Journal of Cell Science 104, 1061-1071 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Diverse mechanisms for cell attachment to platelet thrombospondin

Josephine C. Adams* and Jack Lawler

Room LMRC-414, Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 221, Longwood Avenue, Boston, MA 02115, USA

*Author for correspondence

SUMMARY

Thrombospondin-1 is a component of the extracellular matrix which is thought to play important roles in cell migration and proliferation, during embryogenesis and wound repair. To understand the basis for these activities, we are mapping the regions of the molecule with cell adhesive activity. Here, we use antagonists of specific cell binding sites, adhesion-perturbing thrombospondin monoclonal antibodies and proteolytic fragments of platelet thrombospondin, to investigate the adhesive mechanisms used by G361 melanoma cells, human intestinal smooth muscle cells (HISM), epidermal keratinocytes and MG-63 osteosarcoma cells. When attached to the same preparations of platelet thrombospondin, HISM and MG-63 cells underwent spreading, whereas G361 cells and keratinocytes did not. Attachment of all four cell types involved the carboxy-terminal domain. The type 1 repeats and the amino-terminal heparin binding domain were important for stable attachment of G361, HISM and MG-63 cells, but were not involved in keratinocyte attachment. GRGDSP peptide caused near complete inhibition of HISM and MG-63 cell attachment, partially inhibited G361 attachment, but did not inhibit keratinocyte attachment. Attachment of HISM and MG-63 cells involved the $\alpha_v\beta_3$ integrin. The integrity of the thrombospondin molecule was important for its adhesivity towards G361, HISM, and MG-63 cells, whereas keratinocytes attached to the 140 kDa tryptic fragment as effectively as they did to the intact molecule. These results show that cell attachment to platelet thrombospondin typically involves multiple binding interactions, but the exact profile of interactions is cell type specific. Usage of particular cell-binding sites does not predict whether cells will undergo spreading or not. These data may, in part, explain some of the current controversies surrounding the mechanisms of cell attachment to thrombospondin.

Abbreviations: TSP-1, thrombospondin-1; TSP-2, thrombospondin-2; TBS, Tris-buffered saline; DMEM, Dulbecco's modified Eagle's medium

Key words: cell attachment, thrombospondin, RGD peptide, heparin-binding peptide

INTRODUCTION

The thrombospondins form a family of related, multi-domain glycoproteins (Lawler and Hynes, 1986; Bornstein et al., 1991; LaBell et al., 1992; Vos et al., 1992; Lawler et al., 1993a; Adams and Lawler, 1993). The first gene product to be identified, now termed thrombospondin-1, (TSP-1), is a 420 kDa trimeric glycoprotein which is present in platelet α-granules and is secreted by a variety of cell types in culture (reviewed by Majack and Bornstein, 1987). Proteins immunologically related to thrombospondin-1 are present in the extracellular matrix and basement membranes of various tissues, being particularly widely expressed during development (Wight et al., 1985; O'Shea and Dixit, 1988), and in situations of tissue repair (Raugi et al., 1987; Watkins et al., 1990). In vitro assays have shown that thrombospondin purified from platelets typically supports cell attachment, but not cell spreading, and also promotes cell migration and proliferation. These observations have led to the hypothesis that thrombospondin-1 acts as an anti-adhesive and growth stimulatory component of the extracellular matrix in situations of tissue remodelling (reviewed by Sage and Bornstein, 1991).

To understand how thrombospondin-1 affects cell behaviour, it is important to understand the molecular basis of its interaction with cells. Each subunit of the thrombospondin-1 trimer consists of multiple domains which comprise globular amino- and carboxy-terminal domains, a region with homology to procollagen, and three types of repeated sequence motifs (Lawler and Hynes, 1986; Fig. 1). Currently, four regions of the molecule have been identified as adhesive sites. These are the amino-terminal globular domain, which contains heparin-binding sequences (Roberts et al., 1987; Lawler et al., 1992), the CSVTCG sequences within the type 1 repeats (Prater et al., 1991), the RGD sequence within the last type 3 repeat (Lawler et al., 1988) and a less precisely defined site within the carboxy-terminal globular domain (Kosfeld et al., 1991). Different types of adhesive receptors have been identified as binding to these sites: cell-surface heparan sulphate pro-

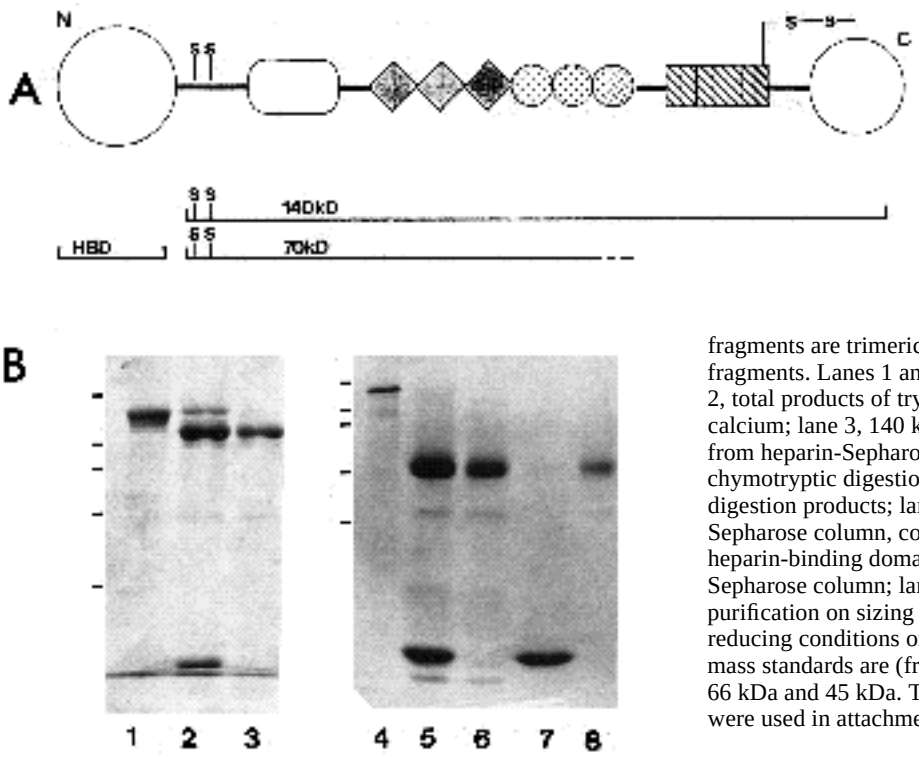

Fig. 1. Thrombospondin-1 and proteolytic fragments. (A) Schematic diagram of the thrombospondin-1 molecule, indicating the globular N- and C-terminal domains; the sites of the intersubunit disulphide bonds; the procollagen homology region (⬭) and the type 1 (◈), type 2 (○), and type 3 repeats (𝍸). Locations of the proteolytic fragments are indicated below. Note that the 70 kDa and 140 kDa fragments are trimeric. (B) SDS-PAGE analysis of proteolytic fragments. Lanes 1 and 4, intact platelet thrombospondin; lane 2, total products of tryptic digest, made in the presence of calcium; lane 3, 140 kDa fragment purified as flow-through from heparin-Sepharose column; lanes 5 to 8, products of chymotryptic digestion in the absence of calcium; lane 5, total digestion products; lane 6, flow through from heparin-Sepharose column, containing the 70 kDa fragment; lane 7, heparin-binding domain, purified by salt elution from heparin-Sepharose column; lane 8, 70 kDa fragment after further purification on sizing column. All samples were resolved under reducing conditions on 7% polyacrylamide gels. Molecular mass standards are (from the top) 200 kDa, 116 kDa, 97 kDa, 66 kDa and 45 kDa. The fragments shown in lanes 3, 7 and 8 were used in attachment assays.

teoglycans, such as syndecan, interact with the amino-terminal domain, and possibly the CSVTCG site (Sun et al., 1989; Guo et al., 1992); the $\alpha_v\beta_3$ integrin recognises the RGD site (Lawler et al., 1988), and CD36 has been implicated in the recognition of the carboxy-terminal domain and the CSVTCG sequences (Asch et al., 1987; Asch et al., 1992).

