Sense and Antisense cDNA Transfection of CD36 (Glycoprotein IV) in Melanoma Cells

ROLE OF CD36 AS A THROMBOSPONDIN RECEPTOR*

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Thrombospondin (TSP) is a multifunctional matrix and platelet glycoprotein that interacts with cell surfaces and may play a role in mediating cell adhesion, platelet aggregation, platelet-monocyte interactions, cell proliferation, angiogenesis, tumor metastasis, and protease generation. To clarify and confirm the function of CD36 (glycoprotein IV) as a TSP receptor, we now describe a transfected cell model using human melanoma cells genetically manipulated by sense or antisense cDNA transfection to express either high or near zero levels of CD36. Surface expression was confirmed by flow cytometry with monoclonal anti-CD36 IgG and quantified by measuring radiolabeled antibody binding. Bowes melanoma cells, which in their wild type did not express CD36 and did not bind radiolabeled TSP when transfected with the sense construct bound TSP in a 1:1 stoichiometric ratio with CD36 expression. Conversely, C32 melanoma cells, which in their wild type expressed high levels of CD36 and bound radiolabeled TSP at a 1:1 stoichiometric ratio, did not express CD36 and did not bind TSP when transfected with an antisense construct. In addition, transfected Bowes cells and wild type C32 cells, unlike wild type Bowes cells, adhered to activated platelets in a TSP-dependent manner. These data, i.e. the gain of function with sense cDNA transfection and loss of function with antisense transfection, strongly support the TSP receptor function of CD36. The distribution of this protein in vascular cells and tissues and observations that it may participate in signal transduction events suggest that TSP-CD36 interactions may play a role in mediating some of the pathophysiological processes associated with TSP.

Thrombospondin (TSP) is a large trimeric glycoprotein (1) produced in a regulated fashion (2) and incorporated into extracellular matrix (3) by a variety of cells, including those of the vascular endothelium (4) and smooth muscle (5), as well as certain tumor cells (6). TSP is also present in platelet α-granules (7), from which it is secreted upon platelet activation. TSP is a multifunctional protein with a complex biology. Based on in vitro data, it has been proposed that TSP may play a role in fibrin and matrix structure and function (8) and in mediating cell adhesion (9–11), platelet aggregation (12), platelet-monocyte interactions (13), cell proliferation (14), angiogenesis (15), tumor metastasis (16), and plasmin generation (17, 18). Many of the functions of this molecule may depend upon interactions with specific cellular receptors.

The identity of the TSP receptor is controversial. At least three potential cell recognition domains have been identified in the TSP molecule. There are three copies of an RGDα sequence (19), a potential integrin binding site, which Lawler et al. (20) have demonstrated may interact with αvβ3 integrin (the vitronectin receptor). The cellular distribution and density of this receptor, however, cannot account for all of the activity of TSP. TSP also contains at its N terminus a heparin binding domain (21) which has been shown to interact with heparan sulfate proteoglycans and perhaps sulfated glycolipids on cell surfaces (22, 23). Mosher and colleagues (24) have shown that TSP binding and uptake by fibroblasts and endothelial cells were inhibited by heparin and that mutant Chinese hamster ovary cell lines deficient in surface proteoglycans did not bind TSP (25). However, we and others have shown that TSP binding to certain cells, including monocytes and melanoma cells, could not be blocked by either heparin (13, 26) or RGD-containing peptides and that a proteolytic fragment of TSP missing the heparin binding domain was capable of supporting TSP adhesion to tumor cells (27). Prater et al. (28) have recently found that synthetic peptides based on the properdin-like type I repeats of TSP promote attachment of tumor cells.

