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A non receptor-mediated mechanism for internalization of molecular chaperones

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Abstract

The evolving realization that stress proteins, which have for many years been considered to be exclusively intracellular molecules under normal conditions, can be released from viable cells via a number of potential routes/pathways has prompted interest into their extracellular biology and intercellular signaling properties. That the stress proteins Hsp60, Hsp70 and gp96 can elicit both pro- and anti-inflammatory effects suggests that these molecules play a key role in the maintenance of immunological homeostasis, and a better understanding of the immunobiology of extracellular stress proteins might reveal new and more effective approaches for controlling and managing infectious disease, inflammatory disease and cancer. A number of cell surface receptors for stress proteins have been identified, and the intracellular consequences of these cell surface receptor-ligand interactions have been characterized. To date, studies into the intercellular signaling properties of stress proteins and their interactions with antigen presenting cells have focused on specific receptor-mediated uptake, and have not considered the fact that such cells can also take up proteins via non-specific endocytosis/pinocytosis. Herein we present a methodological approach for assessing receptor-mediated and non-receptor mediated uptake of gp96 by rat bone marrow-derived dendritic cells.

1. Introduction

Much research is now being focused on the intercellular signaling properties of stress proteins. These efforts have been prompted, in the main, by the observations that intracellular stress proteins can be released by viable cells via what appear to be active release mechanisms [1-7], and that stress proteins with putative pro-inflammatory properties (e.g. Hsp60, Hsp70 and gp96) are present in the peripheral circulation [8-20]. In the case of Hsp60 and Hsp70, some studies have reported that the circulating levels of these proteins are the same as, or in excess of those that have been shown to elicit pro-inflammatory immune responses in vitro (>1000 ng/ml; [8-10,12,18]). Although extracellular stress proteins are commonly perceived to be inflammatory mediators and 'danger' signals to the immune system [21-30], there is also a wealth of evidence to indicate that proteins such as Hsp60, Hsp70 and gp96 can exhibit anti-inflammatory properties by attenuating inflammatory conditions [31-34] and prolonging the survival of transplanted organs and tissue [35-37].

As part of the quest to better understand the immunobiology of stress proteins, studies have focused on characterizing the cell surface receptors on antigen presenting cells (APCs) such

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as dendritic cells (DCs) and monocytes/macrophages with which such proteins interact. In these, the binding of fluorescently labeled or biotinylated stress proteins to cell surface receptors has been monitored using flow cytometry. In the main, these experiments have been performed at 4°C in order to prevent receptor-mediated uptake of the bound protein. An alternative approach has been to determine the binding of stress proteins to non-APCs that have been induced to transiently express given putative receptors [38,39]. Receptor usage by stress proteins has been reviewed in detail elsewhere [40]. CD14 [21] and the Toll-like receptors (TLRs) -2 and -4 [41,42] have been identified as receptors for Hsp60. Although CD14 [24], CD40 [43], CD91 [44], the scavenger receptor LOX-1 [45] and TLR-2 and TLR-4 [25,46] have been identified as receptors for Hsp70, a recent study has shown Hsp70 to bind to LOX-1, but not to CD91, CD40, TLR-2 and TLR-4 [38]. Gp96 has been identified as a ligand for the CD91 molecule [47], TLR-2 and TLR-4 [40,48,49] and scavenger receptor-A [50].

Stress proteins have also been shown to bind to non-APCs. Hsp60 binds to and activates immunoregulatory CD4⁺ T cell populations via TLR-2 [31] and Hsp70 binds to receptors on endothelial/epithelial cells [38]. Receptors for Hsp70 have also been identified on the surface of natural killer (NK) cells, and these have been shown to recognize Hsp70 expressed on the surface of cells [51-55]. We have shown that gp96 binds to CD4⁺ and CD8⁺ T cell populations, via as yet unidentified receptors [56].

A common perception is that a protein must interact with its target cell population via specific cell surface receptors for it to elicit a biological effect. However, with regards to APCs such as DCs this is not necessarily so, as it is well known that immature DCs can take up antigens via pinocytosis in clathrin-coated pits and by fluid-phase pinocytosis [57]. It is of particular importance to note that antigenic peptides derived from pinocytosed exogenous material can be presented to T cells by DCs (reviewed in [58]). It would therefore appear that in vivo, extracellular stress proteins are continually being taken up ('sampled') by DCs via non receptor-mediated endocytosis, and the immunological consequences of this uptake might critically depend on the pathway into which these proteins are delivered and the presence of co-existent signals that the DC receives from the stress protein or other inflammatory or anti-inflammatory molecules in the surrounding microenvironment.

