HSP70 Natively and Specifically Associates with an N-terminal Dermcidin-derived Peptide That Contains an HLA-A*03 Antigenic Epitope*

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Tumor cells very often have elevated expression of HSP70, the anti-apoptotic properties of which contribute to overall tumor survival. Independent of its anti-apoptotic properties, HSP70 was also suggested to be involved in the antigen presentation process by chaperoning cytosolic peptides, thus protecting them from rapid degradation and securing the peptide pool for further processing. In this study, we identified a 33-amino acid N-terminal dermcidin (DCD)–derived peptide from the repertoire of in vivo HSP70–associated peptides isolated from a leukemic cell line, K562. The DCD peptide has been previously shown to be involved in tumorigenesis, to increase tumor survival rate, to improve tumor stress resistance, and to aid growth. We show that HSP70 is a specific binding partner for the DCD prosurvival peptide and define an ATP-dependent DCD-binding site (GNPCH). We also identify an HLA-A*03 antigenic epitope within the DCD peptide, which follows and partially overlaps the HSP70-binding site (CHEASAAQK). This study describes the interaction between HSP70 and the DCD-derived prosurvival peptide, an interaction that may direct the peptide toward antigen presentation and independently contribute to the prosurvival mechanism mediated by DCD.

HSP70 is an extensively studied protein family that has been proposed to be involved in the antigen presentation process. In the specific experimental setup, tumor purified autologous HSP70 family members, including Hsp72 and Hsc70 (1), have been reported as being capable of eliciting an antigen-specific antitumor immune response (2). The mechanism of the HSP70–mediated antitumor response was suggested to be based on the capacity of HSP70 to bind antigenic peptides and was shown to depend on CD8+ and CD4+ T cells (3, 4). We previously reported a number of natively HSP70–associated peptides that were matched with the reported HLA class I and II epitopes (5). This suggests that HSP70 protects proteasome-generated peptides from further degradation and provides a range of suitably sized peptides for MHC presentation. However, the antitumor response mediated by HSP70 depends not only on the peptide cargo but also on HSP70 itself, i.e. HSP70 has been shown to stimulate natural killer cell responses to tumor cells (6–9). The stimulation of natural killer cells by HSP70 was reported to lead to increased recognition of tumor-expressed MHC class I chain-related (MIC) molecules A and B and to trigger perforin-mediated apoptosis via NKG2D (8).

Although immunologically competent cells exposed to tumor purified HSP70 can stimulate the antitumor reaction, the elevated levels of intracellular HSP70 observed in tumor cells improve their survival. The prosurvival mechanism of HSP70 was linked to its anti-apoptotic property (10, 11). It was shown that HSP70 inhibits JNK and Bax (Bcl-2–associated X protein), which regulate release of cytochrome c (12, 13). When cytochrome c is released, HSP70 interferes with APAF-1 (apoptotic protease-activating factor 1) and antagonizes the formation of a functional apoptosome (14). HSP70 has also been shown to inhibit TNFα-induced apoptosis subsequent to activation of caspases (15). On the other hand, it was recently reported that HSP70 function in tumor cells is not predominantly linked to its anti-apoptotic activity but rather to its role in maintaining protein homeostasis, sustaining functional lysosomes and autophagy (16). Accordingly, the mechanism by which HSP70 mediates tumor survival is not fully understood and remains to be elucidated.

In this study, we analyzed in vivo HSP70–associated peptides and found a dermcidin (DCD)2–derived peptide that was previously shown to have prosurvival functions in tumor cells and an unknown mechanism of action. It was shown that DCD was expressed constitutively only in sweat glands and in some parts of the brain (the pons and the paracentral gyrus of the cerebral cortex); however, up-regulation of DCD was also reported in a range of different human tumors (17–19). It was demonstrated that DCD promoted tumor growth and survival, which depended on the N-terminal fragment of DCD (18, 19). This fragment corresponds to a diffusible survival evasion peptide (DSEP) that was previously isolated from culture medium conditioned with a neural cell line exposed to oxidative stress and associated with increased resistance to oxidative stress and immune evasion (20, 21). Although the pathway this N-terminal DCD-derived peptide utilizes in its prosurvival functions remains elusive, the data presented in this work point at the

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2 The abbreviations used are: DCD, dermcidin; DSEP, diffusible survival evasion peptide; IAA, iodoacetamide; ATP5S, adenosine 5′-(y-thiotriphosphate); mo-iDCs, monocyte-derived immature dendritic cells; PIF, proteolysis-inducing factor.
specific interaction with HSP70 and may provide an insight into the prosurvival mechanism mediated by DCD.

