BRIEF REVIEWS

Structure and Function: Heat Shock Proteins and Adaptive Immunity[1]

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Heat shock proteins (HSPs) have been implicated in the stimulation and generation of both innate and adaptive immunity. The ability of HSPs to bind antigenic peptides and deliver them to APCs is the basis of the generation of peptide-specific T lymphocyte responses both in vitro and in vivo. The different HSP families are genetically and biochemically unrelated, and the structural basis of peptide binding and the dynamic models of ligand interaction are known only for some of the HSPs. We examine the contribution of HSP structure to its immunological functions and the potential "immunological repertoire" of HSPs as well as the use of biophysical techniques to quantify HSP-peptide interactions and optimize vaccine design. Although biochemical evidence for HSP-mediated endogenous processing of Ag has now emerged, the issue of whether HSP-peptide complexes act as physiological sources of Ag in cross-presentation is controversial. We assess the contribution of biochemical studies in this field. The Journal of Immunology, 2007, 179: 2035–2040.

Heat shock proteins (HSPs)[4] are ubiquitous. They are expressed in all organisms and in different subcellular compartments, their predominant function being the folding and unfolding of protein substrates (1). From the immunological viewpoint, HSPs have been implicated in stimulation of the innate and adaptive immune systems (2). The ability of HSPs to bind antigenic peptides and deliver them to APCs forms the basis for their potential role in the generation of peptide-specific T lymphocyte responses. A physiological role for HSPs in Ag presentation, particularly cross-presentation, remains controversial (3). Despite this, as vaccine candidates HSPs are involved in phase II and III clinical trials (4) for cancer immunotherapy. New biochemical and structural studies have recently emerged to support the role of HSPs in some, but not all, models of Ag processing and presentation.

The structural basis of peptide binding and dynamic models of ligand interaction are understood for some HSP family members, notably HSP70 and HSP90, but how the basic biology of HSPs influences their immunological functions remains poorly understood. Recent biochemical evidence highlighting the role of HSP90α in endogenous processing of MHC class I Ags (5) suggests that the search for HSP-peptide interactions in vivo might have been misdirected, as the identified peptides were much longer than previously anticipated.

Little attention has been paid to functional differences between HSPs from different species. Although different HSP families are both genetically and biochemically unrelated, there is significant homology within members of the same HSP family (6).

However, this has lead to the mistaken assumption that all HSPs are created equal and have the same biological effects, and this is clearly not the case. Mycobacterial HSP70 appears to be a more potent stimulator of innate immune responses than human HSP70, emphasizing that phylogenetic variations can be responsible for different biological outcomes (7). This review will focus on how insight into HSP-peptide interactions might influence the understanding of their role in the adaptive immune response.

HSP structure and influence on peptide binding

Structural data are available for HSP70 and HSP90. HSP90 has three structural domains (Fig. 1A): 1) an N-terminal nucleotide binding domain (NBD) that also binds HSP90 inhibitors (8) and may bind peptides (9, 10); 2) a middle segment that interacts with client proteins; and 3) the C terminus, which is implicated in homodimerization (11). In contrast, HSP70 has two domains: an NBD and a substrate binding domain (SBD) (Fig. 1B). The 44-kDa N-terminal NBD has ATPase activity and associates with the HSP70 cochaperone DnaJ. The 27-kDa C terminus is composed of the SBD and a "lid" region (12–14). These two domains are connected by a conserved linker, recently shown to be critical for interdomain communication (15).

The structural basis of peptide binding is best understood for HSP70 because both the crystal (16) and solution (17) structures of the SBD of HSP70 complexed to a model peptide (NRLLLTG) have been solved. HSP70 has a single peptide binding site with a hydrophobic channel flanked by an arch allowing peptide access (Fig. 2).

