

# The Thrombospondin Receptor CD47 (IAP) Modulates and Associates with $\alpha 2\beta 1$ Integrin in Vascular Smooth Muscle Cells

Xue-Qing Wang and William A. Frazier\*

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

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The carboxyl-terminal domain of thrombospondin-1 enhances the migration and proliferation of smooth muscle cells. Integrin-associated protein (IAP or CD47) is a receptor for the thrombospondin-1 carboxyl-terminal cell-binding domain and binds the agonist peptide 4N1K (kRFYVVMWKK) from this domain. 4N1K peptide stimulates chemotaxis of both human and rat aortic smooth muscle cells on gelatin-coated filters. The migration on gelatin is specifically blocked by monoclonal antibodies against IAP and a  $\beta 1$  integrin, rather than  $\alpha v\beta 3$  as found previously for 4N1K-stimulated chemotaxis of endothelial cells on gelatin. Both human and rat smooth muscle cells displayed a weak migratory response to soluble type I collagen; however, the presence of 4N1K peptide or intact thrombospondin-1 provoked a synergistic chemotactic response that was partially blocked by antibodies to  $\alpha 2$  and  $\beta 1$  integrin subunits and to IAP. A combination of anti $\alpha 2$  and IAP monoclonal antibodies completely blocked chemotaxis. RGD peptide and anti $\alpha v\beta 3$  mAb were without effect. 4N1K and thrombospondin-1 did not augment the chemotactic response of smooth muscle cells to fibronectin, vitronectin, or collagenase-digested type I collagen. Complex formation between  $\alpha 2\beta 1$  and IAP was detected by the coimmunoprecipitation of both  $\alpha 2$  and  $\beta 1$  integrin subunits with IAP. These data suggest that IAP can associate with  $\alpha 2\beta 1$  integrin and modulate its function.

## INTRODUCTION

For more than a decade, thrombospondin-1 has been implicated as a positive effector of smooth muscle cell proliferation. Thrombospondin-1 stimulates smooth muscle cell growth in vitro (Majack *et al.*, 1985, 1986, 1988), and the protein is associated with sites of smooth muscle cell proliferation in vivo in atherosclerotic lesions (Liau *et al.*, 1993; Van Zanten *et al.*, 1994) and in wounds (DiPietro *et al.*, 1996). Thrombospondin-1 synergizes with epidermal growth factor to potentiate the growth response of smooth muscle cells (Majack *et al.*, 1986). Platelet-derived growth factor (PDGF) or angiotensin II treatment of smooth muscle cells results in the rapid synthesis of throm-

bospondin-1 on the same time scale as “immediate early” genes such as *myc* (Majack *et al.*, 1987; Scott-Burden *et al.*, 1990; Kobayashi and Yamamoto, 1991). Cycloheximide treatment of smooth muscle cells potentiates the induction of thrombospondin-1 mRNA as it does mRNAs of immediate early genes (Majack *et al.*, 1987). Antibodies and heparin, which inhibit the association of secreted thrombospondin-1 with the cell surface, attenuate the response of the smooth muscle cells to PDGF (Majack *et al.*, 1988). Furthermore, mAb C6.7 directed against the thrombospondin-1 carboxyl-terminal domain can inhibit the stimulation of proliferation of smooth muscle cells by thrombospondin-1 (Majack *et al.*, 1988) and the chemotaxis of smooth muscle cells toward the intact thrombospondin-1 molecule (Yabkowitz *et al.*, 1993). The sum of these experiments establishes thrombospondin-1 as a potentially important factor in the pathogenesis of atherosclerosis and restenosis and provides a rationale for pursuing

\* Corresponding author: Box 8231, Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 S. Euclid Ave, St. Louis, MO 63110.

the mechanism of action of thrombospondin-1 on smooth muscle cells.

