

Identification of an $\alpha_3\beta_1$ Integrin Recognition Sequence in Thrombospondin-1*

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A synthetic peptide containing amino acid residues 190–201 of thrombospondin-1 (TSP1) promoted adhesion of MDA-MB-435 breast carcinoma cells when immobilized and inhibited adhesion of the same cells to TSP1 when added in solution. Adhesion to this peptide was enhanced by a β_1 integrin-activating antibody, Mn^{2+} , and insulin-like growth factor I and was inhibited by an $\alpha_3\beta_1$ integrin function-blocking antibody. The soluble peptide inhibited adhesion of cells to the immobilized TSP1 peptide or spreading on intact TSP1 but at the same concentrations did not inhibit attachment or spreading on type IV collagen or fibronectin. Substitution of several residues in the TSP1 peptide with Ala residues abolished or diminished the inhibitory activity of the peptide in solution, but only substitution of Arg-198 completely inactivated the adhesive activity of the immobilized peptide. The essential residues for activity of the peptide as a soluble inhibitor are Asn-196, Val-197, and Arg-198, but flanking residues enhance the inhibitory activity of this core sequence, either by altering the conformation of the active sequence or by interacting with the integrin. This functional sequence is conserved in all known mammalian TSP1 sequences and in TSP1 from *Xenopus laevis*. The TSP1 peptide also inhibited adhesion of MDA-MB-435 cells to the laminin-1 peptide GD6, which contains a potential integrin-recognition sequence Asn-Leu-Arg and is derived from a similar position in a pentraxin module. Adhesion studies using recombinant TSP1 fragments also localized β_1 integrin-dependent adhesion to residues 175–242 of this region, which contain the active sequence.

Expression of the $\alpha_3\beta_1$ integrin is essential for normal development in the kidney and lungs (1). Targeted mutation of the murine α_3 integrin gene resulted in abnormal branching morphogenesis of kidney capillary loops and lung bronchi. Based on antibody inhibition, this integrin may also be important for branching morphogenesis in mammary epithelia (2). In addition to its essential roles in normal development, the $\alpha_3\beta_1$ integrin may play important roles in disease processes, such as cancer. Loss of integrin α_3 subunit expression is a negative prognostic factor in lung adenocarcinoma (3). Conversely, overexpression of $\alpha_3\beta_1$ integrin in a human rhabdomyosarcoma line suppressed tumor formation in mouse xenografts (4).

The $\alpha_3\beta_1$ integrin has been reported to recognize several extracellular matrix ligands, including some laminins, type IV

collagen, fibronectin, thrombospondin-1, and entactin/nidogen (5–8). Although short peptide recognition motifs have been identified in ligands for some integrins (reviewed in Ref. 9), previous attempts to define recognition sequences for binding of matrix ligands to the $\alpha_3\beta_1$ integrin have produced conflicting results. High affinity binding of recombinant soluble $\alpha_3\beta_1$ could be detected only to laminin-5 (10), so binding to other matrix ligands may be of relatively low affinity. Under specific conditions, this integrin can recognize the common integrin binding sequence RGD in fibronectin (6). However, recombinant entactin with the RGD sequence deleted (11) and synthetic peptides from laminin-1 and type IV collagen that lack the RGD motif (12, 13) also bound specifically to the $\alpha_3\beta_1$ integrin. Laminin peptide GD6 (KQNCLSSRASFRGCVRLRLSR) and the type IV collagen peptide affinity purified $\alpha_3\beta_1$ integrin from cell extracts when immobilized on agarose beads (12, 13), but the active peptides from these two proteins share no apparent sequence homology. These data, combined with the evidence that RGD-dependent and RGD-independent adhesion are differentially regulated in $\alpha_3\beta_1$ integrin (6), have led to the proposal that the $\alpha_3\beta_1$ integrin uses distinct mechanisms to interact with each of its ligands and that no conserved binding motif may exist (6).

We recently found that $\alpha_3\beta_1$ is the major TSP1¹-binding integrin on several human breast carcinoma cell lines (14). We have further examined this interaction and report the identification of a peptide sequence from TSP1 that supports $\alpha_3\beta_1$ -dependent adhesion and chemotaxis and is a potent inhibitor of adhesion to TSP1.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Calcium replete TSP1 was purified from human platelets (15). Synthetic peptides containing TSP1 sequences were prepared as described previously (16–21). Recombinant fragments (provided by Dr. Tikva Vogel) and GST fusion proteins expressing fragments of TSP1 (provided by Dr. Jack Lawler, Harvard University) were prepared as described previously (22, 23). Bovine type I collagen and murine Type IV collagen were obtained from Becton Dickinson Labware division, and human vitronectin was from Sigma. Fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (24). Murine laminin-1 purified from the EHS tumor was provided by Dr. Sadie Aznavoorian (NCI, National Institutes of Health). Recombinant human insulin-like growth factor-1 (IGF1) was from Bachem.

Adhesion Assays—Adhesion was measured on polystyrene or glass substrates coated with peptides or proteins as described previously (16). Inhibition assays were performed using the following function-blocking antibodies: 6D7 ($\alpha_2\beta_1$), P1B5 (Life Technologies, Inc., $\alpha_3\beta_1$), 407279 (Calbiochem, $\alpha_4\beta_1$), and PID6 (Life Technologies, Inc., $\alpha_5\beta_1$). The β_1 integrin-activating antibody TS2/16 (25) was prepared from the hybridoma obtained from the American Type Culture Collection. Immunofluorescence analysis of cell adhesion was performed as described previously, using BODIPY TR-X phalloidin (Molecular Probes, Inc., Eugene, OR) to visualize F-actin or using murine primary antibodies

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¹ The abbreviations used are: TSP, human thrombospondin; GST, glutathione S-transferase; IGF1, insulin-like growth factor-1.