Asch and colleagues have identified CD36 (also known as platelet glycoprotein IV (GPIV) or GPIIb) as a potential TSP receptor, based on the ability of monoclonal anti-CD36 antibodies to block TSP binding (26) and adhesion to tumor cells (27). They have also demonstrated that a synthetic peptide based on the properdin-like type I repeat of TSP may be the recognition domain for this protein (29). We have shown that antibodies to this protein also inhibit TSP binding to monocytes (30). CD36 is a transmembrane glycoprotein of a molecular weight of ≈ 88,000 found on platelets (31), monocytes (32), microvascular endothelial cells, breast epithelium (33), and some tumor lines (26). It may also function as a collagen receptor (34) and as an adhesion "receptor" for falciparum malaria-infected erythrocytes (35). Data supporting the role of CD36 as a TSP receptor are inconclusive, however, in that although CD36 and TSP have been shown to interact in purified systems (26, 36), monoclonal antibody
inhibition studies of TSP-cell interactions have been inconsistent (37), and COS cells transfected with the CD36 cDNA did not bind TSP (38).

To clarify and confirm the function of CD36 as a TSP receptor, we now describe a transfected cell model using human cells genetically manipulated to express either high or near zero levels of CD36. We found that TSP bound to these cells in a manner that correlated exactly with CD36 expression and that TSP binding occurred at a 1:1 stoichiometric ratio with CD36 surface expression.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—C32 amelanotic melanoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD), and Bowes melanoma cells from Dr. D. B. Rifkin, NYU Medical Center, New York. Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO/BRL) + 10% fetal calf serum (Hazelton, Lenexa, KS). Transfected cells were grown in the same medium containing the neomycin analog GM418 (GIBCO/BRL) at 0.25 mg/ml.

cDNA Transfections—A CD36 cDNA clone containing the complete coding sequence in the plasmid vector CDM8 was kindly provided by Dr. J. Barnwell (NYU Medical Center). A 1,759-bp XbaI fragment of this plasmid representing 32 bp from the vector and the initial 1,727 bp of the insert (including the entire coding sequence) was cloned into pUC18 and then both orientations were ligated into the vector and the initial 1,727 bp of the insert (including the entire coding sequence) was cloned into pUC18 and then both orientations of the inserts were determined by restriction mapping. Briefly, cells were digested with EcoRI and HindIII and Aliquots were hybridized for 20 h at 37 °C with 0.5-1.0 ng of either TSP Binds CD36-transfected Melanoma Cells

**RESULTS**

**Flow Cytometry**—Purified murine monoclonal anti-CD36 IgG 8A6 (41) was kindly provided by Dr. J. Barnwell (NYU Medical Center). Melanoma cells (wild type or transfected) were removed from culture dishes with EDTA, washed three times, and resuspended in phosphate-buffered saline containing 0.3% bovine serum albumin (Pentex BSA, Miles Laboratories, Naperville, IL). Cells were then aliquoted into 100-μl volumes containing 106 cells and incubated with the 8A6 IgG (2 μg/ml) or control non-immune mouse IgG, for 30 min at 4 °C. The cells were then pelleted by centrifugation and resuspended in phycocerythrin-conjugated goat anti-mouse IgG (Tago Immunologicals, Burlingame, CA) at a 1:100 dilution in the same buffer for 20 min at 22 °C. The cells were then pelleted, resuspended in 0.5 ml filtered phosphate-buffered saline and immediately analyzed with an Epics flow cytometer (Coulter Instruments, Hialeah, FL) equipped with a MDADS data handling computer and an argon laser. Sizing gates were set to include all nucleated cells, and for each sample at least 105 cells were analyzed.

**RNase Protection Assays**—Washed cells were dissolved in 5 M guanidine thiocyanate in 0.1 M EDTA at 2 × 105 cells/ml. Twenty-μl aliquots were hybridized for 20 h at 37 °C with 0.5-1.0 ng of either sense or antisense CD36 RNA probes labeled to specific activities of ≈ 1-2 × 106 cpm/μg with [32P]UTP. Riboprobes were transcribed with either T7 RNA polymerase (antisense) or T3 RNA polymerase (sense) obtained from Promega (Madison, WI) from a 792-bp 5' BamHI fragment of the CD36 cDNA cloned into Bluescript plasmid (Stratagene, La Jolla, CA). After hybridization, the samples were digested with RNAse A and spleen T1 (Sigma), and the protected fragments were electrophoresed on 5% polyacrylamide gels in Tris borate buffer. Autoradiograms of the dried gels were obtained on Kodak X-OMAT-AR film.

