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MINI REVIEW

Distinguishing integral and receptor-bound heat shock protein 70 (Hsp70) on the cell surface by Hsp70-specific antibodies

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Abstract *Cell Stress & Chaperones* journal has become a major outlet for papers and review articles about anti-heat shock protein (HSP) antibodies. In the last decade, it became evident that apart from their intracellular localization, members of the heat shock protein 90 (Hsp90; HSPC) and Hsp70 (HSPA) family are also found on the cell surface. In this review, we will focus on Hsp70 (HSPA1A), the major stress-inducible member of the human Hsp70 family. Depending on the cell type, the membrane association of Hsp70 comes in two forms. In tumor cells, Hsp70 appears to be integrated within the plasma membrane, whereas in non-malignantly transformed (herein termed normal) cells, Hsp70 is associated with cell surface receptors. This observation raises the question whether or not these two surface forms of Hsp70 in tumor and normal cells can be distinguished using Hsp70 specific antibodies. Presently a number of Hsp70 specific antibodies are commercially available. These antibodies were generated by immunizing mice either with recombinant or HeLa-derived human Hsp70 protein, parts of the Hsp70 protein, or with synthetic peptides. This review aims to characterize the binding of different anti-human Hsp70 antibodies and their capacity to distinguish between integrated and receptor-bound Hsp70 in tumor and normal cells.

Keywords Cell surface Hsp70 · Hsp70 antibody epitope · Integrated Hsp70 · Receptor-bound Hsp70

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Introduction

Under physiological conditions, heat shock proteins (HSPs), which are classified according to their molecular weights, play a pivotal role in the maintenance of protein homeostasis. As intracellular molecular chaperones with newly defined moonlighting functions (Cehovin et al. 2010), they support protein folding of nascent polypeptides and transport of proteins across membranes, and they are involved in the regulation of immune responses including antigen processing and cross-presentation. Following environmental stress, their synthesis is dramatically upregulated in order to protect cells from lethal damage and to avoid the propagation of the insult (De Maio 1999; Lindquist and Craig 1988). Hsp70 (Swiss Prot accession #3303; old nomenclature, Hsp72, Hsp70i, Hsp70-1, HSPA), newly termed as HSPA1A (Kampinga et al. 2009), is among the first and the most prominent proteins which are found in stressed cells.

Compared to normal cells, tumors frequently have elevated basal Hsp70 levels which are further enhanced in response to a number of pathological and environmental stresses such as nutrient deficiency, hypoxia, heavy metals, irradiation, and/or chemotherapeutic agents. Also normal

cells show an increase in the synthesis of Hsp70 following stress in order to mediate protection against lethal damage and to maintain protein homeostasis.