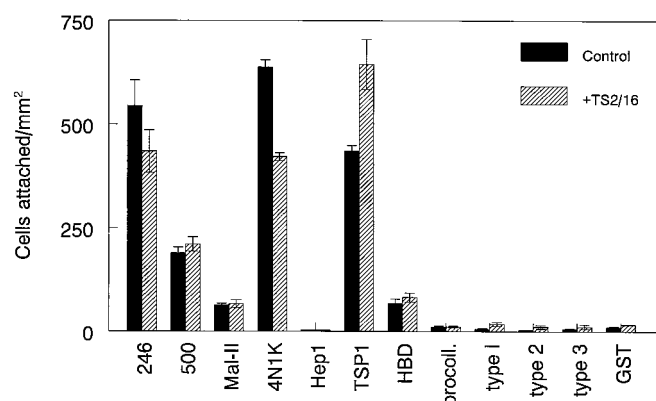


FIG. 1. Adhesion of MDA-MB-435 breast carcinoma cells to recombinant TSP1 fragments and synthetic TSP1 peptides. Adhesion to synthetic TSP1 peptides adsorbed at 10 μ M (246, KRFGQDGG-WSHWSPWSS; 500, NGVQYRNC; Mal II, SPWSSCSVTCGDGVITRIR; 4N1K, KRFFVVMWKK; HepI, ELTGAARKGSGRRLVKGP), TSP1 (0.11 μ M), recombinant 18-kDa heparin-binding domain (HBD) (2.7 μ M), or GST fusion proteins expressing the TSP1 procollagen domain (procoll.), type 1, 2, or 3 repeats, or GST alone (2 μ M) was measured in the absence (solid bars) or presence (striped bars) of 20 μ g/ml of the β_1 integrin-activating antibody TS2/16. Results (mean \pm S.D.) are presented for a representative experiment performed in triplicate.

followed by BODIPY FL anti-mouse IgG to localize integrins, vinculin (Sigma), or focal adhesion kinase (clone 77, Transduction Laboratories) (26).

Motility Assays—Chemotaxis of MDA-MB-435 cells to TSP1 peptides was measured in modified Boyden chambers using polylysine-coated 8 μ m polycarbonate filters as described previously for intact TSP1 (14).

Multiple Sequence Alignment—Protein sequences were compared using MACAW software (National Center for Biotechnology Information, National Library of Medicine, version 2.0.5) by the segment pair overlap and Gibbs sampler methods (27, 28).

RESULTS

In initial attempts to localize the region of TSP1 recognized by the $\alpha_3\beta_1$ integrin, we tested approximately 85% of the TSP1 sequence in the form of synthetic peptides or GST or T7 fusion proteins for promotion of β_1 integrin-dependent adhesion of MDA-MB-435 cells (Fig. 1). Among the recombinant fragments tested, only an 18-kDa fragment of the N-terminal heparin-binding domain had significant adhesive activity, although the recombinant type I repeats had adhesive activity for MDA-MB-435 cells in some experiments (results not shown). A recombinant GST fusion of the type 3 repeats of TSP1 including the RGD sequence had minimal adhesive activity for MDA-MB-435 cells (Fig. 1), in contrast to human melanoma cells, which avidly attached on substrates coated with the same concentrations of this fragment (26). The β_1 integrin-activating antibody TS2/16 did not enhance cell attachment to any of these recombinant fragments but reproducibly stimulated attachment on intact TSP1 (Fig. 1). Synthetic heparin-binding peptides from the type 1 repeats (peptide 246) (16) and the CD47-binding peptide 4N1K (21) also promoted adhesion, but TS2/16 did not enhance adhesion of MDA-MB-435 cells to these peptides. CD36-binding peptides from the procollagen domain (peptide 500) or the type 1 repeats (Mal-II) (29) had weaker adhesive activities and were also insensitive to TS2/16. The focal adhesion disrupting peptide Hep1 from the N-terminal domain of TSP1 (20) did not promote MDA-MB-435 cell adhesion. Although these experiments did not detect a β_1 integrin-dependent adhesive sequence in TSP1, the possibility remains that these regions of TSP1 contain a conformation-dependent recognition motif that is inactive in the recombinant fusion proteins due to misfolding.

A multiple alignment search using MACAW software was

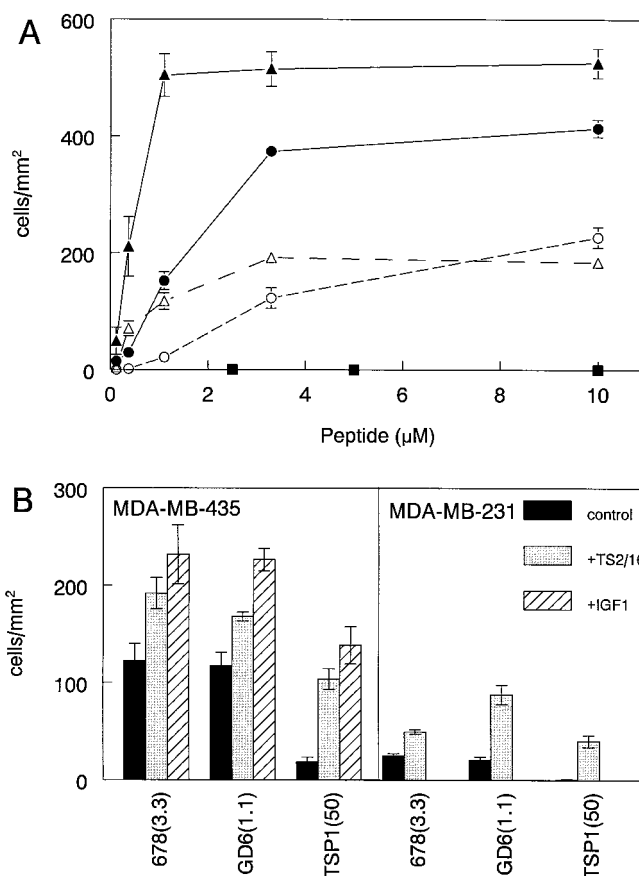


FIG. 2. MDA-MB-435 adhesion to TSP1 peptides and laminin-1 peptide GD6. A, MDA-MB-435 breast carcinoma cell attachment (closed symbols) and spreading (open symbols) was determined on polystyrene substrates coated with the indicated concentrations of TSP1 peptide 678 (FQGVLRNVRVFV) (circles), TSP1 peptide 701 (TPG-QVRTLWHDP) (squares), or the murine laminin-1 peptide GD6 (KQNCSSRSRFRGCVRLRLSR) (triangles). Results are presented as mean \pm S.D. ($n = 3$). B, spreading of MDA-MB-435 or MDA-MB-231 cells on substrates coated with 3.3 μ M TSP1 peptide 678, 1.1 μ M laminin-1 peptide GD6, or 50 μ g/ml TSP1 was determined using untreated cells (black bars) or cells treated with 5 μ g/ml of the β_1 -activating antibody TS2/16 (gray bars) or 3 nM IGF1 (striped bars) (MDA-MB-435 cells only); mean \pm S.D. ($n = 3$).

used to identify TSP1 sequences that might be related to the $\alpha_3\beta_1$ integrin-binding GD6 peptide derived from the A chain of murine laminin-1 (12), which strongly promoted MDA-MB-435 cell adhesion (Fig. 2A). This search identified four TSP1 sequences related to the laminin peptide (Table I). The single peptide identified by the Gibbs sampler method, derived from the C-terminal domain of TSP1 (residues 1059–1077), did not support adhesion or inhibit adhesion of MDA-MB-435 cells to TSP1 or other $\alpha_3\beta_1$ integrin ligands (Fig. 2A and results not shown). Because a synthetic peptide containing the last 12 residues of peptide GD6 (peptide 679, Table I) had similar activity to the intact peptide (see below), we did not test the two peptides identified by segment pair overlap that aligned outside this sequence. Both of these peptides were derived from regions of the type 1 (residues 392–405) or type 2 (residues 598–608) repeat sequences that did not support $\alpha_3\beta_1$ -dependent adhesion when expressed as GST fusion proteins (Fig. 1).