In this study, we show that HSP70 specifically interacts with a DCD-derived prosurvival peptide that contains the HLA-A*03 epitope and has the capacity to induce the T cell response. Therefore, this work explores the ubiquitous chaperoning function of HSP70 benefiting two unrelated cellular processes.

**EXPERIMENTAL PROCEDURES**

**HSP70-associated Peptide Identification** —The K562 leukemic cell line was grown and lysed as described previously by Stocki et al. (5). Approximately 10 g of K562 cell pellet was used for HSP70 purification. HSP70 purification was performed according to the method of Peng et al. (1) with the modifications as outlined by Stocki et al. (5). In brief, HSP70 was purified on an ADP-agarose column (A2810, Sigma) and subjected to buffer exchange on Sephadex™ G-25 (GE Healthcare) to 30 mM ammonium hydrogen carbonate. The samples were freeze-dried and resuspended in 0.1% (v/v) TFA and 50% acetonitrile before drying in a SpeedVac system.

**Peptide Array Affinity Assay** —The peptides were custom-synthesized (GenScript). All peptides had the same amino acid composition with a purity of >95% and a length of 33 residues. Peptides were biotinylated at the N termini with amnicaproic acid as a linker and were amidated at the C termini.

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**CDC Expression at the mRNA Level** —Total RNA was isolated from either the fresh K562 or CCRF-CEM leukemic cell line using an RNeasy mini kit (Qiagen). 1 μg of total RNA was denatured at 65 °C for 5 min. Reverse transcription was performed using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in 1 × Moloney murine leukemia virus reverse transcription buffer (Invitrogen) in RNase-free water (Qiagen) supplemented with 1.5 mM dNTP mixture (Roche Applied Science), 1 unit of RNasin/μl of reaction mixture (Promega), 10 μM random hexamers (GE Healthcare), and 10 mM DTT (Invitrogen). The reverse transcription reaction mixture was incubated for 1 h at 37 °C, followed by a deactivation step for 10 min at 65 °C. PCR was performed using 1 μl of cDNA, 0.1 unit/μl Taq DNA polymerase (New England Biolabs), 1 × GoTaq reaction buffer containing 1.5 mM MgCl₂ (Promega), 0.2 mM dNTP (Fermentas), and a 0.5 μM concentration of each primer. The DCD-specific primers used were 5′-GCATAAAATC-TGCAGGCGTAAGTATACTAAC-3′ and 5′-ACGGTCTGTGGTTGCTATACCA-3′, giving a product of 322 bp. The GAPDH-specific primers 5′-TGGATGAGAAGGAATGTTGGAG-3′ and 5′-CTCC-TTGGGAGGCATAGTGGGCTAT-3′ were used as a control to amplify a 240-bp product. The PCR conditions were as follows: one cycle at 94 °C for 3 min; three cycles at 92 °C for 30 s, 64 °C for 1 min, and 72 °C for 1 min; three cycles at 92 °C for 30 s, 61 °C for 1 min, and 72 °C for 1 min; 35 cycles at 92 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min; and one cycle at 72 °C for 5 min. The PCR products were run on a 2% agarose gel with a 100-bp marker (Promega) to determine the product size.

**CDC Expression at the Protein Level** —Approximately 8 × 10⁵ lysed K562 or CCRF-CEM cells were subjected to 15% SDS-PAGE. Samples were heated for 10 min at 95 °C with standard sample loading buffer at a final concentration of 60 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, and 0.02% (w/v) bromphenol blue.

Where indicated, a cysteine-blocking protocol was used. The cell lysate was incubated with 1 mM tris-(2-carboxyethyl)phosphine for 1 h at room temperature, followed by incubation with 5 mM iodoacetamide (IAA) for 1 h at room temperature. Before running on a gel, the cysteine-blocked samples were heated for 10 min at 95 °C with modified sample loading buffer at a final concentration of 60 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, and 0.02% (w/v) bromphenol blue. Proteins were transferred from the gel using a wet transfer system (Bio-Rad) onto a nitrocellulose membrane (0.2-μm pore size; Bio-Rad).

DCC was detected with goat-specific anti-human primary antibody (1:250 in TBS; clone A-20; sc-27467, Santa Cruz Biotechnology). GAPDH was detected with mouse-specific antibody (MAB374, Millipore), followed by HRP-conjugated anti-goat secondary antibody (1:10,000 in TBS; sc-2020, Santa Cruz Biotechnology) or anti-mouse antibody (1:10,000 in TBS; A5278, Sigma) and visualized with ECL Plus detection reagents (GE Healthcare).

