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Review

Evolution of heat shock protein and immunity

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Abstract

Heat shock proteins (hsps) are among the most abundant intracellular proteins. Their synthesis is rapidly up-regulated by various 'stressors' including temperature, glucose deprivation, infection and cancer. Certain hsps are able to: (i) associate and chaperone a large variety of cellular peptides; (ii) be efficiently internalized by antigen presenting cells (APC) through receptor-mediated endocytosis; (iii) channel antigenic peptides they chaperone in the APC's MHC class I presentation pathway; (iv) and stimulate inflammatory cytokines, chemokines and co-stimulatory molecules through the NFκb signaling pathway. Extracellular release of hsps upon necrotic cell death and their modulated access at the surface of some cells, can be considered as a putative 'danger' signal. Based on the ancient origins and structural conservation of hsps, it has been proposed that, the role of hsps in immunity emerged early in evolution and to be widespread in extant organisms. Data from studies with the frog *Xenopus* support this proposition.

Keywords: *Xenopus*; Hsp70; Gp96; Innate immunity; Molecular chaperone; Immune surveillance; Evolution

1. Introduction

Heat shock proteins (hsps) are evolutionarily ancient and highly conserved intracellular molecular chaperones constituting several multigenic super-families. Hsps are present in all the different subcellular compartments (e.g. nucleus, mitochondria, chloroplast, endoplasmic reticulum, cytosol) of all cell types from prokaryotes and eukaryotes. The initial nomenclature for hsps was based on their apparent molecular weight (i.e. hsp84, 85, 86, etc.) and they were grouped according to their nearest size (e.g. the hsp90 kD family). The availability of nucleotide and deduced amino acid sequences now allows a more systematic classification with a phylogenetic basis. Members in a family exhibit a high degree of sequence identity (even between prokaryote and eukaryote members), whereas there is no homology between families.

Hsps are defined as molecular chaperones that non-convalently bind exposed hydrophobic surfaces of non-native proteins [1]. Although most hsps are constitutively expressed, their expression is up-regulated by various physiological perturbations or stressors (e.g. elevated temperature, hypoxia, ischemia, heavy metals, radiation, calcium increase, glucose deprivation, cancer, and microbial infection). Hsps perform essential biological functions under both physiological and stressful conditions. General

Abbreviations: MHC, major histocompatibility complex; mAb, monoclonal antibody; TCR, T-cell receptor.

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functions attributed to hsps include: (i) preventing protein aggregates under physical stress; (ii) serving as molecular chaperones in protein transport between cell organelles; and (iii) contributing to the folding of nascent and altered proteins [1–3]. Many other more specific functions have been characterized for particular hsp types including a role in immunological processes, the subject of this review.

An increasing body of data suggests that certain hsps play a role in both innate and adaptive immunity [4–7]. Hsps can elicit potent specific cellular adaptive immune responses (e.g. $CD8^+$ cytotoxic T-cell effectors or classic CTLs) based on their ability to chaperone antigenic peptides [4,5]. By mechanisms that are less well understood, hsps can also act independent of chaperoned peptides to directly stimulate innate immune responses [8–10]. In fact, it has been suggested [11] that some hsps may have become specialized as a response modality to 'stress' associated with infection and cancer. Given the ancient origin of hsps, such specialization may have occurred early in evolution and, therefore, may be common to a wide range of extant invertebrate and vertebrate species. However, whereas the interaction of hsps with immune system has become increasingly well studied in mammals, little is known about the phylogeny of hsp in immunity.

This review will focus mainly on two members of the hsp70 family (hsp70 and hsc70) and a member of the hsp90 family, gp96, their involvement in innate and adaptive immunity is well documented. Three major facets of hsp-immune system interactions will be considered with respect to the evolution of immunity: (i) the capacity of hsps to elicit T-cell response specific against antigenic peptide they chaperone; (ii) the ability of hsp to modulate innate response that are independent of chaperoned peptides; and (iii) hsp surface expression. Other immunological aspects of hsp such as their role in autoimmunity and cross-reactive antibody responses have been reviewed elsewhere [12,13] and will not be considered here.

2. Structure and characterization of members of the hsp70 and hsp90 families

Both hsp70 and hsp90 families include members located in different cellular compartments (Fig. 1). In

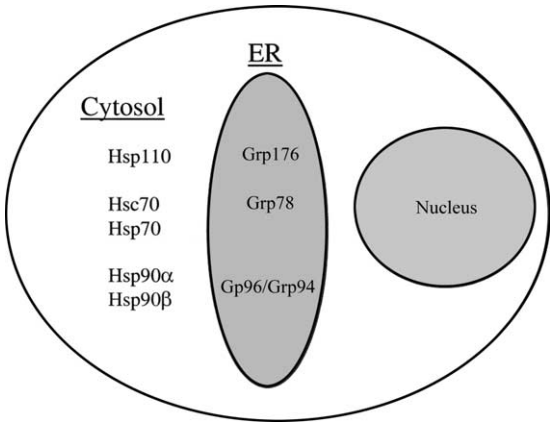

Fig. 1. Intracellular localization of the different hsp70, hsp70-like and hsp90 family members in eukaryotic cells.

vertebrates, two different hsp70 members are expressed in the cytosol. The cognate HSC-70 is constitutively expressed at low levels and is only slightly inducible, whereas HSP-70 is not expressed in most tissues and is highly stress-inducible [2,3]. I will use hsp70 as a nominal term for both the inducible and constitutive cytoplasmic forms. There is a 60% amino acid sequence identity between eukaryotic hsp70s, and a 40% identity between eukaryotic and the *E. coli* hsp70-equivalent DnaK. Hsp70 binds ATP [14,15] and the ATPase domain resides in a 44 kD N-terminus fragment (Fig. 2). An 18 kD fragment containing four-stranded anti-parallel β-sheets and a single α-helix constitutes the peptide-binding domain that can bind unfolded and folded peptides [16].

