Heparin-binding Peptides from Thrombospondins 1 and 2 Contain Focal Adhesion-labilizing Activity*

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The composition of the extracellular matrix plays a significant role in regulating cell shape and adhesion as well as in controlling the activity of various growth factors. Extracellular matrix was initially regarded as an inert scaffolding that held cells in place; however, there is an accumulating body of evidence that certain extracellular matrix glycoproteins can function as "anti-adhesive" molecules for some cell types (for recent reviews see Sage and Bornstein (1991) and Chiquet-Ehrismann (1991). These molecules include thrombospondin, tenasin, and SPARC. In previous work, we showed that these molecules in soluble form cause a disassembly of existing focal adhesion plaques from endothelial cells (Murphy-Ullrich and Höök, 1989; Murphy-Ullrich et al., 1991). Loss of focal adhesions is characteristic of the transition from a stationary to a motile phenotype of cells in culture (Couchman and Rees, 1979). Thus, these matrix molecules may serve as "permissive" factors that destabilize cell-matrix interactions so that they are more responsive to migratory stimuli. These anti-adhesive matrix molecules are potentially significant during processes such as development, wound healing, and metastasis.

Thrombospondin (TSP) is an extracellular matrix glycoprotein that as a substrate has both adhesive and anti-adhesive characteristics depending on the experimental conditions (EDTA versus Ca++; pH 4 versus pH 7) used to immobilize TSP and the cells interacting with TSP (reviewed in Bornstein (1992), Frazier (1991), and Mosher (1990). Many cell types will attach to TSP substrates (implying an interaction of a cellular receptor with a sequence of TSP), whereas TSP is only rarely adhesive (meaning stimulating organization of the cytoskeleton, i.e. spreading and focal adhesion formation, which is triggered by interactions of TSP with receptors). Recent data show that there are at least four distinct but related TSP gene products (Lawler et al., 1991; Lawler et al., 1993; Bornstein et al., 1991a; Vos et al., 1992). TSP1 and TSP2 have a high degree of sequence homology; however, they differ significantly in the amino-terminal heparin-binding domain. As the tissue-specific and developmental patterns of the thrombospondins differ, expression of these gene products is likely to be subject to different regulatory controls (Bornstein et al., 1991b; Laherty et al., 1992; LaBell et al., 1992). To date, most functional studies have been conducted with the platelet form of TSP, most likely a TSP1 homotrimer. In addition to modulating cell adhesion, TSP has been shown to regulate cell proliferation (Majack et al., 1988; Bagavandoss and Wilks, 1990; Taraboletti et al., 1990; Murphy-Ullrich et al., 1992), migration (Taraboletti et al., 1990; Taraboletti et al., 1987), and angiogenesis (Good et al., 1990; Iruela-Arispe et al., 1991). TSP1 also interacts with growth factors (TGF-β) and regulates activation of latent TGF-β (Murphy-Ullrich et al., 1992; Schultz-Cherry and Murphy-Ullrich, 1993).

