Identification of Novel $\beta_1$ Integrin Binding Sites in the Type 1 and Type 2 Repeats of Thrombospondin-1*

Received for publication, June 4, 2004, and in revised form, August 3, 2004
Published, JBC Papers in Press, August 3, 2004, DOI 10.1074/jbc.M406267200

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In addition to the three known $\beta_1$ integrin recognition sites in the N-module of thrombospondin-1 (TSP1), we found that $\beta_1$ integrins mediate cell adhesion to the type 1 and type 2 repeats. The type 1 repeats of TSP1 differ from typical integrin ligands in that recognition is pan-$\beta_1$-specific. Adhesion of cells that express one dominant $\beta_1$ integrin on immobilized type 1 repeats is specifically inhibited by $\alpha_\beta_1$-specific antagonists, whereas adhesion of cells that express several $\beta_1$ integrins is partially inhibited by each $\alpha_\subunit$-specific antagonist and completely inhibited by combining the antagonists. $\beta_1$ integrins recognize both the second and third type 1 repeats, and each type 1 repeat shows pan-$\beta_1$ specificity and divalent cation dependence for promoting cell adhesion. Adhesion to the type 2 repeats is less sensitive to $\alpha$-subunit antagonists, but a $\beta_1$ blocking antibody and two disintegrins inhibit adhesion to immobilized type 2 repeats. $\beta_1$ integrin expression is necessary for cell adhesion to the type 1 or type 2 repeats, and $\beta_1$ integrins bind in a divalent cation-dependent manner to a type 1 repeat affinity column. The widely used TSP1 function blocking antibody A4.1 binds to a site in the third type 2 repeat. A4.1 proximally inhibits $\beta_1$ integrin-dependent adhesion to the type 2 repeats and indirectly inhibits integrin-dependent adhesion mediated by the TSP1 type 1 repeats. Although antibody A4.1 is also an antagonist of CD36 binding to TSP1, these data suggest that some biological activities of A4.1 result from antagonism of these novel $\beta_1$ integrin binding sites.

Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein that modulates cell adhesion, growth, motility, differentiation, and survival. TSP1 interacts with cells via a number of receptors, including several integrins. Locations of four integrin binding sites have been mapped in TSP1. The RGD sequence in the last type three repeat of TSP1 is a ligand for $\alpha_\gamma \beta_3$ and $\alpha_5 \beta_1$ integrins (1–3), although the exposure of this site in native TSP1 may be limited (4–7). At least three $\beta_1$ integrins recognize distinct sites in the N-terminal pentraxin-like domain of TSP1. $\alpha_\gamma \beta_1$ recognizes a sequence near the carboxyl end of the N-module containing the motif NVR (8), but a truncated recombinant N-module lacking this site retains binding to $\alpha_\gamma \beta_1$ and $\alpha_5 \beta_1$ (3, 9). Mutation of Glu (90) abolishes $\alpha_\gamma \beta_1$ but not $\alpha_5 \beta_1$ binding to this region of TSP1 (9). Binding to the latter integrin is at least partially mediated by an LDVP sequence (3).

Although three $\beta_1$ integrin binding sites in TSP1 have now been described, at least two publications have noted $\beta_1$ integrin-dependent activities of TSP1 that cannot be explained by these known sites. DeFreitas et al. (11) report that a TSP1 antibody (A4.1), which was believed to recognize the type 1 repeats (TSR, also known as properdin repeats) (10), inhibited $\alpha_\gamma \beta_1$-dependent neurite outgrowth on TSP1 (11). They further noted that a 50/70-kDa chymotryptic fragment of TSP1 containing the procollagen domain, the three TSRs, and part of the EGF-like type 2 repeats supported neurite outgrowth, which was completely inhibited by a $\beta_1$ antibody. Approximately 80% of this response was inhibited by $\alpha_5 \beta_1$-specific antibodies. Furthermore, adhesion of osteosarcoma cells to a similar 70-kDa core fragment of TSP1 produced by limited proteolysis with chymotrypsin in the absence of calcium was $\alpha_\gamma \beta_1$-dependent (12). Notably, none of these proteolytic fragments contain the N-module or the RGD sequence of TSP1.