Several HSP-70 genes are located in the class III regions of the human, mouse, and frog (*Xenopus*) MHC [17–20]. Hsp70 in the cytosol interacts with various factors including other hsps (hsp90, hsp40) and regulatory components such as Hip and Hop that act as adaptors between hsp70 and hsp90 [1]. Additional hsp70 family members include a mitochondrial form, hsp75 and a glucose-regulated protein member located in the ER known as grp78 or Bip [2,3]. Based on sequence homology, two hsp70-like proteins have been characterized: the cytosolic hsp110 and the ER-resident, glucose-regulated grp170 [21].

There are also two cytosolic hsp90 members in vertebrates (Fig. 1), hsp90α and hsp90β that are likely

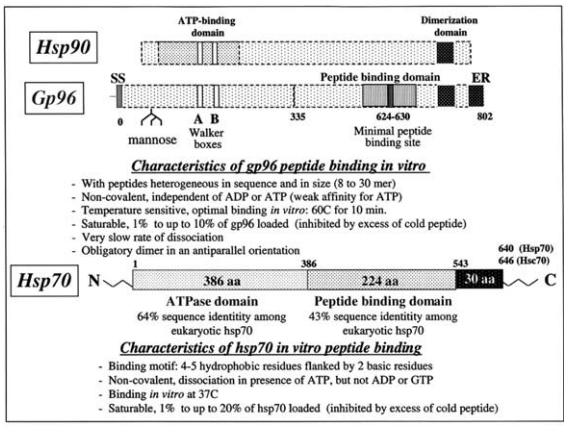

Fig. 2. Scheme of the main structural features and peptide-binding characteristics of hsp90 and hsp70 members. Presumed ATP-binding sites with conserved Walker boxes (A,B) and the dimerization domain of aligned hsp90 and gp96 are indicated. Gp96 specific features include: N-terminal signal sequence (SS), C-terminal endoplasmic reticulum retention signal (ER), the high-mannose oligosaccharide moiety of Asn-196 residue and the recently mapped minimal peptide-binding site with the surrounding 200 amino acid defining an hydrophobic pockets [65]. Residue numbers are from mouse gp96. The general structure of Hsp70 and HSC70 adapted from Ref. [3] include a N-terminal 44 kD ATPase domain, a 18 kD peptide-binding domain and a 10 kD C-terminal domain carrying the EEVD highly conserved terminal sequence present in all eukaryotic hsp70. Residue numbers are from human hsp70.

the result of a gene duplication event coincident with the emergence of vertebrates [22–24]. The other hsp90 member, gp96, resides in the lumen of the ER (mainly in the rough ER; [25]) and is the most abundant ER protein [26]. The primary structure of gp96 is very similar to its hsp90 cytoplasmic counterpart (50% amino acid sequence homology). Unlike hsp90, however, gp96 contains an N-terminal signal sequence (70 amino acid), characteristic of the ER-targeted proteins, and a carboxy terminal KDEL sequence (Lys-Asp-Glu-Leu), which is a retention/retrieval signal from the golgi to the ER [26]. Global alignment of all available hsp90 sequences and a phylogenetic study of the different homologues clearly indicate that cytosolic hsp90 and ER-resident gp96 comprise a paralogous gene family [24]. Hsp90 and gp96 homologues have been identified in virtually all metazoan taxa (Fig. 3) including slime molds (*Dictyostelium discoideum*; [27]), plants [24,28], invertebrates (*C. elegans*. [6]; *S. purpuratus*, Robert unpublished), and vertebrates. A prokaryotic hsp90 homologue, called high temperature protein G (HtpG), displays 40% amino acid sequence identity with eukaryotic hsp90 members [24,29]. With respect to unicellular eukaryotes, however, gp96 homologues have thus far been found only in *Leishmania major* [30], and an extensive search of the yeast *Saccharomyces cervisia* database indicates the absence of gp96 in this species [6]. The phylogenetic tree of available gp96 sequences gives an idea of the extent of the structural conservation of this molecule (Fig. 2). The gene duplication giving rise to hsp90 and gp96 paralogues, therefore, is contemporary to the emergence of eukaryotes.

Like the hsp90 and its prokaryotic counterpart HtpG, gp96 is a phosphorylated anti-parallel rod-like homodimer [31]. The degree of phosphorylation varies with the cell type [32,33]. Dimerization is promoted by hydrophobic interactions, but it can be further stabilized under oxidizing conditions by a disulfide-bridge between Cysteine 117 of the two monomers [34,35]. Furthermore, gp96 forms oligomers, and associates with numerous other proteins, including different protein kinases [1], calreticulin, calnexin [36], grp78/Bip [37], and grp170 [38]. Recently, an intriguing physical association of g96 with Toll-like receptors (TLR) in a murine B-cell line has been reported [11]. Gp96 is also a calcium-binding protein [39], and unlike hsp90, a glycoprotein. Under normal condition, it is N-glycosylated at Asn-196 with a high-mannose oligosacharide moiety [35]. The gp96 dimerization domain has been mapped in the C-terminal region (Fig. 3), and its deletion abrogates dimerization [31].

