Cell Attachment to Thrombospondin: The Role of ARG-GLY-ASP, Calcium, and Integrin Receptors

Jack Lawler, Robert Weinstein, and Richard O. Hynes

Department of Biomedical Research and Medicine, Division of Hematology/Oncology, St. Elizabeth's Hospital of Boston, Tufts University School of Medicine, Boston, Massachusetts 02135; and The Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Thrombospondin is a 420,000-D glycoprotein that has recently been shown to have several properties in common with the members of a class of adhesive proteins. To characterize further the adhesive properties of thrombospondin, we have studied its ability to support cell attachment. Thrombospondin adsorbed to plastic dishes supports the attachment of human endothelial and smooth muscle cells and the monocyte-like cell line (U937) as well as normal rat kidney cells. The majority of attached cells do not spread on the solid-phase thrombospondin. The attachment of all four cell types to thrombospondin is abolished if the assay is performed in the presence of EGTA, although the cells still attach to fibronectin. If thrombospondin is adsorbed to the dishes in the presence of EGTA and then washed with buffer containing calcium before addition of the cells, attachment is still markedly inhibited, indicating that calcium affects the conformation and function of thrombospondin. Attachment of all four cell types is also markedly inhibited by the synthetic peptides gly-arg-gly-asp-ser-pro (GRGDSP) and gly-arg-gly-asp-ala-cys (GRGDAC) but not by the control peptide gly-arg-gly-glu-ser-pro (GRGESP). Affinity chromatography of n-octylglucoside extracts of surface-labeled endothelial cells or smooth muscle cells on thrombospondin-Sepharose and GRGDSP-Affigel columns was used to identify an integrin complex related to glycoprotein IIb-IIIa as an RGD-dependent receptor for thrombospondin. In addition, a monoclonal antibody (LM609) that blocks attachment of endothelial cells to vitronectin, fibrinogen, and von Willebrand factor also inhibits attachment of endothelial cells to thrombospondin. These data indicate that the attachment of cells to thrombospondin is mediated by RGD and calcium-dependent mechanisms and is consistent with the hypothesis that the GRGDAC sequence in thrombospondin is a site for interaction with an integrin receptor of the β3 subclass.

Thrombospondin is a high molecular weight glycoprotein that is synthesized and secreted by various cells in culture (see Lawler, 1986 for a review). Thrombospondin is also secreted by blood platelets at the site of vascular damage (Lawler, 1986). In terms of its structure and functions, thrombospondin is similar to the members of the class of adhesive proteins. Our recent determination of the amino acid sequence of thrombospondin revealed the presence of the sequence arg-gly-asp-ala (RGDA) in a region of the molecule designated as the last type 3 repeat (Lawler and Hynes, 1986). The type 3 repeats are homologous with the calcium binding sites of calmodulin and parvalbumin (Lawler and Hynes, 1986). Thrombospondin has been shown to bind calcium by a cooperative mechanism with an apparent dissociation constant of 120 μM (Lawler and Simons, 1983). In addition, a higher affinity (0.1 μM) interaction can be detected with monoclonal antibodies (Dixit et al., 1986). The binding of calcium to thrombospondin results in a profound conformational change (Lawler and Simons, 1983; Lawler et al., 1985; Galvin et al., 1985; Dixit et al., 1986). The localization of this conformational change, by electron microscopy, agrees well with the location of the type 3 repeats based on amino acid sequence data (Lawler et al., 1985; Galvin et al., 1985; Lawler and Hynes, 1986; Dixit et al., 1986).

The RGD sequence has been shown to be present in the members of a class of adhesive proteins that mediate cell attachment (see Ruoslahti and Pierschbacher, 1986 for a review). A family of cell surface receptor complexes, designated integrins, has been identified which bind proteins containing this sequence (see Hynes, 1987, and Ruoslahti and Pierschbacher, 1987 for reviews). Cell attachment mediated by the integrin family of receptors is frequently specifically inhibited by synthetic peptides which include the RGD sequence (Pierschbacher and Ruoslahti, 1984a,b; Yamada and Kennedy, 1984; Ruoslahti and Pierschbacher, 1986). The integrins are composed of α and β subunits that
form complexes through noncovalent associations. There are three distinct β subunits each of which can associate with several different α subunits (Hynes, 1987; Ginsberg et al., 1988; Phillips et al., 1988). The members of the β3 or cytoadhesive subfamily have the same β subunit, designated integrin β3 or GPIIIa. At least two distinct α subunits that associate with the integrin β3 subunit have been identified to date. The vitronectin receptor α subunit is present on MG63 osteosarcoma cells (Pytel et al., 1986). When this receptor is purified and incorporated into liposomes, the liposomes bind specifically to vitronectin. GPIIb is another α subunit that associates with GPIIIa and is found exclusively on platelet membranes (Ginsberg et al., 1988; Suzuki et al., 1987). The GPIIb-IIIa complex is capable of interacting with fibronectin, fibrinogen, vitronectin, and von Willebrand factor (Plow et al., 1984, 1985, 1987). These two α subunits of the β3 subfamily are highly homologous at the amino acid sequence level (Fitzgerald et al., 1987; Poncz et al., 1987). They are each composed of a heavy chain with an apparent molecular mass of 125,000-130,000 Da and a light chain with a molecular mass of 25,000 Da (Phillips et al., 1988).

