agarose eluate fraction from MDA-MB231 cells and a 12-mer random peptide phage display library (PhD-12; New England Biolabs Inc., Ipswich, MA; Arnaiz et al. 2006). The common recogniser motifs include LNV and WPPH isolated from MCF-7 HSP70-PCs and TQRPXK isolated in round 5 from MDA-MB231 HSP70-PCs (Table 2 and ESM Table 1). With the exception of APN, the peptides are non-polar and positively charged (Fig. 2). The interaction of the selected peptides with HSP70-PC was demonstrated by measuring the binding of HSP70-PC to peptides linked to paramagnetic beads (data not shown).

The IST peptide interacts with GRP78 and the translation factors, eIF-3 and EF1A

Since just a single unique peptide was recovered in the biopanning against HSP70-PCs from MDA-MB-231 cells, this peptide was selected for further characterisation. Whilst the IST peptide showed a strong interaction with HSP70-PC, the true cellular target of this binding was unknown as many cellular proteins are sequestered and processed by HSP70 either for repair or degradation and become part of the HSP70-PC complex. We devised a ‘pull-down’ assay in which the IST peptide was used to ‘fish out’ cellular proteins that it binds to and characterised these proteins by MALDI-TOF. It should be noted that HSP70 and its binding partners are held together through hydrophobic interactions between non-polar amino acids. Such non-polar amino acids may become exposed due to unfolding, denaturation or misfolding. Since the IST peptide has four non-polar amino acids, it may also interact with one or

Fig. 2 Amino acid composition of recogniser peptides. Peptides isolated after ten rounds of biopanning were categorized by amino acid composition. Non-polar amino acids (white), polar amino acids (black), positively charged (grey), negatively charged (mottled). The percentage of each category for each peptide is shown on the Y-axis

more peptides associated with HSP70 through hydrophobic interactions.

The IST peptide, flanked by cysteine residues and a triglycine linker at the C-terminus, was chemically synthesised. The presence of the cysteine residues enables the peptide to retain its cyclic nature as displayed on the pIII virion protein. The peptide was covalently linked to paramagnetic beads and incubated with total soluble protein from MDA-MB-231. The unbound proteins were removed by extensive washing. The bound proteins were eluted from the paramagnetic beads by solubilisation in the SDS sample buffer. As a negative control, the protein extract was incubated with paramagnetic beads without the IST peptide. In addition to the nonspecific protein bands evident in the control samples (Fig. 3, lane 3), a number of unique proteins were evident in the sample eluted from the IST peptide-linked beads. The major proteins, (Fig. 3, lane 2; bands labelled 1–9) and the nonspecific co-migrating bands present in the control unlinked beads (Fig. 3, lane 3; bands labelled 4–8) were excised from the gel and digested *in situ* with trypsin and subjected to MALDI-TOF peptide mass fingerprinting. Proteins were identified from the Swiss-Prot

database using the Mascot search tool. The most significant hits for each of the isolated proteins are shown in Table 3.

The peptide mass fingerprinting of the proteins pulled down with the IST peptide identified three subunits of the eukaryotic translation initiation factor 3 (eIF3A, B and L; Fig. 3, lane 2, bands 1, 2 and 4, and Table 3) and the elongation factor EF1A-3 (Fig. 3, lane 2, band 7, and Table 3). Additionally, GRP78, a member of the HSP70 family, was also pulled down by the IST beads (Fig. 3, lane 2, band 3). A number of proteins common to both the IST beads and the negative control beads were also identified (Fig. 3, lanes 2 and 3, bands 5, 6 and 8, and Table 3). These represented cytoskeleton-associated proteins such as tubulin, actin and vimentin. Two other proteins pulled down by the negative control beads (Fig. 3, lane 3, bands labelled 4 and 7) were putatively identified as contaminating keratin (Table 3), whilst no spectrum was obtained for the protein labelled band 9 (Table 3). It appeared that the negative control beads without the IST peptide pulled down a number of constitutively expressed cellular proteins, perhaps reflecting their abundance. It is pertinent to note that the ‘pull-down’ assays done here used a crude cellular lysate as the starting material, and this may have increased the chance of bringing down some nonspecific proteins. The proteins identified with the IST bead ‘pull-down’ assay were specific to this peptide and were not present in similar ‘pull-down’ assays with unrelated 7-mer peptides (data not shown).

