

Calreticulin, a Peptide-binding Chaperone of the Endoplasmic Reticulum, Elicits Tumor- and Peptide-specific Immunity

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Summary

Calreticulin (CRT), a peptide-binding heat shock protein (HSP) of the endoplasmic reticulum (ER), has been shown previously to associate with peptides transported into the ER by transporter associated with antigen processing (Spee, P., and J. Neefjes. 1997. *Eur. J. Immunol.* 27: 2441–2449). Our studies show that CRT preparations purified from tumors elicit specific immunity to the tumor used as the source of CRT but not to an antigenically distinct tumor. The immunogenicity is attributed to the peptides associated with the CRT molecule and not to the CRT molecule per se. It is further shown that CRT molecules can be complexed in vitro to unglycosylated peptides and used to elicit peptide-specific CD8⁺ T cell response in spite of exogenous administration. These characteristics of CRT closely resemble those of HSPs gp96, hsp90, and hsp70, although CRT has no apparent structural homologies to them.

Key words: heat shock protein • stress protein • antigen presentation • cytotoxic T lymphocyte response • cancer immunity

Peptide-binding heat shock proteins (HSPs)¹ of the cytosol (hsp70, hsp90) and the endoplasmic reticulum (ER) (gp96) have been shown to elicit antigen-specific cellular immunity to tumor antigens (1–9), viral antigens (10), minor histocompatibility antigens (11), and model antigens (12, 13) expressed by the cells from which the HSPs are isolated. The basis of the general ability of HSP preparations to immunize specifically lies in the observation that hsp70, hsp90, and gp96 are associated with a broad repertoire of peptides, including the antigenic peptides generated in any given cell or tissue (6, 10, 12). Immunogenic HSP-peptide complexes can also be reconstituted in vitro by complexing a given peptide with hsp70 or gp96 (13, 14). The mechanism through which HSP-peptide complexes elicit peptide-specific immunity has been partially elucidated: HSP-peptide complexes are taken up by professional APCs and the peptides are processed and re-presented on the surface of the APC by the MHC I molecules of the APC, which then stimulate antigen-specific CD8⁺ T lymphocytes (15, 16).

The physiological function of the HSP-peptide complexes within the cells of origin is unclear. It has been pro-

posed that HSPs constitute a relay-line of chaperones that transport peptides from the point of their generation in the cytosol to their being loaded onto the MHC I molecules in the ER (17). Specifically, hsp90 and hsp70, the most abundant cytosolic chaperones, have been proposed to transport peptides to the heterodimeric transporters associated with antigen processing (TAPs), whereas gp96, a major chaperone of the lumen of the ER, has been proposed to facilitate assembly of the MHC I-β2 microglobulin-peptide complexes in the ER. Recent studies by Spee and Neefjes (18) have shown that TAP-transported peptides associate with gp96, and indicate that another ER-resident chaperone, calreticulin (CRT), is a recipient of the TAP-transported glycosylated peptides. The studies reported here were undertaken to test whether CRT would, by virtue of its peptide-binding ability and because it is an HSP, elicit tumor immunity if purified from tumor cells, and peptide-specific immunity if complexed with antigenic peptides in vitro.

Materials and Methods

Mice and Antibodies. BALB/cJ and C57BL/6 mice were obtained from The Jackson Laboratory. Antibodies to CRT and protein disulfide isomerase (PDI) were purchased from Affinity BioReagents, and antibodies to gp96 were obtained from NeoMarkers. A rabbit serum to a synthetic peptide derived from the sequence of human Erp57 was a gift from Peter Cresswell (Yale University, New Haven, CT).

¹Abbreviations used in this paper: CRT, calreticulin; ER, endoplasmic reticulum; HSP, heat shock protein; PDI, protein disulfide isomerase; TAP, transporter associated with antigen processing; VSV, vesicular stomatitis virus.

Tumor Cell Lines. Methylcholanthrene-induced fibrosarcomas Meth A and CMS5 (BALB/c) have been described previously (2). Meth A was maintained in ascites formed in BALB/cJ mice by weekly intraperitoneal passage of cells.