A GPIIb-IIIa-like integrin complex is found on the surface of many cell types, including endothelial cells, smooth muscle cells, and melanoma cells (Thiagarajan et al., 1988; Fitzgerald et al., 1988; Leeksm et al., 1986; Newman et al., 1986; Plow et al., 1986; Charo et al., 1986). The β subunit of this complex is also GPIIa or integrin β3 (Ginsberg et al., 1987). Some of the antibody preparations that have been raised against GPIIb-IIIa also react with the GPIIb-IIIa-like molecules on endothelial cells and inhibit the attachment of endothelial cells to fibrinogen, vitronectin, and von Willebrand factor (Charo et al., 1987; Cheresch, 1987; Chen et al., 1987). Thus, in terms of ligand specificity, the adhesion receptor on endothelial cells is similar to platelet GPIIb-IIIa in that it can interact with fibrinogen, von Willebrand factor, and vitronectin.

Several lines of investigation have demonstrated that thrombospondin is capable of interacting with cell surfaces. Thrombospondin binds to a low number of receptors in resting platelets (Wolff et al., 1986). After thrombin treatment of the platelets, a 10-fold increase in the number of receptor sites is observed (Wolff et al., 1986). Maximal binding activity is observed in the presence of calcium (Wolff et al., 1986; Aiken et al., 1987). Thrombospondin has also been shown to bind to mouse peritoneal macrophages and to cells of the monocyte-like human cell line U937 (Silverstein and Nachman, 1987). The binding of thrombospondin to these cell types is divalent cation-dependent (Silverstein and Nachman, 1987). Recently, an 88,000-D membrane glycoprotein that is present in platelets, endothelial cells, monocytes, and several human tumor cell lines has been reported to function as a receptor for thrombospondin (Asch et al., 1987).

Platelet thrombospondin has been reported to support attachment of human squamous carcinoma cells, human melanoma cells, human platelets, human fibroblasts, bovine endothelial cells, and porcine epithelial cells (Varani et al., 1986; Roberts et al., 1987; Tuszyński et al., 1987). Cell spreading on the thrombospondin substrates varies considerably for the various cell types and lines (Varani et al., 1986; Roberts et al., 1987; Tuszyński et al., 1987). Human G36Cl melanoma cells spread on thrombospondin substrates (Roberts et al., 1987). Roberts et al. (1987) have found that the heparin-binding domain must be present for thrombospondin to support spreading. By contrast, the 18,000-D COOH-terminal portion of the molecule has been found to be essential for cell attachment (Roberts et al., 1987). In this paper, we report that thrombospondin supports attachment of human endothelial cells, human smooth muscle cells, human U937 cells, and normal rat kidney (NRK) cells by RGD and calcium-dependent mechanisms. In addition, the glycoprotein IIb-IIIa-like molecules on endothelial cells and smooth muscle cells are identified as a receptor for RGD-dependent binding of thrombospondin.

**Materials and Methods**

**Preparation of Protein Substrates**

Human platelet thrombospondin and human plasma fibronectin were purified and iodinated as described previously (Engvall and Ruoslahti, 1977; Lawler et al., 1985). Human vitronectin was obtained from Calbiochem-Behring Corp. (La Jolla, CA) and human fibrinogen was obtained from Kabi Vitrum (Stockholm, Sweden). BSA (Sigma RIA grade) was dissolved in 10 mM Hepes (pH 7.2), 135 mM NaCl, 3 mM KCl, and 0.5 mM MgCl2 (HBS) at a concentration of 2 mg/ml and heated to 70°C for 1 h. After heat treatment, 1 mM CaCl2 or 0.5 mM EGTA was added to the BSA solutions. The synthetic polypeptides GRGDSP, GRGESP, and GRGDAE were obtained from Peninsula Laboratories Inc. (Belmont, CA).

Thrombospondin, fibronectin, vitronectin, or fibrinogen were diluted to 20-40 µg/ml in HBS containing either 1 mM CaCl2 or 0.5 mM EGTA and adsorbed to the surface of 35-mm dishes (No. 1008; Falcon Labware, Oxford, CA) at 37°C for 2 h. To determine the amount of thrombospondin that is adsorbed to the surface of the dishes, thrombospondin was radiolabeled with 125I-Bolton-Hunter reagent using protocols that are provided by the supplier (DuPont Co., Wilmington, DE). A thrombospondin concentration of 0.02 mg/ml resulted in the adsorption of 1.4 ± 0.3 µg/cm² in either the presence of 1 mM CaCl2 or 0.5 mM EGTA.