Fig. 3 IST peptide-binding proteins. The peptide IST was covalently linked to amino-coated paramagnetic beads and incubated with total cell lysate from MDA-MB-231 cells. Following washing of the column, the bound beads were eluted by boiling in SDS protein sample buffer. *Lane M* Molecular weight marker. *Lane 1* ADP agarose eluate fraction (10 μ g). *Lane 2* Total eluate from IST peptide-linked paramagnetic beads. *Lane 3* Total eluate from paramagnetic beads only

IST binds to breast tumour cell lines and tumour tissue

The ability of the IST peptide to bind to breast tumourigenic (MDA-MB-231) and non-tumourigenic (MCF-12A) cell lines was next investigated. From the pull-down assay, it appeared that IST recognises some constitutively expressed cytoplasmic proteins. Therefore, one would have predicted that IST peptide-stained tissue sections may have a total staining of the cytoplasm. Indeed, the IST peptide produced a distinctive cytoplasmic staining pattern (Fig. 4). In many cells, a crescent-shaped perinuclear staining pattern was evident (Fig. 4, top panels). The observed staining appeared specific to the IST peptide as little or no staining was seen with a control scrambled peptide (Fig. 4, bottom panels). However, there did not appear to be any discernible difference in staining between the tumourigenic and non-tumourigenic cells (compare left and right panels in Fig. 4). It should be noted that the anti-HSP70 monoclonal antibody also produces a predominantly cytoplasmic staining pattern in the two cell lines used here and in other human tumourigenic cell lines (Arnaiz, James and Bond, unpublished results; also see images at www.abcam.com;brm-22 and references therein). As a preliminary exercise to determine whether the peptide was capable of binding to human tumour tissues, immunohistochemical

Table 3 Significant hits for each of the isolated proteins

Band	MW	Identified protein	Swiss-Prot name	CI (%)
1	166,468	Eukaryotic translation initiation factor 3 subunit A	EIF3A	100
2	92,433	Eukaryotic translation initiation factor 3 subunit B	EIF3B	99.99
3	72,288	78-kDa glucose-regulated protein	GRP78	100
4	66,684	Eukaryotic translation initiation factor 3L	IF3EI or EIF3L	100
4 ^a	65,978	Keratin, type II cytoskeletal 1	K2C1	68.9
5	53,619	Vimentin	VIME	100
5 ^a	53,619	Vimentin	VIME	100
6	50,119	Tubulin alpha-1B chain	TBA1B	100
6 ^a	49,639	Tubulin beta chain	TBB5	100
7	50,153	Elongation factor 1-alpha 3	EF1A3	100
7 ^a	65,978	Keratin, type II cytoskeletal 1	K2C1	0
8	41,709	Actin, cytoplasmic 1	ACTB	100
8 ^a	41,709	Actin, cytoplasmic 1	ACTB	100
9		No spectrum obtained		

CI confidence interval

^aFrom control beads

staining was carried out on a tumour tissue microarray which contained a number of breast tumour tissue sections. Since HSP70 has been shown to be up-regulated in a number of tumours (Calderwood 2010; Tan et al. 2011), the distribution of HSP70 within the tissues was also examined using an anti-HSP70 antibody. For direct comparison, staining with the IST peptide and anti-HSP70 was carried out on sequential sections of the same tumour tissue. In

theory, one would expect similarities in the staining patterns obtained with both anti-Hsp70 antibody staining sites and the IST peptide as the peptide should recognise HSP70-associated peptides. We can also expect staining to the parent protein(s) from which the HSP70-PC was derived, such as the ones identified by the ‘pull down’ assay. The staining patterns obtained with both the anti-HSP70 antibody and the IST peptide are illustrated in Fig. 5. As expected, the HSP70 antibody showed positive staining in many tumour epithelial cells. Staining with the IST peptide was weaker, but the same areas of the tumour, which were positive with HSP70, also stained with the IST peptide (Fig. 5b, panels A–B). The observed weaker staining by IST may be attributed to the size and affinity of the peptide to the targets within the cell.