With regard to regulation of its expression, synthesis of gp96, like other glucose-regulated ER proteins (grp78, grp170), is rapidly up-regulated under glucose deprivation [1]. For example, culture of the *Xenopus* A6 fibroblast cell line in medium in which glucose has been replaced by a deoxyglucose analogue, results in an induced and transiently enhanced synthesis of grp78 and gp96 protein with a maximal level occurring by 12–24 h of culture [40]. Enhanced synthesis also occurs in response to hypoxia and reduction. Interestingly, in mice and humans, γ-interferon (IFN-γ)-responsive elements are present in the gp96 gene promoter, and IFN-γ-induced up-regulation of gp96 transcription has been shown [41].

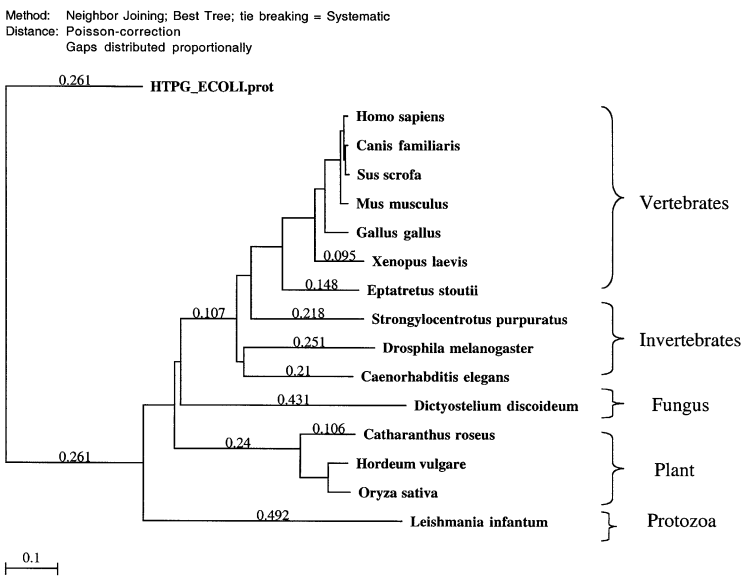

Fig. 3. Phylogenetic analysis of available complete deduced amino acid sequence of gp96 homologues. Deduced amino acid sequences were aligned using CLUSTALW algorithm. The relative sequence divergence between gp96 homologues was analyzed by the neighbor joining matrix method with Poisson-correction (MacVector software, Oxford Molecular, WI). The tree was rooted with the prokaryotic hsp90 homologue HtpG. The accession number of the sequence analyzed are: *H. sapiens*, P14625 (SwissProt); *C. familiaris*, P41148 (SwissProt); *S. scrofa*, Q29092 (GenBank); *M. musculus*, PO8113 (SwissProt); *G. gallus*, P08110 (SwissProt); *X. laevis*, (xxx); *E. stoutii*, (xxx); *S. purpuratus*, (xxx); *C. elelgans*, Z69751 (GenBank); *D. discoidium*, AB040814 (GenBank); *C. roseus*, L14594 (GenBank); *H. vulgare*, S31862 (GenBank); *O. sativa*, AB037681 (GenBank); *L. infantum*, AF253053 (GenBank); *E. coli* tpG, P10413 (SwissProt); *X. laevis* (AY187545); *E. stoutii* (AY187546); S.purpuratus (AY187547).

Schematically, the immunogenicity of hsps results from two different properties: (i) a peptide-dependent capacity to chaperone and elicit adaptive CTL responses against antigenic peptides, and (ii) a peptide-independent immunomodulatory capacity. Each of these properties will now be considered in detail.

3. Peptide-dependent immunogenicity of hsps

Thanks in large part to the pioneering work of Srivastava and his colleagues [4,5,42,43] as well as to recent data from other labs [6,7], it is now well established that hsps can elicit a potent specific cellular adaptive immune response that depends on its ability to chaperone a large variety of peptides through the cellular milieu.

Immunization of mice with tumor-derived hsp generates protective immunity against a subsequent tumor challenge. For example, immunization of BALB/c mice by subcutaneous injections of as little as 10 μg gp96 purified from 'syngeneic' methylcholanthrene-induced sarcomas (Meth-A tumors) specifically protects animals from subsequent challenges with live Meth-A tumor cells. Other hsp family members like hsp70 and hsp90 from mouse tumors [44] have been reported to elicit immune protection similar to that reported for gp96. The potency of anti-tumor response elicited by hsps has been extended to a therapeutic model in which regression of existing tumors can be seen following injection of tumor-derived hsps [8]. Generation of specific anti-tumor response by hsps is not restricted to mice since preliminary phase I and II clinical immunotherapy trials appear promising [45,46]. Germane to this review is the fact that we have demonstrated, by using a model of transplantable tumors in the frog *Xenopus*, that anti-tumor immunity elicited by gp96 and hsp70 is evolutionarily conserved [47,48]. Furthermore, in mice, hsps generates protective immunity against influenza virus [49] as well as *Mycobacterium*

tuberculosis and *Listeria monocytogenes* [50]. Hsp-mediated anti-tumor murine immune responses in vivo critically involves cytotoxic CD8 T-cell effectors, and is abrogated when CD8 lymphocytes are depleted either during the priming or the effector phase [51]. In vivo depletion studies also suggest an important involvement of macrophages during the priming phase [51].

