Calreticulin Is a Binding Protein for Muramyl Dipeptide and Peptidoglycan in RK_{13} Cells†

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ABSTRACT: Calreticulin (CRT) was isolated and identified as a protein in rabbit kidney RK_{13} cells that binds the apoptogenic bacterial cell wall (BCW) components, muramyl dipeptide (MDP) and peptidoglycan (PG). Mannan-agarose purified RK_{13} cell CRT (rCRT) selectively bound sepharose-immobilized L,D-MDP and PG, but not L,L-MDP or D,D-MDP. Purified rCRT and bovine CRT (bCRT) also bound free PG and L,D-MDP demonstrated in bioassays of RK_{13} cell apoptosis. The results suggest that, in RK_{13} cells, (a) CRT is a specific binding protein for both L,D-MDP and PG and (b) CRT binding L,D-MDP or PG is dependent on the stereoisomeric configuration of the dipeptide (L-alanyl-D-isoglutamine) moiety. In addition, the results also suggest that, in RK_{13} cells, the binding of L,D-MDP, L,L-MDP, D,D-MDP, or PG to CRT correlates with their capacities of inducing apoptosis.

Bacterial cell wall (BCW)[1] components [muramyl dipeptide (MDP), peptidoglycan (PG), and lipopolysaccharide (LPS)] act in concert to induce the inflammatory symptoms of acute bacterial infection (*1*) in experimental animal models and presumably in man. MDP or *N*-acetylmuramyl-L-alanyl-D-isoglutamine (L,D-MDP), represents the smallest, nonantigenic BCW component that can exert multiple biological activities and mimic the acute inflammatory response induced by injection of Gram-positive and Gram-negative BCW. L,D-MDP is edemagenic (*2*), pyrogenic (*3*), apoptogenic (*4*), somnogenic (*5*) and immunostimulatory (*6*). Injection of L,D-MDP can induce uveitis (*7*, *8*), meningitis (*9*), arthritis (*10*), and shock (renal, liver, and spleen dysfunction) (*11*). L,D-MDP is cytotoxic to tracheal (*12*), fallopian (*13*), and renal cells and can cause death in aged rabbits that fail to excrete L,D-MDP (*4*). L,D-MDP can affect other cellular functions such as enhancing human Langhans-type cell formation (*14*), stimulation of Ca^{2+} release and bone resorption (*15*), increasing catabolism in chondrocytes (*16*), inducing prostaglandin synthesis, and inhibiting glutamate transporter activity and production in vivo (*7*, *8*). Central to this investigation is the observation that the potency of L,D-MDP's biological activity is dependent upon the stereoisomeric configuration of the dipeptide moiety (*6*, *8*, *9*, *15*−*19*) and that the dipeptide moiety configuration (L-alanyl-D-isoglutamine) is essential for induction of rapid death of RK_{13} cells (*4*). Therefore, the apoptogenic signal induced by L,D-MDP appears to be due to a stereoisomer specific interaction with a regulatory protein.

In the present investigation, a RK_{13} cell protein binding apoptogenic synthetic L,D-MDP and bacterial PG was isolated and identified as calreticulin (CRT) [a ubiquitous multicompartmental and multifunctional protein (*20*−*22*)]. Purified RK_{13} cell CRT (rCRT) selectively bound sepharose-immobilized L,D-MDP and PG, but not L,L-MDP or D,D-MDP. Purified rCRT or bovine CRT (bCRT) was also shown to bind free L,D-MDP and free PG and thus inhibited RK_{13} cell apoptosis induced by PG or L,D-MDP. The results suggest that CRT is an RK_{13} cell protein that specifically binds both L,D-MDP and PG.

MATERIALS AND METHODS

Reagents. Stereoisomers of MDP (L,D-MDP, L,L-MDP, D,D-MDP), peptidoglycan (PG) from *Staphylococcus aureus* (Sa) and *Bacillus subtilis* (Bs), Tris·HCl, SDS (sodium dodecyl sulfate), $CaCl_2$, DTT (dithiothreitol), PBST (phosphate buffer saline-Tween-20), PMSF (phenylmethylsulfonyl fluoride), glycine, Triton X-100, Triton X-114, glycerol, BSA (bovine serum albumin), Dulbecco's modified essential medium (DMEM), antibiotic and antimycotic solution, phenol solution, phenol:chloroform:isoamyl alcohol (25:24:1), ethidium bromide, cyanogens bromide (CNBr) activated sepharose 6MB (Seph-6MB), horseradish peroxidase (HRP) conjugated secondary goat anti-rabbit IgG (Fab specific), and bovine liver calreticulin (bCRT) were purchased from Sigma (St. Louis, MO). Ethanol and methanol were obtained from Fisher Biotech (Fair Lawn, NJ). Bovine calf serum (BCS) was obtained from BioWhittaker (Walkersville, MD). Rabbit