Fig. 4 Immunofluorescent staining with IST peptide. MDA-MB-231 and MCF12A cells were fixed with paraformaldehyde and incubated with 100 µg/mL of biotinylated IST peptide (*top panels*) or a control peptide (LSTSTV) (*bottom panels*). The cells were washed and incubated with ExtrAvidin-Cy3. Cells were counterstained with DAPI at a concentration of 1 µg/mL. Slides were examined using either a Nikon Eclipse E400 microscope or an Olympus confocal microscope. Magnification, $\times 200$. Similar results were obtained in a number of independent staining experiments

Discussion

Dysregulation of cellular protein homeostasis is associated with tumour development (Blagden and Willis 2011). HSP70 has indispensable roles in many intracellular pathways participating in cellular protein homeostasis. In its role as a major chaperone protein, HSP70 interacts with a large repertoire of proteins through its peptide-binding domain, either assisting in the folding of nascent polypeptides, unfolding of aberrantly folded protein, in protein transport and localisation and, finally, through its interaction with the proteasome, in protein degradation (Odunuga et al. 2004). The collection of peptides and polypeptides associated with HSP70 in tumour cells represents a unique profile indicative of the protein turnover. Previous studies have demonstrated that the HSP70-PC pool contains many antigenic peptides. HSP70 directly presents these peptides to antigen-presenting cells where they are represented by

Fig. 5 Immunohistochemical staining of tissue microarrays with IST peptide. *Top panel* Breast tumour tissues incubated with anti-HSP70 antibody. *Bottom panel* Sequential tissue sections incubated with biotinylated IST peptide and then anti-biotin antibody. Antibody binding

was detected using the Bond Polymer Refine Detection Kit (Leica Microsystems) and visualized using DAB. **a** and **b** are the same case. Magnification, $\times 100$ (**a**), $\times 400$ (**b**), and $\times 200$ (**c**). Similar results were obtained in at least four independent patient tumour tissue sections

MHC class I molecules to elicit T cell responses against the tumour (Srivastava et al. 2009). A number of researchers, notably Srivastava et al. (2009), have exploited this property of HSP70-PC for cancer therapy with varying rates of success.

The pool of peptides associated with HSP70 in tumour cells may also be a novel untapped source of tumour biomarkers for diagnostic purposes. Over-expression of HSP70 and many other proteins in certain tumours have been documented. Some of the abnormally expressed proteins as well as those over-expressed in tumour cells may have a higher chance of being sequestered by the HSP70 chaperone system and therefore may become part of the HSP70-PC in tumour cells. Despite their significance, both as biomarkers and as tumour antigens, the physical quantity of HSP70-PC in a cell is very limited for recovery and experimentation. For therapeutic purposes and good prognosis, the tumour has to be detected at the earliest stage where the amount of tissue available for biochemical fractionation is very limited. Furthermore, the composition of the peptides in the HSP70-PC of cells may be dynamic, and the detection and identification of many of these peptide pools may turn out to be a long and arduous process. In one of the approaches pioneered by Stocki and colleagues (Stocki et al. 2010, 2011), low-molecular-weight proteins and peptides ($<10,000$ kDa) from tumour cells were extracted and finger printed through mass spectrometry, and a synthetic array of these peptides were assayed for their ability to bind to HSP70. With the necessary controls, they identified 44 peptides associated with HSP70 from the leukaemia cell line, K562. One of the identified

peptides, which contains a HLA-A*3 antigenic epitope, was derived from dermcidin, a protein that purportedly promotes tumour cell survival (Stocki et al. 2011).