The cell adhesion regulating extracellular matrix glycoprotein, thrombospondin (TSP), causes a loss of focal adhesion plaques from spread endothelial cells and fibroblasts. To localize the site on TSP that has focal adhesion-labilizing activity, we initially tested proteolytic fragments of TSP for activity. The heparin-binding fragment has significant focal adhesion-labilizing activity, whereas the nonheparin-binding 140-kDa fragment had no significant activity. These results were consistent with previous data that showed that both a monoclonal antibody to the heparin-binding domain of TSP (A2.5) and heparin neutralized TSP activity. Peptides from putative heparin-binding sequences of the amino-terminal heparin-binding domain of TSP were synthesized and tested for their ability to cause loss of focal adhesions. The hep I peptide (amino acids 17–35) caused maximal loss of focal adhesions and was active at 0.1 μM, whereas peptide hep II (74–95) and peptide hep III (170–189) were inactive. The activity of the hep I peptide was neutralized by the addition of heparin and heparan sulfate but not by chondroitin sulfate. The basic amino acids in the hep I sequence appear to be required for focal adhesion-labilizing activity, because modification of the lysine residues at amino acids 24 and 32 rendered the peptide completely inactive. In addition, a peptide from the analogous sequence of mouse TSP 2, in which basic residues are conserved, was nearly as active as hep I from TSP1. These data show that the anti-adhesive activity of TSP is conserved in both TSP1 and TSP2 and that the active site is located in a 18-amino acid sequence in the heparin-binding domain of TSPs.
There are multiple receptors for TSP, and the variability in cell adhesive properties of TSP may be partially attributed to differences in expression patterns of each of these TSP receptors (Asch et al., 1991; Frazier, 1991). These receptors include heparan sulfate proteoglycans (Murphy-Ullrich and Mosher, 1987; Murphy-Ullrich et al., 1988; Sun et al., 1989), CD36 (Asch et al., 1987; Silverstein, 1992), αβ integrins (Lawler et al., 1988), and a 105-80 kDa heterodimeric membrane protein (Yabkowitz and Dixit, 1991). Because different domains of TSP interact with these various receptors, purification conditions that alter “accessibility” of certain domains of the TSP molecule also influence the adhesive properties of the molecule (Kaesberg et al., 1989; Sun et al., 1992; Murphy-Ullrich and Mosher, 1987).

In previous work (Murphy-Ullrich and Höök, 1989), we showed that TSP acts as an anti-adhesive matrix protein, causing the disassembly of focal adhesion plaques from spread endothelial cells. The anti-adhesive activity could be neutralized by a monoclonal antibody to the heparin-binding domain of TSP and by heparin (Murphy-Ullrich and Höök, 1989). In these experiments, the bacteria were passaged through the use of partially purified TSP and synthetic peptides that the anti-adhesive activity of TSP is localized to the heparin-binding domain of the molecule and that the basic amino acids are functionally important for TSP’s bioactivity. Furthermore, we identified an active sequence from the TSP2 molecule.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased: Dulbecco’s modified Eagle’s medium (DMEM) (Cell-Gro, Mediatech, Herndon, VA); fetal bovine serum (Hyclone Laboratories, Logan, UT); 500 μg/ml trypsin, 2.2 mM EDTA (Life Technologies, Inc.); bovine serum albumin (BSA); thrombin, heparin, and 863 base pairs (obtained from Owens, Monsanto Co., St. Louis, MO). A new NcoI restriction site was introduced by oligonucleotide-directed mutagenesis, which also created a HindIII site. The construct was expressed in E. coli JM101 by growth of cultures in either LB or complete M9 medium to an A660 nm of 0.6-0.8 followed by 1 h of induction with naladixic acid (Sigma) at 50 μg/ml. Bacteria were harvested by centrifugation.

The bacterial pellet was lysed by sonication in the presence of protease inhibitors, and the recombinant heparin-binding domain was purified by a modification of the method of Yabkowitz et al. (1989). Briefly, the heparin-binding domain is solubilized from the lysed bacterial pellet in 8 mM urea, diluted 40-fold in 25 mM Tris-HCl, pH 7.9, with 0.2 mM phenylmethylsulfonyl fluoride, 15 mM MgCl2 and centrifuged to clarify. The supernatant is applied to a Q-Sepha-rose fast flow column in 25 mM Tris-HCl, pH 7.6, and then applied directly to a heparin-Sepharose column (Pharmacia) and washed further with buffer containing 150 mM NaCl. The heparin-binding domain was eluted from the column at 0.6 M NaCl and stored at 4 °C. The recombinant heparin-binding domain migrated as a single band at ~29 kDa on SDS-polyacrylamide gel electrophoresis.

Cells—BAE cells were provided by Dr. Tommy Brock, Hypertension Research Unit, University of Alabama at Birmingham, or isolated in this laboratory from bovine aortas obtained at a local abattoir. All cultures were characterized for the presence of Factor VIII-related antigen and for uptake of acetylated low density lipoproteins. Cells were routinely cultured in DMEM supplemented with 4.5 g/liter glucose, 2 mM glutamine, and 10% fetal bovine serum. Cells were passaged when confluent with trypsin-EDTA and used between passages 3 and 12. Cultures were negative for mycoplasma.