After protein adsorption, the dishes were washed three times with 3 ml of HBS containing either 1 mM CaCl2 or 0.5 mM EGTA and 1.5 ml of HBS containing 2 mg/ml BSA, and either 1 mM CaCl2 or 0.5 mM EGTA were added. Synthetic peptides were added at this point to some of the dishes. In some experiments, the proteins were incubated in the dishes at 37°C for 2 h in the presence of 0.5 mM EGTA. The dishes were then washed twice in HBS containing 0.5 mM EGTA and then washed once in HBS containing 1 mM CaCl2 before addition of the cells. In some experiments, 1.5 ml of HBS containing 1 mM CaCl2 and 2 mg/ml BSA were added to the thrombospondin-coated dishes and then 2.25 ml of HBS, preimmune or anti-thrombospondin antibodies (0.6-0.8 mg/ml) were incubated overnight at 4°C in the dishes. The preimmune and anti–human platelet thrombospondin antisera were fractionated by protein A-Sepharose affinity chromatography as described previously (Lawler et al., 1985). After incubation, the dishes were emptied and 1.5 ml of HBS containing 1 mM CaCl2 and 2 mg/ml BSA were added before the addition of 1.5 ml of cell suspension.

**Attachment Assay**

Human umbilical vein and arterial endothelial cells and human arterial smooth muscle cell were grown in culture as described previously (Weinstein and Wenc, 1986). Human U937 cells were kindly provided by Dr. Livingston Van der Water (Beth Israel Hospital, Boston, MA) and were grown in DME containing 4.5 g/liter glucose and 10% FCS. The NRK cells were grown in DME containing 4.5 g/liter glucose and 5% FCS. The endothelial, smooth muscle and NRK cells were washed once with 5 ml of HBS and were removed form the flasks with 5 ml of HBS containing 0.1 mg/ml TPCK-trypsin (Worthington Biochemical Corp., Freehold, NJ) at 37°C for 5 min with continuous agitation. The cell suspension was removed, mixed with 5 ml of HBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) and centrifuged at 400 g for 5 min. The cells were washed three times in HBS containing 0.5 mg/ml soybean trypsin inhibitor and then resuspended in a volume of HBS to give a final cell count of 2×10⁶ cells/ml.

1. Abbreviation used in this paper: NRK, normal rat kidney.
of 0.5–3.3 × 10^6 cells/ml. The U937 cells were grown in suspension and were harvested by centrifugation at 400 g for 5 min followed by three washes in HBS. The cell suspension (1.5 ml) was added to each protein-coated dish and the dishes were incubated at 37°C for 2 h. After incubation, the dishes were washed three times with 3 ml of HBS alone or with HBS containing 1 mM CaCl_2 or 0.5 mM EGTA. The cells were fixed with 3% paraformaldehyde, stained with Giemsa, and photographed. The number of cells bound was determined by counting representative areas of the photomicrograph.

To determine the effect of the monoclonal antibody LM609 (kindly provided by Dr. David Cheresh, La Jolla Cancer Research Foundation, La Jolla, CA) on attachment, endothelial cells were incubated with varying concentrations of antibody in a total volume of 200 μl at 4°C for 1 h. The antibody was purified from ascites by affinity chromatography on a column of protein A-Sepharose (Sigma Chemical Co.). After the incubation, the cell suspension was centrifuged at 400 g for 6 min. The cell pellet was resuspended in 1.5 ml of HBS and added to the protein-coated dishes which were then incubated at 37°C for 1 h, washed, and fixed as described above.

**Affinity Chromatography**

Endothelial cells or smooth muscle cells in four 100-mm dishes were washed three times in PBS (with 1 mM CaCl_2 and 1 mM MgCl_2). The cell layers were then incubated with 0.5 ml of PBS containing 1 μCi 125I, 20 μg/ml lactoperoxidase, and 0.1 U/ml glucose oxidase. The dishes were rocked continuously for 10 min at 22°C. The iodination mixture was removed and the cell layers were washed three times with PBS containing 1 mM NaI. The cell layers from all four dishes were solubilized with 10 μl of PBS containing 200 mM n-octylglucoside (Boehringer Mannheim Biochemicals, Indianapolis, IN). The extract was incubated at 0°C for 15 min and then centrifuged for 15 min at 3,000 g. The supernatant was decanted and applied to either a column (0.8 × 4 cm) of thrombospondin–Sepharose or a column (0.8 × 1 cm) of GRGDSP–Affigel 10. After the sample had entered the columns, the flow was stopped for 1 h. The columns were eluted with 60 ml PBS containing 50 mM n-octylglucoside (buffer A), followed by 2.5 ml of buffer A containing 1 mg/ml of the peptide GRGES, and finally 2.5 ml of buffer A containing 1 mg/ml of the peptide GRGDSP.

