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Identification of a New Cell Adhesion Motif in Two Homologous Peptides from the COOH-terminal Cell Binding Domain of Human Thrombospondin*

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Thrombospondin-1 (TS1) contains at least four domains that support cell attachment. The COOH-terminal cell binding domain (CBD) was first identified with a monoclonal antibody against TS1 that blocked secretion-dependent platelet aggregation. Subsequently, this domain of TS1 has been found to bind a number of normal and transformed cells. We have localized attachment sites for human melanoma cells (G361) within the CBD to two noncontiguous 30-residue peptides designated C4 and C7 (Kosfeld, M. D., and Frazier, W. A. (1991) *J. Biol. Chem.* 267, 16230–16236). Here we report studies to define the active sequences within C4 and C7. An octapeptide, RFYVVMWK (4N1-1), from C4 and a pentapeptide, IRVVM (7N3-1), from C7 were found to support attachment of G361 melanomas, K562 erythroleukemia cells, HT1080 fibrosarcomas, C32 amelanotic melanomas, and endothelial cells. These peptides also inhibit the adhesion of cells to the recombinant CBD of TS1. The hexapeptide RFYVVM (4N1-2) also inhibits cell attachment. The inhibitory effect of combinations of C4- and C7-derived peptides is synergistic. The sequences 4N1-1 and 7N3-1 of TS1 share homology with two cell adhesive peptides from laminin (LM), LMF9 and LMPA22-2, respectively. These TS1 and LM peptides are interchangeable in inhibiting the adhesion of G361 cells to LM or TS1, suggesting a possible sharing of receptors by LM and TS1. K562 cells, however, bound only to TS1, and this binding was inhibited preferentially by the TS1 CBD peptides, indicating a receptor specific for TS1 which does not recognize LM. The active TS1 peptides are highly conserved among five species and four isoforms of TS1. Homologs of the TS1 peptides are found in tenascin, a matrix protein that shares several properties with TS1 and in factor VIII, α_2-macroglobulin, and von Willebrand factor.

The interaction of cells with extracellular matrix molecules is a complex process from which cells derive a wealth of information about their environment. This information is processed in a number of ways that ultimately affect cell motility, shape, proliferation, and gene expression (Hynes, 1992). Extracellular matrix macromolecules such as fibronectin, laminin, vitronectin, and collagen have been shown to mediate cell adhesion, a process that includes cell attachment and spreading. Like these proteins, thrombospondin 1 (TS1)[1] promotes adhesion of a number of normal and transformed cell types (Frazier, 1991; Roberts *et al.*, 1987), a function which underlies many effects that TS1 exerts in several biologically complex systems. These effects include stabilizing platelet aggregation (Leung *et al.*, 1984; Dixit *et al.*, 1985), regulating cell growth (Majack *et al.*, 1988; Good *et al.*, 1990), specifying the differentiation phenotype of certain cells (Castle *et al.*, 1991), wound healing (Raugi *et al.*, 1987), and the migration of tumor cells (Tuszynski *et al.*, 1987) and polymorphonuclear neutrophils (Mansfield *et al.*, 1990). A good example of the regulation of several aspects of cellular behavior by TS1 is the inhibition of angiogenesis *in vivo* and of endothelial cell migration and proliferation *in vitro* (Good *et al.*, 1990; Taraboletti *et al.*, 1990).

There are at least four TS isogenes, TS1, 2, and 3 (Bornstein *et al.*, 1991; LaBell *et al.*, 1992; Laherty *et al.*, 1992; Vos *et al.*, 1992) and cartilage oligomeric matrix protein or COMP (Oldberg *et al.*, 1992) whose products are related, but decidedly different. Of these, platelet TS (which is pure TS1) is the best characterized, and serves as a prototype for this growing family. Distinct activities can be assigned to certain domains. For example, the amino-terminal domain of TS1 induces spreading of G361 cells while the COOH-terminal cell binding domain (CBD) of TS1 promotes haptotaxis and attachment of these cells (Taraboletti *et al.*, 1987; Roberts *et al.*, 1987). TS1 contains at least four domains that support cell attachment: the amino-terminal heparin binding domain (Murphy-Ullrich *et al.*, 1989), the type I repeats of about 60 amino acid residues containing the CSVTCG sequence (Prater *et al.*, 1991), the RGDA sequence in the last of the type 3 calcium-binding repeats (Lawler *et al.*, 1988) and the COOH-terminal ~210 residues termed the "cell binding" domain (Kosfeld *et al.*, 1991). We have previously selected a monoclonal antibody (mAb) called C6.7 which binds to this CBD and blocks its interaction with cellular receptors (Dixit *et al.*, 1985). Using this mAb it has been shown that the CBD is essential for binding of TS1 to platelets (Dixit *et al.*, 1985), many transformed cells (Varani *et al.*, 1986), and human melanoma cells (Taraboletti *et al.*, 1987). We have expressed in bacteria the CBD of TS1 (rCBD) exclusive of the upstream RGD sequence and demonstrated its attachment activity for human melanoma cells (Kosfeld *et al.*, 1991). By synthesizing and testing a series of eight overlapping synthetic peptides (30 mers)

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[1] The abbreviations used are: TS1, thrombospondin-1; COMP, cartilage oligomeric matrix protin; CBD, cell binding domain; mAb, monoclonal antibody; TBS, Tris-buffered saline; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; LM, laminin.

spanning the entire 212-residue CBD, we localized the cell attachment activity of the CBD to two nonoverlapping 30-residue synthetic peptides, designated C4 and C7 (Kosfeld and Frazier, 1992). Peptides immobilized on plastic wells promoted melanoma cell attachment, and soluble peptides added to the medium inhibited cell attachment to TS1 and rCBD-coated surfaces. Furthermore, both active peptides blocked the attachment of cells to either peptide suggesting that they share a common receptor on the melanoma cells (Kosfeld and Frazier, 1992).