**125I-TSP Binding Assays**—Purified human calcium-replete TSP was prepared from thrombin-induced platelet releasates as described previously (42) by sequential heparin-Sepharose affinity chromatography and anion exchange chromatography using a Mono Q column and a fast pressure liquid chromatography system (Pharmacia LKB Biotechnology Inc.). TSP was labeled with Na125I using immobilized chloramine T (IODO-BEADS) obtained from Pierce Chemical Co. Melanoma cells were removed from culture dishes with EDTA, washed three times, and resuspended at 7 × 105 cells/ml in HEPES-buffered saline (20 mM HEPES, 0.15 mM NaCl, pH 7.4) containing 1 mM CaCl2 and 2 mM mg BSA (HBBS/BSA) prior to use. The cells were then aliquoted into 1.5-ml polypropylene microcentrifuge tubes (0.08 ml/tube) containing radiolabeled TSP (final volume 0.1 ml) diluted in the same buffer. For concentration isotherms, increasing amounts of a fixed ratio of labeled and unlabeled TSP (specific activity 0.1 μCi/μg) were added. After rotating end-over-end for 45 min at 4 °C, the cell suspensions were layered onto 0.4 ml of silicone oil (60/40 v/v 12° C) (Cornell Medical College, contains 20 ml of silicone oil (60/40 v/v 12° C) (Cornell Medical College, contains 20 ml of silicone oil (60/40 v/v 12° C) (Cornell Medical College, contains 20 ml of silicone oil (60/40 v/v 12° C) and 3' Moloney leukemia virus long terminal repeat sequences flanking a neomycin resistance gene and the EcoRl/HindIII cloning site. Orientation of the inserts was confirmed by restriction mapping: and two clones were isolated, pMV7/CD36A, containing the insert in the sense orientation, and pMV7/CD36D, containing the insert in the antisense orientation. C32 melanoma cells, which express high levels of CD36, and Bowes melanoma cells, which do not express CD36, were transfected with supercoiled plasmid DNA (antisense and sense constructs, respectively) by calcium phosphate co-precipitation (40). Briefly, cells were plated on 60-mm dishes at ≈ 40% confluence, twenty-four h later (at ≈ 60% confluence) they were washed and overlaid with the DNA-containing precipitates (7.5-10 μg/dish) for 5 min at 22 °C. Cold fresh medium was then added and selection begun 24 h later with the addition of GM418 (250 μg/ml) to the medium. Transfected cells were maintained continuously in GM418-containing medium. After selection, the Bowes cells, GM418-resistant cells were pooled, expanded by passage twice at 1:3, and then selected for high CD36 expression by two rounds of adsorption on anti-CD36 monoclonal antibody 8A6-conjugated magnetic beads (Dynabeads M-450, Dynal Inc., Great Neck, NY). Quantitative TSP and anti-CD36 binding studies were done 3-4 days after selection and the clones were passed in culture for a total of 4-10 times. Cells were periodically monitored by flow cytometry for surface expression of CD36, by polymerase chain reaction for the presence of the transfected DNA, and by RNase protection assay for presence of RNA transcripts.

**Establishment of Cell Lines**—Several human melanoma cell lines were analyzed by flow cytometry for CD36 expression. One that did not express CD36 (Bowes) and one that expressed high levels of CD36 (C32) were subsequently chosen for transfection experiments. Transfection was accomplished using a CD36 cDNA clone into a plasmid expression vector (pMV7) 5' to the coding sequence for the bacterial neomycin phosphotransferase gene. Transcription is driven by Moloney leukemia virus long terminal repeats. GM418-resistant Bowes
cells were enriched for CD36 expression by two rounds of immunoselection on magnetic beads coupled to a murine monoclonal anti-CD36 IgG (8A6). As shown in Fig. 1, a stable line of transfected cells was generated which expressed high levels of CD36 as assessed by flow cytometry. Surface labeling of these cells with 

\[ ^{32} \text{P} \text{IgG} \] followed by immunoprecipitation with 8A6 anti-CD36 demonstrated that the recombinant CD36 expressed on the surface of these cells co-migrated with native CD36 immunoprecipitated from C32 cells (data not shown).

