Thrombospondin Mediates Focal Adhesion Disassembly through Interactions with Cell Surface Calreticulin*

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Thrombospondin induces reorganization of the actin cytoskeleton and restructuring of focal adhesions. This activity is localized to amino acids 17–35 in the N-terminal heparin-binding domain of thrombospondin and can be replicated by a peptide (hep I) with this sequence. Thrombospondin/hep I stimulate focal adhesion disassembly by thrombospondin/hep I. A 60-kDa protein from endothelial cell detergent extracts has homology and immunoreactivity to calreticulin, binds a hep I affinity column, and neutralizes thrombospondin/hep I-mediated focal adhesion disassembly. Calreticulin on the cell surface was confirmed by biotinylation, confocal microscopy, and by fluorescence-activated cell sorting analyses. Thrombospondin and calreticulin potentially bind through the hep I sequence, since thrombospondin-calreticulin complex formation can be blocked specifically by hep I peptide. Antibodies to calreticulin and preincubation of thrombospondin/hep I with glutathione S-transferase-calreticulin block thrombospondin/hep I-mediated focal adhesion disassembly and phosphoinositide 3-kinase activation, suggesting that calreticulin is a component of the thrombospondin-induced signaling cascade that regulates cytoskeletal organization. These data identify both a novel receptor for the N terminus of thrombospondin and a distinct role for cell surface calreticulin in cell adhesion.

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 (1–3). These proteins, which include tenascin-C and SPARC, in addition to thrombospondin, have been termed “matricellular” proteins to reflect their cell regulatory properties (4). 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 (5–7). Vinculin and α-catenin, but not the αβ integrin, are selectively redistributed from the restructured focal adhesions in response to thrombospondin (5, 8). A 19-amino acid sequence (amino acids 17–35) in the N-terminal heparin-binding domains of both TSP-1 and TSP-2, referred to as the hep I peptide, has been determined to be sufficient for focal adhesion disassembly (9). The signaling events stimulated by thrombospondin/hep I interactions with cells are only partially understood. It is known that thrombospondin/hep I binding to endothelial cells stimulates activation of phosphoinositide 3-kinase (PI3K) and generation of phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)P3) (8). Basal levels of cyclic GMP-dependent protein kinase activity are also necessary for thrombospondin-mediated focal adhesion disassembly (10).

The receptor molecule that binds the hep I sequence of thrombospondin and mediates the generation of these intracellular signals involved in cytoskeletal regulation has not been identified. There are a number of molecules that act as cell surface binding molecules for the N-terminal heparin-binding domain of thrombospondin. These include heparan sulfate proteoglycans (11–13), LDL receptor-related protein (14, 15), and more recently, the αβ integrin (16). Previously, we showed that heparitinase-treated BAE cells are competent to signal hep I-induced focal adhesion disassembly, suggesting that heparan sulfate proteoglycans are not the receptors that mediate this activity of thrombospondin. The integrin-binding sequence in the heparin-binding domain of thrombospondin has been localized to a sequence distinct from that of the hep I peptide (16). Therefore, we sought to identify the receptor that mediates focal adhesion disassembly in response to the hep I sequence of thrombospondin.

Calreticulin is a widely expressed calcium-binding protein found mainly in the endoplasmic reticulum but also in other cellular compartments. In the ER, calreticulin acts as a molecular chaperone and regulates calcium homeostasis (17). The localization of calreticulin to other cellular compartments, including the cell surface, as secreted forms, and possibly in the cytoplasm and nucleus, is prompting reconsideration of calreticulin as a candidate receptor for thrombospondin/hep I.
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Calreticulin as a mediator of a broader array of cellular functions. Calreticulin apparently plays a critical role in the development of the myocardium as calreticulin knock-out animals exhibit severe cardiac deformities (18). Support for important physiologic roles for calreticulin is provided by recent data showing that calreticulin inhibits angiogenesis and suppresses tumor cell growth (19, 20).

Calreticulin can regulate cell adhesion by a number of different mechanisms. Intracellular calreticulin levels have been shown to regulate levels of vinculin and N-cadherin expression, implicating calreticulin in both cell-substrate and cell-cell adhesion (21). Cells overexpressing calreticulin have higher levels of vinculin expression and are consequently more adhesive. The mechanism whereby calreticulin in the ER regulates gene expression is unknown. Calreticulin can also regulate cell adhesion by modulating the affinity of integrin for its ligand through transient interactions with the cytoplasmic domain of the integrin α subunit (22–25). Consistent with this putative function is the observation that calreticulin-deficient ES cells have impaired cell adhesion (23, 26). Calreticulin on the cell surface is reported to have a lectin-like function and to mediate cell spreading on glycosylated laminin (27). However, the existence of cell surface forms of calreticulin are only beginning to be appreciated, and the role of this form of calreticulin as a modulator of cell adhesion has been obscure.

By using a hep I affinity purification approach, we isolated a 60-kDa protein from detergent extracts of BAE cells with N-terminal sequence homology and immunoreactivity to the calcium-binding protein calreticulin. Here we report 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 PI3K.

EXPERIMENTAL PROCEDURES

Materials—The following items were purchased: Dulbecco’s modified Eagle’s medium (DMEM), Cell-Gro, Mediatech; fetal bovine serum (FBS, HyClone Laboratories); 500 μg/ml trypsin, 2.2 mM EDTA (Life Technologies, Inc.—cultured cell products, Grand Island, NY); G-Sepharose beads (Amersham Pharmacia Biotech); stained and prestained molecular weight markers (Bio-Rad); chemiluminescence PerkinElmer Life Sciences detection kit; EZ-Link Sulfo-NHS-biotin, avidin, and horseradish peroxidase-conjugated biotin (Pierce).