In the present study, we focus on the amino acid sequence(s) within the peptides C4 and C7 to identify the essential residues for cell attachment activity. We assayed the cell attachment-promoting activities of a number of overlapping subpeptides derived from C4 and C7 and examined the ability of these subpeptides to inhibit cell attachment to the CBD. We have found two peptides, a pentamer from C4 and an octamer from C7, which reproduce the cell adhesive properties of the CBD and inhibit melanoma cell attachment to the CBD of TS1. These TS-1 peptides have stringent homologs in other TS1 isoforms and interesting similarities in other proteins including laminin, tenascin, von Willebrand factor, and other serum proteins which interact with cells.

MATERIALS AND METHODS

The generation and characterization of mAb C6.7 has been described and its epitope localized to peptide C7 within the CBD of TS1 (Dixit *et al.*, 1985, Kosfeld *et al.*, 1991). All cell lines used were obtained from ATCC: human melanoma G361 (CRL 1424), K562 human erythroleukemia cells (ATCC CCL 241), HT-1080 human fibrosarcomas (ATCC CCL 121), and C32 amelanotic human melanoma (ATCC CRL 1585). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 5% CO_2 (Roberts *et al.*, 1987). All reagents were obtained from Sigma unless specified otherwise.

The rCBD of TS1—The rCBD was expressed as previously described (Kosfeld *et al.*, 1991). The rCBD was found in the soluble fraction of bacterial lysates and was purified by chromatography on a column of Q-Sepharose "fast flow" (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl (TBS). The column was eluted with a linear gradient of NaCl (0.15–1 M) in the Tris buffer. Fractions with the highest concentration of the protein A-CBD fusion protein were identified by SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide gels, followed by staining with Coomassie Blue and Western blotting using alkaline phosphatase-conjugated IgG to locate bands containing the protein A moiety. The rCBD fractions were dialyzed against PBS and stored at −70 °C until used. We have previously shown that the protein A moiety has no attachment activity for these cells.

Peptide Synthesis—Peptides whose sequences correspond to portions of peptide C4 (Fig. 1A) or C7 (Fig. 2*A*) of the CBD of TS1 were synthesized and purified by the Protein Chemistry Facility as described previously (Prater *et al.*, 1991). Briefly, peptides were made on an Applied Biosystems model 3804 solid phase peptide synthesizer. The resultant peptides were cleaved and deblocked by Immunodynamics (San Diego, CA) and purified by reversed phase HPLC (Waters) using acetonitrile, 0.1% trifluoroacetic acid solvent systems. Purity was tested with analytical HPLC. Primary structures of peptides were confirmed by amino acid composition on a Beckman model 6300 amino acid analyzer, and by sequencing with an Applied Biosystems model 477A Sequencer. All peptide preparations were tested for cytotoxicity on G361 cells and were not toxic at the concentrations employed in these experiments.

Cell Adhesion Assay—Cell adhesion was performed in 96-well plates as previously described (Prater *et al.*, 1991; Kosfeld and Frazier, 1992). Synthetic peptides were solubilized with 6 N guanidine HCl because some hydrophobic peptides were insoluble at concentrations approaching 1 mM in TBS. The peptides were coated onto plastic 96-well plates (Nunc Immuno Plate Maxisorp) by incubating 50 μl of peptide solutions per well at the indicated concentrations. After incubating overnight at room temperature, wells were rinsed with TBS and blocked with 10 mg/ml bovine serum albumin (BSA) for 30 min at room temperature. Peptides were also coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacture's instructions. Approximately 2 mg of peptide were used per ml of the Sepharose. The efficiency of coupling of all peptides was greater than 90% as judged by UV absorbance. The peptide-coated beads were then blocked with BSA as above. IgG-coated beads were also prepared for use as controls.

After blocking, cells were allowed to attach for 1 h at 37 °C to substrate-coated wells or beads. In some experiments, inhibition was determined by adding peptide inhibitors of cell attachment to each well along with the cells. After removal of nonadhering cells, cells attached to immobilized substrates were quantitated with endogenous cellular phosphatase activity which is measured by absorption at 410 nm as previously described (Prater *et al.*, 1991). Each assay was carried out in duplicate and each peptide was tested in three separate experiments at three or more different concentrations.

Peptide-BSA Conjugates—In addition to the free peptides, peptides conjugated to protease-free BSA (Humphries *et al.*, 1987) were also used in the cell binding assay. Peptides were synthesized with an NH_2-terminal cysteine for covalent coupling to BSA via the heterobifunctional cross-linking reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). BSA was activated at room temperature with SPDP at a molar ratio of 50:1 (SPDP:BSA) for 30 min. Unreacted SPDP was separated from derivatized BSA by gel filtration on a Sephadex G-25SF column equilibrated with PBS. The activated BSA was then added to dry peptide at a molar ratio of 1:20 (BSA:peptide). The reactants were mixed overnight at room temperature and unconjugated peptides removed by dialysis against PBS, pH 7.4. Peptide-BSA conjugates were stored frozen at −20 °C until used.

RESULTS

As described in previous studies, two nonoverlapping synthetic peptides designated C4 and C7 represent two potent cell attachment sites within the COOH-terminal CBD (Kosfeld and Frazier, 1992). In this study, through a reductionist approach, we have further defined the activity within these peptides by systematically examining shorter peptides derived from the active peptides C4 and C7.