Previously, we have used phage display biopanning to circumvent the limitation of the available HSP70-PC in a cell and to design molecular probes to study the HSP70-PC (Arnaiz et al. 2006). Our long-term goal is to generate a library of 'recogniser peptides' and to use them to probe for putative mimic peptides of HSP70-PC with a view to use them in adoptive immunotherapy (Arnaiz et al. 2006). The conventional method of ADP agarose chromatography for purifying HSP70-PCs at least co-purifies three low-molecular-weight contaminating proteins, namely, actin and nucleoside diphosphate kinases A and B; all are known ADP-binding proteins. Here, we introduced either stand-alone or in-line HSP70 immunoaffinity purification steps in the biopanning setup to remove the contaminating ADP-binding proteins and thereby any phages binding to them. Thus, the protocol adapted in this study is an improvement of previous biopanning approaches (Arnaiz et al. 2006) and more likely to generate HSP70-PC-specific 'recogniser peptides'. Rigorous steps were taken to rule out the selection of phages binding to the HSP70 protein itself. We also used immunoaffinity-purified HSP70-PCs from two different breast epithelial tumour cell lines, MDA-MB-231 and MCF-7, for biopanning. The antibody used for immunoaffinity, brm-22, recognises the major constitutive and inducible isoforms of HSP70 and thus provided the broadest selection of HSP70-PCs in the cell. With the availability of antibodies to specific isoforms of HSP70, it may be possible in the future to identify peptides interacting

with a more specific HSP70-PC population. The identification of unique ‘recogniser’ peptides for HSP70-PCs from each cell line indicates that there are differences amongst the pool of peptides associated with HSP70 in each cell line and supports the view that the isolated peptides are not recognising HSP70, the backbone of the HSP70-PC complexes. Interestingly, some of the newly identified recogniser peptides have partial sequence identity to two peptides independently isolated previously from an earlier biopanning scheme using the ADP agarose eluate fraction from MDA-MB231 cells and a 12-mer random peptide phage display library (PhD-12; New England Biolabs Inc.; Arnaiz et al. 2006). Such similarities in recogniser peptide motifs between different cell lines and phage display libraries clearly demonstrate the unique ability of the phage biopanning technology to reproducibly identify protein–protein interactions and provide veracity to the use of phage display biopanning as an approach to identifying biologics that interact specifically with Hsp70-PCs. Recently, a yeast two-hybrid-based approach was used to identify small peptide inhibitor molecules (aptamers) of HSP70 from HeLa cells (Rérole et al. 2011). None of the peptides identified in that study were identical or similar to the peptides reported in this study. This may reflect the selectivity of the assay, indicating that the ‘recogniser’ peptides are more likely derived from the associated peptides and not to the HSP70 protein itself, as in the case of the peptide aptamers.

Sequencing of the DNA of the phage populations enriched after ten rounds of biopanning against MDA-MB-231 cell line-derived HSP70-PCs identified just a single peptide, ISTHGPL, although other peptide motifs were enriched in earlier rounds of biopanning. As in the case of aptamer selection, a serial enrichment of the peptide motifs, THXPL and IXTH, was evident after the fourth and sixth rounds of biopanning, respectively (see ESM Table 1). Using MCF-7 cell line-derived HSP70-PCs as a target, a more diverse set of peptides were identified in the final round of biopanning. Again, a serial enrichment of amino acid motifs found in the final pool of peptides was evident in earlier rounds of biopanning (ESM Table 1). In general, the peptides present in the final round of biopanning were non-polar, neutral or positively charged.

The IST peptide was characterised in more detail as it was the single peptide present in the final round of biopanning against MDA-MB-231 cells. A biotin-tagged IST peptide and cy3-streptavidin was used to locate the cellular components that it binds to. The IST interactions were limited to the cytoplasm and more specifically produced a halo-like arc-shaped perinuclear staining in some cells. Following a ‘pull-down’ assay using immobilized IST and peptide mass fingerprinting of the products, two classes of proteins were identified as IST-interacting.