These publications suggested that $\alpha_\gamma \beta_1$ and $\alpha_5 \beta_1$ recognize additional sites in the central stalk region of TSP1, possibly in the TSRs. Using recombinant regions of TSP1, we have now verified that activated $\alpha_\gamma \beta_1$ and $\alpha_5 \beta_1$ recognize secondary sites in this region of TSP1. We report here evidence for three such sites in the second and third TSRs and in the type 2 repeats of TSP1. However, we find that the specificity of these sites differs from those of most previously described integrin ligands in that they are pan-$\beta_1$-specific. We further show that a widely used TSP1 function blocking antibody, A4.1, inhibits $\beta_1$ integrin recognition of both repeats in TSP1.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—TSP1 was purified from human platelets (13). Recombinant proteins containing various domains of TSP1 or TSP2 (summarized in Fig. 1A) were prepared as described (14–16). Vitrogen type I collagen was from Cohesion Technologies (Palo Alto, CA).

Antibodies and Antagonists—A $\beta_1$ integrin function-blocking antibody (mAb13) and a fibronectin antibody (13G12) were provided by Dr. Ken Yamada (NIDCR, National Institutes of Health, Bethesda, MD) (17, 18). The $\beta_1$ integrin-activating antibody TS2/16 (19) and the anti-TSP1 antibody HB8432 were produced from hybridoma cell lines obtained from the American Type Culture Collection (Manassas, VA). The anti-$\alpha_5$ antibody 6D7 was provided by Dr. Harvey Gralnick (20). TSP1

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†† The abbreviations used are: TSP, thrombospondin; TSR, thrombospondin type 1 repeat; BSA, bovine serum albumin; EGF, epidermal growth factor; phLDVP, (4-(2-methylphenyl)aminocarbonyl)-aminophenyl)acetyl-LDVP; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; TBS, Tris-buffered saline.
antibody A4.1 was provided by Dr. Bill Frazier (Washington University School of Medicine, St. Louis, MO). TSP1 antibody 5G11 was obtained from Biodesign International (Saco, ME). The function-blocking integrin antibodies FB12 (anti-α1), P1B5 (anti-α1), P4C2 (anti-α1), P1D6 (anti-α3), and GoH3 (anti-αv) were obtained from Chemicon (Temecula, CA). The αvβ3 integrin antagonist SB229245 was provided by Dr. William Miller (GlaxoSmithKline) (22). The disintegrins VLO-5 (αvβ3, integrin-specific) and obtustatin (αvβ3, integrin-specific) were prepared as described (23, 24).

Cells—Human umbilical vein endothelial and human dermal microvascular cells were obtained from Clonetics BioWhittaker Inc. (Walkervill, MD). Human umbilical vein endothelial cells were maintained in M199 containing 20% fetal bovine serum, 2 mM glutamine, 80 μg/ml endothelial cell mitogen (Biomedical Technologies, Inc., Stoughton, MA), 10 μg/ml heparin, 50 units/ml penicillin, and 50 μg/ml streptomycin. Human mesangial cells (25) were maintained in Dulbecco’s modified Eagle’s medium supplemented with Glutamax (Invitrogen), 10% fetal bovine serum and 2 mM glutamine. All cell cultures were grown at 37 °C in 5% CO2. The breast carcinoma cell line MDA-MB-231 (American Type Culture Collection) was maintained in RPMI 1640 containing 10% fetal bovine serum.

Adhesion Assays—TSP1, recombinant proteins derived from TSP1 (summarized in Fig. 1A), or type 1 collagen was adsorbed (triplicates of 8–12 drops) onto polystyrene dishes (Falcon 1008) by incubating overnight at 4 °C. The dishes were removed, and the dishes were blocked with 1% BSA, Dulbecco’s phosphate-buffered saline for 30 min. Cells were dissociated with 2 mM EDTA in PBS and resuspended in RPMI, 0.1% BSA (Jurkat, MDA-MB-231 and mesangial cells) or M199, 0.1% BSA (human umbilical vein endothelial and human dermal microvascular cells) at 5 × 10^6 cells/ml. For activation cells were treated with 5–10 μg/ml TS2/16 antibody, 20 ng/ml PMA, or 0.1 mM MnCl2. For inhibition TSP1 antibodies A4.1 or HB8432, specific α-integrin-blocking antibody A4.1 was added at a concentration of 10−5 M. Jurkat T cells were also activated with 1 μg/ml TS2/16 antibody and to a lesser extent by phorbol ester. The latter treatment was further indicating that Jurkat cells do not express detectable levels of this receptor. Neither was fibronectin involved, because Jurkat cells do not express detectable levels of fibronectin (28), 1 integrin ligand fibronectin (28), 1 integrin-deficient mutant A1 (26) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. All cell cultures were grown at 37 °C in 5% CO2.