Proteins—Thrombospondin was isolated from fresh human platelets purchased from the American Red Cross. It was purified according to established protocols using heparin affinity and gel filtration chromatography (6). Tenasin-HBL (high molecular weight form of tenasin-C) was a gift of Harold Erickson, Duke University. SPARC (two sets of 100 μg) was a gift of Dr. Helene Sage, Hope Heart Institute, Seattle. Hep I (ELT-GAARRGSGRLVRGPD) and GammaBind G-Sepharose beads (Amersham Pharmacia Biotech) were purchased at the University of Alabama at Birmingham Comprehensive Cancer Center/Peptide Synthesis and Analysis shared facility.

Antibodies—Mouse anti-TSP 133 antibody was raised against stripped TSP and developed using the monoclonal antibody Core facility at the University of California at Los Angeles (28). Rabbit polyclonal anti-GST antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-calreticulin antisera was purchased from Affinity Bioreagents, Inc. Mouse anti-cytochrome c antibody was purchased from Pharmingen. Rabbit anti-N-terminal calreticulin antibody was raised in rabbits inoculated with the purified recombinant human N-domain (amino acids 1–180) of calreticulin that had been expressed in Escherichia coli. Animals were immunized by intramuscular injection of 50 μg of protein emulsified with 0.5 ml of Freund’s complete adjuvant in a total volume of 1 ml over 3 monthly intervals. The IgG fraction of antisera was prepared by sodium sulfate precipitation followed by protein-A affinity purification from a 4-month post-immunization bleed.

Cells—BAE cells were isolated and cultured in DMEM containing 4.5 g/liter glucose, 2 mM glutamine, and 20% fetal bovine serum (FBS) as described previously (9). Cells were used between passages 4 and 12.

Detergent Extraction of BAE Cells—BAE cells were grown to near confluence in 12–24 150-mm diameter glass Petri dishes. Cells were washed three times with cold PBS, 0.9 mM CaCl2, 0.8 mM MgSO4, with a mixture of protease inhibitors (PI) (2 μg/ml pepstatin A, 10 μg/ml aprotonin, 2 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride), scraped, and pooled. Cells were pelleted by centrifugation (12,000 rpm, 4 min), washed 4 times, and pellets were pooled twice. After the final wash, cells were sonicated twice for 15 s each on ice and resuspended in 2 ml of PBS/PI with 200 mM N-octylglycosonanide (Inalco SPA, Milan, Italy). Detergent-soluble proteins were extracted on ice for 60 min. This mixture was sonicated twice for 30 s each time. Insoluble proteins were separated by centrifugation (12,000 rpm) for 15 min at 4 °C. Detergent-soluble membrane proteins and soluble cytoplasmic proteins were collected in the supernatant and stored at −20 °C until used for affinity purification. In some experiments, cells were washed, scraped, and pooled as above with the following modifications: cells were disrupted by homogenization (>20 strokes) in a tissue grinder, insoluble proteins were pelleted by centrifugation, and soluble cytoplasmic proteins were removed by multiple washes in PBS with calcium and magnesium prior to extraction of membrane proteins with 200 mM N-octylglycosonanide.

Purification of Hep I-binding Proteins—Hep I-binding proteins were isolated by affinity chromatography. Since the basic amino acids are critical for hep I activity (9), hep I was synthesized with a C-terminal cysteine, reduced by passing through a dithiothreitol column (Pierce), and coupled via the free sulfhydryl group to a Sulfo-Link resin (Pierce). Octylglycosonanide-soluble proteins were first applied to an uncoupled Sulfo-Link resin that had been blocked by preincubation with 50 mM cysteine according to the manufacturer’s protocol in order to pre-clear the sample of nonspecific resin-binding proteins. The pre-cleared sample was then applied to the hep I affinity column (2-ml bed volume) in PBS with 0.9 mM CaCl2 and 0.5 mM MgSO4 and incubated for 15 min at 4 °C. Unbound proteins were washed extensively with PBS with calcium and magnesium. Specifically bound proteins were eluted with a 0.15 to 1 x NaCl gradient (total volume 50 ml) in PBS with calcium and magnesium. Eluted proteins were analyzed by silver staining of SDS-PAGE and for the ability to neutralize hep I activity in focal adhesion assays. Fractions with neutralizing activity were pooled and further purified by gel filtration chromatography on a Sephacryl 100 HR resin (4 ml, 50 ml). Elution fractions were analyzed by SDS-PAGE and for the ability to neutralize hep I activity in focal adhesion assays. Proteins from fractions with hep I inhibitory activity were separated on SDS-PAGE and transferred to PVDF membranes for N-terminal amino acid sequencing at the University of Alabama at Birmingham Protein Microsequencing Facility.

Focal Adhesion Assays—Focal adhesion assays were performed according to the protocols described by Murphy-Ullrich and Hóok (6). Briefly, BAE cells were grown to near confluence for 20–24 h on 12-mm glass coverslips in DMEM with 20% FBS. After preincubation under serum-free conditions for 30 min, cells were cultured in 1 ml of DMEM. Cells were examined for the presence of focal adhesions by interference reflection microscopy (IRM) with a specially equipped Zeiss Axiosvert 10 Microscope. A minimum of 400 cells/condition were evaluated for the presence of focal adhesions. Cells that are positive usually have ≥15–30 plaques/cell. Cells with less than 3–5 plaques/cell were scored as negative. Experiments were repeated a minimum of 3 times.