3.1. Peptide binding

In humans, mice, and frogs, hsp-mediated T-cell responses depend on, and are specific for, the chaperoned peptide. The ability of gp96 and hsp70 to bind peptides was first postulated, and then demonstrated, by Srivasatava [4,5]. The initial indirect evidence showed that protection against a particular tumor could be obtained by immunization with hsp derived from this tumor but not from another tumor type or from normal tissue [44]. More direct evidence revealed that peptide depletion abolishes the tumor-protective capacity of hsp70 [52]. A large variety of peptides, heterogeneous both in size (4–40 mer) and in sequence, can be eluted from gp96 by acid treatment [53–57] and from hsp70 in presence of ATP [52,57]. In a few cases, specific antigenic MHC class I-restricted epitopes such as vesicular somatitis virus [54,58] and ovalbumin, [57,59], have been purified from, and identified in, the peptide pool eluted from gp96, hsp90 and hsp70 from VSV-transformed or ovalbumin-transfected stable cell lines.

Synthetic peptides can be complexed to hsp in vitro [60] by moderate heat denaturation for gp96 and ATP treatment for hsp70. The extent of reconstitution usually ranges between 1 and 10% of hsp molecules loaded with exogenous peptides; but up to 20% was obtained with hsp70 using an optimized peptide containing four hydrophobic amino acids flanked by two regions enriched in basic residues [61,62]. There is still little known concerning peptide-binding specificity by gp96. The use of nonamer peptide sets, systematically altered at position 2 and 9, suggest a bias of gp96 for uncharged residues at these positions [56]. Although the association of peptides with hsp is non-covalent, it is remarkably stable, persisting many days in the cold [63] and resisting denaturation (without heat treatment) by SDS–PAGE [60].

The structural features of the mammalian hsp peptide-binding domain and the crystal structure of the bacterial hsp70 homologue DnaK, have been resolved [64]. The peptide-binding pocket of hsp70 identified consists of four-stranded anti-parallel β-sheets and a single α-helix. This conformation is different from the MHC peptide-binding domain, making it unlikely (unless additional exon shuffling is considered) that the MHC peptide-binding domain originates from an ancestral hsp70 gene [65]. For gp96, a putative minimal peptide-binding site at the amino acid position 624–630, adjacent to the dimerization domain (Fig. 3), has been mapped using a peptide tagged with fluorescent probes [66]. The surrounding region of over 200 amino acids defines hydrophobic pockets whose tri-dimensional modeling suggest a conformation similar to the MHC class I peptide-binding domain. Although seductive, this speculative model awaits to be supported by experimental data, and the localization of gp96 peptide-binding site need to be corroborate to rule out for example possible artifactual binding of synthetic probes. This is particularly important since studies from several laboratories [31,63] suggest that dimeric rather than monomeric gp96 is the true peptide-binding structure. Sequence alignment of gp96 homologues in the portion involved in dimerization and the peptide-binding site are highly conserved. Therefore, it is likely that peptide-binding is an evolutionary ancient property of gp96. In this regard, it is also important to recall that the yeast proteasome can generate peptides that fit in murine MHC class I molecules [67], and therefore, that peptides are potentially available to load hsp in any eukaryotes. We have shown that *Xenopus* gp96 can also form complexes in vitro with synthetic peptides (8 and 19 mers; [47]) as stably and efficiently as can mouse or human gp96.

3.2. Interaction of hsp with antigen presenting cells

The unique ability of hsps to generate a robust T-cell response against minute amounts of chaperoned antigenic peptides is thought to result mainly from the specific interaction of hsps with antigen presenting cells (APCs). A large body of data from in vitro studies using murine and human cells [5] indicates that APCs (e.g. peritoneal macrophages and dendritic

cells (DC)) rapidly internalize purified or artificially reconstituted hsp–peptide complexes by receptor-mediated endocytosis. The antigenic peptide carried by hsp is then channeled into the endogenous processing pathway and presented to, and recognized by, T-cells as a peptide–MHC class I complex [68]. This process is not observed with B-cells or fibroblasts [68]. Specific in vitro CTL responses have been obtained against a large variety of chaperoned antigenic peptides including those from tumors [4,8,69], virus-infected cells [54,70], minor histocompatiblity [71] and model antigens [55,68,71], and with purified native [4,69] as well as in vitro reconstituted [61,70] hsp–peptide complexes.

Although MHC class I pathway classically presents endogeneous peptides, APC such as DC have the capacity to take up, process and present exogeneous antigens in association with MHC class I molecule. This pathway is referred as cross-presentation and the resulting $CD8^+$ T-cell priming as cross-priming [72]. The capacity of hsp to deliver exogenous antigenic peptide into the MHC class I presentation pathway is now recognized as an important mechanism of cross-presentation [62,73]. In mice, hsp purified from virally infected cells of one MHC haplotype can cross-prime anti-viral immune responses of mice of another haplotype through the re- or cross-presentation of antigenic peptide by the host MHC. In contrast to MHC molecules, hsps are non-polymorphic and they bind peptides more heterogeneous in size and sequences. Therefore, the repertoire of cellular peptides they bind is unlikely to be restricted by MHC haplotype.

The high degree of structural conservation of hsp and its potential to cross-present antigen is further exemplified by our comparative studies. We have shown [47] that pulsing mouse macrophages with *Xenopus* gp96 complexed in vitro with an antigenic peptide results in the processing and re-presentation of the chaperoned peptide to mouse CTLs as efficiently as a peptide chaperoned by murine gp96. This strongly suggests that the hsp–APC interaction is phylogenetically conserved, including the surface receptor that binds hsps (see earlier). The re-presentation of peptide chaperoned by hsp is approximately 400–200 × more efficient than loading antigenic peptide directly to class I on live cells [68].

