Cell-Type Specific Adhesive Interactions of Skeletal Myoblasts with Thrombospondin-1

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Thrombospondin-1 (TSP-1) is an extracellular matrix glycoprotein that may play important roles in the morphogenesis and repair of skeletal muscle. To begin to explore the role of thrombospondin-1 in this tissue, we have examined the interactions of three rodent skeletal muscle cell lines, C2C12, G8, and H9c2, with platelet TSP-1. The cells secrete thrombospondin and incorporate it into the cell layer in a distribution distinct from that of fibronectin. Myoblasts attach and spread on fibronectin- or thrombospondin-coated substrates with similar time and concentration dependencies. Whereas cells adherent on fibronectin organize actin stress fibers, cells adherent on TSP-1 display prominent membrane ruffles and lamellae that contain radial actin microspikes. Attachment to thrombospondin-1 or the 140-kDa tryptic fragment is mediated by interactions with the type 1 repeats and the carboxy-terminal globular domain. Attachment is not inhibited by heparin, GRGDSP peptide, or VTCT peptide but is inhibited by chondroitin sulphate A. Integrins of the $\beta 1$ or αv subgroups do not appear to be involved in myoblast attachment to TSP-1; instead, this process depends in part on cell surface chondroitin sulphate proteoglycans. Whereas the central 70-kDa chymotryptic fragment of TSP-1 does not support myoblast attachment, the carboxy-terminal domain of TSP-1 expressed as a fusion protein in the bacterial expression vector, pGEX, supported myoblast attachment to 30% the level of intact TSP-1. Thrombospondin-4 (TSP-4) is also present in skeletal muscle and a fusion protein containing the carboxy-terminal domain of TSP-4 also supported myoblast adhesion, although this protein was less active on a molar basis than the TSP-1 fusion protein. Thus, the carboxy-terminal domain of TSP-1 appears to contain a primary attachment site for myoblasts, and this activity is present in a second member of the thrombospondin family.

INTRODUCTION

The thrombospondins form a gene family of structurally related extracellular glycoproteins. The five family members identified to date have been designated thrombospondin-1 (TSP-1)¹ (Lawler and Hynes, 1986), thrombospondin-2 (TSP-2) (Bornstein *et al.*, 1991; LaBell *et al.*, 1992), thrombospondin-3 (TSP-3) (Vos *et al.*, 1992), thrombospondin-4 (TSP-4) (Lawler *et al.*, 1993b), and cartilage oligomeric matrix protein (Oldberg *et al.*, 1992; see also reviews by Bornstein, 1992; Adams and Lawler, 1993a). TSP-1 is a homotrimeric protein, each

subunit of which consists of globular amino- and carboxy-terminal domains, a domain with homology to procollagen, and three types of repeated sequences, termed the type 1, type 2, and type 3 repeats (Lawler and Hynes, 1986). This structural organization is shared by TSP-2, whereas TSP-3, TSP-4, and COMP each have distinct amino-terminal domains and lack the procollagen homology domain and type 1 repeats. These three proteins thus form a separate subgroup of the family (Adams and Lawler, 1993a; Lawler *et al.*, 1993a).

TSP-1 was first isolated from platelet α -granules (Lawler *et al.*, 1978), but it subsequently became apparent that protein(s) immunologically related to TSP-1 were expressed in the extracellular matrices and basement membranes of many developing or repairing

¹ Abbreviations used: TBS, Tris-buffered saline; TSP-1, thrombospondin-1; TSP-2, thrombospondin-2; TSP-4, thrombospondin-4.

tissues (Wight *et al.*, 1985; Raugi *et al.*, 1987; O'Shea and Dixit, 1988; Watkins *et al.*, 1990; Corless *et al.*, 1992). One tissue for which this has been thoroughly documented is skeletal muscle. In mouse embryos, thrombospondin protein is present within the forming somites from embryonic day 8.5, being located on the surface of undifferentiated myoblasts and, at lower levels, on the surface of myotubes (O'Shea and Dixit, 1988; Corless *et al.*, 1992). Expression decreases toward birth but increases again in the endomysium of murine muscle during postnatal development (O'Shea and Dixit, 1988; Hantai *et al.*, 1991). Thrombospondin is present at low levels within the endomysium of adult human muscle (Wight *et al.*, 1985; Rao *et al.*, 1992), and its expression is upregulated after crush injury (Watkins *et al.*, 1990). Thrombospondin protein is also upregulated in the endomysium of patients suffering from amyotrophic lateral sclerosis, a fatal neuromuscular disorder (Rao *et al.*, 1992).

The presence of thrombospondin protein in the extracellular matrix of skeletal muscle thus correlates with episodes of myoblast proliferation and migration. These observations are in accordance with the general hypothesis that extracellular TSP-1 serves as an anti-adhesive and growth-regulatory matrix component in situations of tissue remodelling (Sage and Bornstein, 1991). Northern blotting and in situ hybridization studies have now identified TSP-1 and TSP-4 as the predominant thrombospondins in skeletal muscle (Bornstein *et al.*, 1991; Laherty *et al.*, 1992; Oldberg *et al.*, 1992; Lawler *et al.*, 1993b; Iruela-Arispe *et al.*, 1993; Tucker, 1993).

To advance our understanding of the role of thrombospondins in skeletal muscle, we have examined the interactions of skeletal myoblasts with platelet TSP-1 and a recombinant domain of TSP-4. The TSP-1 molecule contains multiple cell-binding sites, including heparin- and sulphatide-binding sites within the amino terminal globular domain and type 1 repeats (Roberts, 1988; Holt *et al.*, 1990; Asch *et al.*, 1991; Guo *et al.*, 1992a,b; Lawler *et al.*, 1992), the sequence CSVTCG that occurs twice within the type 1 repeats, (Prater *et al.*, 1991; Tuszynski *et al.*, 1992; 1993) and an RGD sequence located in the last type 3 repeat (Lawler *et al.*, 1988). The carboxy-terminal globular domain also possesses adhesive activity for platelets and melanoma cells (Roberts *et al.*, 1987, 1988; Kosfeld *et al.*, 1991; Kosfeld and Frazier, 1992, 1993). How these sites work together to support cell adhesion is not well understood at present, and few studies have systematically examined the mechanisms of attachment used by appropriate nontransformed cell types. Our results also provide the first evidence that TSP-4 can support cell adhesion and are discussed in the context of current ideas concerning the mechanisms of cell adhesion to TSP-1.