Apart from their intracellular localization, Hightower and Guidon reported about an ER–Golgi-independent release of Hsp70 from viable cells with intact cell membranes already in 1989 (Hightower and Guidon 1989). Extracellular HSPs are considered as molecules with immunomodulatory functions (Pockley and Multhoff 2008; Pockley et al. 2008) either as cross-presenters of immunogenic peptides (Srivastava 1997; Asea et al. 2000) or in a peptide-free version as chaperokines (Asea et al. 2002) or stimulators of innate immune responses (Multhoff et al. 1995). Despite these well-documented immunological functions, the mechanisms how HSPs are exported from cells are still controversial since cytosolic HSPs lack a consensus signal for secretion. However, apart from Hsp70, other molecules lacking a secretory signal such as IL-1alpha, IL-1beta, and high mobility group box 1 (HMBG-1; Eder 2008; Nickel and Seedorf 2008) are also found outside of cells. Hsp70 also has been found to be located on the cell surface although it lacks a transmembrane domain (Multhoff and Hightower 1996). Membrane Hsp70 might help to maintain stability of tumor cells and thus might protect tumors from lethal damage induced by environmental stress (Horvath and Vigh 2010; Horvath et al. 2008). The pioneering work of the group of Antonio De Maio has demonstrated an interaction of members of the Hsp70 family with artificial membranes containing phosphatidylserine (PS; Arispe and De Maio 2000; Arispe et al. 2002; Vega et al. 2008; De 2010). My group reported on an interaction of Hsp70 with the sphingolipid globoyltriaosylceramide (Gb3) in the plasma membrane of non-stressed human GIST tumors (Gehrmann et al. 2008a). Gb3 is found in cholesterol-rich microdomains, also termed as lipid rafts, which serve as signal transduction platforms. Following irradiation or hypoxia-induced stress Hsp70 was found to be associated predominantly with PS outside of lipid rafts in the plasma membrane of tumor cells (Schilling et al. 2009). These data indicate that environmental stress might result in a re-organization of the lipid bilayer and might modulate the interaction of Hsp70 with lipid components. Surprisingly, only tumors but not the corresponding normal tissues were found to be membrane Hsp70 positive when the staining was performed using the IgG1 mouse monoclonal antibody (mAb) cmHsp70.1. In contrast, other Hsp70-specific antibodies failed to bind to membrane Hsp70 on viable tumor cells (Stangl et al. 2010, accepted for publication). The discovery that neither high/low salt concentrations nor changes in the pH were able release Hsp70 from the plasma membrane of tumor cells (Vega et al. 2008; Gehrmann et al. 2008a) confirmed our hypothesis that in tumor cells Hsp70 is an integral membrane protein which can associate with raft (Gb3) and non-raft (PS) lipid components.

Cell surface expression of Hsp70 in tumor and normal cells—is this contradictory?

The cmHsp70.1 mAb (multimmune GmbH, Munich, Germany), which selectively binds to membrane Hsp70 on tumor cells was generated by immunization of mice with the 14-mer peptide TKDNNLLGRFELSG, termed "TKD", comprising amino acids 450–461 ($aa_{450–461}$) in the C terminus of the inducible Hsp70. Since the human and murine "TKD" sequences only differ in one amino acid (Zhang et al. 2007; human T**K**DNNLLGRFELSG; mouse T**R**DNNLLGRFELSG), the cmHsp70.1 mAb shows a cross-reactivity for human and mouse tumors. Screening of nearly 1,000 primary human tumor biopsies and the corresponding normal tissues has indicated that human carcinomas, but none of the tested normal tissues, frequently present Hsp70 on their cell surface (Multhoff et al. 1995; Multhoff 2007). A membrane Hsp70-positive tumor phenotype has been found to be associated with a significantly decreased overall survival in tumor patients. Therefore, the expression of this molecule could serve as a negative prognostic marker (Pfister et al. 2007). The "TKD" sequence which is exposed to the extracellular milieu of tumors resides in the C-terminally localized oligomerization domain which is part of the substrate binding domain of the Hsp70 molecule (Fouchaq et al. 1999). In contrast to other commercially available Hsp70 antibodies, the cmHsp70.1 mAb uniquely identifies the membrane form of Hsp70 on viable human and mouse tumor cells not only in vitro but also in vivo in tumor-bearing mice (Stangl et al. 2010).

Furthermore, therapeutic intervention such as radiochemotherapy enhances the cell surface density of Hsp70 selectively on tumors (Gehrmann et al. 2005, 2008b; Kleinjung et al. 2003; Farkas et al. 2003), and thus, integral membrane Hsp70 provides a tumor-specific recognition structure for therapeutic interventions. In contrast to cmHsp70.1 mAb, other commercially available mouse antibodies directed against Hsp70 fail to stain the surface of viable tumor cells, although they could be used for the detection of Hsp70 by Western blot and immunohistological analysis. A list of Hsp70-specific antibodies, their cross-reactivity, immunogen, specificity, and epitope is summarized in Table 1. Apart from Hsp70, a global profiling of cell surface-bound proteins revealed an abundance of other stress proteins on the plasma membrane of tumors (Ferrarini et al. 1992; Shin et al. 2003). However, no antibodies are available at present to determine their membrane expression by flow cytometry.