The uptake of hsp–peptide complexes is mediated by receptors expressed at the surface of APC [62,74,75] (see section 4). One such receptor interacting with gp96 has been identified as CD91, the receptor discovered for its ability to bind α2-macroglobulin (α2-M; [76]). CD91 is a member of the low-density lipoprotein (LDL) family of scavenger proteins. Interestingly, evidence that CD91 also mediates the uptake of calreticulin, hsp70 and hsp90 by APCs and the re-presentation of peptides has been obtained recently by inhibition experiments with α2-M or anti-CD91 antibody [77]. Based on these results, CD91 has been proposed to be the only receptor involved in cross-presentation of hsp-chaperoned peptides. Although a critical role of CD91 in hsp cross-presentation is well documented, recent studies suggest that this process is more complex and may involve other receptors. First, a putative CD91-independent internalization pathway has been suggested for gp96 [78], although the involvement of another receptor awaits further study. Second, antigen cross-presentation by hsp70 at least in humans has been shown to involve another scavenger receptor LOX-1 [79] rather than CD91, and evidence suggests that CD40 is also implicated in the uptake of hsp70–peptide complex by human APC [80]. Finally, beside endocytic receptors, several signaling receptors have been proposed to interact with hsps and activate APCs (Section 4), including CD36 [81], Toll-like receptors TRL-2/4 [82,83] and their co-factor CD14 [84,85], and CD40 [80]. TLR-2/4 has been shown not only to bind hsp60 [86] but also gp96 [82] and hsp70 [83].

While receptors mediating endocytosis of hsp such as CD91 and LOX-1 are clearly required for representation of peptides chaperoned, they do not appear to have signaling ability. It has been suggested [79] therefore that the T-cell response elicited through APC's activation and representation of antigenic peptide bound by hsp results from a cooperation between endocytic (CD91, LOX-1) and signaling (CD14, TRL-2/4) receptors. Experiments are required to determine if and how such receptor cooperation occurs. How these receptors recognize ligands of heterologous molecules (i.e. gp96, hsp70, α2-M) also remains unresolved issues. Further processing of internalized chaperoned peptides depends on a functional proteasome [77] and is usually TAP-dependent, although other pathways appear to be involved in channeling peptides into the ER [62].

However, the whole process of hsp–peptide complex processing and peptide representation is still poorly understood. This is a particularly important issue since the cross-presentation ability of hsps constitutes their main attractiveness as vaccines against intracellular infections.

4. Peptide-independent immunomodulatory capacity of hsps (chaperokine)

The characterization of a receptor-mediated uptake of hsp–peptide complexes by APCs partially explains the potency of hsp to elicit a specific response. However, it has become evident that the initiation of an immune response depends on the proper activation of APCs through up-regulation of co-stimulatory molecules and the release of various cytokines and chemokines [86]. In this regard, a newly emerging facet of hsps immunological properties should be considered. Several in vitro studies have revealed that, independent of their bound peptides, gp96, hsp90 and hsp70 are able to induce macrophages to produce the proinflammatory cytokines Il-1β, TNFα, Il-12, and GM-CSF [10] as well as C–C chemokines such as MCP-1, MIP-1 and RANTES [87,88]. Furthermore, hsps induce DC maturation as determined by their up-regulation of MHC class II and the co-stimulatory molecules CD86 (B7-2) and CD40 [10,73], and promote their accumulation in draining lymph node in vivo [73,89]. Interaction of gp96 [10] with murine DC or hsp70 with human monocytes [83–85] also triggers translocation of NFκB, a key signaling transduction pathway in immune responses. Evidence of hsp-mediated stimulation of innate immune responses also comes from antibody-depletion experiments showing that NK cells are critical for protective immunity to tumors [8]. Enhanced secretion of IL-12 following the interaction between hsp and APCs is a likely explanation for this observation [5].

One primary concern about these immunomodulatory properties of hsp is that they could be due to trace contaminants of bacterial lipopolyssaccharide (LPS), a very potent inflammatory agent. Several lines of evidence, however, argue against this possibility. First, hsp70, hsp90, and gp96 that have been heat-denatured completely lose their immunomodulatory activity. Second, in contrast to LPS, gp96-mediated activation of DC is strictly dependent on endocytosis and, therefore, is impaired in the presence of the endocytosis inhibitor MDC [82]. In contrast, hsp-mediated activation of DC and macrophages is unaffected by the LPS-inhibitors polymyxin B [83] and the LPS-antagonist Rslp [10]. Third, highly purified hsps, with an undetectable level of LPS using the sensitive limulus amebocyte lysate assay, remains fully active [10]. Fourth, hsp70, but not LPS, induces a rapid intracellular calcium flux in monocytes [84,85]. Further support of a bona fide immunomodulatory ability of hsp comes from the observation that transformed cells expressing recombinant gp96 targeted to the cell surface by the addition of a transmembrane domain, induces efficient DC maturation following cell-to-cell contact [90]. Finally, the following recent preliminary data obtained in our *Xenopus* system provides evolutionary validation of the involvement of hsp in innate immune responses. Injection of gp96, but not LPS, generates long lasting (1 week) increased NK activity in adults frogs, as well as the induction of IL-1β expression both in larvae and adults (Robert et al., unpublished). These observations further support the hypothesis of a true involvement of hsp, rather than an LPS contaminant, in innate immune responses. *Xenopus* B-cells are, in fact, poorly responsive to LPS in vitro [91], and, the intraperitoneal injection of 0.5 mg of LPS, which would be lethal for a human or mice, is well tolerated by *Xenopus* tadpoles and adults.

