The Anti-adhesive Activity of Thrombospondin Is Mediated by the N-terminal Domain of Cell Surface Calreticulin*

Received for publication, March 6, 2002, and in revised form, June 19, 2002
Published, JBC Papers in Press, July 29, 2002, DOI 10.1074/jbc.M202200200

Silvia Goicoechea‡, Manuel Antonio Pallero‡, Paul Eggleton§, Marek Michalak¶, and Joanne E. Murphy-Ullrich***

From the ‡Department of Pathology, Division of Molecular and Cellular Pathology and Cell Adhesion and Matrix Research Center, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019, the §Medical Research Council Immunochemistry Unit, University of Oxford, Oxford and Peninsula Medical School, Devon OX1 3QU, United Kingdom, and the ¶Canadian Institutes of Health Research Membrane Protein Research Group and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Thrombospondin (TSP) induces reorganization of the actin cytoskeleton and restructuring of focal adhesions through binding of amino acids (aa) 17–35 (hep I peptide) of thrombospondin to a cell surface form of calreticulin (CRT). In this report we provide further evidence for the involvement of calreticulin in thrombospondin signaling and characterize thrombospondin-calreticulin interactions. Wild type but not crt⁻⁻ cells respond to hep I/TSP. Responsiveness can be restored by incubation of cells with exogenous calreticulin or by transfection with calreticulin. Thrombospondin forms complexes with the CRT-N-domain that are enhanced by physiologic levels of calcium and zinc. Consistent with thrombospondin/CRT-N-domain binding, only the CRT-N-domain blocks hep I- and thrombospondin-stimulated focal adhesion disassembly. A series of glutathione S-transferase-N-domain mutants were used to map the sequence within the N-domain that interacts with TSP/hep I. A construct containing aa 1–43 but not a construct of aa 1–31 supported thrombospondin binding and focal adhesion disassembly. A series of overlapping peptides were used to further map the thrombospondin-binding site. Peptides spanning aa 19–36 (RWIESKHKSDFGK-FVLLS) blocked hep I-stimulated focal adhesion disassembly, indicating that the TSP/hep I-binding site is located in this sequence in calreticulin. A mutant fusion protein lacking aa 19–36 (glutathione S-transferase-CRTA(hep I) failed to restore responsiveness to hep I in crt⁻⁻ cells, bind thrombospondin, or competitively block focal adhesion disassembly, providing evidence for the role of this calreticulin sequence in mediating thrombospondin signaling.

Calreticulin is a major intracellular calcium-binding protein that was first identified in skeletal muscle sarcoplasmic reticulum (1). It is a widely expressed protein that was thought to function primarily as an endoplasmic reticulum (ER) chaperone and regulator of calcium homeostasis (2–5). However, numerous reports have implicated calreticulin in a diverse number of functions and cellular locations. Outside the ER, calreticulin modulates cell adhesion (6–10), integrin-dependent calcium signaling (11), and steroid-sensitive gene expression (12–14). It is also involved in blood function and development (15–17).

Calreticulin can regulate cell adhesion by a number of different mechanisms from both inside and outside the cell. Recent reports indicate that calreticulin may influence cell adhesion indirectly from the ER lumen via modulation of gene expression of adhesion-related molecules such as vinculin and β-catenin (8–10). It has also been shown that calreticulin associates transiently with the cytoplasmic domains of integrin α subunits during spreading and that this interaction can influence integrin-mediated cell adhesion to extracellular matrix (6, 11, 18–20). Calreticulin can also modulate cell adhesion from the cell surface. It has been reported to have a lectin-like function and mediate cell spreading on glycosylated laminin (3, 4, 21, 22). Recently we showed that thrombospondin-induced focal adhesion disassembly is mediated by cell surface calreticulin (23).

Based on the amino acid sequence of the protein, calreticulin can be divided into three distinct structural and functional domains (1). The N-domain (aa 1–180), which corresponds to the highly conserved N-terminal half of the molecule, has a globular β-sheet structure. This domain contains a low affinity, high capacity zinc-binding site ($K_d = 310 \mu M$ and 14 mol of zinc/mol CRT) (24–26). The N-domain is followed by a proline-rich sequence, the P-domain (aa 181–290), and the C-terminal quarter of the protein, the C-domain (aa 291–400). The C-domain of calreticulin is acidic and binds $Ca^{2+}$ with high capacity and low affinity, whereas the P-domain binds $Ca^{2+}$ with low capacity and high affinity (27). It has been shown recently that calcium and zinc ions induce strikingly different changes in the biochemical and structural properties of calreticulin, suggesting the possible importance of these metal ions in modulating calreticulin functions (28, 29).