Biotinylation and Salt Extraction of Cell Surface Proteins—BAE cells were grown to near confluence in 11 150-mm diameter glass Petri dishes. Cells were scrapped in cold PBS, 0.9 mM CaCl2, 0.8 mM MgSO4, with a mixture of protease inhibitors (PI)(2 μg/ml pepstatin A, 10 μg/ml aprotonin, 2 μg/ml leupeptin and 2 mM phenylmethylsulfonyl fluoride), and pooled. Cells were then resuspended in 1 ml of EZ-Link Sulfo-NHS-biotin (Pierce) (0.5 mg in PBS) and incubated for 30 min at room temperature. After biotinylation, cells were washed three times with PBS to remove the unreacted biotin and pelleted by centrifugation (1500 rpm, 4 min, at 4 °C). After the final wash, cells were disrupted by homogenization (25 strokes) in a tissue grinder. Insoluble proteins were pelleted by centrifugation and resuspended in 1 ml NaCl for 5 min. Salt extracts were centrifuged (14,000 rpm, 10 min, at 4 °C), and soluble protein fractions were collected and stored at −80 °C until used for affinity purification.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Samples were separated by SDS-polyacrylamide gel electrophoresis (% of acrylamide is indicated in the figure legends) under reducing conditions. After electrophoresis, gels were stained with either silver or Coomassie Blue or transferred electrophoretically to PVDF membranes.
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(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). Membranes were then incubated with primary antibodies diluted in TBST (dilutions are specified in figure legends) followed by 3 washes for 15 min each in TBST. Preparative gels were transferred with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), resuspended in reducing Laemmli buffer, analyzed by SDS-PAGE (10%), transferred to a PVDF membrane, and detected with rabbit anti-glyceraldehyde-3-phosphate dehydrogenase, rabbit anti-actin, rabbit anti-calreticulin N-terminal IgG, and rabbit anti-calreticulin C-terminal IgG (1/150 dilutions) according to the manufacturer's instructions. The gels were then incubated overnight with 0.1% BSA in PBS. This solution was transferred to 12 x 75-mm Falcon tubes. The lysates were then pre-cleared with 30 μl of beads from the immunoprecipitation were washed 2 times with PBS and resuspended in 0.5 ml of PBS + 1% BSA and resuspended in 0.5 ml of PBS + 1% BSA. This solution was transferred to 12 x 75-mm Falcon tubes, and samples were analyzed by fluorescence-activated cell sorting using a Becton Dickinson FACSCalibur instrument with CellQuest software. Membranes were then incubated with avidin (10 μg/ml) followed by incubation with horseradish peroxidase-conjugated biotin (2.5 μg/ml). After 3 washes of 15 min each in TBST, blots were developed using enhanced chemiluminescence PerkinElmer Life Sciences according to the manufacturer's instructions.

Identification of Biotinylated Proteins—After hep I affinity chromatography of NaCl-extracted proteins, bound proteins (50 μl) were separated by SDS-polyacrylamide gel electrophoresis (10%) under reducing conditions. After electrophoresis, gels were transferred electrophoretically to PVDF 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). Membranes were then incubated with avidin (10 μg/ml) followed by incubation with horseradish peroxidase-conjugated biotin (2.5 μg/ml). After 3 washes of 15 min each in TBST, blots were developed using enhanced chemiluminescence PerkinElmer Life Sciences according to the manufacturer's instructions.

Immunofluorescence—BAE cells were grown to confluence for 20–24 h on 12-mm glass coverslips in DMEM with 20% FBS. After washing in DMEM, cells were incubated in serum-free DMEM for 30 min with serum-free DMEM, DMEM containing 0.9 mM CaCl2 and 0.8 mM MgSO4) for 10 min at room temperature, and then washed three times with PBS. Cells to be permeabilized were treated with 0.1% Triton X-100 in PBS for 3 min at room temperature. Permeabilized and non-permeabilized cells were then incubated overnight with 0.1% BSA in PBS at 4 °C to block sites of nonspecific binding. Coverslips were then incubated for 60 min with 100 μl of polyclonal anti-calreticulin antisera (1/500) or with rabbit anti-N-terminal calreticulin IgG at 250 μg/ml, followed by three washes with PBS and a 30-min incubation with goat anti-rabbit IgG conjugated to fluorescein (1/70). After washing three times with PBS, cells were mounted in Vectashield mounting medium for fluorescence microscopy (H-1000 (Vector Laboratories) and examined using a LEICA TCS NT laser confocal microscope at the University of Alabama at Birmingham High Resolution Imaging Facility.

FACS Analysis—BAE cells were grown to approximately 80% confluence in 24 x 100-mm polystyrene culture plates. Cells were then washed twice in PBS and detached from the plate by adding PBS + 0.005% trypsin + 0.05 mM EDTA. Trypsinization was stopped by adding 5 ml of DMEM + 10% FBS, and the cells were collected. Cells were washed twice with ice-cold PBS + 1% BSA and filtered through 70-μm nylon mesh. Cells were then aliquoted to sample tubes at approximately 2 million cells per condition, and incubated with primary antibody for 30 min on ice, followed by three washes in PBS + 1% BSA. When primary antibody was omitted, cells were incubated in PBS + 1% BSA. Cells were fixed for 15 min in PBS + 2% paraformaldehyde and washed twice in PBS + 1% BSA to remove fixing solution. Cells were then resuspended in 2 ml of secondary antibody: FITC-conjugated goat α-rabbit and FITC-conjugated goat α-mouse antibodies (Jackson ImmunoResearch Laboratories) were both used at a 1/150 dilution. Rabbit anti-CRT N-terminal IgG was used at 250 μg/ml. Cells were then washed three times in PBS + 1% BSA and resuspended in 0.5 ml of PBS + 1% BSA. This solution was transferred to 12 x 75-mm Falcon tubes and samples were analyzed by fluorescence-activated cell sorting using a Becton Dickinson FACSCalibur instrument with CellQuest analysis software at the Multipurpose Arthritis and Musculoskeletal Disease Core Facility at University of Alabama at Birmingham. A positive signal was set as any fluorescence level above that observed in cells alone. Cells were also stained with mouse monoclonal antibody to β2 integrin as a marker for cell surface proteins and with antibody to cytochrome c as a marker for an intracellular antigen.

