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Integrin-associated Protein Is a Receptor for the C-terminal Domain of Thrombospondin*

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The C-terminal "cell-binding domain" (CBD) of thrombospondin-1 (TS1) is a binding site for many cell types. Cell-binding peptides based on the sequence RFYVVM from the CBD of TS1 affinity label a 52-kDa cell surface glycoprotein, which we show is integrin-associated protein (IAP or CD47). IAP associates with $\alpha_v\beta_3$ and thereby modulates the activity of several integrins. Cells that express IAP bind strongly to TS1, the CBD, and its active cell-binding peptides while IAP negative cells do not. The 52-kDa protein is affinity labeled on IAP-positive but not IAP-negative cells, and monoclonal antibodies against IAP specifically immunoprecipitate the affinity-labeled 52-kDa protein from lysates of IAP-positive cells. Consistent with the association of IAP with $\alpha_v\beta_3$ integrin, the labeled 52-kDa protein is immunoprecipitated by an anti-$\alpha_v\beta_3$ antibody. Endothelial cells exhibit chemotaxis toward TS1 (at concentrations above 10 nM) and RFYVVM peptides. Chemotaxis to both agents is specifically inhibited by a function blocking anti-IAP monoclonal antibody. These data establish IAP (CD47) as a receptor for the CBD of TS1 and suggest a mechanism for the well established effects of the CBD on cell motility.

The thrombospondins (TS)[1] are a family of proteins implicated in the regulation of the motility, proliferation, and differentiation of many cell types (1, 2). For example, thrombospondin 1 (TS1) blocks angiogenesis by inhibiting the chemotaxis and proliferation of endothelial cells and promoting their differentiation into capillaries (1, 3). These effects of TS1 on cells are mediated through the binding of a number of domains and peptide sequences of TS to several classes of cellular receptors including heparan sulfate proteoglycans, sulfatides, CD36 (platelet glycoprotein IV or IIIb), and a number of integrins of the β3 and β1 families (1, 2, 4). Platelet thrombospondin (TS1) is the prototypic member of this family, which now consists of five isogenes and encoded isoforms (2, 4). We have mapped cell-binding sites to peptide sequences within seven of the eight domains of TS1 (5–8), including the C-terminal "cell-binding domain" (CBD) (6, 7), first identified as such with monoclonal antibody (mAb) C6.7, which inhibits secretion-dependent platelet aggregation (8, 9), the attachment of many different cell types to TS1 (10, 11), the chemotaxis toward TS1 of monocytes, polymorphonuclear monocytes (12), tumor cells (13), and smooth muscle cells (14) and a Ca^{2+} influx stimulated by TS1 (15).

Given the important functional role of the CBD in TS1-cell interactions, we sought to determine the essential features of the CBD for cell binding and the identification of the receptor(s) through which the CBD exerts its effects on cells. Expression of recombinant CBD demonstrated that it contained a unique cell-binding site(s) independent of other sequences in TS1, including the RGD sequence, which is not contained within the CBD (6). Using overlapping synthetic peptides (30-mers), we identified two homologous cell-binding sequences: RFYVVM within the fourth peptide (C4) and FIRVVM within the seventh (C7) (7). The RFYVVM sequence is highly conserved in all TS family members (2, 7). The C4- and C7-derived peptides competed with one another in cell adhesion assays suggesting that they bound a common receptor. Using derivatives of these peptides we affinity-labeled a 52-kDa protein on K562 cells (16) and many other cells and tissues. All of the properties of this hydrophobic, cell surface glycoprotein were consistent with those of a receptor for the CBD of TS1.

Integrin-associated protein (IAP) was first identified by Brown and co-workers (17, 18) due to its association with $\alpha_v\beta_3$ integrin purified from placenta. IAP is expressed on many mammalian cells, has an M_r of ~50 kDa, and consists of an N-terminal (extracellular) IgG variable type domain followed by five potentially membrane-spanning, hydrophobic helices (18, 19). IAP was recently shown to be identical to the 1D8 antigen and CD47, which is reduced on Rh_{null} erythrocytes (19). IAP appears to be involved in signal transduction by α_v and perhaps other integrins since mAbs directed to IAP block integrin-stimulated phagocytosis (20), an entactin-stimulated oxidative bust in neutrophils and monocytes (21), the inward calcium current in endothelial cells (22, 23), and fibroblasts (15) adhering to vitronectin or fibronectin. The wide expression of IAP suggests that it may have a general role in integrin-mediated signal transduction (17, 21, 24, 25); however, studies of the IAP mechanism and its physiological role have been hampered by the fact that its natural ligand is unknown. The biochemical properties of IAP precisely mirror those found for the 52-kDa protein affinity-labeled by the CBD peptides. We report here that IAP is a receptor for the TS1 CBD and its VVM-containing peptides and that a function-blocking anti-IAP mAb inhibits the chemotactic response to TS1 and its CBD peptides in endothelial cells.