The remaining sequence is from a region of the N-terminal domain of TSP1 (residues 188–199) that was not covered by the recombinant fragments tested in Fig. 1 and conserves most of the hydrophilic residues in the laminin-1 GD6 peptide that could mediate protein-protein interactions. This sequence also overlaps with a region identified in a screen of N-terminal

TABLE I
TSP1 sequences related to murine laminin-1 peptide GD6

The amino acid sequences for human and murine TSP1 and laminin-1 peptide GD6 were compared by multiple alignment using MACAW.

Peptide origin	Sequence	MP score vs. GD6	p value
Laminin GD6	KQNCLSSRASFRGCVNRLRLSR		
Laminin p679	FRGCVNRLRLRLSR		
TSP1 (598–608)	NCLPCPPRFTG	42.0 ^a	5.9×10^{-8}
TSP1 (188–199)	DNFQGVLLQNVRF	39.0 ^a	5.9×10^{-7}
TSP1 (392–405)	NNRCEGSSVQTRTC	35.0 ^a	4.5×10^{-4}
TSP1 (1059–1077)	RNALWHTGNTPGQVRTLWH	43.3 ^b	2.1×10^{-8}

^a Alignment scores were determined by segment pair overlap.

^b Alignment by Gibbs sampler method.

TSP1 peptides as having heparin-independent adhesive activity (30). A synthetic peptide containing this TSP1 sequence (peptide 678) had strong adhesive activity for MDA-MB-435 cells (Fig. 2A). Spreading of two breast carcinoma cell lines on this peptide, laminin peptide GD6, and TSP1 was enhanced in the presence of the β_1 integrin-activating antibody TS2/16 (Fig. 2B). We previously found that IGF1 strongly stimulated β_1 integrin-mediated adhesion to TSP1 (14). IGF1 similarly stimulated spreading of MDA-MB-435 cells on the TSP1 peptide 678 and to the laminin peptide GD6 (Fig. 2B).

The TSP1 peptide 678 strongly inhibited spreading of MDA-MB-435 cells on TSP1 and murine EHS tumor-derived laminin-1/entactin but did not inhibit spreading of the same cells on the $\alpha_5\beta_1$ integrin ligand fibronectin or on type IV collagen (Fig. 3A). The TSP1 peptide in solution strongly inhibited MDA-MB-435 cell attachment to itself and to GD6 (Fig. 3B), a known $\alpha_3\beta_1$ integrin-binding peptide from murine laminin-1 (12). In contrast, the laminin peptide was a relatively weak inhibitor of adhesion to either peptide or TSP1 when tested in solution ($IC_{50} = 700 \mu M$, data not shown).

The TSP1 peptide 678 sequence was not in the recombinant N-terminal fragment tested in Fig. 1, but the previously reported 28-kDa N-terminal fragment of TSP1 contains this sequence (22). Adhesion assays using MDA-MB-231 (Fig. 3C) and MDA-MB-435 breast carcinoma cell lines (data not shown) verified that the larger fragment, expressing residues 1–242, contains a β_1 integrin-dependent adhesion sequence that is not present in residues 1–174. Adhesion to the longer fragment was stimulated by the β_1 -activating antibody TS2/16 and inhibited by peptide 678 (Fig. 3C). Therefore, a β_1 integrin-binding site is present in residues 175–242 of TSP1 and is functional when expressed as a recombinant protein.

To verify that the TSP1 peptide 678 contains an $\alpha_3\beta_1$ integrin recognition sequence, integrin α -subunit antibodies were tested for blocking adhesion to the peptide (Fig. 4). The α_3 -specific blocking antibody P1B5, which we have shown to inhibit adhesion of the same cells to intact TSP1 (14), partially inhibited adhesion of MDA-MB-435 cells on peptide 678 and completely reversed the enhancement of MDA-MB-435 cell adhesion to the same peptide stimulated by the β_1 integrin-activating antibody TS2/16. In a further control experiment, the $\alpha_2\beta_1$ -blocking antibody 6D7 inhibited adhesion of MDA-MB-435 cells to type I collagen but not to peptide 678 (Fig. 4B). Function-blocking antibodies for $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins also had no effect on adhesion to peptide 678 (data not shown). Therefore, the peptide does not support adhesion mediated by $\alpha_4\beta_1$ or $\alpha_5\beta_1$ integrins or inhibit adhesion to other integrin ligands.

Divalent cation dependence is also characteristic for binding of integrin ligands. Although Mn^{2+} but not Ca^{2+} induced the expected increase in MDA-MB-435 cell spreading on TSP1 peptide 678 and intact TSP1 (Fig. 4C), addition of EDTA only