The amino acid sequences of the subpeptides synthesized from C4 are shown in Fig. 1*A*. As a first step to locate the essential amino acid sequence(s) within C4, two peptides with overlapping sequences, 4N and 4C, were synthesized and their activities assessed using the cell attachment assay. The results in Fig. 1*B* show that peptide 4N, but not 4C, displayed high

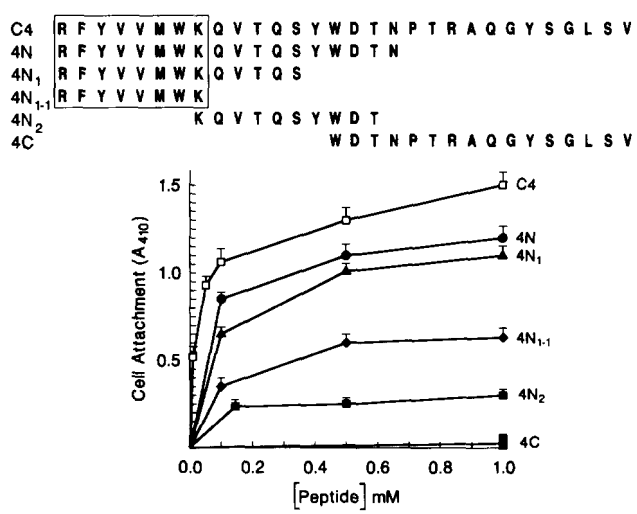

FIG. 1. *A*, amino acid sequences of the subpeptides derived from peptide C4 of the CBD of TS1. The *boxed letters* indicate amino acid residues that are common in all active subpeptides. *B*, direct attachment of G361 cells to C4 subpeptides. Equimolar concentrations of peptides shown in *A* were evaluated as attachment factors for G361 cells. Peptides C4 (□), 4N (●), 4N1 (▲), 4N1-1 (♦), 4N2 (■) and 4C (▮) were adsorbed to microtiter plates at the indicated concentrations. Cells were added to the wells and incubated for 1 h at 37 °C. The attached cells were quantitated as described under "Materials and Methods," and the actual absorbance due to endogenous cellular phosphatase hydrolysis of *p*-nitrophenyl phosphate at 410 nm is plotted.

activity. In order to further locate the active sequence(s) within 4N, three shorter overlapping peptides spanning 4N (Fig. 1*A*) were tested. Peptide 4N1 showed significantly greater cell binding activity than peptide 4N2. 4N1-1, the amino-terminal sequence of 4N1, retained 60% of the maximum activity of 4N1. These results indicate the importance of the sequence common to both active peptides, 4N1 and 4N1-1, the octapeptide RFYVVMWK.

The amino acid sequences of the C7 subpeptides is shown in Fig. 2*A* and their cell attachment activities in Fig. 2*B*. First, we synthesized two peptides, 7N and 7C, which together represent the entire length of C7. 7C actually extends beyond the COOH terminus of C7 (*arrow* in Fig. 2). The amino-terminal peptide, 7N, showed significant cell attachment activity while the COOH-terminal peptide, 7C, exhibited none. Next, 7N was divided into three overlapping subpeptides designated 7N1, 7N2, and 7N3. Of these, only 7N2 and 7N3 had significant activity, the activity of 7N2 being less than half of that of 7N3. Based on this result, 7N3 was further dissected into two pentapeptides, 7N3-1 with the sequence IRVVM and 7N3-2 with the sequence YEGKK. Peptide 7N2 was also divided into two smaller subpeptides, 7N2-1 (RPKTGF) and 7N2-2 (KTGFIR). The 7N2-2 sequence contains the COOH-terminal sequence of the 7N2 and the amino-terminal sequence of the 7N3-1. The maximal cell attachment activity of 7N3-1 was comparable to that of 7N3, and nearly as high as that of 7N and the parent peptide C7. Peptides 7N2-1, 7N2-2, and 7N3-2 on the other hand, exhibited negligible attachment-promoting activities. These results localize the highest cell attachment activities to peptides containing the central region of C7 such as 7N, 7N3, and 7N3–1, indicating that the critical residues for activity lie in the IRVVM sequence (7N3-1). Thus both the active sequence from C4, RFYVVMWK and from C7 IRVVM contain the VVM sequence, the only sequence shared by C4 and C7. This result is consistent with the mutual competition reported earlier for C4 and C7 (Kosfeld and Frazier, 1992).

To ascertain that the activities of the peptides are not a function of their association with the plastic surface, peptides linked to BSA or Sepharose beads were also used in cell binding assays. Again the same peptides that are shown to be active in Figs. 1 and 2 also promote substantial cell attachment when conjugated to BSA and then coated on plastic wells, or when covalently attached to Sepharose beads (not shown). These observations confirm the sequences RFYVVMWK from C4 and IRVVM from C7 as the primary determinants of the activity of the CBD of TS1.

To be sure that the active peptides from C4 and C7 contain the sequences that are relevant for attachment of cells to the CBD, the shorter, more soluble active peptides were tested as soluble inhibitors of the binding of G361 cells to rCBD immobilized on plastic wells. The short peptide, 7N3-1, in contrast to its larger homolog 7N3, is highly soluble, which makes it possible to test it at the high concentrations often required for inhibition of cell attachment. In contrast, 4N1-1 and 4N1, the active subpeptides for C4, are insoluble at concentrations higher than 0.2 mM and thus must be tested as soluble inhibitors at concentrations below this solubility limit. It should be noted that inclusion of peptides in the cell attachment assay at these concentrations had no adverse effects on the cells. The ability of the active subpeptides from C4 and C7 to inhibit G361 melanoma cell adhesion to rCBD is shown in Fig. 3. The results of this complementary bioassay confirmed those of the direct cell adhesion assays (Figs. 1 and 2). We found that peptides 4N1 and 7N3 interfere with the attachment of G361 cells to rCBD-coated surfaces by about 30 and 50%, respectively (Fig. 3*B*), while the shorter peptides 4N1-1 and 7N3-1 were inhibitory by 30 and 25%, respectively (Fig. 3*A*). The inhibition was dose-dependent at peptide concentrations <0.1 mM for 4N1 and 7N3 and <0.2 mM for 4N1-1 and 7N3-1. 7N3-1 tested at 2 mM exhibits no significant increase in inhibitory effect compared to that at 0.2 mM (not shown). Thus the shortest active subpeptides from C4 or C7, when added individually to cells, only partially inhibit cell attachment to the rCBD. A combination of these subpeptides, however, had a synergistic effect as shown in the *triangles* in Fig. 3, *A* and *B*. Each peptide is present at one-half the indicated concentration. In the case of 4N1 plus 7N3, the inhibition was nearly complete (87%). In contrast, the effect of 4N1-1 plus 7N3-1 was less than that of the longer 4N1 plus 7N3 peptides, suggesting that some active amino acid residues might be missing from the shorter peptides. In contrasting Fig. 3, *A* to *B*, the activity exhibited by 4N1-1 and 4N1 was

