Identification of cell adhesive active sites in the N-terminal domain of thrombospondin-1

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Using a series of fusion proteins that span almost all of the thrombospondin-1 (TSP-1) molecule, we observed in this study that Chinese hamster ovary (CHO) K1 cells strongly attached to the N-terminus but not to the other domains of TSP-1 (e.g. the C-terminus, and type 1, type 2 and type 3 repeats). In addition, attachment to the N-terminus of CHO S745 cells defective in cell-surface glycosaminoglycans (GAGs) was decreased by 47% compared with that observed with CHO K1 cells, indicating the presence of GAG-dependent cell adhesive sites. With the aim of identifying these cell adhesive sites, a series of synthetic peptides, overlapping heparin-binding sequences ARKGSGRR (residues 22–29), MKKTRG (residues 79–84) and TRDLASIALRLIA-KGVNDNF (residues 170–189), were synthesized and tested for their ability to support CHO cell attachment. Using both centrifugation and cell-attachment assays, MKKTRG-containing peptides promoted CHO K1 cell adhesion, while ARKGSGRR-containing peptides and peptide TRDLASIALRLIA-KGVNDNF did not. CHO S745 cell attachment to MKKTRG-containing peptides was partially decreased. A 36% decrease in CHO K1 cell attachment to the N-terminus was also observed when the heparin-binding consensus sequence KKTR was mutated to QNTR. In addition, peptide MKKTRG partially inhibited (25% inhibition) CHO K1 cell attachment to the N-terminus. However, peptide MKKTRG was not sufficient to

fully promote cell attachment to the N-terminus of TSP-1. Peptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS also supported CHO K1 cell attachment in a GAG-dependent and -independent manner respectively. Moreover, CHO K1 cell attachment to MKKTRG was found to be markedly enhanced when flanked with the sequences VDAVRTEKGFLLLASLRQ and TLLALERKDHS. Peptide VDAVRTEKGFLLLASLRQ-MKKTRG nearly abolished (98% inhibition) CHO K1 cell attachment to the N-terminus, while peptides MKKTRG, MKKTRGTLLALERKDHS and VDAVRTEKGFLLLASLRQ had only a moderate inhibitory effect (25, 27 and 53% inhibition respectively). These data indicate that the sequence VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHS (residues 60–94) constitutes a GAG-dependent cell adhesive site in the N-terminus of TSP-1. Moreover, a GAG-independent site, encompassing residues 189–200 (FQGVLPQNVRFVF), has been identified. These two adhesive sites supported the attachment of a wide variety of cells (human breast carcinoma, melanoma and osteosarcoma cells), and a high degree of sequence homology was found between TSP-1 and TSP-2 between residues 60 and 94 (48% identity) and 189–200 (67% identity), further suggesting the functional importance of these two cell adhesive sites in the N-terminus of TSP-1.

INTRODUCTION

Thrombospondin (TSP) is a 450 kDa trimeric extracellular matrix glycoprotein synthesized and secreted by a wide range of normal and malignant cells *in vitro* and *in vivo* [1–3]. Five distinct genes have been described that encode for four structurally different TSPs (TSP-1, TSP-2, TSP-3 and TSP-4) and cartilage oligomeric matrix protein (COMP) [4,5]. However, most functional studies have been performed with TSP-1 isolated from human blood platelets. The functions of TSP-2, TSP-3, TSP-4 and COMP are largely unknown. TSP-1 functions in modulating cellular adhesion, migration, proliferation and differentiation [6]. Molecular cloning of TSP-1 revealed that each subunit of the trimer is made up of several domains, including N-terminal, C-terminal and procollagen homology domains, and type 1, type 2 and type 3 repeats [4]. The cell adhesive properties of TSP-1 involve several of these domains working together to support adhesion in a cell-type-dependent fashion [7–10], and separate receptors are used in a cooperative manner to support cell

adhesion [8,9,11,12]. Five regions of TSP-1 are involved in cell adhesion: (a) the heparin binding N-terminal domain; (b) a WSPWS sequence that occurs within the type 1 repeats and binds cell surface heparan sulphate proteoglycans [7,13,14]; (c) the sequence CSVTCG that occurs twice within type 1 repeats and binds to sulphatides [15,16], CD36 [17,18] and CSVTCG binding protein (CBP) [19]; (d) the sequence RGD located in the last type 3 repeat that binds to $\alpha_v\beta_3$ and $\alpha IIb\beta_3$ integrins [20,21]; and (e) the RFYVVM sequence that occurs within the C-terminal domain and binds CD47 [22]. In addition, the C-terminal domain also binds to a 105/80 kDa cell-membrane receptor [23].

The N-terminal domain of TSP-1 is essential to support attachment of various cultured cells, including many transformed cells [7,8,11], Chinese hamster ovary (CHO) cells [13] and smooth muscle and endothelial cells [11,24]. However, the adhesive active(s) site(s) located within the N-terminal domain are not known. The N-terminal domain of TSP-1 contains three regions of high affinity for heparin: the amino acid sequences ARKGSGRR (residues 22–29), MKKTRG (residues 79–84) and

Abbreviations used: TSP, thrombospondin; GST, glutathione S-transferase; CHO, Chinese hamster ovary; GAG, glycosaminoglycan; COMP, cartilage oligomeric matrix protein; BCA, bicinchoninic acid.