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[4] Abbreviations used in this paper: HSP, heat shock protein; NBD, nucleotide binding domain; SBD, substrate binding domain.
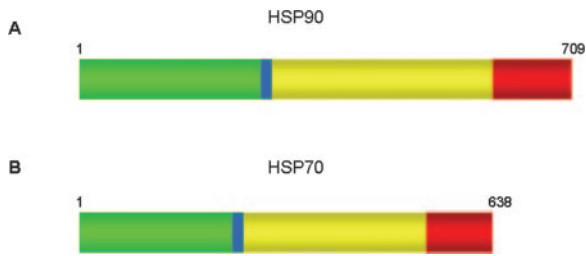
FIGURE 1. Schematic of the domain structures of yeast HSP90 (*A*) and *E. coli* HSP70 (*B*) as determined by structural and limited proteolysis studies. HSP90 has an N-terminal NBD (green) that may also contain a peptide binding element. The middle segment (yellow) interacts with client proteins and also contributes a loop that catalyzes ATP hydrolysis. The C-terminal domain (red) is implicated in homodimerization. HSP70 has two major domains: a 44-kDa N-terminal NBD (green) and a 27-kDa C-terminal substrate binding domain (yellow and red). This region comprises the main substrate binding domain (yellow; 18 kDa) that consists of two times four anti-parallel β-strands and four connecting loops and makes contact with the bound substrate, as well as an α-helical lid region (red; 10kDa). Both molecules have a linker region (blue) that is thought to mediate communication between the major subdomains.

From the initial crystal structure of the SBD of DnaK (*Escherichia coli* HSP70) with a bound peptide, Hendricksen and colleagues proposed a model for peptide binding depending on allosteric actions mediated by nucleotide exchange (16). The recent publication of the first complete HSP70 molecule confirms this model (15). Binding of ATP opens the helical lid and allows access of the peptide to the substrate binding cavity. Following hydrolysis of ATP to ADP the lid is closed, resulting in slower peptide on and off rates. It would be incorrect to think of the ATP-bound state as "open" and the ADP-bound state as "closed" because both states exist with either nucleotide bound, but the dynamic equilibrium favors "open" (low peptide affinity) in the ATP-bound state and "closed" (high peptide affinity) in the ADP-bound state (see Fig. 3 and Ref. 18). Preparation of HSP70-Ag complexes for use in immunization studies have been performed using ADP as opposed to ATP affinity purification (19, 20) to maximize the yield of intact complexes.

Several types of interaction contribute to HSP70 substrate binding. Hydrogen bonds formed between the peptide backbone and two of the loops of the SBD (16) mediate recognition
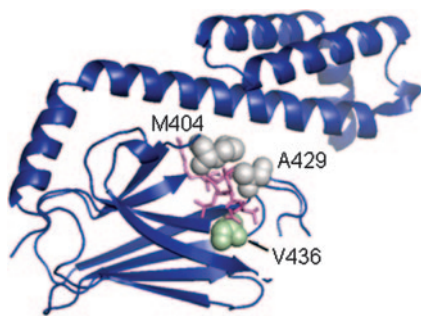

FIGURE 2. Schematic of the SBD of DnaK. This secondary structure representation of the SBD of DnaK in a partial space-filling and ribbon representation is derived from the crystal structure of the C-terminal domain bound to the peptide NRLLLTG (shown in pink; Ref. 13). The SBD is composed of β-pleated sheets comprising the peptide binding cleft and an α-helical lid region. An arch formed by residues M404 and A429 (gray) encloses the peptide backbone and a deep pocket formed by residue V436 (green) accommodates hydrophobic side chains.
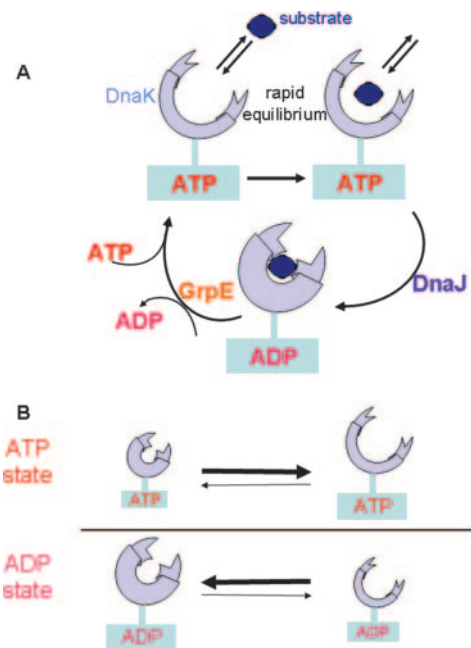