The abbreviations used are: ER, endoplasmic reticulum; TSP, thrombospondin; CRT, calreticulin; BAE, bovine aortic endothelial; MEF, mouse embryonic fibroblast(s); DMEM, Dulbecco’s modified Eagle’s medium; IRM, interference reflection microscopy; GST, glutathione S-transferase; TBST, Tris-buffered saline containing Tween 20; LRP, low density lipoprotein receptor-related protein; aa, amino acid(s); Fmoc, N-9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography.
Thrombospondin (TSP) is a member of a group of extracellular matrix proteins that exist in both soluble and extracellular matrix forms and that variably regulate cellular adhesion (30–34). When exposed to cells in its soluble form, thrombospondin has primarily anti-adhesive effects characterized by a reorganization of stress fibers and loss of focal adhesion plaques as ascertained by interference reflection microscopy (32, 33, 35). A 19-amino acid sequence (aa 17–35) in the N-terminal heparin-binding domain of thrombospondin, referred to as the hep I peptide, has been shown to be sufficient for focal adhesion disassembly (36). In earlier studies, we showed that thrombospondin binds calreticulin and that a cell surface form of calreticulin mediates the ability of thrombospondin or the hep I peptide to stimulate focal adhesion disassembly and activation of phosphoinositide 3-kinase (23). In this paper, we report that interactions between calreticulin and thrombospondin are Zn$^{2+}$- and Ca$^{2+}$-dependent and involve the RWIESKHSDFGKFVLS$^{35}$ sequence in the N-terminal region of the N-domain of calreticulin.

EXPERIMENTAL PROCEDURES

Materials—The following items were purchased: Dulbecco’s modified Eagle’s medium (DMEM; Cell-Gro, Mediatech); fetal bovine serum (HyClone Laboratories); 500 µg/ml trypsin and 2.2 mM EDTA (Life Technologies, Inc.); glutathione-Sepharose 4B and GammaBind G-Sepharose beads (Amersham Biosciences); stained and prestained molecular weight markers (Bio-Rad), and a chemoluminescence detection kit (PerkinElmer Life Sciences).

Proteins, Peptides, and DNA—Thrombospondin was isolated from fresh human platelets purchased from the American Red Cross. It was purified in the presence of 0.1 mM CaCl$_2$ according to established protocols using heparin affinity and gel filtration chromatography (35). The identification of the 19-amino acid active site in TSP1 (hep I; aa 17–35) has enabled us to substitute the peptide for the TSP1 molecule in several assays. Hep I (ELTGAAARKGSGRRLVKGPD) peptide was synthesized, purified, and analyzed by the University of Alabama at Birmingham Comprehensive Cancer Center/Peptide Synthesis and Analysis shared facility. Overlapping peptides, 15 amino acid residues long, spanning amino acids 13–48 of N-terminal domain of human calreticulin were synthesized as described by Kovacs et al. (37). Briefly, the peptides were synthesized by Fmoc-based solid phase peptide synthesis with a BT7400 manual peptide synthesizer (Biotech Instruments Ltd., Kippton, UK). After lyophilization, the peptides were analyzed by reverse-phase HPLC (Gilon, Anachem, Luton, UK). The details (nucleotide sequences, restriction sites, vectors, etc.) for construction of the GST-CRT domains (N-, P-, and C-domains) have been published (27).