Expression and Purification of Recombinant Calreticulin—cDNA for GST-calreticulin was a gift from Dr. Marek Michalak, University of Alberta, Edmonton, Alberta, Canada. Expression in Baculovirus was achieved in Sf9 cells using a baculovirus expression system (Invitrogen). Expression was monitored using an anti-CRT antibody (ICN Biomedicals). Purification was performed using affinity chromatography on a GST-capture column was performed using affinity chromatography on a GST-capture column followed by a phosphotyrosine affinity column. The protein complexes were then incubated with recombinant GST-calreticulin (0.75 μg/ml) and recombinant GST-calreticulin (0.75 μg/ml) were incubated together in a total volume of 300 μl of PBS for 1 h at 4 °C. Binding of thrombospondin to GST-calreticulin and precipitation of GST-CRT 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 (15 μg/ml) in PTO buffer (0.1% ovalbumin, 0.5% Tween 20, 0.1% BSA), washed once with RIPA buffer and analyzed by Western blotting with rabbit anti-actin antibody (1/1000 dilution) followed by incubation with peroxidase-conjugated anti-rabbit IgG (1/15000). Blots were then developed using enhanced chemiluminescence PerkinElmer Life Sciences as indicated under "Experimental Procedures." Inhibition of Calreticulin-Thrombospondin Complex Formation with Hep I Peptide—Native thrombospondin and recombinant GST-calreticulin were incubated together as indicated above. The incubation was performed in the presence or absence of either hep I or a modified hep I peptide in which the lysine residues were substituted with alanine. This modified peptide was inactive in the focal adhesion disassembly assays (data not shown). Peptides were used at 10–1000-fold excess to thrombospondin. In the presence of the peptides, GST-calreticulin was preincubated with the peptide for 1 h at 4 °C. Thrombospondin was then added to the protein/peptide mixture, and this mixture was then incubated for 1 h at 4 °C. The protein complexes were then incubated with GST-Sepharose bound to monoclonal anti-TSP (monoclonal antibody 133) (15 μg/ml) in PTO buffer (0.1% ovalbumin, 0.5% Tween 20 in PBS). Immune complexes were washed with RIPA buffer and analyzed by Western blotting with rabbit polyclonal anti-GST antibodies as indicated above.

Assay for Phosphoinositide 3-Kinase Activity—P3K activity was determined by quantification of the product of this kinase as measured by phosphoinositide 3-kinase activity as described (5). BAE cells were grown to approximately 80% confluence in 24 x 100-mm polystyrene culture plates. The cells were then incubated under low serum conditions in DMEM with 0.2% FBS for 12 h in order to lower background levels of active P3K. To test the effect of anti-calreticulin antibodies on thrombospondin/hep I-induced P3K activation, the cells were incubated with a 1/500 dilution of either rabbit anti-calreticulin antisera or rabbit anti-immune serum for 20 min. The cells were then washed with serum-free DMEM. The cells were grown to approximately 80% confluence in 24 x 100-mm polystyrene culture plates. The cells were then incubated with antibodies to phosphotyrosine (PY20) for 2 h on ice to precipitate P3K that is activated by binding of its SH2 domain to a tyrosine-phosphorylated protein, followed by a 1-h incubation with 30 μl of protein A-Sepharose on a rocker tray. The tubes were washed 3 times with lysis buffer. Tubes containing 30 μl of beads from the immunoprecipitation were washed 2 times with kinase buffer (10 mM Hepes, pH 7.2, 20 mM β-glycerophosphate, 0.8 mM Na2VO4, and 30 mM NaCl). Lipids were prepared by adding 0.2 μl of 20% Lipid Resuspension Buffer (1 μl of kinase buffer, 3.5 μl of 1 M dithiothreitol) to a pre-dried mixture of 15 μg of Ptdlns(4,5)P2 (American Radiolabeled Chemicals, St. Louis, MO) and 150 μg of phosphatidylinositol (Sigma). The mixture was briefly sonicated to resuspend the lipid. 20 μl of lipids were added to each tube, vortexed, and incubated for exactly 10 min at 37 °C. Next, 20 μl of kinase buffer containing 17.5 μM ATP, 50 μM of [γ-32P]ATP, and 17.5 mM MgCl2 was added to each tube, vortexed, and incubated for 10 min at 37 °C. The reaction was stopped by adding 160 μl of methanol/chloroform (1/1). Lipids were extracted by adding 80 μl of 1 M HCl, briefly centrifuging, and then resuspending the lower organic phase in acetic acid. The resuspending solution was then spotted on silica gel plates precoated with 1% potassium oxalate. The plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7), dried, and exposed for autoradiography. The intensity of the bands migrating at the position of phosphoinositide 3,4,5-trisphosphate was quantified by densitometry using the Scanalytics OneScan 1.31 program.
RESULTS

A 60-kDa Hep I-binding Protein Isolated from BAE Extracts Has Sequence Homology to Calreticulin—The hep I peptide of thrombospondin (amino acids 17–35) has been shown to cause loss of focal adhesions from spread BAE cells (9). To identify hep I-binding proteins that mediate focal adhesion disassembly, we used an affinity purification approach. Supernatants from octylglucopyranoside-extracted BAE cells were fractionated using a hep I affinity column, and fractions eluted with increasing concentrations of sodium chloride were analyzed for the ability to block focal adhesion disassembly by hep I following dilution to reduce the salt concentration to 0.15 M (Fig. 1A).