We have previously reported a strategy for generating a population of immature rat bone marrow-derived DCs (BMDCs) [59,60], and demonstrated that these do not express cell surface receptors for gp96 [56]. However, these cells do internalize gp96 via non receptor-mediated endocytosis in the absence of any overt effect on their phenotype [56]. It might therefore be that the re-presentation of antigens associated with internalized gp96 to responding T cell populations by immature DCs, and thereby in the absence of essential co-stimulatory molecules, in part at least explains the ability of donor tissue-derived gp96 to prolong the survival of rat cardiac transplants [37].

Gp96 is a 94-96 kDa member of the Hsp90 family of molecular chaperones/stress proteins which resides within the lumen of the endoplasmic reticulum to which have been attributed a number of pro- [26-30,61-63] and anti-inflammatory [33,36,37,56,62] properties. This study describes approaches for the purification and FITC-labeling of gp96, the generation of a defined population of rat BMDCs and the determination of receptor-mediated binding and non receptor-mediated uptake of gp96 by these cells using multicolor flow cytometry.

2. Methods

2.1. Preparation and analysis of purified gp96

Gp96 can be purified from Wistar rat and C57BL/6 mouse livers as described below [64]. All laboratory equipment used in the purification procedure should be baked overnight at 220°C

and all solutions should be prepared using autoclaved ultrapure H₂O (e.g. Elga PURELAB® Ultra Genetic Polishing Unit, Vivendi Water Systems, High Wycombe, UK).

2.1.1. Purification of gp96

1. Livers are homogenized in phosphate buffered saline (PBS) containing the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF, 0.2 mM, Sigma-Aldrich Company Ltd., Gillingham, UK) (60 ml buffer/30 g tissue) using a Waring Laboratory Science laboratory blender and stainless steel mini container (Christison Particle Technologies Ltd., Gateshead, UK).
2. The homogenate is centrifuged for 10 min at 2,000g at 4°C to remove unbroken cells, nuclei and other debris. The supernatant is then ultracentrifuged at 100,000g for 90 min after the removal of the lipid layer.
3. The supernatant is brought to 50% w/v saturation with NH₂SO₄ and the resultant precipitate is removed by centrifugation at 5,000g for 20 min. The supernatant is then diluted 1:1 with PBS containing 2 mM Ca²⁺ and 2 mM Mg²⁺ and applied to a concanavalin A (Con A)-Sephacel bead column (Amersham Biosciences UK Ltd., Little Chalfont, UK).
4. The bound fraction is eluted with 10% w/v α -methyl mannopyranoside (Sigma-Aldrich Company Ltd.), and the eluted fraction is passed through a PD10 size exclusion column (Amersham Biosciences UK Ltd.).
5. The protein-containing eluate is applied to a DEAE-Sephacel bead column (DEAE Sephacel™, Amersham Biosciences UK Ltd.) equilibrated with 5 mM sodium phosphate buffer (pH 7.0). Seven 2 ml fractions are collected - only those eluting between 450 mM and 500 mM NaCl (fractions 3 and 4) contain detectable levels of protein when measured using the Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), and gp96 when assessed using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and ECL™ Western blot analysis.

2.1.2. Analysis of purified gp96 by SDS-PAGE

1. Samples (1 μ g total protein) in sample loading buffer (62.5 mM Tris-HCL pH 6.8, 2% w/v SDS, 10% v/v glycerol) containing dithiothreitol and bromophenol blue (total volume 40 μ l) are vortex mixed and denatured at 99°C for 5 min.
2. Samples are run on pre-cast BioRad 10% w/v SDS gels for SDS-PAGE at 180V for ~60 min using a Mini Gel Criterion Gel System in running buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS).
3. Gels are silver stained or electrophoresed proteins are transferred to nitrocellulose membranes (N-bond) using a BioRad Mini Trans-Blot Electrophoretic Transfer Cell for ECL™ Western blot analysis.