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[1] Abbreviations: BCW, bacterial cell wall; MDP, muramyl dipeptide; L,D-MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; L,L-MDP, *N*-acetylmuramyl-L-alanyl-L-isoglutamine; D,D-MDP, *N*-acetylmuramyl-D-alanyl-D-isoglutamine; PG, peptidoglycan; CRT, calreticulin; rCRT, rabbit RK_{13} cell CRT; bCRT, bovine CRT; BP, binding protein; MDPBP, L,D-MDP binding protein; PGBP, PG binding protein.

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anti-human CRT antiserum was obtained from Affinity Bioreagents (Golden, CO). SwellGel blue albumin remove kit and metal enhanced DAB substrate kit were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Hybond-P PVDF membrane and ECL+Plus chemiluminescent kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden**)**. TMB peroxidase EIA substrate kit, Precision Plus Protein Dual Color Standards, Quantum prep freeze 'N squeeze DNA gel extraction spin columns, Microspin columns, and Micro Bio-Spin 6 columns were obtained from Bio-Rad (Hercules, CA). Perfect Protein Markers (15−150 kD) were purchased from Novagen (Madison, WI). Polysorp Nunc-Immuno plates were purchased from Nalge Nunc International (Rochester, NY). Tissue culture dishes (6-well, 12-well, and 96-well) and 175 cm^2 culture flasks were obtained from Sarstedt, Inc. (Newton, NC).

Tissue Culture. Rabbit kidney RK_{13} cells were provided by Dr. Dennis O'Callaghan (Department of Microbiology and Immunology, LSU Health Sciences Center, Shreveport, LA). Cells were maintained in DMEM supplemented with 10% BCS and antibiotics/antimycotics as previously described (*4*). For experiments, cell suspensions in culture medium were pipetted into 6-well culture dishes (1×10^6 cells/mL) and incubated at 37 °C in a humidified 5% CO_2 atmosphere.

DNA Ladder Analysis for Apoptotic Cells. Apoptotic DNA ladders were identified as previously described (*4*). Briefly, 0.4 mL of cell lysis buffer (50 mM Tris·Cl, 10 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, pH 7.6) was added to cells in 6-well culture plate cultures and stored at −70 °C. The cell lysate was phenol extracted, and nucleic acids were precipitated by ethanol and recovered by centrifugation at 12000*g*. The pellets were washed with 70% ethanol and 100% ethanol in succession and air-dried for 5 min. The nucleic acid fraction was dissolved in RNase A digestion buffer (50 mM Tris·Cl, 5 mM EDTA, 60 mg/mL RNase A, pH 7.5). Samples were incubated at 37 °C for 60 min to allow complete RNA digestion, and the samples were run on 1.2% agarose gel and DNA bands visualized with ethidium bromide under ultraviolet light. Digital images were obtained using a Bio-Rad gel documentation system and Quantity One software.

Sepharose-Immobilized MDP and PG. The coupling of L,D-MDP, L,L-MDP, D,D-MDP, and PG to Sepharose 6MB was performed by CNBr-activated sepharose 6MB as previously described (*23*).

Isolation of MDP Binding Proteins from RK_{13} Cells Using Sepharose-Bound MDP and PG. For protein isolation and purification, cells were grown in 175 cm^2 culture flasks at 37 °C until confluent. Trypsinized RK_{13} cells from 25 175-cm^2 culture flasks were washed with TSE buffer (10 mM Tris·Cl, 150mM NaCl, 1 mM EDTA, pH 7.4) and lysed with 50 mL of 1× cell lysis/binding buffer [50mM Tris·Cl, 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1% Triton X-100, 0.1% Triton X-114, 0.1% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4] for 30 min at 4 °C with gentle end-over-end rotation. The cell lysate was centrifuged at 10000*g* at 4 °C for 10 min to recover the supernatant (protein sample I). Sample I was adjusted to pH 10 by 10 N NaOH for 5 min before being adjusted back to pH 7.4 by concentrated HCl and then centrifuged to remove any precipitate and recover the supernatant (protein sample II). Protein sample I or II was then applied to sepharose-bound MDP and PG columns, which had been equilibrated by 1× cell lysis/binding buffer. After binding at 4 °C overnight, the columns were washed with 10 volumes of 1× cell lysis/binding buffer, 5 volumes of washing buffer I (1× cell lysis/binding buffer with NaCl adjusted to 0.5 M), and 5 volumes of wash buffer II (1× cell lysis/binding buffer with NaCl adjusted to 0.8 M). Affinity bound proteins were then eluted with L,D-MDP elution buffer (10 μM L,D-MDP, 50 mM Tris·Cl, 100 mM NaCl, 1 mM PMSF, pH 7.4) or gradient pH elution buffers (50 mM Tris·Cl, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, pH 3.5−12.0).