Thrombospondin Purification—Thrombospondin was purified from fresh (24-48 h) human platelets purchased from the Birmingham American Red Cross by using heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.) affinity chromatography, followed by gel permeation chromatography on a column of A0.5m resin (Bio- Rad) in buffers containing 0.1 mM CaCl2, glutaraldehyde, glycine, cetoxime, and TLCK-treated chymotrypsin (Sigma); bovine kidney heparan sulfate and heparitinase (Seikagaku America Inc., Rockville, MD). Monoclonal antibodies to thrombospondin were kindly provided by Dr. Vishva Dixit, Department of Pathology, University of Michigan (Galvin et al., 1985).

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lucrual Probes, Inc., Eugene, OR. Rhodamine-conjugated goat anti-mouse IgG was purchased from Jackson Laboratories, West Grove, PA.

RESULTS

Heparin-binding Domain of TSP Contains Focal Adhesion-labilizing Activity—Previous work (Murphy-Ullrich and Höök, 1989), which showed that the TSP-mediated loss of focal adhesions from spread BAE cells was neutralized by heparin and by monoclonal antibody A2.5 to the heparin-binding domain of TSP, suggested that the active region of TSP was located in the heparin-binding domain of the molecule. Therefore, to localize this activity, we tested TSP fragments for focal adhesion-labilizing activity (Fig. 1A). The 25-kDa heparin-binding fragment caused a loss of focal adhesions from spread BAE cells and was slightly more effective than the intact TSP trimer when tested at equimolar concentrations (0.4 μM). The nonheparin-binding fragments of TSP, consisting primarily of 140- and 120-kDa fragments, had no activity (Fig. 1A). In other experiments not shown, the 70-kDa chymotryptic fragment from the stalk region of TSP also showed no activity. When the heparin-binding domain was incubated with the 140/120-kDa fragment, there was no further reduction in focal adhesion positive cells as compared with the cells incubated with the heparin-binding domain alone. These data strongly suggest that the active site is located in the heparin-binding domain.

To exclude effects from possible associated platelet proteins that might co-purify with the heparin-binding fragment, a recombinantly expressed form of the heparin-binding domain was purified from E. coli lysates and also found to have focal adhesion-labilizing activity identical to that of the natural heparin-binding domain (Fig. 1B).

Anti-adhesive Activity of TSP Is Localized to a 19-Amino Acid Sequence in the Heparin-binding Domain—To further localize the anti-adhesive activity of TSP, three peptides with potential heparin binding activity, i.e. clusters of basic amino acids, were synthesized, purified, and tested in focal adhesion assays. The sequences of the peptides are presented in Table I. Note that hep II contains two BXXB putative heparin binding sequences (Cardin and Weintraub, 1989), whereas hep I and hep III lack this configuration of basic amino acids. Of the three potential heparin binding sequences, hep I consistently showed anti-adhesive activity, whereas hep II and hep III were inactive (Fig. 2A). Loss of focal adhesions in cells treated with hep I was maximal between 0.1-10 μM (Fig. 2A). The loss of focal adhesions from spread cells was a partial effect and was similar to what was observed with TSP and other anti-adhesive matrix proteins, tenascin and SPARC (Murphy-Ullrich and Höök, 1989; Murphy-Ullrich et al., 1991). In 24 separate experiments, the mean number of focal adhesion-positive cells in samples treated with 0.1 μM hep I was 41 ± 8% and, in controls, 67 ± 9% cells were positive for focal adhesions. The activity is specific for hep I, because a peptide with a scrambled amino acid sequence of hep I has no activity (Fig. 2B and Table I).

There was no significant loss of focal adhesions when cells were treated with peptides from other domains of TSP (amino acids 368–387 from the first type I repeat (Prater et al., 1991) and amino acids 1115–1130 from the COOH-terminal domain (Kosfeld and Frazier, 1992)).