FIGURE 3. Dynamic model of interaction of DnaK with substrates. *A*, The peptide's initial interaction with DnaK in the "open" conformation stimulates ATP hydrolysis by DnaJ and trapping of the peptide. A nucleotide exchange facilitated by GrpE allows the rebinding of ATP and an interaction with a new substrate. *B*, Model of ATP and ADP states of DnaK. In both states the substrate binding domain opens and closes periodically, the principal difference being the rate of transition between the two states. The size of the symbols represents the relative frequency of a given conformation within a population of DnaK proteins.

of the extended peptide conformation. Van der Waals interactions between the peptide side chain residues and the substrate binding cleft confer the specificity of the HSP70-peptide interaction. These are strongest at a single hydrophobic residue (V436 in *E. coli*) that builds a deep pocket tailored to accommodate large hydrophobic side chains such as leucine (designated position 0, L_4 (boldface) in NRL**L**LTG (16)). An arch formed by residues M404 and A429 encloses the bound peptide backbone through contact with residues in positions −1 and +1 (Fig. 2). There are some similarities to peptide binding by MHC class I molecules, as HSP70 also binds peptides in an open, extended conformation. However, MHC class I molecules have two strongly determining pockets for peptide binding, accommodating anchor residues at either end of the peptide and ensuring that peptide length is necessarily limited. HSP70 has only one such pocket (V436 in *E. coli*), which allows it to bind peptides of between seven and many hundreds of amino acids in length.

An important consideration is that HSP70 interacts preferentially with unfolded polypeptide chains, but not their native counterparts, with a few exceptions such as the tumor suppressor gene p53 (21). Within an unfolded polypeptide chain, HSP70 binds short peptide sequences comprising a core of five hydrophobic residues flanked preferentially by basic residues (see below). These sequences occur every 30–40 residues in most proteins but in their native folded state are inaccessible to HSP70, being buried within the hydrophobic core. Therefore, for HSP70 to be involved in Ag processing and presentation the majority of its ligands need to be peptides or unfolded polypeptide chains, i.e., processed as opposed to native proteins. This

becomes important when the physiological role of HSPs in Ag presentation is considered.

Peptide binding repertoire of HSP70

The critical proposed function of HSPs in the generation of adaptive immunity is the binding of peptide Ags and their delivery to APCs. Does HSP70 bind all peptide sequences equally well, and what is the potential immunological repertoire of HSP70? The preferred amino acid residues recognized by HSP70 have been determined for a few HSPs, notably DnaK, as well as bovine brain Hsc70 and yeast BiP (the endoplasmic domain-resident homologue of HSP70). The methods used have included affinity panning of peptide sequences expressed by phage display (22–24), panning of a random peptide mixture (25), and examination of DnaK binding to cellulose-bound peptide libraries (26). An advantage of the latter approach was that peptide sequences were generated from naturally occurring proteins rather than randomly generated sequences.

Given the variety of techniques used in different HSP70 species, it is not surprising that the results of these studies are not entirely consistent. Gething's group proposed that HSP70 binds heptamers with alternating hydrophobic and aromatic amino acid residues (22), and this is at odds with other studies that found aromatic residues to be generally excluded from the HSP70 peptide binding motif. The crystal structure of DnaK suggests its peptide binding pocket cannot easily accommodate such large residues (16). The present consensus is that the HSP70 binding motif is defined as a core of hydrophobic residues, particularly leucine, flanked by basic residues (24, 26, 27). Acidic and aromatic residues were not favored for binding DnaK (26).

The effects of HSP70 mutations on its peptide binding profile have defined the critical parameters of HSP70 structure that determine its binding preferences and may be further optimized for vaccine design. These studies may also provide insight into how HSP70 species differences can modify immunological functions. Deletion of the lid reduces affinity but does not affect the repertoire of peptides bound (28). Pharmacological approaches using recombinant HSPs would favor using the smallest peptide binding unit. A minimal peptide binding unit of mycobacterial HSP70 (136 amino acids, residues 359–494) binds Ag with only a 2-fold reduction in affinity compared with wild-type mycobacterial HSP70 (29). When complexed to antigenic peptide, this polypeptide is able to generate Ag-specific CTL with only a minor reduction in efficiency (29).