MATERIALS AND METHODS

Materials

TSP-1 was prepared from human platelets as previously described (Lawler *et al.*, 1985) with the modification of passing material eluted from the heparin-Sepharose column over an anti-vitronectin antibody column. The flow-through from this column was free of vitronectin, as measured by Western blotting. The integrity of the TSP-1 preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). GRGDSP or GRGESD synthetic peptides were obtained from Telios Pharmaceuticals (San Diego, CA). ³⁵S-methionine (specific activity 10 mCi/ml) was from Amersham (Arlington Heights, IL). VTCG and GVCT synthetic peptides were prepared by Dr. C. Dahl, Harvard Medical School (Boston, MA). Peptide purity was 80%, measured by analytical high-performance liquid chromatography. Chondroitin sulphates A, B, and C, heparin, heparan sulphate, fucoidan, dextran sulphates molecular weight (MW) 5000 and 500 000, o-nitrophenol β -D-xylopyranoside, p-nitrophenol β -D-xylopyranoside, and heparinase II were obtained from Sigma (St. Louis, MO). Chondroitin sulphate A was also obtained from Calbiochem (San Diego, CA). Chondroitinase ABC was obtained from Seikagaku America (Rockville, MD).

Antibodies

A rabbit polyclonal antiserum to human platelet thrombospondin was raised in the laboratory. Monoclonal antibodies to thrombospondin included mouse antibodies MAI and MA II (Lawler *et al.*, 1985), MA III and MA IV (Lawler, unpublished data), antibodies ESTs 10 and ESTs12 (1.2) (the generous gifts of Dr. N.R. Hunter of the Scottish National Blood Transfusion Service, National Science Laboratory, Edinburgh, UK), antibody 4.1 (Prater *et al.*, 1991) and antibody C6.7 (Dixit *et al.*, 1985) (the gifts of Dr. W. Frazier, Washington University School of Medicine, St. Louis, MO), and the rat antibody 5G11 (Legrand *et al.*, 1988) (Amac, Westbrook, ME). Polyclonal antisera directed against human fibronectin, human fibronectin receptor, and human vitronectin receptor were obtained from Telios Pharmaceuticals.

Cell Lines

HT1080 human fibrosarcoma cells, human intestinal smooth muscle cells (Graham *et al.*, 1984), G361 human melanoma cells, C2C12 mouse skeletal myoblasts (Blau *et al.*, 1985), G8 mouse skeletal myoblasts (Christian *et al.*, 1977), and H9c2 rat embryonic myoblasts (Kimes and Brandt, 1976) were obtained from the American Type Culture Collection (Rockville, MD). Intestinal smooth muscle cells, G361 cells, and H9c2 cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS) (Hyclone Labs, Logan, Utah); C2C12 and G8 cells were maintained in DMEM containing 20% FCS, and HT1080 cells were maintained in MEM containing 10% FCS. All cells were kept at 37°C in a humidified atmosphere containing 5% CO₂.

Metabolic Labeling and Immunoprecipitation

Cells were plated at 8×10^5 to 10×10^5 cells per 60-mm tissue culture dish in serum-containing medium and allowed to attach for 5 h. The medium was then changed to DMEM containing 5% dialyzed FCS and 50 μ Ci/ml ³⁵S-methionine. After overnight incubation (18–20 h), the medium was collected, clarified by centrifugation, and phenylmethylsulfonyl fluoride added to 2 mM. Aliquots of medium equalized on the basis of trichloroacetic acid (TCA)-precipitable counts (typically 3×10^6 cpm per precipitation) were precleared by incubation with 2 μ l of rabbit nonimmune serum and 30 μ l Protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) used as a 1:1 (wt/wt) suspension for 1 h on ice. The supernatants were incubated with non-immune rabbit serum or a rabbit polyclonal serum to human platelet TSP-1 for 2 h on ice then mixed end-over-end at 4°C for 45 min with 40 μ l of Protein A-Sepharose. Beads and associated immune complexes

were washed as previously described (Adams and Watt, 1988), suspended in SDS-PAGE sample buffer containing 100 mM dithiothreitol, boiled, and resolved on 7.5% polyacrylamide gels according to the method of Laemmli (1970). Gels were fixed and stained then treated with Amplify fluorographic reagent (Amersham). Dried gels were exposed to Kodak (Rochester, NY) XAR-5 X-ray film at -70°C .

Indirect Immunofluorescence

For staining with anti-thrombospondin or anti-fibronectin antibodies, cells were plated on glass coverslips, grown for 24 h, and fixed in 3.7% formaldehyde in tris(hydroxymethyl) aminomethane (Tris)-buffered saline (TBS). Cells were incubated with primary antibody for 1 h, washed extensively in TBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (ICN Biomedicals, Irvine, CA) for 45 min. For staining with TRITC (Texas Red isothiocyanate)-phalloidin (Sigma Chemical, St. Louis, MO), cells were allowed to attach for 2 h at 37°C to glass coverslips coated with 50 nM thrombospondin or 50 nM rat plasma fibronectin (Telios) and then fixed in 3.7% formaldehyde for 10 min, followed by 10 min in 50 mM MES pH 6.1, 5 mM MgCl_2 , 3 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid, 100 mM KCl, and 0.2% Triton X-100 (O'Neill *et al.*, 1990). The permeabilized cells were incubated with TRITC-phalloidin for 1 h at room temperature. After further washing, all preparations were mounted in Vectastain mounting medium (Vector Laboratories, Peterborough, UK) and examined under epifluorescence using a Nikon Microphot-FXA (Garden City, NY) microscope.

Cell Attachment Assay

Thrombospondin was diluted in TBS containing 2 mM CaCl_2 and allowed to adsorb to bacteriological plastic 96-well trays (Flow 76-232-05) at 4°C overnight. Wells were then washed and blocked with 1 mg/ml heat-denatured bovine serum albumin (BSA) for 1 h at room temperature. Cells were trypsinized from stock cultures, using 0.01% trypsin in versene, washed once in DMEM containing 10% FCS to inactivate the trypsin, washed twice in serum-free DMEM, and plated at 10^4 cells/16 mm well in a final volume of 100 μl . This procedure allowed a recovery period of ~ 25 min. In experiments involving anti-thrombospondin antibodies, the antibodies were incubated with the blocked wells for 2 h at room temperature, then the wells were washed with TBS, and the cells added. Other putative competitors were added to the wells at the same time as the cells. Attachment was carried out at 37°C , typically for 90 min or 120 min. Nonadherent cells were removed by washing with TBS containing 2 mM CaCl_2 ; adherent cells were fixed in 3.7% formaldehyde and stained with Giemsa for direct observation. To quantitate adhesion, stained cells were solubilized in 0.2% SDS and the absorbance at 650 nm read in a Biorad model 3550 (Richmond, CA) microplate reader (Plantefaber and Hynes, 1989).

Proteolytic Digests of Thrombospondin

Five-microgram aliquots of platelet thrombospondin in TBS (15 mM Tris-HCl pH 7.6, 140 mM NaCl, 2 mM CaCl_2) were digested with chymotrypsin or trypsin (Worthington, Freehold, NJ) on ice for 16 to 20 h in the presence or absence of 5 mM EDTA. Digests were carried out at enzyme:substrate ratios of 1:10 000, 1:1000, 1:100, or 1:10 (wt/wt). For cell adhesion assays, digests were ended and the enzyme removed by addition of soybean trypsin inhibitor coupled-agarose (Pierce, Rockford, IL). Either the total digestion products were coated onto plastic, or the 140 kDa tryptic fragment, the 70-kDa chymotryptic fragment, and the 25-kDa chymotryptic fragment were purified as previously described (Adams and Lawler, 1993b). Material used in adhesion assays was routinely analyzed by SDS-PAGE.