SDS−PAGE Analysis of Sepharose-Bound MDPBPs and PGBPs. Samples containing MDPBPs or PGBPs were boiled in sample buffer (50 mM Tris·Cl, 8% sucrose, 2% SDS, 100 mM DTT, 0.02% bromophenol blue) for 5 min and separated on 8−10% SDS polyacrylamide gel ($C = 4\%$). Gels were stained with Coomassie Blue Brilliant R-250 or silver nitrate.

MALDI-TOF Mass Spectrometry and AutoMS-Fit Analysis of Isolated MDPBP. The purified 56 kDa RK_{13} cell protein on SDS polyacrylamide gel was analyzed with porcine trypsin MALDI-TOF mass spectrometry (LSU-S Research Core Facility) by Voyager System 6147 (PE Biosystems). The spectral data were first performed with baseline correction and noise removal at 2 standard deviations using Data Explorer software (version 4.0). AutoMS-Fit analysis was then conducted against NCBI and Genpept protein databases with AutoMS-Fit software (UCSF).

ELISA of Isolated MDP/PG Binding Protein Samples. Protein samples were diluted with coating buffer (15 mM Na_2CO_3−35 mM $NaHCO_3$, pH 9.6), coated on Polysorp Nunc-Immuno plates at 4 °C overnight, and then washed 3 times with PBST. Rabbit anti-human CRT antiserum and other antibodies in PBST were added and reacted at room temperature for 2 h. Secondary goat anti-rabbit IgG Fab conjugated with horseradish peroxidase was used after primary antibody reaction and washing (3 times, PBST). The plates were then washed 3 times with PBST before colorization was done with TMB peroxidase EIA substrate kit.

Purification of RK_{13} Cell CRT (rCRT) and Bovine CRT (bCRT). rCRT was purified with mannan-agarose affinity chromatography from RK_{13} cells by the methods of White et al. (*24*). Sigma's bCRT protein was further purified with SwellGel blue albumin remove kit according to the manufacturer's instructions.

Western Blotting Analysis. Purified CRT and affinity eluted protein samples were separated by 8−10% SDS−PAGE and transblotted onto Hybond-P PVDF membranes. After blots were probed with primary antibodies and corresponding HRP-conjugated secondary antibodies, ECL+Plus chemiluminescent kit or metal enhanced DAB substrate kit was used to identify the CRT protein.

Bioassay of CRT Binding Free L,D-MDP or PG. Purified rCRT and bCRT samples or BSA control was first reacted with/without a final concentration of 0.4 mM free MDP or 30 μg/mL PG (Sa) in 50 μL of CRT binding buffer (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 1 mM PMSF, pH 7.4) at 37 °C for 1−1.5 h. Each reaction mixture was then transferred into the medium (2 mL) of a 6-well culture of RK_{13} cells. After overnight incubation (16−20 h), the cells were harvested for DNA ladder analysis.