Fractions that eluted with 0.275–0.4 M NaCl (fraction numbers 11–17) partially blocked focal adhesion disassembly activity. These fractions were further purified by gel filtration chromatography on a Sephacryl S-300 HR column. A peak eluting prior to the void volume of the column and two discrete peaks (peaks I and II) were identified, but only fractions from peak II ($K_v = 0.097$) were able to inhibit hep I-mediated focal adhesion disassembly (Fig. 1B). These fractions were analyzed for the ability to block focal adhesion disassembly by hep I. Incubation of 100 μl of several fractions from peak II with hep I inhibited the ability of hep I to stimulate focal adhesion disassembly by 80%.

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Fig. 1. Calreticulin from BAE extracts binds to a hep I affinity column. A, BAE cell extracts were prepared as described under “Experimental Procedures” and fractionated on a hep I affinity column. Absorbance at 280 nm (--) was used to monitor elution. Specifically bound proteins were eluted with a 0.15–1 M NaCl gradient (---). Various fractions were then analyzed for their ability to neutralize hep I activity in focal adhesion assays (solid bars). Fractions themselves had no effect on basal levels of focal adhesion-positive cells. B, 5 ml of hep I affinity chromatography eluate (pooled fractions 11–17; $A_{280 nm} = 0.1$) was fractionated on a Sephacryl S-300. Absorbance at 280 nm (--) was used to monitor elution. Selected fractions were then analyzed for their ability to neutralize hep I activity in focal adhesion assays (solid bars). Fractions had no effect on basal levels of focal adhesion-positive cells. C, proteins in pooled peak II were resolved by SDS-PAGE and Western blot. Lane 1, silver staining of SDS-PAGE (12%). Lane 2, identification of immunoreactive calreticulin in peak II as detected by Western blot analysis with rabbit anti-CRT antiserum.
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The components of pooled peak II were analyzed by SDS-PAGE under reducing conditions. Three bands were detected at approximately 60, 34, and 32 kDa (Fig. 1C). However, one or more of the components of peak II appears to migrate as a higher molecular weight complex since peak II elutes from the Sephacryl column prior to the elution of bovine serum albumin ($K_v = 0.41$). The 60-kDa band (SDS-PAGE) was reproducible over multiple preparations, although the appearance of the lower molecular weight bands was variable in different preparations. Similarly, a 60-kDa protein was also isolated in other experiments in which bound proteins were eluted with hep I peptide instead of a sodium chloride gradient (data not shown).

In order to obtain the identity of these proteins, they were electrophoretically transferred to PVDF membranes and subjected to N-terminal amino acid analysis. The N-terminal sequence of the 60-kDa protein was XPTVYFKEQF, which corresponds to the amino acid sequence of calreticulin. This sequence identified a match in 8 of 9 residues with human and rabbit calreticulin and in 9 of 9 residues with bovine calreticulin (29–31) (Table I). The identity of the 60-kDa eluted protein as calreticulin was further confirmed by its reactivity with anti-calreticulin antibodies using Western blot analysis (Fig. 1C).

Subsequent to the identification of calreticulin, we repeated these studies using an alternative isolation strategy. Since a major source of calreticulin is the lumen of the endoplasmic reticulum, we modified our extraction procedure to minimize possible contamination of plasma membrane proteins with cytoplasmic components. Cells were homogenized, pelleted, and washed three more times to remove the bulk of cytoplasmic proteins. The remaining membrane and cytoskeletal pellet was then extracted with N-octylglucopyranoside as above. Extracts prepared in this manner similarly contained immunoreactive calreticulin that bound to the hep I affinity column and that blocked focal adhesion disassembly (data not shown).

Tenascin-C and SPARC also stimulate the loss of focal adhesions from ~50% of the cells with preformed adhesion plaques (32, 33). In order to test the specificity of the inhibitory activity of peak II, we also tested whether proteins from peak II could similarly inhibit tenascin-C and SPARC stimulation of focal adhesion disassembly. The effects of the proteins in peak II appear to be specific for hep I-mediated focal adhesion disassembly as this peak did not inhibit the ability of either tenascin-C or SPARC to induce focal adhesion disassembly (Fig. 2). In addition, these data suggest that the inhibitory effect of peak II on hep I-mediated focal adhesion disassembly is not due to a general deleterious effect on the cells themselves.

Calreticulin Interacts with Thrombospondin—In order to determine whether there is a direct interaction between calreticulin and thrombospondin, recombinantly expressed GST-calreticulin and purified thrombospondin were incubated together and complexes immunoprecipitated with an anti-thrombospondin antibody as described under “Experimental Procedures.” Thrombospondin complexed with GST-calreticulin was detected by Western blot analysis using anti-GST antibodies.

| Table I |
| Comparison of the N-terminal amino acid sequence of the 60-kDa hep I-binding protein and calreticulins |
| Residues common to bovine, human, and rabbit calreticulin (29–31) are indicated in bold. The first amino acid in the Hep I-binding protein was unable to be identified definitively. |
| Bovine hep I-binding protein | XPTVYFKEQF |
| Bovine calreticulin | EPVYFKEQF |
| Human calreticulin | EPVYFKEQF |
| Rabbit calreticulin | EPVYFKEQF |

These studies show that GST-calreticulin forms complexes with thrombospondin (Fig. 3A). Binding was not due to the GST portion of the protein since there was no detectable binding of GST protein to thrombospondin (Fig. 3A). Thrombospondin-calreticulin complex formation was enhanced in buffers containing physiologic concentrations of calcium (2–3 mM), suggesting that this interaction might be modulated by calcium (data not shown).