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[1] The abbreviations used are: TS, thrombospondin; TS1, human thrombospondin-1; IAP, integrin-associated protein (CD47); mAb, monoclonal antibody; CBD, cell-binding domain of human thrombospondin-1; rCBD, recombinant cell-binding domain of human thrombospondin-1; HUVEC, human umbilical vein endothelial cells; HLA, human lymphocyte antigen; TNF, tumor necrosis factor. All peptides are indicated in single-letter amino acid code, and peptides referred to by trivial names are defined in the legend to Fig. 1.

EXPERIMENTAL PROCEDURES

Monoclonal antibodies LM609 (anti-$\alpha_v\beta_3$) and P1F6 (anti-$\alpha_v\beta_5$) were the generous gifts of Dr. D. Cheresh, Scripps Research Institute. B6H12 and 2D3, anti-human IAP mAbs, have been previously described (17–21). OV10 cells are a subclone of OVM1 human ovarian carcinoma (kindly provided by Dr. W. H. Stimson, Strathclyde University, Glasgow), which fail to express IAP. They were transfected with pIAP45 (18), an IAP expression construct, and IAP-positive clones were selected by fluorescence-activated cell sorting. K562 cells expressing $\alpha_v\beta_3$ are described in Blystone *et al.* (24). Human platelet TS1 was purified as described (8); the rCBD was expressed as a His^6-tagged protein in *Escherichia coli* using pQE30 (Qiagen) and purified by nickel-nitriloacetate chromatography. Peptides were synthesized, purified, and characterized by the Washington University Protein and Nucleic Acid Chemistry Laboratory as described (7, 16).

Cell adhesion assays were performed and quantified with endogenous cellular phosphatase activity as described in Prater *et al.* (5). Affinity labeling of cells and detergent lysates with ^{125}I-4N1K peptide (KRFYVVMWKK) was performed as described in Gao and Frazier (16). Immunoprecipitations employed the indicated primary mouse mAb and anti-mouse IgG-agarose (Sigma) and were done as described in Brown *et al.* (17). Iodinated samples were dissolved in boiling SDS sample buffer with reduction and electrophoresed on 10% SDS gels, which were dried and autoradiographed (16). Chemotaxis assays were performed in modified Boyden chambers (Neuroprobe, Cabin John, MD) using 8.0-μm pore size gelatin-coated polycarbonate filters as described by Tolsma *et al.* (26). Antibodies (purified IgG) were added to the cells at the indicated concentrations before placing the cells into the wells of the chamber.

RESULTS

To test whether IAP was necessary for the binding of cells to the CBD, we first had to find a cell line deficient in IAP expression to serve as a negative control. Previous studies with anti-IAP mAbs (17–21, 28) indicated that IAP has an extremely broad cell and tissue distribution. The ovarian carcinoma cell line OVM1 was found to have low levels of IAP expression, and a subclone designated OV10 expressed essentially no IAP by flow cytometric and Western blotting criteria.[2] Suspensions of OV10 (IAP negative) and OV10$^+$ (IAP transfected) cells were incubated in plastic 96-well plates coated with intact TS1, rCBD, and three versions of the C4 peptide. Fibronectin-coated wells served as a positive control (OV10 cells express $\alpha_5\beta_1$) and bovine serum albumin-coated wells as the negative control. Fig. 1 shows that both OV10 and OV10$^+$ cells adhered equally well to fibronectin-coated wells, but twice as many OV10$^+$ cells expressing IAP adhered to TS1 as IAP-deficient OV10. Adhesion to the rCBD and all three C4 peptides showed an even greater preference for the IAP-expressing cells. Like most cells, OV10s probably express receptors for several domains of intact TS1 (1, 2, 4). Both cell lines adhered poorly to peptides from the calcium-binding domain of TS1 (CaIII and CaVII), although, interestingly, the OV10$^+$ cells adhered better to CaVII, which contains the RGD sequence of TS1. This may be due to an effect of IAP expression on the function of the low levels of $\alpha_v\beta_3$ expressed by OV10 cells (not shown). Peptide Mal II is a cell-binding peptide from the type 1 repeats of TS1 (5), and both cell types adhered to it to the same extent. Thus, expression of IAP confers on the OV10$^+$ cells the ability to adhere to the VVM peptide site(s) within the CBD of TS1.