2.1.2.1. Silver staining of electrophoresed gels: Deionized H₂O with a conductivity of ~18 m Ω is used throughout the procedure and all reagents are used at room temperature in order to optimize the staining. The oxidizer (potassium dichromate and nitric acid) and silver reagent (silver nitrate) are diluted with deionized H₂O on the day of staining (1:10). The developer (sodium carbonate and paraformaldehyde) is also prepared in deionized H₂O (16 g in 500 ml). Reagents can be obtained from BioRad Laboratories (Hemel Hempstead, UK). It is worth noting that silver staining is 10-50 fold more sensitive than Coomassie brilliant blue R-250, but that Coomassie is suitable for the quantitative measurement of protein abundance by image analysis techniques.

1. Electrophoresed gels are fixed (40% v/v methanol/10% v/v acetic acid) for 24 h at 4°C, after which they are mixed for 15 min in 10% v/v ethanol/5% v/v acetic acid fixative on a rocking platform.
2. The fixative is removed and gels are incubated with fresh fixative for a further 15 min.
3. The fixing solution is removed and gels are then incubated with oxidizer (potassium dichromate and nitric acid) for 5 min. The resultant yellow color is removed from the gel by washing three times in distilled H₂O.
4. Gels are treated with silver reagent (silver nitrate) for 20 min, after which they are washed in distilled H₂O.
5. Gels are then placed in developer solution (sodium carbonate and paraformaldehyde) until clear bands appear (approximately 30 sec). The resultant brown smoky precipitate is removed and fresh developer is applied for 5 min.
6. This procedure is repeated until the desired staining intensity has been achieved. The reaction is stopped by the addition of 5% v/v acetic acid in order to maintain the level of staining. Gel images can then be obtained using a gel imager (e.g. BioDoc-it Systems, Ultra-Violet Products Ltd., Cambridge, UK).

2.1.2.2. Immunodetection of purified gp96 by ECL™ Western blot:

1. Nitrocellulose membrane optimized for nucleic acid transfer (N-bond) is washed in methanol to enhance binding of proteins and then transferred to blotting buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol).
2. A sandwich is made by placing a pad onto the black anode of the transfer cell. The resolving gel is pre-soaked in the blotting buffer and placed onto the filter paper, followed by the membrane. A second pre-soaked pad follows the filter paper. An ice pad and flea are placed into the tank and the gel is blotted for 60 min at 300 mA.
3. Membranes are blocked by incubating with 5% w/v non-fat milk powder in Tris-buffered saline/Tween 20 (TBS/T) for 1 h, and then incubated with a rat anti-gp96 monoclonal antibody (mAb, Assay Designs, Ann Arbor, USA; catalog number SPA-850) diluted 1:1000 in 5% w/v milk powder TBS/T overnight on a rocker. Negative controls are incubated with 5% w/v milk powder TBS/T alone.
4. Membranes are washed in 5% w/v milk powder TBS/T for 30 min and incubated for 45 min at room temperature with a horseradish peroxidase (HRP)-conjugated F (ab')₂ rabbit anti-rat IgG polyclonal antibody (1/1000, AbD Serotec Ltd., Kidlington, UK; catalog number STAR21B).
5. Membranes are washed three times for 10 min each in 5% w/v milk powder TBS/T and finally for 10 min in TBS/T.
6. Enhanced chemiluminescence (ECL™) detection reagents 1 and 2 (Amersham Biosciences, catalog number RPN 2106) are mixed in equal parts and poured onto the membrane for 1 min with agitation.
7. Excess liquid is removed and the membrane is placed on Saran Wrap in a photographic X-ray film cassette.
8. The membrane is fixed in place, air bubbles are removed with a roller and the film cassette is closed.
9. Autoradiography film is exposed to the gel for an appropriate amount of time (typically 1 min), after which the film is developed, washed in H₂O, fixed, washed

again and allowed to dry. The positions of the molecular weight markers are annotated onto the developed film. Gels can also be visualized directly using an appropriate gel documentation and analysis system (e.g. ChemiGenius², Syngene, Cambridge, UK).

This purification approach reproducibly yields approximately 30-60 µg gp96 per gram wet weight of liver tissue with an endotoxin content which is routinely less than 0.001 EU (0.1 pg per µg gp96 [64]. Analysis of gp96 preparations using 2-D gel electrophoresis and liquid chromatography electrospray ionization tandem mass spectrometry reveals that the procedure for purifying gp96 is reproducible, as similar protein profiles are observed in replicate gels of gp96 preparations [64]. The purity of the preparations is typically around 70%, with minor co-purified proteins of varying molecular weights and mobilities being present [64].