Production of Glutathione S-Transferase (GST) Fusion Proteins

The carboxy-terminal domains of human TSP-1 and human TSP-4 were expressed as fusion proteins with GST, using the pGEX vector (Pharmacia). In preliminary experiments, we found that the stability of the TSP-1 fusion protein was greatly improved if the last segment of the type 3 repeats was included with the carboxy-terminus. The TSP-1 fusion protein therefore contained amino acid residues 877–1152 of human TSP-1 (Lawler and Hynes, 1986). In this protein, the RGD site was rendered nonfunctional by a point mutation that converted the amino acid sequence to IGD. Site-directed mutagenesis was carried out by standard procedures, using an M13 mutagenesis kit (Amersham). The TSP-4 fusion protein contained amino acid residues 713–931 of human TSP-4 (Lawler *et al.*, 1993a). Expression and purification of the fusion proteins and of unfused GST were carried out according to standard procedures (Smith and Johnson, 1988). Briefly, bacterial cultures were grown to an optical density at 600 nm of 0.6–0.8, and fusion protein expression was induced by addition of isopropyl β -thiogalactopyranoside to 1 mM for 1 h. The bacteria were pelleted by centrifugation at 4200 rpm for 15 min in a Sorvall RC-3B centrifuge (Newton, CT) and resuspended in 10 ml phosphate-buffered saline. The pellets were lysed by sonication at 4°C . Triton X-100 was added to 1% and the insoluble fraction pelleted by centrifugation for 5 min at $10\ 000 \times g$. The supernatant was mixed end-over-end with 2 ml of 50% (vol/vol) glutathione-agarose beads (Sigma Chemical) for 20 min at 4°C . The beads were washed three times in TBS, packed into a column, and eluted with 10 mM glutathione (Sigma). One-milliliter fractions were collected and their protein content measured by Bradford assay (Bradford 1976) using the Bio-Rad kit. Peak fractions were pooled, treated with 8 M urea and 100 mM 2-mercaptoethanol to denature and refold the proteins (Kohn *et al.*, 1990), and dialyzed for 30 h at 4°C against TBS. The purity of the recombinant proteins was confirmed by SDS-PAGE and the purified material was stored at -80°C .

RESULTS

Muscle Cell Lines Secrete Thrombospondin and Incorporate It into the Extracellular Matrix

Subconfluent cultures of C2C12 or H9c2 myoblasts were metabolically labeled with ^{35}S -methionine and immunoprecipitated with a polyclonal antiserum raised against human platelet TSP-1. A polypeptide of apparent molecular weight 185 kDa, which comigrated

Figure 1. Myoblast cell lines synthesize and secrete thrombospondin. C2C12 (lanes 1 and 2) and H9c2 (lanes 3 and 4) cultures were labeled overnight with ^{35}S -methionine. Aliquots of media were equalized on the basis of TCA-precipitable counts and immunoprecipitated using rabbit nonimmune serum (lanes 1 and 3) or rabbit serum to thrombospondin (lanes 2 and 4). Reduced samples were resolved on 7.5% polyacrylamide gels. The arrowhead indicates the migration position of platelet thrombospondin. Molecular weights of protein standards are given in kDa.

Figure 2. Localization of fibronectin and thrombospondin in myoblast cultures. H9c2 myoblasts were grown for 24 h, fixed in 3.7% formaldehyde, and stained with antibodies to fibronectin (a), and thrombospondin (b). Bar, 50 μ m.

with platelet TSP-1, was specifically precipitated by the antiserum from the conditioned media of both cell lines (Figure 1). A 140-kDa, proteolysed form of thrombospondin is found in the conditioned medium of BHK21 cells (Good *et al.*, 1990); however, a protein of this molecular weight was not detected in the immunoprecipitates made from muscle cell-conditioned media. These results indicate that myoblasts in culture synthesize and secrete thrombospondin.

To determine if thrombospondin becomes incorporated into the myoblast extracellular matrix, the same polyclonal serum to platelet TSP-1 was used to stain fixed but not permeabilized H9c2 cells after 24 h in culture. For comparison, the cells were also stained with an antibody to fibronectin. Fibronectin was distributed in long streaks and arrays of fibrils around the margins of the cells, and all cells stained positively for fibronectin (Figure 2a). In contrast, thrombospondin was localized in discrete patches on the apical surfaces of cells, which did not coincide with the fibronectin-containing fibrils (Figure 2b). About 80% of the cells stained positively for thrombospondin. Thus, thrombospondin becomes incorporated into the extracellular layer but does not codistribute with fibronectin.

Myoblasts Adhere to TSP-1

The ability of the myoblasts to adhere to substrates coated with platelet TSP-1 was examined under serum-

free conditions. C2C12, G8, and H9c2 myoblasts all attached and spread extensively on thrombospondin-coated substrates (shown for C2C12 and H9c2) (Figure 3, a and b). This response was in marked contrast to that of other cell types such as HT1080 cells or human epidermal keratinocytes, which attached to TSP-1 but did not spread (Figure 3c) (see also Adams and Lawler, 1993b). This cell-type specificity of response was obtained when the same batch of platelet TSP-1 was used

Figure 3. Morphology of cells attached to thrombospondin. C2C12 cells (a), H9c2 cells (b), and HT1080 cells (c) were allowed to attach to plastic surfaces coated with 30 μ g/ml of thrombospondin for 2 h at 37°C. Adherent cells were washed, fixed, and stained with Giemsa. Bar, 100 μ m.

Figure 4. Comparison of myoblast adhesion to thrombospondin and fibronectin. (a and b) Concentration dependence. (c and d) Time dependence. C2C12 (a and c) or H9c2 (b and d) cells were plated into wells coated either with thrombospondin (open symbols) or fibronectin (filled symbols). In a and b, the adhesion time was 2 h; in c and d wells were coated with 50 nM thrombospondin or fibronectin. Each point is the mean of duplicate experiments. Bars indicate SEM.

in parallel assays of the three cell types and was reproducible between different batches of TSP-1. For these assays, single cell suspensions were prepared by trypsin/EDTA treatment; however, cell suspensions prepared by EDTA treatment alone showed the same responses to TSP-1. Because the formation of cell aggregates during the assay was a problem under these conditions, particularly for C2C12 cells, trypsin/EDTA treatment was used in all subsequent experiments. Cells plated on TSP-1 for 2 h could be trypsinized and replated in serum-containing medium without loss of viability.