To determine if IAP was acting directly as the CBD receptor, we affinity-labeled cells with peptide 4N1K, a derivative of the C4 peptide, with the sequence KRFYVVMWKK. This peptide has been shown to specifically label a 52-kDa plasma membrane protein on intact cells and in detergent lysates (16). When OV10 cells were affinity-labeled with 4N1K, no labeled proteins were found (Fig. 2, *lanes 3* and *4*). In contrast, labeling of OV10$^+$ cells revealed a 52-kDa protein along with the previously characterized 37-kDa proteolysis product (*lane 1*). Affinity labeling of both bands could be inhibited completely by excess cold peptide, demonstrating the specificity of labeling (*lane 2*). These bands are identical to those labeled on K562 cells (*lanes 5* and *6*). Immunoprecipitation of labeled OV10 cell lysates with two anti-IAP mAbs revealed no 52-kDa protein (*lanes 7* and *8*) while the labeled protein was abundant in anti-IAP precipitates of OV10$^+$ cells (*lanes 9* and *10*) but not in control anti-HLA (*lane 11*) or anti-TNF receptor (*lane 12*) precipitates. Thus in IAP-negative OV10 cells, transfection of IAP confers binding to the CBD, and IAP is specifically labeled by the CBD peptide. These data demonstrate a direct interaction of IAP with the CBD. The direct binding of the CBD peptides to IAP is confirmed by the observation that the extracellular IgGv domain of IAP, when expressed as an Fc fusion protein,[3] binds specifically to the cell binding VVM containing C4 and C7 peptides but not to six other 30-mers representing the rest of the CBD sequence (data not shown) (27).

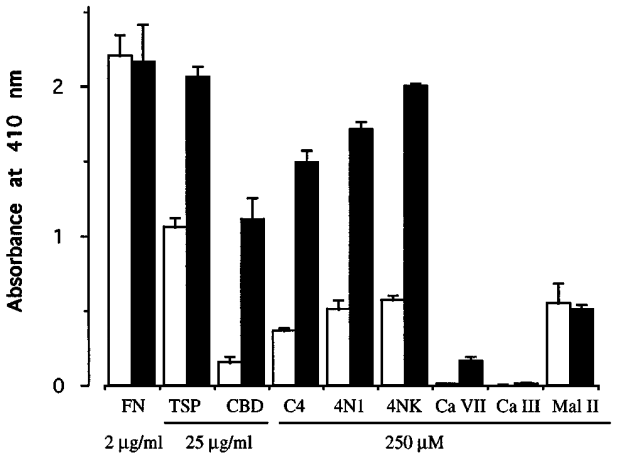

FIG. 1. **Adhesion of OV10 cells to TS1, CBD, and its peptides.** IAP-negative, vector-transfected OV10 cells and pIAP45-transfected OV10$^+$ IAP positive cells were allowed to adhere for 90 min to plastic wells coated with the indicated proteins and peptides. The wells were washed and adhesion-quantified by endogenous phosphatase activity with *p*-nitrophenyl phosphate as a substrate (5). □, vector-transfected OV10 cells; ■, IAP-transfected OV10 cells. Peptides are: C4, RFYVVMWKQVTQSYWDTNPTRAQGYSGLSV; 4N1, RFYVVMWKQVTQSYWDTN; 4NK, KRFYVVMWKQVTQSKKY; CaVII, KDNCRLVPNPDQKDSDGDGRGDACK; CaIII, NCPYNHNPDQADTDNNGEGDAC; Mal II, SPWSSASVTAGDGVITRIR. *FN*, fibronectin; *TSP*, thrombospondin.