RESULTS

Three Domains of TSP1 Are Recognized by β1 Integrins—In addition to the three known β1 integrin binding sites in the N-module of TSP1 (3, 8, 9), a survey of cell adhesion to other recombinant regions of TSP1 in the absence or presence of a β1 integrin-activating antibody revealed that the type 1 and type 2 repeats contain β1 integrin-dependent adhesion sites (Fig. 1). Of several cell types examined, mesangial cells attached and spread most avidly to both the type 1 and type 2 repeats, which was enhanced after activation of β1 integrins using the antibody TS2/16 (Fig. 1B). At equimolar concentrations, NoC1 supported slightly less adhesion than intact TSP1, and three constructs containing all or only the third TSP supported adhesion at ~50% of this level. The type 2 repeats alone were weakly active at this concentration, whereas the C-terminal regions of TSP1 were inactive. Results were qualitatively similar using TS2/16-activated Jurkat T cells (Fig. 1C), although these cells do not spread due to their limited cytoplasmic volume. Dose-response curves showed that NoC1 is ~3-fold less active than intact TSP1 for mediating cell attachment, and CTSR123 is 13-fold less active. Endothelial cells also recognized the TSPs but did not adhere at significant levels on the type 2 repeats (Fig. 1, D and E, and results not shown). As noted for the other cell types, adhesion was stimulated by the β1 antibody TS2/16 (results not shown). Adhesion of endothelial cells on the third TSP (TSR3) was completely inhibited by a β1-specific blocking antibody mAb13 (Fig. 2A), indicating that the type 1 repeats are recognized by β1, but not by β2 or β3 integrins, which are also expressed on Jurkat cells (26). Activation-dependent adhesion was absent in the A1 somatic mutant lacking β1 (26), further indicating that β1 and β2 integrins do not recognize the TSPs. Therefore, β1 integrins are necessary for T cell adhesion on the TSPs.

Because the TSP1 TSPs are known to interact with CD36, which associates with at least two β1 integrins (27), and with the β1 integrin ligand fibronectin (28), β1 integrin-dependent adhesion to the TSPs could be indirect. However, detergent-solubilized β1 integrins bound to an affinity column of TSR123 and were specifically eluted by chelating divalent cations (Fig. 2B). Based on immunoprecipitation with the antibody TS2/16, the major proteins eluted under these conditions corresponded to β1 integrin with a molecular mass around 130 kDa, which in Jurkat cells associates primarily with α5 and αv subunits (140 and 135 kDa, respectively, under reducing conditions). Indirect interaction of integrins with the TSPs mediated by CD36 can be excluded because Jurkat cells do not express detectable levels of this receptor. Neither was fibronectin involved, be-
cause immunoprecipitation of biotinylated Jurkat cells using a specific fibronectin antibody did not detect this protein in the cell lysate (data not shown).

Phorbol ester treatment and TS2/16 similarly induced Jurkat T cell adhesion to E123, containing the three type 2 repeats of TSP1 (Fig. 2C). PMA-stimulated adhesion to the type 2 repeats was completely inhibited by the \( \beta_1 \) antibody mAb13, indicating specificity for \( \beta_1 \) integrins. The Jurkat A1 mutant was inactive, demonstrating that \( \beta_1 \) integrins are also necessary for adhesion of T cells on the EGF-like repeats of TSP1.

**TSP1 Type 1 Repeats Are pan-\( \beta_1 \) Integrin Ligands**—Several \( \alpha \beta_1 \) dimer-specific blocking antibodies and antagonists were tested to determine which \( \beta_1 \) integrins recognize the TSRs (Fig. 3). Remarkably, the \( \beta_1 \) integrin specificity for adhesion on TSP1 type 1 repeats was cell-type-specific. Jurkat cell adhesion on the type 1 repeats was partially inhibited by an \( \alpha \beta_1 \) integrin-antagonist, but \( \alpha_2 \), \( \alpha_5 \), and \( \alpha_6 \) inhibitors were inactive (Fig. 3A). The potent \( \alpha_4 \) antagonist philDVP inhibited adhesion on the type 1 repeats by \(~40\%\), whereas it inhibited adhesion on the N-terminal region of TSP1 by more than \( 80\%\), suggesting that additional \( \beta_1 \) integrins may contribute to T cell adhesion on the TSRs, although \( \alpha \beta_1 \) is the most abundant \( \beta_1 \) integrin expressed by Jurkat T cells (26). Notably, none of the specific \( \beta_1 \) integrin blocking antibodies or antagonists except mAb13 significantly inhibited activation-dependent Jurkat cell adhesion on the type 2 repeats (Fig. 3A).