**Immunoprecipitation**

Immunoprecipitation of the various fractions was performed as described by Marcantonio and Hynes (1988). Protein A-purified monoclonal antibody LM142 (kindly provided by Dr. David Cheresh) was coupled to Sepharose 4B following standard procedures suggested by the supplier (Pharmacia Fine Chemicals, Piscataway, NJ). 100 μl of the antibody–Sepharose suspension (∼20 μl of packed beads) was mixed with 50–500 μl of sample. After immunoprecipitation, the pellet was solubilized in sample buffer and was subjected to electrophoresis on 3.5–10% acrylamide gradient gels (Lawler et al., 1985).

**Results**

**Attachment of Cells to Thrombospondin**

Human endothelial cells, smooth muscle cells, and U937 cells, as well as NRK cells, attach to thrombospondin which is adsorbed to plastic surfaces in the presence of 1 mM CaCl_2 (Figs. 1 and 3). The effect of varying the concentration of thrombospondin used to coat the dishes was assayed with NRK cells. The number of cells that attach increases as the concentration of the thrombospondin solution used to coat the dishes increases up to 25 μg/ml (data not shown). From 25 to 75 μg/ml the number of cells that attached remained constant. A thrombospondin concentration of 20 μg/ml was used for the remainder of the experiments described in this report. All four of the cell types studied fail to attach to heat-treated BSA (Fig. 1 b). Less than 5% of the attached (anchorage-dependent) cells (endothelial, smooth muscle, and NRK) spread on the thrombospondin substrate (Figs. 1–3). By contrast, the majority of the attached cells spread on fibronectin substrates, when 1 mM CaCl_2 is present (Figs. 1 and 2). Preincubation of the thrombospondin-coated dishes with protein A-purified polyclonal anti–thrombospondin antibodies reduced the number of attached endothelial, smooth muscle, or NRK cells to 20 ± 6.4, 9.4 ± 2.1, or 21 ± 5.8% of control, respectively. Preincubation of the thrombospondin-coated dishes with the preimmune immunoglobulin did not reduce the number of cells attached (99 ± 6% of control averaged over the three cell types). U937 cells contain immunoglobulin receptors and could not be readily studied in this system (Fleit et al., 1982).

**The Effect of Calcium on the Attachment of Cells to Thrombospondin**

Since calcium modulates the structure of thrombospondin, the effect of calcium on the attachment of the four cell types...
The effect of calcium on the attachment of smooth muscle cells to (a and c) thrombospondin (TSP) and (b and d) fibronectin (FN). The dishes were coated with protein (0.02 mg/ml) in HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d) for 2 h at 37°C. The dishes were washed three times with HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d). BSA solution (2 mg/ml) prepared in HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d) was added before addition of the cells that were resuspended in HBS. Bar, 100 μm.

Figure 2. The effect of calcium on the attachment of smooth muscle cells to (a and c) thrombospondin (TSP) and (b and d) fibronectin (FN). The dishes were coated with protein (0.02 mg/ml) in HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d) for 2 h at 37°C. The dishes were washed three times with HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d). BSA solution (2 mg/ml) prepared in HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d) was added before addition of the cells that were resuspended in HBS. Bar, 100 μm.

was investigated. When the thrombospondin is adsorbed to the dishes in the presence of 0.5 mM EGTA and the cell attachment assay is performed in the presence of 0.25 mM EGTA, the attachment of all four cell types is abolished (Fig. 2). Note that divalent cations are present in this system, which contains 0.25 mM Mg++. The loss of attachment activity is not due to an inability of thrombospondin to bind to the plastic surface in the presence of EGTA since an equivalent amount of radiolabeled thrombospondin bound to the dishes in either 1 mM CaCl₂ or 0.5 mM EGTA (data not shown). Under these experimental conditions the cells are still capable of attaching to fibronectin, albeit at a decreased level (Fig. 2). The percentage of cells that spread on the fibronectin substrate is markedly reduced in the presence of EGTA. These data indicate that the effect of EGTA may be, at least in part, due to an effect on the cells. To distinguish the effect of calcium on the thrombospondin substrate from its effect on the cells, the thrombospondin was adsorbed to the dishes in the presence of 0.5 mM EGTA and then washed with buffer containing 1 mM CaCl₂ before the incubation of the cells in the presence of 0.5 mM calcium. The attachment of endothelial cells, smooth muscle cells, U937 cells, and NRK cells to this substrate was reduced to 1.1 ± 0.8, 11 ± 2, 0.4 ± 0.7, and 20 ± 3% of control values, respectively (Fig. 3). By contrast, equivalent numbers of attached and spread NRK cells were observed on fibronectin substrates prepared in the presence of 1 mM CaCl₂ or 0.5 mM EGTA, when the cells were added in the presence of 0.5 mM CaCl₂ (data not shown). Thus, some of the effect of calcium depletion is on the thrombospondin.