Similarly, C32 cells were stably transfected with the same plasmid, in which the CD36 sequence was placed in the monoclonal anti-CD36 IgG (8A6). As shown in Fig. 2, unlike the parent wild type cells, these transfected cells expressed little or no CD36. RNase protection assays were performed on the wild type and transfected cells to detect the presence of native and/or transgenic CD36 transcripts. As shown in Fig. 3, transcripts (i.e. protected species of appropriate size) were seen in the transfected Bowes cells (lane E) and wild type C32 cells (lane C), but not the wild type Bowes cells (lane F), consistent with the data obtained on analysis of protein expression. Differences in transcript level in the transfected Bowes compared with the wild type C32 may reflect differences in message stability or may be because RNA was obtained from the Bowes cells prior to immunoselection. The antisense-transfected C32 cells contained a markedly reduced level of native transcripts (lane D) but a high level of antisense message detected with a sense probe (lane B). This is also consistent with the protein expression data and suggests that the inhibition of CD36 expression on these cells was a result of the intracellular production of antisense mRNA by the transgene.

**CD36-dependent Binding of TSP to Melanoma Cells**—We have shown previously that TSP bound specifically to C32 melanoma cells and that the binding was inhibited by monoclonal antibodies reactive with CD36 (25). As shown in Fig. 4, we now show that TSP bind only minimally to Bowes melanoma cells, a line that does not express CD36 protein (Fig. 1) or message (Fig. 3). This binding was inhibited by 100 \( \mu \text{g/ml} \) heparin sulfate. When this line was genetically altered by transfection to express CD36, however, markedly increased specific binding capacity for TSP was gained. As shown in Fig. 4, the transfected cells bound \( ^{125} \text{T}-\text{TSP} \) in a concentration-dependent, saturable manner. Saturation was attained at \( \approx 200 \text{nM} \) TSP. Binding was completely reversible at 4 °C and

**Fig. 1.** Flow cytometric demonstration of CD36 expression on Bowes melanoma cells. Wild type (dotted line) or CD36 sense cDNA-transfected cells (solid line) were incubated in suspension with murine monoclonal anti-CD36 IgG 8A6 (2 \( \mu \text{g/ml} \)) in HBS/BSA for 30 min at 4 °C. Cells were then pelleted, resuspended in phycoerythrin-conjugated goat anti-mouse IgG for 20 min at 22 °C, and analyzed by flow cytometry. Histograms demonstrate 8A6 binding (i.e. CD36 expression) on the transfected cells, not the wild type. Transfected cells incubated with control mouse IgG (dashed line) did not show fluorescence.

**Fig. 2.** Flow cytometric demonstration of CD36 expression on C32 melanoma cells. Wild type (dashed line) or CD36 antisense cDNA-transfected cells (solid line) were incubated in suspension with murine monoclonal anti-CD36 IgG 8A6 (2 \( \mu \text{g/ml} \)) in HBS/BSA for 30 min at 4 °C. Cells were then pelleted, resuspended in phycoerythrin-conjugated goat anti-mouse IgG for 20 min at 22 °C, and analyzed by flow cytometry. Histograms demonstrate 8A6 binding (i.e. CD36 expression) on the wild type cells, with minimal expression on the transfected cells. Wild type cells incubated with control mouse IgG (dotted line) did not show fluorescence.