**Peptide Array Affinity Assay** —Customized peptide arrays were ordered from JPT Peptide Technologies. Peptides were synthesized at 5 nmol/spot with acetylated N termini and covalently bound by C termini with a polyethylene glycol linker to the cellulose membrane. Two isoforms of HSP70 were analyzed in the affinity assays: recombinant Hsp72 (NSP-555, Assay Designs) and recombinant Hsc70 (SPP-751, Assay Designs). The peptide arrays were blocked with 3% (w/v) skim milk powder in TBS for 3 h and then incubated with 100 nM Hsp72/Hsc70 in TBS overnight at 4 °C. The binding of the proteins to the peptides was detected after a 1-h incubation with mouse anti-Hsp72 (1:5000 in TBS; SPA-810, Assay Designs) or rabbit anti-Hsc70 (1:5000 in TBS; SPA-816, Assay Designs) primary antibody and a 1-h incubation with HRP-conjugated anti-mouse (1:10,000 in TBS; SAB-100J, Assay Designs) or anti-rabbit (1:10,000 in TBS; A0545, Sigma) secondary antibody. The membranes were washed three times for 10 min with TBS after primary antibody incubation. After secondary antibody incubation, the membranes were washed three times for 5 min with 1% (w/v) SDS and 7% (v/v) 2-mercaptoethanol in TBS, and washing was continued if the background signal was not eliminated. A negative control was performed for each experiment by excluding Hsp72/Hsc70 from the incubation protocol. The detection was performed with ECL Plus detection reagents. The membranes were black-to-white-inverted, and the intensities of the spots were analyzed with ProScan software (PerkinElmer Life Sciences).

**Biotinylated Peptide Affinity Assay** —The peptides were custom-synthesized (GenScript). All peptides had the same amino acid composition with a purity of >95% and a length of 33 residues. Peptides were biotinylated at the N termini with amnicaproic acid as a linker and were amidated at the C termini.
15 μl (~9 × 10^5 cells) of K562 cell lysate (adjusted to 120 mM NaCl and 10 mM Tris-HCl (pH 7.6)) or 26.4 pmol (~1.85 μg) of recombinant Hsp72 was incubated with 396 pmol (~1.4 μg) of each peptide in TBS for 10 min before running on a native polyacrylamide gel. Where indicated, the reaction was supplemented with 10 mM ATP, ADP, or ATPγS.

Native Gel Electrophoresis—Native gels (4% stacking and 15% resolving) were prepared without SDS. Native sample loading buffer at a final concentration of 60 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.02% (w/v) bromphenol blue was added before the samples were loaded onto a gel. Proteins were transferred from the gel onto a nitrocellulose membrane (0.2-μm pore size). For detection of biotinylated peptides, HRP-conjugated streptavidin (1:5000 in TBS; ab5439, Abcam), followed by HRP-conjugated antimouse secondary antibody (1:10,000 in TBS), was used. Visualization was performed with ECL Plus detection reagents.

Generation of Peptide-responsive T Cells—Healthy donors were serologically typed for HLA-A*03 (ab31572, Abcam). T cells from HLA-A*03-positive donors were isolated using a T cell enrichment mixture (STEMCELL Technologies). Three peptides were commercially synthesized at a purity of >95% (GenScript) and tested for their antigenic potential: KDI (KDILPRHIQ), DCDp (CHEASAAQK), and Pmel-17/gp100 (ALLAVGATK). Autologous monocyte-derived immature dendritic cells (mo-iDCs) were generated (24) in serum-free CellGro dendritic cell medium (CellGenix), pulsed with a 10 μM concentration of each peptide, and used to stimulate T cells at a 1:10 ratio. The cells were plated at a concentration of 0.7 × 10^6/ml in 6-well plates with the addition of 0.5 IU/ml IL-2. After 2 days of incubation, 20 IU/ml IL-2 and a 5 μM concentration of either peptide were added to the culture and further incubated for 8 days. The cells were then restimulated every 7 days with feeder cells, autologous peripheral blood mononuclear cells pulsed with either peptide (10 μM) overnight, and then irradiated with 20 gray. The T cells were mixed with the feeder cells at a 1:1 ratio in the presence of 10 μM peptide and 20 IU/ml IL-2. The cells were cultured in RPMI 1640 medium (Invitrogen) containing 100 IU/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 2 mM l-glutamine (Invitrogen) supplemented with 10% human AB serum (PAA Laboratories). After 6 weeks, the cells were tested using a FACScan flow cytometer (BD Biosciences) for the activated CD25-expressing CD8+ T cells. The antibodies used were obtained from BD Biosciences: antigen CD8 (clone SK1) and antigen CD25 (clone 2A3). For each test, 100,000 events were acquired. Data were analyzed using FlowJo software (Tree Star Inc.).