The Effect of Synthetic Peptides on the Attachment of Cells to Thrombospondin

The hexapeptide GRGDSP inhibits the attachment of all four cell types to thrombospondin in a dose-dependent manner (Figs. 4 and 5). At a concentration of 0.4 mM GRGDSP, the inhibition was >94% for all the four cell types. By contrast, the synthetic peptide GRGESP had only a small inhibitory effect at the highest concentration (0.4 mM) tested (Figs. 4 and 5).

Based on the thrombospondin sequence, the peptide GRGDAC was synthesized and its effect on attachment of the four cell types to thrombospondin was determined. This peptide also inhibited attachment of all four cell types to thrombospondin in a dose-dependent manner (Fig. 5). A concentration of 0.4 mM GRGDAC reduced the number of endothelial, smooth muscle, U937, or NRK cells, which attached to thrombospondin, to 15 ± 7.3, 4.3 ± 3.6, 1.7 ± 1.0, or 27 ± 2% of control, respectively.

Identification of a RGD-dependent Receptor

To identify the receptor for thrombospondin, endothelial,
The effect of calcium on the attachment of NRK (a, c, and e) and U937 (b, d, and f) cells to thrombospondin. The dishes were coated with thrombospondin (0.02 mg/ml) in HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c-f). The dishes were washed three times in HBS containing either 1 mM CaCl₂ (a and b) or 0.5 mM EGTA (c and d) as in Fig. 2. Two additional dishes (e and f) were washed twice in HBS containing 0.5 mM EGTA followed by one wash in HBS containing 1 mM CaCl₂. BSA solution (2 mg/ml) prepared in HBS containing either 1 mM CaCl₂ (a, b, e, and f) or 0.5 mM EGTA (c and d) was added before addition of the cells that were resuspended in HBS.

Bar, 200 μm.
attachment of endothelial cells to vitronectin and fibrinogen was also studied. The cellular attachment to vitronectin was inhibited by antibody LM609 to an extent that was quantitatively equivalent to the inhibition of attachment to thrombospondin (Fig. 9). By contrast, endothelial cell attachment to fibrinogen was completely abolished by the antibody LM609 at all concentrations studied (Fig. 9).

**Discussion**

The data presented show that thrombospondin can act as a cell attachment factor for a variety of cells. The attachment to thrombospondin of all four cell types included in this study is abolished if the thrombospondin is adsorbed to the dishes in the presence of EGTA and the cells are incubated in the dishes in the presence of EGTA. That is, attachment to thrombospondin is Ca\(^{++}\)-dependent whereas attachment to fibronectin is not. If dishes coated with thrombospondin in the absence of Ca\(^{++}\) are washed with buffer containing 1 mM CaCl\(_2\) before the addition of the cells, the attachment activity is partially recovered (10–20% of control) for the NRK and smooth muscle cells, but not for the endothelial or U937 cells (<2% of control). These results show that the major effect of Ca\(^{++}\) depletion is on the thrombospondin, not the cells, and indicate that, for the most part, the thrombospondin that is adsorbed to the plastic in the presence of EGTA does not revert to the calcium-dependent form upon calcium restoration. The low level of attachment that is observed on restoration of calcium may be due to the ability of some of the thrombospondin molecules to recover or may be due to an effect on the cell surface receptors. In the latter case, a calcium-dependent increase in the receptor's affinity for thrombospondin may partially compensate for the fact that the conformation of the thrombospondin molecules is not correct.

The data presented here also suggest that the GRGDAC sequence of thrombospondin is the functional site for the attachment of the four cell types studied. This sequence occurs in the last type 3 repeating sequence of thrombospondin (Lawler and Hynes, 1986; Lawler and Hynes, 1987). The sequences of the type 3 repeats are homologous with the calcium-binding sites of calmodulin and parvalbumin. The presence of the GRGDAC sequence in the calcium-binding region of thrombospondin is consistent with the observation that the attachment of cells to thrombospondin is calcium dependent. Calcium may be affecting the activity of the RGD
Figure 5. The effect of synthetic peptides on the attachment to thrombospondin of smooth muscle cells, U937 cells, NRK cells, and endothelial cells. The dishes were coated with thrombospondin (0.02 mg/ml dissolved in HBS containing 1 mM CaCl₂). Before addition of the cells, either no peptide (0), 0.2 mM GRGDSP, 0.4 mM GRGDSP, 0.2 mM GRGDAC, 0.4 mM GRGDAC, or 0.4 mM GRGESP was added to the dishes.