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TRDLASIALRLIAKGVNDNF (residues 170–189) [25,26]. The sequence MKKTRG contains the BBXB (B is a basic and X is any amino acid) consensus glycosaminoglycan (GAG)-binding sequence (KKTR) identified in a number of heparin-binding proteins [27]. Sequences ARKGSRR and TRDLASIALRLIAKGVNDNF lack this BBXB configuration but contain highly positively charged residues (RKGSRR and RLRIAK) responsible for binding to heparin [25,26]. Because heparin and heparan sulphate inhibit cell adhesion to TSP-1 [8,9,11–14], it is possible that these heparin-binding sites may mediate cell attachment to TSP-1 by binding to cell surface heparan sulphate proteoglycans. However, cell adhesion activity of the N-terminal domain of TSP-1 does not correlate simply with heparin affinity. Synthetic peptides mimicking two of these high-affinity heparin-binding sites (RKGSRR and RQMKKTR) do not support cell attachment of human A2058 melanoma cells [14]. Moreover, Murphy-Ullrich et al. [28] have shown that a synthetic peptide (residues 17–35) containing the amino acid sequence ARKGSRR has, on the contrary, an anti-adhesive activity.

With the aim of identifying the adhesive active site(s) present within the N-terminal domain of TSP-1, we have used CHO K1 cells and mutant CHO cell lines, with varying deficiencies in GAG synthesis, to study their attachment to a series of fusion proteins and synthetic peptides that span almost all of the N-terminal domain of TSP-1. The results reported here indicate that at least two sites (residues 60–94 and residues 189–200) in the N-terminal domain of TSP-1 can support cell attachment in a GAG-dependent and -independent manner respectively. The functional importance of these two cell adhesive sites is further suggested by their high degree of conservation in TSP-1 and TSP-2 from all known species.

EXPERIMENTAL PROCEDURES

Cell lines

The CHO parent line (K1) and mutant lines (S745 and S677), selected for deficiencies in GAG synthesis, were kindly provided by Dr. D. Mosher (University of Wisconsin, Madison, WI, U.S.A.), and have been described previously [13,26]. Human breast carcinoma (MCF-7, Hs578T, MDA-MB-231), melanoma (Bowes) and osteosarcoma (MG-63) cell lines, and the murine NIH 3T3 fibroblast cell line were obtained from the American Type Culture Collection. These cells were cultured in RPMI medium supplemented with 10 % (v/v) fetal calf serum.

TSP-1 and fusion proteins

Human platelet TSP-1 was purified by Mono Q anion-exchange chromatography as described previously [29]. Alternatively, purified human platelet TSP-1 was obtained from Serbio (Gennevilliers, France). Glutathione S-transferase (GST) fusion proteins were produced from the portions of cDNA clones designated M9, M10L, M4, M5 and M3 that encode for the N-terminal amino acid residues 1–90, the type 1 amino acid residues 385–522, the type 2 amino acid residues 559–669, the type 3 amino acid residues 784–932 and the C-terminal amino acid residues 877–1152 respectively [30]. These cDNAs were subcloned, in frame, into the *EcoRI* site of the bacterial expression vector, pGEX1. Expression and purification of unfused or fused GST with the N-terminus, type 1, type 2 and type 3 repeats and the C-terminus were carried out as previously described [9]. Mutagenesis of heparin-binding sequences in the N-terminal domain of TSP-1 were produced as previously described [25]. Two adjacent lysine residues (80 and 81) in the heparin-binding consensus sequence KKTR were mutated to QNTR [construct

NH₂ (80,81)]. This mutation was combined with the substitution of two adjacent arginine residues (28 and 29) in the sequence ARKGSRR to obtain ARKGSQNQ [construct NH₂ (80,81 + 28,29)]. A fusion protein encompassing amino acid residues 1–174 of the N-terminal domain of TSP-1 [31] was kindly provided by Dr. Chantal Legrand (INSERM Unit Research 353, Hôpital Saint Louis, Paris, France).

Peptide synthesis

Peptides were synthesized on an Applied Biosystems model 433A solid-phase peptide synthesizer using an *N*-(9-fluorenylmethoxycarbonyl (Fmoc) amide resin functionalized with the KNORR linker [4-(2',4'-dimethoxyphenyl-Fmoc-aminoethyl)-phenoxyacetamidoethyl resin]. The crude peptides were purified by reverse-phase HPLC using a C₁₈ column (Kromasil), and the purified peptides (purity > 93 %) were submitted for amino acid analysis. Each peptide was synthesized with and without a C-terminal tyrosine for coupling to BSA using the carbodi-imide or the bisdiazobenzidine method. The conjugation efficiency was determined by amino acid analysis.