In contrast to T cells, MDA-MB-231 breast carcinoma cell attachment on the TSRs was inhibited by \( \alpha_4\beta_1 \) and \( \alpha_2\beta_1 \) function-blocking antibodies but not by the \( \alpha_4\beta_1 \) antagonist (Fig. 3B). Neither \( \alpha_4\beta_1 \) nor \( \alpha_2\beta_1 \) antibodies completely inhibited MDA-MB-231 cell attachment. In the case of the \( \beta_1 \) antibody, this was not due to a limiting antibody concentration because cell attachment on type I collagen was inhibited to a much greater extent. Combining the \( \alpha_2 \) and \( \alpha_4 \) antibodies, however, produced a specific additive effect for adhesion on TSR123 but not on NoC1 or type I collagen, suggesting that both integrins recognize the TSRs. However, the \( \alpha_4 \) antagonist, which inhibited T cell adhesion to the same protein (Fig. 3A), was inactive and showed no additive activity with the \( \alpha_4\beta_1 \) antibody. Lack of activity of the \( \alpha_4 \) antagonist presumably is due to absence of \( \alpha_4 \) expression, because the \( \alpha_4 \) antagonist also had no effect on the known \( \alpha_4\beta_1 \) ligand NoC1, which was recognized exclusively by \( \alpha_4\beta_1 \) in the breast carcinoma cells (Fig. 3B). Based on these results, \( \alpha_4\beta_1 \) and \( \alpha_2\beta_1 \), but not \( \alpha_4\beta_1 \), are adhesion receptors in MDA-MB-231 cells for the TSP1 TSRs. Notably, \( \alpha_2\beta_1 \) and \( \alpha_3\beta_1 \) are the most abundant integrins expressed by MDA-MB-231 cells (29).

Mesangial cells exhibited a third phenotype (Fig. 3C). Antibodies to several \( \beta_1 \) integrins, including \( \alpha_3\beta_1 \), \( \alpha_4\beta_1 \), and \( \alpha_6\beta_1 \), partially inhibited mesangial cell spreading on the type 1 repeats of TSP1. Small molecule antagonists of \( \alpha_4\beta_1 \) and \( \alpha_3\beta_1 \) integrins were also partially inhibitory. However, the \( \beta_1 \) antibody completely inhibited spreading (Fig. 3C) and inhibited overall cell attachment by \(~50\%\) (results not...
shown). Consistent with this result and the partial inhibition by antagonizing individual integrins, additive effects were observed by combining the $\alpha_4\beta_1$ antibody and the $\alpha_v$ antagonist. Therefore, $\alpha_4\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_5\beta_1$, and $\alpha_v$ inhibitors all contribute to mesangial cell adhesion on the TSP1 TSRS.

Although apparently contradictory, the disparate data for these three cell lines can be rationalized by considering the relative expression levels of the various $\beta_1$ integrin in each cell line. Adhesion to the TSRS was inhibited by blocking the most
abundant $\beta_1$ integrins in cells that express a limited integrin repertoire, $\alpha_6\beta_1$ in Jurkat cells (26) and $\alpha_5\beta_1$ and $\alpha_6\beta_1$ in MDA-MB-231 cells (29, 30). Mesangial cells express a broader range of $\beta_1$ integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_8\beta_1$, and $\alpha_\beta_1$ (25, 31, 32), and most $\alpha$-specific antagonists affected adhesion of these cells to the TSRs. This suggests that the TSRs are pan-specific ligands for $\beta_1$ integrins.

Both TSR2 and TSR3 Are Recognized by $\beta_1$ Integrins—The above results could be explained either by a single promiscuous $\beta_1$ integrin binding site in the TSRs or by the presence of distinct binding sites for each integrin. To further localize the integrin binding site(s) in the TSRs of TSP1, recombinant forms of the second and third TSRs were tested for adhesion activity (Fig. 4). TS2/16 stimulated mesangial cell adhesion on both the second and third TSRs, although the third TSR was ~2-fold more active (Fig. 4A). However, these two TSRs alone were less active than a construct containing all three TSRs (TSR123) or intact TSP1. In the absence of activation, mesangial cells also showed significant dose-dependent adhesion to TSR2, TSR123, and intact TSP1. The absence of adhesion of unstimulated cells to TSR3 may be an artifact of the limited concentration of this fragment that could be tested or its relatively inefficient adsorption on polystyrene. Adhesion to the two individual TSRs exhibited the divalent cation dependence typical of integrin-mediated adhesion (Fig. 4B). The addition of 0.4 mM Mn$^{2+}$-stimulated adhesion and the addition of 10 mM EDTA abolished mesangial cell spreading and cell attachment on both TSR2 and TSR3. Spreading on TSR123 was also abolished by EDTA, but attachment was only partially inhibited by EDTA. Attachment on type I collagen that was even stronger than to TSR123 was completely inhibited by EDTA, suggesting that some mesangial cell attachment on the full-length type 1 repeats is divalent cation-independent.