Several observations corroborated that myoblasts attached directly to TSP-1. First, <10% of the myoblasts attached to BSA-coated substrates and none of the attached cells spread within 2 h, indicating that spreading was not caused by secretion of endogenous matrix components. Secondly, myoblast adhesion was inhibited by 85% to 90% if thrombospondin-coated substrates were preincubated with polyclonal IgG to TSP-1, whereas preincubation with polyclonal antisera to

vitronectin or fibronectin decreased cell adhesion by ~10%. Thirdly, addition of 5 mM EDTA, either during the substrate adsorption period, or during the cell adhesion assay, inhibited cell adhesion. Substratum dependence on calcium is highly characteristic of TSP-1, because the molecule undergoes an irreversible conformational change when calcium is chelated (Lawler *et al.*, 1985, 1988).

The time and concentration dependence of myoblast attachment to TSP-1 was examined in comparison with adhesion to plasma fibronectin, an adhesive glycoprotein that mediates the spreading of many cell types (reviewed Hynes, 1990). For each cell line, the parameters of adhesion to TSP-1 or fibronectin were very similar. At 2 h, maximal attachment of myoblasts to both substrates was observed at coating concentrations of 50 nM (Figure 4, a and b). On substrates coated with 50 nM of fibronectin or TSP-1, C2C12 cells reached maximal levels of adhesion within 2 h, whereas H9c2 cells took 1.5 h (Figure 4, c and d). In the case of C2C12 and G8 cells, maximal adhesion represented attachment of

~90% of the cells, whereas ~60–70% of the H9c2 cells attached at maximum. The adhesive properties of G8 myoblasts were similar to those of C2C12 cells (unpublished data).

Adhesion to TSP-1 or Fibronectin Result in Different States of Actin Microfilament Organization

To compare the adhesive response of myoblasts to TSP-1 and to fibronectin in more detail, the organization of the actin cytoskeleton was examined by using Texas red-conjugated phalloidin to visualize polymerized actin. Adhesion assays were carried out under conditions that gave maximal cell adherence to TSP-1 or to fibronectin and the cells were examined after 2 or 4 h.

In comparison with cells plated on fibronectin, myoblasts plated on TSP-1 tended to have polarized morphologies rather than polygonal shapes. Whereas some cells showed a single, well-spread leading lamella and a trailing tail, others had a more irregular cell margin with several areas of lamellae. These differences were most pronounced in the case of H9c2 cells (see Figure 3, a and b) for morphologies of myoblasts plated on TSP-1).

These morphological differences were also clearly apparent in terms of actin microfilament organization. Cells plated upon TSP-1 tended to display a polarized subcellular distribution of stress fibers. Many cells exhibited extensive areas of lamellae, which contained intensely staining, radial actin microspikes (arrowed in Figure 5, a and d). In contrast, C2C12 and H9c2 myo-

blasts plated upon fibronectin contained longitudinal and circumferential arrays of actin stress fibers. Lamellae-containing actin microspikes were not observed; instead, some cells displayed well developed microfilament bundles parallel to the cell perimeter (Figure 5, b and d). These substratum-dependent differences in cytoskeletal organization were transient. After 4 h adhesion to TSP-1, cell shape and actin filament distribution more closely resembled that of cells adherent on fibronectin for 2 h; that is, cells had become less irregular in shape, contained more prominent actin stress fibers and no longer contained radial actin microspikes (Figure 5c and 5f). Because by this time the cells adherent on BSA-coated substrata had also begun to spread, these changes are likely to be because of secretion of endogenous matrix components. However, these results clearly show that initial spreading of cells on fibronectin or TSP-1 lead to different states of organization of the actin cytoskeleton.

Mapping of Myoblast-binding Sites in TSP-1

To map the regions of the TSP-1 molecule that are involved in myoblast attachment, a panel of monoclonal antibodies to TSP-1 were tested for their ability to prevent cell spreading or cell attachment. In these experiments, thrombospondin-coated substrata were incubated with antibody for 2 h, then washed and used for cell adhesion assays. Of the eight monoclonal antibodies tested, antibodies MAII, MAIV, and 5G11, which recognize epitopes within the amino-terminal heparin-binding domain (Lawler *et al.*, 1985; Legrand *et al.*,

Figure 5. Actin microfilament organization in adherent myoblasts. C2C12 cells (a–c) and H9c2 cells (d–f) were plated on substrates coated with 50 nM thrombospondin (a, c, d, and f) or 50 nM fibronectin (b and e) for 2 h (a, b, d, and e) or 4 h (c and f). All cells were stained with TRITC-phalloidin. Arrows in a and d indicate areas of membrane ruffles and lamellae that contain actin microspikes. Bar, 50 μ m.

1988), MAI, which recognizes an epitope proximal to the carboxy-terminal globular domain (Lawler *et al.*, 1985, 1986), and C6.7, which recognizes an epitope at the extreme carboxy-terminus of the molecule (Dixit *et al.*, 1985), did not inhibit myoblast adhesion. However, antibodies 4.1, ESTs 10, and ESTs 12 (1.2) completely prevented the adhesion of all three myoblast cell lines in a concentration dependent manner (Figure 6 and unpublished data). Inhibition of adhesion by Mab ESTs 12 (1.2) required somewhat higher concentrations of IgG; however, inhibition was cell type dependent, because this antibody did not inhibit the attachment of human epidermal keratinocytes to TSP-1 when used at the same concentrations (Figure 6b) (Adams and Lawler, 1993b).

Monoclonal antibody 4.1 recognizes an epitope located within the procollagen homology domain or the type 1 repeats (Prater *et al.*, 1991), and we have previously shown that the binding sites for antibodies ESTs 12 (1.2) and ESTs 10 are located in the type 1 repeats and the carboxy-terminal globular domain, respectively (Adams and Lawler, 1993b). Thus, two separate regions of the TSP-1 molecule contain myoblast attachment sites. Individually, 3 $\mu\text{g}/\text{ml}$ of ESTs 10 and 5 $\mu\text{g}/\text{ml}$ of ESTs 12 (1.2) caused 20% or 10% inhibition of myoblast adhesion, respectively. However, preincubation of TSP-1 with both antibodies at these concentrations caused a 60% decrease in myoblast adhesion, suggesting that the two sites may act synergistically (unpublished data; see also Figure 8C).

The Roles of Proteoglycans and Integrins in Myoblast Adhesion to TSP-1

To confirm and extend the antibody mapping experiments, we examined a series of soluble antagonists and anti-receptor reagents for their ability to block myoblast adhesion. Soluble heparin, at concentrations between 2 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$, prevents the attachment of certain cell types to TSP-1 (Roberts *et al.*, 1987; Neugebauer *et al.*, 1991); however, the adhesion of C2C12, G8, or H9c2 myoblasts was not inhibited even in the presence of 500 $\mu\text{g}/\text{ml}$ heparin. Synthetic VTCC peptide was used to examine the possible involvement of the CSVTCG cell binding sites within the type 1 repeats (Rich *et al.*, 1990; Prater *et al.*, 1991; Tuszyński *et al.*, 1992). The peptide, used at 1 mM, did not prevent myoblast attachment, although it did cause a 50% inhibition in the attachment of G361 cells, which are known to recognize the CSVTCG site (Prater *et al.*, 1991; Adams and Lawler, 1993b). Myoblast adhesion was also insensitive to the combination of 1 mM VTCC peptide and 500 $\mu\text{g}/\text{ml}$ heparin (unpublished data).