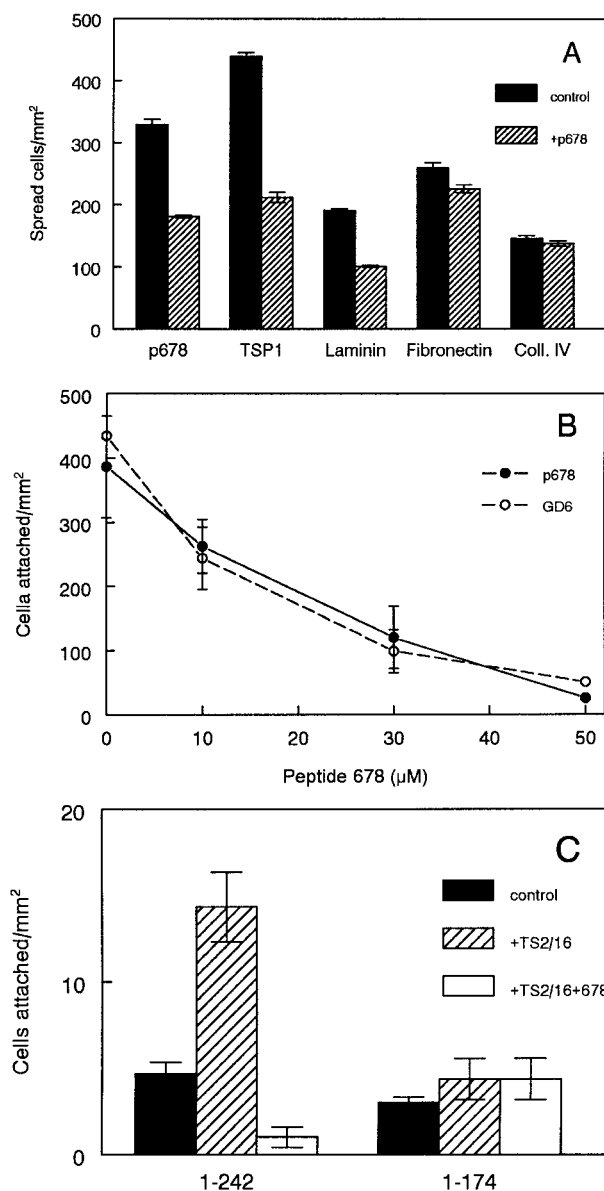


FIG. 3. Inhibition of breast carcinoma cell spreading on matrix proteins by peptide 678. A, MDA-MB-435 cell spreading was determined in the absence (solid bars) or presence (striped bars) of $10 \mu M$ TSP1 peptide 678 on substrates coated with $10 \mu M$ peptide 678, $40 \mu g/ml$ TSP1, $10 \mu g/ml$ murine laminin-1, $10 \mu g/ml$ human plasma fibronectin, or $10 \mu g/ml$ type IV collagen. Cell spreading is presented as mean \pm S.D. ($n = 3$). B, inhibition of MDA-MB-435 cell adhesion to surfaces coated with $10 \mu M$ peptide 678 (●) or laminin peptide GD6 (○) was measured in the presence of the indicated concentrations of peptide 678 added in solution. C, adhesion of MDA-MB-231 cells to the indicated recombinant TSP1 fragments was measured in RPMI medium containing 0.1% bovine serum albumin (black bars) or the same medium containing $5 \mu g/ml$ of the β_1 -activating antibody TS2/16 (striped bars) or TS2/16 plus $20 \mu M$ peptide 678 (open bars). Cell attachment is presented as mean \pm S.D. for triplicate determinations.

minimally inhibited basal spreading on peptide 678. EDTA completely inhibited the spreading on TSP1 observed in medium containing Mg^{2+} as the sole divalent cation, although it did not inhibit cell attachment on TSP1 (Fig. 4C and results not shown). This residual adhesion probably results from the significant contribution of proteoglycans to adhesion of MDA-MB-435 cells on TSP1 (14). Spreading on peptide 678 with Mg^{2+} as the divalent cation became partially sensitive to EDTA, however, in the presence of the β_1 -activating antibody TS2/16. Addition of Mn^{2+} further stimulated spreading on peptide 678

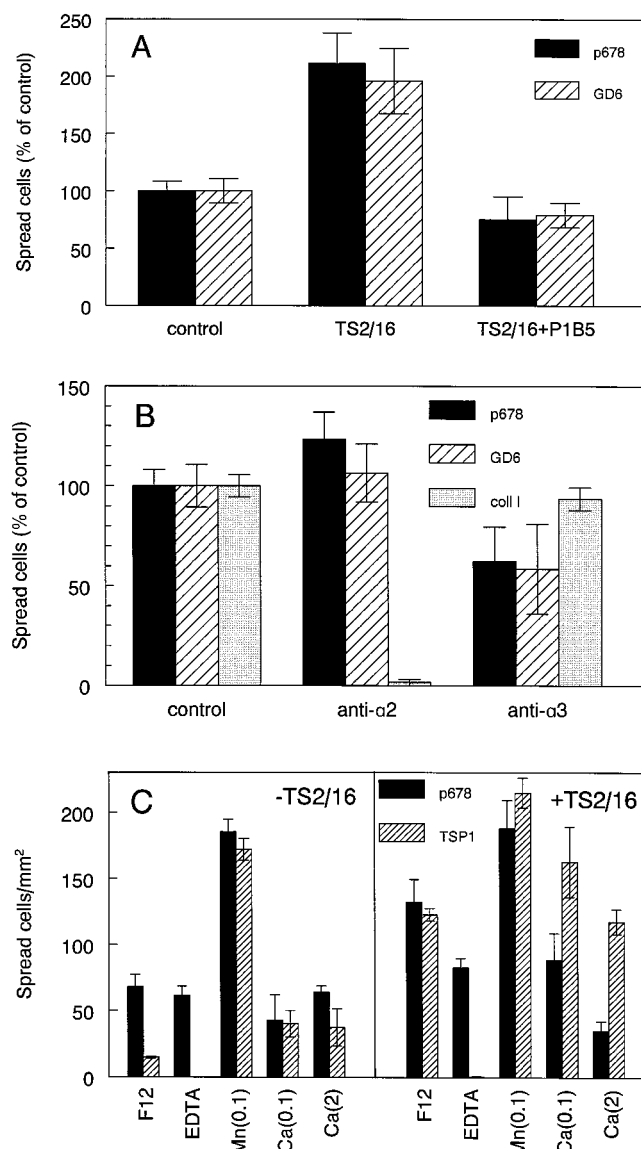


FIG. 4. The $\alpha_3\beta_1$ integrin mediates adhesion to TSP1 peptide 678 and laminin-1 peptide GD6. A, MDA-MB-435 cell spreading on TSP1 peptide 678 (solid bars) or laminin-1 peptide GD6 (striped bars) was determined with no additions (control), in the presence of 5 $\mu\text{g/ml}$ of β_1 -integrin antibody TS2/16, or in the presence of 5 $\mu\text{g/ml}$ each of antibody TS2/16 and the $\alpha_3\beta_1$ -blocking antibody P1B5. Results are normalized to the control and are presented as mean \pm S.D. ($n = 3$). B, MDA-MB-435 cell spreading on substrates coated with 10 μM TSP1 peptide 678 (black bars), 5 μM laminin-1 peptide GD6 (striped bars), or 5 $\mu\text{g/ml}$ type I collagen (gray bars) was determined in the presence of 5 $\mu\text{g/ml}$ of the $\alpha_3\beta_1$ -blocking antibody 6D7 (anti- α_2) or the $\alpha_3\beta_1$ -blocking antibody P1B5 (anti- α_3). Results are normalized to untreated controls and presented as mean \pm S.D. ($n = 3$). C, divalent cation dependence for adhesion on TSP1 peptide 678 and intact TSP1. MDA-MB-435 cells were suspended in calcium-free Ham's F-12(K) medium containing 2 mM magnesium and the indicated concentrations of divalent cations or 2.5 mM EDTA. Cell spreading on substrates coated with 5 μM peptide 678 (solid bars) or 40 $\mu\text{g/ml}$ TSP1 (striped bars) was determined in the absence or presence of 5 $\mu\text{g/ml}$ of the β_1 integrin-activating antibody TS2/16.

and intact TSP1 in the presence of TS2/16, but addition of Ca^{2+} produced a dose-dependent inhibition of spreading on both substrates. Specific inhibition by Ca^{2+} is consistent with previous data for the $\alpha_3\beta_1$ integrin (31). These results suggest that integrin binding to peptide 678 is partially independent of divalent cations, but MDA-MB-435 cell spreading on this peptide may involve both $\alpha_3\beta_1$ integrin binding and divalent cation-independent interactions with another cell surface molecule.