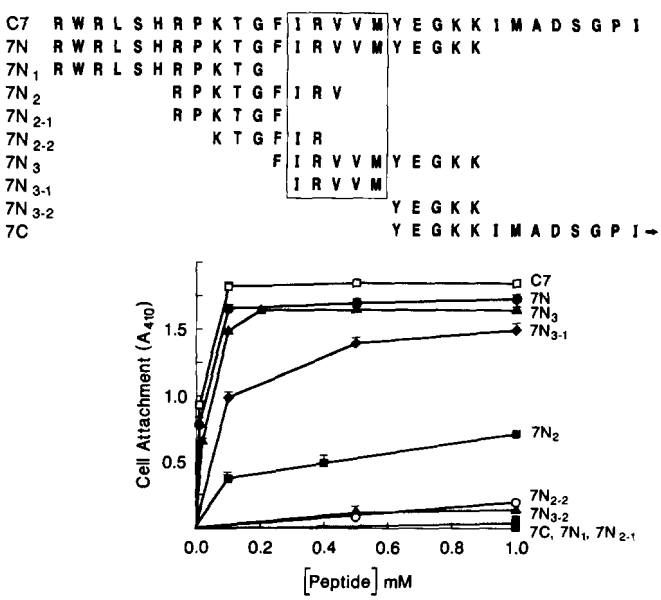

FIG. 2. *A*, amino acid sequences of subpeptides derived from peptide C7 of the CBD of TS1. The *boxed letters* indicate the amino acid residues that are in common in all active subpeptides. *B*, direct attachment of G361 cells to subpeptides of peptide C7. Microtiter wells were adsorbed with peptides C7 (□), 7N (●), 7N3 (▲), 7N3-1 (◆), 7N2 (■), 7N2-2 (○), 7N3-2 (▲), and 7C, 7N1, or 7N2-1 (■) at various concentrations, and the attachment of G361 cells was determined. The numbers of cells attached to these peptides were correlated with the cellular phosphatase activity which is expressed as absorbance at 410 nm.

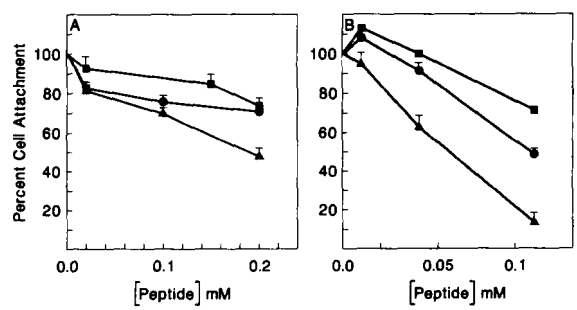

FIG. 3. **Effect of the active subpeptides of C4 and C7 on G361 cell attachment to the rCBD of TS1.** To microtiter wells containing immobilized rCBD (10 μg/ml), G361 cells were added with (*A*) 4N1-1 (●), 7N3-1 (■), 4N1-1 plus 7N3-1 (▲) and (*B*) 4N1 (■), 7N3 (●), and 4N1 plus 7N3 (▲). The cells were incubated for 1.5 h in the wells, and cell attachment was determined. The effects of these subpeptides on cell attachment are expressed as percent relative to the maximum cell attachment for each experiment where 100% is the number of cells binding to rCBD-coated wells in the absence of peptide inhibitors.

comparable while peptide 7N3-1 was not as active as 7N3. This suggests that 7N3-1 does not contain all of the active residues. To evaluate the contribution of adjacent sequences to activity, peptide 7N2-2, which contained the NH_2-terminal sequence of 7N3-1, and 7N3-1 were examined in inhibition studies. While less inhibitory that 7N3-1, both peptides showed significant inhibitory activity, suggesting the importance of adjacent sequences in modifying the activity of the 7N3-1 peptide (not shown). Peptides that are distant from the active sequences of C4 and C7 (4C, 4N2, 7N1) showed no inhibition of cell attachment to the rCBD (not shown), indicating that the inhibition is specific and not due to cytotoxic effects of the peptide preparations.

In the course of these inhibition experiments, a shorter peptide derived from 4N1-1 with the sequence RFYVVM (designated 4N1-2) was tested. This peptide had little or no activity in the direct cell adhesion assays (Fig. 1) yet in the inhibition assays was as potent as the longer 4N1-1. This may represent a case of a peptide that either binds very poorly to the plastic wells or, when bound, assumes a configuration on the plastic that prevents it from interacting with cellular receptors. We have also synthesized peptides which alter the sequence of 7N3-1 or IRVVM to test the importance of the isoleucine, arginine, and methionine residues in binding to cellular receptors. These peptides, GRVVM, IEVVM, and IRVVG, were tested as inhibitors of cell adhesion to the rCBD. All were inactive (not shown).