2.2. FITC-labeling of gp96 and bovine serum albumin (BSA)

Gp96 and BSA can be labeled with FITC using the FluoReporter® FITC Protein Labeling Kit (Molecular Probes via Invitrogen Ltd., Paisley, UK). Purified protein should be at a concentration of 1-10 mg/ml in a buffer which does not contain any ammonium ions or primary amines (e.g. Tris or glycine). Proteins in unsuitable buffers should be exchanged into PBS by dialysis or centrifugation in appropriate spin columns. Proteins can also be labeled using the FluoroTag™ FITC protein conjugation kit (Sigma-Aldrich Company Ltd.).

2.2.1. FITC-labeling using the FluoReporter® FITC Protein Labeling Kit

1. Transfer 200 µl of protein (1 mg) to a 2 ml reaction tube containing a stir bar on a magnetic stirrer.
2. To each tube, add 20 µl 1 M NaHCO₃ buffer, pH 9.0 (0.84 g NaHCO₃ in ~9 ml distilled H₂O. Adjust pH to 9.0 with NaOH and make up the volume to 10 ml with distilled H₂O) with 5 µl of reactive FITC dye in dimethyl sulphoxide (DMSO), after which wrap tubes in aluminum foil (to protect them from light) and stir the reaction mixture for 1 h at room temperature. The amount of reactive dye to be used for each labeling reaction depends on the concentration of protein to be used. For the *FluoReporter*® approach, the volume of the stock reactive dye solution (10 mg/ml) to be added is calculated thus:

$$\text{Volume of dye stock solution}(\mu\text{l}) = \frac{\text{mg} / \text{ml protein} \times 0.2 \text{ ml}}{\text{MW}_{\text{protein}}} \times 389 \times 100 \times \text{MR}$$

in which 0.2 ml is the volume of protein, 389 is the molecular weight (MW) of the reactive dye and 100 is a unit conversion factor. $\text{MW}_{\text{protein}}$ is the molecular weight of the protein to be labeled and MR is the molar ratio of dye to protein in the reaction mixture.

3. Two spin columns from the FITC labeling kit are centrifuged at 1100g for 3 min at 20°C to remove excess buffer.
4. Remove the contents for the conjugation reaction from the mixing tube and carefully add to the spin column drop-wise until it is completely absorbed by the size exclusion resin.
5. Place collection tubes below the spin columns and centrifuge the unit at 1100g for 5 min at 20°C.
6. Discard the spin columns and collect the eluates containing gp96-FITC or BSA-FITC. Labeled proteins are diluted in PBS.

7. Successful labeling can be confirmed by observing an approximate 2 kDa shift in the molecular weight of the proteins following SDS polyacrylamide gel electrophoresis and silver stain analysis (Fig. 1).

2.3. Generation of rat bone marrow-derived dendritic cells (BMDCs)

Immature rat BMDCs can be generated as described below [60].

1. Bone marrow is flushed from the tibias and femurs of rats using a 25-gauge needle.
2. Bone marrow cells are washed twice in RPMI 1640 medium supplemented with 10% v/v fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin (designated RPMI*, Invitrogen Ltd.).
3. Cells (3×10^6 cells, 5 ml) are cultured in T25 Nunc™ tissue culture flasks (Nalge Europe Ltd., Hereford, UK) with recombinant rat GM-CSF (100 ng/ml, 1000 U/ml; Invitrogen Ltd.; PeproTech EC Ltd., London, U.K.) with recombinant rat IL-4 (5 ng/ml, 33 U/ml; PeproTech EC Ltd.).
4. On day 3, 5 ml RPMI* containing the appropriate cytokines is added and on day 7, non-adherent cells are removed and adherent cells are harvested by gentle scraping. Scraping is used, as this better maintains viability and results in the highest cell yield. Viable cell counts are determined using trypan blue (Sigma-Aldrich Company Ltd.) and a modified Neubauer counting chamber.