In order to determine whether the hep I sequence of thrombospondin is important for thrombospondin binding to calreticulin, increasing concentrations of the hep I peptide in molar excess were used to inhibit complex formation between thrombospondin and calreticulin (Fig. 3B). The hep I peptide significantly (90%) inhibited complex formation between calreticulin and thrombospondin in a dose-dependent manner with ~80% inhibition at a 10-fold molar excess (7.5 μM of hep I peptide).

The ability of hep I to inhibit thrombospondin-calreticulin interactions appears to be specific since a peptide in which the lysine residues of hep I had been modified to alanine residues was unable to inhibit calreticulin-thrombospondin interactions and did not stimulate focal adhesion disassembly. Taken together, these data suggest that thrombospondin interacts with calreticulin through the sequence represented by hep I peptide, consistent with a role for calreticulin in mediating the effects of hep I on focal adhesion disassembly.

Calreticulin Is Present on the Cell Surface of BAE Cells—Calreticulin is a calcium-binding protein found predominantly in the lumen of the endoplasmic reticulum. Its localization to other cellular compartments has been somewhat controversial; although in recent years there have been numerous reports of calreticulin expression on the surfaces of fibroblasts, lymphocytes, B16 melanoma cells, neurons, and neutrophils (27, 34–41). The identification of calreticulin in fractions of BAE extracts and the ability of calreticulin to block focal adhesion disassembly (see below) suggest that calreticulin might indeed be expressed on the surface of BAE cells where it could mediate focal adhesion disassembly by thrombospondin.

To determine the presence of calreticulin on the cell surface of BAE cells, surface proteins were labeled using a membrane-
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Fig. 3. The hep I peptide inhibits TSP and calreticulin complex formation. A, 0.75 μM of GST-calreticulin and 0.75 μM of purified TSP were incubated together, and complexes were co-immunoprecipitated with anti-TSP antibody (15 μg/ml). Binding of TSP to GST and immunoprecipitation of GST-CRT in the absence of TSP were tested as controls. The complexes were analyzed by SDS-PAGE and Western blot. Results are representative of three experiments. B, the interaction between TSP and GST-calreticulin was analyzed as described in A, except that GST-calreticulin was incubated with 7.5, 75, and 750 μM of hep I peptide (10^2–10^3-fold molar excess to TSP monomer) prior to the incubation with thrombospondin. A modified hep I peptide (Lys → Ala), which is inactive in focal adhesion disassembly, was also tested for its ability to inhibit thrombospondin-calreticulin complex formation.

Immunolocalization by confocal microscopy and fluorescence-activated cell sorting (FACS) were used to further confirm the cell surface localization of calreticulin. The localization of calreticulin in both permeabilized and non-permeabilized BAE cells was examined by confocal microscopy of BAE cell cultures treated with anti-calreticulin antiserum. The staining patterns for calreticulin in non-permeabilized and permeabilized cells are distinct. Staining of non-permeabilized cells is characterized by a diffuse to small punctate distribution over the cell surface (Fig. 5A and B). In contrast, staining for calreticulin in permeabilized cells exhibits the typical perinuclear and endoplasmic reticular pattern (Fig. 5C). Similar results were obtained using two different anti-calreticulin antibodies, including one specific for the N-terminal domain of calreticulin (Fig. 5B). This staining pattern was not detected in samples in which the primary antibodies had been omitted or in which preimmune serum was substituted for primary antibody (data not shown).

As another means of determining the cell surface expression of calreticulin, mildly trypsinized BAE cells were immunostained for calreticulin using two different anti-calreticulin antibodies and fluorescein-conjugated secondary antibody and then analyzed for surface-bound immunofluorescence using FACS analysis (Fig. 6). Polyclonal anti-calreticulin antiserum reacted with ~28% of the cells with a mean fluorescence nearly 2.5-fold greater than secondary antibody controls (Table II). Interestingly, a rabbit antibody raised against the N-terminal peptide of calreticulin stained a greater percentage of the cell population (~66%). The major portion of the calreticulin staining observed in these studies appears to be localized to the cell surface, since only 7% of cells stained with an antibody to an intracellular antigen, cytochrome c. Fluorescence intensity of calreticulin staining was variable over the cell population (Fig. 6), an observation that was not readily appreciated by immunofluorescence localization on adherent cells. Alternatively, there may be a subpopulation of cells with calreticulin that is more susceptible to either trypsinization or loss during cellular manipulations. However, under these same conditions 97% of the cells stained positive for the β3 integrin.

Calreticulin Blocks Hep I and TSP-mediated Focal Adhesion Disassembly—In order to determine whether cell surface calreticulin is important for hep I and thrombospondin stimulation of focal adhesion disassembly, BAE cells were pretreated with antibodies to calreticulin to determine whether they could block focal adhesion disassembly by hep I. Preincubation of cells with rabbit polyclonal anti-calreticulin antiserum blocked the ability of hep I to stimulate focal adhesion disassembly. Non-immune serum did not affect the activity of hep I. Anti-serum alone or non-immune rabbit serum did not affect the basal number of cells positive for focal adhesions (Fig. 7A). The ability of anti-calreticulin to block hep I-mediated focal adhesion disassembly does not appear to be the result of nonspecific steric factors, since antibody to protein disulfide isomerase, a
Immunofluorescence identification of cell surface calreticulin by confocal microscopy. Non-permeabilized BAE cells were fixed in 3% paraformaldehyde for 10 min and processed for immunofluorescence with anti-calreticulin antiserum (A) or rabbit anti-N-terminal calreticulin IgG (B). Staining of non-permeabilized cells was compared with staining of fixed cells permeabilized with 0.1% Triton X-100 with rabbit anti-calreticulin (C). Cells were viewed using laser confocal microscopy. Bar = 20 μm.