How these adhesive sites are used by cells is not well understood at present. The heparin-binding domain and RGD site have been the most extensively studied cell-binding sites, and different investigators have obtained contradictory results regarding the importance of these sites, even for the same cell type (e.g. Taraboletti et al., 1990; Lawler et al., 1988). In part, these contradictions appear to arise because of differences in the procedures used to purify platelet thrombospondin, which may alter the exposure of the RGD site (Lawler et al., 1988; Sun et al., 1992). However, it does appear that cell attachment to thrombospondin generally involves the use of more than one adhesive site. The contributions of all four adhesive sites have only been assessed for a couple of cell types: the data obtained indicate that G361 melanoma cells recognise all four sites (Roberts et al., 1987; Asch et al., 1991; Prater et al., 1991; Kosfeld et al., 1991), whereas 11B squamous carcinoma cells do not utilise the RGD site (Varani et al., 1986, 1988; Yabkowitz and Dixit, 1991). It is also unclear how individual adhesive sites work together to support cell attachment. Whilst there are reports that antagonists of single adhesive sites can completely prevent cell adhesion (e.g. Lawler et al., 1988), others have shown that it is necessary to block cellular interactions with at least two adhesive sites simultaneously to prevent cell adhesion (Asch et al., 1991; Stomski et al., 1992).

With the aim of clarifying these issues, we have used three separate approaches to identify the adhesive sites used by four different cell types. The cell types used all secrete thrombospondin (Majack and Bornstein, 1987; Wikner et al., 1987; Clezardin et al., 1989) and are derived from human tissues which contain thrombospondin (Wight et al., 1985; Gehron-Robey et al., 1989). Since thrombospondin has been implicated in both normal tissue biology and oncogenesis (Tuszynski et al., 1987), both non-transformed and transformed cell types were examined. Keratinocytes and smooth muscle cells were chosen as examples of cells reported to differ in RGD-sensitivity, which undergo migratory or proliferative responses to thrombospondin (Varani et al., 1986, 1988; Majack and Bornstein, 1987; Nickoloff et al., 1988; Lawler et al., 1988). G361 melanoma cells have been extensively used as a model for cell adhesive interactions with platelet thrombospondin (see above) and therefore provided a form of control for our experiments. Cell-bound thrombospondin is functionally important for platelet aggregation by MG-63 cells, an event which may be of importance in metastasis (Clezardin et al., 1991). The results we have obtained show that cell attachment to thrombospondin-1 typically involves recognition of multiple cell-binding sites and that there are distinct ways in which this can be achieved. We discuss these results in the context of current controversies regarding cell adhesive mechanisms to platelet thrombospondin, and with regard to the biological roles of thrombospondin.

MATERIALS AND METHODS

Materials

Thrombospondin-1 was prepared from human platelets as described (Lawler et al., 1985), with the modification of passing

material eluted from the heparin-Sepharose column over an anti-vitronectin antibody affinity column. Heparin (porcine intestinal sodium salt) was purchased from the Sigma Chemical Co. (St. Louis, MO). GRGDSP and GRGESP synthetic peptides, and antibodies P1B5 and P1D6, directed against the α_3 and α_5 integrin subunits, respectively (Wayner and Carter, 1987), were obtained from Telios Pharmaceuticals Inc, (San Diego, CA). Antibody 5E8, directed against the α_2 integrin subunit (Chen et al., 1991) was a gift from Dr. R. Bankert, Roswell Park Memorial Institute, Buffalo, NY). Antibody GoH3, directed against the α_6 integrin subunit (Sonnenberg et al., 1986) was obtained from CLB (Amsterdam, The Netherlands). Antibodies LM142, to the α_v integrin subunit, and LM609 to the $\alpha_v\beta_3$ complex (Cheresh, 1987), were generous gifts from Dr D. Cheresh, Research Institute of Scripps Clinic, San Diego, CA. Antibody Mab 13, to the β_1 integrin subunit (Akiyama et al., 1989) was a gift from Dr K. Yamada, NCI, Bethesda, MD. VTCG and GVCT peptides were synthesised by Dr C. Dahl, Harvard Medical School. Anti-thrombospondin monoclonal antibodies 4.1 (Prater et al., 1991) and C6.7 (Dixit et al., 1985) were gifts from Dr W. Frazier, Washington University, St. Louis, MO; the ESTs antibodies were generous gifts from Dr N. Hunter, Scottish National Blood Transfusion Service, Edinburgh, UK. Monoclonal antibodies MAI and MAII have been previously described (Lawler et al., 1985).

Cells

Human G361 melanoma cells, and HISM cells (Graham et al., 1984), were obtained from the ATCC. MG-63 osteosarcoma cells were a gift from Dr. S. Goodbourn, Imperial Cancer Research Fund, UK. The 3T3 cell lines transfected with human TSP-1 constructs have been previously described (Lawler et al., 1992). These cells were cultured in DMEM containing 10% fetal calf serum. Human epidermal keratinocytes (passages 4 to 8), were isolated from neonatal foreskin and cultured on a feeder layer of mitomycin-treated 3T3 cells in FAD medium as described (Adams and Watt, 1988). All cells were grown at 37°C in a humidified, 5% CO_2 atmosphere.

Cell adhesion assays

Polystyrene 96-well plates (Falcon # 76-232-05) were coated with thrombospondin or proteolytic fragments thereof, in Tris-buffered saline (TBS) containing 2 mM $CaCl_2$, for 16 h at 4°C. Wells were blocked with TBS, containing 2 mM $CaCl_2$ and 1 mg/ml heat-denatured BSA, for 1 h at room temperature. Cells were trypsinised from stock cultures, washed once in DMEM containing 10% fetal calf serum and twice in serum-free medium, resuspended at a concentration of 10^5 cells/ml, and 100 μl aliquots added to each 16 mm well. Attachment was carried out at 37°C for periods of between 1.5 h and 4 h, and then the non-adherent cells were removed by gentle rinsing. Adherent cells were fixed in 3.7% formaldehyde and stained with Giemsa. Photographs were taken using a Nikon Microphot microscope and Kodak T-MAX 100 film. For quantitation of adhesion assays, cell counts were made from photographs. At least three fields were scored for each replicate well, and mean values were calculated from replicate experiments. This mode of scoring permitted accurate assessment of cell morphologies under different experimental conditions.

Putative competitors of cell adhesion were added to the wells at the same time as the cells. In experiments using anti-thrombospondin antibodies, antibodies were incubated in the coated, blocked wells for 2 h at room temperature. The wells were then rinsed and adhesion assays carried out in the usual manner. The coating of thrombospondin fragments onto plastic was confimed by ELISA assay.

Epitope mapping of thrombospondin antibodies

A panel of wild type and mutant human TSP-1 proteins, stably expressed in 3T3 cells (Lawler et al., 1992) were used to identify the binding sites of monoclonal antibodies ESTs10 and ESTs12 (1.2). The cell lines were plated in 60 mm tissue culture dishes and grown for 24 h in DMEM containing 10% FCS, until nearly confluent. The cells were then metabolically labelled for 18 h in DMEM containing 5% dialysed FCS and 40 μCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL; 15 mCi/ml). The media were collected and clarified by centrifugation and PMSF added to 2 mM. Aliquots, equalised on the basis of TCA-precipitable counts, were then incubated with thrombospondin antibodies for 2 h on ice. Rabbit anti-mouse IgG serum (ICN Biomedicals, Irvine, CA) was then added and the incubation continued for a further 1 h. To collect the immune complexes, 30 μl of a 1:1 (v/v) suspension of Protein A-Sepharose (Pharmacia Biosystems Inc., Piscataway, NJ) was added and the samples mixed end-over-end at 4°C for 45 min. The beads and associated immune complexes were then pelleted by centrifugation and washed as previously described (Adams and Watt, 1988). Proteins were released by boiling for 5 min in SDS-PAGE sample buffer containing 100 mM dithiothreitol, and resolved on 7.5% polyacrylamide gels according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue, destained, and treated with 'Amplify' fluorographic reagent before drying. Dried gels were exposed to Kodak XAR-5 X-ray film at −70°C.