Antibodies—Mouse anti-TSP 133 antibodies were raised against TSP1 depleted of associated transforming growth factor-$eta$ and developed using the Hybridoma Core facility at the University of Alabama at Birmingham (38). Rabbit polyclonal anti-GST antibodies were pur-
complexes were immunoprecipitated with anti-TSP antibody (15 μg/ml) and analyzed by SDS-PAGE and Western blot using anti-GST antibody (1:1000). B, GST-CRT (0.75 μM) and GST-N-domain (0.75 μM) of calreticulin were incubated with purified TSP (0.75 μM) in the absence and the presence of 50 μM ZnCl2. The immune complexes were immunoprecipitated with anti-TSP antibody (15 μg/ml) and analyzed by SDS-PAGE and Western blot using anti-GST antibody (1:1000). These results are representative of three experiments. C, GST-CRT (0.75 μM) and GST-N-domain (0.75 μM) of calreticulin were incubated with purified TSP (0.75 μM) in the absence and the presence of 50 μM ZnCl2. The immune complexes were immunoprecipitated with anti-TSP antibody (15 μg/ml) and analyzed by SDS-PAGE and Western blot using anti-GST antibody (1:1000). These results are representative of three experiments. IP: immunoprecipitated; IB: immunoblot.
adhesions by interference reflection microscopy (IRM) with a specially equipped Zeiss Axiovert 10 microscope. A minimum of 300 cells/condition was evaluated for the presence of focal adhesions. The number of focal adhesions was determined by IRM. The focal adhesions were not affected by the addition of GST or GST-CRT domains alone. The results are expressed as the mean percentages of cells positive for focal adhesions ± S.D. (n = 3). B, BAE cells were preincubated for 30 min with anti-CRT (1/250), anti-N-domain (1/250), or anti-C-domain (1/250) antibody before addition of hep I (1 μM) or TSP (67 nM). The number of focal adhesions was determined by IRM. The focal adhesions were not affected by the addition of anti-CRT, anti-N-domain, or anti-C-domain antibody alone. The results are expressed as the mean percentages of cells positive for focal adhesions ± S.D. (n = 6).

Fig. 4. The N-domain of calreticulin and the anti-N-domain antibody block hep I- and TSP-mediated focal adhesion disassembly. A, TSP (67 nM) was preincubated for 20 min with 1.8 μM of CRT domains (N, P, or C) before addition to BAE cells. The number of focal adhesions was determined by IRM. The focal adhesions were not affected by the addition of GST or GST-CRT domains alone. The results are expressed as the mean percentages of cells positive for focal adhesions ± S.D. (n = 3). B, BAE cells were preincubated for 30 min with anti-CRT (1/250), anti-N-domain (1/250), or anti-C-domain (1/250) antibody before addition of hep I (1 μM) or TSP (67 nM). The number of focal adhesions was determined by IRM. The focal adhesions were not affected by the addition of anti-CRT, anti-N-domain, or anti-C-domain antibody alone. The results are expressed as the mean percentages of cells positive for focal adhesions ± S.D. (n = 6).

RESULTS
Effect of TSP and hep I on Calreticulin-null and Calreticulin Rescued Mouse Embryonic Fibroblasts—We previously reported that TSP/hep I binds calreticulin and that a cell surface form of calreticulin mediates the ability of TSP/hep I to stimulate focal adhesion disassembly (23). To further demonstrate

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—The samples were separated by SDS-polyacrylamide gel electrophoresis (the percentages of acrylamide are indicated in the figure legends) under reducing conditions. After electrophoresis, the gels were stained with either silver or Coomassie Blue or transferred electrophoretically to polyvinylidene difluoride membranes (2 h, 100 V, at 4 °C). Nonspecific protein-binding sites present in the membranes were blocked by incubation with 1% casein in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membranes were then incubated with primary antibodies diluted in TBST (dilutions are specified in figure legends) followed by three 15-min washes in TBST. Antibody binding was detected with appropriate peroxidase-conjugated secondary antibodies and developed by enhanced chemiluminescence according to the manufacturer’s instructions (PerkinElmer Life Sciences).