Proliferation and IFNγ Secretion Assays—The T cells that were stimulated with the tested peptides were examined for their response when stimulated with the corresponding peptide-pulsed autologous mo-iDCs. The ratio used was 1:10 (0.5 × 10^5 T cells versus 0.5 × 10^4 mo-iDCs). The proliferation assay was performed on 96-well round-bottomed plates (200 μl/well; Greiner Bio-One). On day 4, 50 μl of the medium was collected for IFNγ analysis. The remaining culture was pulsed with [3H]thymidine (TRA310, GE Healthcare) at a concentration of 37 kBq/ml. The cells were further incubated for 16 h, and [3H]thymidine incorporation by proliferating cells was determined using a β-counter (Matrix 9600, Packard Instrument Co.). Each experiment was performed in triplicate. IFNγ secretion was analyzed using a cytometric bead array (BD Biosciences) and analyzed using FCAP Array software (BD Biosciences).

RESULTS

HSP70 Natively Associates with a DCD-derived Peptide Linked to Prosurvival Function in Tumor Cells—HSP70 was purified from the chronic myelogenous leukemic cell line K562. HSP70 was purified using ADP affinity chromatography, which allowed isolation of HSP70 with associated peptides (1, 5). The purification resulted in the isolation of a heterogeneous mixture of at least two cytosolic members of the HSP70 family: inducible Hsp72 and constitutive Hsc70 (data not shown) (5). Peptides isolated with HSP70 were analyzed by mass spectrometric techniques. The raw data analysis was performed with Mascot, which identified a 33-amino acid peptide derived from DCD. The fragmentation spectra of this 3210-Da peptide were further validated by de novo sequencing (Fig. 1 and Table 1). The peptide was derived from the N termini of DCD and localized between residues 20 and 52 of the full protein (gi:16751921) (Fig. 2). The sequence of the N-terminal DCD-derived peptide isolated with HSP70 overlaps with the reported prosurvival peptide DSEP, which is positioned between residues 20 and 49 of DCD (gi:16751921) (Fig. 2). In comparison with DSEP, the peptide isolated with HSP70 is 3 amino acids longer on the C-terminal end. The HSP70-isolated N-terminal DCD-derived peptide also shares residual homology with the proteolysis-inducing factor (PIF) peptide. PIF is, however, 13 amino acids shorter on the C-terminal end compared with the DCD-derived peptide isolated with HSP70. In addition, PIF has one residual substitution in position 15 (cysteine for serine) in comparison with DSEP or the DCD peptide reported for serine) in comparison with DSEP. Although DCD has been reported to be expressed in a number of tumors, it has not been previously shown to be expressed.

FIGURE 1. Fragmentation spectrum of the N-terminal DCD-derived peptide isolated with HSP70. HSP70 was purified from the K562 cell line and fragmented with a Finnigan LTQ-FT mass spectrometer. *, the most intense peaks that correlated with the DCD sequence (Table 1). m/z is the mass-to-charge ratio of ions. The DCD-derived peptide sequence was determined by de novo sequencing to be YDPEASAPGGNPECHEASAAQKENAGEDPGLA (Table 1). Other HSP70-associated peptides were described previously by Stocki et al. (5).
by the K562 cell line (17–19). Therefore, to exclude the possibility of contamination or misinterpretation of the mass spectrometric data, the expression of DCD in the cell line was analyzed. Reverse transcription followed by PCR with specific primers was shown to produce a 322-bp band, as was expected for the DCD product (Fig. 3A). GAPDH-specific primers were used as a control and gave the PCR product of the desired size (Fig. 3A). Another leukemic cell line, CCRF-CEM, was tested using PCR and was shown not to express DCD (Fig. 3A).

The expression of DCD at the protein level was also investigated. The expected size of DCD is ~12 kDa with a signal secretory peptide or 9 kDa without (Fig. 2). Under standard SDS-PAGE conditions, followed by Western blotting, multiple bands were detected, but no bands at 12 or 9 kDa were seen (Fig. 3B). Bands of ~66, 52, 41, 34, and 24 kDa appeared repeatedly with different intensities of the corresponding bands depending on the primary antibodies used (data not shown).

The biochemical properties of DCD were extensively studied previously and showed that DCD predominantly localizes at ~20–24 kDa (21, 25–27) and 66–69 kDa (20, 25, 26) when run on a reducing SDS-polyacrylamide gel. The 20–24 kDa band was shown previously to be the glycosylated form of DCD, whereas 66–69 kDa band was found to be unglycosylated and in a complex with albumin (25). Our observations of DCD species distribution were in accordance with the published data, thus suggesting post-translational modification of DCD and/or formation of oligomers.