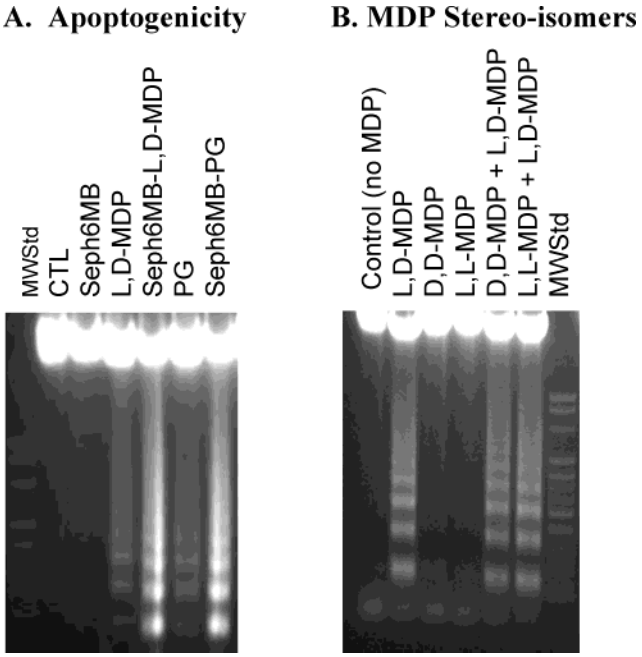

FIGURE 1: The apoptogenic activity of resin-bound MDP or PG. (A) Apoptotic DNA ladders in rabbit RK_{13} cells treated with free and Sepharose 6MB bound MDP and PG. Apoptotic DNA ladders were detected in RK_{13} cells (EB stained) treated with 10 μg/mL L,D-MDP, 30 μg/mL PG_{Sa} (*S. aureus*) for 24 h. (B) Stereoisomer specific apoptogenic activity of MDP. Note that apoptotic DNA was not detected in rabbit RK_{13} cells treated with 10 μg/mL L,L-MDP and D,D-MDP, but was detected in RK_{13} cells treated with 500 ng/mL L,D-MDP. Also, 10 μg/mL L,L-MDP and D,D-MD did not inhibit apoptosis induced by 500 ng/mL L,D-MDP.

RESULTS

Apoptogenicity of Sepharose-Immobilized MDP and PG. Previously we showed that free L,D-MDP was capable of inducing the apoptosis of rabbit kidney RK_{13} cells and that its dipeptide moiety configuration (L-alanyl-D-isoglutamine) was essential for the induction of RK_{13} cell apoptosis (*4*). MDP or L,D-MDP is the structural unit of bacterial cell wall component peptidoglycan (PG). Therefore, the apoptogenic activity of free and resin-bound MDP or PG was investigated. Synthetic L,D-MDP and PG from *S. aureus* (and *B. subtilis*; not shown) immobilized on Sepharose 6MB (Seph6MB) induced apoptosis in rabbit RK_{13} cells demonstrated by apoptotic DNA ladders (Figure 1A). Apoptotic DNA ladder was detected in RK_{13} cells treated for 6 h with just 500 ng/mL L,D-MDP, but not in RK_{13} cell cultures treated with as much as 10 μg/mL L,L-MDP or D,D-MDP for 16 h (Figure 1B). Moreover, 20-fold of L,L-MDP or D,D-MDP did not inhibit L,D-MDP-induced RK_{13} cell apoptosis. The results confirm that the apoptotic signal is stereoisomer specific for the dipeptide (L-alanyl-D-isoglutamine) moiety configuration of L,D-MDP, and suggest that (a) the PG likely contained apoptogenic L,D-MDP and (b) Seph6MB-L,D-MDP and Seph6MB-PG columns may be used for isolating RK_{13} cell proteins that bind MDP and PG.

Isolation of the L,D-MDP and PG Binding Proteins (BP). In order to isolate the RK_{13} cell protein(s) that bind(s) both MDP and PG, solubilized RK_{13} cell proteins (cell lysate) were applied to Seph6MB-L,D-MDP or Seph6MB-PG columns and washed with 800 mM NaCl wash buffer, and the bound proteins were eluted with 10 μM L,D-MDP. No Coomassie-stained protein was eluted from the control Seph6MB column, but 4 Coomassie-stained proteins were eluted from Seph6MB-L,D-MDP column (i.e., 29, 42, 56, and 66 kDa proteins), while 5 Coomassie-stained proteins (45, 56, 66, 72, and 85 kDa proteins) were eluted from Seph6MB-PG column by L,D-MDP (Figure 2A, lane 3 or 5). The results suggest that (a) the above 7 proteins (29, 42, 45, 56, 66, 72, and 85 kDa proteins) are not nonspecifically bound to Seph6MB resin and (b) the 56 kDa and 66 kDa proteins may be the RK_{13} binding protein(s) for a molecule containing L,D-MDP structure since they were eluted from both columns.