Viable membrane Hsp70-positive tumors have been found to actively release lipid vesicles that present Hsp70 on their surface like the tumors from which they were derived (Gastpar et al. 2005; Bausero et al. 2004; Lancaster and Febbraio 2005). An ATP binding cassette-dependent

Table 1 List of commercially available mouse monoclonal (mAb) directed against human Hsp70 indicating the immunogen, specificity, epitope, and cross-reactivity

Name/isotype (company)	Immunogen	Specificity	Epitope	Cross-reactivity
cmHsp70.1/IgG1 (multimmune)	Human Hsp70 peptide aa 450–461	Hsp70 Integral membrane	aa 450–461	Mouse
C92F3A-5/IgG1 (Stressgen)	Human Hsp70 (HeLa)	Hsp70	aa 436–503	Broad
SPM254/(Abcam)	Human Hsp70 (HeLa)	Hsp70, Hsc70	aa 383–447	Broad
W27/IgG2a (Acris)	Human Hsp70 (HeLa)	Hsp70, Hsc70	?	Broad
5A5/IgG (Abcam)	Human Hsp70 ATPase domain (aa 122–246)	Hsp70, Hsc70, Grp78	aa 122–264	Broad
2A4/IgM (Abcam)	Human recombinant Hsp70	Hsp70, Hsc70, Grp78	aa 437–479	Broad
N27F3–4/(Abcam)	Human recombinant Hsp70/Hsc70	Hsp70, Hsc70	?	Broad

lysosomal/endosomal pathway has been discussed for the export of lipid vesicles (Nylandsted et al. 2004; Mambula and Calderwood 2006). Immunostimulatory capacities are well established for these exocytosed vesicles that carry a number of immunostimulatory molecules such as major histocompatibility complex (MHC) class I, adhesion molecules, and HMBG1 (Liu et al. 2006). Apart from this vesicular export of Hsp70 from viable tumor cells, another source of extracellular HSPs could be cell lysis (Basu et al. 2000). The presence of Hsp70– and Hsp90–peptide complexes in the circulation is most likely due to the secretion by necrotic tissues.

The group of Calderwood convincingly demonstrated that extracellular Hsp70 can exert immunological effects in a receptor-mediated manner (Murshid et al. 2010; Calderwood et al. 2007; Theriault et al. 2005). Type F scavenger receptors such as scavenger receptor expressed by endothelial cells type 1 (SREC-1)/SCARF-1 could be identified on antigen-presenting cells such as dendritic cells, as potential binding partners for Hsp70– and Hsp90–peptide complexes. Most recently, the GPI-AP-enriched early endosomal compartment was described to mediate internalization of HSP–peptide complexes to recycling endosomes. Peptides with the proper length (8–10 aa) could be loaded directly onto MHC class I in endosomes. Cross-presentation of HSP-chaperoned peptides through a c-Src kinase-dependent pathway to CD8+ T lymphocytes was found to be more efficient than that of chaperone-free peptides.

Since Hsp70–peptide complexes are bound from outside to antigen-presenting cells via cell surface receptors, the Hsp70 protein is accessible for almost any Hsp70-specific antibody (Fig. 1). It is most likely that any commercially available antibody would be able to detect receptor-bound Hsp70 on the cell surface of antigen-presenting cells, whereas in case of the integrated Hsp70 in the plasma membrane of tumor cells, only a minor part of the C-terminal substrate domain ($aa_{450–461}$) Hsp70 molecule is accessible for antibodies from outside (Fig. 1). At present, only cmHsp70.1 mAb, which was generated using this peptide sequence as an immunogen, is able to detect the integrated membrane form of Hsp70 on viable tumor cells (Table 1).