Heparin-agarose affinity chromatography

The strength of binding of peptides with heparin was assessed by loading 100 µg of each peptide on a 1 ml heparin-agarose column linked to an FPLC system (Pharmacia), and determining the salt concentration at which the peptide eluted using a 0–1 M NaCl gradient in 0.01 M Tris, pH 7.4, containing 0.3 mM CaCl₂. The absorbance of each fraction eluted from the column was measured at a wavelength of 206 nm using a spectrophotometer (Kontron Instruments).

Centrifugal cell-attachment assay

In this assay, cells were spun in the U-shaped wells of 96-well plates (Falcon) that had been precoated with the proteins to be tested and blocked with excess BSA. This assay is based on the fact that there is an equilibrium between the centrifugal force that drives cells to the bottom of the well and the force of the cell-substrate adhesion [32–35]. On a non-adhesive substrate the centrifugal force predominates and the cells are driven to the bottom of the well to form a tight pellet. As the strength of the cell-substrate adhesion increases, the cells become more likely to bind to the substrate as they contact it, resulting in a ring pattern around the sides of the well. The inside diameter of the ring (corresponding to the cell-free area inside the ring) is then used as a measure of the adhesivity of the substrate. The more the cells adhere to a substrate, the more the inside ring diameter increases.

Increasing concentrations of TSP-1, fusion proteins and peptides coupled to BSA were diluted in HBS buffer [10 mM Hepes (pH 5.3)/0.135 M NaCl/3 mM KCl/0.5 mM MgCl₂/2 mM CaCl₂] and added to the U-shaped wells of a 96-well plate (40 µl/well). Duplicate or triplicate wells at each protein concentration were prepared, depending on the experiments. After overnight incubation at 4 °C, wells were washed three times with HBS buffer containing 0.2 % (w/v) heat-inactivated BSA (200 µl/well). The last wash was left for 30 min at room temperature. Trypsin/EDTA-treated cells were resuspended in HBS buffer containing 0.2 % (w/v) heat-inactivated BSA (200 000 cells/ml), left for 30 min at room temperature to recover from trypsin treatment, and added to each well (100 µl/well). The plate was immediately centrifuged at 800 *g* for 5 min. In some experiments, albumin- and TSP-coated wells were pre-incubated for 30 min with medium containing increasing concentrations of an anti-TSP-1 oligoclonal antibody (made of a mixture

of monoclonal antibodies) (50 μ l/well); cells were then added to the wells (400 000 cells/ml; 50 μ l/well). Alternatively, increasing concentrations of GAGs (heparin, heparan sulphate, dermatan sulphate, chondroitin sulphate; obtained from Sigma) (50 μ l/well) were preincubated in TSP-coated wells and cells were added directly to the wells (400 000 cells/ml; 50 μ l/well). The pattern of the cells in each well was observed using darkfield microscopy, and the ring diameter was measured with a concentric-circle eyepiece reticle (Zeiss, plate no. VI), previously calibrated with a stage micrometer (Graticule Ltd, Townbridge, U.K.).

Gravity cell-attachment assay

In the gravity assay, the cell suspension is allowed to settle for a fixed period of time onto a surface that had been precoated with the proteins to be tested and blocked with excess BSA. After washing to remove non-attached cells, attached cells are counted.

Cell gravity assays were performed in 60-mm bacteriological Petri dishes (Falcon), dotted with the proteins to be tested, as described by Grzesik and Gehron Robey [36] with minor modifications. Increasing concentrations of peptides coupled to BSA were diluted in HBS and applied (10 μ l/dot) to the Petri dishes. Each peptide concentration consisted of four replicates. After overnight incubation at 4 °C, the fluid was aspirated and 10 μ l of 60% methanol was added to each dot. After 2 h incubation at 4 °C, plates were washed twice with HBS/CaCl₂, followed by washing three times with RPMI medium containing 0.2% (w/v) BSA. The last wash was left for 30 min at room temperature. Trypsin/EDTA-treated cells were resuspended in RPMI medium containing 0.2% (w/v) heat-inactivated BSA (0.24 $\times$ 10⁶ cells/ml). After 30 min incubation at 37 °C to allow cells to recover from trypsin/EDTA treatment, cells were plated in Petri dishes (5 ml/dish) and further incubated for 3 h at 37 °C in a CO₂/air (1:19) incubator. At the end of the incubation, non-attached cells were removed by washing three times with PBS, and attached cells were fixed for 20 min in PBS containing 0.25% glutaraldehyde. Cells were then observed by microscopy and counted microscopically using an eyepiece reticle (Zeiss, plate no. VII).

Quantification of proteins and peptides immobilized on the plastic wells

Proteins and peptides coupled to BSA were immobilized on plastic wells using the procedure for the centrifugal cell-attachment assay. Bound peptides were quantified using a micro-bicinchoninic (BCA) protein assay as described by Kosfeld and Frazier [10]. The amount of each peptide bound was determined with a standard curve for known amounts of the same peptide.