To examine the involvement of proteoglycans in more detail, we tested a series of soluble glycosaminoglycans for their ability to inhibit myoblast attachment to thrombospondin. Each reagent was added to the at-

Figure 6. Inhibition of myoblast adhesion to thrombospondin by anti-thrombospondin antibodies. (a) C2C12 cells were plated onto wells coated with 30 $\mu\text{g}/\text{ml}$ thrombospondin that had been preincubated for 2 h with the indicated concentrations of Mab 4.1 IgG (●) or ESTs 10 IgG (■). (b) C2C12 (■), H9c2 (●), or human epidermal keratinocytes (▲) were plated on wells coated with 30 $\mu\text{g}/\text{ml}$ thrombospondin that had been preincubated for 2 h with the indicated concentrations of ESTs 12 (1.2) IgG. Adhesion times were 2 h, and the data is plotted as the percentage of adherent cells relative to untreated (UT) cells plated on thrombospondin in the absence of IgG. Each point is the mean of duplicate experiments. Bars indicate SEM.

tachment assay at a concentration of 500 $\mu\text{g}/\text{ml}$. Heparin, heparan sulphate, fucoidan, chondroitin sulphate B, chondroitin sulphate C, and low molecular weight dextran sulphate were all inactive, but chondroitin sulphate A and high molecular weight dextran sulphate both inhibited cell attachment by 90% (Figure 7A). If thrombospondin-coated wells were preincubated with 100 $\mu\text{g}/\text{ml}$ of chondroitin sulphate A for 30 min at room temperature then washed and used for attachment assays, cell attachment was inhibited by 90% (unpublished data). The inhibition of attachment by chondroitin sulphate A was concentration dependent;

Figure 7. Involvement of chondroitin sulphate proteoglycans in myoblast adhesion to thrombospondin. (A) Inhibition of myoblast attachment to TSP-1 by a series of glycosaminoglycans. Lane 1, heparin; lane 2, fucoidan; lane 3, chondroitin sulphate A; lane 4, dermatan sulphate; lane 5, chondroitin sulphate C; lane 6, heparan sulphate; lane 7, dextran; lane 8, dextran sulphate MW 5000; lane 9, dextran sulphate MW 500 000. Each glycosaminoglycan was used at a final concentration of 500 μ g/ml and was added at the time of plating the cells. Each bar represents the mean of triplicate experiments. (B) Concentration dependence of inhibition of attachment by chondroitin sulphate A (●), heparin (▲), or fucoidan (■). Each point represents the mean of duplicate experiments. (C) Inhibition of myoblast attachment by β -xylosides. Cells were treated for 20 h with 2.5 mM (●) or 5 mM (▲) p-nitrophenol β -D-xylopyranoside, or 5 mM o-nitrophenol β -D-xylopyranoside (■). The attachment assays were carried out in the continued presence of these reagents. In B and C, the data are plotted as the percentage of adherent cells relative to the adherence of untreated (UT) control cells. Each point is the mean of triplicate experiments. In each set of experiments, the attachment time was 90 min; for the experiments shown in A and B, the wells were coated with 50 nM TSP-1.

maximal inhibition was achieved at a concentration of 100 μ g/ml (Figure 7B). Three preparations of chondroitin sulphate A obtained from different commercial sources were tested; all had inhibitory activity although the maximal level of inhibition varied between 70% and 96%.

To further test the possible involvement of chondroitin sulphate-containing proteoglycans in myoblast adhesion to TSP-1, we treated C2C12 myoblasts for 20 h with p-nitrophenol β -D-xylopyranoside, a competitive inhibitor for the addition of glycosaminoglycan chains to core proteins (Schwartz, 1977). Incubation with 2.5 mM p-nitrophenol β -D-xylopyranoside did not affect the ability of C2C12 cells to attach to TSP-1, but 5 mM p-nitrophenol β -D-xylopyranoside caused a 60% decrease in the maximum number of adherent cells. Incubation of cells for 20 h in the presence of 5 mM o-nitrophenol β -D-xylopyranoside, an inactive isomer (Schwartz, 1977), did not prevent cell adhesion to TSP-1 (Figure 7C).

We next examined the effect of chondroitinase ABC treatment on C2C12 adhesion to TSP-1. In a series of experiments, treatment of myoblasts with 2 U/ml of enzyme for 30 min at 37°C caused 28% ($\pm$ 5%) inhibition of cell attachment. Treatment of C2C12 cells with heparinase II or boiled chondroitinase ABC caused a 7% ($\pm$ 3%) decrease in the number of adherent cells. These differences are significant at $P < 0.05$ (unpublished data). To examine the role of proteoglycan sulphation in myoblast adhesion, C2C12 cells were grown in the presence of 30 mM sodium chlorate for 20 h to prevent the sulphation of glycosaminoglycans (Rapraeger *et al.*, 1991). This treatment did not alter the ability of cells to adhere to TSP-1 (unpublished data). Taken together, these results indicate that cell surface chondroitin sulphate proteoglycans, rather than heparan sulphate proteoglycans, are involved in myoblast adhesion to TSP-1. Sulphate groups do not appear to be critical components of the thrombospondin-binding carbohydrate structures.

To examine the role of the GRGDA cell binding site located within the type 3 repeats (Lawler *et al.*, 1988), GRGDSP peptide was used as a soluble competitor. As previously reported, 1 mM GRGDSP peptide inhibited the attachment of smooth muscle cells to TSP-1 (Lawler *et al.*, 1988; Adams and Lawler, 1993b); however, the peptide did not prevent the adhesion of C2C12, G8, or H9c2 myoblasts. We also investigated whether the combinations of 500 μ g/ml heparin and 1 mM GRGDSP peptide, or 1 mM VTCC and 1 mM GRGDSP peptides might synergistically inhibit myoblast adhesion; however, no inhibition of cell adhesion was observed in either case (unpublished data).

Because myoblast attachment could not be inhibited by GRGDSP peptide, it appeared that the α v β 3 integrin did not play a major role in myoblast adhesion to TSP-1 and indeed, a polyclonal serum raised against human

placental vitronectin receptor did not prevent myoblast adhesion to TSP-1. By several criteria this antiserum acts as an adhesion-perturbing reagent. It inhibits attachment of human cells to vitronectin (Suzuki *et al.*, 1986), and in our hands blocked adhesion of human smooth muscle cells to TSP-1, inhibited C2C12 adhesion to vitronectin and caused rounding of H9c2 cells on vitronectin (unpublished data).