An alternative approach was also performed for the purpose of isolating the RK_{13} cell protein(s) that bind(s) both MDP and PG. Gradient pH elution buffers (50 mM Tris·Cl, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, pH 3.5−12.0) were first used to elute Seph6MB-L,D-MDP bound RK_{13} cell protein(s). The eluates were immediately neutralized to pH 7.4 and used in a free L,D-MDP binding bioassay. The neutralized pH 9.5 to 10.3 eluates were capable of binding free L,D-MDP as indicated by their abilities to inhibit L,D-MDP induced RK_{13} cell apoptosis (data not shown). The result suggests that (a) the MDPBP(s) of RK_{13} cells were in the pH 9.5−10.3 eluates and (b) pH 9.5−10.3 buffer elution/neutralization did not damage the L,D-MDP binding capability of RK_{13} cell MDPBP(s). On the basis of these data, the pH of RK_{13} cell lysate was increased to pH 10 for 10 min and then neutralized back to pH 7.4. The high pH pretreated RK_{13} cell lysate (soluble proteins) was then applied to Seph6MB, Seph6MB-L,D-MDP, and Seph6MB-PG columns and washed with 800 mM NaCl wash buffer, and the bound proteins were eluted with 10 μM L,D-MDP. This pH 10 pretreatment resulted in binding only the 56 kDa protein by Seph6MB-L,D-MDP and Seph6MB-PG (Figure 2A, lanes 4 and 6, respectively). The result indicates that the 56 kDa RK_{13} protein is both a MDPBP and a PGBP.

Moreover, the neutralized pH 10 eluate from Seph6MB-L,D-MDP column was directly or indirectly rebound to Seph6MB-L,D-MDP. In the case of direct rebinding, all the proteins in the eluate were readsorbed to the column. However, in the case of indirect rebinding, the neutralized eluate was first reacted with excess free L,D-MDP and then rebound to Seph6MB-L,D-MDP, and only one band of Coomassie-stained protein (56 kDa) was left in the pass-through while all other proteins were readsorbed to the column (data not shown). This result indicates that the 56 kDa RK_{13} protein is capable of binding free L,D-MDP, i.e., it is a specific RK_{13} cell MDPBP.

Characterization of the 56 kDa MDPBP or PGBP. The AutoMS-Fit analysis against NCBI and Genpept protein databases indicated that the MALDI-TOF mass spectrometry of the above 56 kDa protein eluted by L,D-MDP was most similar to the 55 kDa rabbit CRT (i.e., 62% intensity score). This is because (a) 8 of the 21 tryptic peptides of the protein matched the tryptic peptides of rCRT in the databases with 62% intensity matched; (b) 7 of the 21 tryptic peptides of the protein matched the tryptic peptides of rCRT precursor in the databases with 59% intensity matched; (c) 5 of the 21 tryptic peptides of the protein matched the tryptic peptides of bCRT in the databases with 53% intensity matched (Table 1). In addition, the MOWSE scores of rCRT, rCRT precursor, and bCRT were higher (MOWSE = 1180, 445, and 201, respectively) than the scores for enterococcal Na^+-ATPase

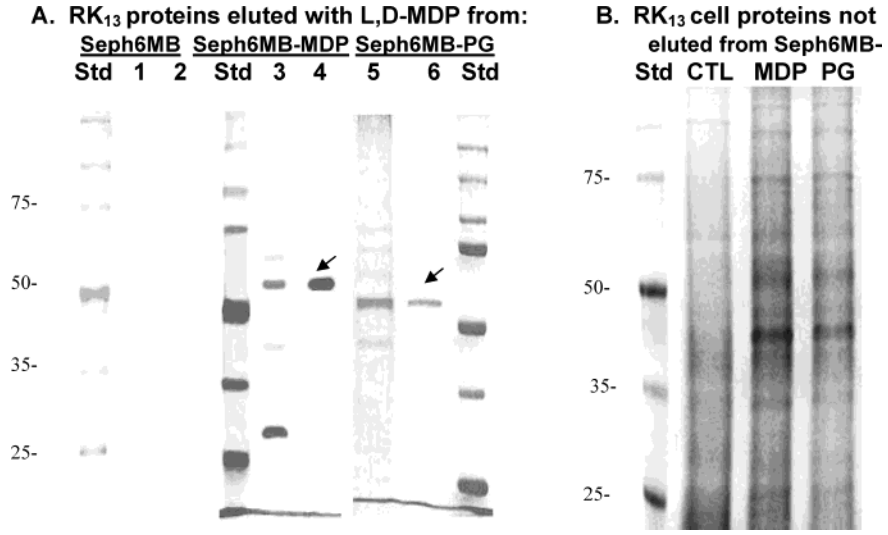

FIGURE 2: SDS−PAGE analysis of RK_{13} cellular proteins that bind MDP and PG. (A) Proteins in RK_{13} cell lysates (pH 7.5) were reacted with Seph6MB, Seph6MB-L,D-MDP, and Seph6MB-PG columns. After washing with 0.8 M NaCl buffer, 0, 4, or 5 Coomassie blue stained proteins were eluted with L,D-MDP (lanes 1, 3 or 5). On the basis of empirical experimental data, proteins in RK_{13} cell lysates were subjected to pH 10 treatment, quickly neutralized to pH7.4, and loaded onto Seph6MB, Seph6MB-L,D-MDP, or Seph6MB-PG columns. After washing with 0.8 M NaCl buffer, no or only one Coomassie blue stained protein (56 kDa; arrows) was eluted with L,D-MDP (lanes 2, 4, or 6). (B) Proteins bound to Seph6MB and the Seph6MB affinity columns that were not eluted with L,D-MDP.