Soluble Complex Formation and Immunoprecipitation—Immunoprecipitation experiments were performed using a monoclonal anti-TSP antibody (monoclonal antibody 133). Native thrombospondin and recombinant GST-CRT, GST-CRT-N-domain, GST-CRT-P-domain, GST-CRT-C-domain, or GST-CRT-N-domain fragments were incubated together in a total volume of 300 μl of DMEM with 0.5% Tween 20 for 1 h at 4 °C. Binding of thrombospondin to GST protein and precipitation of GST-CRT, GST-CRT-N-domain, GST-CRT-P-domain, GST-CRT-C-domain, or GST-CRT-N-domain alone and GST-CRT-N-domain fragments alone were used as controls. The protein complexes were incubated for 1 h at 4 °C with GammaBind G-Sepharose conjugated with anti-TSP antibody (7 μg/ml) in PTO buffer (0.1% ovalbumin, 0.5% Tween 20 in DMEM). Immune complexes were washed with washing buffer (DMEM containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), resuspended in reducing Laemmli buffer, analyzed by SDS-PAGE (10%), transferred to a polyvinylidene difluoride membrane, and detected with rabbit anti-GST antibodies (1:1000) followed by incubation with peroxidase-conjugated anti-rabbit IgG (1:15000). The blots were then developed using the enhanced chemiluminescence as indicated under “Experimental Procedures.”

CRT N-domain Mediates TSP-induced Focal Adhesion Disassembly
the role of calreticulin as a surface receptor for TSP/hep I-induced focal adhesion disassembly, we tested MEF from *crt*<sup>−/−</sup> (K42) and wild type (K41) embryos for their ability to respond to TSP/hep I (39). Because K42 cells do not express calreticulin, they provide an excellent tool to investigate the role of calreticulin in thrombospondin and hep I-stimulated focal adhesion disassembly. As expected, wild type K41 cells were responsive to TSP and hep I-induced focal adhesion disassembly (Fig. 1A). In contrast thrombospondin or hep I did not induce focal adhesion disassembly in *crt*<sup>−/−</sup> cells. Responsiveness to TSP/hep I was restored by stable transfection of *crt*<sup>−/−</sup> cells with calreticulin (K42CRT) (Fig. 1A) (40). These K42CRT cells exhibited cell surface staining for calreticulin (data not shown). Tenascin-C and SPARC also stimulate the loss of focal adhesions but do not appear to utilize calreticulin to signal disassembly (23, 41, 42). To test the whether the K42 cells had generally lost the ability to restructure focal adhesions, we also tested whether SPARC or tenascin-C could induce focal adhesion disassembly in *crt*<sup>−/−</sup> cells. Fig. 1B shows that SPARC and tenascin-C-induced focal adhesion disassembly were unaffected by the lack of calreticulin, indicating that loss of calreticulin does not cause a general unresponsiveness and that the failure to respond is specific for TSP/hep I.

The lack of responsiveness of *crt*<sup>−/−</sup> cells could possibly be secondary to alterations in protein processing as a function of the chaperone activities of calreticulin and not as a direct consequence of the lack of calreticulin on the cell surface. Therefore, we incubated K42 cells with exogenous recombinant calreticulin for a short time to ascertain whether readdition of calreticulin directly to the cell surface could restore responsiveness to hep I/TSP. These experiments showed that the K42 cells are rescued by short incubations with exogenous recombinant GST-calreticulin prior to the addition of hep I (2). GST alone did not rescue calreticulin-null cells. These results are consistent with our earlier observations that signaling in response to hep I occurs through calreticulin on the cell surface and is unrelated to its effects as an ER chaperone (23).

**Thrombospondin Interacts in Vitro with the N-domain of Calreticulin**—To identify the specific region of calreticulin involved in the interaction with TSP/hep I, three regions of calreticulin were expressed as GST fusion proteins and used in this study: the GST-N-domain (aa 1–180); the GST-P-domain (aa 181–290), and the GST-C-domain (aa 290–401). GST was used as a control. We examined direct binding between TSP and CRT domains. Binding studies were assessed by incubating 0.2 μM of each calreticulin domain with 0.2 μM purified TSP or SPARC in DMEM, immunoprecipitating with anti-TSP antibody, and detecting bound GST-CRT domain by Western blotting with anti-GST antibody. There is strong complex formation between the N-terminal domain of calreticulin and thrombospondin and no significant binding to the P- or C-domains (Fig. 3A).