This approach induces the differentiation of large numbers of a homogeneous population of adherent cells (typically 1.2×10^6 adherent cells/flask), all of which exhibit the morphological appearance of DCs and 90% of which stain positive with the OX62 mAb [59]. The purity of each population can be confirmed by multicolor flow cytometry thus:

1. Surface antigen expression by BMDCs can be determined by 2-color flow cytometry using a PE-conjugated, murine mAb specific for the rat DC lineage marker OX62, in combination with FITC-conjugated mAbs specific for rat MHC class II, CD11b, CD54 (ICAM-1), CD80 (B7.1), CD86 (B7.2), CD68 (clone ED1) and CD3, all of which can be obtained from AbD Serotec Ltd. OX62 recognizes the integrin α_{E2} subunit (150 kDa) and was originally described as being expressed on most, if not all, DCs in the rat [65]. Subsequent data indicate that 80% of spleen DCs express the epitope recognized by OX62, whereas only 50% of lymph node DCs [66] and Flt-3 ligand differentiated, loosely- and non-adherent BMDCs [67] do. Plasmacytoid DCs are OX62 negative [68]. An anti-CD40 mAb is available from BD Biosciences (Oxford, UK).
2. For staining, harvested BMDCs are incubated for 30 min at 4°C with 10 µl FITC- and PE-conjugated mAb, or appropriate isotype-matched, non-reactive negative control murine immunoglobulin (AbD Serotec Ltd.) in 12×75 mm polycarbonate tubes (Falcon, BD Biosciences). Cells are washed twice in PBS containing 1% w/v BSA (Sigma-Aldrich Company Ltd.) and the cell pellet is re-suspended in the residual volume (~100 µl). Cells are analyzed immediately, or can be fixed by the addition of CELLFix™ (100 µl/tube, BD Biosciences) and analyzed the following day. The volume of mAb to be used should be optimized in preliminary experiments.

2.4. Assessment of protein binding and uptake by flow cytometry

Binding of FITC-labeled gp96 and BSA (as control) to the surface of BMDCs (receptor-mediated binding) and their uptake (non-specific endocytosis/pinocytosis) is determined by two-color flow cytometry. For the determination of specific, receptor-mediated interactions all instruments, solutions and incubations are performed at 4°C. For the determination of receptor-

mediated endocytosis and/or non-specific uptake via pinocytosis, all instruments and solutions are maintained at room temperature and incubations are performed at 37°C [56].

1. Harvested BMDCs are washed with PBS containing 2% v/v mouse serum (4°C/room temperature as appropriate), counted, re-suspended at 1×10^6 /ml and incubated for 30 min with an appropriate concentration of gp96-FITC or BSA-FITC (e.g. 50, 100 µg/ml).
2. Cells are washed four times with PBS containing 1% w/v BSA, and gp96-FITC or BSA-FITC binding is immediately determined by flow cytometry. Cells can also be co-stained with PE-conjugated phenotypic markers such as the OX62 mAb. Propidium iodide (PI) is also added to cells immediately prior to flow cytometry, and PI⁺ (non-viable cells) are excluded from the analysis. It is important to ensure that instrument compensation is adjusted in order to exclude spectral overlap in the FL1 (FITC) and FL2/FL3 (PI) channels.

As we have reported previously, no fluorescent staining of this population of immature rat BMDCs [59,60] is observed when incubations are performed at 4°C, whereas cells do become positive when incubations are performed at 37°C (Fig. 2). These findings confirm that stress proteins can be internalized by DCs via a non receptor-mediated mechanism.

3. Conclusion

To date, the possibility that stress proteins can be internalized via non receptor-mediated interactions has received little attention, despite the fact that it is well known that immature DCs can take up antigens via pinocytosis in clathrin-coated pits and by fluid-phase pinocytosis [57], and that antigenic peptides derived from pinocytosed exogenous material can be presented to T cells by DCs (reviewed in [58]). It is also known that TLRs, for which stress proteins such as Hsp60, Hsp70 and gp96 have been identified as ligands [40,42,46,48,49,69] are located inside cells and can be activated intracellularly [70]. This alternative route for entering DCs might explain why gp96 is able to influence the morphology of BMDCs in the absence of any apparent evidence that it binds to specific cell surface receptors [56]. Non receptor-mediated uptake of stress proteins might have implications for the immunoregulatory properties of these molecules and should therefore be considered in greater detail.

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Fig. 1. Silver stained gels of electrophoresed unlabeled and FITC-labeled gp96 preparations (A), and unlabeled and FITC-labeled BSA preparations (B).

Fig. 2. Flow cytometric fluorescent histograms of rat BMDCs alone, and rat BMDCs following incubation with FITC-labeled BSA and FITC-labeled gp96 at 4°C (upper panel) and 37°C (lower panel). Histograms are representative of 2 independent experiments.