Neurite outgrowth on TSP-1 is mediated by $\beta 1$ integrins but is not inhibited by GRGDSP peptide (Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991). We therefore tested the effect of polyclonal serum directed against human fibronectin receptor on myoblast adhesion to TSP-1. This reagent caused rounding of myoblasts spread on fibronectin or vitronectin but did not affect myoblast adhesion to TSP-1 (unpublished data). Thus, it appears unlikely that $\beta 1$ or α_v -containing integrins are involved in the initial attachment of myoblasts to TSP-1.

Ability of Proteolytic Fragments of Thrombospondin-1 Fragments to Support Myoblast Adhesion

To determine if the central and carboxy-terminal regions of TSP-1 directly support myoblast adhesion, proteolytic fragments of platelet TSP-1 were used as adhesive substrata. In initial experiments, TSP-1 was digested with chymotrypsin or trypsin in the presence of 2 mM calcium. Various enzyme/substrate ratios were used to generate a range of fragments (Lawler *et al.*, 1985), and the entire digests were coated onto plastic. The major product of tryptic digests made at a 1:10 000 enzyme/substrate ratio is a trimeric fragment, termed 140 kDa, which lacks the heparin binding domain (Lawler *et al.*, 1985). Such digests retained adhesive activity, whereas digests made at a 1:100 enzyme/substrate ratio, which contain smaller fragments, were not adhesive. Chymotryptic digests made at a 1:1000 enzyme/substrate ratio contain a 120–18 kDa disulphide-bonded fragment (Dixit *et al.*, 1985) and were also nonadhesive (unpublished data). These data suggested that an intact carboxy-terminal globular domain was crucial for myoblast adhesion. Indeed, in assays using purified fragments, the 140-kDa tryptic fragment was as effective on a molar basis as intact TSP-1 in mediating myoblast attachment and spreading, whereas the central 70-kDa chymotryptic fragment or the 25-kDa, amino-terminal heparin-binding chymotryptic fragment were not adhesive (Figure 8A and 8B).

The panel of antibodies, peptides, and soluble glycosaminoglycans were used to examine the mechanism of myoblast adhesion to the 140-kDa fragment. As found for intact TSP-1, adhesion was not inhibited by 500 μ g/ml heparin or 1 mM VTCG peptide but was decreased by 90% in the presence of 100 μ g/ml chondroitin sulphate A. In contrast to adhesion to intact TSP-

1, 1 mM GRGDSP peptide caused a 40% decrease in cell attachment, and cells that did attach tended to be less well spread. One millimolar GRGESp had no effect on cell adhesion to the 140-kDa fragment. Cell attachment was inhibited by antibodies 4.1, ESTs 10, or ESTs 12 (1.2) individually, whereas low concentrations of antibodies ESTs 10 and ESTs 12 (1.2) exhibited synergistic inhibition of adhesion. (Figure 8C, shown for C2C12 cells only). Because the central 70-kDa fragment did not support myoblast adhesion, these data indicate either that the carboxy-terminal domain contains the primary adhesive site for myoblasts or that sites within the type 1 repeats and carboxy-terminal globular domain act synergistically when presented within the same polypeptide.

The Carboxy-terminal Domains of TSP-1 and TSP-4 Support Myoblast Adhesion

To directly investigate the adhesive activity of the carboxy-terminal globular domain of TSP-1, we expressed this domain as a carboxy-terminal fusion with GST in the bacterial expression vector, pGEX (Smith and Johnson, 1988). Because this domain exhibits the highest degree of sequence conservation between the different thrombospondin family members (Adams and Lawler, 1993a; Lawler *et al.*, 1993a) and TSP-4 is expressed by skeletal muscle (Lawler *et al.*, 1993b), we also expressed a GST fusion protein containing the carboxy-terminal globular domain of human TSP-4. These fusion proteins were termed hTSP-1C and hTSP-4C, respectively.

To purify the fusion proteins, the soluble fractions of bacterial lysates were passed over glutathione-agarose affinity columns and, after extensive washing, eluted with 10 mM glutathione. The eluted proteins were dialyzed against TBS and examined on SDS-PAGE gels under reducing conditions. Unfused GST resolved with an apparent molecular weight of 27 kDa. The hTSP-1C protein has a calculated molecular weight of 57.4 kDa and was resolved on SDS-PAGE gels with an apparent molecular weight of 60 kDa. The hTSP-4C protein has a calculated molecular weight of 51.6 kDa and resolved with an apparent molecular weight of 53 kDa (Figure 9). Typical yields of purified proteins were 15 mg/l for unfused GST; 10 mg/l for hTSP-1C, and 4 mg/l for hTSP-4C.

We have previously shown that hTSP-1C is recognized by monoclonal antibody ESTs 10, which is reactive with native platelet TSP-1 (Adams and Lawler, 1993b). Thus, the conformation of the hTSP-1C protein is structurally similar to that of the carboxy-terminal domain of native TSP-1. Because antibodies to intact TSP-4 are not available at present, the hTSP-4C protein could not be examined in this way.

We next examined the ability of the fusion proteins to support myoblast adhesion. When coated on plastic at equimolar concentrations, GST was nonadhesive,

Figure 8. Adhesion of myoblasts to proteolytic fragments of thrombospondin. C2C12 (A) or H9c2 (B) myoblasts were allowed to attach for 2 h to wells coated with intact thrombospondin (■) or the 140-kDa tryptic fragment (◆). The 70-kDa chymotryptic fragment and the 25-kDa heparin-binding fragment were both nonadhesive (●). Each point is the mean of duplicate experiments. Bars indicate SEM. (C) Adhesion of C2C12 myoblasts to the 140-kDa tryptic fragment, used at a coating concentration of 50 nM in the presence of 500 μ g/ml heparin (lane 1), 100 μ g/ml chondroitin sulphate A (lane 2), 1 mM VTCG peptide (lane 3), 1 mM GRGDSP peptide (lane 4), 1 mM GRGESD peptide (lane 5), 25 μ g/ml ESTs 10 IgG (lane 6), 3 μ g/ml ESTs 10 IgG (lane 7), 25 μ g/ml ESTs 12 (1.2) IgG (lane 8), 5 μ g/ml ESTs 12 (1.2) IgG (lane 9), 3 μ g/ml ESTs 10 IgG plus 5 μ g/ml ESTs 12 (1.2) IgG (lane 10), or 10 μ g/ml 4.1 IgG (lane 11). Data are plotted as the percentage of adherent cells relative to the adherence of untreated (UT) control cells.

whereas both hTSP-1C and hTSP-4C supported the attachment and spreading of C2C12 and G8 myoblasts (Figure 10A, shown for C2C12 cells only). A coating concentration of 2 μ M hTSP-1C supported maximal adhesion, and this represented 30% of the maximal level of adhesion achieved on intact platelet TSP-1. On a molar basis the hTSP-4C protein was less active than the hTSP-1C protein. A coating concentration of 2 μ M supported cell attachment to 20% of maximal attachment to platelet TSP-1.