Calreticulin has two distinct Ca<sup>2+</sup>-binding sites: a high capacity site (>25 mol Ca<sup>2+</sup>/mol of protein) and a high affinity site (*K<sub>D</sub>* < 10 μM) (27, 43). In addition to Ca<sup>2+</sup>, calreticulin binds other ions including Zn<sup>2+</sup> (24, 44). In this study, we investigated the effects of Ca<sup>2+</sup> and Zn<sup>2+</sup> on TSP/CRT interactions. These experiments showed that binding of thrombospondin to the N-domain is enhanced in the presence of physiologic levels of Ca<sup>2+</sup> (2 mM) (Fig. 3B). Zn<sup>2+</sup> similarly enhanced binding to the N-domain (Fig. 3C). Thrombospondin did not interact with recombinant GST control in the presence or the absence of Ca<sup>2+</sup> or Zn<sup>2+</sup> (data not shown). We conclude that in this in vitro system, thrombospondin interacts with the N-terminal domain of calreticulin and that this interaction is modulated by divalent cations.

**N-domain of Calreticulin Mediates TSP-stimulated Focal Adhesion Disassembly**—We then investigated whether interactions between calreticulin N-domain and TSP mediate thrombospondin activity. The ability of thrombospondin to stimulate focal adhesion disassembly was examined following preincubation with calreticulin domains (Fig. 4A). Consistent with the binding studies, the N-domain blocks focal adhesion disassembly by thrombospondin. The isolated P- and C-domains have no effect on thrombospondin activity, although these data do not eliminate the possibility that sites within the P- and C-domains can be involved but are not accessible in the absence of the N-domain.

To determine whether the N-terminal domain of calreticulin is important for hep I and thrombospondin-stimulated focal adhesion disassembly as it is expressed on the cell surface, BAE cells were pretreated with antibodies to the N- and C-terminal domains to determine whether they could block

---

**Fig. 5.** N-domain fragments. A, schematic representation of full-length CRT-N-domain and N-domain fragments expressed in E. coli as GST fusion proteins. GST is depicted as a shaded box. The numbers above each box denote the amino acid numbers of mature calreticulin. B, 12% SDS-PAGE of recombinant purified N-domain mutants. The proteins were expressed in a bacterial expression system as GST fusion proteins and purified as described under "Experimental Procedures." MW, molecular weight standards; N, GST-CRT-N-domain (aa 1–180); N1, GST-CRT-N1 mutant (aa 1–134); N2, GST-CRT-N2 mutant (aa 1–83); N3, GST-N3 mutant (aa 1–43); N5, GST-N6 mutant (aa 1–20); N6, GST-N11 (aa 1–31); N11, GST-N5 (aa 43–180).
focal adhesion disassembly by hep I. Preincubation of cells with a rabbit antibody raised against the N terminus blocked the ability of hep I to stimulate focal adhesion disassembly. Antibody raised against the C terminus did not affect the activity of hep I. Antiserum alone did not affect the basal number of cells positive for focal adhesions (Fig. 4B). These data show that thrombospondin interactions with the N-domain of calreticulin are important for mediating focal adhesion disassembly.

Identification of TSP/hep I-binding Site Present in the N-terminal Domain of Calreticulin—To determine the sequence within the N-domain of calreticulin that binds TSP/hep I, a series of GST-N-domain truncation mutants were constructed. Six different overlapping constructs were used in this study: GST-N1 domain (aa 1–134), the GST-N2 domain (aa 1–83), the GST-N3 domain (aa 1–43), GST-N5 (aa 43–180), GST-N6 (aa 1–20), and GST-N11 (aa 1–31) (Fig. 5A). These mutants were expressed in E. coli, purified as described under “Experimental Procedures” and analyzed by SDS-PAGE (Fig. 5B). N-domain mutants were first tested for thrombospondin binding in GST pull-down assays. The results shown in Fig. 6A indicate that GST-N1, GST-N2, and GST-N3 fusion proteins bind thrombospondin. However, constructs consisting of aa 1–20 (GST-N6), aa 1–31 (GST-N11), and aa 43–180 (GST-N5) failed to bind to thrombospondin.

To confirm the binding results, CRT-N-domain mutants were tested for their ability to block focal adhesion disassembly by hep I. Consistent with binding studies, only GST-N1, GST-N2, and GST-N3 blocked hep I-induced focal adhesion disassembly. These data suggest that the binding site is localized within aa 21–42. Three constructs consisting of aa 1–20 (GST-N6), aa 43–180 (GST-N5), and aa 1–31 (GST-N11) did not have any effect on hep I or thrombospondin-induced focal adhesion disassembly. Therefore, thrombospondin binding to aa 13–48 of the N-domain of calreticulin was evaluated with a series of overlapping peptides, each 15 amino acid residues long.