The critical hydrophobic HSP70 residue that directly contacts bound peptide is conserved in all HSP70s (V436 in DnaK). Mutation of this residue to a bulky phenylalanine prevents access of peptide to the substrate binding cleft, resulting in decreased peptide binding (18, 29, 30). The same mutation in mycobacterial HSP70 (V410F) retains the innate stimulation properties of the wild-type parent HSP70 but loses its ability to generate adaptive immunity because it no longer binds peptide (30).

The residues comprising the HSP70 arch show no species conservation and vary within species according to subcellular compartmentalization. The arch mutants of DnaK, M404A and A429W, show a similar configuration to mammalian HSP70 (i.e., A404 and Y429) and were more tolerant of acidic residues than wild-type DnaK (28).

Quantitation of HSP-peptide interactions and implications for immunity

Knowing how much peptide is bound to HSP70 addresses the important issue of how much Ag, complexed to HSP, is required for delivery to APC to generate immunity i.e., does it fall within the "physiological" or "pharmacological" range? Furthermore, quantitation of the HSP-peptide interaction allows the relative efficiency of different forms of HSP-Ag complexes to be compared, both for pharmacological studies and for optimization of vaccine design.

HSP-peptide interactions have been studied using environmentally sensitive fluorescent peptides (31), fluorescence anisotropy (32, 33), radiolabeled peptides (34), and tryptophan fluorescence (35). These techniques have been used to quantify Ag delivery by HSPs, although the extremely hydrophobic nature of many antigenic peptides compared with "model" HSP binding peptides limits the methodology. Initial studies used radiolabeled or fluorescently labeled peptides and either gel electrophoresis or size exclusion chromatography to separate free peptide from HSP-bound peptide and were performed under conditions where the peptide concentration far exceeded the HSP concentration. These conditions favored maximal peptide binding, and 1–30% of HSP was found to be loaded with peptide (36–39). Because the experimental conditions favored peptide loading of HSP, these studies suggested that the interaction was weak because, at most, one-third of the HSP molecules were occupied by Ag. However, the HSP-peptide interaction was not quantified nor was the minimum quantity of Ag required for an immune response ascertained.

Surface plasmon resonance has been used to both qualitatively (40, 41) and quantitatively (42) describe peptide binding to HSP70. At 1 nM the derived affinity of the peptide NRLLLTG was several orders of magnitude higher than previously published (29, 34) and might have been due to the solid phase anchoring of peptide in surface plasmon resonance, resulting in an overestimation of affinity compared with solution phase methods such as isothermic calorimetry (43).

Further studies used fluorescent techniques to quantify the HSP-Ag interaction (30, 44). Using fluorescence anisotropy (Fig. 4), we determined the equilibrium affinity of HSP70 for antigenic peptides to be in the range of 1–12 μM and found that picomolar concentrations of mycobacterial HSP70-peptide complexes were sufficient to generate in vitro viral CTL responses (30). These data suggest that generation of CTL by cross-presentation of HSP-peptide could be achieved by physiological concentrations of Ag. Mutant mycobacterial HSP70, with amino acid substitutions in the critical peptide binding cleft, attenuated peptide binding and thus allowed the two immunological functions of HSP70, stimulation of innate vs stimulation of adaptive immunity, to be separated.

Optimizing peptide binding to HSPs provides an interesting strategy to improve the efficiency of generating immune responses. Fletchner et al. designed hybrid antigenic peptides with two modifications at the N terminus of the "javelin" peptide sequence (44). Amino acid residues (e.g., NRLLLTG) that bind well to HSP70 were followed by a linker region (FFRK) that was predicted to enhance proteasomal and protease cleavage and, presumably, the availability of the antigenic epitope for cross-presentation. Use of the modified "javelin" antigenic peptides enhanced affinity of epitopes for HSP by several orders of
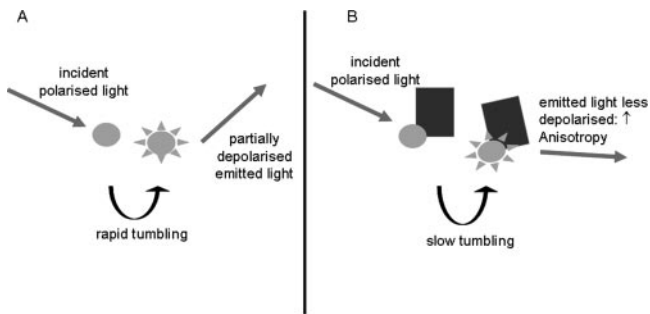
FIGURE 4. Schematic of fluorescence anisotropy to calculate HSP-peptide affinity. *A*, When a fluorescent peptide (circle) is excited by polarized incident light, rapid tumbling of the fluorophore during its fluorescence-excited state lifetime, τ, results in partial depolarization of the emitted fluorescent light. *B*,. Binding of the peptide to its ligand, HSP70 (square), results in slower tumbling and thus decreased depolarization of the emitted fluorescence. This is measured as an increase in anisotropy. The increase in anisotropy for different HSP70 concentrations allows the calculation of the HSP-peptide affinity.