Production of glutathione S-transferase fusion proteins

A cDNA designated M3, encoding human TSP-1 amino acid residues 913-1152 (Lawler and Hynes, 1986), was subcloned, in frame, into the *Eco*RI site of the expression plasmid, pGEX1 (Pharmacia LKB Biotechnology). Expression and purification of the M3 fusion protein and of unfused glutathione S-transferase were carried out according to standard procedures (Smith and Johnson, 1988). The purity of the recombinant proteins was confirmed by SDS-PAGE.

Enzyme-linked immunosorption assay

Polystyrene 16 mm wells were coated with 5 μg/ml solutions of fusion proteins in TBS, overnight at 4°C. Wells were washed in TBS and blocked for 1 h at room temperature with 0.5% BSA in TBS. After washing, a dilution series of the primary antibody was incubated in the wells for 2 h. After further washing, wells were incubated with a 1:1000 dilution of peroxidase-conjugated goat anti-mouse IgG serum for 2 h. Wells were then washed, colour developed using a TMB peroxidase substrate kit (Kirkegaard and Perry Labs. Inc., Gaithersburg, MD), and the A_{450} was read using a Biorad microwell plate reader.

Preparation of proteolytic fragments of thrombospondin

The locations of the proteolytic fragments within the TSP-1 molecule are shown schematically in Fig. 1A. To prepare the 140 kDa fragment, platelet thrombospondin was digested for 20 h at 4°C with trypsin (Worthington) in the presence of 2 mM $CaCl_2$, at a 1:500 enzyme:substrate ratio (Lawler et al., 1985). Digestion was terminated by the addition of diisopropyl fluorophosphate (DFP) to 1 mM. The digest was passed over a 0.8 cm × 3.0 cm column of heparin-Sepharose (Pharmacia Biosystems Inc.) and the 140 kDa fragment was collected in the flow-through. To prepare the 25 kDa amino-terminal heparin-binding domain and the 70 kDa fragment, thrombospondin was digested for 20 h at 4°C with chymotrypsin (Worthington) in the presence of 5 mM EDTA, at an enzyme: substrate ratio of 1:100 (Dixit et al., 1985; Lawler et al., 1986). Digestion was terminated by the addition of DFP to 1 mM, and

the chymotrypsin was removed by filtering the digest through soybean trypsin inhibitor-coupled agarose (Pierce Chem. Co., Rockford, IL). The digest was then applied to the heparin-Sepharose column; the 70 kDa fragment was recovered in the flow-through and further purified by gel filtration through a 1.4 cm × 26 cm column of Sepharose 4B, equilibrated in 15 mM Tris-HCl, pH 7.6, 140 mM NaCl, 2 mM $CaCl_2$, containing 0.02% sodium azide. Peak fractions were pooled and concentrated by centrifugation through Centricon-10 microconcentrator units (Amicon Corp., Danvers, MA). The 25 kDa heparin-binding fragment was eluted from the heparin-Sepharose column with TBS containing 0.55 M NaCl. Protein concentrations were determined by the Bradford assay, using the Bio-Rad kit, and the purity of all the fragments was confirmed by SDS-PAGE, as shown in Fig. 1B.

RESULTS

Cell attachment to thrombospondin

The morphology of cells allowed to attach for 2 h at 37°C to surfaces coated with 50 μg/ml (120 nM) of platelet thrombospondin displayed cell type-dependent differences. Epidermal keratinocytes remained rounded, as did about 94% of the G361 cell population, although the other 6% underwent some spreading and assumed a fusiform morphology. In contrast, HISM and MG63 cells underwent spreading, with HISM cells becoming particularly well-spread and flattened. However, rather than adopting a smooth-edged, polygonal shape, HISM and MG-63 cells spread in an irregular manner, displaying multiple cytoplasmic processes (Fig. 2).

These differing morphological responses were reproducibly observed in cells adherent on four separate preparations of thrombospondin. Rounded or spread morphologies did not reflect differences in the speed of cell adhesion to thrombospondin, since even after 4 hours keratinocytes and the majority of G361 cells remained rounded. G361 cells, prepared for the attachment assays by EDTA treatment rather than by trypsinisation, also remained rounded. For each cell type, the percentage of cells attached depended on the coating concentration of thrombospondin used: maximal cell attachment was achieved at coating concentrations of 25 μg/ml (60 nM) and represented about 40% of the keratinocytes plated, 90% of the G361 cells, 65% of the HISM cells and 85% of the MG63 cells. Even at higher coating concentrations, keratinocytes and the majority of G361 cells remained unspread. Thus, the morphologies observed reflect cell type dependent differences.

Attachment was specifically to thrombospondin, as judged by several criteria. Firstly, cell attachment to BSA-coated substrata over the same time period was never more than 7% of the cells plated, and none of the cell types underwent spreading on BSA. Secondly, polyclonal antibodies to fibronectin or vitronectin did not prevent cell attachment, whereas a series of monoclonal antibodies to thrombospondin prevented cell attachment in a cell type-dependent manner (see below). Thirdly, attachment was prevented when thrombospondin was treated with 5 mM EDTA prior to coating (data not shown). Calcium-dependent adhesive activity is highly characteristic of thrombospondin, but is not a feature of fibronectin (Lawler et al., 1988). Thus, for all four cell types, attachment appeared to

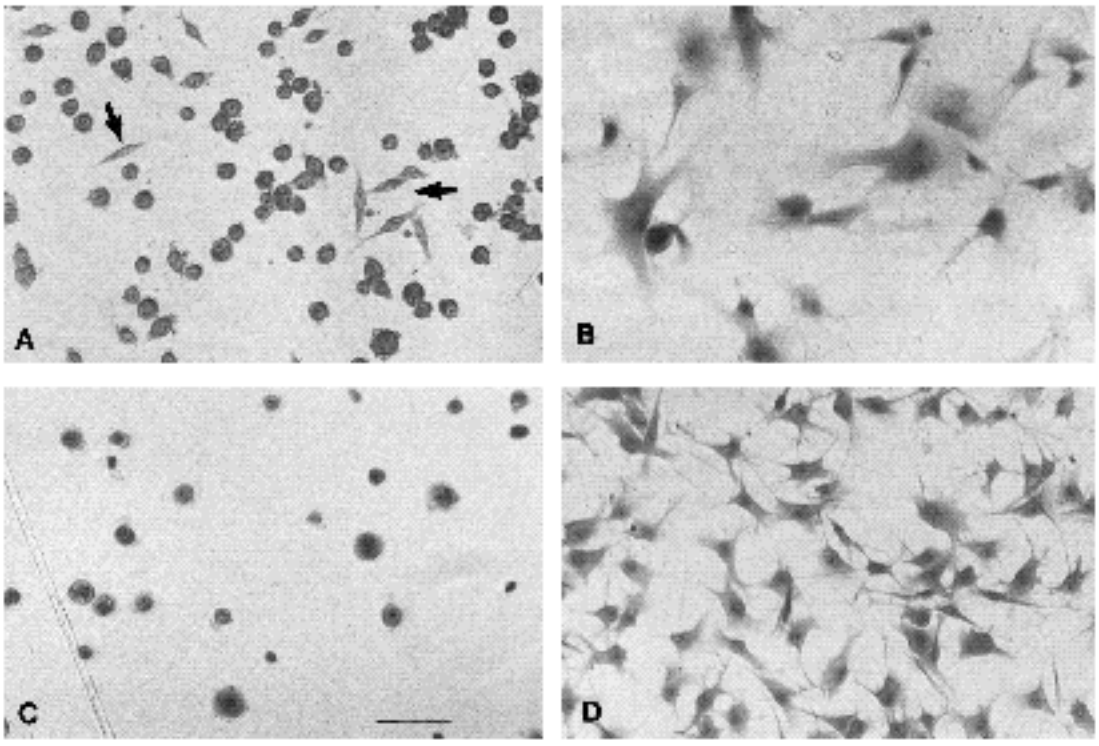

Fig. 2. Morphologies of cells attached to platelet thrombospondin. (A) G361 melamona cells (arrows indicate the few cells which underwent spreading); (B) human intestinal smooth muscle cells; (C) epidermal keratinocytes; (D) MG-63 osteosarcoma cells. Bar, 50 μm.

be directly to thrombospondin, and the spreading of MG-63 cells and HISM cells did not appear to be caused by the secretion of endogenous matrix.