(A Table I). As with the GST fusion proteins, the peptides were incubated with hep I prior to the addition to cells to determine which peptides competitively inhibit hep I activity. Two peptides encompassing the amino acids RWIESKHKSDFGKFV-5-36 blocked hep I-induced focal adhesion disassembly, suggesting that the TSP/hep I-binding site is located at this site in calreticulin (Fig. 7).

A Calreticulin Lacking the hep I-binding Site Sequence Does Not Mediate Focal Adhesion Disassembly—To confirm that this sequence in calreticulin (amino acids 19–36) is indeed the thrombospondin-binding site responsible for focal adhesion disassembly, we generated a recombinant GST-CRT mutant lacking the N-domain 19–36 amino acids (GST-CRTΔhep I). This mutant was expressed in E. coli and purified as described under “Experimental Procedures.” Coomassie Blue staining of the protein demonstrated that the migration of the mutant corresponds to the expected molecular weight (not shown). GST-CRTΔhep I was tested for its ability to competitively block focal adhesion disassembly by hep I and thrombospondin in BAE cells (Fig. 8A, upper panel and lower panel, respectively).
Although GST-CRT blocked the anti-adhesive activity of hep I and thrombospondin, GST-CRT\textsubscript{hep} I did not have any effect on hep I- or thrombospondin-induced focal adhesion disassembly. In further experiments, the ability of GST-CRT\textsubscript{hep} I to restore responsiveness of \( \text{crt}^{-/-} \) cells to TSP/hep I was examined. These studies show that \( \text{crt}^{-/-} \) cells are not rescued by incubation with exogenous GST-CRT\textsubscript{hep} I prior to the addition of hep I or thrombospondin (Fig. 8D). Together, these data show that aa 19–36 of calreticulin are involved in thrombospondin binding and are required for calreticulin signaling of TSP/hep I-induced focal adhesion disassembly.

DISCUSSION

Previously, we reported the identification of calreticulin as a receptor for thrombospondin and elucidated a role for calreticulin in mediating focal adhesion disassembly (23). In the present study, we showed that interactions between calreticulin and thrombospondin involve the N-terminal domain of calreticulin. We identified an 18-amino acid sequence as the putative thrombospondin-binding site in calreticulin: amino acids 19–36 (RWIESKHKSDFGKFLVLS). We also showed that interactions between calreticulin and thrombospondin are \( \text{Ca}^{2+} \)- and \( \text{Zn}^{2+} \)-dependent.

We have used calreticulin-null mouse embryonic fibroblasts to confirm the role of calreticulin in thrombospondin-mediated focal adhesion disassembly. Not only do calreticulin-null cells fail to respond to TSP/hep I, but cells rescued either by stable transfection of calreticulin or by short incubation of calreticulin-null cells with exogenous calreticulin recover the ability to respond to hep I in the focal adhesion disassembly assays. These latter experiments are important because they show that calreticulin is acting through binding thrombospondin at the cell surface and not through its chaperone functions from the ER.

Using immunoprecipitation studies we confirmed that thrombospondin interacts with the calreticulin N-terminal domain. Furthermore, the ability of calreticulin domains fusion proteins to block TSP/hep I-mediated focal adhesion disassembly suggests that the N-domain of calreticulin is accessible when calreticulin is on the cell surface and available for binding to thrombospondin. Amino acids 19–36 of the N-terminal domain of calreticulin are required for mediating thrombospondin binding. Analysis of the secondary structure of the sequence (amino acid 19–36) spanning the active peptides (RWIESKHKSDFGKFLVLS) suggests that amino acids 20–23 (WIES) and 32–34 (FVL) are in a helical structure. Both of these sequences appear to be necessary for optimal focal adhesion disassembly because peptides containing only one of these short sequences have suboptimal activity (see peptides 16–30 and 25–39 in Fig. 7). Furthermore, the FVLSS sequence (aa 32–36) in the thrombospondin-binding site appears to be critical for thrombospondin binding because the calreticulin fusion protein (aa 1–31), which lacks this portion of the binding site, was not sufficient to block focal adhesion disassembly. A hydrophathy analysis of aa 19–36 of calreticulin produces a pattern that is clearly inverted to the hydrophathy pattern of the hep I peptide when aligned in parallel such that Glu\textsuperscript{22} of calreticulin corresponds with Glu\textsuperscript{17} of the hep I portion of thrombospondin (Fig. 9). Interestingly, lysines present in both hep I and calreticulin are integral for both biologic activity and hydrophathic integrity of the molecules involved. Such hydrophathic inversion will predispose molecules toward interaction according to the molecular recognition theory of Blalock (45). This is based on the idea that hydrophobic residues will tend to congregate toward the interior of the macromolecule, whereas hydrophilic amino acids will stick out toward the aqueous environ. When hydropathies are inverted, the extrusion of the hydrophilic residues from one protein will correlate with hydrophobic inclusions on the other, and the surface contours will thus have a topography amenable to the coordination and interdigitation of these molecules. Such interactions have been documented, with varying binding affinities in at least 40 different systems (46) including earlier work also involving TSP (47). This analysis together with the current data strongly suggest that these two regions may form the basis of interaction between calreticulin (N-terminal) and thrombospondin.