Soluble hTSP-1C was tested for its ability to inhibit myoblast adhesion to intact TSP-1. Fifty micromolars hTSP-1C inhibited adhesion to intact thrombospondin-1 by 50% and to coated hTSP-1C by 62%, indicating that the cell binding site in hTSP-1C corresponded to that in the intact molecule (Figure 10B). The panel of peptides, glycosaminoglycans, and monoclonal antibodies to TSP-1 were tested for their ability to prevent adhesion to hTSP-1C. The only antibody that inhibited cell adhesion was ESTs10; the maximum level of inhibition observed was 68%. Chondroitin sulphate A (100 μ g/ml), 100 μ g/ml heparin, 1 mM VTCG peptide, or 1 mM GRGDSP peptide did not inhibit adhesion to hTSP-1C (unpublished data).

DISCUSSION

Thrombospondin protein is present within the ECM of developing and repairing skeletal muscle tissue and has been hypothesized to play roles in the proliferative, adhesive, and migratory events involved in myoblast fusion, the development of neuromuscular junctions, and muscle regeneration (Watkins *et al.*, 1990; Hantai *et al.*, 1991; Rao *et al.*, 1992). The aim of this series of experiments was to analyze the mechanisms by which skeletal myoblasts interact with thrombospondin-1.

To date it has remained unclear whether myocytes themselves are the source of thrombospondin in muscle. In one previous study, rat myoblasts were found not to synthesize thrombospondin (Dreyfus and Lahav, 1988), whereas others have demonstrated that thrombospondin is synthesized by the myogenic C2 cell line at the myotube stage (Hantai *et al.*, 1991). In this study, we have shown by metabolic labeling and immunoprecipitation that C2C12 and H9c2 skeletal myoblasts do indeed synthesize thrombospondin. As reported for other cell types, thrombospondin deposited extracellularly into the cell layer does not form fibrils and has a different distribution to fibronectin (Raugi *et al.*, 1982; Wikner *et al.*, 1987; Dreyfus and Lahav, 1988; Murphy-Ullrich *et al.*, 1988; Clezardin *et al.*, 1989).

Many cell types attach to TSP-1 (reviewed Frazier, 1991), but only certain cell types undergo cell spreading (Roberts *et al.*, 1987; Varani *et al.*, 1988; Asch *et al.*, 1991; Adams and Lawler, 1993b), and in some cases, conflicting results have been obtained by different laboratories (Murphy-Ullrich and Hook, 1989; Tarabotti

Figure 9. SDS-PAGE analysis of thrombospondin fusion proteins. Purified fusion proteins were resolved on a 12.5% polyacrylamide gel under reducing conditions. Lane 1, unfused GST; lane 2, hTSP-4C fusion protein; lane 3, hTSP-1C fusion protein. Molecular weights of protein standards are given in kDa.

et al., 1990). These differences may result in part from the variations in the position and number of disulphide bonds within the type 3 repeats that have been identified in different preparations of platelet TSP-1 (Speziale and Detwiler, 1990). Alterations in the numbers of disulphide bonds affect the exposure of the RGD site and thereby the adhesivity of TSP-1 preparations (Sun *et al.*, 1992). However, cells of different tissue origins, both normal and transformed, exhibit different morphological responses when plated on the same preparations of platelet TSP-1 (Adams and Lawler, 1993b). Thus cell type-dependent factors also contribute to the observed variability. Such factors could involve the ligation of different sets of thrombospondin receptors or the activation of different signal transduction pathways. Skeletal myoblasts provide an example of a nontransformed cell type that spreads extensively on TSP-1.

Four regions within the TSP-1 molecule possess adhesive activity (reviewed Frazier, 1991; Adams and Lawler, 1993a). To identify the sites recognized by myoblasts, we used a panel of soluble inhibitors, antibodies to TSP-1, and proteolytic fragments of platelet TSP-1. We found that adhesive activity involved the type 1 repeats and the carboxy-terminal globular domain. Of the other cell types in which the involvement of all four adhesive sites of TSP-1 have been systematically examined, G361 melanoma cells, MG-63 osteosarcoma cells, and smooth muscle cells attach through concurrent interactions with all four sites (Roberts *et al.*, 1987; Asch *et al.*, 1991; Kosfeld *et al.*, 1991; Prater *et al.*, 1991; Adams and Lawler, 1993b), whereas 11B carcinoma cells do not recognize the RGD site (Varani *et al.*, 1986, 1988; Yabkowitz and Dixit, 1991a).

These data contrast with the less complex mechanism of adhesion that we have identified for skeletal myoblasts, in which the heparin-binding domain and RGD site are not involved. However, other nontransformed cell types, such as keratinocytes, haematopoietic progenitor cells, and glomerular mesangial cells, also undergo adhesive interactions that are not inhibited by heparin or GRGDSP peptide (Varani *et al.*, 1988; Long and Dixit, 1990; Taraboletti *et al.*, 1992; Adams and

Lawler, 1993b). Haematopoietic progenitor cells, T cells, and neuronal cells principally interact with the type 1 repeats (Long and Dixit, 1990; Osterhout *et al.*, 1992; Yabkowitz *et al.*, 1993), whereas glomerular mesangial cells and keratinocytes interact with the carboxy-terminal globular domain (Taraboletti *et al.*, 1992; Adams and Lawler, 1993b). The mechanism used by skeletal myoblasts that involves both the type 1 repeats and carboxy-terminal globular domain is thus, to date, unique.

The carboxy-terminal domain was the only portion of the TSP-1 molecule that supported myoblast attachment in isolation and thus appears to contain the primary myoblast attachment site. However, in terms of cell numbers, adhesion to the fusion protein was always lower than adhesion to intact TSP-1 or the 140 kDa fragment. Thus, as suggested by the antibody inhibition experiments, the carboxy-terminal domain may function in synergy with the type 1 repeats. It has previously been reported that G361 melanoma cells attach to a

Figure 10. Attachment of myoblasts to fusion proteins. (A) C2C12 myoblasts were allowed to attach for 90 min to wells coated with unfused glutathione S-transferase (■), hTSP-1C (●), or hTSP-4C (▲). Data are plotted as the percentage of adherent cells, relative to maximal adhesion to intact thrombospondin. (B) C2C12 cells were allowed to attach for 90 min to substrates coated with 50 nM platelet thrombospondin (■) or 5 μ M hTSP-1C fusion protein (▼) in the presence of the indicated concentrations of soluble hTSP-1C. Each point is the mean of duplicate experiments. Bars indicate SEM.

recombinant carboxy-terminal domain of TSP-1 (Kosfeld *et al.*, 1991; Kosfeld and Frazier, 1992, 1993). It is not clear if these cells recognize the same site(s) as the myoblasts, because antibody C6.7, which binds to an epitope located at the extreme carboxy-terminus of TSP-1 (Dixit *et al.*, 1985), prevents G361 attachment (Kosfeld *et al.*, 1991) but does not inhibit myoblast attachment. Complete inhibition of myoblast attachment to intact TSP-1 is achieved at lower concentrations of antibody ESTs 10 than inhibition of G361 cell attachment (Adams and Lawler, 1993b).