These include subunits of the translation initiation and elongation factors eIF3 and EF1 α 3, respectively, and GRP78, a member of the HSP70 family of proteins. A rate-limiting step in eukaryotic translation is initiation. The process involves many eukaryotic initiation factors (eIFs), and it is known that altering the expression level or function of eIFs may influence the synthesis of some proteins and may cause abnormal cell growth and malignant transformation. The largest of the 13 subunits of eIF3 is the 170-kDa protein (eIF3p170; eIF3A), and it has been shown to be elevated in human breast, cervical, esophageal and lung cancers. eIF3A expression is cell cycle-regulated, peaking in the S-phase and is a key regulator of a subset of mRNAs including p27 (kip1), tyrosinated α -tubulin and ribonucleotide reductase M2 subunit, which all play central roles in the regulation of the cell cycle (Dong et al. 2004; Silvera et al. 2010). The over-expression of individual subunits of eIF3 appears to be sufficient to result in the malignant transformation of the immortal NIH3T3 cell line (Zhang et al. 2007). Taking these data together, it appears that eIF3A is a major contributor of malignant transformation and/or cell growth control (Saletta et al. 2010).

The translation elongation factor EF1 α 3 is a member of the EF1 α family and is nearly identical to EF1 α 1 (99%) and EF1 α 2 (96%). Both EF1 α 1 and EF1 α 2 have been identified as oncoproteins and are also over-expressed in breast and ovarian tumour cells (Sharma et al. 2007; Tomlinson et al. 2005). The IST peptide-binding sites within the cell as revealed by indirect staining patterns are consistent with them being associated with the protein translational machinery at the endoplasmic reticulum.

The third IST-interacting protein identified in this study is GRP78, a member of the HSP70 family of proteins. It plays a major role in the endoplasmic reticulum shock and unfolded protein responses. Like eIF3 and the EF1 α family, GRP78 is also over-expressed in a number of cancers, and the presence of autoantibodies to GRP78 in cancer patient sera is associated with higher pathological tumour grades, poor prognosis and drug resistance in breast cancers (Tan et al. 2011; Zhang et al. 2006). There is a possibility, *albeit* remote, that GRP78 may have been brought down in the pull-down assay due to its similarity to HSP70 or that the IST peptide may recognise certain HSP70 isoforms. Our view is that this is unlikely as no other HSP70 isoform was brought down in this assay. The fact that all three IST-interacting proteins (eIF3, EF1 α and GRP78) identified in this study have a common link to the translational machinery in the cell fits the paradigm that these proteins are HSP70-PC clients and/or that they may have the same structural requirements to associate with the IST peptide. If this is the case, then, synthetic ligands that recognise the IST peptide may mimic those structural requirements of HSP70-PC clients. As stated earlier, mimics of recogniser peptides derived from HSP70-PC can stimulate

the innate and adaptive immune system (Arnaiz et al. 2006); thus, such mimics would be functionally equivalent to anti-idiotypic molecules and may be developed to target immune responses against client proteins such as eIF3, EF1a and GRP78 in tumour cells.

As a preliminary exercise to determine whether the IST peptide could be developed as a diagnostic probe for tumour detection, we examined the binding of the peptide to breast epithelial cell lines and a number of breast tumour tissue samples. The IST peptide showed a cytoplasmic staining pattern in both the tumourigenic MDA-MD-231 and the non-tumourigenic but immortalised MCF12A cell lines, but we did not discern a noticeable difference in the staining patterns between the two cell lines. This may be partly due to the fact that all of the identified cellular ligands of the IST peptide are constitutively expressed in all mammalian cells, although there may be quantitative differences in the level of expression which is not detectable by fluorescence staining. We also demonstrated the staining of a number of patient breast tumour tissue sections with the IST peptide. The peptide showed a similar staining pattern as the anti-HSP70 antibody in breast tumour tissue. A future goal in this project will be to assess the presence or absence of IST staining in a more extensive range of human tumour tissues pre- and post-drug therapy.

Taken together, the data indicate that HSP70-associated peptides represent a reservoir of peptides unique to each cell, which, through their association with over-expressed HSP70, can serve as a useful source of cancer biomarkers. The prototype peptide IST interacts with a number of key cellular proteins that are over-expressed in cancer cells and binds to the tumour tissue from which it was derived. The methodology employed here can be used in cancer research to identify novel short peptides for diagnostic and therapeutic uses.

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