In standard loading buffer for SDS-PAGE, 2-mercaptoethanol is used as a reducing agent to break disulfide bonds. However, on a running SDS-polyacrylamide gel, the 2-mercaptoethanol might be diluted to a suboptimal reducing concentration, thus allowing disulfide bonds to reform and hence the production of DCD complexes. The possibility that DCD forms complexes under standard reducing SDS-PAGE conditions was tested. To avoid disulfide bond reformation when running a SDS-polyacrylamide gel, cysteines were permanently blocked with 5 mM IAA, which alkylated the sulphydryl groups of cysteines and therefore prevented disulfide bond reformation. Permanent blocking of cysteines with IAA produced a band of ~9 kDa and reduced the intensity of the band at 66 kDa (Fig. 3B).

This experiment was repeated with different DCD-specific antibodies, each time producing a 9-kDa band when sulphydryl groups were alkylated with IAA (data not shown). This suggests that DCD is a highly reactive protein that creates oligomeric forms when run on a SDS-polyacrylamide gel. The CCRF-CEM cell line lysate was used as a negative control for Western blotting and showed no nonspecific reactivity with the antibodies (Fig. 3B). Thus, the expression of DCD was confirmed at both the mRNA and protein levels in the K562 cell line (Fig. 3).

**Specific and ATP-dependent Interaction of HSP70 with DCD—** A peptide array was designed with 19 DCD-derived peptides synthesized and bound to a cellulose membrane. The peptides were 20 amino acids in length, with each subsequent peptide on the array moved by 5 amino acids in the sequence toward the C-terminal end. This produced an array of 19 peptides, each with a 15-amino acid overlap of the preceding peptide. The N-terminal DCD-derived peptide, which was isolated with HSP70, was distributed across 11 peptides, starting from the peptide in position 1 (Fig. 4B). The peptide array was incubated with recombinant Hsp72 or Hsc70. The ability of these proteins to bind was detected with specific primary and HRP-conjugated secondary antibodies (Fig. 4A). Both Hsp72 and Hsc70 showed the same binding specificity across the array, and both were observed to bind to peptides 4–7, although Hsp72 did

![FIGURE 2. DCD protein sequence and positions of the HSP70-associated N-terminal DCD-derived peptide, DSEP, and PIF. Shown is the DCD protein sequence (gi:16751921) and the sequences of DSEP and PIF previously published (20, 21, 25). The DCD protein is shown divided into the signal peptide (secretory signal sequence), DSEP (the reported prosurvival peptide; underlined) (20, 21), the propeptide (the DCD fragment localized between two reported functional peptides), and DCD-1 (the reported antimicrobial peptide; underlined) (17), PIF, a glycosylated N-terminal DCD-derived peptide, was suggested to be involved in the development of cachexia (27). Shown in **boldface** is the amino acid mismatch compared with the DCD sequence.](12806_F2.png)