Thus far, we have identified the octapeptide 4N1-1 (RFYVVMWK) and the pentapeptide IRVVM (7N3-1) as important sequences for the cell attachment activity of the TS1 CBD. Furthermore, the peptide 4N1-2 (RFYVVM) is active as an inhibitor of cell binding to the rCBD. These peptides share the tripeptide VVM and both contain an arginine upstream. We then looked for sequences related to these peptides in other cell adhesion and extracellular matrix proteins (Yamada, 1991). This search revealed two peptides from laminin (LM) having sequences similar to 4N1 and 7N3. The sequence homology between these peptides is shown in Fig. 4. The peptide designated LMF9, from the F9 fragment of LM (Skubitz *et al.*, 1990), shares five identical residues and two conservative substitutions with 4N1. LM22-2, from peptide PA22-2 of LM (Tashiro *et al.*, 1989), has sequence homology (not identity) with 7N3, particularly within the IKVAV portion, the active region of the LM peptide. We

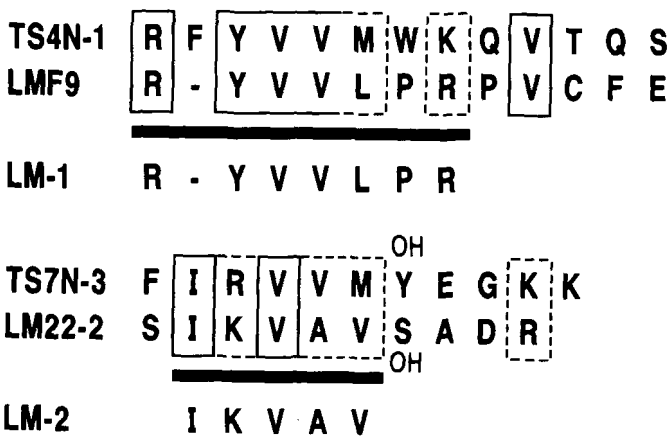

FIG. 4. **Cell attachment sites in TS1 and LM.** Amino acid sequence comparison between a LM peptide designated LMF9 with 4N-1 and LM PA22-2 with 7N3 is shown. Amino acid residues which are identical are shown in *solid boxes* while conservative substitutions are shown in *broken boxes*. The alignments produced were modified by the addition of a gap in the LMF9 in order to maintain the best alignment.

synthesized two peptides modeled on these residues of LM (Fig. 4) and tested them as substrates for attachment of G361 cells. LM-1 (the LMF9 analog of TS1 4N1-1) had little cell attachment activity while LM-2 (the PA22-2 analog of TS1 7N3-1) bound cells but to a lesser degree than the 7N3-1. These results were confirmed using peptides linked to BSA and Sepharose beads (not shown).

We also tested the ability of these peptide homologs to substitute for each other in inhibiting G361 cell binding to the TS1 rCBD or LM-coated surfaces (Fig. 5). G361 cells bound to LM in a concentration-dependent manner (Fig. 5*A*) and this attachment activity was inhibited by LM1 (21%), LM2 (28%) (Fig. 5*B*), 4N1-1 (32%), and 7N3-1 (50%) (Fig. 5*C*). LM1 and LM2 also inhibited cell binding to the CBD of TS1, 15 and 34%, respectively (Fig. 5*D*). Quantitatively, LM peptides were less effective than their TS1 homologs. Other adhesive peptides of LM such as LGTIPG, PGAIPG, and YIGSR were also tested as inhibitors of the CBD and LM but showed no detectable activity (Fig. 5, *B* and *D*). These results suggest that TS1 and LM may be able to share a common receptor.

To further address this issue, we tested several cell types and lines for attachment to LM and TS1 to see if any cells attached to one protein but not the other. Fig. 6 shows attachment to TS1 and LM of four cell types. These include G361 human melanomas (Fig. 6*A*), K562 human erythroleukemia cells (Fig. 6*B*), HT1080 human fibrosarcomas (Fig. 6*C*), and C32 human melanoma (Fig. 6*D*). It is of interest to note that C32 and K562 cells attached to TS1 much more avidly than to LM while G361 and HT1080 cells bound well to both proteins. The K562 cells also bound well to the rCBD and its active sequences (not shown). Next we tested the effect of 4N1-1, 7N3-1, and their LM homologs on K562 cell attachment to the rCBD. Since these cells demonstrated

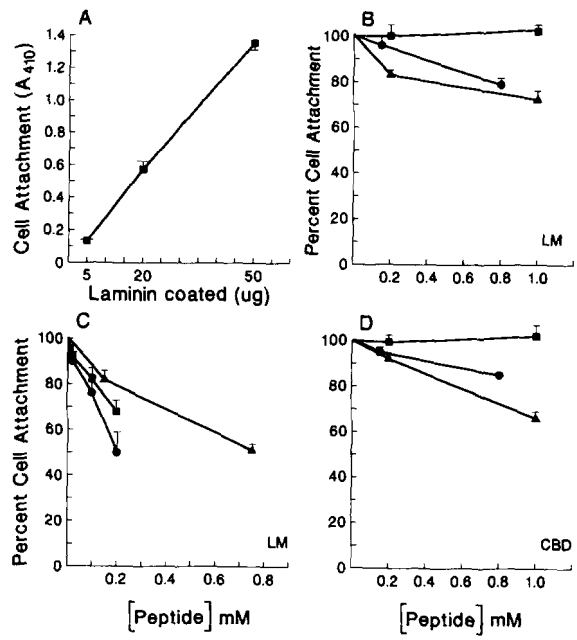

FIG. 5. **Effects of soluble peptides of TS1 or LM on G361 cell attachment to immobilized TS1 or LM.** *A*, in the standard assay, G361 cells alone (no inhibitors) were added to microtiter wells coated with LM at the indicated concentrations. In inhibition studies, soluble subpeptides were included with G361 cells at the indicated concentrations. Soluble LM peptides LGTIPG, PGAIPG, YIGSR (■), LM1 (●), and LM2 (▲) were added to LM-coated wells (*B*) or rCBD-coated surface (*D*). Soluble TS1 subpeptides 4N1-1 (■), 7N3-1 (▲), 4N1-1 plus 7N3-1 (●) were tested on (*C*) LM-coated wells. Control attachment (100%) is that with no added peptides. Data are shown for concentrations of each peptide below its solubility limit.