RESULTS

CHO K1 cells specifically attach to TSP-1 in the centrifugation assay

Using this cell-adhesion assay, attachment of CHO K1 cells was proportional to TSP-1 concentration, reaching a plateau between 25 and 50 μ g/ml (Figure 1A). Increasing concentrations of an anti-TSP oligoclonal antibody (made of a mixture of monoclonal antibodies) dose-dependently inhibited the attachment of CHO K1 cells to TSP-1 (25 μ g/ml; 0.05 μ M) (Figure 1B), but not to collagen-coated wells (results not shown). Adhesion to TSP-1 was inhibited by 50% when the anti-TSP antibody was used at 0.015 μ M, and over 80% when added at 0.07 μ M. Because heparin and dermatan sulphate inhibit CHO K1 cell adhesion to

Figure 1 CHO K1 cells specifically attach to TSP-1 in the centrifugation assay

(A) Substrates were prepared by incubation with increasing concentrations of TSP-1. Trypsin/EDTA-treated cells were added to each U-shaped well and the plate was immediately centrifuged at 800 *g* for 5 min. The inside diameter of the ring was then used as a measure of the adhesivity of TSP-1 and was plotted versus the concentration of protein added to the wells. CHO K1 cells attached to TSP-1 in a dose-dependent manner. Results are the means $\pm$ S.D. of 11 separate experiments. (B) CHO K1 cells were directly added into TSP-coated wells (25 μ g/ml; 0.05 μ M) incubated with increasing concentrations of an anti-TSP oligoclonal antibody ($\square$), heparan sulphate ($\circ$) or dermatan sulphate ($\triangle$). The anti-TSP antibody and GAGs dose-dependently inhibited cell attachment to TSP-1. Results are the means $\pm$ S.D. of at least three separate experiments.

TSP-1 in a gravity assay [13], the ability of GAGs to inhibit CHO K1 cell adhesion to TSP-1 was determined in the centrifugation assay. Heparin (results not shown), heparan sulphate and, to a lesser extent, dermatan sulphate inhibited in a dose-dependent manner the attachment of CHO K1 cells to TSP-1 (Figure 1B). Half-maximal inhibition with heparan sulphate and dermatan sulphate was obtained at concentrations of 8 and 25 μ M respectively. As previously reported in the gravity assay [13], chondroitin sulphate did not inhibit adhesion.

N-terminal domain is essential to support CHO K1 cell attachment to TSP-1

The adhesive activity of the cell-recognition domains of TSP-1 was directly investigated using the N-terminal domain, type 1, type 2 and type 3 repeats and the C-terminal domain fused with GST. To normalize the attachment of CHO K1 cells to each of these TSP-1 domains, the diameter of the ring of cells in the centrifugation assay was plotted with respect to mol of fusion proteins actually bound to the wells (3.1–200 pmol/well). CHO K1 cells strongly attached to the N-terminal domain but not to

Figure 2 The N-terminal domain is essential to support CHO K1 cell attachment to TSP-1

Top: schematic diagram showing the different structural domains of the TSP-1 subunit. Bottom: the diameter of the ring of cells was plotted with respect to moles of TSP domains fused with GST which are bound to the wells. Attachment of CHO K1 cells to the N-terminus, the C-terminus, and to type 1, type 2 and type 3 repeats of TSP-1, are compared with GST. Results are the means $\pm$ S.D. of *n* separate experiments.

the C-terminal domain, and to type 1, type 2 and type 3 repeats (Figure 2). Using similar experimental conditions, NIH 3T3 fibroblast cells attached to the N-terminal domain, type 3 repeats and the C-terminal domain when compared with GST (Figure 3A). Bowes melanoma cells attached to the N-terminal domain, type 1 repeats and the C-terminal domain (Figure 3B). CHO K1 cell attachment to recombinant N-terminal proteins, encompassing amino acid residues 1–174 and 1–90, was compared. No significant decrease in cell attachment to a recombinant N-terminal protein encompassing amino acid residues 1–90 was observed compared with that obtained with fusion protein 1–174 (2.32 ± 0.6 versus 2.17 ± 0.5 mm (S.D., *n* = 4) at 100 pmol/well respectively), indicating that the cell adhesive activity of TSP-1 for CHO K1 cells was mainly located within amino acid residues 1–90.

Cell surface GAGs mediate CHO K1 cell attachment to the N-terminal domain of TSP-1

Because CHO K1 cell attachment to TSP-1 was dependent on the N-terminal domain (Figure 2), and because cell surface heparan sulphate proteoglycans on CHO K1 cells act as TSP cell adhesion receptors [13,26], the attachment to both TSP-1 and the recombinant N-terminal domain (residues 1–90) of CHO mutant cells defective in cell surface GAGs was studied. When compared with CHO K1 cells, the attachment of CHO S745 cells (6 % of GAGs with decreased heparan sulphate and chondroitin sulphate proteoglycans) to TSP-1 and its N-terminal domain was reduced by 43 and 47 % respectively (Table 1). The attachment of CHO S677 cells (50 % of GAGs with decreased heparan sulphate but increased chondroitin sulphate proteoglycans) to the N-terminal domain was decreased by 34 %. The non-specific cell attachment to GST did not differ significantly between CHO mutant cells and the parent cell line (Table 1). These findings, taken together with the fact that heparan sulphate inhibited CHO K1 cell

Figure 3 TSP-1 supports adhesion in a cell type-dependent fashion

The diameter of the ring of cells was plotted with respect to moles of TSP domains fused with GST which are bound to the wells. (A) NIH 3T3 fibroblast cells attached to the N-terminal domain, type 3 repeats and the C-terminal domain when compared with GST. (B) Bowes melanoma cells attached to the N-terminal domain, type 1 repeats and the C-terminal domain. Results are the means $\pm$ S.D. of *n* separate experiments.