Inhibition of cell attachment by antagonists of defined adhesive sites

Three cell binding sites within the thrombospondin-1 molecule have been defined in detail: these are the amino-terminal heparin binding domain, the CSVTCG sites within the type 1 repeats, and the RGDA site within the last type 3 repeat. Heparin, VTCG peptide or GRGDSP peptide, respectively, can be used as antagonists of the individual sites. To test the ability of these reagents to inhibit cell attachment, attachment assays were carried out in which each reagent was added at a range of concentrations, at the time of plating the cells, in wells coated with 50 nM thrombospondin.

The four cell types differed widely in their sensitivity to heparin: whilst attachment of epidermal keratinocytes was insensitive to heparin, the attachment of MG-63 cells, G361 cells and HISM cells could be inhibited in a concentration-dependent manner, but the maximal level of inhibition achieved was cell type-dependent: 500 μg/ml heparin caused a 40% to 50% inhibition of G361 or HISM cell adhesion, respectively, whereas a 70% to 80% inhibition of MG-63 cell adhesion was caused by heparin at concentrations above 100 μg/ml (Fig. 3A). The residual HISM or MG-63 cells which did attach tended to be rounded rather than spread.

VTCG synthetic peptide was tested at concentrations of up to 3 mM, and only G361 cells were sensitive to this reagent. The peptide caused a 50% inhibition of G361 cell attachment (Fig. 3B). The scrambled control peptide, GVCT, also used at 3 mM, did not prevent G361 cell attachment (data not shown).

Synthetic GRGDSP peptide, used at concentrations of up to 1 mM, did not prevent attachment of epidermal keratinocytes, but did inhibit the attachment of the other cell types in a concentration-dependent manner. The extent of inhibition by GRGDSP peptide varied in a cell type-dependent manner: 1 mM peptide caused a 40% decrease in G361 cell attachment, a 75% decrease in HISM attachment and a greater than 90% inhibition of MG-63 cell attachment (Fig. 3C). The residual attached cells tended to be rounded rather than spread. 1 mM GRGESP peptide did not prevent the attachment of any of these cell types (data not shown). In a variety of cell types, the thrombospondin RGD site serves as a ligand for the $\alpha_v\beta_3$ integrin (Lawler et al., 1988). To test whether this integrin was responsible for the RGD-dependent interactions of G361 cells, HISM cells and MG-63 cells with thrombspondin, a series of anti-integrin antibodies were tested for their ability to prevent cell adhesion. A function-blocking antibody, LM609, which is directed against the $\alpha_v\beta_3$ complex (Cheresh, 1987), prevented HISM and MG-63 cell attachment, and had a small effect on G361 cell attachment. An inactive antibody directed against the α_v subunit, LM142, was used as a negative control and did not prevent cell attachment (Fig. 3D). Function perturbing

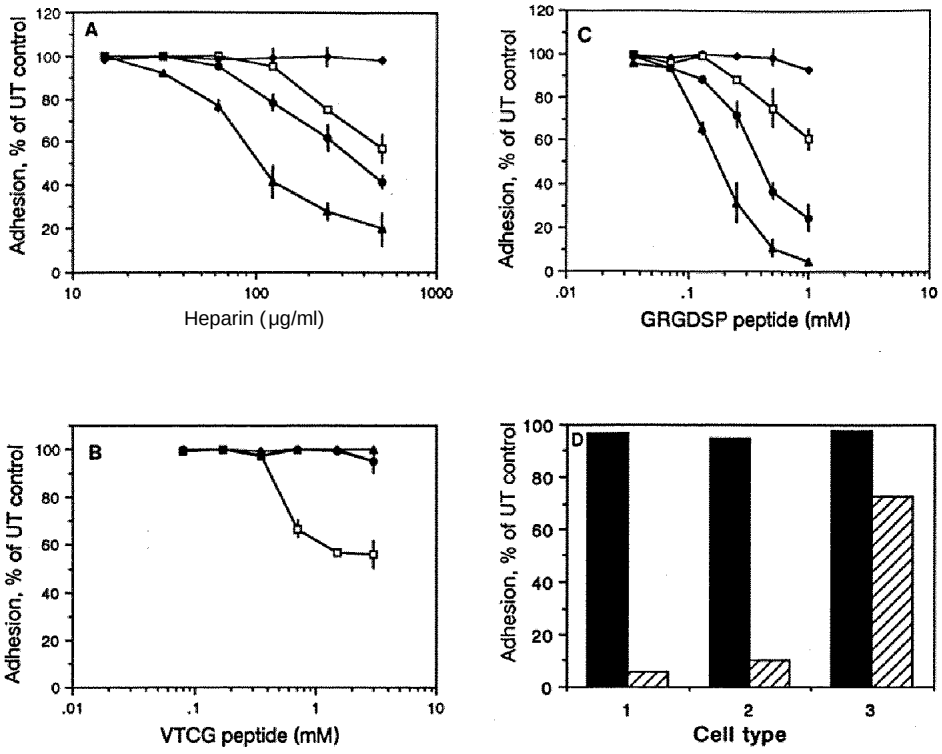

Fig. 3. Inhibition of cell adhesion to platelet thrombospondin by specific antagonists. G361 (□), HISM (●), keratinocytes (◆) or MG-63 cells (▲) were added to wells coated with 50 nM thrombospondin, in the presence or absence of heparin (A), VTCG peptide (B) or GRGDSP peptide (C). In D assays were carried out on HISM (1), MG-63 (2) or G361 cells (3) in the presence of 50 μg/ml of antibody LM142 (■), or antibody LM609 (▨). Data points are the means of duplicate determinations, bars indicate s.e.m.

antibodies directed against the α_2, α_3, α_5 and α_6 subunits (5E8, P1B5, P1D6 and GoH3, respectively), did not prevent cell adhesion. Mab 13, a function-perturbing antibody to the β_1 integrin subunit (Akiyama et al., 1989), had no effect on G361 or HISM cell attachment and caused a 25% decrease in MG-63 cell attachment (data not shown). HISM and MG-63 cells both express the $\alpha_v\beta_3$ integrin (Pytela et al., 1985; Adams, unpublished observation), and these data indicate that this integrin mediates RGD-dependent cell attachment to thrombospondin, as it does in other cell types (Lawler et al., 1988).

Inhibition of cell attachment by anti-thrombospondin antibodies

To investigate the involvement of the central and carboxy-terminal domains of the thrombospondin molecule in cell attachment, we examined the attachment-perturbing activities of 4 monoclonal antibodies which recognise epitopes within these regions of thrombospondin. The binding site of antibody 4.1 maps to a 50 kDa fragment which comprises the procollagen homology domain and the type 1 repeats (Prater et al., 1991), and antibody C6.7 recognises an epitope located near the carboxy terminus, between amino acid residues 1031 and 1152 (Dixit et al., 1985; Lawler and Hynes, 1986). The binding sites of antibodies ESTs12 (1.2) and ESTs10 were mapped using a panel of murine 3T3 cell lines, stably transfected with human TSP-1 expression vectors (Lawler et al., 1992). Neither antibody recognised murine thrombospondin (Fig. 4, lanes 1, 6 and 9), but both antibodies precipitated full-length human TSP-1 from the conditioned media of metabolically labelled cells (Fig. 4, lanes 2 and 8). Antibody ESTs12 (1.2) was incapable of precipitating TSP-1 deleted for the type 1 repeats (Fig. 4, lane 11), indicating that the epitope for this antibody depends on the presence of amino acid residues 361 to 530. Antibody ESTs10, however, also precipitated expressed human TSP-1 proteins deleted for either the procollagen homology region, the type 1 repeats or the type 2 repeats (Fig. 4, lanes 3 to 5), indicating that the epitope for this antibody lay outside the central region of the thrombospondin molecule. Since the antibody recognised the carboxy-terminal 140 kDa fragment of thrombospondin, but not the amino-terminal heparin-binding domain, in ELISA assays (data not shown), the epitope recognised by antibody ESTs10 must be located at the carboxy terminus of the thrombospondin molecule, between amino acid residues 673 and 1152. To map the binding site of this antibody more finely, a bacterially expressed glutathione S-transferase fusion protein containing the carboxy-terminal domain of TSP-1 was used in ELISA assays. The antibody specifically recognised the fusion protein containing human TSP-1 derived sequence, showing that the epitope for antibody ESTs10 lies between amino acid residues 913 and 1152 (Fig. 5). Thus, antibodies 4.1 and ESTs12 (1.2) serve as probes for the central region of the molecule, and antibodies C6.7 and ESTs10 are probes for the carboxy-terminal domain.