To determine whether the apparent pan $\beta_1$ integrin specificity is intrinsic to each repeat or results from each TSR recognizing different $\beta_1$ integrins, we compared their activities to promote adhesion of T cells and breast carcinoma cells (Fig. 4, C–D). Adhesion of Jurkat T cells to TSR2 or TSR3 was inhibited strongly by blocking $\alpha_5\beta_1$ and weakly by blocking $\alpha_6\beta_1$ (Fig. 4C). Adhesion of MDA-MB-231 cells to TSR2 or TSR3 was inhibited partially by blocking $\alpha_5\beta_1$ or $\alpha_6\beta_1$ and completely by blocking both integrins (Fig. 4C). This additivity was not observed for adhesion of the same cells on NoC1, which MDA-MB-231 cells recognize exclusively via $\alpha_\beta_1$ (33). Based on the ability of both TSR2 and TSR3 to be recognized by at least three different $\beta_1$ integrins in a cell-specific manner, we conclude that at least the second and third TSRs of TSP1 have pan-specific $\beta_1$ integrin binding sites.

Several functional peptide sequences have been identified in the TSRs that interact with other known TSP1 ligands or receptors. To determine whether any of these known functional sequences contribute to integrin-mediated adhesion on the TSRs, we examined the effects of synthetic peptides on the adhesion of mesangial cells (Fig. 5). VTCGGSQKRSRL (peptide 245) is a CD36-binding peptide from the third TSP1 TSR that inhibits angiogenesis (34). VTCGSGDVT (peptide 205) and SPWSSCSVTCDGSSVTR (peptide 616) are CD36-binding peptides from the second TSR (34). KRKFQDGWHSVSPWSS (peptide 246) is a heparin-, transforming growth factor-$\beta$-, and fibronectin-binding peptide from the border of the first and second TSRs (28, 35, 36). GPWSPWDICSVT (pep-
tide 186) is a heparin-binding peptide from the third TSR (35). A related WSXW peptide from SCO-spondin was recently implicated in mediating $\alpha_\beta_1$ integrin-dependent neurite outgrowth (37). However, none of the TSR1 peptides containing this sequence or the CD36 binding sequences inhibited adhesion of TS2/16-activated cells alone or in the presence of 25 $\mu$m peptides derived from the indicated positions in the TSRs: heparin binding peptides KRFKQDGQGWSHSPWS (peptide 246) and GPWSWPDICSVT (peptide 186); CD36 binding peptides VTCGGGV-QKR (peptide 245), VTCGDVQVR (peptide 205), and SPWSWPDICSVT (peptide 616). Results are mean ± S.D., n = 3.

The Epitope for TSP1 Antibody A4.1 Is in the Third Type 2 Repeat—The TSP1 antibody A4.1 blocks several cellular responses to TSP1, and its epitope was initially mapped to the TSRs (10). However, A4.1 did not recognize CTSR123–I, which contains all three TSRs, on a Western blot (Fig. 6A, lane b). Rather, A4.1 recognized the EGF-like repeats and bound to all such constructs that contain the E3 module (lanes c–f). A4.1 did not bind to full-length recombinant TSP2 but bound to full-length murine TSP1 (lanes h and i).

Specificity of A4.1 for E3 of TSP1 was confirmed using a competitive enzyme-linked immunosorbent assay (Fig. 6, B and C). All constructs containing the E3 module equally inhibited A4.1 binding to human platelet TSP1, whereas the N-terminal region of TSR1, NoC1, and a region of TSR2 containing its EGF-like repeats (E123CaG2) were inactive. Therefore, A4.1 specifically recognizes an epitope in E3 of TSR1.