The stimulatory capacity of innate immune responses by hsps in conjunction with their ability to generate antigen-specific T-cell responses, has important implications at the level of immune surveillance. Several studies suggest that by virtue of their abundance hsps are the major protein species released in the extracellular compartment when cells die by necrosis but not by apoptosis [10,92,93]. According to this view, hsps could constitute a 'danger' [94] signal of non-programmed cell death such as viral infection or cancer [4].

5. Evolutionary study of hsp immunogenicity in *Xenopus*

The experimental model we have developed in the frog *Xenopus* is characterized by naturally MHC class

I-deficient but immunocompetent larvae, T-cell-deficient thymectomized animals, minor and major histocompatibility-defined syngeneic cloned frogs, and MHC classical class I-negative and positive transplantable lymphoid tumor cell lines. Thus, we can investigate the phylogenetic conservation of some of the immunologically relevant features of hsps. The immune system of adult *Xenopus*, a genus who last shared a common ancestor with mammals ~350 million years ago [95], is fundamentally similar to that of mammals (e.g. rearranging TCR and Ig genes, MHC class I- and II-restricted T-cell recognition [96], NK [97] and CD8 NK/T-cells [98] have been characterized). Relative to mammalian systems, however, the *Xenopus* model allows the study of tumor immunity either in the presence or in the complete absence of MHC class I molecules [99,100]. Although both larval and adult *Xenopus* are immunocompetent and have $CD8^+$ T-cells, larval cell-mediated immunity cannot involve classical MHC class I antigens since these molecules are not expressed until metamorphosis [101,102]. In fact, since neither classical class I, nor non-classical class Ib, nor LMP7 mRNAs can be detected in the thymus until metamorphosis [102], a strong case can be made for the absence of class I education during larval life. Spontaneously arising thymic lymphoid tumors in *Xenopus* have been characterized, and transplantable cell lines developed [103–105]. By taking advantage of these tumors, we have shown that like mammals, adult frogs can detect tumor-specific antigenic determinants and generate an anti-tumor thymus-dependent protective immunity [105,106]. Moreover, the immune system of MHC class I-negative larvae is also able to recognize (and develop a long-lived memory against) tumor antigens. Nevertheless, the larval immune system appears to lack a fully operational effector system. Whether such 'weakness' is related to the absence of MHC class I surface expression is unknown.

We have shown that *Xenopus* gp96 complexed in vitro with exogenous antigenic peptides, can interact with mouse macrophages, leading to the cross-representation of the antigenic peptide by class I molecules of murine macrophages, and peptide-specific activation of a MHC-restricted mouse $CD8^+$ T-cell line [47]. To obtain more evidence of the ability of Hsps to generate MHC-restricted CD8 T-cell mediated responses against chaperoned exogenous antigenic peptides in absence of *Xenopus* MHC-restricted antigen-specific T-cell clones or lines, we took advantage of several minor histocompatibility (H) alloantigen disparate *Xenopus* clones [48,100]. Both hsp70 and gp96 can generate a thymus-dependent, adaptive, specific, cellular immune response against chaperoned minor H antigenic peptides that effects an accelerated rejection of minor H-locus disparate skin grafts in vivo. Furthermore, hsp70 and gp96 immunization elicited a CTL in vitro response specific against minor H-antigens. As in the mouse, neither *Xenopus* peptide-free hsp70, nor cognate gp96-peptide complexes elicit any CTL activity. In addition, CTL generated by gp96 or hsp70–peptide complexes kill only MHC-compatible targets derived from the same minor H-locus-disparate genotype as the frog clone from which the Hsps were purified. This strongly suggests that the CTL response has been generated against chaperoned minor-H antigenic peptides, and that these exogenous peptides have been channeled in the antigen presentation pathway of the host. Thus the immunological properties of gp96 and hsp70 extend beyond mammals to ectothermic (cold-blooded) vertebrates like *Xenopus*.

Immunization of *Xenopus* with gp96 purified from a highly tumorigenic MHC class I negative 15/0 tumor generates potent anti-tumor immunity as measured by a significant delay in the appearance and diminished size of tumors after challenge [47]. Weaker but nevertheless reproducible reduction in the size of tumors has been observed with gp96 from normal tissues, suggesting that as in mammals, gp96 can generate anti-tumor responses in the frog that are both peptide-specific (adaptive) and non-peptide-specific (innate). This conclusion has been further substantiated by the similar anti-tumor responses obtained by priming with a purified *Xenopus* hsp70–tumor peptide complex, and a more limited effect (i.e. reduced tumor size) with tumor-derived hsp70 that is free of peptide [47]. In the absence of MHC class I expression by 15/0 tumors (both mRNA and protein), the peptide-specific anti-tumor response elicited by gp96 and hsp70 cannot be due to classical MHC-restricted CTL response. Among other possible effectors, we are focusing on NK cells, MHC-unrestricted cytotoxic $CD8^+$ T-cells, and the recently

characterized NK/T-cells [98]. Since classical class Ib [102,104] and β2-microglobulin mRNAs (Robert and Horton, unpublished data) are detectable in 15/0 tumor cells, a non-classical class Ib restricted response is possible.