The four cell types displayed differing patterns of sensitivity to these antibodies. Attachment of G361 cells was completely prevented by antibodies 4.1, ESTs12 (1.2) and ESTs10, although somewhat higher concentrations of the latter antibodies were required for maximal inhibition. Antibody C6.7 caused a 30% decrease in cell attachment when used at 200 μg/ml (Fig. 6A). The attachment of HISM cells or MG-63 cells was also prevented by antibodies ESTs10, ESTs12 (1.2) or 4.1, with antibody 4.1 again being somewhat more effective (Fig. 6B and 6D). In contrast, keratinocyte attachment was substantially inhibited only by antibody ESTs10: even at high concentrations, the other three antibodies caused only a 20% to 25% decrease in the

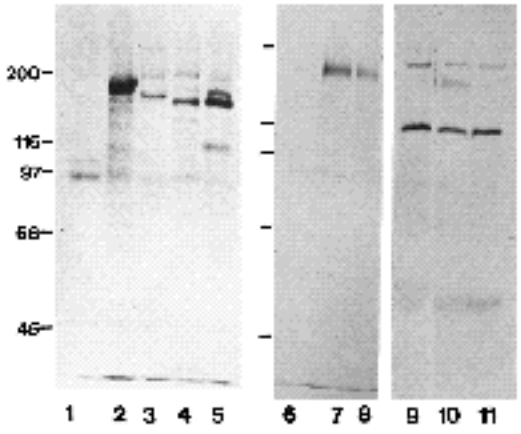

Fig. 4. Epitope mapping for monoclonal antibodies ESTs10 and ESTs12 (1.2). The conditioned media of metabolically labelled 3T3 cells transfected with pSV2neo plasmid (lanes 1,6 and 9), or pSV2neo plasmid plus pLEN plasmid containing cDNA encoding human TSP-1 (lanes 2,7 and 8); human TSP-1 deleted for the procollagen homology region (lane 3), the type 1 repeats (lanes 4,10 and 11), or the type 2 repeats (lane 5), were immunoprecipitated using antibodies ESTs10 (lanes 1-5), antibody MAI, (lanes 7 and 10), or antibody ESTs12 (1.2), (lanes 6,8,9 and 11). Precipitates were resolved on 7.5% polyacrylamide gels under reducing conditions. Protein standards are indicated in kDa.

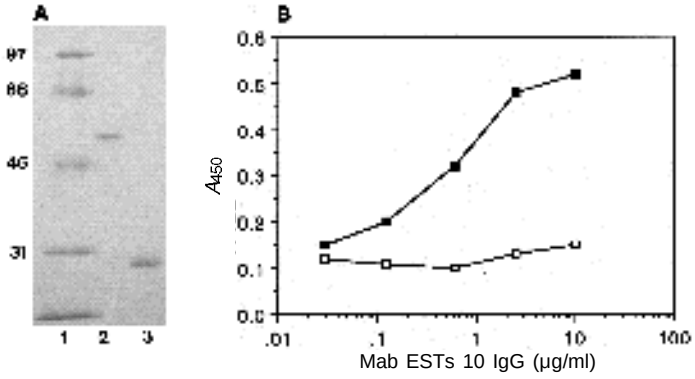

Fig. 5. The epitope for antibody ESTs10 lies within the carboxy-terminal domain. (A) Purified recombinant proteins used for ELISA assay, resolved on a 12% polyacrylamide gel. Lane 1, protein standards; lane 2, M3-GST fusion protein; lane 3, unfused GST. (B) Reactivity of antibody ESTs10 with M3-GST (■) or GST (□), measured by ELISA assay. Each point is the mean of triplicate determinations.

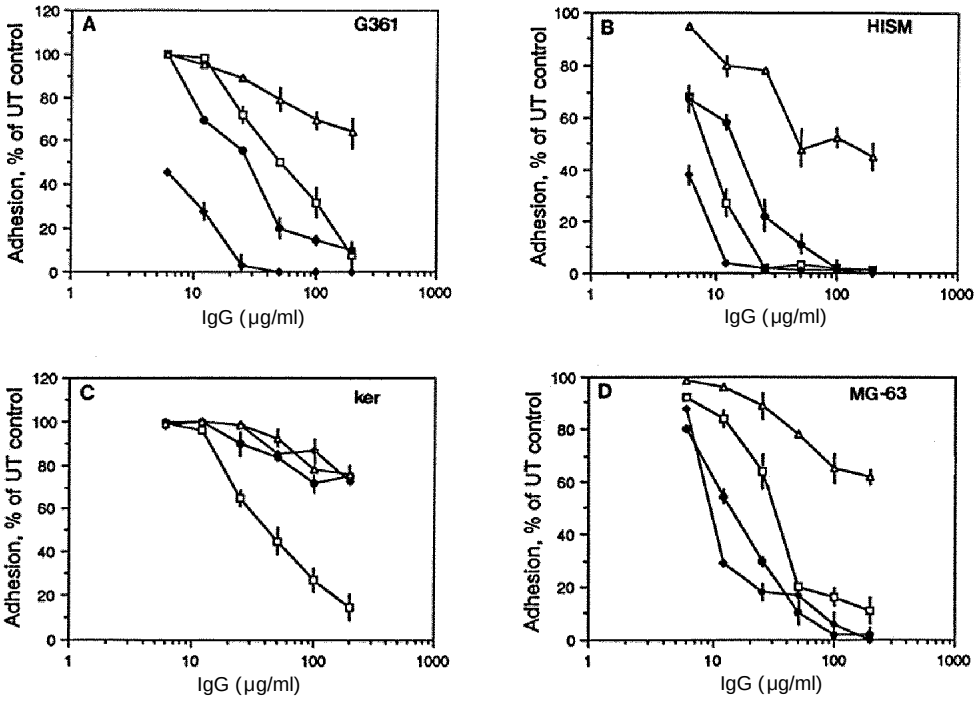

Fig. 6. Inhibition of cell adhesion to platelet thrombospondin by anti-thrombospondin monoclonal antibodies directed against the central or carboxy-terminal domains. G361 (A), HISM (B), keratinocytes (C) or MG-63 cells (D), were added to wells coated with thrombospondin which had been preincubated with monoclonal antibodies 4.1 (◆), ESTs12 (1.2) (●), C6.7 (△) or ESTs 10 (□). Data are plotted as the percentage of attached cells, relative to the numbers adherent on thrombospondin in the absence of antibodies (% UT). Data points are the mean of duplicate determinations, bars indicate s.e.m.

numbers of attached cells (Fig. 6C). Overall, these results indicate that the attachment of all four cells to TSP-1 involves the carboxy-terminal globular domain, while the central domain is also important for the attachment of G361, HISM and MG-63 cells. Two antibodies directed against the heparin-binding domain (MAII and MAIV) were also tested; neither of these antibodies prevented the attachment of any of the four cell types. None of the antibodies increased the spreading of keratinocytes or G361 cells, although antibody MAIV did increase the spreading of HISM cells in some experiments (data not shown).

Adhesion of cells to proteolytic fragments of platelet thrombospondin

To directly examine the abilities of particular adhesive domains to mediate cell attachment, we examined the ability of the four cell types to attach to wells coated with equimolar concentrations of platelet thrombospondin, the 140 kDa tryptic fragment, the 70 kDa chymotryptic fragment, or the 25 kDa heparin binding domain. The ability of the four cell types to attach to the 140 kDa fragment varied greatly. In Fig. 7, the data are plotted as the ratio of the number of adherent cells on a given concentration of 140 kDa fragment relative to the number adherent on the eqimolar concentration of intact thrombospondin. Thus, for G361 cells and HISM cells, a 10-fold higher concentration of the 140 kDa fragment was required to support cell attachment, and the number of adherent cells was about 5-fold less than observed on intact thrombospondin. There was less difference in the concentration dependency of attachment to the 140 kDa fragment or intact thrombospondin for MG-63 cells, but the number of adherent MG-63 cells was, at most, 40% of that observed on intact thrombospondin, and the attached cells did not spread. In contrast, as previously reported (Varani et al., 1988) the 140 kDa fragment was as effective on a molar basis as intact thrombospondin in supporting keratinocyte attachment, indicating that the adhesive sites recognised by keratinocytes are entirely contained within the 140 kDa fragment.