Purification of gp96 and CRT. A 40-ml Meth A cell pellet (or equivalent wet weight of mouse liver) was homogenized in 160 ml of hypotonic buffer (30 mM NaHCO₃, 1 mM PMSF, pH 7.1) and a 100,000 g supernatant was obtained. Solid ammonium sulfate was added to bring the solution to 50% saturation. This was centrifuged at 14,000 rpm for 30 min. The precipitate was discarded and the supernatant was subjected to subsequent fractionation at 80% ammonium sulphate. After centrifugation at 14,000 rpm for 30 min the precipitate was solubilized in PBS containing 2 mM Ca²⁺ and 2 mM Mg²⁺. This was applied to a Con A-Sepharose column (Pharmacia). The Con A-bound proteins were used for purification of gp96 as previously described (2). The buffer of Con A unbound material was changed to 25 mM Na citrate buffer, pH 5.3, by PD-10 column (Sephadex G-25; Pharmacia Biotech.). It was then applied to the CM-Sephadex C-50 column. The buffer of unbound of CM-Sephadex was then changed to 19 mM Na-phosphate buffer, pH 6.1 by PD-10 columns. It was then applied to the DEAE-sephacel column and eluted with a linear gradient of 0.15–0.5 M NaCl in 20 mM Na-phosphate buffer. The CRT-containing fractions were identified by immunoblots.

Tumor Rejection Assay. 6-wk-old female BALB/cJ mice were immunized subcutaneously with gp96 or CRT or PBS in a 200 μl vol twice at weekly intervals. Mice were challenged intradermally 1 wk after last immunization with 100,000 live tumor cells. Tumor diameter was measured every 2–3 d.

Complexing In Vitro of Peptide to gp96 or CRT. Gp96 or CRT was mixed with vesicular stomatitis virus (VSV)19 peptide in a 50:1 peptide/protein molar ratio in 0.7 M NaCl in Na-phosphate buffer (for gp96) or 20 mM Na-phosphate buffer (for CRT) and heated at 50°C for 10 min, then incubated at room temperature for 30 min. Excess free peptide was removed with PBS using microcon 10 (Amicon, Inc.).

Induction of Cytotoxic T Cells. C57BL/6 mice were immunized intraperitoneally with 50 μg of gp96 or 27.5 μg of CRT complexed with VSV19 peptide. 10 d later, recipient spleens were removed and splenocytes were stimulated with VSV8 synthetic peptide at 1 μM concentration. After 5 d, mixed lymphocyte tumor cultures were tested for cytotoxicity in a Cr-release assay using EL4 cells alone and EL4 cells pulsed with VSV8 peptide as targets.

Results

Tumor-specific Immunogenicity of Tumor-derived gp96 and CRT Preparations. Apparently homogenous preparations of gp96 and CRT were obtained from Meth A cells. The two proteins were recognized by the respective antibodies on immunoblots (Fig. 1). As CRT has a mol wt very similar to two other chaperones, PDI (18) and Erp57 (28), CRT preparations were immunoblotted with antibodies to each, as described in Materials and Methods. Although the antibodies recognized the cognate proteins in control blots, neither antibody detected a corresponding protein in purified CRT preparations (Fig. 2). BALB/cJ mice were immunized twice at weekly intervals with a 20-μg dose of purified gp96 or an equimolar dose (11 μg and a lower dose of 5.5 μg) of CRT and were challenged with 100,000 live Meth A cells 1 wk after the second immunization. Tu-

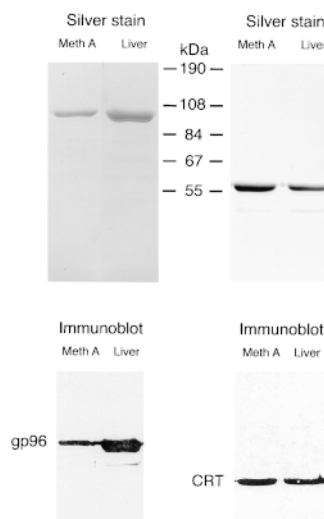


Figure 1. Gp96 and CRT preparations. Gp96 and CRT purified from Meth A sarcoma and liver of BALB/cJ mice were resolved by SDS-PAGE, silver-stained, or immunoblotted using antibodies as described in Materials and Methods.

mors grew progressively in all buffer-immunized mice, but mice immunized with the Meth A-gp96 or Meth A-CRT preparation were equally protected from tumor growth. The immunogenicity of CRT was dose dependent, as mice immunized with half the effective dose succumbed to tumor challenge. Furthermore, mice immunized with Meth A-CRT were found to be not resistant to challenge with the antigenically distinct BALB/cJ fibrosarcoma CMS5, indicating the tumor-specificity of immunogenicity of Meth A-CRT. In a further demonstration of specificity, immunization with homogenous preparations of gp96 or CRT from BALB/cJ mouse liver did not elicit immunity to challenge with Meth A sarcoma at any dose tested (Fig. 3).