The population of hep I-treated cells with fewer than 3 focal adhesion plaques/cell (defined as negative) was morphologically similar to those cells treated with either TSP or tenascin (Murphy-Ullrich and Höök, 1989; Murphy-Ullrich et al., 1991). Hep I-treated focal adhesion negative cells, as observed by interference reflection microscopy (IRM), were gray in appearance and lacked the central distribution of focal adhesion plaques characteristic of control BAE cells (Fig. 3). Staining for vinculin and in hep I-treated cells corresponded to the interference reflection images. Vinculin-staining plaques were generally absent from the central cell body and occasionally found at the cell periphery in negative cells (Fig. 4). The F-actin-containing microfilaments in hep I-treated cells tended to be more peripheral in distribution than in control cells (Fig. 4).

The relative ability of these peptides to bind heparin was tested using a heparin-Sepharose affinity column (Fig. 5A). The hep I peptide has a heterogeneous affinity for heparin, eluting from the heparin column as two peaks at 0.22 and 0.26 M NaCl. These two peaks when reapplied separately to the column again eluted at NaCl concentrations identical to the original preparation, suggesting the presence of two distinct classes of binding sites on the heparin affinity matrix for the hep I peptide, rather than heterogeneity of the peptide itself. Similar heterogeneity of binding of the hep II and hep III peptides to the affinity matrix was also observed. However, these peptides eluted from the heparin-Sepharose column at a lower NaCl concentration than did hep I, indicating that the major binding site for hep I on the heparin affinity matrix has a higher affinity than do the major binding sites for hep II and hep III.

The amino acid sequence of hep I does not appear to influence binding to the heparin matrix because a scrambled version of hep I (Table I) eluted from the heparin-Sepharose with similar affinities as the native hep I sequence (Fig. 5B). Modification of the side chains of the lysine residues in the dihydroxypropylation-treated hep I eliminated the highest affinity binding. A similar elution profile was obtained for the
TABLE I

Sequences of peptides from amino-terminal TSP heparin-binding domain

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Domain</th>
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<tbody>
<tr>
<td>Hep I (huTSP1)</td>
<td>ELTGARKSGRRLVKGPD</td>
<td>amino acids 17-35</td>
</tr>
<tr>
<td>Hep I (muTSP2)</td>
<td>SISINRKTIGAKFRGPD</td>
<td>amino acids 17-35</td>
</tr>
<tr>
<td>Scrambled (hep I huTSP1)</td>
<td>RSKAGTLGERLKGARVG</td>
<td>amino acids 17-35</td>
</tr>
<tr>
<td>Hep II</td>
<td>ASLRQMKTRGLALAERKQSD</td>
<td>amino acids 74-95</td>
</tr>
<tr>
<td>Hep III</td>
<td>TRDLASIALRIRAKGRVQDF</td>
<td>amino acids 170-189</td>
</tr>
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Fig. 2. The hep I peptide contains focal adhesion-labilizing activity and modification of lysines 24 and 32 or scrambling the sequence abolishes hep I activity. A, BAE cells grown overnight on coverslips were incubated with increasing concentrations of three peptides (hep I, amino acids 17-35; hep II, amino acids 74-95; hep III, amino acids 170-189) from the heparin-binding domain of TSP and assayed for percent of cells positive for focal adhesions by IRM. Results are expressed as the means of five separate experiments ± S.D. B, BAE cells were incubated with BSA (30 μg/ml), 0.1 μM hep I, 0.1 μM lysine-modified hep I (hep I−K) or a scrambled hep I sequence, and assayed for focal adhesions. Results are expressed as the means of three separate experiments ± S.D.

Hep I Activity Is Neutralized by Heparin or Heparan Sulfate, but Activity Is Independent of Cell-surface Heparan Sulfate Glycosaminoglycans—Because TSP anti-adhesive activity is neutralized by heparin and monoclonal antibody A2.5 to the heparin-binding domain (Murphy-Ullrich and Höök, 1989), it was determined whether hep I activity was also sensitive to sulfated glycosaminoglycans. Heparin and heparan sulfate from bovine kidney partially neutralized hep I activity when tested at 10–100 μg/ml; however, chondroitin 6-sulfate had no significant neutralizing activity (Fig. 6A). This is consistent with previous observations using TSP.