We propose that the calcium-binding sites of thrombospondin are loop structures based on their sequence homology with the calcium-binding sites of other proteins of established structure and based on secondary structural analysis using a predictive algorithm (Chou and Fasman, 1978; Kretsinger, 1980). A schematic diagram of the structure of the type 3 repeat which contains the RGD sequence is shown in Fig. 10. The α-helix and β-sheet potentials, calculated using the Chou and Fasman (1978) algorithm, are low throughout the region. By contrast, the loop potential is high in this region as indicated by the averages of the normalized frequencies of amino acid occurrence in omega loops (Leszczynski and Rose, 1986). A detailed algorithm for the prediction of loops has not been published to date. β-turns with relatively high probability are predicted at the beginning and end of the sequence shown in Fig. 10 (Chou and Fasman, 1978). The remainder of the sequence is proposed to form two loops around each calcium ion (Fig. 10). In this model the oxygen atoms of the D residues at the octahedral vertices x, y, z, -x, and -z form the calcium-binding site as they do in parvalbumin and related proteins (Kretsinger, 1980). There is one additional amino acid between the -x and -z coordinates in the first loop. A similar insertion occurs in the calcium-binding site of the gamma-subunit of fibrinogen (Dang et al.,

Figure 6. Isolation of a thrombospondin receptor from human endothelial cells. Endothelial cell membranes were radiolabeled with 125I-lactoperoxidase, extracted with 200 mM n-octylglucoside, and applied to a column of thrombospondin-Sepharose. The elution of the column is described in Material and Methods. The quantity of the 100,000-D protein was determined from densitometric scan of the autoradiograph. The initial lanes could not be quantitated because of the high level of background radioactivity. Note small increase in all of the radioactive binds after fraction 17 is probably due to a decrease in the flow rate that is done to conserve peptides.
The second loop brings the two C residues into close proximity. We propose that these C residues form a disulfide bond which stabilizes the loop.

In the model proposed in Fig. 10, the D residue of the RGD cell binding domain is one of the residues proposed to be involved in calcium binding. Assuming that calcium does, in fact, bind to this residue, there must be a mechanism that enables the D residue also to participate in the interaction with the cell surface receptor. This could be accomplished by a simple, competitive mechanism if the receptor affinity is high enough. Alternatively, it is possible that a calcium or magnesium ion also participates in the interaction with the cell surface receptor such that a calcium-binding site is formed by sequences from thrombospondin and its receptor. The α-subunits of the integrin family of adhesive protein receptors have calcium-binding sites that are similar in terms of amino acid sequence to those found in thrombospondin (Fitzgerald et al., 1987; Suzuki et al., 1987; Poncz et al., 1987).

The location of the RGD sequence within the type 3 repeat may have some implications for its evolutionary origin in thrombospondin. The RGD sequence occurs at positions 12–14 within the repeat (Lawler and Hynes, 1986). The GD sequence occurs at positions 13 and 14 of all of the type 3 repeats, suggesting that these residues are important for calcium binding. The 12 position is occupied by five different
amino acids, suggesting that this position can vary without loss of calcium-binding activity. We hypothesize that the seven type 3 repeats were formed by a series of endoduplications of a primordial gene that encoded a calcium-binding motif. The amino acids that were not specifically involved in calcium binding were free to diverge. When an R residue arose in position 12 of the last type 3 repeat, thrombospondin obtained the ability to interact with cell surfaces via an existing family of receptors.

The data presented here indicate that the GPIIb–IIIa-like complex on the surface of endothelial and smooth muscle cells functions as an RGD-dependent receptor for thrombospondin. These results and the data of Cheresh (1987) suggest that thrombospondin, vitronectin, fibrinogen, and von Willebrand factor interact with a common integrin receptor on endothelial cells. However, this conclusion needs to be forwarded with caution. Since the members of the β3 subfamily are very similar to each other and share a common β subunit, it is common for antibodies that have been raised against one member to cross react with other members of the subfamily. Antibodies that were raised against platelet GPIIb–IIIa inhibit the binding of thrombospondin to platelets (Plow et al., 1985). However, thrombasthenic platelets bind normal levels of thrombospondin (Aiken et al., 1986). This raises the possibility that the vitronectin, thrombospondin, fibrinogen, and von Willebrand receptors are similar but distinct entities. The monoclonal antibody LM609 reacts with the β3 integrin complex found on endothelial cells, smooth muscle cells, and melanoma cells (Cheresh, 1987; Cheresh and Spiro, 1987). This antibody does not immunoprecipitate the GPIIb–IIIa complex on platelets or the vitronectin receptor on MG63 osteosarcoma cells (Cheresh, 1987; Cheresh and Spiro, 1987).