In summary, these observations indicate that a comparative immunofluorescence staining using different Hsp70 antibodies are needed to distinguish between integral and receptor-bound Hsp70. The cmHsp70.1 mAb qualifies for the detection of integral membrane Hsp70, whereas other Hsp70 antibodies listed in Table 1 rather detect receptor-bound Hsp70. A comparative staining of surface Hsp70 by cmHsp70.1 and by other Hsp70-specific antibody might shed light in this issue.

Commercial ELISA kits made by Enzo Life Sciences that detect Hsp70 are most often used by authors of papers that appear in *Cell Stress & Chaperones*. Our assessment is that there are likely to be three kits in existence. The oldest is Stressgen kit EKS-700 that contains an anti-Hsp70A1A antibody for detection that reproducibly recognizes human, native extracellular Hsp70 (eHsp70) in plasma and serum. The behavior of this antibody suggests that it recognizes an epitope shared by intracellular and eHsp70. Starting in 2005, when Assay Designs and Stressgen merged, a new detection antibody was produced, tested, and packaged as kit EKS-700B. Both kits recognized similar levels of Hsp70A1A in cell lysates, but the new detection antibody

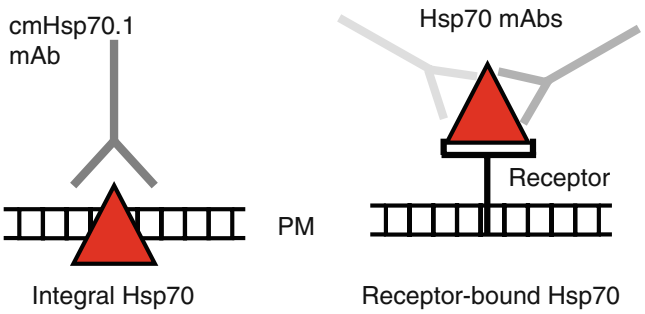

Fig. 1 Schematic representations of integral and receptor-bound Hsp70 in the membranes of tumor and normal cells. The *red triangle* represents the positioning of Hsp70 in the lipid bilayer in the case of tumor cells and on a cell surface receptor such as SREC-1 on antigen-presenting cells

did not reproducibly recognize eHsp70. Therefore, according to their products manager, Assay Designs certified kit EKS-700B only for cell lysates. Subsequently, Assay Designs produced a new anti-Hsp70 antibody that detects reproducibly levels of eHsp70. This detection antibody is packaged in kit ADI-EKS-715 (Enzo Life Sciences), and it is now the certified kit for detection of eHsp70 in plasma and serum of human, mouse, or rat origin (90 pg/ml sensitivity). ADI-EKS-700B kit is certified for use in detection of Hsp70 in cell lysates and tissue of human, mouse, or rat origin (200 pg/ml sensitivity).

An immediate problem that sometimes arises when manuscripts are submitted for review containing measurement data for eHsp70 is failure to identify the ELISA kit used. With the changes in the Hsp70 kits that have occurred, it is necessary to have the kit number and the date purchased to determine if the appropriate kit was used. Also, it is essential to know the source of the samples used as a source of Hsp70 and how they were prepared. Among the Enzo kits, we recommend to authors to use only kit EKS-715 for measurement of eHsp.

Concluding remarks

Viable human tumors, but not the corresponding normal tissues, frequently present Hsp70 on their plasma membranes which can be specifically detected using the cmHsp70.1 mAb. Other commercially available Hsp70-specific antibodies fail to recognize the integral membrane form of Hsp70 on viable tumor cells.

In the case of antigen-presenting cells, Hsp70–peptide complexes can be bound from outside to scavenger receptors and thus become endocytosed and loaded onto MHC class I molecules for an effective cross-presentation to CD8+ lymphocytes (Murshid et al. 2010). Receptor-bound Hsp70 can be recognized by all listed Hsp70-specific antibodies.

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