To date, cell surface receptors for the carboxy-terminal globular domain have not been fully characterized. A 105/80-kDa nonintegrin heterodimer has been isolated from 11B squamous carcinoma cells (Yabkowitz and Dixit, 1991b), but because antibodies to this complex are not yet available we were unable to test its role in myoblast adhesion. However, the thrombospondin-binding proteins of 11B cells are trypsin-sensitive (Yabkowitz and Dixit, 1991a), whereas myoblasts will attach and spread on TSP-1 after trypsin treatment. Although C2C12, G8, and H9c2 myoblasts express the $\alpha v \beta 3$ integrin and an array of $\beta 1$ -containing integrins (von der Mark *et al.*, 1991; Rosen *et al.*, 1992; Adams, unpublished data), integrins did not appear to be the primary myoblast thrombospondin receptors. The small inhibitory effect of RGD peptide on cell attachment to the 140-kDa fragment suggests that this site is cryptic in the intact molecule. The $\alpha v \beta 3$ integrin is expressed at low levels in developing mouse muscle and does not colocalize with thrombospondin protein (Corless *et al.*, 1992).

Instead, our data indicate that chondroitin sulphate proteoglycans may play a role in myoblast attachment. Soluble chondroitin sulphate A inhibited myoblast adhesion, as did pretreatment of cells with p-nitrophenol β -D-xylopyranoside or chondroitinase ABC. Because chondroitin sulphate A inhibited attachment to thrombospondin or the 140-kDa chymotryptic fragment but not to the carboxy-terminal fusion protein, we hypothesize that cell surface chondroitin sulphate proteoglycans interact with the type 1 repeats. However, because the 70-kDa chymotryptic fragment was non-adhesive, it appears that the interactions with the type 1 repeats are insufficient to support myoblast adhesion. By analogy with fibronectin, such interactions with the type 1 repeats may serve to stabilize the higher affinity interaction with the carboxy-terminal domain (Woods *et al.*, 1986).

Although TSP-1 has principally been characterized as a heparin-binding protein, chondroitin sulphate A also binds to TSP-1 (Holt *et al.*, 1990; Pancake *et al.*, 1992), and chondroitin sulphate proteoglycans participate in the binding and endocytosis of thrombospondin (Murphy-Ullrich *et al.*, 1988). The attachment of neuronal cells to F-spondin, a protein that contains six thrombospondin type 1 repeats, is inhibited by both

chondroitin sulphate and heparin (Klar *et al.*, 1991). Direct measurement of heparin binding by peptides derived from the type 1 repeats of TSP-1 has shown the WSXWS sequence motif to be critical for activity (Guo *et al.*, 1992a,b). The adhesive interactions of myoblasts with the type 1 repeats thus differ from those described for melanoma cells, which undergo heparin-sensitive attachment to the 70-kDa chymotryptic fragment, and to peptides containing the CSVTCG sequence (Prater *et al.*, 1991). Furthermore, heparan sulphate proteoglycans from G361 cells bind to thrombospondin-agarose with higher affinity than do chondroitin sulphate proteoglycans (Roberts, 1988). Several cell surface proteoglycans that carry chondroitin sulphate glycosaminoglycan chains have now been characterized. These include CD44 (Underhill, 1992) and syndecan (Bernfield and Sanderson, 1990). Indeed, syndecan and thrombospondin colocalize in developing skeletal muscle (Corless *et al.*, 1992).

Although the myoblasts attached equally well to TSP-1 and fibronectin, attachment resulted in markedly different subcellular distributions of actin microfilaments, suggesting that distinct secondary cellular responses were evoked. Cells attached to TSP-1 tended to have a more angular outline, were polarized and elongated rather than polygonal in shape, and displayed prominent lamellae and actin microspikes instead of stress fiber bundles. These results suggest that myoblasts adherent on TSP-1 form close contacts, structures that are characteristic of motile cells (Couchman and Rees, 1979; Kolega *et al.*, 1982, reviewed Burridge *et al.*, 1988). Thrombospondin disrupts the focal contacts of adherent endothelial cells (Murphy-Ullrich and Hook, 1989) and has haptotactic effects on various cell types (Taraboletti *et al.*, 1987; Mansfield *et al.*, 1990); clearly, it may be evoking similar responses from myoblasts and this may be important for its function in developing or traumatized muscle tissue (Watkins *et al.*, 1990).

Our results provide the first evidence that TSP-4 can also act as a cell adhesion molecule. The carboxy-terminal globular domain is the region of highest sequence homology between different members of the thrombospondin family (Adams and Lawler, 1993a and references therein; Lawler *et al.*, 1993a,b), and the carboxy-terminal globular domains of human TSP-1 and TSP-4 display 57% amino acid identity. The hTSP-4C fusion protein supported myoblast adhesion but was less active on a molar basis than the TSP-1C protein. These results support the hypothesis that these homologous regions encode shared functions but also indicate that they have different levels of adhesive activity. Because TSP-4 does not contain the type 1 repeats, any secondary adhesive interactions with the intact TSP-4 molecule are likely to be mediated by a different molecular mechanism. Such differences may ensure that individual thrombospondin family members have distinct roles in the extracellular matrix. In this respect, it

is interesting that TSP-1 and TSP-2 differ markedly in their heparin-binding activity (O'Rourke *et al.*, 1992).

In conclusion, we have shown that skeletal myoblasts can act as a source of thrombospondin-1 and have documented a novel mechanism used by these cells to adhere to TSP-1. The diversity of attachment mechanisms that have now been described for TSP-1 emphasizes the importance of analyzing individual cell types derived from the tissues in which TSP-1 is expressed. The anti-adhesive and growth-regulatory activities of TSP-1 both map to the amino-terminal heparin-binding domain (Majack *et al.*, 1986, 1988; Murphy-Ullrich and Hook, 1989); however, for a variety of cell types attachment to TSP-1 does not involve this domain (Long and Dixit, 1990; Neugebauer *et al.*, 1991; O'Shea *et al.*, 1991; Adams and Lawler, 1993b). It is now also established that cell attachment to TSP-1 can result in cell spreading and organization of the actin cytoskeleton (Asch *et al.*, 1991; Stomski *et al.*, 1992; and this study). Thus, although TSP-1 has some anti-adhesive properties in common with the glycoproteins tenascin and SPARC (Sage and Bornstein, 1991), it may be more appropriate to consider the protein as encoding a wide repertoire of adhesive signals, the interpretation of which depends on the receptors expressed by a particular cell type (Chiquet-Ehrismann, 1991). Further analysis of cell-binding sites and cell surface receptors for TSP-1 will shed more light on this question and on the roles of other members of the thrombospondin gene family in tissue organization.

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