Because Con A chromatography was used in purification of both gp96 and CRT and because Con A is known to stimulate monocytes and T lymphocytes, the possibility that the immunogenicity of CRT and gp96 preparations observed here derives from a Con A contamination of our preparations should be considered. Three observations rule out this possibility: the specificity of immunogenicity of tumor-derived HSP preparations, as has been demonstrated repeatedly in several studies from our laboratory (1, 2) and by other groups (11) argues against such a nonspecific effect. Furthermore, in a routine, periodic screening of gp96 preparations with anti-Con A antibodies over the years, such contamination has not been observed, nor has inclusion of α-methyl pyranoside in the immunizing preparations reduced the immunogenicity of HSP preparations (data not shown).

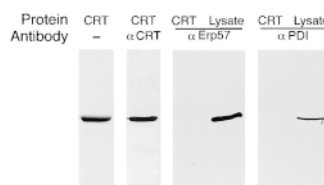


Figure 2. CRT preparation is free of other peptide-binding proteins of similar size. CRT preparations from Meth A sarcoma were resolved on SDS-PAGE and were immunoblotted with antibodies to Erp 57 or PDI (as described in Materials and Methods). The lane marked – was silver-stained directly and was not immunoblotted. Meth A cell lysate was used as source of protein for each immunoblot.

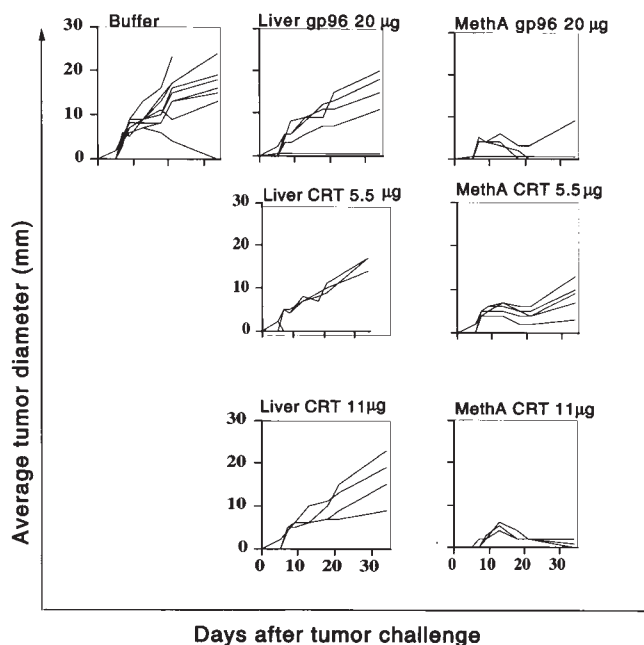


Figure 3. Immunogenicity of CRT and gp96 preparations from Meth A sarcoma. Mice were immunized with 20 µg of gp96 or 5.5 and 11 µg of CRT (molar equivalent of 10 and 20 µg of gp96) preparations from Meth A sarcoma or normal liver of BALB/c mice as indicated. Immunization was carried out in 200 µl vol subcutaneously twice at a weekly interval. Mice were challenged with 100,000 live Meth A cells intradermally 1 wk after the last immunization. Each line represents the kinetics of tumor growth in one mouse.