There was no observed cell adhesion to the 25 kDa heparin binding domain, which was used at coating concentrations of up to 500 nM; or to the 70 kDa chymotryptic fragment, which was used at coating concentrations of up to 200 nM. In a further set of experiments, the 25 kDa and 70 kDa chymotryptic fragments were co-coated in equimolar amounts, but HISM and MG-63 cells were still unable to attach, indicating that these two domains were unable to complement each other. To investigate whether

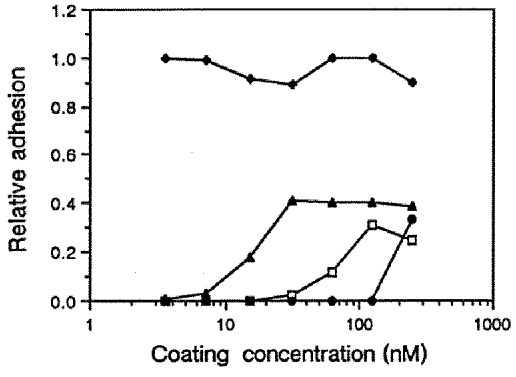

Fig. 7. Cell adhesion to the 140 kDa fragment of platelet thrombospondin. G361 (□), HISM (●), keratinocytes (◆) or MG-63 (▲) were added to wells coated with the indicated concentrations of the 140 kDa tryptic fragment. Adhesion is plotted relative to the number of cells which attached to wells coated with equimolar concentrations of intact thrombospondin. Each point is the mean of duplicate determinations.

it was necessary for all the cell binding sites to be present within a single molecule, thrombospondin was digested with trypsin or chymotrypsin under mild conditions in the presence of calcium ions (Lawler et al., 1985, 1986), and the digestion products coated as a mixture. Even under conditions which yielded the 140 kDa fragment and the 30 kDa heparin-binding domain, the ability of G361, HISM and MG-63 cells to attach was reduced by at least 70% (data not shown).

DISCUSSION

In this paper we have investigated the mechanisms of attachment to TSP-1 used by four cell types. Our results offer three major conclusions. First, cell attachment to TSP-1 typically depends on the recognition of multiple cell-binding sites. Secondly, each cell type displays a different profile of sensitivity to individual adhesion-perturbing reagents, suggesting that each cell type has a unique adhesive phenotype. Thirdly, usage of particular cell binding sites does not predict whether a cell will spread on TSP-1 or not. Therefore, the current controversy over the mechanisms of cell attachment to TSP-1 can, in part, be attributed to cell type-specific mechanisms of interaction. These findings are of significance for considerations of the biological roles of thrombospondin.

The importance of the amino-terminal heparin-binding domain for cell attachment was assessed both by using soluble heparin as a competitive inhibitor of cell attachment and by comparing cell attachment to intact platelet TSP-1 with attachment to the 140 kDa fragment, which lacks the heparin-binding domain. The fact that heparin prevented the attachment of three of the cell types, but did not affect keratinocyte attachment, indicated that its inhibitory activities were not simply due to non-specific charge effects. The importance of the heparin-binding domain for the attachment of G361, HISM and MG-63 cells was corroborated by the poor adhesivity of the 140 kDa tryptic fragment towards these cell types. However, since the isolated heparin-binding domain did not support cell attachment either, it appears to function in stabilising other adhesive interactions. Previous studies have also indicated a secondary, stabilising role for the heparin-binding domain, and led to the suggestion that this domain mediates cell spreading rather than direct attachment (Roberts et al., 1987; Asch et al., 1991). Our results do not wholly support this idea, since although the heparin-binding domain was involved in the adhesion of G361 cells, these cells did not spread on intact TSP-1. However, the partial spreading of MG-63 cells was indeed dependent upon this domain, since cells attached to the 140 kDa fragment remained rounded.

The central region of the thrombospondin molecule has been implicated in adhesive activity, but the molecular basis for this activity is presently poorly defined. The sequence motif CSVTCG, present within the type 1 repeats of TSP-1 and TSP-2, and also found in malarial circumsporozoite proteins, complement components and F-spondin (Prater et al., 1991; Klar et al., 1992), has been identified as an attachment site for G361 cells (Prater et al., 1991), and our experiments confirm these results. However, attachment of the other cells was not sensitive to inhibition by VTCG synthetic peptide, although the attachment of all four cell types was inhibited by monoclonal antibodies directed against the type 1 repeat region, suggesting that this region was indeed involved in cell attachment. Other possible cell binding sites within the type 1 repeats include BBXB heparin-binding sites (where B represents a basic residue, X, any residue; Cardin and Weintraub, 1989) and the WSXWS sequences, which form a novel type of heparin-binding motif (Guo et al., 1992) and which have also been implicated in protein-protein interactions (Miyazaki et al., 1991). These sequence motifs are conserved in TSP-1 from all species examined to date (Lawler et al., 1993b). Since we did not observe cell attachment to the 70 kDa fragment, which comprises the procollagen domain, the type 1 and type 2 repeats (Lawler et al, 1986), it may be that any cell binding sites within this domain are of low affinity and that interactions with other portions of the TSP-1 molecule are required for stable cell attachment. The substantial inhibitory effects of antibodies which bind to this domain could therefore be caused by steric hinderance of cellular interactions with the amino- or carboxy-terminal portions of TSP-1.

The type 3 repeats and the carboxy-terminal globular domain are thought to form one functional domain within the intact TSP-1 molecule (Lawler et al., 1985). The RGD site appeared to be involved in the adhesion of HISM and MG-63 cells, but not in the adhesion of keratinocytes. These results are in agreement with those obtained by others (Lawler et al., 1988; Varani et al., 1988). Keratinocytes do not express the $\alpha_v\beta_3$ integrin, defined as a receptor for this adhesive site (Adams and Watt, 1991; Lawler et al., 1988), whereas MG-63 and HISM cells both express this integrin (Pytela et al., 1985; J. C. Adams, unpublished observation) and an antibody to $\alpha_v\beta_3$ specifically prevented cell attachment to thrombospondin. There have been conflicting reports as to the RGD sensitivity of G361 cell adhesion to thrombospondin (Roberts et al., 1987; Asch et al., 1991); in our hands the peptide caused a partial inhibition of adhesion, whereas it completely prevented MG-63 and HISM cell adhesion. It has recently been shown that the degree of exposure of the RGD site can greatly alter the adhesivity of thrombospondin (Sun et al., 1992); however, in our experiments, the same preparations of thrombospondin were used under the same experimental conditions and so it is clear that cell type-specific differences do exist. The ligand binding activity of integrins can be regulated (Hynes, 1992), and so the differences in RGD-sensitivity we observe may reflect differences in the affinity of the ligand/receptor interaction (Felding-Habermann et al., 1992).

Interactions with the carboxy-terminal portion of the thrombospondin molecule were investigated through use of antibodies ESTs10 and C6.7, and by comparing the adhesive activities of the 140 kDa and 70 kDa proteolytic fragments. The antibodies had some attachment-perturbing activity against all four cell types, although the extent of inhibition varied. For keratinocytes and MG-63 cells there appeared to be significant adhesive activity within the 140 kDa fragment, which was not present within the 70 kDa fragment, showing that these two cell types recognise binding site(s) present within the carboxy terminus. For keratinocytes, it is clear that this adhesive activity does not

Table 1. Summary of mechanisms of cell adhesion to thrombospondin-1

Cell	Spreading on TSP-1	Involvement of TSP adhesive sites: Heparin-binding domain	Central region	RGD site	Carboxy-terminus
G361	−	+	+*	+	+
HISM	+	+	+	+	+
Keratinocyte	−	−	−#	−	+
MG-63	+	+	+	+	+

The table summarises the data obtained by using GRGDSP peptide, VTCG peptide, heparin, and antibodies to thrombospondin to inhibit cell adhesion to TSP-1, using proteolytic fragments of TSP-1 as adhesive substrata. Key: +, involved in cell adhesion; −, not involved. *, only G361 cells appear to recognise the VTCG site; #, antibodies to the central domain have only a small inhibitory effect on keratinocyte adhesion.

involve the RGD site. Neuronal cells also undergo RGD-independent attachment to the 140 kDa fragment of thrombospondin (O'Shea et al., 1991). Interestingly, although G361 cells and HISM cells attached very poorly to the 140 kDa fragment, their attachment to intact thrombospondin was inhibited by antibodies C6.7 and ESTs10, suggesting that the carboxy terminus indeed contained binding sites for these cell types, although it was unable to support cell attachment in the absence of the heparin-binding domain.