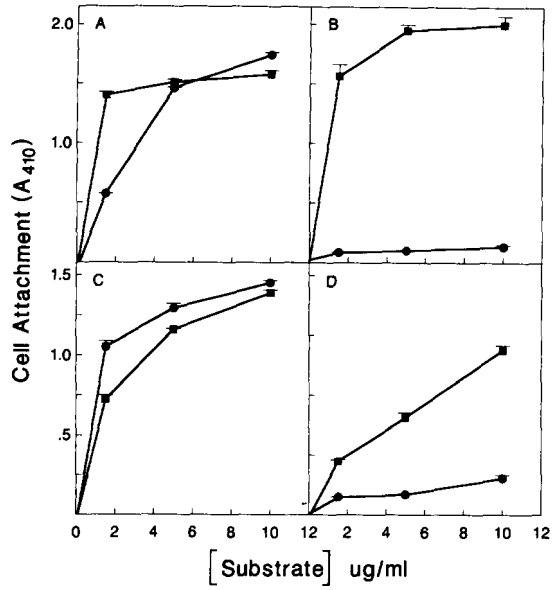

FIG. 6. **Attachment of four cell types to immobilized TS-1 and LM.** Wells were coated with the indicated concentrations of TS1 (■) or LM (●). Assays were performed as described under "Materials and Methods" with G361 human melanomas (*A*), K562 erythroleukemia cells (*B*), HT1080 fibrosarcomas (*C*), and C32 amelanotic melanomas (*D*) added to the wells.

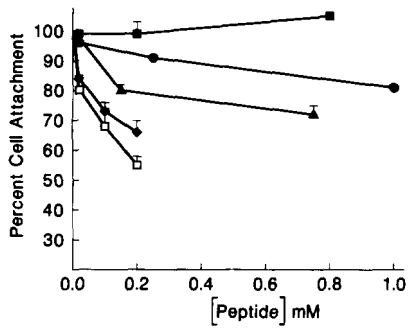

FIG. 7. **Effect of peptides from TS1 and LM on K562 cell attachment to immobilized rCBD of TS1.** Wells were coated with 10 μg/ml rCBD/well. Prior to addition of K562 cells, indicated concentrations of peptide LM1 (■), LM2 (●), 4N1-1 (♦), 7N3-1 (▲), and 4N1-1 plus 7N3-1 (□) were added to the wells. Control attachment (100%) is that with no added peptides.

specific attachment to TS1 and not to LM, no LM receptor should contribute to the binding of the TS1 CBD. As expected, the TS1 peptides showed significant inhibitory activity (36% for 4N1-1, 28% for 7N3-1) while the LM peptides exhibited an undetectable (LM1) or a lower (19% for LM2) level of inhibition (Fig. 7). In agreement with previous results, the combination of 4N1-1 and 7N3-1 increased the inhibition of rCBD-mediated cell attachment (45%). The TS1 peptides thus, are better competitors than the LM peptides in the absence of a LM receptor interaction. K562 cells therefore provide a valuable model in which only the cellular interactions with TS1 can be evaluated. Thus it appears that a receptor exists which binds the rCBD of TS1 with a high degree of specificity which excludes an interaction with LM.

If the active sequences in the CBD are critical for cell attachment, then one would expect these residues to be conserved among different species. Fig. 8 shows the alignment of the homologs of peptides 7N3 and 4N1 from all known isoforms and species of TS1. While peptide 7N3 is highly conserved only in human and mouse TS1, similar residues are retained in human, mouse, and chicken TS2. In mouse TS3

A. Peptide 7N-3 Homologs in TS Isoforms

HUMAN-1	F	I	R	V	V	M	Y	E	G	K	K
MOUSE-1	Y	I	R	V	V	M	Y	E	G	K	K
HUMAN-2	Y	I	R	V	L	V	H	E	G	K	Q
MOUSE-2	Y	M	R	V	L	V	H	E	G	K	Q
CHICKEN-2	L	I	K	V	L	V	Y	E	G	K	Q
MOUSE-3	Y	I	R	V	K	L	Y	E	G	P	Q
COMP	Y	I	R	V	R	F	Y	E	G	P	E

B. Peptide 4N-1 Homologs in TS Isoforms

HUMAN-1	R	F	Y	V	V	M	W	K	Q	V	T
MOUSE-1	R	F	Y	V	V	M	W	K	Q	V	T
HUMAN-2	R	F	Y	V	V	M	W	K	Q	V	T
MOUSE-2	R	F	Y	V	V	M	W	K	Q	V	T
CHICKEN-2	R	F	Y	V	L	M	W	K	Q	V	T
MOUSE-3	R	F	Y	V	V	M	W	K	Q	T	E
COMP	S	F	Y	V	V	M	W	K	Q	M	E

FIG. 8. **Conserved cell binding regions of the CBD of known TS1 isoforms.** In *A*, residues identical among the TS1 sequences are shown in the *box*. In *B*, only residues which differ are *boxed*. References for each sequence are indicated in the text.

A. TENASCIN

Human/Mouse TS-1 7N3	F	I	R	V	-	V	M	Y	E	G	K K
Human/Mouse Tenascin	F	I	R	V	F	A	I	L	E	N	K K

B. SERUM PROTEINS

Human/Mouse TS-1	I	R	V	V	M
Human/Mouse Laminin A	I	K	V	A	V
Porcine vWF, Factor VIII					
Rat α-2 Macroglobulin	I	R	V	A	V
Human vWF	V	R	V	A	V

FIG. 9. *A*, region of human and mouse tenascin is compared with peptide 7N3 of the CBD of TS1. *Boxes* enclose identical residues. *B*, list of other extracellular proteins that contain sequences homologous to the IRVVM sequence of TS1.

and in bovine and rat COMP, positively charged residues are rearranged. Peptide 4N1, however, shows an extremely high degree of conservation in all species and isoforms of TS1 sequenced to date. In fact within the region of the shortest active peptide, 4N1-1, there are only two substitutions in all known sequences. This conservation of sequence supports the notion that this is a region of the TS1 CBD responsible for a critical function that is retained through evolution and in various TS1 isoforms.