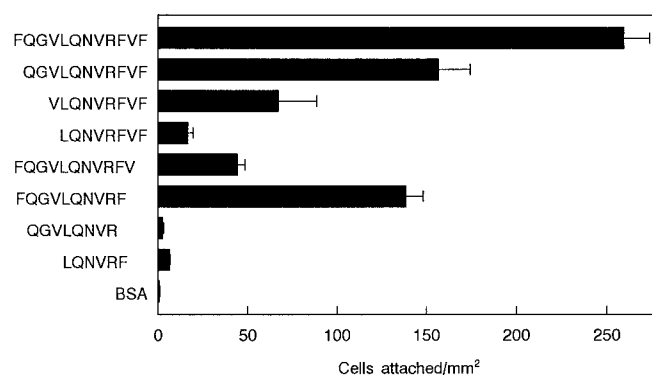


FIG. 5. Determination of the minimal active TSP1 sequence to promote breast carcinoma cell adhesion. MDA-MB-435 cell adhesion was determined to polystyrene coated with 10 μM of the indicated peptides or with bovine serum albumin (BSA). Cell attachment is presented as the mean \pm S.D. for triplicate determinations.

Truncated peptides that contained portions of peptide 678 were synthesized to identify essential residues (Fig. 5). Truncation of the N-terminal Phe or the C-terminal Val-Phe only moderately decreased adhesive activity, but further truncations from either end of the peptide greatly diminished its activity. Inhibition assays confirmed that the loss of adhesive activity reflected loss of integrin binding rather than loss of ability to adsorb on the substrate (Table II). As found in the direct adhesion assays, peptides without the N-terminal Phe or the C-terminal Val-Phe retained significant inhibitory activities, but all shorter peptides were weak inhibitors or inactive. These results imply that the integrin recognizes an extended sequence, but this approach could not discriminate conformational effects of flanking sequences from a direct contribution to integrin binding.

To better define those residues involved in $\alpha_3\beta_1$ integrin binding, we systematically substituted Ala residues into the peptide 678 and tested each for adhesive activity (Fig. 6). Based on the complete loss of adhesion activity for MDA-MB-435 cells following its substitution, only Arg-198 was essential for adhesive activity of peptide 678 (Fig. 6). Replacement of Arg-198 with a His also dramatically reduced adhesive activity. Ala substitutions at several other positions significantly decreased adhesive activity, except for the two N-terminal residues, which only slightly decreased adhesive activity.

Although only the Arg residue was essential for direct adhesion, substitution of several additional residues with Ala markedly decreased or abolished inhibitory activity of the respective soluble peptides in solution to block $\alpha_3\beta_1$ -dependent adhesion to immobilized peptide 678 (Table III). These experiments showed that Arg-198, Val-197, and Asn-196 are essential for inhibitory activity of the peptides in solution. Substitution of Phe-199 and Phe-201 decreased the inhibitory activities of the respective peptides 5–8-fold, indicating that these flanking residues also contribute to activity of the peptides in solution. In contrast, peptides with Ala substitutions at four of the six N-terminal residues in this sequence had inhibitory activities equivalent to that of the native TSP1 sequence. Therefore, NVR is the essential sequence for binding to the $\alpha_3\beta_1$ integrin, but flanking residues may be necessary for inducing the proper conformation of this minimal sequence in peptide 678.

The specificity for an Arg residue at position 198 was further examined using conservative amino acid substitutions (Table III). Substitution with Lys decreased activity approximately 2-fold, whereas substitution with Gln, to retain hydrogen-bonding ability while removing the positive charge, abolished the inhibitory activity. A His substitution showed intermediate activity, indicating that a positive charge rather than a large

TABLE II
Inhibition of MDA-MB-435 cell adhesion to immobilized peptide 678 by fragments of the active peptide

Mean doses to achieve 50% inhibition of control adhesion (IC_{50}) to polystyrene coated with 5 μM peptide 678 were determined from at least three independent experiments, each performed in triplicate. Peptides were tested at up to 300 μM or to the solubility limit for each peptide, where lower limits for inhibitory activity are indicated.

Peptide	Sequence	IC_{50}
		μM
678	FQGV LQNVR FVF (TSP1)	3.5
682	FQGV LQNVR F	6
683	QGV LQNVR	>300
685	QGV LQNVR FVF	24
688	VLQNVR FVF	>100
684	LQNVR FVF	300
681	ac-LQNVR F-am	700

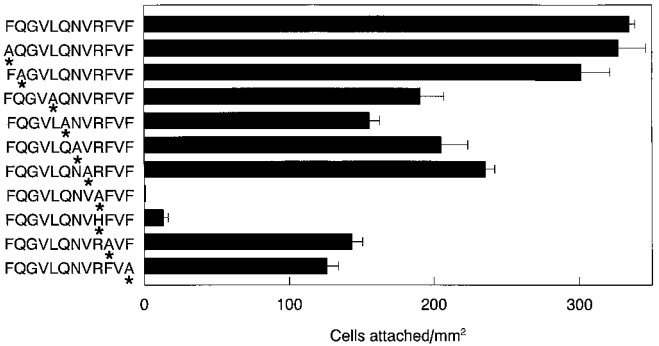


FIG. 6. Effect of systematic substitution of Ala residues on adhesive activities of the TSP1 sequence 190–201 for breast carcinoma cells. Cell attachment was determined to substrates coated with each peptide at 10 μM and is presented as mean \pm S.D. ($n = 3$). Residues substituted in the native TSP1 sequence are indicated with an asterisk.

TABLE III
Mapping of essential residues for inhibition of MDA-MB-435 cell adhesion to immobilized peptide 678

Mean doses to achieve 50% inhibition of control adhesion to 5 μM peptide 678 (IC_{50}) were determined from at least three independent experiments, each performed in triplicate. Residues substituted in the native TSP1 sequence are underlined.

Peptide	Sequence	IC_{50}
		μM
678	FQGV LQNVR FVF (TSP1)	3.5
697	AQGV LQNVR FVF	5
696	FAGV LQNVR FVF	1.8
695	FQGV LQNVR FVF	5
687	FQGV LQNVR FVF	3
686	FQGV LQNV R FVF	>300
691	FQGV LQNV R FVF	>300
690	FQGV LQNV R FVF	>300
702	FQGV LQNV R FVF	6
694	FQGV LQNV R FVF	54
703	FQGV LQNV R FVF	>100
692	FQGV LQNV R FVF	18
693	FQGV LQNV R FVF	27

side chain with hydrogen bonding ability is required at this position.