Taken together, our results show that cells attach to TSP-1 by recognising multiple cell-binding sites (Table 1). However, each cell type examined displayed a unique profile in terms of its sensitivity to individual reagents; for example, HISM and MG-63 cells are similar in utilising all four binding sites, but differ in their sensitivity to heparin and GRGDSP peptide. At present, we do not know whether this reflects differences in the exact cell-binding sites recognised; in the profile of receptors expressed; or in post-receptor, intracellular events. Further definition of attachment sites will be a first step towards resolving these issues. Since reagents directed against individual sites or domains substantially inhibit cell attachment, our results support a model in which cell attachment to TSP-1 is mediated by a series of binding interactions, the disruption of any one of which can prevent cell attachment.

From these studies we can distinguish two distinct types of mechanism for cell attachment to thrombospondin-1. For some cell types, such as keratinocytes and skeletal myoblasts (Adams and Lawler, unpublished) attachment occurs independently of the heparin-binding and RGD sites; for other cell types (G361, HISM, MG-63), both of these sites are involved in cell attachment. All cell types appear to recognise sites within the carboxy-terminal globular domain, which is the region most highly conserved in TSP-1 from different species and between thrombospondin family members (Lawler et al., 1993b; Adams and Lawler, 1993). Keratinocytes attach to the 140 kDa tryptic fragment, whereas the adhesive activity of TSP-1 towards the other cell types is severely decreased by proteolysis. Since we did not observe trans-complementation between individual adhesive domains when co-coated, it appears that, for certain cell types, the conformation of the TSP-1 molecule is critical in presenting cell-binding sites in an active form. These different modes of interaction with TSP-1 may be of biological significance, since the heparin-binding domain has been implicated in the rapid turnover and endocytosis of thrombospondin (Murphy-Ullrich and Mosher, 1987), in chemotactic cell migration (Taraboletti et al., 1897) and in the ability of TSP-1 to disrupt focal contacts in cells attached to other matrix molecules (Murphy-Ullrich and Hook, 1989). It is therefore likely that cells such as keratinocytes, which do not bind to this domain, will process and respond to TSP-1 in different ways. In addition, the heparin-binding domain is the region most labile to proteolysis (Lawler et al., 1982). As we have shown for some cell types, such proteolysis causes profound changes in the adhesiveness of TSP-1, which could be of significance in mediating changes in cell adhesive and migratory behaviour in vivo. It will therefore be important to identify the attachment mechanisms utilised by cells which are exposed to TSP-1 during early development.

We thank Mark Duquette and Kathy MacHenry for preparing the platelet thrombospondin, Dr Ian Dransfield for helpful discussions, and Drs R. Bankert, D. Cheresh, W. Frazier, N. Hunter, and K. Yamada for generous gifts of antibodies. This work was supported by grants HL28749 from the National Institutes of Health and HL42443 from the National Heart, Lung and Blood Institute (to J.L.) and Long Term fellowships from the European Molecular Biology Organisation and, latterly, the International Human Frontiers of Science Program Organisation (to J.C.A.).

REFERENCES

Adams, J. C. and Lawler, J. (1993). The thrombospondin family. *Curr. Biol.* (in press).

Adams, J. C. and Watt, F. M. (1988). An unusual strain of human keratinocytes which do not stratify or undergo terminal differentiation in culture. *J. Cell Biol.* **107**, 1927-1938.

Adams, J. C. and Watt, F. M. (1991). Expression of β_1, β_3, β_4 and β_5 integrins by human epidermal keratinocytes and non-differentiating keratinocytes. *J. Cell Biol.* **115**, 829-841.

Akiyama, S. K., Yamada, S. S., Chen, W.-T. and Yamada, K. M. (1989). Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly and cytoskeletal organisation. *J. Cell Biol.* **109**, 863-875.

Asch, A. S., Barnwell, J., Silverstein, R. L. and Nachman, R. L. (1987). Isolation of the thrombospondin membrane receptor. *J. Clin. Invest.* **79**, 1054-1061.

Asch, A. S., Silbiger, S., Heimer, E. and Nachman, R. L. (1992). Thrombospondin sequence motif (CSVTCG) is responsible for CD36 binding. *Biochem. Biophys. Res. Commun.* **182**, 1208-1217.

Asch, A. S., Tepler, J., Silbiger, S. and Nachman, R. L. (1991). Cellular attachment to thrombospondin: cooperative interactions between receptor systems. *J. Biol. Chem.* **266**, 1740-1745.

Bornstein, P., O'Rourke, K., Wikstrom, K., Wolf, F. W., Katz, R., Li, P. and Dixit, V. M. (1991). A second, expressed thrombospondin gene (*Thbs2)* exists in the mouse genome. *J. Biol. Chem.* **266**, 12821-12824.

Cardin, A. D. and Weintraub, H. J. R. (1989). Molecular modelling of protein-glycosaminoglycan interactions. *Atherosclerosis* **9**, 21-32.

Chen, F. A., Repasky, E. A. and Bankert, R. B. (1991). Human lung tumor-associated antigen identified as an extracellular matrix adhesion molecule. *J. Exp. Med.* **173**, 1111-1119.

Cheresh, D. A. (1987). Human endothelial cells synthesise and express an Arg-Gly-Asp-directed receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc. Nat. Acad. Sci. USA* **84**, 6471-6475.

Clezardin, P., Jouishomme, H., Chavassieux, P. and Marie, P. J. (1989). Thrombospondin is synthesised and secreted by human osteoblasts and osteosarcoma cells. *Eur. J. Biochem.* **181**, 721-726.

Clezardin, P., Serre, C.-M., Trzeciak, M.-C., Drouin, J. and Delmas, P. D. (1991). Thrombospondin binds to the surface of human osteosarcoma cells and mediates platelet-osteosarcoma cell interaction. *Cancer Res.* **51**, 2621-2627.

Dixit, V. M., Haverstick, D. M., O'Rourke, K. M., Hennessy, S. W., Grant, G. A., Santoro, S. A. and Frazier, W. A. (1985). A monoclonal antibody against human thrombospondin inhibits platelet aggregation. *Proc. Nat. Acad. Sci. USA* **82**, 3472-3476.

Felding-Habermann, B., Ruggeri, Z. M. and Cheresh, D. A. (1992). Distinct biological consequences of integrin $\alpha_V\beta_3$-mediated melanoma cell adhesion to fibrinogen and its plasmic fragments. *J. Biol. Chem.* **267**, 5070-5077.

Gehron-Robey, P., Young, M. F., Fisher, L. W. and McLain, T. D. (1989). Thrombospondin is an osteoblast-derived component of mineralised extracellular matrix. *J. Cell Biol.* **108**, 719-727.

Graham, M. F., Diegelmann, R. F., Elson, C. O., Bitar, K. N. and Ehrlich, H. P. (1984). Isolation and culture of human intestinal smooth muscle cells. *Proc. Soc. Exp. Biol. Med.* **176**, 503-507.

Guo, N.-H., Krutzsch, H. C., Nègre, E., Vogel, T., Blake, D. A. and Roberts, D. D. (1992). Heparin- and sulfatide-binding peptides from the type 1 repeats of human thrombospondin promote melanoma cell adhesion. *Proc. Nat. Acad. Sci. USA* **89**, 3040-3044.

Hynes, R. O. (1992). Integrins: versatility, modulation and signalling in cell adhesion. *Cell* **69**, 11-25.

Klar, A., Baldassare, M. and Jessell, T. M. (1992). F-spondin: a gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension. *Cell* **69**, 95-110.