Table 1 CHO subline adhesion to TSP and its N-terminal domain

TSP, the N-terminal domain fused with GST and unfused GST were coated at 50 pmol/well. The total glycosaminoglycan content present on the surface of S677 and S745 cells is 50 and 6 % of the amount present on the parent cell line (K1) respectively. Results (in mm) are the means $\pm$ S.D. of *n* separate experiments as indicated.

Adsorbed protein	CHO		
	K1	S677	S745
TSP	1.42 ± 0.26 (<i>n</i> = 11)	Not done	0.81 ± 0.19 (<i>n</i> = 3)
NH ₂	1.55 ± 0.18 (<i>n</i> = 4)	1.03 ± 0.1 (<i>n</i> = 4)	0.82 ± 0.1 (<i>n</i> = 4)
GST	0.5 ± 0.24 (<i>n</i> = 4)	0.64 ± 0.28 (<i>n</i> = 4)	0.45 ± 0.25 (<i>n</i> = 4)

attachment to TSP-1 (Figure 1), suggested the presence of heparan sulphate-dependent adhesive sites within the N-terminal domain of TSP-1. To examine this hypothesis, different synthetic peptides overlapping heparin-binding sequences ARKGSRR (residues 22–29), MKKTRG (residues 79–84) and TRDLAS-

Table 2 Relative strength of binding of synthetic peptides from the N-terminal domain of TSP to heparin-agarose

Heparin-binding sites present within synthetic peptides are indicated in bold characters. The numbers in parentheses correspond to the positions of the residues in TSP-1. *The strength of binding of peptides to heparin-agarose was assessed by elution from the column of bound peptides using a 0–1 M NaCl gradient. Peptides eluted at the same salt concentration whether coupled or not coupled to BSA. †The capacity of adsorption of each peptide was quantified using the BCA protein assay.

Peptide	Sequence	Salt concentration at which the peptide eluted (mM)*	Capacity of adsorption to plastic (pmol/well)†
1	(17)ELTGA ARKGSGRR LVKGDPDP(36)	280	62
2	ARKGSGRR	400	60
3	(60)VDAVRTEKGFLLLASLRQM KKTRG T(84)	300	75
4	(60)VDAVRTEKGFLLLASLRQ	110	65
5	KKTRG	350	50
6	KKTRG TLLALERKDHS(94)	350	95
7	TLLALERKDHS(94)	50	55
8	(98)FSVVSNGKAGTLDL(111)	0	65
9	(117)GKQHVVSVEEALLATGQWK(135)	200	60
10	(177) RLRIAKGGVNDN (188)	380	50
11	(189)FQGVLLQNVRFVF(200)	200	30
12	(201)GTTPEDILRNKGSS(214)	80	15

IARLRIAKGVNDNF (residues 170–189) were synthesized and tested for their ability to support cell attachment.

CHO K1 cells attach to GAG-dependent and -independent cell adhesive sites in the N-terminal domain of TSP-1

Sequences of the different synthetic peptides used in this study are shown in Table 2. BSA-peptide conjugates 2, 3, 4, 5, 6, 7, 9 and 11 showed a dose-dependent cell adhesive activity for CHO K1 cells (Figure 4), while BSA-peptide conjugates 1, 8, 10 and 12 exhibited none, using peptide concentrations up to 1 mM (results not shown). No cell attachment was observed when BSA activated with the cross-linking reagent was used instead of peptide-BSA conjugates. Lack of adhesion to peptides 1, 8, 10 and 12 was not due to inability of the peptides to bind to the well surface. Using a BCA protein assay, the capacity of adsorption of these peptides to plastic wells was very similar to that obtained with cell adhesive peptides 2, 3, 4, 5, 6, 7, 9 and 11 (Table 2), indicating that peptides 1, 8, 10 and 12 are inherently inactive. Such a BCA protein assay has already been used successfully to quantify immobilized synthetic peptides derived from the C-terminal domain of TSP-1 [10]. Both the BCA assay and the ELISA using antisera against the peptides gave very similar coating efficiencies for a given peptide [10], demonstrating that the BCA assay is a reliable method for quantifying immobilized peptides. The apparent discrepancy in the maximum cell adhesive activities between these peptides (Figure 4) and TSP-1 (Figure 1) may be a reflection of their density on the plastic surface. Synthetic peptides provide a higher cell adhesive substrate density than TSP-1. Such an explanation has been proposed with other active peptides from the C-terminal domain of TSP-1 [10].