Table 1: AutoMS-Fit Protein Identification of MDP-BP

	protein name	species	MOWSE	no. hit/ no. submitted	MW/p*I*	% intensity matched	av ± SD |λppm|
1	CRT	rabbit	1180	8/21	46652/4.3	62	15 ± 15
2	CRT precursor[a]	rabbit	445	7/21	48275/4.3	59	11 ± 10
3	CRT	bovine	201	5/21	46382/4.3	53	12 ± 12
4	Na^+-ATPase[b]	enterococcus	200	5/21	51142/5.2	11	13 ± 8
5	v-a NSP[c]	murine	179	5/21	65365/7.1	11	31 ± 4

[a] *Oryctola* (rabbit) calreticulin precursor (J05138). [b] Na^+-ATPase subunit B (D17462). [c] Virion-associated nuclear-shuttling protein (AY012159).

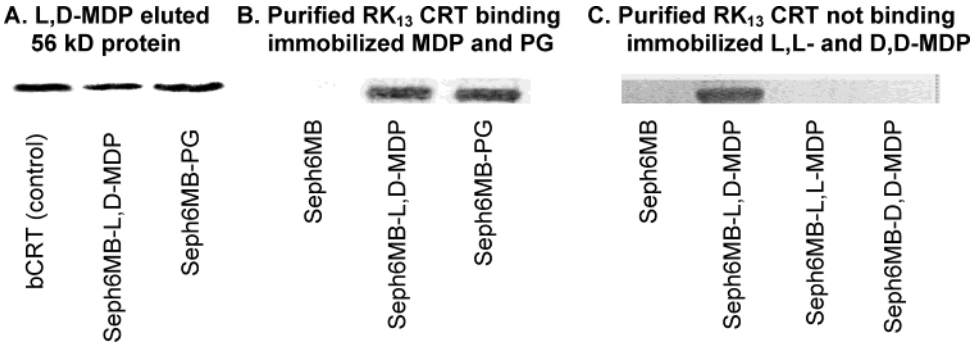

FIGURE 3: RK_{13} CRT (rCRT) binds Sepharose 6MB immobilized L,D-MDP and PG. **(**A**)** L,D-MDP eluted 56 kDa protein from Seph6MB-L,D-MDP and Seph6MB-PG bound with high pH pretreated RK_{13} solubilized proteins. Pure bovine CRT (bCRT) obtained from Sigma was used as positive control. **(**B**)** The mannan-agarose purified rCRT (56 kDa) bound with Seph6MB, Seph6MB-L,D-MDP, and Seph6MB-PG, and eluted by L,D-MDP. (C) The mannan-agarose purified rCRT (56 kDa) bound with Seph6MB, Seph6MB-L,D-MDP, Seph6MB-L,L-MDP, and Seph6MB-D,D-MDP, and eluted by L,D-MDP. The primary antibody used on Western blotting (A, B, and C) was rabbit anti-human CRT antibody.

subunit B protein (MOWSE = 200) and virion-associated nuclear shuttling protein (v-a NSP; MOWSE = 179) (Table 1). Enzyme linked immunoassay and Western blot analysis with rabbit anti-human CRT antibody confirmed that the 56 kDa protein was CRT (Figure 3A). These results suggest that CRT is the specific protein that binds L,D-MDP and PG in RK_{13} cells.

rCRT Binds Immobilized L,D-MDP and PG but Not L,L-MDP and D,D-MDP. In order to determine whether pure rCRT binds resin-immobilized L,D-MDP and PG, rCRT was first purified by mannan-agarose affinity chromatography (*24*) and applied onto Seph6MB, Seph6MB-L,D-MDP, Seph6MB-PG, Seph6MB-L,L-MDP or Seph6MB-D,D-MDP. The columns were then thoroughly washed with 800 mM NaCl wash buffer. The rCRT in the 10 μM L,D-MDP eluates from the columns was detected by rabbit anti-human CRT antibody on Western blotting. Eluates from Seph6MB-L,D-MDP and Seph6MB-PG columns (Figure 3B), but not control Seph6MB, Seph6MB-L,L-MDP, and Seph6MB-D,D-MDP columns (Figure 3C), had L,D-MDP elutable rCRT. The results suggest that (a) purified rCRT can specifically bind resin-immobilized PG and L,D-MDP but not L,L-MDP