Kosfeld, M. D., Pavlopoulos, T. V. and Frazier, W. A. (1991). Cell attachment activity of the carboxy-terminal domain of human thrombospondin expressed in E. coli. *J. Biol. Chem.* **266**, 24257-24259.

LaBell, T. L., McGookey Milewicz, D. J., Disteche, C. M. and Byers, P. H. (1992). Thrombospondin II: partial cDNA sequence, chromosome location and expression of a second member of the thrombospondin gene family in humans. *Genomics* **12**, 421-429.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lawler, J., Chao, F. C. and Cohen, C. M. (1982). Evidence for calcium sensitive structure in platelet thrombospondin. *J. Biol. Chem.* **257**, 12257-12265.

Lawler, J., Connolly, J. E., Ferro, P. and Derick, L. H. (1986). Thrombin and chymotrypsin interactions with thrombospondin. *Ann. N.Y. Acad. Sci.* **485**, 273-287.

Lawler, J., Derick, L. H., Connolly, J. E., Chen, J.-H. and Chao, F. C. (1985). The structure of human platelet thrombospondin. *J. Biol. Chem.* **260**, 3762-3772.

Lawler, J., Duquette, M., Urry, L., McHenry, K. and Smith, T. (1993b). The evolution of the thrombospondin gene family. *J. Mol. Evol.* (in press).

Lawler, J., Duquette, M., Wittaker, C., Adams, J. C., McHenry, K. and DeSimone, D. (1993a). Identification and characterisation of thrombospondin-4, a new member of the thrombospondin gene family. *J. Cell Biol.* **120**, 1059-1067.

Lawler, J., Ferro, P. and Duquette, M. (1992). Expression and mutagenesis of thrombospondin. *Biochemistry* **31**,173-1179.

Lawler, J. and Hynes, R. O. (1986). The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* **103**, 1635-1648.

Lawler, J., Weinstein, R. and Hynes, R. O. (1988). Cell attachment to thrombospondin: the role of Arg-Gly-Asp, calcium and integrin receptors. *J. Cell Biol.* **107**, 2351-2361.

Majack, R. A. and Bornstein, P. (1987). Thrombospondin: a multifunctional platelet and extracellular matrix glycoprotein. In *Cell Membranes*, vol. 3 (ed. E. Elson, W. Frazier and L. Glaser), pp. 55-57. Plenum Publishing Corp., New York.

Miyazaki, T., Maruyama, M., Yamada, G., Hatakeyama, M. and Taniguchi, T. (1991). The integrity of the conserved 'WS motif' common to IL-2 and other cytokine receptors is essentail for ligand binding and signal transduction. *EMBO J.* **10**, 3191-3197.

Murphy-Ullrich, J. E. and Hook, M. (1989). Thrombospondin modulates focal adhesions in endothelial cells. *J. Cell Biol.* **109**, 1309-1319.

Murphy-Ullrich, J. E. and Mosher, D. F. (1987). Interactions of thrombospondin with cells in culture: rapid degradation of both soluble and matrix thrombospondin. *Sem. Thromb. Hemostasis* **13**, 343-351.

Nickoloff, B. J., Mitra, R. S., Riser, B. L., Dixit, V. M. and Varani, J. (1988). Modulation of keratinocyte motility: correlation with production of extracellular matrix molecules in response to growth promoting and antiproliferative factors. *Amer. J. Path.* **132**, 543-551.

O'Shea, K. S and Dixit, V. M. (1988). Unique distribution of the extracellular matrix component thrombospondin in the developing mouse embryo. *J. Cell Biol.* **107**, 2737-2748.

O'Shea, K. S., Liu, L.-H. and Dixit, V. M. (1991). Thrombospondin and a 140 kDa fragment promote adhesion and neurite outgrowth from embryonic central and peripheral neurons and from PC12 cells. *Neuron* **7**, 231-237.

Prater, C. A., Plotkin, J., Jaye, D. and Frazier, W. A. (1991). The properdin-like type 1 repeats of human thrombospondin contain a cell attachment site. *J. Cell Biol.* **112**, 1031-1040.

Pytela, R., Pierschbacher, M. D. and Ruoslahti, E. (1985). A 125/115 kDa cell surface receptor specific for vitronectin interacts with the Arg-Gly-Asp adhesion sequence derived from fibronectin. *Proc. Nat. Acad. Sci., USA* **82**, 5766-5770.

Raugi, G. J., Olerud, J. E. and Gown, A. M. (1987). Thrombospondin in early human wound tissue. *J. Invest. Dermatol.* **89**, 551-554.

Roberts, D. D., Sherwood, J. A. and Ginsburg. V. (1987). Platelet thrombospondin mediates attachment and spreading of human melanoma cells. *J. Cell Biol.* **104,** 131-139.

Sage, E. H. and Bornstein, P. (1991). Extracellular proteins that modulate cell-matrix interactions. *J. Biol. Chem.* **266**, 14831-14834.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Sonnenberg, A., Daams, H., van der Valk, M. A., Hilken, J. and Hilgers, J. (1986). Development of mouse mammary gland: identification of stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *J. Histochem. Cytochem.* **34**, 1037-1046.

Stomski, F. C., Gani, J. S., Bates, R. C. and Burns, G. F. (1992). Adhesion to thrombospondin by human embryonic fibroblasts is mediated by multiple receptors and includes a role for glycoprotein 88 (CD36). *Exp. Cell Res.* **198,** 85-92.

Sun, X., Mosher, D. F. and Rapraeger, A. (1989). Heparin sulphate-mediated binding of epithelial cell surface proteoglycan to thrombospondin. *J. Biol. Chem.* **264**, 2885-2889.

Sun, X., Skorstengaard, K. and Mosher, D. F. (1992). Disulphides modulate RGD-inhibitable adhesive activity of thrombospondin. *J. Cell Biol.* **118**, 693-701.

Taraboletti, G., Roberts, D. D. and Liotta, L. A. (1987). Thrombospondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains. *J. Cell Biol.* **105**, 2409-2415.

Taraboletti, G., Roberts, D., Liotta, L. A. and Giavazzi, R. (1990). Platelet thrombospondin modulates endothelial cell adhesion, motility and growth: a potential angiogenesis regulatory factor. *J. Cell Biol.* **111**, 765-772.

Tuszynski, G. P., Gasic, T. B., Rothman, V. L., Knudsen, K. A., and Gasic, G. J. (1987). Thrombospondin, a potentiator of tumor cell metastasis. *Cancer Res.* **47**, 4130-4133.

Varani, J., Dixit, V. M., Fligiel, S. E. G., McKeever, P. E. and Carey, T. E. (1986). Thrombospondin-induced attachment and spreading of human squamous carcinoma cells. *Exp. Cell Res.* **167**, 376-390.

Varani, J., Nickoloff, B. J., Riser, B. L., Mitra, R. S., O'Rourke, K. and Dixit, V. M. (1988). Thrombospondin-induced adhesion of human keratinocytes. *J. Clin. Invest.* **81**, 1537-1544.

Vos, H. L., Devarayalu, S., de Vries, Y. and Bornstein, P. (1992). Thrombospondin 3 (*Thbs3*), a new member of the thrombospondin gene family. *J. Biol. Chem.* **267**, 12192-12196.

Watkins, S. C., Lynch, G. W., Kane, L. P. and Slayter, H. S. (1990).

Thrombospondin expression in traumatised skeletal muscle. Correlation of appearance with post-trauma regeneration. *Cell Tiss. Res.* **261**, 73-84.

Wayner, E. A. and Carter, W. G. (1987). Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J. Cell Biol*. **105**, 1873-1884.

Wight, T. M., Raugi, G. J., Mumby, S. M. and Bornstein, P. (1985). Light microscopical immunolocalisation of thrombospondin in human tissues. *J. Histochem. Cytochem.* **33**, 295-302.

Wikner, N. E., Dixit, V. M., Frazier, W. A. and Clark, R. A. F. (1987). Human keratinocytes synthesise and secrete the extracellular matrix protein, thrombospondin. *J. Invest. Dermatol.* **88**, 207-211.

Yabkowitz, R. and Dixit, V. M. (1991). Human carcinoma cells express receptors for distinct domains of thrombospondin. *Cancer Res.* **51**, 1645-1650.

(Received 22 December 1992 - Accepted 8 January 1993)