Direct binding of synthetic peptides to heparin by heparin-agarose affinity chromatography demonstrated that the cell adhesive activity of these peptides did not correlate simply with heparin affinity (Table 2). Peptides 2, 3, 5 and 6, containing heparin-binding sequences **ARKGSGRR** or **KKTR**, exhibited both cell adhesive activity and high affinity for heparin (Figure 4 and Table 2). However, peptides 1 and 10, which also contain a heparin-binding sequence, had no cell adhesive activity despite their high affinity for heparin. In addition, peptides 4, 7, 9 and 11 exhibited cell adhesive activity, and bound poorly to heparin-agarose. It must be kept in mind that centrifugal cell-attachment assays were performed in the presence of 150 mM NaCl. The

relative strength of binding of peptides 9 and 11 to heparin-agarose (both eluted at 200 mM NaCl) was therefore regarded as low with respect to their cell adhesive activities. Finally, peptides 8 and 12 neither had cell adhesive activity nor affinity for heparin-agarose.

To assess whether high-affinity heparin-binding peptides interacted with cell-surface GAGs to mediate cell adhesion, the attachment to cell adhesive peptides of mutant CHO S677 and S745 cells was investigated (Figure 4). CHO S677 cell attachment to high-affinity heparin-binding peptides 3 and 6 was slightly decreased, while its attachment to peptides 2 and 5 remained unaffected compared with that obtained with the parent cell line (Figure 4). CHO S745 cell attachment to peptides 3 and 6 was further decreased compared with that observed with CHO S677 cells. In addition, a moderate decrease in CHO S745 cell attachment to peptides 2 and 5 was observed. Although peptide 4 does not contain any known BBXB consensus GAG sequence, and had no heparin-binding affinity (Table 2), CHO S677 cell attachment to peptide 4 was significantly decreased compared with that observed with CHO K1 cells, and CHO S745 cell attachment was even nearly abolished (Figure 4). These results correlate with the fact that CHO K1 cell attachment to peptide 4 was inhibited in the presence of increasing concentrations of heparan sulphate with IC_{50} (25 nM) very similar to that observed for CHO K1 cell attachment to heparin-binding peptide 3 (IC_{50} = 10 nM) (results not shown). In contrast to peptide 4, peptides 9 and 11 bound poorly to heparin-agarose (Table 2), and did not exhibit significantly different adhesive activities whether CHO cells were deficient or not deficient in cell-surface GAGs (Figure 4).

To further examine the contribution of high-affinity heparin-binding sites **ARKGSGRR** and **KKTR** in CHO K1 cell attachment to the N-terminal domain of TSP-1, cell attachment experiments were performed with an N-terminal fusion protein in which two adjacent lysine residues (80 and 81) in the heparin-binding consensus sequence **KKTR** were mutated to **QNTR** [construct NH₂ (80,81)]. In addition, cell attachment experiments were performed with a construct in which mutation **QNTR** was combined with the substitution of two adjacent arginine residues (28 and 29) in the sequence **ARKGSGRR** to obtain **ARKGSGNQ** [construct NH₂ (80,81 + 28,29)]. CHO K1 cell attachment to construct NH₂ (80,81) was decreased by 36% compared with that observed with the native N-terminal domain

Figure 4 CHO cells attach to synthetic peptides spanning GAG-binding sequences in the N-terminal domain of TSP-1

Peptides 3, 5 and 6 contain the BBXB (B is a basic and X any amino acid) consensus GAG-binding sequence (KKTR) identified in a number of heparin-binding proteins [27]. Peptide 2 lacks this BBXB configuration but contains highly positively charged residues responsible for binding to GAGs. For other peptides see Table 2. Substrates were prepared by incubation with increasing concentrations of synthetic peptides coupled to BSA. CHO cells were added to U-shaped wells and the plate was immediately centrifuged. The inside diameter of the ring (in mm) was then used as a measure of the adhesivity of synthetic peptides and was plotted versus the amount of peptides added to the wells. CHO S677 and S745 cells have 50 and 6% of the amount of cell-surface GAGs respectively as CHO K1 cells. Results are the means $\pm$ S.E.M. of *n* separate experiments.

(Figure 5). CHO K1 cell attachment to construct NH₂ (80,81 + 28,29), which combines mutations in the two heparin-binding motifs, was not further decreased (31% inhibition) (Figure 5).

The inhibitory effect of KKTR-containing peptides 3 and 6, and subpeptides 4, 5 and 7 on CHO K1 cell attachment to the N-terminal domain of TSP-1 was therefore tested. Figure 6 shows that peptide 3 drastically inhibited cell attachment ($97.5 \pm 3.5\%$ inhibition), while peptide 6 had only a moderate inhibitory effect ($27 \pm 14\%$ inhibition). Subpeptide 4, the N-terminal sequence of peptide 3, retained most of the inhibitory activity of peptide 3 ($53 \pm 9.6\%$ inhibition), while subpeptide 7, the C-terminal sequence of peptide 6, had almost no inhibitory effect ($5 \pm 0.9\%$ inhibition). Subpeptide 5, the KKTRGT sequence shared by peptides 3 and 6, exhibited an inhibitory activity ($24.5 \pm 2.1\%$ inhibition) similar to that observed with peptide 6. The inhibitory effect of ARKGSGRR-containing peptides 1 and 2 on CHO K1 cell attachment to the N-terminal domain of TSP-1 was also tested. Peptides 1 and 2 had only a slight inhibitory effect ($16 \pm 2.8\%$ and $5 \pm 7.3\%$ inhibition respectively) (results not shown).