The N-terminal domain of calreticulin, which includes aa 1–180, is the most conserved domain in calreticulin and has no reported homology to other protein sequences (1, 48). The N-domain is capable of interacting with multiple ligands, including the DNA-binding domain of the glucocorticoid receptor \textit{in vitro} (12), rubella virus RNA (49–51), integrin \( \alpha \) subunits (18), the C1q recognition subunit of the first component of the classical complement pathway (52), protein-disulfide isomerase...
(25), and ER protein 57 (Erp57) (53). However, only the precise binding site for rubella virus RNA has been identified (50). It has been shown that the N-terminal 10 amino acids of calreticulin N-domain are necessary for its RNA binding activity, whereas a region between amino acids 60 and 180 of the N-domain contribute to autophosphorylation activity (50). Interestingly, a fragment of the N-domain (aa 1–180), termed vasostatin, has been purified from an Epstein-Barr virus-immortalized cell line and shown to have anti-angiogenic activity. This function of calreticulin N-domain is apparently mediated by its interactions with laminin (54–56).

Although the tertiary structure of the N- and C-domains of calreticulin has not yet been described, it has been shown recently that the protein adopts an elongated shape in solution (57, 58), which can be attributed to the extended hairpin structure of the P-domain (59, 60). It has also been shown that the structural properties of calreticulin can be significantly modulated by interactions with divalent metal ions, which could affect its functions and its ability to interact with other proteins (28, 29). The results from our studies (Fig. 3, B and C) showed that binding of thrombospondin to calreticulin and to the N-terminal domain of calreticulin is enhanced in the presence of physiologic levels of Ca$^{2+}$, but there is still detectable binding at subphysiologic levels of cation. Binding of Ca$^{2+}$ ions to calreticulin affects the tertiary structure of the protein as indicated by calcium-dependent changes in calreticulin sensi-

---

**FIG. 8.** Soluble GST-CRT/hep I inhibits focal adhesion disassembly and does not rescue CRT-null cells. A, hep I (25 nM) (upper panel) or TSP (67 nM) (lower panel) were preincubated with a 10-fold molar excess of GST-CRT and GST-CRT/hep I for 30 min before addition to BAE cells, respectively. The number of focal adhesions was determined by IRM. The focal adhesions were not affected by the addition of GST-CRT or GST-CRT/hep I alone. The results are expressed as the mean percentages of cells positive for focal adhesions ± S.D. (n = 3). B, K42 (CRT-null) MEFs were incubated with equimolar amounts of GST-CRT or GST-CRT/hep I for 30 min at 37 °C before addition of hep I (25 nM, upper panel) or TSP (67 nM, lower panel) to cells for 30 min. Untreated cells were used as controls. The cells were fixed, and the number cells positive for focal adhesions ± S.D. (n = 3).
tivity to protease digestion (28, 29). Li et al. (29) have suggested that Ca\(^{2+}\) ions may serve to spatially organize and stabilize the highly negatively charged C-domain, which was shown to be more conformationally flexible and destabilized in the absence of added Ca\(^{2+}\) ions. Ca\(^{2+}\) dependence of the interaction between thrombospondin and the calreticulin N-domain were unexpected, because the Ca\(^{2+}\)-binding site in the N-terminal domain has not been identified. Although the hep I binding site in aa 19–36 does not contain a typical Ca\(^{2+}\)-binding sequence, it is possible that divalent cation interactions with other portions of the N-domain modify the conformation/accessibility of the thrombospondin-binding sequence.