Strikingly, gp96 is able to generate responses against MHC class I-negative tumors in naturally class I-deficient but immunocompetent larvae [48, 100]. In this case, however, the response does not appear particularly dependent on the presence of antigenic chaperoned peptide since immunization with gp96 purified from normal tissue is just as effective as tumor-derived gp96 in evoking inhibition of tumor growth. In addition, immunization with either tumor-derived or non-tumor-derived gp96 results in a significantly prolonged survival of tadpoles challenged with a lethal tumor. Given the absence of MHC class I antigens in pre-metamorphic larvae, this suggests a prominent contribution of an innate-type of response. The peptide non-specific anti-tumor responses generated by non-tumor-derived gp96 in naturally class I-deficient larvae further suggests that the MHC class I re-presentation pathway of exogenous hsp-chaperoned peptides is crucial for eliciting a specific adaptive cellular immune response. It is unclear whether hsp-mediated larval anti-tumor responses involve cytotoxic effectors. A minor population of NK cells can be detected in the spleen of pre-metamorphic stages taploles in parallel with the first detection some class I^+ splenocytes (Horton et al., submitted). However, no NK-like cytotoxicity could be detected using a sensitive DNA fragmentation JAM assay (Horton et al, submitted), even after immunization with gp96 (Goyos and Robert, unpublished).

6. Surface expression of hsps

One poorly understood aspect of the immunological properties of hsp70 and gp96 concerns their expression at the surface of certain cells. Surface expression of hsp70 [107,108], BiP [109], hsp90 [107, 110] and gp96 [42,111] has been observed for a variety of human and murine tumor cells, but not for the limited number of transformed or normal cells that have been examined (human EBV-transformed B-cells, peripheral blood leukocytes, and fibroblasts). It has been reported that the inducible form of hsp70 expressed on the cell surface of some tumors may directly interact with γδ-T-cells [108,112,113] and/or NK-cells [9]. Gp96 also has been reported to be expressed on the surface of some mouse tumor cells but not on normal embryonic mouse fibroblasts [111], despite the fact that the gp96 on the cell surface contains an ER-retention KDEL sequence. Surface expression of several ER-resident molecular chaperones, including gp96, found on a subset of murine immature thymocytes [114] as well as LPS-activated B-cells [115], reveals that this phenomenon might be not restricted to tumor cells.

We [116] have demonstrated that, as in mice [117], a fraction of the *Xenopus* gp96 synthesized by thymus-derived lymphoid tumor cells is actively and specifically directed to the surface of these cells. However, in contrast to mice, our observations have also revealed that some normal *Xenopus* B-lymphocytes also express surface gp96, whereas we have been unable to detect gp96 on the surface of normal non-transformed *Xenopus* erythrocytes, splenic and peritoneal macrophages, and fibroblasts. The C-terminus of surface gp96 contains the KDEL motif involved in the retention of resident protein by the endoplasmic reticulum. Gp96 is tightly bound to the plasma membrane of these cells, and the possibility of its adventitious deposition from extracellular compartments has been ruled out for both *Xenopus* [116] and mice [111]. It is unclear, however, how in absence of a transmembrane domain, gp96 can be expressed at the cell surface. The wide occurrence of this phenomenon suggests a biological relevance. We have found a similar surface expression on some, but not all, T- and B-cell lines from the channel catfish as well as lymphoid-like cells of the hagfish [115]. In *Xenopus* as in fish and hagfish, gp96 is directed to the surface by an active process dependent on translocation of protein from the ER to the Golgi (i.e. a Brefeldin A-sensitive process). Most interestingly, we have recently been able (Robert and Smith, unpublished) to extend the restricted cell surface expression of gp96 homologues to sea urchin. We have characterized a rabbit anti-mouse gp96 polyclonal anti-serum that specifically cross-reacts with gp96 from *S. purpuratus* by Western blotting (i.e. inhibition by pre-incubation with purified mouse gp96). Approximately 50% of freshly harvested coelomic

phagocytes were brightly surface stained with anti-gp96 mAb, whereas other coelomic cell types were negatively stained. This surface signal was not due to cell death as controlled for by propidium iodide. Moreover, no surface signal was detected at the surface of phagocytes with anti-C3 mAb that intensely stains fixed cells.

Recent pulse/chase experiments with *Xenopus* B-cells (Morales and Robert, unpublished data) suggest that gp96 is constitutively directed to the surface of lymphoid tumor cells and freshly harvested normal B-cells by a process involving a rapid turnover (gp96 appears within 4–5 h at the surface, disappears after 1 h). A scant amount of labeled gp96 was detected in the culture medium in parallel with its appearance at the cell surface of B-cells suggesting a possible secretion, although the rapid disappearance of the whole surface gp96 signal may also involve an active re-internalization. Interestingly, stimulation of *Xenopus* B-cells with heat killed *E. coli* bacteria up-regulates gp96 mRNA and, intracellular and surface gp96 protein (Morales et al., manuscript in preparation). Given the dual ability of gp96 to chaperone antigenic peptides and to stimulate innate immune responses, such gp96-induced cellular modulations may play a role in immune surveillance by allowing B-cells to access and probe the extracellular milieu as well as to trigger immune responses. In this regard, in vitro evidence in mouse suggests that gp96 surface expression by B-cell upon LPS-activation may function as a Th2-specific co-stimulatory molecule [115]. Generally, increased hsp surface expression by stressed, tumor, and activated immune cells may constitute a way other than necrosis to expose APC to hsps.