Taken together, these results suggested that CHO K1 cell attachment to the N-terminal domain of TSP-1 was partly dependent on the heparin-binding site KKTR, but not on the

motif ARKGSGRR, and that KKTR-containing peptides 3 and 6 constitute a GAG-dependent cell adhesive site in the N-terminal domain of TSP-1, while peptides 9 and 11 may represent two potential GAG-independent cell adhesive sites.

GAG-dependent and -independent adhesive sites in the N-terminal domain of TSP-1 also support gravity cell attachment

To further determine the relative functional importance of each of these cell adhesive peptides, CHO cell adhesion experiments were performed using a gravity assay in which weakly adherent cells are frequently detached [32–35]. Peptide 3, and to a lesser extent peptides 6 and 11, were found to efficiently support CHO K1 cell attachment (Table 3). In contrast, no cell attachment to peptides 2, 4, 5, 7 and 9 was observed (results not shown). CHO K1 cells attached to peptides 3, 6 and 11 in a dose-dependent manner. However, maximal cell attachment was obtained at peptide concentrations 10-fold higher than that required in the centrifugal cell-attachment assay. These results further indicate that peptides 3 and 6 constitute a cell adhesive active site in the N-terminal domain of TSP-1. This cell adhesive active site is GAG-dependent because CHO S745 cell attachment to peptide 3 was decreased by 60% compared with that observed with CHO

Figure 5 CHO K1 cell attachment to the N-terminal domain of TSP-1 with mutations in the high-affinity heparin-binding sites

CHO K1 cells were added to U-shaped wells coated with the recombinant N-terminal domain 1–90 (100 pmol/well) in its native form (NH₂) or with mutations of the heparin-binding site KKTR [construct NH₂ (80,81)] combined with or without mutations of the heparin-binding site ARKGSGRR [NH₂ (80,81 + 28,29)]. Results are the means $\pm$ S.D. of 4–7 separate experiments. Statistical analysis was performed using the Mann–Whitney test.

K1 cells, and even nearly abolished to peptide 6 (Table 3). By contrast, CHO K1 and S745 cells attached to peptide 11 to a similar extent, confirming that peptide 11 represents a GAG-independent cell adhesive site (Table 3).

If these cell adhesive active sites in the N-terminal domain are critical for CHO K1 cell attachment, then one would expect these peptides to be active for other cell types known to attach to TSP-1. To address this issue, we tested several cell lines for attachment to these peptides, including human MCF-7, Hs578T and MDA-MB-231 breast carcinoma cells, human Bowes melanoma cells, murine NIH 3T3 fibroblast cells and human MG-63 osteosarcoma cells (Table 3). As observed for CHO K1 cells, all of these cell lines strongly attached to peptide 3 and, to a lesser extent, to peptide 6. Attachment of human breast carcinoma and osteosarcoma cells to peptide 11 was markedly increased compared with the other cell lines.

Table 3 Cell attachment activities of peptides from the N-terminal domain of TSP-1 in the gravity assay

Peptides 3 and 6 were used at a concentration of 100 μ M, while peptide 11 was used at 500 μ M. Data are expressed as the mean $\pm$ S.D. of *n* separate experiments.

Cell type	Peptide ...	Adherent cells (cells/mm ²)			
		3	6	11	None
CHO K1		298 $\pm$ 123 (<i>n</i> = 10)	76 $\pm$ 61 (<i>n</i> = 7)	46 $\pm$ 27 (<i>n</i> = 3)	3 $\pm$ 1 (<i>n</i> = 10)
CHO S745		126 $\pm$ 48 (<i>n</i> = 8)	5 $\pm$ 3 (<i>n</i> = 7)	36 $\pm$ 25 (<i>n</i> = 3)	2 $\pm$ 1 (<i>n</i> = 8)
MCF-7		369 $\pm$ 97 (<i>n</i> = 2)	70 $\pm$ 11 (<i>n</i> = 2)	280 $\pm$ 68 (<i>n</i> = 2)	4 $\pm$ 1 (<i>n</i> = 2)
Hs578T		146 $\pm$ 18 (<i>n</i> = 2)	27 $\pm$ 12 (<i>n</i> = 2)	135 $\pm$ 10 (<i>n</i> = 2)	6 $\pm$ 2 (<i>n</i> = 2)
MDA-MB-231		408 $\pm$ 79 (<i>n</i> = 2)	129 $\pm$ 74 (<i>n</i> = 2)	223 $\pm$ 214 (<i>n</i> = 2)	4 $\pm$ 3 (<i>n</i> = 2)
Bowes		165 $\pm$ 78 (<i>n</i> = 6)	72 $\pm$ 35 (<i>n</i> = 6)	68 $\pm$ 16 (<i>n</i> = 4)	2 $\pm$ 1 (<i>n</i> = 6)
NIH 3T3		180 $\pm$ 43 (<i>n</i> = 4)	91 $\pm$ 43 (<i>n</i> = 4)	91 $\pm$ 37 (<i>n</i> = 2)	2 $\pm$ 1 (<i>n</i> = 4)
MG-63		455 $\pm$ 72 (<i>n</i> = 2)	156 $\pm$ 18 (<i>n</i> = 2)	310 $\pm$ 86 (<i>n</i> = 2)	5 $\pm$ 4 (<i>n</i> = 2)