Inhibition of Integrin Recognition by TSP1 Antibody A4.1—Antibody A4.1 inhibits CD36-dependent motility responses of endothelial cells to TSP1, and this activity has been attributed to inhibiting recognition of the TSRs by CD36 (36–41). To determine whether A4.1 also inhibits recognition of the TSRs by $\beta_1$ integrins, we examined its effects on mesangial cell adhesion (Fig. 7). A4.1 inhibited mesangial cell adhesion only on constructs containing the type 2 repeats (Fig. 7A). A4.1 inhibited cell attachment on delNo and E123 and completely inhibited spreading on delNo and on E123 but not on TSR123 or NoC1. HB8432, a second TSP1 antibody that binds to the two most N-terminal type 2 repeats,² had a similar profile but was less inhibitory than A4.1 (Fig. 7A). The ability of A4.1 to completely block spreading on delNo implies that the antibody can sterically block integrin binding to the TSRs as well as proximally inhibit recognition of the type 2 repeats. To test this hypothesis we compared adhesion on the third TSR expressed alone or fused to the type 2 repeats (Fig. 7B). A4.1 dose-dependently inhibited mesangial cell adhesion on TSR3E123 but not on TSR3 or TSR123. The TSP1 antibody 5G11, which recognizes TSR3,² blocked adhesion on TSR3 and TSR3E123 but not E123, indicating that TSR3 contributes substantially to the adhesive activity of TSR3E123 (results not shown). The same conclusion is supported by activity of the $\alpha_4$ antagonist to inhibit Jurkat cell adhesion on TSR3E123 but not E123 (results not shown). Considering these results, the data presented in Fig. 7B indicate that A4.1 proximally blocks $\beta_1$-dependent adhesion to the type 2 repeats and also indirectly inhibits $\beta_1$-dependent adhesion to the TSRs.

Disintegrins Inhibit Adhesion on TSP1 Type 2 Repeats—Although adhesion on the type 2 repeats is resistant to most specific integrin antibodies and antagonists, it is sensitive to some disintegrins (Fig. 8). Mesangial cell adhesion to type 2 repeats was more resistant to individual $\beta_1$ integrin antagonists than was adhesion of the same cells on the type 1 repeats, but the disintegrins VLO-5 and obtustatin significantly inhibited spreading on E123 (Fig. 8A). At lower concentrations of obtustatin, additive effects were observed with an $\alpha_2\beta_1$ antibody (Fig. 8B). The activity of VLO-5 may not be due to its specific antagonism of $\alpha_4\beta_1$ in mesangial cells because the dose response was much broader than for inhibiting T cell adhesion to the known $\alpha_4\beta_1$ ligand NoC1 (Fig. 8C). Furthermore, Jurkat T cell attachment on E123 was completely resistant to VLO-5 inhibition, although attachment of the same cells on NoC1 was inhibited as expected. This suggests that $\alpha_4\beta_1$ does not mediate adhesion on E123 and that activity of the disintegrin VLO-5 for mesangial cells may be due to antagonism of other integrins, possibly including $\alpha_2\beta_1$ (24).

² D. S. Annis and D. F. Mosher, unpublished results.
DISCUSSION

Our data confirms prior indirect evidence for \( \beta_1 \) integrin-mediated adhesion to the type 2 repeats and directly inhibits \( \beta_1 \)-dependent adhesion to the type 1 repeats. However, we could not reproduce the reported specificity of these sites for individual \( \beta_1 \) integrins (11, 12). Rather, we find that pan-\( \beta_1 \) integrin recognition sites are present in the second and third TSRs and in the type 2 repeats of TSP1. Adhesion mediated by the TSRs is inhibitable by function-blocking antibodies specific for the major \( \alpha_\beta_1 \) dimers present in any given cell type as well as by small molecule antagonists of the respective integrins. Although we could not test all of the known \( \beta_1 \) integrins, at least seven of these recognize the TSRs. This suggests that the TSP1 TSRs are pan-specific ligands for \( \beta_1 \) integrins. In contrast, no \( \alpha_\beta_1 \)-specific antagonists have been identified that strongly block adhesion to the type 2 repeats, although \( \beta_1 \) is necessary, and the latter activity is sensitive to a \( \beta_1 \) blocking antibody and some disintegrins. Thus, the type 1 and type 2 repeats of TSP1 contains additional cell adhesion sites, and their distinct interactions with \( \beta_1 \) integrins may play significant but previously unrecognized roles in the many biological activities that have been mapped to these domains of TSP1.

Although we cannot strictly exclude binding to \( \beta_1 \) integrin-associated molecules, our data strongly support a direct inter-

FIG. 6. **A4.1 recognizes EGF-like module 3 (E3).** A, approximate mol eq (10 pmol) of TSP1 and TSP1-derived proteins were resolved on a 4–20% SDS-PAGE gradient gel under non-reducing conditions. The proteins were subsequently transferred to polyvinylidene difluoride membrane. A4.1-positive bands were detected by peroxidase conjugated antimouse \( \gamma \)-chain-specific antibodies and enhanced chemiluminescence technology. Lanes with no reactivity even upon overexposure of the membrane to film are marked at the top of the gel: lane a, NoC1; lane b, CTSR123-1; lane c, E123-1; lane d, E3; lane e, E3CaG-1; lane f, delNo-1. These proteins were produced using the pAcGP67.coco (coco) baculovirus system and contain six histidine tags. Human TSP1 (g), hTSP-2 (h), and mTSP-1 (i) generated in baculovirus do not include His tags. B, competitive enzyme-linked immunosorbent assay for A4.1 binding to immobilized platelet TSP1. The indicated concentrations of E3-1, E123-1, E3CaG1, E123CaG1, and E123CaG2 were used. C, competitive enzyme-linked immunosorbent assay as in B using NoC1 (•), TSR3E123-1 (△), or E3CaG1 (○).