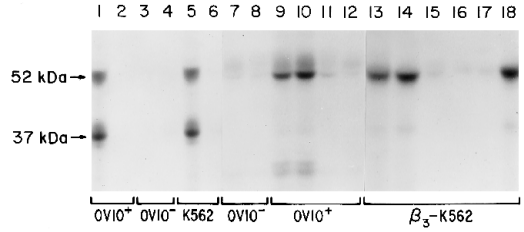

FIG. 2. **Affinity labeling of IAP with CBD peptide.** Peptide ^{125}I-KRFYVVMWKK (4N1K) was used to affinity label intact OV10$^+$ cells expressing IAP (*lanes 1* and *2*), parental OV10 cells (OV10$^-$) (*lanes 3* and *4*), and K562 cells (*lanes 5* and *6*) as described by Gao and Frazier (16). Unlabeled 4N1K peptide was present in reactions for *lanes 2*, *4*, and *6*. Detergent lysates of OV10$^-$ (*lanes 7* and *8*) or OV10$^+$ (*lanes 9–12*) cells were affinity-labeled and then precipitated with the following mAbs: anti-IAP B6H12 (*lane 9*); anti-IAP 2D3 (*lane 10*); anti-HLA W6/32 (*lane 11*); anti-TNF receptor (*lane 12*). Detergent lysates of K562 cells expressing $\alpha_v\beta_3$ (β_3-K562) were labeled and precipitated with the following mAbs: B6H12 (*lane 13*); 2D3 (*lane 14*); W6/32 (*lane 15*); anti-TNF receptor (*lane 16*); anti-$\alpha_v\beta_5$ P1F6 (*lane 17*); and anti-$\alpha_v\beta_3$ LM609 (*lane 18*).

[2] F. P. Lindberg and E. J. Brown, unpublished data.

[3] F. P. Lindberg and E. J. Brown, manuscript in preparation.

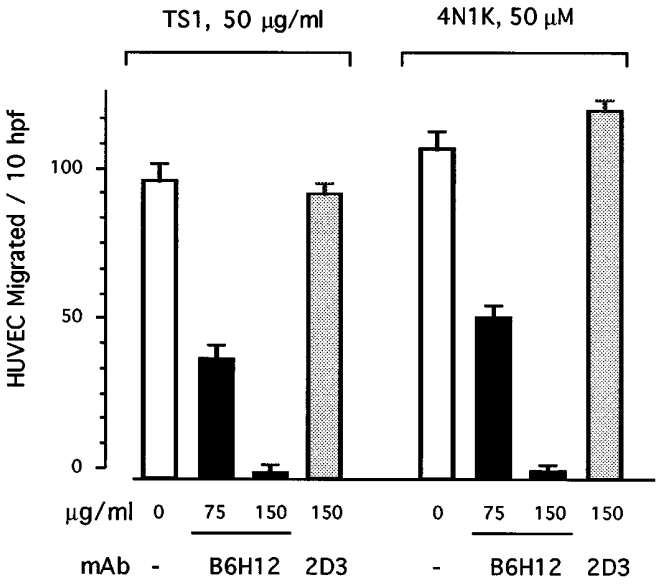

FIG. 3. **Effect of anti-IAP mAbs on endothelial chemotaxis.** Endothelial (HUVEC) chemotaxis was stimulated by TS1 or the CBD peptide 4N1K in the presence of the indicated antibodies. Chemotaxis was performed as described by Tolsma *et al.* (26). After staining, cells were counted in 5 high power fields (*hpf*) in each of the triplicate wells for each concentration of mAb. The number of cells migrating randomly in the absence of chemoattractant has been subtracted from all data points.

Because IAP associates with $\alpha_v\beta_3$ integrin (17), we examined 4N1K labeling of IAP in $\alpha_v\beta_3$-transfected K562 cells (20). This cell line was used since OV10 cells express little $\alpha_v\beta_3$ (not shown). When a detergent lysate was first labeled with 4N1K and then immunoprecipitated with anti-IAP mAbs B6H12 (*lane 13*) or 2D3 (*lane 14*) the labeled 52-kDa IAP band was recovered in the precipitate as with the $OV10^+$ cells. No labeled band was recovered in precipitates of anti-HLA or anti-TNF receptor (another ~55-kDa protein) or anti-$\alpha_v\beta_5$ mAbs (*lanes 15–17*). Interestingly, immunoprecipitation of labeled cell lysates with $\alpha_v\beta_3$ antibody (*lane 18*) coprecipitated labeled IAP, suggesting that IAP can interact simultaneously with $\alpha_v\beta_3$ and the CBD peptide.

The CBD of TS1 stimulates cell motility via haptotaxis, chemotaxis, and chemokinesis as judged by inhibition of these processes in several cell types by mAb C6.7 (2, 10, 12–14). We have previously reported that intact TS1 at concentrations above 10 nM could stimulate the chemotaxis of endothelial cells (26). We recently found that the CBD VVM-containing peptides act as attachment factors for several types of endothelial cells including HUVEC and that the C4 peptides affinity label a 52-kDa membrane protein on these cells with properties identical to those reported (16) for the protein from K562 cells. HUVECs express high levels of IAP as determined by flow cytometry (not shown). Furthermore, the CBD peptides are able to stimulate chemotaxis and chemokinesis of endothelial cells.[4] Thus the effects of anti-IAP mAbs on chemotaxis to TS1 and the CBD peptide 4N1K were determined using HUVEC as the test cell (Fig. 3). The anti-IAP mAb B6H12, previously characterized as a function-blocking mAb (17, 20, 23, 28), inhibited, in a concentration-dependent way, chemotaxis of endothelial cells toward both TS1 and the 4N1K peptide while 2D3, in other assays a nonblocking anti-IAP mAb (17, 23, 28), had no effect. These data indicate that IAP is the CBD receptor responsible for the stimulation of cell migration.