7. Possible role of hsp in the evolution of the immune system

The capacity to control invasion by pathogens and neoplastic cells is certainly a major selection pressure for any organism. A strong positive selection for any defense and immune surveillance mechanisms should have been maintained throughout evolution leading to multiple redundant systems. As already mentioned, hsps are very ancient and conserved structures. The receptors that mediate their internalization are also likely to be ancient and conserved. For example, highly conserved α2-M homologues are known in most vertebrate taxa, and *C. elegans* [117], and CD91 receptor homologues are also likely to be well-conserved. TLR homologues are also of ancient origin [86,118]. Macrophage-like cells are also a common ancestral-type of immune cells. Therefore, a sensor system based on detecting hsps released in the extracellular environment following stress and necrotic death could constitute the archetype of an ancestral system of immune surveillance. In addition to providing general inflammatory or danger signals [94] that trigger innate immune responses, hsps would have the potential, at least in mammals and frogs, of generating specific adaptive immune responses against the peptides they chaperone. Such a system may be very ancient and may still be active in a wide array of extant organisms. In addition, an early specialization of certain hsps in immune surveillance may have led to a subsequent diversification of their functions and interactions. It is possible, for example, that in different organisms, hsps interact with different cell types and/or different receptors.

Based on the convergent ability of hsp and MHC molecules to bind peptides, and their expression at the cell surface, we [119] and others [43,120] have hypothesized that hsps are part of an ancestral pathway that is antecedent to, and independent of the antigen presentation pathway that uses MHC molecules. Specifically, we have proposed that the surface expression of gp96 (and perhaps hsp70) may have provided a primitive mechanism for presentation of cell surface antigens. Interestingly, a potentially analogous non-immunological system using surface hsp70 has been proposed for the ascidian *Ciona intestinalis* to prevent auto-fertilization of hermaphroditic animals [121].

Based on extensive molecular studies [122], the evolution of the vertebrate adaptive immune system appears to have occurred abruptly with the simultaneous emergence of all the different gene elements in the ancestors of Gnathostomata (jawed fish). Indeed, T-and B- lymphocyte receptors as well as MHC class I and II genes have been identified in the living representatives of all the classes of gnathostomes whereas none have been found in agnathans and prochordates despite repeated attempts to look for them. Although the emergence of a functional adaptive immune system may have resulted from

an accelerated evolutionary rate, it is unlikely to happen in a single step. In agreement with a strict Darwinian viewpoint where production of phenotypic diversity precedes natural selection, Du Pasquier and Flajnik [122] proposed a scenario in which a primitive repertoire of lymphocyte receptors, probably TCR-like, was first generated by somatic rearrangement (possibly as originally proposed by Marchalonis et al. [123], as a result of a horizontal transfer of the rearrangement machinery. The characterization of transposon activity by Rag1 and 2 genes [124,125] support this possibility. During the second step, MHC genes evolved to select a more efficient lymphocyte receptor repertoire and to avoid autoimmunity. It is tempting to speculate that hsps served as a kind of surrogate for the MHC during the period required for the assembly of MHC genes.

There are some aspects of the immunological properties of hsps that maybe relevant for applied immunobiology (Table 1). Important efforts are being made to control or prevent viral and bacterial infections of economically important organisms (i.e. fish, shrimp, etc.) by developing new treatment or vaccines. Hsps have been shown to be potent in generating specific anti-tumor, anti-viral and anti-bacterial protective immunity in mammals. Among the advantages of developing hsp-based vaccines is that there is no need to identify and purify antigens prior to therapy. The purification of hsp70 and gp96 (together with their bound peptides) from infected tissues is relatively easy. Moreover, it is likely that since purified hsps carry more than one antigenic peptide, they would elicit an immune response against multiple antigens. Given the success of hsp–peptide immunization in mammals, hsp vaccination may also be efficient in other vertebrate species including fish and amphibians. In this regard, accumulating evidence suggests that fungal (chytrid fungus) and viral (iridoviruses) agents are implicated in the worldwide declines of amphibian populations and species [126,127]. Hsp-mediated vaccines, especially against iridoviruses, may represent a useful strategy to protect endangered captive (zoo) amphibian specimens. In the case of invertebrates, the possibility of enhancing innate immunity by hsp stimulation merits serious evaluation.

Finally, hsp70, hsp90, and gp96 expression levels may serve as useful biomarkers of the health of a tissue or a cell population. Gp96 is up-regulated upon any disturbance of the ER function; hsp70 and hsp90 are also up-regulated upon tissue injury and infection. Hsp70 has been shown for example, to provide a suitable biomarker of effect of pollutants on the earthworm [128]. The increasing number of differential gene expression studies (i.e. micochips, proteomics) in various invertebrate and vertebrates species could provide potentially interesting background information to look for correlation between hsps and other immune-related gene expression.

Table 1
Immune-related characteristics of mammalian and amphibian Hsps

Hsp70 and gp96	Mammals	Amphibian
Peptide binding	+	+
Peptide chaperoning	+	+
Peptide-specific immunogenicity against		
Minor H-Ag	+	+
Tumor	+	+
Virus	+	+
Bacteria	+	?
Effectors		
MHC-restricted CD8 T-cells	+	+
MHC-unrestricted CD8 T-cells	?	+
NK cells	+	+
Tumor immunity in absence of class I presentation	?	Yes
Surface expression		
Tumors	+	+
IgM^+ B-cell subset	+	+

In conclusion, the recent realization that hsps play a key role in mammalian immunity, has implications for evolutionary and comparative immunobiology that should be thoroughly evaluated. Involvement of hsps in immunity is likely to have emerged early and diversified during evolution. It is my personal conviction that more extensive comparative studies will reveal novel and potentially unique features of hsp–immune system interactions.

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