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Protein expressed at the membrane that can bind calreticulin and thrombospondin, does not block focal adhesion disassembly in response to hep I (data not shown). The antibody to calreticulin was similarly able to block focal adhesion disassembly in response to thrombospondin, suggesting that the action of thrombospondin itself on BAE cells is also mediated by cell surface calreticulin (Fig. 7B). The ability of cell surface calreticulin to mediate focal adhesion disassembly appears to be specific for thrombospondin since the anti-calreticulin antiserum had no effect on either SPARC 2.1 or tenascin-mediated focal adhesion disassembly (Fig. 7C).

The ability of cell surface calreticulin to mediate focal adhesion disassembly by thrombospondin is not limited to BAE cells, since anti-CRT antiserum also blocks hep I-mediated focal adhesion disassembly in a uterine smooth muscle cell line (ELT-3) (63) and in bovine embryonic fibroblasts. These cells also stain for cell surface calreticulin (data not shown).

If thrombospondin/hep I interactions with BAE cells occur through binding to calreticulin, then preincubation of hep I or thrombospondin with recombinant GST-calreticulin should competitively block the ability of hep I and thrombospondin to interact with calreticulin on the cell surface and to signal focal adhesion disassembly. In order to test this, the ability of either hep I or thrombospondin to stimulate focal adhesion disassembly was examined following preincubation with increasing concentrations of GST-calreticulin (Fig. 8). It was found that GST-calreticulin blocked the ability of either hep I or thrombospondin to stimulate focal adhesion disassembly in a dose-dependent manner. The IC₅₀ value of calreticulin required to block 1 nM hep I activity was approximately 1.5 nM, suggesting a 1:1 interaction between this peptide and calreticulin. On the other hand, ~0.1 nM GST-calreticulin was the IC₅₀ value for inhibition of nearly 2,000-fold greater molar amounts of thrombospondin monomer (167 nM). This suggests that the hep I site might not be in an active conformation in the majority of thrombospondin molecules in this preparation. GST control protein had no effects on the ability of hep I to stimulate focal adhesion disassembly (data not shown). GST-calreticulin by itself had no effect on the stability of focal adhesions in endothelial cells (Fig. 8).

Anti-calreticulin Antiserum and Preincubation with Calreticulin Blocks the Ability of Hep I and Thrombospondin to Stimulate Activation of Phosphoinositide 3-Kinase—Thrombospondin and the hep I peptide stimulate an increase in the activity of the lipid kinase, PI3K, and the generation of the product of this kinase, PtdIns(3,4,5)P₃ (5). Stimulation of PI3K is required for focal adhesion disassembly in response to thrombospondin or the hep I peptide. If hep I is mediating focal adhesion disassembly through its interactions with calreticulin on the cell surface, then blocking thrombospondin/hep I binding to calreticulin should similarly block the ability of thrombospondin/hep I to activate PI3K. The generation of PtdIns(3,4,5)P₃ in response to stimulation with either thrombospondin or hep I was examined in the presence and absence of anti-calreticulin antibodies. These studies demonstrate that the ability of thrombospondin and hep I to stimulate activation of PI3K is inhibited by incubation of the BAE cells with anti-calreticulin antiserum but not by non-immune rabbit serum (Fig. 9). The ability of insulin to activate PI3K was not blocked with anti-calreticulin antiserum, suggesting that the effect of this antiserum is specific for calreticulin-mediated signaling and not a general effect of incubating the cells with this antiserum. These data are consistent with the conclusion that calreticulin is a component of the cell surface receptor complex that mediates the signaling of PI3K-dependent focal adhesion disassembly in response to the hep I sequence of thrombospondin. In addition, preincubation of the GST-calreticulin protein with either thrombospondin or hep I blocked stimulation of PI3K-dependent generation of PtdIns(3,4,5)P₃ (Fig. 10). Incubation of thrombospondin or the hep I peptide with GST had no effect on PI3K activity.

Discussion

To our knowledge, this is the first report to identify calreticulin as a receptor for thrombospondin and to elucidate a role for calreticulin in mediating focal adhesion disassembly. This
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FIG. 6. Identification of cell surface calreticulin by FACS analysis. BAE cells were pretreated as described for the PI3K assay. After a brief trypsinization, BAE cells were incubated with rabbit anti-calreticulin antibody (1/500). Cells were fixed followed by incubation with FITC-conjugated goat anti-rabbit IgG (1/150). Fluorescence associated with plasma membrane was analyzed by FACS. Cells treated with secondary antibody alone were used to determine the background fluorescence signal. Positive signal was determined as any fluorescence above that seen with secondary antibody alone. Results shown are representative of three separate experiments.