Zn\(^{2+}\) similarly enhanced the binding of thrombospondin to calreticulin and to the N-terminal domain of calreticulin. Upon binding Zn\(^{2+}\) ions, calreticulin adopts a more loosely packed and thermally destabilized structure (28, 29). It has been reported that protein-disulfide isomerase and calreticulin interactions are Zn\(^{2+}\)-dependent (25). The precise binding site of Zn\(^{2+}\) to calreticulin has not been determined, but the five histidine residues in the N-terminal region of calreticulin are essential for Zn\(^{2+}\) binding to the protein (25).

Our data also show that calreticulin signals TSP/hep I-mediated focal adhesion disassembly from the peripheral membrane to the inside of the cell. In the absence of stimulation with TSP/hep I, calreticulin binding either to calreticulin-expressing BAE cells or to calreticulin-null mouse embryonic fibroblasts does not in itself signal focal adhesion disassembly. This suggests that expression of calreticulin at the cell surface is not in itself sufficient to signal. Rather, it is likely that interactions with thrombospondin are necessary to "activate" calreticulin so that it can signal. This binding to calreticulin might alter the conformation of calreticulin at the membrane, potentially facilitating association with a transmembrane protein that then can act as a co-receptor and transmit signals. It has been shown that calreticulin co-localizes with low density lipoprotein receptor-related protein (LRP), (CD91) on cells (61, 62). In fact, we now have evidence that LRP acts as a co-receptor with calreticulin to mediate TSP/hep I-stimulated focal adhesion disassembly and that hep I binding to calreticulin enhances its association with LRP in cells.\(^2\)

These studies present further evidence that TSP/hep I-mediated focal adhesion disassembly occurs through interactions with a cell surface form of the calcium-binding protein, calreticulin. We also established that thrombospondin binds to aa 19–36 in the N terminus of the calreticulin N-domain and that this interaction is Ca\(^{2+}\)- and Zn\(^{2+}\)-dependent. Further investigation will be important to assess the role of other calreticulin domains in signaling focal adhesion disassembly, perhaps through binding to LRP. In addition, it will be interesting to determine how ion-dependent conformational changes regulate the physiological function of calreticulin. Knowledge of these events and factors will help us to better understand the significance of cell surface calreticulin in regulation of cell de-adhesion and will provide us new insights into how calreticulin mediates signaling as a result of binding thrombospondin.

Acknowledgments—We thank Drs. Patricia L. Jackson (Department of Physiology and Biophysical Optics, University of Alabama at Birmingham) and Nathaniel M. Weathington (Medical Scientist Training Program, University of Alabama at Birmingham) for the development of the hydrophathy plot. We also thank Dr. Claudia Oliva (University of Alabama at Birmingham) for the HPLC purification of GST-N3 and GST-N6 fragments and Dr. Harold Erickson (Duke University) for the gift of recombinant tenascin-CfIII-A-D, and Dr. Helene Sage (Hope Heart Institute) for the SPARC peptide.

REFERENCES
1. Michalak, M., Milner, R. E., Burns, K., and Opas, M. (1992) Biochem. J. 285, 681–692
2. Mery, L., Messali, N., Michalak, M., Opas, M., Lew, D. P., and Krause, K. H. (1996) J. Biol. Chem. 271, 9332–9339
3. Krause, K. H., and Michalak, M. (1999) Cell 88, 439–443
4. Labriola, C., Cazzulo, J. J., and Parodi, A. J. (1999) Mol. Biol. Cell 10, 1381–1394
5. Michalak, M., Corbett, E. F., Messali, N., Nakamura, K., and Opas, M. (1999) Biochem. J. 344, 281–292
6. Leung-Hagestein, C. Y., Milanovk, K., Michalak, M., Wilkins, J., and Dedhar, S. (1994) J. Cell Sci. 107, 589–600

\(^2\) C. Pedraza, A. W. Orr, M. A. Pallero, D. Strickland, and J. E. Murphy-Ullrich, manuscript submitted.