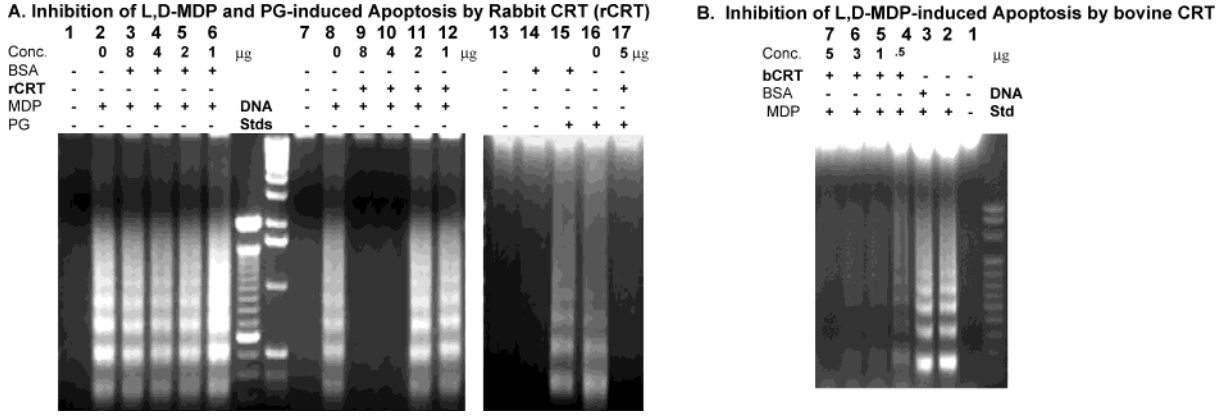

FIGURE 4: Bioassay of purified rCRT and bCRT binding L,D-MDP and PG. (A) Inhibition of L,D-MDP-induced apoptosis by purified rabbit RK_{13} cell CRT (rCRT). L,D-MDP and PG with serial dilutions of purified rCRT or alone were incubated on RK_{13} cells. Note that different amounts of albumin had no effect (lanes 3−6), while 8 and 4 mg/mL purified rCRT (lanes 9 and 10) inhibited L,D-MDP (50 ng/mL; 0.1 nM) induced apoptotic ladders (lane 8) (i.e., compared to no apoptotic DNA ladders in CTL; lane 7). Also, 5 mg/mL albumin had no effect, but 5 mg/mL rCRT (lane 17) inhibited PG (25 mg/mL) induced apoptosis (lane 16). (B) Inhibition by bovine liver CRT (bCRT). Similarly, L,D-MDP mixed with serial dilutions of purified bCRT (0.6 to 5 mg/mL; lanes 4−7, respectively) reduced or inhibited L,D-MDP induced RK_{13} cell apoptosis (lane 2).

and D,D-MDP and (b) the dipeptide moiety configuration of L,D-MDP is essential for L,D-MDP or PG to be bound to rCRT.

rCRT and bCRT Binds Free MDP and PG. To determine if pure rCRT or bCRT binds free L,D-MDP and PG, bioassay of L,D-MDP or PG was performed with purified rCRT or bovine liver CRT (bCRT). The addition of 4 and 8 μg/mL purified rCRT inhibited the RK_{13} cell apoptosis induced by free L,D-MDP, while BSA [serum protein control previously reported not to bind to MDP (*25*)] did not inhibit MDP-induced apoptosis (Figure 4A). Similarly, 5 μg/mL purified rCRT strongly inhibited PG induced RK_{13} cell apoptosis. In comparison, 0.6 to 2.5 μg/mL purified bovine liver CRT significantly reduced and 5 μg/mL strongly inhibited L,D-MDP induced apoptosis in RK_{13} cells (Figure 4B). These results suggest that (a) purified rCRT and bCRT can bind free L,D-MDP and PG and (b) the bindings are dose-dependent.