The active peptides strongly promoted formation of filopodia in MDA-MB-435 cells (Fig. 7A) similar to those induced by attachment on intact TSP1 (14). Addition of IGF1 enhanced spreading and increased formation of lamellipodia on the same peptide (Fig. 7B). Phalloidin staining demonstrated organization of F-actin at the cell periphery but no organization of stress fibers across the cell body (Fig. 7C). Using antibodies recognizing vinculin (Fig. 7D) and focal adhesion kinase (data not shown) as markers of focal adhesion formation, we could not

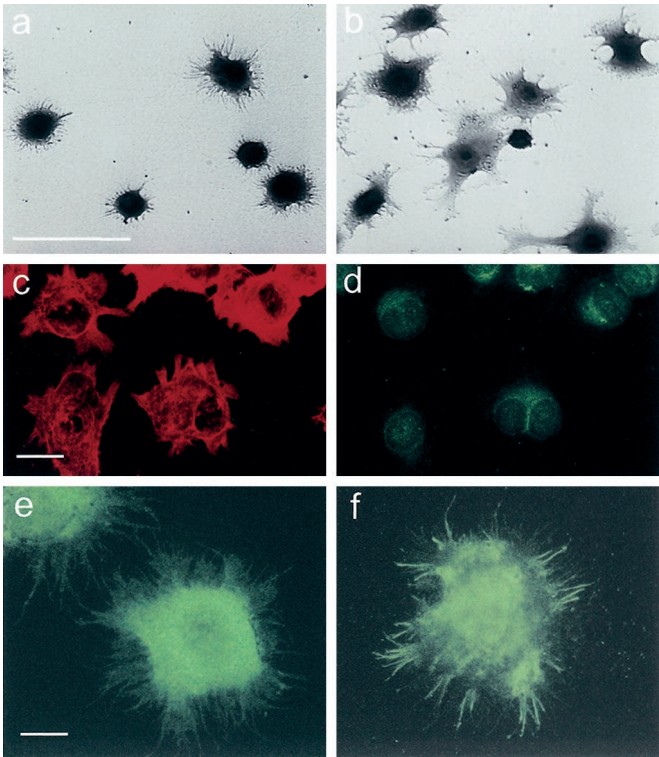


FIG. 7. Morphology of MDA-MB-435 cells attaching on TSP1 peptide 678. a, direct adhesion on TSP1 peptide 678 stimulates formation of filopodia (bar, 50 μm). b, IGF1 stimulates increased spreading with formation of lamellipodia. c, staining of F-actin using BODIPY TR-X phalloidin (bar, 20 μm). d, double labeling of the field in c with anti-vinculin antibody. e, immunolocalization of β_1 integrin subunits in cells attached on peptide 678 using antibody TS2/16 (bar, 10 μm). f, immunolocalization of α_3 integrin subunits using antibody P1B5.

detect any induction of focal adhesions in MDA-MB-435 cells attaching on these peptides, although the same markers showed typical focal adhesion staining patterns in the cells when attaching on vitronectin or fibronectin substrates (results not shown). Staining for the $\alpha_3\beta_1$ integrin was punctate and prominently localized in filopodia extended by MDA-MB-435 cells on immobilized peptide 678 (Fig. 7F), whereas total β_1 integrin staining was more diffuse and concentrated over the cell body.

TSP1 stimulates chemotaxis of MDA-MB-435 cells, and this response is inhibited by the $\alpha_3\beta_1$ -blocking antibody P1B5 (14). Peptide 678 also stimulated chemotaxis of MDA-MB-435 cells (Fig. 8). Chemotaxis to peptide 678 was dose-dependent with a maximal response at 10 μM (Fig. 8A). This response was specific in that peptide 690 was inactive. In agreement with the observations that IGF1 stimulated β_1 integrin-dependent chemotaxis of MDA-MB-435 cells to TSP1 (14) and adhesion of the same cells to peptide 678 (Figs. 2 and 7), the chemotactic response of MDA-MB-435 cells to peptide 678, but not to peptide 690, was increased in the presence of IGF1 (Fig. 8B).

DISCUSSION

Based on examination of synthetic peptides and recombinant fragments representing approximately 90% of the TSP1 sequence, only the sequence FQGV LQNVR FVF from the N-terminal domain exhibited activities that are expected for an $\alpha_3\beta_1$ integrin binding sequence in TSP1. A recombinant fragment of TSP1 containing this sequence also promoted β_1 integrin-dependent adhesion. In solution, this peptide specifically inhibited adhesion to TSP1 but not to ligands recognized by other integrins. Adhesion to this peptide and to TSP1 was stimulated by IGF1 receptor ligands that stimulate integrin-dependent

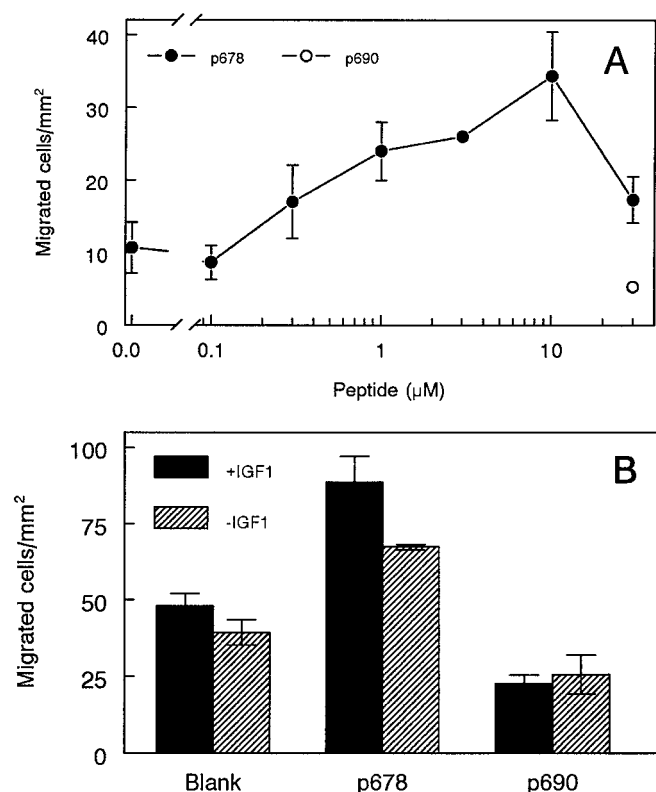


FIG. 8. TSP1 peptide 678 promotes breast carcinoma cell chemotaxis. A, dose dependence for stimulation of MDA-MB-435 cell motility by peptide 678 added to the lower well of a modified Boyden chamber. Cells that migrated to the lower surface of an 8- μm -pore polycarbonate filter were quantified microscopically after 7 h; the data shown are mean \pm S.D. ($n = 3$) for a representative experiment. B, MDA-MB-435 cell chemotaxis was measured to medium alone (Blank) or to 10 μM TSP1 peptide 678 or 10 μM inactive analog peptide 690 added to the lower chamber. Chemotaxis of untreated cells (striped bars) or cells treated with 10 nM IGF1 in the upper chamber (solid bars) was determined after 7 h and is presented as mean \pm S.D. ($n = 3$).

adhesion to intact TSP1, by Mn^{2+} , and by a β_1 integrin-activating antibody and partially inhibited by an $\alpha_3\beta_1$ function-blocking antibody. Based on systematic amino acid substitutions in the active sequence, NVR appears to be the essential core sequence in this TSP1 peptide for recognition by the $\alpha_3\beta_1$ integrin.