To assess whether interactions of hep I with cell-surface heparan sulfate glycosaminoglycans are necessary to mediate the loss of focal adhesions, BAE cells were pretreated with heparitinase to specifically degrade the heparan sulfate glycosaminoglycan chains and then tested with hep I. Heparitinase-treated cells retained sensitivity to the focal adhesion labilizing effects of hep I (Fig. 6B), suggesting that the actual
cell-surface binding molecule for hep I may not be a heparan sulfate glycosaminoglycan.

The Basic Amino Acids Are Important for Hep I Anti-adhesive Activity—To determine the functional importance of the positively charged residues of hep I, a modified peptide subjected to dihydroxypropylation reduction treatment to block the side chains of lysines at residues 24 and 32 was tested for activity. In repeated experiments, the lysine-modified peptide at concentrations up to 1 μM, failed to demonstrate any focal adhesion-labilizing activity (Fig. 2B), and suggested that one or both of the lysine residues are essential for activity. However, the corresponding sequence from mouse TSP2 (in which lysine 24 is conserved but lysine 32 is substituted for an arginine) is nearly as active as hep I from human TSP1 (Fig. 7 and Table I). This could mean that while the presence of a basic amino acid in position 32 is required, a lysine or arginine could fulfill this requirement equally well or that it is the lysine in position 24 that is critical for the anti-adhesive activity. Experiments with peptides modified (lysine to norleucine) either at residue 24 or 32 support the former, a basic residue is required at position 32.

DISCUSSION

These studies show that the amino-terminal high affinity heparin-binding domain of TSP is responsible for the TSP-mediated loss of focal adhesions from cells. Furthermore, these data show that residues 17–35 from TSP1 and TSP2 contain the active site, because synthetic peptides mimicking these sequences displayed activity. In addition, the presence and the arrangement of the basic amino acid lysine are critical for activity. The effective concentration of the hep I peptide (~0.1 μM) corresponded to the effective dose of TSP trimer (0.3 μM) required to achieve a similar response (Murphy-Ullrich and Höök, 1989). The morphology of the affected cells correlated with the changes we previously observed in cells treated with TSP trimer, tenasin, or SPARC, a loss of focal adhesions from the central cell body and a loss of vinculin-containing plaques and rearrangement of actin microfilaments. Similar to cells treated with TSP trimer or tenasin, there was a subpopulation of cells (~40%) that was refractory to treatment with these anti-adhesive proteins (Murphy-Ullrich and Höök, 1989; Murphy-Ullrich et al., 1991). As with the TSP trimer, loss of focal adhesion activity mediated by hep I could be neutralized with heparin and heparan sulfate but not chondroitin sulfate. Thus, these data indicate that residues 17–35 of TSP contain the anti-adhesive active site.

The amino-terminal domain of TSP has been shown to have interactions with heparin, heparan sulfate, and sulfatide (Murphy-Ullrich et al., 1988; Sun et al., 1989; Dixit et al., 1984; Roberts et al., 1985; Roberts, 1988) that can mediate multiple activities, melanoma cell spreading (Roberts et al., 1987), chemotaxis of tumor cells (Taraboletti et al., 1987), CHO cell attachment (Kaesberg et al., 1989), and binding and...
internalization of TSP (Murphy-Ullrich and Mosher, 1987; Murphy-Ullrich et al., 1988). In addition, we now confirm that the anti-adhesive activity of TSP is also located in the heparin-binding domain.

The hep I peptide binds heparin-affinity matrices with moderate affinity, its activity can be neutralized by heparin and heparan sulfate, and the basic lysines at positions 24 and 32 are required for activity. These data would seem to suggest that hep I interactions with cell-surface heparan sulfate proteoglycans are important for bioactivity. However, focal adhesion-labilizing activity does not correlate simply with heparin binding to the cell surface, because heparitinase digests of ICs, of P2 (residues 24-33) for inhibiting deactivation-labilizing activity does not correlate simply with heparin binding to the cell surface.

REFERENCES