DISCUSSION

MDP is the smallest component of Gram-positive and Gram-negative PG that induces uveitis, apoptosis of rabbit kidney RK_{13} cells, and death of aged rabbits (*4*, *7*, *8*). The uveitic and apoptogenic activities of MDP are stereoisomer specific for L,D-MDP containing the specific dipeptide moiety (*4*, *8*) because muramic acid (the muramyl moiety of L,D-MDP), like L,L-MDP and D,D-MDP, neither induced RK_{13} cell apoptosis nor inhibited the L,D-MDP induced RK_{13} cell apoptosis (data not shown). This stereoisomer specificity suggests that the apoptogenic signal is the dipeptide (L-alanyl-D-isoglutamine) moiety of L,D-MDP or PG, which specifically interacts with a cell protein(s) that modulates a gene(s) or function(s) critical to cell viability. Therefore, the purpose for this investigation was to isolate and identify the RK_{13} cell protein(s) that specifically binds this dipeptide (L-alanyl-D-isoglutamine) moiety of L,D-MDP or PG.

Although 7 Coomassie blue stained proteins were eluted by L,D-MDP from Seph6MB-L,D-MDP and Seph6MB-PG affinity columns, only the 56 and 66 kDa proteins were eluted from both columns. This suggests that the other 5 proteins (29, 42, 45, 72, and 85 kDa) may be in a complex(es) with these 2 proteins or bind to the other moiety of L,D-MDP or PG. Therefore, the 56 and 66 kDa proteins became our target proteins. The pH 10 pretreatment of RK_{13} cell lysates reduced the number of L,D-MDP elutable proteins from both Seph6MB-L,D-MDP and Seph6MB-PG columns to just one protein (the 56 kDa protein), suggesting that the 66 kDa protein is not a binding protein for both L,D-MDP and PG. All the proteins in the neutralized pH 10 eluate from Seph6MB-L,D-MDP column, after reacting with excessive free L,D-MDP, rebound to the Seph6MB-L,D-MDP column except the 56 kDa protein found in the pass-through. This suggests that the 56 kDa protein could not rebind to the L,D-MDP column because it already bound free L,D-MDP. The result confirms that the 56 kDa protein is a specific MDPBP. Taken together, the above results exclude the 66 kDa protein, along with the other proteins (29, 42, 45, 72, and 85 kDa), as a binding protein for both L,D-MDP and PG. An explanation for the above results is that the 56 kDa protein might have been in a complex with the 66 kDa protein, which resulted in their coelution from Seph6MB-L,D-MDP and Seph6MB-PG columns by L,D-MDP, but the pH 10 pretreatment was capable of dissociating a complex(es) containing the 56 kDa protein.

The above 56 kDa RK_{13} protein was characterized as CRT by mass spectrometry and Western blot analysis. CRT binding was specific for the L-alanyl-D-isoglutamine dipeptide structure because purified rCRT bound Seph6MB-L,D-MDP and Seph6MB-PG, but did not bind Seph6MB-L,L-MDP and Seph6MB-D,D-MDP. More importantly, purified rabbit and bovine CRT bound free L,D-MDP or free PG (and inhibited RK_{13} cell apoptosis induced by L,D-MDP and PG). Therefore, in RK_{13} cells, CRT stereospecifically binds the L-alanyl-D-isoglutamine structure that exists in both L,D-MDP and PG. Moreover, our results suggest that, in RK_{13} cells, the binding of L,D-MDP, L,L-MDP, D,D-MDP, or PG to CRT correlates with their capacities of inducing apoptosis (Figures 1 and 3). However, experiments with the CRT from other cell lines, such as human conjunctival HCC cells or HeLa cells, indicated that not all types of cellular CRT can bind L,D-MDP and PG (data not shown). The reason for this phenomenon is still under investigation. CRT is a

multifunctional 55 kDa Ca^{2+}-binding, lectin-like chaperone for newly synthesized proteins, and appears to play a role in the modulation of cell sensitivity to apoptosis (*26*−*28*). CRT may be found on the cell surface or in the lumen of the endoplasmic reticulum (ER) (*20*, *27*, *29*, *30*). Therefore, CRT-dependent RK_{13} cell apoptosis induced by L,D-MDP or PG may be mediated by the following interactions: (a) interaction of L,D-MDP or PG with CRT in the cytoplasm or ER might modulate the ER form of rCRT, which may further affect normal protein synthesis or activate Ca^{2+}-dependent apoptotic pathways (*26*, *28*); (b) interaction of L,D-MDP or PG with CRT on the cell surface might activate certain apoptotic signaling pathway(s). Induction of RK_{13} cell apoptosis by Sepharose 6MB immobilized L,D-MDP or PG suggests that a MDPBP/PGBP or its complex should act as the receptor of L,D-MDP or PG on the RK_{13} cell surface. At present, whether there is a RK_{13} surface CRT acting as the receptor for L,D-MDP or PG and how the binding of L,D-MDP or PG to CRT transduces the apoptotic signal in RK_{13} cells are being investigated in this laboratory.

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