**HSP70 Natively Binds Dermcidin-derived Antigenic Peptide**

| Table 1: De novo sequencing of the DCD peptide isolated with HSP70 from K562 cells |
| --- |
| **y**<sup>+</sup>, y<sup>2+</sup>, and y<sup>3+</sup> are C-terminal fragmentation ions, and b<sup>+</sup>, b<sup>2+</sup>, and b<sup>3+</sup> are N-terminal fragmentation ions in charge states +1, +2, and +3, respectively. The masses of ions are shown as m/z ratios. “No.” indicates the amino acid (aa) position in the peptide sequence. The peaks that correlated with the DCD sequence on the fragmentation spectrum (Fig. 1) are shown in boldface. |
| b<sup>+</sup> | b<sup>2+</sup> | b<sup>3+</sup> | No. | aa | No. | y<sup>+</sup> | y<sup>2+</sup> | y<sup>3+</sup> |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1 | Y | 33 |  |  |  |  |  |  |
| 2 | D | 32 |  |  |  |  |  |  |
| 3 | P | 31 | 1467.15 | 978.44 |  |  |  |  |
| 4 | E | 30 | 1418.63 |  |  |  |  |  |
| 5 | A | 29 | 1354.10 |  |  |  |  |  |
| 6 | A | 28 | 1318.59 |  |  |  |  |  |
| 7 | S | 27 | 1283.07 |  |  |  |  |  |
| 8 | A | 26 | 1239.55 |  |  |  |  |  |
| 9 | P | 25 | 1204.03 |  |  |  |  |  |
| 10 | G | 24 | 1168.50 |  |  |  |  |  |
| 11 | S | 23 | 1103.46 |  |  |  |  |  |
| 12 | G | 22 | 1064.44 |  |  |  |  |  |
| 13 | N | 21 | 1054.97 |  |  |  |  |  |
| 14 | P | 20 | 997.95 |  |  |  |  |  |
| 15 | C | 19 | 949.42 |  |  |  |  |  |
| 16 | H | 18 | 948.65 |  |  |  |  |  |
| 17 | E | 17 | 892.41 |  |  |  |  |  |
| 18 | A | 16 | 891.43 |  |  |  |  |  |
| 19 | S | 15 | 890.43 |  |  |  |  |  |
| 20 | A | 14 | 889.44 |  |  |  |  |  |
| 21 | A | 13 | 888.46 |  |  |  |  |  |
| 22 | Q | 12 | 887.47 |  |  |  |  |  |
| 23 | K | 11 | 886.49 |  |  |  |  |  |
| 24 | E | 10 | 885.49 |  |  |  |  |  |
| 25 | N | 9 | 884.51 |  |  |  |  |  |
| 26 | A | 8 | 883.51 |  |  |  |  |  |
| 27 | G | 7 | 882.52 |  |  |  |  |  |
| 28 | E | 6 | 881.52 |  |  |  |  |  |
| 29 | D | 5 | 880.53 |  |  |  |  |  |
| 30 | P | 4 | 879.53 |  |  |  |  |  |
| 31 | G | 3 | 878.54 |  |  |  |  |  |
| 32 | L | 2 | 877.54 |  |  |  |  |  |
| 33 | A | 1 | 876.55 |  |  |  |  |  |

<sup>**a** These are the most intense peaks observed on the fragmentation spectrum (as shown by an asterisk in Fig. 1) that were correlated with the DCD sequence. Other HSP70-associated peptides were described previously by Stocki et al. (5).</sup>
have a higher relative affinity compared with Hsc70 (Fig. 4A). A negative control was performed with primary and secondary antibodies only and showed no binding to the peptides. The analysis of peptides 4–7 revealed a common 5-amino acid fragment (Fig. 4B). This short fragment (GNPCH) first appeared in peptide 4 and was removed by peptide 8 (Fig. 4). The GNPCH fragment is approximately in the middle of the DCD peptide eluted from HSP70 (Fig. 2). These experiments confirmed specific binding of HSP70 to DCD in vitro and suggest the specific interaction site to be GNPCH in position 31–35 of the DCD sequence.

The DCD-specific interaction site for HSP70 was further examined using custom peptides. Three different 33-amino acid peptides were produced: original (peptide O), derived from the N-terminal end of DCD with the same sequence as isolated from HSP70; inverted GNPCH (peptide I), with an inverted middle fragment (HCPNG); and scrambled (peptide S), with the same residual abundance but in a random order (Fig. 5D). All peptides were biotinylated at the N-terminal end. Peptides O, I, and S were incubated with K562 cell lysate shortly before running on a native gel. Detection of biotinylated peptides was performed with HRP-conjugated streptavidin. Peptide localization was observed exclusively on a protein of 72 kDa, which is the approximate mass of HSP70, with the strongest signal given for peptide O and a much weaker signal for peptides I and S (Fig. 5A).

The same pattern of co-localization was observed for the biotinylated peptides when incubated with recombinant Hsp72 (Fig. 5B). Recombinant Hsp72 was run on a native gel after incubation with each of the peptides at a 15-fold molar excess over the protein. The strongest binding signal was observed for peptide O and the weakest for peptides I and S. The same binding pattern and localization on the native gel were observed when peptides O, I, and S were incubated with cell lysate (Fig. 5A). This suggests that HSP70 specifically recognizes the N-terminal DCD-derived peptide (Fig. 5A). It was not possible to recreate the results with Hsc70 when the same concentration as Hsp72 was used (data not shown).

The mechanism of HSP70 interaction with substrates is dependent on ATP. To determine whether the N-terminal DCD-derived peptide binds to the substrate-binding region of HSP70, the influence of ATP, ADP, and ATPγS (non-hydrolyzable analog of ATP that "locks" HSP70 in the open inert state, as it cannot be hydrolyzed to ADP) on peptide binding was tested. Recombinant Hsp72 was supplemented with 10 mM ATP, ADP,
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or ATPγS upon peptide O addition and run on a native gel (Fig. 5C). The molar concentration of the peptide was 15-fold higher than that of recombinant Hsp72. As observed, the addition of ATP or ADP had little or no influence on the binding of the peptide by Hsp72; however, ATPγS completely abolished the interaction. This shows that HSP70 with the ATPγS open binding groove did not bind to the peptide, which would normally capture. This experiment indicates that the N-terminal DCD-derived peptide binds to the substrate-binding site of HSP70 in an ATP-dependent manner. The almost unaffected binding in the presence of ATP can be explained by its hydrolysis to ADP and dilution on a running gel.