Association of CRT Molecules with Peptides In Vivo and In Vitro. The immunogenicity of tumor-derived but not normal tissue-derived CRT is reminiscent of similar observations with the HSPs gp96, hsp90, and hsp70. In those instances, the immunogenicity was demonstrated to derive from HSP-associated peptides and not the HSPs per se. The possibility that CRT molecules are associated with peptides was considered. Because CRT is an ATP-binding protein (19, 20), and as hsp70-ATP interaction has been shown to result in dissociation of peptides from hsp70 (6), the ability of ATP to dissociate peptides from CRT was tested. It was observed that treatment of homogeneous preparations of CRT with ATP did not dissociate peptides from CRT under conditions that led to dissociation of peptides from hsp70 (Fig. 4). Interestingly, treatment of gp96 with ATP also did not result in elution of associated peptides, although gp96 is an ATP-binding protein (21). These observations must be considered with the caveat that ATP binding by CRT might require conditions other than those used for hsp70, hsp90, or gp96. Although this possibility is considered unlikely because of the conserved ATP-binding domains present in ATP-binding proteins (22), our observations do not rule it out.

The possibility that the peptides bound to CRT in vivo can be exchanged with exogenous peptides at high temperatures was tested. The procedure shown to be effective with other HSPs (13) was tested. CRT preparations, and gp96 preparations as a positive control, were incubated

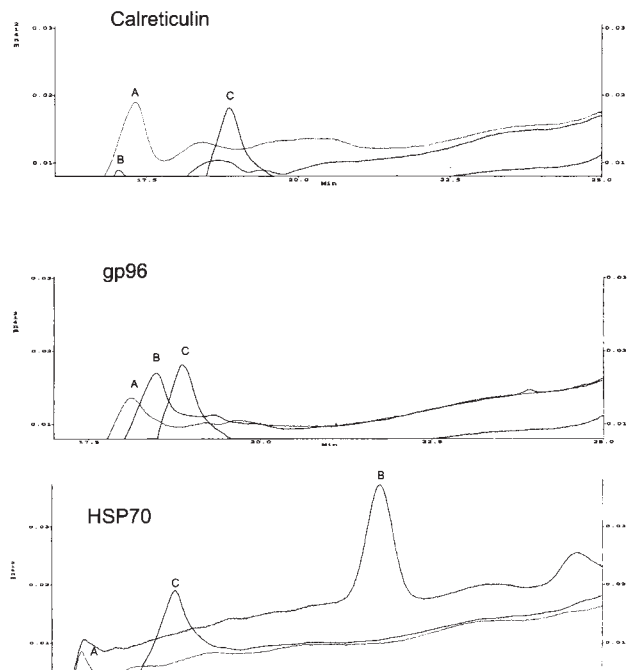


Figure 4. Interaction of CRT, gp96, and hsp70 with ATP or ADP. CRT, gp96, or hsp70 were purified from Meth A cells and were incubated with Tris-buffer containing 150 mM NaCl and 1 mM dithiothreitol at pH 8.0 or the above buffer containing 10 mM ATP or ADP and 2 mM Mg²⁺ and 0.5 mM Ca²⁺ at room temperature for 2 h. The mixtures were centrifuged through centricon 10 and the eluates were applied to a reverse phase column R2H. The x-axis represents elution time and the y-axis represents the absorbance at 214 nm. Specific peaks were obtained from hsp70 treated with ATP but not with ADP or with buffer alone. Peptides could not be eluted from gp96 or CRT after treatment with ATP or ADP. A, buffer; B, plus ATP; C, plus ADP.

with radio-iodinated peptide VSV19 (NH₂-Ser-Leu-Ser-Asp-Leu-Arg-Gly-Tyr-Val-Tyr-Gln-Gly-Leu-Lys-Ser-Asn-Val-Ser-COOH which is extended on the NH₂ and COOH termini of K^b-binding VSV nucleocapsid protein epitope VSV8, underlined) at 25°C, 37°C, or 50°C, followed by 30 min at room temperature. Equimolar quantities of each protein were used so that the molar ratio of peptide to protein was the same in each lane. The samples were analyzed by SDS-PAGE followed by Coomassie staining (to determine protein content) and by autoradiography (to determine the bound peptide) (Fig. 5). It was observed that CRT associated with exogenous radiolabeled peptides in a temperature-dependent manner and that CRT-peptide complexes remained stable under conditions of SDS-PAGE. Quantitative analysis of bound peptides indicated that under the conditions used for peptide-exchange in vitro, ~2% of the CRT molecules received exogenous peptides at 50°C, and at a lower temperature such as 37°C only half as many CRT molecules could associate with the exogenous peptide. When gp96 and CRT were used together, such that they had to compete for the same pool of peptides under the same conditions, gp96 was observed to bind peptides two to three times more efficiently than CRT.