magnitude. Although the affinity of the native epitopes for HSP70 varied greatly, ranging from 40 to 2500 μM, the affinities of the modified epitopes were all within one order of magnitude ($\sim$1 μM) (44). The advantage of this methodology is that it facilitates the binding of multiple different antigenic epitopes to HSP70 without favoring one epitope over another due to differing HSP70 affinities. This approach of combining structural and biochemical data to optimize Ag design and quantifying the efficiency of the immunological response provides a potentially significant advance in the design of HSP vaccines.

Are HSP-peptide complexes physiological sources of Ag for cross-presentation?

As in vivo intracellular chaperones, HSPs bind a range of substrates (45). HSP-peptide complexes can clearly cross-present Ag in vitro (30, 46–48) and are being used for the generation of autologous tumor vaccines and for enhancing immunity to defined antigenic peptides. Do they have a physiological role in cross-presentation/priming?

HSPs and cross-presentation

HSPs have been implicated in cross-presentation at several different points of the Ag presentation pathway (2): 1) HSP-peptide complexes may be formed and released into the extracellular milieu upon necrotic cell death where they could interact with both signaling and uptake HSP receptors on APCs; 2) as peptide chaperones, HSPs may "protect" peptides that have entered the cytosol until they are transported by TAP into the lumen of the endoplasmic reticulum for loading onto MHC class I molecules; 3) the release of microbial HSPs upon phagocytosis and killing of the pathogen make HSP-peptide complexes available within the APC for cross-presentation. Definitive evidence for some of these models is only now emerging through biochemical and structural studies, >10 years after the initial observation that HSP70-associated peptides cross-prime Ags (20).

The role of gp96 in generalized adaptive immunity to murine tumors was challenged by Nicchitta and colleagues who found that the progress of chemically induced murine tumors was equally retarded by both gp96 (an ER-resident HSP90) and a truncated mutant of gp96 that lacked the C-terminal peptide binding domain of the molecule (49–51). However, other

groups identified an N-terminal peptide binding site for gp96 (9, 10), albeit at a low affinity of 400 μM, (10) potentially explaining these findings.

Whether there is a direct role for HSP-chaperoned peptides in cross-priming remains controversial. Shen and Rock engineered transgenic fibroblast cell lines that expressed OVA in different subcellular compartments under different steady-state stabilities (52). All constructs generated similar amounts of the MHC class I OVA epitope SIINFEKL in association with K^b as measured by the clonotypic Ab 25.D1.16. The ability to cross-prime was related to steady-state levels of intact OVA, and the deletion of intact proteins by specific Abs prevented cross-priming. Yewdell and colleagues argued that cross-priming is directly related to protein stability (53) because the expression of proteins engineered to be rapidly degraded generated large quantities of peptides, but these were not associated with cross-priming except following treatment with the proteasome inhibitor lactacystin. Transfection of cells with minigenes expressing only the minimal antigenic epitope failed to cross-prime, arguing that intact proteins rather than peptides are the sources of Ag in cross-presentation.

Opposing these arguments, Albert and colleagues provided evidence for processed rather than intact Ag as the source of material for cross-priming, at least from apoptotic cells (54). They postulate that defective ribosomal initiation products (DriPs; Ref. 55), which, as denatured polypeptides, would have high affinity for HSPs, are the source of this processed Ag. Moreover, Binder and Srivastava found that HSP-associated peptides are necessary and sufficient for cross-priming (56) because the depletion of four different HSPs (HSP70, gp96, HSP90, and calreticulin) from cell lysates reduced the efficiency of cross-priming, implying a certain redundancy in the system. Some of Rock's observations might have been due to the artifactual dissociation of HSP-peptide through the use of nitrogen cavitation for cell lysis (19) and the number of cell equivalents used.