Sequence GNPCH of DCD Sufficiently Facilitates HSP70 Binding—We further investigated the DCD interaction site, GNPCH, by performing alanine substitutions in this sequence to determine the crucial residues responsible for facilitating the interaction with HSP70. The peptide array analysis revealed that proline-to-alanine substitution had the greatest impact on the interaction (GNPCH to GNACH); however, glycine and asparagine substitutions were also shown to have a detrimental effect (Fig. 6, A and C). On the other hand, a cysteine replacement did not produce any affinity variations, whereas histidine substitution was shown to only marginally increase relative affinity for recombinant Hsp72 (Fig. 6, A and C).

Next, we tested if the GNPCH fragment is sufficient to facilitate HSP70 interaction with peptides. We speculated that the addition of the HSP70 interaction site to antigenic peptides might target them to HSP70 and increase their presentation on MHC molecules and thus be beneficial for antigen-based anti-tumor therapy. We examined two known HLA-A*03 antigens: Pmel-17/gp100 (Fig. 6C, peptide 7) and influenza A virus nucleoprotein-derived peptide (peptide 10). Either the GNPCH or GNACH fragment was added to the peptides at their N termini, and the relative affinity for Hsp72 was tested. The addition of GNPCH was shown to improve the relative peptide binding to Hsp72 by approximately 4- and 10-fold for Pmel-17/gp100 and influenza A virus nucleoprotein-derived peptide, respectively (Fig. 6, B and C). GNACH-extended variants of these peptides showed only a marginal change in the peptide affinity (Fig. 6, B and C).

HSP70 Interaction Site in DCD (GNPCH) Is Followed by the HLA-A*03 Antigen (CHEASAAQK), Capable of Inducing the T Cell Response—HSP70 has been shown to be involved in the antigen presentation process; therefore, we investigated if the DCD-derived peptide, which was found to associate in vivo with HSP70, has any antigenic potential. A computational approach using the SYFPEITHI algorithm allowed us to identify, within
the DCD-derived peptide purified with HSP70, a putative HLA-A*03 antigen, CHEASAAQK (DCDp) (Fig. 7D). DCDp scored 21 using SYFPEITHI, which correlates with a moderate presentation of this epitope on HLA-A*03 (Fig. 7D). In comparison, the known antigenic peptide Pmel-17/gp100 produced a score of 38, whereas the non-antigenic peptide KDI scored only 8 (Fig. 7D).

We further tested the antigenic potential of DCDp in vitro using cells obtained from HLA-A*03-positive donors. Purified T cells were stimulated with peptide-pulsed autologous feeder cells and tested for activated (CD25+) CD8+ T cell expansion. The KDI peptide was used as a negative control, with Pmel-17/gp100 as a positive control. After six rounds of restimulation with the peptides, the cells were tested by flow cytometry and showed that 95.9% of CD8+ T cells stimulated with Pmel-17/gp100 were activated as they coexpressed the activation marker CD25+ (Fig. 7A). 73.7% of CD8+ T cells were shown to be activated when stimulated with DCDp and 40.4% when stimulated with the KDI peptide (Fig. 7A). These results correlated with the observed expansion of each of the stimulated cell populations as well as with the number of clusters formed when stimulated with the pulsed feeder cells (data not shown).

The response of the peptide-stimulated T cells was measured in the proliferation and IFNγ secretion assays. Autologous mo-iDCs were pulsed with one of the peptides (Pmel-17/gp100, DCDp, or KDI) and used as stimulatory cells for the corresponding cell populations. The background proliferation and IFNγ secretion were assessed with non-pulsed mo-iDCs, and the values were subtracted from the readouts obtained when peptide-pulsed mo-iDCs were used. KDI-stimulated T cells showed neither proliferative nor secretory responses to the peptide-pulsed autologous mo-iDCs (Fig. 7, B and C). In contrast, DCDp and Pmel-17/gp100 responded to the peptide-pulsed mo-iDCs; however, Pmel-17/gp100 stimulation was considerably higher compared with DCDp stimulation (Fig. 7, B and C).