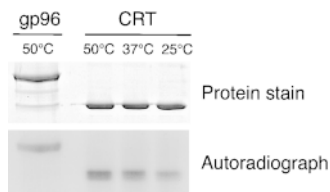


Figure 5. CRT binds to peptides *in vitro*. CRT or gp96 (40 pmol) were incubated with iodinated synthetic peptide (2 nmol, NH₂-Ser-Leu-Ser-Asp-Leu-Arg-Gly-Tyr-Val-Tyr-Gln-Gly-Leu-Lys-Ser-Asn-Val-Ser-COOH) in phosphate buffer in 20 μ l reaction volume at 25°C for 20 min,

37°C for 1 h, or 50°C for 10 min. After a brief centrifugation, the mixtures were incubated at 25°C for another 30 min. Samples were analyzed by SDS-PAGE and staining followed by autoradiography of the stained gel.

Generation of VSV-specific CTLs in Mice Immunized with CRT-peptide Complexes Reconstituted In Vitro. The ability of CRT to act as a CD8⁺ response-eliciting adjuvant was tested. The VSV19 peptide was complexed *in vitro* with either gp96 or CRT at 50°C for 10 min, followed by incubation at room temperature for 30 min. C57BL/6 mice were immunized with the reconstituted complexes (50 μ g of gp96 or 27.5 μ g of CRT complexed with VSV19), or VSV19 alone, or gp96 or CRT alone without complexing. Spleen cells of immunized mice were cultured with the VSV8 and tested for cytotoxic activity on ⁵¹Cr-labeled EL4 cells or EL4 pulsed with the VSV8 peptide as targets. It was observed that spleen cells of mice immunized with CRT-peptide complexes showed peptide-specific CTL activity, whereas splenocytes of mice immunized with peptide or CRT alone showed no cytotoxic activity (Fig. 6). Similar results were obtained with gp96-peptide complexes. Based on the quantity of peptides bound to CRT, these results suggest that \sim 20 ng of VSV19 or 5.7×10^{12} molecules of VSV19 coupled to CRT are sufficient to elicit a CD8⁺ CTL response. The *in vitro* complexing studies were carried out with two variations: those where the CRT used for complexing with peptides was derived from syngeneic C57BL/6 mice and those where it was derived from allogeneic BALB/cJ mice. It was observed that CRT of either haplotype could be complexed to VSV19 and could be used to immunize C57BL/6 mice to elicit an H-2^b-restricted CTL response against VSV8 (Fig. 6).

Discussion

CRT is a calcium-binding protein of the ER and has been shown to be involved in the folding and assembly of MHC I molecules. Recent observations by Spee and Neefjes (18) demonstrate that peptides transported into the lumen of the ER by the TAP molecule associate with gp96 as well as with PD1 and CRT. That study showed the general ability of CRT to bind to proteins as well as to glycosylated peptides of specific sequences inside lumen of the ER. Association of CRT with peptides as well as the evidence of CRT to be associated with MHC I molecule during its folding and assembly (23–25) makes CRT a potential candidate of the relay line of HSPs during antigen processing and presentation by MHC I molecules, as we had suggested previously (17).