[4] A-G. Gao, S. S. Tolsma, M. B. Finn, P. J. Polverini, N. Bouck, and W. A. Frazier, submitted for publication.

DISCUSSION

The data presented here identify IAP as a novel receptor for the CBD of TS1 and probably other TS isoforms as well since the RFYVVM sequence in peptide C4 of the CBD is highly conserved (2, 7). This conclusion is based on: (i) the marked enhancement of OV10 cell adhesion to TS1, the rCBD, and the CBD-derived peptides by transfection of OV10 cells with IAP; (ii) affinity labeling of IAP with the 4N1K peptide; and (iii) inhibition of TS1- and 4N1K-stimulated endothelial cell chemotaxis by a function-blocking but not by a non-inhibitory anti-IAP mAb. Interestingly, it seems that IAP, which is known to physically associate with β_3 integrins (17), can maintain its integrin association even when bound to the CBD peptide, since anti-$\alpha_v\beta_3$ will immunoprecipitate affinity-labeled IAP. This observation, along with the proximity of the RGD sequence of TS1 to the CBD (1, 2, 6), raises the intriguing possibility that TS1 can simultaneously engage both IAP and its associated β_3 integrin. This could occur in an ordered fashion, thus providing a physiological mechanism for the exposure of the normally cryptic RGD sequence of TS1 (29) and an explanation for the reports that $\alpha_v\beta_3$ and platelet $\alpha_{IIb}\beta_3$ can act as TS1 receptors (30, 31).

Based on the inhibitory action of mAb C6.7, the CBD of TS1 has been thought to stimulate the motility of a number of cell types (11–14). The C4 CBD-derived peptides are chemotactic for endothelial cells (this paper).[4] Blockade by an anti-IAP mAb establishes this chemotactic response as a direct biological consequence of the interaction of the CBD peptide with IAP. Two possible mechanisms for IAP involvement in chemotaxis are suggested by other data. First, an anti-IAP mAb has been shown to block the Ca^{2+} influx of endothelial cells (23) adherent to RGD-containing substrates. If the natural ligand, TS1, or its peptides were to stimulate Ca^{2+} influx via IAP, this could provide a mechanism for directed motility. That such a stimulation occurs has been shown by Tsao and Mousa (15), who found that TS1 and the C4 peptide RFYVVMWK stimulated a transient Ca^{2+} influx in fibroblasts, which was partially blocked not only by mAb C6.7 (in the case of TS1) but also B6H12, the same function blocking anti-IAP mAb used in our studies. These data confirm that a known function of IAP, *i.e.* stimulation of Ca^{2+} entry (23), can be activated by the C4 peptide RFYVVMWK (15).

The second possible mechanism for stimulation of cell motility by IAP derives from the association of IAP with $\alpha_v\beta_3$ and the role of this integrin in the motility of cultured endothelial cells (32). Thus CBD binding to IAP may modulate the affinity or avidity of $\alpha_v\beta_3$, which is used for adhesion and traction on the gelatin-coated (RGD-containing) filter (32). In this way the CBD or its C4 peptides, all of which lack an RGD sequence, could act on $\alpha_v\beta_3$ through binding to IAP. The IAP-$\alpha_v\beta_3$ association is also interesting in light of recent data implicating $\alpha_v\beta_3$ in angiogenesis where it acts, at least in part, by protecting endothelial cells from apoptosis (33, 34). TS1 plays an important regulatory role in endothelial cells, where it promotes a differentiated phenotype (1–3) and antagonizes the initial events of angiogenesis (26). It is interesting to speculate that TS1-IAP interaction may modulate the function of $\alpha_v\beta_3$ during angiogenesis. Moreover, the IAP-$\alpha_v\beta_3$ complex has been shown to regulate the function of other integrins (19–21, 24, 25). It is possible that TS1 may exert many of its apparently diverse biological effects (1–4, 11–15) through modulation of this signaling complex.

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