The sequences 4N1-1, 7N3, and 7N3-1 were used as probe sequences in a computer search of all available data bases. No perfect matches were found for the 4N1-1 sequence or its shorter inhibitory form 4N1-2 (RFYVVM). Similarly, the sequence IRVVM appears to be unique to TS1 isoforms among extracellular proteins. However, searching for homologs of the 7N3 sequences revealed that human and mouse tenascin contain a related sequence as shown in Fig. 9. Since we have obtained evidence (above) that the LM sequence IKVAV can at least partially substitute for the TS1 sequence IRVVM, we searched for combinatorially interchanged sequences, such as IKVVM, IRVAV, and VRVAV. As shown in Fig. 9*B*, several interesting extracellular proteins contain related sequences including human and mouse LM, human and porcine von Willebrand factor, porcine factor VIII, and rat α_2-macroglobulin. These proteins have been singled out because they interact with cells through receptors, some of which have not yet been identified. It is interesting to speculate that the TS1 homologous sequences may represent occurrences of a similar receptor binding motif in these proteins.

DISCUSSION

By screening a series of overlapping subpeptides from C4 and C7 for their ability to support attachment of G361 melanoma cells and inhibit attachment of these cells to rCBD, we have pinpointed two sequences, RFYVVMWK (4N1-1) from C4 and IRVVM (7N3-1) from C7 which retain the activity of the parent 30-mer peptides and faithfully reflect the activity of the TS1 CBD. We had noted earlier that both C4 and C7 contain the sequence VVM, and that they competed for cell binding to one another suggesting that the two peptides bind to a common receptor. The two minimal active peptides revealed in this study both contain the VVM sequence in different contexts. This along with the fact that the C4 sequence is extremely well conserved in all species and isoforms of TS1 while the C7 sequence appears to be subject to more variability may indicate that these two act in concert. The C4 VVM site could provide the "core" recognition site while the C7 region could provide a fine tuning of specificity. In the case of the C7 peptides, the pentamer IRVVM (7N3-1) retains more than 70% of the maximal cell binding activity of the parent 30-mer C7, and this does not take into account possible differences in the plastic coating efficiency of the two peptides. In the extreme case, the inability of a short peptide to attach well to plastic might result in failure to identify an active subpeptide. To avoid this problem, we also tested all peptides as inhibitors of the attachment of cells to the rCBD. The two assays identified the same subpeptides as containing active sequences. For the C4 subpeptides, we identified the sequence RFYVVMWK (4N1-1) as the active region. It displays about 40% of the maximal activity of the parent 30-mer, with a substantial drop in activity occurring between peptides 4N1 and 4N1-1 in which the sequence QVTQS is deleted from the COOH terminus of 4N1. Two explanations can be offered for this observation. It may be that these residues contain a part of the structure necessary for high affinity recognition of the more VVM proximal residues. Or, alternatively, it has been established that the sequence VTCG, which occurs in the type 1 repeats of all TS1 isoforms, is a cell adhesive site. We have shown that G361 cells have receptors for and attach to peptides derived from this region of TS1 (Prater *et al.*, 1991) and that this interaction is partially inhibited by the peptide VTCG.[2] It may be that the VTQS sequence in peptide 4N1 has a weak interaction with these receptors and this contributes to the cell binding activity of the peptide. Loss of this sequence in 4N1-1 would then result in weaker cell attachment. This explanation would appear to be unlikely in that VTCG inhibits adhesion of these cells to the rCBD only weakly (10% or less inhibition). The existence of residual cell attachment in peptide 4N2 supports the first possibility. In a similar way, the YEGKK sequence (7N3-2), which is on the COOH-terminal end of 7N3, improved the inhibitory activity of 7N3-1 (not shown). These observations may explain the lower activity of 4N1-1 and 7N3-1 relative to their longer homologs sequences, 4N1 and 7N3, and indicate the importance of the surrounding amino acid sequences in enhancing either affinity or specificity of the interactions at these sites.

As reported earlier, mAb C6.7 inhibited cell attachment to the CBD (Kosfeld *et al.*, 1991). Recently, we have located the epitope for this mAb in peptide C7 and demonstrated its inhibitory effect on cell attachment to C7 (Kosfeld and Frazier, 1992). In this study, we have further localized the epitope of mAb C6.7 to the central region of C7 within 7N3 which includes the sequence IRVVM (data not shown). These results support this sequence as part of the active site of the CBD and further indicate that, even though the VVM sequence itself is hydrophobic, it is probably accessible on the surface of intact TS1 and thus available to cell surface receptors.

Because of the proximity and similar spectrum of activities of 4N1-1 and 7N3-1, it is likely that these sequences act in concert in the native TS1 molecule. This possibility was raised in a previous report (Kosfeld and Frazier, 1992) and is further supported in this study where the inhibitory effects of 7N3 and 4N1 or 7N3-1 and 4N1-1 were shown to be synergistic. The sequence VVM shared by 4N1-1 and 7N3-1 may be essential for the recognition of the CBD of TS1 by cell surface receptors. This situation would be strikingly reminiscent of the RGDS sequence of FN in which the critical residues RGD are contained in a number of sequence contexts in other proteins which may confer different specificities (Hynes, 1992). Furthermore, the occurrence of two versions of a related recognition sequence at nonadjacent sites in the linear sequence is also documented for the IIICS region of FN. This alternatively spliced type III repeat contains the sequence REDV (Humphries *et al.*, 1987) at its COOH terminus and the related recognition sequence LDV (Komoriya *et al.*, 1991) at its NH_2 terminus. These two peptides are separated by 77 residues of linear sequence. The RFYVVM and IRVVM sequences of TS1 are separated by 96 residues. Both of these sequences lie downstream of the RGD sequence of TS1 as do the LDV and REDV sequences of FN. In addition, Bowditch *et al.* (1991) have reported that a site which strengthens the binding of RGD to an integrin receptor lies at least 55 residues upstream of the RGD sequence. Thus this sort of tandem array of cell binding sites may not be an unusual phenomenon. It is difficult to visualize how two distinct sequences could occupy the same binding site on a receptor, thus leading to the speculation that the receptor may have more than one peptide binding domain. An even more attractive possibility is that receptor monomers may be engaged by the two peptide sites leading to aggregation of receptor monomers. Receptor aggregation or oligomerization is a well documented mode of transmembrane signaling (Ullrich and Schlessinger, 1990).