**Fig. 3.** CD36 RNA transcripts in melanoma cells. Washed cells were dissolved in 5 M guanidine isothiocyanate and aliquots hybridized for 20 h at 37 °C with 0.5–1.0 ng of either sense (lanes A and B) or antisense (lanes C–F) CD36 \( ^{32} \text{P} \)-labeled RNA probes. Probes were transcribed from a 792-bp BamHI fragment of the CD36 cDNA cloned into Bluescript. After hybridization, the samples were digested with RNase A and RNase T1 and the protected fragments electrophoresed on 5% polyacrylamide gels in Tris borate buffer. Autoradiograms of the dried gels were obtained on Kodak X-OMAT-AR film. Protected species of the appropriate size were seen with wild type C32 cells (C) and sense-transfected Bowes cells (E) hybridized to antisense RNA. Protected species were seen with C32 cells transfected with antisense cDNA (B) probed with sense RNA. A faint native (sense) transcript was detected in the antisense-transfected C32 cells with the antisense probe (D). No transcripts were detected in wild type Bowes cells hybridized to antisense probes (F) or wild type C32 hybridized to sense probes (A).
we also studied adhesion of these tumor cells to thrombin-
to 1:1. The number of TSP binding sites on the transfected cells at
saturation was 1.2
in Fig. 4. 125I-TSP binding to Bowes melanoma cells. Melano-
ma cells (≈ 7 × 10⁶ cells/ml) suspended in HBS/BSA were ali-
quotted into 1.5-ml polystyrene microcentrifuge tubes (0.08 ml/tube) containing radiiodinated TSP (final volume 0.1 ml) diluted in
the same buffer. After rotating end-over-end for 45 min at 4 °C, the
cell suspensions were centrifuged through silicone oil and cell-bound
radioactivity counted. A representative isotherm is shown demon-
strating saturable, concentration-dependent binding of TSP to the
transfected cells (■) but not to the wild type cells (□). Specific (i.e.
EDTA-inhibitable) binding is plotted, with each point representing
the mean of duplicate tubes. Nonspecific binding averaged <0.5% of
input radioactivity. Cell viability was assessed by dye exclusion and
was >98%.

was inhibited by adding EDTA or a 10-fold molar excess of
unlabeled TSP to the reaction mix, demonstrating specificity.
No differences were seen when binding was measured at 37 °C.
The number of TSP binding sites on the transfected cells at
saturation was 1.2 × 10⁵/cell. 125I-8A6 binding to these cells
was done in parallel to the TSP binding to estimate the number of CD36 molecules expressed on each cell. As shown
in Fig. 5, the transfected Bowes cells expressed 1.27 ± 0.10 × 10⁵
8A6 binding sites/cell. Thus, the stoichiometric ratio of
TSP binding to 8A6 binding (i.e. CD36 expression) was close
to 1:1.

To correlate TSP binding with a change in cell behavior,
we also studied adhesion of these tumor cells to thrombin-
stimulated platelets. We demonstrated previously that mon-
ocyte adhesion to activated platelets was at least partially
dependent on TSP (13) and CD36 (30). As shown in Table I,
wild type C32 cells and CD36-transfected Bowes cells, but not
wild type Bowes cells, adhered to monolayers of thrombin-
stimulated platelets. Approximately 20–30% of the added
melanoma cells bound to the wells. This adhesion was cal-
culated to be at least partially dependent on TSP (13) and CD36 (30).

### Table I

<table>
<thead>
<tr>
<th>Cells added</th>
<th>Treatment</th>
<th>Adherent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C32</td>
<td>None</td>
<td>1,217 ± 113</td>
</tr>
<tr>
<td>Bowes (wild type)</td>
<td>None</td>
<td>33 ± 17</td>
</tr>
<tr>
<td>Bowes (CD36 transfected)</td>
<td>Monoclonal anti-TSP</td>
<td>908 ± 130</td>
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<tr>
<td>Bowes (CD36 transfected)</td>
<td>Polyclonal anti-TSP</td>
<td>54 ± 22</td>
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<tr>
<td>Bowes (CD36 transfected)</td>
<td>None</td>
<td>61 ± 32</td>
</tr>
<tr>
<td>Bowes (CD36 transfected)</td>
<td>Nonimmune mouse</td>
<td>775 ± 82</td>
</tr>
<tr>
<td>Bowes (CD36 transfected)</td>
<td>Nonimmune rabbit</td>
<td>815 ± 58</td>
</tr>
</tbody>
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Fig. 4. 125I-TSP binding to Bowes melanoma cells. Melano-
ma cells (≈ 7 × 10⁶ cells/ml) suspended in HBS/BSA were ali-
quotted into 1.5-ml polystyrene microcentrifuge tubes (0.08 ml/tube) containing radiiodinated TSP (final volume 0.1 ml) diluted in
the same buffer. After rotating end-over-end for 45 min at 4 °C, the
cell suspensions were centrifuged through silicone oil and cell-bound
radioactivity counted. A representative isotherm is shown demon-
strating saturable, concentration-dependent binding of TSP to the
transfected cells (■) but not to the wild type cells (□). Specific (i.e.
EDTA-inhibitable) binding is plotted, with each point representing
the mean of duplicate tubes. Nonspecific binding averaged <0.5% of
input radioactivity. Cell viability was assessed by dye exclusion and
was >98%.