DISCUSSION

The centrifugation assay allows the observation of interactions that are undetectable in the gravity assay where weakly adherent cells are frequently detached [32–35]. Several laboratories have previously used centrifugation assays to demonstrate the binding of various cultured cells (e.g. NIH 3T3, neuroblastoma, glioma and mammary epithelial cells) to collagens [32], fibronectin [34], laminin [33] and tenascin [33,35]. Using this cell-adhesion assay, CHO K1 cells attached to TSP-1 by binding to the N-terminus but not to the other domains of TSP-1 (e.g. the C-terminus, and type 1, type 2 and type 3 repeats). The demonstration of the importance of the N-terminus in CHO K1 cell attachment extends previous findings showing that 84% of CHO K1 cells attached to intact TSP-1, while only 5% attached to a cleavage fragment of TSP-1 that was missing the N-terminal domain [13]. In contrast to CHO K1 cells, we have shown that NIH 3T3 cells

Figure 6 Inhibition of CHO K1 cell attachment to the N-terminal domain of TSP-1 by soluble synthetic peptides containing GAG-binding sequences

CHO K1 cells were added to U-shaped wells coated with the recombinant N-terminal domain 1–90 (60 μ g/ml, 1 μ M) in the presence of peptides (500 μ M) spanning the heparin-binding consensus sequence KKTR. Bar graphs correspond to the inhibitory effect of each peptide on CHO K1 cell attachment to the N-terminal domain. Results are the means $\pm$ S.D. of two separate experiments.

	Peptide 3										
											Peptide 6
	Peptide 4					Peptide 5					Peptide 7
											</

markedly enhanced when flanked with the heparin-binding consensus sequence KRFK (KRFGGWSHWSPWSS), while the motif KRFK was not sufficient to promote cell adhesion [14]. Moreover, we have shown that peptide VDAVRTEKGFLLLASLRQMKKTRG nearly abolished (98 % inhibition) CHO K1 cell attachment to the N-terminal domain of TSP-1, while peptides MKKTRG, MKKTRGTLLALERKDHS and VDAVRTEKGFLLLASLRQ had only a moderate inhibitory effect (25, 27 and 53 % inhibition respectively). Finally, we have observed that CHO K1 cells attached to peptide VDAVRTEKGFLLLASLRQ. Surprisingly, although peptide VDAVRTEKGFLLLASLRQ does not contain any known BBXB consensus GAG sequence and has no heparin binding affinity, CHO K1 cell attachment to this peptide was inhibited by heparan sulphate and CHO S745 cell attachment was nearly abolished. Kosfeld and Frazier [37] found that heparan sulphate was a more potent inhibitor of cell attachment to peptide C4 than to peptide C6, two synthetic peptides derived from the C-terminal domain of TSP-1. This was unexpected because peptide C6 contains a heparin-binding sequence (RWR), while peptide C4 lacks any known GAG binding sequence. It may be that peptide VDAVRTEKGFLLLASLRQ behaves like C4. Alternatively, the presence of highly positively charged amino acids (RTEK and LRQ) in peptide VDAVRTEKGFLLLASLRQ could contribute to the heparan sulphate inhibition and the decreased attachment of CHO S745 cells to this peptide.

Overall, our study provides, for the first time, evidence that the N-terminal domain of TSP-1 supports cell attachment through a GAG-dependent cell adhesive site encompassing residues 60–94 (sequence VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHS), while residues 189–200 (sequence FQGVQLQNVRFVF) constitute a GAG-independent site. If these active sequences are critical for cell attachment to the N-terminal domain of human TSP-1, then one would expect these residues to be conserved within human TSP-2. Although the N-terminal domain of human TSP-1 is only 37 % identical with human TSP-2 [38], a higher degree of homology exists between TSP-1 and TSP-2 between residues 60 and 94 (48 % identity) and 189–200 (67 % identity) (Figure 7). Moreover, these two cell adhesive sites are also highly conserved in all known species of TSP (mouse TSP-1, mouse TSP-2 and chicken TSP-2) (Figure 7). The functions of TSP-2 are largely unknown. Mouse recombinant TSP-2, like human TSP-1, supported adhesion of MG-63 cells, and heparin inhibited adhesion of MG-63 cells to both human TSP-1 and mouse TSP-2 [8,39]. In addition, MG-63 cells attached to TSP-1 through the N-terminal domain of the molecule [8]. Because MG-63 cells strongly attached to residues 60–94 and 189–200 of TSP-1 (Table 3), and a high degree of homology exists between TSP-1 and TSP-2 over these cell adhesive sequences (Figure 7), it is conceivable that homologous sequences in TSP-2 also mediate cell attachment.

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