Adhesive activities of the immobilized peptides imply that only Arg-198 may directly participate in this interaction, although the partial resistance to inhibition by an $\alpha_3\beta_1$ integrin antibody and EDTA suggest that the peptides with Arg may also support adhesion independent of integrin binding. The context surrounding the Arg is important, however, because other peptides with similar sequences (such as peptide 701, with a QVRT sequence) had no activity, and Ala substitutions of the flanking residues in peptide 678 eliminated or markedly decreased its inhibitory activity in solution. The essential amino acid residues are completely conserved in human, murine, bovine, and *Xenopus* TSP1, but in chicken TSP1, a His replaces the Arg. A similar motif is found in murine and human TSP2, with a His residue replacing the Arg. As a free peptide, the TSP1 sequence with a His substitution was much less active, so it is not clear whether the TSP2 sequence could be recognized by $\alpha_3\beta_1$ integrin. Activity of the latter sequence may be increased in an environment that increases protonation of the imidazole in His.

Previous publications have not identified a consensus $\alpha_3\beta_1$ integrin recognition sequence in its ligands. One hypothesis is that different ligands have unrelated binding sequences, which

is supported by a recent mutagenesis study (32). However, other recent data have raised questions about whether all of the proteins reported to mediate $\alpha_3\beta_1$ -dependent adhesion are true $\alpha_3\beta_1$ ligands (33). LamA2 and LamA3 were verified to bind $\alpha_3\beta_1$ integrin. These have potential binding motifs based on our data, but human LamA1, which was found not to bind $\alpha_3\beta_1$ with high avidity, has an Ala in the position occupied by the essential Arg in the TSP1 sequence. Substitution of Ala for the Arg in the TSP1 sequence abolished all activity of the synthetic TSP1 peptide. Among the five G domain modules of LamA3, G2 has a better consensus sequence based on our results (NLK) than does G4 (NFQ) or G5 (NIH). Expressed as recombinant proteins, only the G2 module promoted $\alpha_3\beta_1$ -dependent adhesion (34). Although RGD was reported to be an $\alpha_3\beta_1$ ligand, the RGD in entactin is not required for recognition, and the RGD in the type 3 repeats of TSP1 is not recognized by this integrin. A binding site for the $\alpha_3\beta_1$ integrin in entactin was mapped to the G2 domain (residues 301–647) (11). Multiple alignment of this region of entactin against the TSP1 sequence and the murine laminin-1 peptide GD6 identified a related sequence, FSGIDE-HGHLTI, but this sequence lacks all of the essential residues in the TSP1 sequence. This domain of entactin also contains two NXR sequences: NNRH and NGRQ. It remains to be determined whether either of these can function as an $\alpha_3\beta_1$ integrin recognition sequence.

The absence of an Asp residue in peptide 678 may account for its partial independence of divalent cations. An Asp residue is usually considered an essential element for integrin peptide binding, the divalent cation participates directly in binding an Asp-containing peptide ligand (reviewed in Ref. 37). Thus an integrin peptide ligand without a carboxyl side chain cannot coordinate with a bound divalent cation and therefore may not have a divalent cation requirement for binding to the integrin. The alternate model, proposing an indirect role of divalent cations in integrin activation (37), would be consistent with the observed stimulation of cell spreading on peptide 678 by Mn^{2+} but not Ca^{2+} and the partial inhibition following chelation of divalent cations.

Another interpretation of the partial divalent cation independence for the adhesive activity of peptide 678 is that ionic interactions of the Arg side chain in the TSP1 peptide with the negatively charged cell surface contribute to the adhesive activity of this peptide. Weak ionic interactions could promote adhesion to the immobilized peptide through multivalent interactions with negatively charged glycoproteins and proteoglycans on the cell but would not significantly contribute to binding of the same monovalent peptide to the cell in solution. This hypothesis would explain why the Arg-containing peptides 686 and 691, in which the essential Val or Asn residues were substituted with Ala, lacked activity in solution to inhibit adhesion to $\alpha_3\beta_1$ ligands but retained some adhesive activity when immobilized. Thus, inhibitory activities in solution may provide a more reliable assessment of integrin binding specificity for Arg-containing peptides.

Spreading of MDA-MB-435 breast carcinoma cells on intact TSP1 (14) or the $\alpha_3\beta_1$ integrin-binding peptide 678 induces formation of filopodia. In cells plated on peptide 678, these structures are enriched in the α_3 integrin subunit, suggesting that engagement of this integrin by TSP1 triggers formation of filopodia. Formation of filopodia or microspikes has been noted during attachment of other cell types on TSP1 (38). This response may be mediated by the $\alpha_3\beta_1$ integrin, because lamellar spreading rather than formation of filopodia was typically observed on melanoma cells that predominantly use the $\alpha_v\beta_3$ integrin receptor for spreading on TSP1 (26).

Using multiple sequence alignment, the N-terminal domains of thrombospondins were recently shown to contain a module related to pentraxins and to the G domain modules of laminins (39). Based on this alignment, both the $\alpha_3\beta_1$ integrin-binding sequence from TSP1 identified here and the GD6 sequence of laminin are located at the C terminus of a pentraxin module. The known three-dimensional structures of other members of the same superfamily (40, 41) lead to the prediction that both potential integrin binding sequences are located in the last β -strand of a pentraxin module and therefore may be presented with similar topologies on the laminin G domain and the N-terminal domain of TSP1. This observation suggests an evolutionary relationship between the thrombospondin N-terminal domains and laminin G domains that is consistent with their proposed common function as recognition sites for a β_1 integrin receptor.