**DISCUSSION**

HSP70 was suggested to take part in the antigen presentation process by capturing peptide products of the proteasome and protecting them from further degradation, thus securing a peptide library for presentation on MHC molecules (2). The interaction of HSP70 with peptides may go beyond the antigen presentation process and could provide a more general mechanism of peptide protection from degradation. Accordingly, HSP70 interaction with the DCD-derived peptide should not be considered restricted to antigen presentation only but also might contribute to unrelated processes mediated by DCD, such as its prosurvival function (19–21).
HSP70 Natively Binds Dermcidin-derived Antigenic Peptide

Here, we have observed an interaction between the cytosolic protein and the peptide derived from the secretory protein. The DCD-derived HSP70-associated peptide was found to have a cleaved signal sequence, which strongly suggests that the peptide was initially translocated into the endoplasmic reticulum. We therefore speculate that HSP70 gained access to the DCD peptide by one of the two following mechanisms: after the protein was retrotranslocated to the cytosol, following the endoplasmic reticulum-associated degradation pathway and proteasomal proteolysis (28), or after secreted DCD was endocytosed and targeted to lysosomes, where HSP70 is known to reside (29). Regardless of the mechanism, the HSP70-bound peptide acquired protection from further degradation and was consequently found to be associated with HSP70. We theorize that the DCD-derived peptide utilizes an interaction with HSP70 to extend its survival, thus providing a means to its prosurvival action.

The DCD-derived peptide was found to be associated with HSP70 and shares sequence homology with the previously described functional peptide, DSEP. DSEP is the N-terminal DCD-derived peptide that was independently identified to be associated with prosurvival functions. It was shown in a rat model that the direct application of the DCD peptide to the cerebral cortex after lesion induction reduced cortical neuron atrophy and rescued pyramidal neurons that would otherwise deteriorate (20, 21). DCD itself was suggested to be an oncogene (18), and when expressed, it was shown to increase tumor survival rates (19–21). Prostate cancer cells stably transfected with DCD showed a higher proliferation rate and survival compared with sham transfections (19). The DCD-transfected cells were also reported to be more resistant to oxidative stress and hypoxia (19). The fragment of DCD that is critical for its prosurvival function was previously reported to be at the N-terminal end and to contain, herein identified, the HSP70 interaction site (19). It was also previously observed that substitution of asparagine with glutamine at position 32 (GNPCH to GQPCH), which is localized in the HSP70 recognition site, abrogates the prosurvival function of DCD (30). This therefore shows that the HSP70 interaction fragment is essential for maintaining the prosurvival properties of DCD and consequently suggests an important role for HSP70 in the prosurvival mechanism of DCD action.

Normally, human tissues have a very limited DCD expression; however, its up-regulation was reported in a variety of different tumors, and it was suggested to be an oncogene (18). This characteristic potentially makes DCD a good candidate for antigen-specific antitumor immunotherapy. It was shown previously that HSP70-associated peptides can be a source of antigens and induce a generation of tumor-specific T cells that lead to tumor regression. Although the K562 cell line does not normally express HLA class I molecules, the downstream processes of antigen presentation, such as proteasomal generation of peptide epitopes and their interaction with cytosolic chaperones, remain independent of HLA expression. Consequently, we investigated if the DCD-derived peptide associated with HSP70 has any HLA antigens. Assuming that the type of HLA expressed does not determine the specificity of interaction between HSP70 and cytosolic peptides, the antigen search was not restricted to any particular human HLA type. The computational approach revealed a putative 9-amino acid HLA-A*03 antigen at positions 35–43, which was preceded by the HSP70 interaction site located in region 31–35. The HSP70-binding site, preceding DCDp on N termini, may allow cytosolic trafficking of the DCD peptide to TAP (transporter associated with antigen processing), and when transported into the endoplasmic reticulum, it can be removed by endoplasmic reticulum aminopeptidases, revealing DCDp. This peptide was shown experimentally, in the in vitro assays, to have an antigenic potential and to stimulate T cell responses. Consequently, this shows that HSP70 binds antigenic precursors in vivo, and therefore, it can be assumed that HSP70 takes part in the initial cytosolic stage of the antigen presentation process. We speculate that the function of HSP70 in this process is, however, accidental rather than direct and specific. Thus, chaperoning by HSP70 might consequently drive the DCD peptide toward two unrelated processes: antigen presentation and DCD-mediated prosurvival mechanism.

In conclusion, we have shown that HSP70 is associated in vivo with an N-terminal DCD-derived peptide. We have identified the interaction site, which interestingly was previously linked to the prosurvival function of DCD, thus suggesting a role for HSP70 in the mechanism of its action. Moreover, we found within the DCD sequence an antigenic epitope preceding the HSP70 interaction site, which provides further insight into the role of HSP70 in the initial step of the antigen presentation process. This study also explored the general ability of HSP70 to associate with cytosolic peptides, which appears to have significant and diverse consequences on cellular processes.

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