Whereas the data presented here are consistent with previous studies in establishing that thrombospondin can support attachment of cells, most previous studies have not demonstrated an RGD effect (Varani et al., 1986; Roberts et al., 1987; Tuszynski et al., 1987). Santoro (1987) has reported that the attachment of platelets GPIIb–IIIa inhibit the binding of thrombospondin to platelets (Plow et al., 1985). However, thrombasthenic platelets bind normal levels of thrombospondin (Aiken et al., 1986). This raises the possibility that the vitronectin, thrombospondin, fibrinogen, and von Willebrand receptors are similar but distinct entities. The monoclonal antibody LM609 reacts with the β3 integrin complex found on endothelial cells, smooth muscle cells, and melanoma cells (Cheresh, 1987; Cheresh and Spiro, 1987).
types included in this study (Asch et al., 1987). However, the interaction with isolated GPIV is not inhibited by the synthetic peptide RGDS. Several hypotheses can be proposed to reconcile these data. The purification of GPIV may have induced a conformational change that has resulted in the loss of RGD specificity. Alternatively, another component of the membrane may bind to GPIV in much the same way GPIIb and GPIIIa form a complex. Such an additional component might confer RGD specificity. A third possibility is that GPIV is not functioning as the receptor in cell attachment assays. GPIV has been isolated from C32 melanoma cells by immunoaffinity columns prepared with the monoclonal antibody OKM5 (Asch et al., 1987). Roberts et al. (1987) have reported that antibody OKM5 does not inhibit attachment of C32 melanoma cells to thrombospondin.

The results presented here indicate that the RGD sequence of thrombospondin is active in supporting cell attachment. Furthermore, the ability of the RGD sequence to support attachment is dependent upon the preservation of calcium-dependent structures in thrombospondin. Whereas divalent cations have been shown to be important to the interaction of adhesive proteins with the integrin family of cell surface receptors, the divalent cations generally bind to the $\alpha$-subunits of these receptors. Thrombospondin represents the first example of divalent cation modulation of the RGD in the ligand. It is currently unclear how divalent cation concentration could be regulated in an extracellular environment. One possibility is that glycosaminoglycans could bind divalent cations and affect their local concentration. Alternatively, calcium binding may not be a regulatory mechanism for these proteins. The calcium ion may be a component of the native molecule that confers conformational stability or resistance to proteolysis.

We wish to thank David Cheresh for the generous supply of the monoclonal antibodies LM142 and LM609. The U937 cell line was kindly provided by Livingston Van der Water. We also wish to thank Gene Marcantonio and Gene Yee for advice and support. Expert technical assistance was provided by Paula Ferro, Karen Wenc, Mark Duquette, and Joseph Connolly. The art was done by Joan Joos, and the manuscript was edited by Sami Lawler and typed by Patsy Bustos.

This work was supported by National Institutes of Health Research Award HL-28749 (to J. Lawler) and CA-26712 (to R. O. Hynes), and by a grant by Paula Ferro, Karen Wenc, Mark Duquette, and Joseph Connolly. The art was designed by Patsy Bustos.

Received for publication 30 June 1988.

References

Aiken, M. L., M. H. Ginsberg, and E. F. Plow. 1986. Identification of a new class of adhesion receptors on platelets: thrombospondin interacts with platelets via a GPIIb-IIIa-independent mechanism. J. Clin. Invest. 78:1713-1716.

Aiken, M. L., M. H. Ginsberg, and E. F. Plow. 1987. Divalent cation-dependent and independent surface expression of thrombospondin on thrombin-stimulated human platelets. Blood. 69:58-64.

Asch, A. S., J. Barnwell, R. L. Silverstein, and R. L. Nachman. 1987. Isolation of the thrombospondin membrane receptor. J. Clin. Invest. 79:1054-1061.

Charo, I. F., L. A. Fitzgerald, B. Steiner, S. A. Nemeth, and L. S. Bekeart. 1988. Platelet glycoprotein Ib and IIB: evidence for a family of immunologically and structurally related glycoproteins in mammalian cells. Proc. Natl. Acad. Sci. USA. 83:8351-8355.

Chen, C. S., P. Thangarajan, S. M. Schwartz, J. M. Harlan, and R. L. Heimark. 1987. The platelet glycoprotein IIb/IIIa-like protein in human endothelial cells promotes adhesion but does not mediate attachment to extracellular matrix. J. Cell Biol. 105:1885-1892.

Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed receptor involved in attachment to vitronectin, fibrinogen and von Willebrand factor. Proc. Natl. Acad. Sci. USA. 84:6471-6475.

Cheresh, D. A., and R. C. Spiro. 1987. Biosynthetic and functional properties of an Arg-gly-asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand factor. J. Biol. Chem. 262:17703-17711.

Chou, P. Y., and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. 47:45-148.

Dang, C. V., R. P. Ebert, and W. R. Bell. 1985. Localization of a fibrinogen binding site between membrane proteins 311 and 336 by terbutylamine fluorescence. J. Biol. Chem. 250:9713-9719.

Dixit, V. M., J. K. Galvin, K. M. O'Rourke, and W. A. Frazier. 1986. Monoclonal antibodies that recognize calcium-dependent structures of human thrombospondin. J. Biol. Chem. 261:1962-1968.

Engvall, E., and E. Ruoslahti. 1977. Binding of the solvable form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer. 20:201-205.