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REFERENCES

- Kreidberg, J. A., Donovan, M. J., Goldstein, S. L., Rennke, H., Shepherd, K., Jones, R. C., and Jaenisch, R. (1996) *Development* **122**, 3537–3547
- Stahl, S., Weitzman, S., and Jones, J. C. R. (1997) *J. Cell Sci.* **110**, 55–63
- Adachi, M., Taki, T., Huang, C., Higashiyama, M., Doi, O., Tsuji, T., and Miyake, M. (1998) *J. Clin. Oncol.* **16**, 1060–1067
- Weitzman, J. B., Hemler, M. E., and Brodt, P. (1996) *Cell Adhes. Commun.* **4**, 41–52
- DeFreitas, M. F., Yoshida, C. K., Frazier, W. A., Mendrick, D. L., Kypta, R. M., and Reichardt, L. F. (1995) *Neuron* **15**, 333–343
- Elices, M. J., Urry, L. A., and Hemler, M. E. (1991) *J. Cell Biol.* **112**, 169–181
- Hemler, M. E., Elices, M. J., Chan, B. M., Zetter, B., Matsuura, N., and Takada, Y. (1990) *Cell Differ. Dev.* **32**, 229–238
- Wu, C., Chung, A. E., and McDonald, J. A. (1995) *J. Cell Sci.* **108**, 2511–2523
- Yamada, K. M. (1991) *J. Biol. Chem.* **266**, 12809–12812
- Eble, J. A., Wucherpfennig, K. W., Gauthier, L., Dersch, P., Krukonis, E., Isberg, R. R., and Hemler, M. E. (1998) *Biochemistry* **37**, 10945–10955
- Gresham, H. D., Graham, I. L., Griffin, G. L., Hsieh, J. C., Dong, L. J., Chung, A. E., and Senior, R. M. (1996) *J. Biol. Chem.* **271**, 30587–30594
- Gehlsen, K. R., Sriramara, P., Furcht, L. T., and Skubitz, A. P. (1992) *J. Cell Biol.* **117**, 449–459
- Miles, A. J., Knutson, J. R., Skubitz, A. P., Furcht, L. T., McCarthy, J. B., and Fields, G. B. (1995) *J. Biol. Chem.* **270**, 29047–29050
- Chandrasekaran, S., Guo, N., Rodrigues, R. G., Kaiser, J., and Roberts, D. D. (1999) *J. Biol. Chem.* **274**, 11408–11416
- Roberts, D. D., Cashel, J., and Guo, N. (1994) *J. Tissue Culture Methods* **16**, 217–222
- Guo, N. H., Kruttsch, H. C., Nègre, E., Vogel, T., Blake, D. A., and Roberts, D. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3040–3044
- Guo, N. H., Kruttsch, H. C., Nègre, E., Zabrenetzky, V. S., and Roberts, D. D. (1992) *J. Biol. Chem.* **267**, 19349–19355
- Guo, N., Kruttsch, H. C., Inman, J. K., and Roberts, D. D. (1997) *J. Peptide Res.* **50**, 210–221
- Prater, C. A., Plotkin, J., Jaye, D., and Frazier, W. A. (1991) *J. Cell Biol.* **112**, 1031–1040
- Murphy-Ullrich, J. E., Gurusiddappa, S., Frazier, W. A., and Höök, M. (1993) *J. Biol. Chem.* **268**, 26784–26789
- Gao, A.-G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1996) *J. Biol. Chem.* **271**, 21–24
- Vogel, T., Guo, N. H., Kruttsch, H. C., Blake, D. A., Hartman, J., Mendelovitz, S., Panet, A., and Roberts, D. D. (1993) *J. Cell. Biochem.* **53**, 74–84
- Legrand, C., Thibert, V., Dubernard, V., Begault, B., and Lawler, J. (1992) *Blood* **79**, 1995–2003
- Akiyama, S. K., and Yamada, K. M. (1985) *J. Biol. Chem.* **260**, 4492–4500
- Hemler, M. E., Sanchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A., and Strominger, J. L. (1984) *J. Immunol.* **132**, 3011–3018
- Sipes, J. M., Kruttsch, H. C., Lawler, J., and Roberts, D. D. (1999) *J. Biol. Chem.* **274**, 22755–22762
- Schuler, G. D., Altschul, S. F., and Lipman, D. J. (1991) *Prot. Struct. Funct. Genet.* **9**, 180–190
- Lawrence, C. E., Altschul, S. F., Boguski, M. S., Liu, J. S., Neuwald, A. F., and Wootton, J. C. (1993) *Science* **262**, 208–214
- Dawson, D. W., Pearce, S. F., Zhong, R., Silverstein, R. L., Frazier, W. A., and Bouck, N. P. (1997) *J. Cell Biol.* **138**, 707–717
- Clezardin, P., Lawler, J., Amiral, J., Quentin, G., and Delmas, P. (1997) *Biochem. J.* **321**, 819–827
- Weitzman, J. B., Pasqualini, R., Takada, Y., and Hemler, M. E. (1993) *J. Biol. Chem.* **268**, 8651–8657
- Krukonis, E. S., Dersch, P., Eble, J. A., and Isberg, R. R. (1998) *J. Biol. Chem.* **273**, 31837–31843
- Delwel, G. O., de Mleker, A. A., Hogervorst, F., Jaspars, L. H., Fles, D. L. A., Kuikman, I., Lindblom, A., Paulsson, M., Timpl, R., and Sonnenberg, A. (1994) *Mol. Biol. Cell* **5**, 203–215
- Mizushima, H., Takamura, H., Miyagi, Y., Kikkawa, Y., Yamanaka, N., Yasumitsu, H., Misugi, K., and Miyazaki, K. (1997) *Cell Growth Differ.* **8**, 979–987
- Aota, S., and Yamada, K. M. (1995) *Adv. Enzymol. Relat. Areas Mol. Biol.* **70**, 1–21
- Ruoslahti, E. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 697–715
- Fernandez, C., Clark, K., Burrows, L., Schofield, N. R., and Humphries, M. J. (1998) *Frontiers Biosci.* **3**, 684–700
- Adams, J. C. (1995) *J. Cell Sci.* **108**, 1977–1990
- Beckmann, G., Hanke, J., Bork, P., and Reich, J. G. (1998) *J. Mol. Biol.* **275**, 725–730
- Shrive, A. K., Cheetham, G. M. T., Holden, D., Myles, D. A. A., Turnell, W. G., Volanakis, J. E., Pepys, M. B., Bloomer, A. C., and Greenhough, T. J. (1996) *Nat. Struct. Biol.* **3**, 346–353
- Emsley, J., White, H. E., O'Hara, B. P., Olivia, G., Srinivasan, N., Tickle, I. J., Blundell, T. L., Pepys, M. B., and Wood, S. P. (1994) *Nature* **367**, 338–345

Identification of an $\alpha_3\beta_1$ Integrin Recognition Sequence in Thrombospondin-1

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