The use of peptides to map cell binding and other active sites is particularly appropriate in a situation like that of TS in which a number of isoforms exist and in which alternative splicing has been reported (Frazier, 1991). By identifying minimal peptide sequences which possess certain activities one can then simply ask if a particular sequence exists in other isoforms or species of TS1. In the case of the two sequences that we have identified in this study the answer to this question is a definite yes for the C4 peptides and a resounding maybe for the C7 sequence. The 4N1-1 sequence RFYVVMWK is conserved in all three isotypes of TS1 in humans and in mice. In chicken TS2 there is a single substitution of a leucine for a valine. In rat and bovine cartilage oligomeric matrix protein, a recently described TS isoform thought to be different from TS3 (Oldberg *et al.*, 1992; Bornstein, 1992), the arginine is replaced by serine, but the rest of the sequence is unaltered. The C7 sequence is less well conserved and seems to be divided into two regions of conservation. As seen in Fig. 8, the IRV is well conserved as is the YEG(K) sequence, but these are separated by two variable residues. This pattern of conservation supports the notion derived from the peptide inhibition studies that the YEGKK sequence (7N3-2) has a role in receptor binding. It is tempting to speculate that the greater divergence of the C7 site is related to fine tuning of specificity in different TS isoforms. For example, the VVM sequence occurs only in TS1 while all the TS2 isoforms have the sequence VLV at this position (Fig. 8).

Cell adhesive peptides from laminin, LMF9 and LMPA22-2, are similar to the sequences 4N1-1 and 7N3-1 of TS1, respectively. These TS1 and LM peptides were nearly inter-

[2] M. D. Kosfeld and W. A. Frazier, unpublished data.

changeable in inhibiting the adhesion of G361 cells to LM or TS1, confirming the activity of these sequences and suggesting a sharing of receptors by LM and TS1. These results imply a single recognition mechanism that cells may use for interacting with several adhesive proteins. This type of situation is known to exist where a single receptor such as the $\alpha_v\beta_3$ integrin binds several different protein ligands (Hynes, 1992). However, K562 cells bind to TS1, the rCBD, C4, and C7 peptides, but not to LM (Fig. 6, and data not shown). Thus the TS1 CBD receptor on these cells is not also a LM receptor. This binding was inhibited preferentially by the CBD peptides compared to the LM-derived peptides. There must, therefore, be a receptor specific for the CBD of TS1 which does not recognize LM. It is unclear, however, if all LM receptors which bind the IKVAV sequence of LM are also TS1 receptors. These results raise the possibility that related receptors may exist for the IKVAV sequence of LM and the VVM sequences of TS1.

In addition to LM, the peptide 7N3 of TS-1 has interesting similarities with other proteins including tenascin, von Willebrand factor, and other serum proteins (Fig. 9). The biological significance of the IRVVM-like sequences in these proteins is not clear at present (Tashiro *et al.*, 1989). Of these proteins, the glycoprotein tenascin is of most immediate interest because it shares many features with TS1. Both proteins serve as attachment factors for cells, but the attachment often occurs without spreading (Chiquet-Ehrismann *et al.*, 1988; Sage and Bornstein, 1991). Both have been called "anti-adhesive" (Lotz *et al.*, 1989; Murphy-Ullrich *et al.*, 1989; Spring *et al.*, 1989). Neither TS1 nor LM are widespread structural components of adult matrix (Erickson and Bourdon, 1989) but both proteins are highly but transiently expressed in embryonic morphogenesis (Aufderheide *et al.*, 1987; Chiquet-Ehrismann *et al.*, 1986), wound healing (Mackie *et al.*, 1988), and some cases of oncogenesis (Bourdon *et al.*, 1983; Mackie *et al.*, 1987; Chiquet-Ehrismann *et al.*, 1986; Castle *et al.*, 1991). In the case of tenascin, only an RGD sequence (Bourdon and Ruoslahti, 1989) and a chondroitin sulfate binding domain (Erickson and Bourdon, 1989) have been implicated as cell binding sites. We suggest that the region of homology with TS1 may be a candidate for another cell binding site in tenascin. If this is the case, the interaction between cells and sequences related to IRVVM might be a manifestation of a more widely distributed recognition mechanism in which the receptors for these sequences may be related to each other and the critical aspects of recognition conserved. In this regard it is interesting that several other extracellular proteins that bind to cells contain homologs of the IRVVM sequence (Fig. 9).

The identification of peptides IRVVM (7N3-1) and RFYVVM (4N1-2) as cell binding sites in the CBD of TS1 has several important implications. 1) The sequences, particularly RFYVVM, are highly conserved in all TS1 isoforms suggesting that the sequence VVM may form the "core" of a novel recognition motif. 2) TS1 may bind to a receptor that is related in some way to a LM receptor for the IKVAV (in LM peptide PA22-2) sequence of LM. 3) Homologs of the active TS1 sequence IRVVM have been found in other matrix and serum proteins suggesting additional roles for this sequence motif in other biological contexts. 4) The peptide sequences identified in this report will be instrumental as site specific reagents in the study of cell surface receptors of TS and in dissecting, in complex biological systems, those functions of TS1 which reside in the CBD.

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