Fitzgerald, L. A., I. F. Charo, and D. R. Phillips. 1985. Human and bovine endothelial cells synthesize membrane proteins similar to human platelet glycoproteins IIb and IIIa. J. Biol. Chem. 260:10893.

Fitzgerald, L. A., M. Poncz, B. Steiner, S. C. Rall, Jr., J. S. Bennett, and D. R. Phillips. 1987. Comparison of cDNA-derived protein sequences of the human fibronectin and vitronectin receptor a-subunits and platelet glycoprotein IIb. Biochemistry. 26:8158.

Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fcgamma receptor distribution and structure. Proc. Natl. Acad. Sci. USA. 79:3275-3279.

Galvin, N. J., V. M. Dixit, K. M. O'Rourke, S. A. Santoro, G. A. Grant, and W. A. Frazier. 1985. Mapping of epitopes for monoclonal antibodies against human platelet thrombospondin with electron microscopy and high sensitivity amino acid sequence. J. Biol. Chem. 260:1434-1441.

Ginsberg, M. H., J. C. Loftus, and E. R. Plow. 1988. Cytoadhesion, integrins and platelets. Throm. Haemostasis. 59:1-6.

Ginsburg, M. H., J. C. Loftus, J. Rychwarta, M. Pierschbacher, R. Pytela, E. Ruoslahti, and E. F. Plow. 1989. Immunochemical and amino-terminal sequence comparison to two cytoadhesins indicates they contain similar or identical $\beta$ subunits and distinct $\alpha$ subunits. J. Biol. Chem. 262:5437-5440.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. Cell. 59:455-554.

Kretsinger, R. H. 1980. Structure and evolution of calcium-modulated proteins. CRC Crit. Rev. Biochem. 1:119-174.

Lawler, J. 1986. The structural and functional properties of thrombospondin. Blood. 67:1197-1209.

Lawler, J., and R. O. Hynes. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. J. Cell Biol. 103:1635-1648.

Lawler, J., and R. O. Hynes. 1986. Structural organization of the thrombospondin molecule. Semin. Thromb. Hemostasis. 13:245-254.

Lawler, J., and E. Simons. 1983. Cooperative binding of calcium to thrombospondin: the effect of calcium on the circular dichroism and limited tryptic digestion of thrombospondin. J. Biol. Chem. 258:12098-12101.

Lawler, J., J. H. Derick, J. E. Connolly, J. H. Chen, and F. C. Chao. 1985. The structure of human platelet thrombospondin. J. Biol. Chem. 260:3762-3774.

Leekie, L., H. B. Kanzig, J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.

Leekie, L., and J. C. Unkeless. 1982. Human neutrophil Fcγ receptors. Curr. Develop. Immunol. 1:15-19.
Poncz, M., R. Eisman, R. Heidenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb: homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. J. Biol. Chem. 262:8476-8482.

Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science (Wash. DC). 231:1559-1562.

Roberts, D. D., J. A. Sherwood, and V. Ginsburg, 1987. Platelet thrombospondin mediates attachment and spreading of human melanoma cells. J. Cell Biol. 104:131-139.

Ruoslahti, E., and M. D. Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. Cell. 44:517-518.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. Science (Wash. DC). 238:491-497.

Santoro, S. A. 1987. Thrombospondin and the adhesive behavior of platelets. Semin. Thromb. Hemosisssus. 13:280-297.

Silverstein, R. L., and R. L. Nachman. 1987. Thrombospondin binds to monocytes-macrophages and mediates platelet-monocyte adhesion. J. Clin. Invest. 79:867-874.

Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor alpha-subunit and comparative expression of adhesion receptor mRNAs. J. Biol. Chem. 262:14080-14085.

Thiagarajan, P., S. S. Shapiro, E. Levine, L. DeMarco, and A. Yalcin. 1985. A monoclonal antibody to human platelet glycoprotein IIb detects a related protein in cultured human endothelial cells. J. Clin. Invest. 75:896-901.

Tuszynski, G. P., V. Rothman, A. Murphy, K. Siegler, L. Smith, S. Smith, J. Karczewski, and K. A. Knudsen. 1987. Thrombospondin promotes cell-substratum adhesion. Science (Wash. DC). 104:131-139.

Varani, J., V. M. Dixit, S. E. G. Fligiel, P. E. McKeever, and T. E. Carey. 1986. Thrombospondin-induced attachment and spreading of human squamous carcinoma cells. Exp. Cell Res. 167:376-390.

Weinstein, R., and K. Wenc. 1986. Growth factor responses to human arterial endothelial cells in vitro. In Vitro Cell. Dev. Bio. 22:549-556.

Wolff, R., E. F. Plow, and M. H. Ginsberg, 1986. Interaction of thrombospondin with resting and stimulated human platelets. J. Biol. Chem. 261:6840-6846.

Yamada, K. M., and D. W. Kennedy. 1984. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. J. Cell Biol. 99:29-36.