What is the evidence for HSP-peptide complexes in vivo? HSP70 has high affinity for peptides in the ADP state, but binding in vitro takes several hours. Although binding in the ATP state is rapid, the affinity is too low to be physiological (57). For binding to substrates in vivo, HSP70 requires several cochaperones, notably DnaJ (Fig. 3). DnaJ also exhibits peptide binding in vitro and acts as a "scanning factor" for HSP70, identifying which substrates HSP70 will bind. However, the affinity of DnaJ for short as opposed to longer polypeptides is low (57), making binding of HSP70 to short peptides in vivo less likely. The extrapolation of in vitro studies of HSP-peptide interactions using small model peptides is thus problematic for the determination of in vivo functions. Recent proteomic analysis of tumor cell lysates has identified a number of HSP70-associated peptides (58), providing evidence that such interactions do occur in vivo.

A clear role for HSP90-associated peptide in Ag presentation has now been demonstrated (5). Recombinant forms of OVA with lysines flanking the SIINFEHL epitope (the lysine at position 8 is mutated to a histidine to prevent protease-dependent cleavage) allowed biochemical identification of proteolytic intermediates of Ags that had previously been elusive. Kunisawa and Shastri previously identified small (<10 kDa) peptides that associated with HSP90 (59) but were nonimmunogenic. They found that HSP90α associates with larger (between 10 and 30

kDa in size), proteolytically processed polypeptides that are immunogenic. The discovery of immunogenic, HSP-associated peptides was a major advance in support of a physiological role for HSPs in adaptive immunity, albeit the peptides were much larger than previously postulated.

The mechanism by which HSP70 might associate with antigenic peptides in vivo has yet to be demonstrated, and at least one study, (60) failed to find small (<10 kDa) immunogenic peptides that associated with HSP70. Earlier structural and biochemical studies used small model HSP70-associated peptides, and it is therefore possible that the actual HSP70 antigenic substrate is larger than previously thought. Indeed, although a few HSP70-associated peptide intermediates have been previously identified (61), the methodology used specifically excluded larger (>10 kDa) peptides. These larger, processed intermediates could also possess higher affinities for DnaJ cochaperones, allowing rapid association between HSP70 and the peptides.

Unanswered questions and future directions

Many questions therefore remain about the role of HSPs in adaptive immunity. Although there is now direct evidence of a role for HSP90 in "protecting" peptides derived from endogenously processed proteins before further processing and loading onto MHC class I molecules (5), a clear physiological role for HSPs in other Ag processing pathways, notably cross-presentation, has yet to be demonstrated. Structural and biochemical studies using small model peptide ligands for HSPs may have misdirected the search for in vivo HSP ligands, which could turn out to be larger than anticipated. On the vaccine front, the increasing use of biophysical techniques coupled with the biochemical characterization of HSP-ligand interactions will assist in the rational use of these molecules for immunotherapy. Although both HSP90 and HSP70 family members have been used in immunological studies, much more is known about the interaction of HSP70 with its ligands than that of HSP90. Structural studies of HSP90 complexed to peptide ligands may allow similar optimization of the use of this protein in vaccines.

The recent identification of human, but not murine, CCR5 as a novel pathogen recognition signaling receptor for mycobacterial HSP70 (7, 62) suggests a mechanism by which mycobacterial HSP70 can bridge the innate and adaptive immune system in its natural host. Indeed, signaling through CCR5 is responsible for the mycobacterial HSP70-dependent formation of functional immune synapses between dendritic cells and T cells. Importantly, this CCR5-mediated calcium response was only seen in mycobacterial and not human HSP70 (our unpublished data). Microbial HSP70s therefore appear to be more immunostimulatory than endogenous human HSP70s, with which they share 35% homology. These findings emphasize that despite the structural conservation across the HSP70 species, there are sufficient differences to give diverse biological outcomes. There is therefore a clear need to distinguish the effects of both different HSP family members within the same species as well as related HSPs from different species. Whether these microbial HSPs are purely adjuvants or are also involved in Ag delivery remains the subject of future work.

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