TABLE II
Quantification of cell surface calreticulin expression demonstrated by FACS analysis

| Gated as % signal | Mean fluorescence |
|-------------------|------------------|
| No antibody       | 0.6 ± 0.3        | 3.3 ± 0.5 |
| FITC goat anti-rabbit IgG | 13.1 ± 3.7 | 7.3 ± 4.0 |
| Rabbit anti-N-terminal calreticulin | 66.2 ± 7.8 | 45.4 ± 24 |
| Rabbit anti-calreticulin | 28.2 ± 2.9 | 18.0 ± 9.0 |
| FITC goat anti-mouse IgG | 5.1 ± 2.8 | 6.2 ± 2.4 |
| Mouse anti-vinculin | 11.9 ± 4.2 | 13.8 ± 6.9 |
| Mouse anti-β₁ integrin | 7.4 ± 2.1 | 8.3 ± 3.7 |
| Mouse anti-β₂ integrin | 97.4 ± 1.9 | 120.4 ± 31 |

newly identified role for cell surface calreticulin as mediator of de-adhesive changes differs from data showing that ER or cytoplasmic calreticulin promotes stable cell adhesion (21, 22, 43–45). Calreticulin in the ER plays a role in the control of cell adhesiveness via regulation of vinculin expression. Both vinculin protein and mRNA levels are increased in L fibroblasts expressing calreticulin and are down-regulated in cells expressing reduced levels of calreticulin (45). Similar down-regulation of vinculin expression is observed in epithelial cells with a diminished level of calreticulin (21). This coincides with an increase of total cellular phosphorytrosine, suggesting that the effects of calreticulin on cell adhesiveness may involve modulation of the activities of protein tyrosine kinases or phosphatases, which can affect the stability of focal contacts (21). On the other hand, 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 (22–24, 26, 46). Calreticulin-deficient embryonic stem cells have impaired integrin-mediated adhesion to fibronectin, although integrin expression is unaltered. Taken together, these results suggest that intracellular calreticulin promotes stable cell adhesion. This is in contrast to this present report in which calreticulin on the cell surface is involved in destabilizing cytoskeletal organization and cell adhesion. However, there are examples of signaling mediators or cell adhesion molecules having differential effects on cell adhesion depending on cell type, the initial adhesive state of the cell, and the particular milieu of receptors and matrix molecules (5).

Expression of calreticulin has been reported on the surface of several types of cells (27, 34–36, 38–42, 47, 48). CRT has an N-terminal signal sequence and thus could be transported to the cell surface (49). Gray et al. (35) showed that cell surface calreticulin on fibroblasts binds to the β chain of fibrinogen mediating its mitogenic activity. White et al. (48) found that calreticulin is expressed on the external cell surface as a putative mannoside lectin that triggers mouse melanoma cell spreading. Recently, Arosa et al. (39) reported finding calreticulin expressed on the cell surface of activated human peripheral blood T lymphocytes, where it is physically associated with a pool of unfolded major histocompatibility complex class I molecules. In addition, it has been reported that calreticulin is also localized on the surface of neutrophils and participates in the pertussis toxin-sensitive signal transduction pathway stimulated by L5, an anti-microbial peptide (40). Calreticulin can also be released from neutrophils during inflammation (37). Numerous studies have reported the presence of calreticulin on endothelial cells (50–53). Calreticulin on the surface of endothelial cells appears to be capable of modulating cell function, because it has been shown that calreticulin is involved in the production of interleukin-8 by human umbilical vein endothelial cells (51). Calreticulin is readily detectable on the surface of resting bone marrow vascular endothelial cells, and its expression is up-regulated in response to inflammatory mediators (53). Finally, there is evidence that calreticulin binds specifically and reversibly to bovine aortic endothelial cells in vitro (Kₐ approximately 7.4 nM) (36), suggesting that secreted calreticulin can also modulate endothelial cell behavior.

It is not clear how calreticulin is able to leave the ER and be transported to the cell surface. However, the expression of other KDEL-containing proteins at the cell surface has recently been shown (41). In fact, newly synthesized calreticulin appears to be preferentially transported to the cell surface where it has a half-life of approximately 12 h (41). These investigators also showed that calreticulin expressed on the surface of neuronal cells is turned over via a lysosomal degradation pathway (41). Interestingly, the N-terminal heparin-binding domain of thrombospondin that contains the hep I sequence also mediates rapid lysosomal degradation of thrombospondin via interactions with heparan sulfate proteoglycans and LDL-receptor-
related proteins (11, 12, 15). It is not known whether the hep I sequence is actually involved in the lysosomal degradation of thrombospondin, although it is possible that calreticulin binding to the hep I sequence in the N-terminal heparin-binding domain of thrombospondin may facilitate degradation of calreticulin.

Antibodies to calreticulin block the ability of thrombospondin or hep I to stimulate activation of PI3K, strongly indicating that calreticulin can signal from the peripheral membrane to the inside of the cell. Yet it is not clear as to how a peripheral membrane protein can induce intracellular signals. Calreticulin binding to cells does not appear to be sufficient to signal focal adhesion disassembly (Fig. 8), suggesting that thrombospondin/hep I binding to cell-associated calreticulin might trigger a ligand-dependent association (activation) or dissociation (de-repression) of calreticulin with a transmembrane molecule. It is also possible that thrombospondin/hep I can bind directly to a pre-existing complex of calreticulin and a transmembrane protein to signal focal adhesion disassembly. It is interesting to note that the focal adhesion disassembly receptor for tenascin-C is also a calcium-binding peripheral membrane protein, annexin II (54). The signaling pathways activated by tenascin-C-annexin II interactions have not yet been determined, although it is known that tenascin-C does not activate PI3K (5).

It will be important to determine what factors regulate specific expression of calreticulin at the cell surface and how these factors correlate with the highly regulated patterns of thrombospondin expression. Both calreticulin and thrombospondin have been shown to be up-regulated under conditions of stress and/or response to injury (55–62). Knowledge of these events and factors will help us to understand better the physiologic significance of thrombospondin-calreticulin interactions in reg-
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