FIG. 7. TSP1 antibody A4.1 proximally inhibits \( \beta_1 \)-integrin-dependent adhesion to the type 2 repeats and indirectly inhibits \( \beta_1 \)-dependent adhesion to the type 1 repeats. A, A4.1 inhibits mesangial cell adhesion only on constructs containing the type 2 repeats. Mesangial cell adhesion was determined on substrates coated using 10 \( \mu \)g/ml NoC1, 12 \( \mu \)g/ml delNo, 15 \( \mu \)g/ml TSR123, or 15 \( \mu \)g/ml E123. TSP1 antibodies A4.1 and HB8432 were used at 10 \( \mu \)g/ml to inhibit adhesion of TS2/16-activated cells. Results are presented as the mean ± S.D. normalized to TS2/16-stimulated adhesion on each substrate as 100% (NoC1 = 145 ± 14 cells/mm², delNo = 131 ± 7, TSR123 = 114 ± 8, E123 = 117 ± 11). B, dose dependence for inhibition by A4.1 of TS2/16-activated mesangial cell adhesion on TSR3E123, TSR123, or TSR3. All proteins were coated at 1 \( \mu \)M.
TSRs or type 2 repeats is limited to $\beta_1$ integrins. $\beta_1$-deficient Jurkat cells express $\beta_2$ and $\beta_3$ integrins, and these are activated by phorbol esters, but we found no PMA-stimulated adhesion of these cells to TSRs. Therefore, $\beta_2$ and $\beta_3$ integrins do not recognize the TSRs. Recognition of the TSRs by integrins containing other $\beta$-subunits has not been examined.

Although our data indicate that $\beta_1$ integrins directly recognize the TSP1 TSRs, several indirect mechanisms were also considered. CD36 also binds to the type 1 repeats (42, 43). Furthermore, CD36 associates with two of the integrins that mediate adhesion to the type 1 repeats, $\alpha_v\beta_1$ and $\alpha_v\beta_3$ (27). However, binding of the type 1 repeats to CD36 seems unlikely to explain our observed pan-$\beta_1$ specificity because CD36 does not associate with several of the integrins implicated by our data, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_7$ (27). Some of the cells that adhere on the type 1 repeats also lack CD36 expression. In addition, several CD36-binding peptides from TSP1 did not inhibit adhesion of cells on immobilized TSRs. In some cases, biological activities of A4.1 were inferred from its blocking CD36 binding to the TSRs (38–41). However, the relevance of A4.1 blocking to CD36 function may need to be reevaluated based on our evidence that A4.1 also blocks recognition of the TSRs by $\beta_1$ integrins and our new data mapping the A4.1 epitope to the third type 2 repeat rather than the TSRs.

We also considered heparan sulfate proteoglycans and fibronectin as potential indirect ligands. However, fibronectin was not present at detectable concentrations in our adhesion assays or the affinity purification experiment. Furthermore, fibronectin should preferentially be recognized by $\alpha_v\beta_1$, antagonists of which were only minimally effective to block adhesion to the TSRs. Although a heparin binding sequence from the TSRs stimulates $\alpha_v\beta_1$-dependent melanoma cell adhesion (2), this peptide had no effect on $\beta_1$-dependent adhesion to immobilized TSRs.

Our data are consistent with prior observations that the TSRs contribute to attachment of G361 melanoma cells (44), which do not express the other known receptor for this region, CD36 (2). Further indirect evidence for such receptor recognition of the TSRs comes from many observations that the TSP1 antibody A4.1 inhibits various cellular responses to TSP1 (10). A4.1 inhibits adhesion of G361 (10), MG-63 (44, 45), HEL (46), keratinocyte and intestinal smooth muscle cells (44), and C2C12 myoblasts (47). A4.1 also inhibits neurite outgrowth (11, 48), macrophage phagocytosis of apoptotic fibroblasts (40), the mitogenic activity of TSP1 and TSP1-stimulated cdk2 activity for vascular smooth muscle cells (49), the antiangiogenic activities of TSP1 in vitro and in vivo (38, 41, 50–53), and TSP1-induced endothelial cell apoptosis (39). The role of $\beta_1$ integrins in each of these interactions should be reexamined.