Fig. 5. 125I-anti-CD36 binding to melanoma cells. Wild type
(wt) or PMV/CD36D (antisense cDNA)-transfected C32 melanoma
cells; or wild type or PMV/CD36A (sense cDNA)-transfected Bowes
melanoma cells were incubated in suspension with 125I-conjugated
8A6 IgG (5 µg/ml). Cell-bound radioactivity was counted after cen-
trifugation through silicone oil. A 10-fold molar excess of nonla-
beled 8A6 was added to some tubes to measure nonspecific binding, which averaged <0.8% of input radioactivity. Anti-CD36-bound to the wild
type C32 cells and the sense-transfected Bowes cells.

Fig. 6. 125I-TSP binding to C32 melanoma cells. TSP binding
to C32 melanoma cells was performed as described in Fig. 4. Saturable,
concentration-dependent binding of TSP was seen to the wild type cells (■) but not to cells transfected with pMV7/CD36D (□), an
expression vector containing the antisense cDNA construct.
observed, a value consistent with the level of CD36 expression determined by 8A6 binding (Fig. 5).

DISCUSSION

Whether CD36 can function as a cellular TSP receptor is controversial. Leung and colleagues (36) and Asch et al. (26) have confirmed the potential for this molecule to function as a TSP receptor by demonstrating that purified CD36 binds TSP in solid phase binding assays and when incorporated into phospholipid liposomes (43). These data, however, are confounded by recent observations that so-called NAK-platelets, i.e. platelets from donors serologically defined as lacking the NAK epitope, do not express CD36 (44) yet bound and expressed normal amounts of TSP (45). Furthermore, Oquendo et al. (38) have found that COS cells transfected with the CD36 cDNA expressed surface CD36 and adhered to falciparum-infected erythrocytes but did not bind TSP. While we and others have shown that monoclonal anti-CD36 antibodies inhibited TSP binding to monocytes (29), and certain tumor lines (26), others have found that OKM5 monoclonal anti-CD36 antibody did not inhibit adhesion of tumor cells to TSP (37).

We now report data derived from human melanoma cells stably transfected with the CD36 cDNA which confirm the potential TSP receptor function of this protein. DNA transfection has been a powerful tool in identifying and confirming the function of membrane proteins, particularly adhesion receptors. We have used this technique to create genetically altered cell lines from human melanoma cells. Wild type Bowes cells do not express CD36 and were shown not to bind TSP. When transfected with the CD36 cDNA, these cells expressed the molecule on their surface (Figs. 1 and 5), bound soluble TSP in a 1:1 stoichiometric fashion (Fig. 4), and adhered to a HEPES-based buffer that is phosphate free. No precipitates formed, which may then interact with the cell surface, since with short term COS transfection generally < 25% of the cells express recombinant protein. However, it is also likely that the monkey-derived COS cells may process the transfected protein product differently from the human cells. Recombinant CD36 immunoprecipitated from the COS cells was < 80,000 molecular weight (38), whereas that from the Bowes melanoma cells was the same molecular weight as from wild type C32 cells (90,000). This suggests that some post-translational event may play a role in CD36 function.

In summary, using sense and antisense cDNA transfection of human melanoma cells we have shown that CD36 can function as a TSP receptor and can mediate TSP-dependent cell-cell interactions. Although CD36 is clearly not the sole TSP receptor, the distribution of this protein in vascular cells and tissues (47) and the observations by us (48) and others (49–51) that it may participate in signal transduction events suggest that TSP-CD36 interactions may play a role in mediating some of the pathophysiological processes associated with TSP.

Acknowledgment—We appreciated the technical assistance of Susan DeLeon.

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