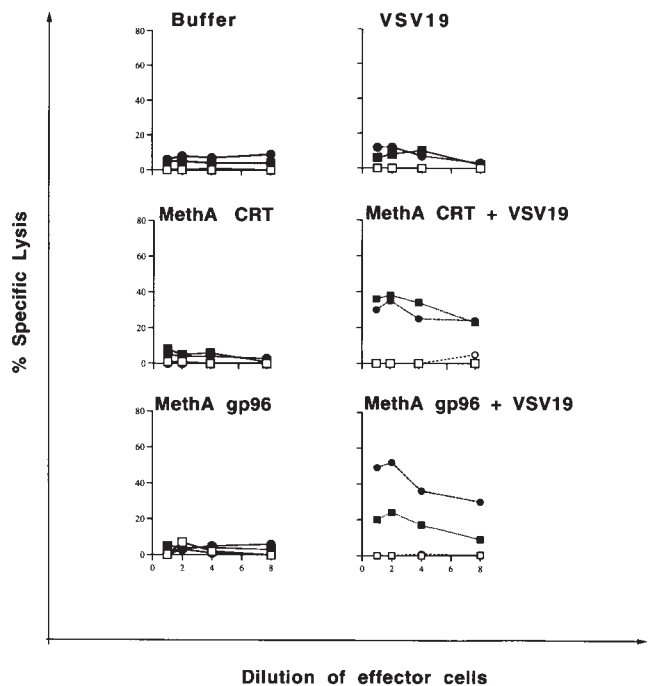


Figure 6. CRT-peptide complex reconstituted *in vitro* primes mice for antigen-specific CTL response. Mice were immunized with gp96 alone (50 μ g), CRT alone (27.5 μ g), peptide alone (50 μ g), gp96-peptide complex (50 μ g), or CRT-peptide complex (27.5 μ g). After 10 d, spleens were removed and stimulated with VSV8 peptide (1 μ M). The lymphocyte cultures were tested for cytotoxic activity on ⁵¹Cr-labeled EL4 cells or EL4 cells pulsed with the VSV8 peptide as targets. Open symbols, EL4; filled symbols, EL4 plus VSV8.

In our studies, CRT and gp96 bind peptide with comparable efficiency *in vitro*, when each is tested alone. In a competition assay, gp96 binds to the longer peptide more efficiently than CRT, but when gp96 is not present in the assay, CRT binds to the peptide as efficiently as gp96. Although Spee and Neefjes (18) demonstrated that only glycosylated peptides are accepted by CRT in their assay, the peptide complexed *in vitro* in the present study is unglycosylated. This suggests either that the requirement for glycosylation of peptides for their acceptance by CRT in the lumen of the ER is not absolute, or that such apparent requirement is a peculiarity of the assay used in the study of Spee and Neefjes (18). The possibility should also be entertained that although CRT has the capacity to bind both glycosylated and unglycosylated peptides, only glycosylated peptides are routed to it *in vivo*, due to the intricacies of the peptide trafficking from the cytosol to the ER. The fact that calnexin, a protein related to CRT, is a chaperone for a wide array of glycosylated integral membrane protein, and has been defined as a lectin (26), is consistent with this possibility. This line of reasoning also raises the question of whether calnexin, as an integral membrane protein or in a soluble luminal form, has the capacity for binding peptides *in vivo* and the capacity to immunize.

The mechanism by which CRT-peptide complex elicits immunity is presently unknown. It is conceivable that it is

similar to the mechanism by which gp96-peptide complexes elicit immunity, which includes a receptor through which gp96 is taken up by the professional APCs and the peptides are re-presented. A crucial question in this regard is whether this is a common receptor for the HSPs or whether the mechanisms are different. This is particularly intriguing since gp96, hsp70, or CRT molecules have no structural homologies.

Binding of peptides by CRT provides evidence of the generality of the peptide-binding property of HSPs and of the immunogenicity of such HSP-peptide complexes. From an evolutionary perspective, considering the broad chaperoning function of HSPs and the association of HSPs with the degradative machinery of cells, this is not surpris-

ing. However, it is interesting to note that the hsp70, hsp90, gp96 and CRT molecules do not share any obvious sequences or other structural homology. The possibility that their peptide binding pockets may share conformational similarities despite the lack of structural homology, clearly exists and will be instructive with regard to evolution of peptide-binding proteins including the MHC molecules (27). Our previous observation that complexes of non-HSPs, such as serum albumin with peptides, do not elicit peptide-specific CTLs (13) suggest that the immunogenicity does not result solely and inevitably from peptide binding and that the immunogenicity of HSP-peptide complexes is unique, deriving perhaps from specific interaction between HSPs and APCs (16).

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Note added in proof. During the course of these studies, we have learned that C. Nicchita, E. Gilboa, and colleagues have independently discovered that calreticulin can elicit, in a cell- and tumor-specific manner, CTL responses (Nair, S., P. Wearsch, D.A. Mitchell, J.J. Wassenberg, E. Gilboa, and C.V. Nicchita. Calreticulin displays in vivo peptide binding activity and can elicit cytotoxic T lymphocyte responses against bound peptides. *J. Immunol.* In press.).

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