Most integrin ligands show relatively high specificity for one or a few integrins, but at least three ligands were previously known to mediate general $\beta_1$-dependent adhesion. Invasin mediates internalization of Yersinia pseudotuberculosis and is a well characterized promiscuous $\beta_1$ integrin ligand (54). Specific residues in the $\beta_1$ subunit are required for invasin binding (55). The disintegrin domains of ADAMs-2 and -3 were also shown to have broad binding specificity for $\beta_1$ integrins (56). Finally, cell adhesion to several fibronectin type 3 repeats that lack the RGD sequence could be stimulated by TS2/16 or by PMA and was sensitive to $\beta_1$ blocking antibodies but not to any $\alpha$-specific blocking antibodies tested (57). These results resemble adhesion on the type 2 repeats of TSP1, which can be blocked only by disintegrins or $\beta_1$ blocking antibody but contrasts with adhesion to the TSP1 TSRs, which is sensitive to inhibition by

**Fig. 8. Adhesion on the type 2 repeats is resistant to most specific integrin antagonists but sensitive to some disintegrins.** A, mesangial cell spreading on TSP1 type 2 repeats is more resistant to individual $\alpha_v\beta_1$ integrin antagonists (antag) that spreading on the type 1 repeats but is inhibited by two disintegrins. The indicated integrin function-blocking antibodies were used at 5 $\mu$g/ml. $\alpha_v$ and $\alpha_\gamma$ antagonists, VLO-5, and obtustatin were used at 1 $\mu$g/ml. Results are expressed as percentage of TS2/16 control (39 ± 5 and 121 ± 7 cells on TSR123 and E123, respectively). B, additive effects of combined obtustatin (330 nM) and anti-$\alpha_\gamma$ integrin antibody 6D7 (5 $\mu$g/ml). Results are expressed as percentage of TS2/16 control (35 ± 1 and 58 ± 2 cells on TSR123 and E123, respectively). C, dose-dependent activity of the $\alpha_v\alpha_\gamma$ disintegrin VLO-5 for inhibiting attachment of T cells on NoC1 (○), mediated by $\alpha_v\beta_3$ or E123 (△) or mesangial cells on E123 (▲).
α-subunit-specific antibodies and small molecule antagonists. These results suggest that the fibronectin type 3 repeats and the TSP1 type 2 repeats may be recognized autonomously by the activated β1 subunit, whereas the TSRs must interact with sites that are close to the known ligand binding sites at the αβ interface that interact with small molecule integrin antagonists (58). The mechanisms by which β1 integrins recognize the TSRs and type 2 repeats of TSP1 remain to be determined.

We have shown that both the second and third TSP1 TSRs are β1 integrin ligands. We have not determined whether the first repeat is also active. Preliminary evidence indicates that TSRs from TSP2 have similar integrin recognition sites. Some evidence suggests that integrin recognition is a more general property of TSRs. SCO-spondin contains TSRs, which mediate neurite outgrowth in a β1 integrin-dependent manner (37). Notably, these authors reported specificity of a peptide containing their SCO-spondin motif for αβ1 and found no significant inhibition using αβ2, αβ3, αβ5, or αβ6 antibodies. However, the observed integrin specificity should be verified using an intact recombinant TSR rather than the synthetic peptide. The observed integrin specificity should be verified using an intact recombinant TSR rather than the synthetic peptide.

EGF-like repeats are also found in many proteins, but their role as integrin ligands is poorly understood. A 39-amino acid β1 integrin binding sequence was identified in an EGF-like repeat of entactin/nidogen (60). Milk fat globule-EGF-factor 8 contains an RGD motif in its second EGF repeat that is recognized by αβ1, αβ2 (61) as does developmental endothelial locus 1 (62). The EGF-like type 2 repeats of TSP1 do not contain any known integrin binding motifs.

In summary, we have identified three additional β1 integrin recognition sites on TSP1. These are less active than the three specific sites previously defined in the N-module of TSP1 but recognize a broader range of β1 integrins. Therefore, cells lacking the integrins that bind to the N-module may still interact with TSP1 via β1 integrins. The mechanisms for integrin recognition of the TSRs and EGF-like repeats and the potential for the unique glycosylation of Trp and conserved Ser/Thr residues in TSRs (63) to modulate this integrin binding remain to be explored.

Acknowledgments—We thank Drs. Yoji Shimizu, William Miller, William Frazier, Christian Hugo, and Ken Yamada for providing reagents.

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Identification of Novel β₁ Integrin Binding Sites in the Type 1 and Type 2 Repeats of Thrombospondin-1

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J. Biol. Chem. 2004, 279:41734-41743.
doi: 10.1074/jbc.M406267200 originally published online August 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406267200

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