An important step in this direction is to determine the receptors on smooth muscle cells with which thrombospondin-1 interacts. This has been hindered by the complex structure of the thrombospondin-1 molecule, which contains several domains harboring peptide sequences that interact with distinct cellular receptors. For example, the N-terminal heparin-binding domain binds sulfated glycosaminoglycans and glycolipids such as sulfatides (Sun *et al.*, 1989; Abedi *et al.*, 1995) and destabilizes focal adhesions in some types of adherent cells (Murphy-Ullrich *et al.*, 1993). The type 1 repeat peptides bind CD36 (Asch *et al.*, 1987, 1992, 1993; Tolsma *et al.*, 1993) and inhibit the stimulatory effects of angiogenic factors such as basic fibroblast growth factor and vascular endothelial growth factor on endothelial cell chemotaxis and proliferation (Dawson *et al.*, 1997). The RGD sequence of thrombospondin-1 resides in the last of the type 3 or calcium-binding repeats and binds to integrins such as  $\alpha v \beta 3$  and  $\alpha IIb \beta 3$  (Lawler and Hynes, 1989). Recently we have localized the cell-binding activity of the carboxyl-terminal cell-binding domain of thrombospondin-1 to two homologous peptides, RFYVVM and IRVVM (Gao and Frazier, 1994). Derivatives of these peptides were used to affinity label a receptor candidate, a membrane glycoprotein of 52 kDa, which proved to be integrin-associated protein or IAP (CD47) (Gao *et al.*, 1996b). IAP is known to associate with  $\beta 3$  integrins and when stimulated with thrombospondin-1, the recombinant cell-binding domain, or a VVM-containing peptide such as 4N1K (kRFYVVM-WKk), IAP initiates a signaling pathway resulting in up-regulation of integrin-mediated functions such as cell spreading (Gao *et al.*, 1996a), cell migration on RGD-containing matrices (Gao *et al.*, 1996b), and platelet aggregation (Chung *et al.*, 1997). All of these functions involve the activation of  $\beta 3$  integrins. However, effects of anti-IAP monoclonal antibodies (mAbs) in assays of leukocyte transmigration (Cooper *et al.*, 1995; Parkos *et al.*, 1996) and phagocytosis (Blystone *et al.*, 1995) suggest that  $\beta 2$  and perhaps  $\beta 1$  integrins could also be modulated by thrombospondin-1-IAP interactions.

The purpose of the present study was to investigate the possible role of IAP in the effects of thrombospondin-1 on smooth muscle cells. In culture, human aortic smooth muscle cells undergo a transition from their *in situ* "contractile" phenotype to the proliferative, migratory "synthetic" phenotype thought to be analogous to the activated state of smooth muscle cells found at sites of vessel injury (Ross and Kariya, 1980; Skinner *et al.*, 1994). This transition includes the down-regulation of the expression of  $\alpha 1 \beta 1$  integrin and the reciprocal up-regulation of  $\alpha 2 \beta 1$  integrin expression, which mediates migration of these cells on

collagen-I (Skinner *et al.*, 1994). Here we have used the thrombospondin-1-derived 4N1K peptide as an agonist of IAP to investigate the role of IAP in modulating smooth muscle cell migration. Curiously, rat and human smooth muscle cells utilize  $\alpha 2 \beta 1$  for adhesion and migration on both gelatin and native collagen-I. Other cell types use  $\alpha v$  integrins when attaching and migrating on gelatin, an RGD-dependent process (Leavesley *et al.*, 1992; Felding-Habermann and Cheresch, 1993; Gao *et al.*, 1996b). While 4N1K ligation of IAP itself is a relatively weak chemotactic stimulus, IAP can modulate the activity of  $\alpha 2 \beta 1$ , resulting in enhanced migration toward soluble collagen. Finally, we present data indicating that IAP can associate with  $\alpha 2 \beta 1$ .

## MATERIALS AND METHODS

### Reagents

All peptides used were synthesized by the Protein and Nucleic Acid Chemistry Laboratory of Washington University School of Medicine as described previously (Kosfeld and Frazier, 1993). Peptides were evaluated by mass spectrometry before and after purification by high-pressure liquid chromatography. The amino acid sequences of the thrombospondin-1 peptides and preparation of human platelet thrombospondin-1 were as described (Santoro and Frazier, 1987). Rat tail collagen-I, human vitronectin, and fibronectin were obtained from Collaborative Biochemical Products (Bedford, MA). Anti-human IAP mAbs, 2D3, B6H12, 1F7, and anti  $\alpha v$  (L230) mAbs were supplied by Dr. E. Brown (Washington University School of Medicine, St. Louis MO) (Brown *et al.*, 1990; Lindberg *et al.*, 1993, 1994). mAbs P4C10 (anti- $\beta 1$ ) and P1E6 (anti- $\alpha 2$ ) were obtained from Life Technologies (Grand Island, NY); anti- $\alpha v \beta 3$  mAb 4C1 was from Monsanto-Searle, St. Louis, MO; mAb BHA2.1 (anti- $\alpha 2 \beta 1$ ) and Western blotting polyclonal antibodies against  $\alpha 2$  and  $\beta 1$  were from Chemicon International (Temecula, CA). Goat anti-rabbit (Fab)<sub>2</sub> antibody conjugated with horseradish peroxidase was from Jackson ImmunoResearch Labs (West Grove, PA). Enhanced chemiluminescence Western blotting detection kit was from Amersham (Arlington Heights, IL). Anti-mouse immunoglobulin G (IgG) agarose and other reagents were from Sigma Chemical (St. Louis, MO).

### Cell Culture

Human arterial smooth muscle cells from the aorta of a 4-y-old boy and rat aorta smooth muscle cells were isolated by the explant method and cultured as described (Ross and Kariya, 1980). Cells were maintained in a humidified 37°C and 5% CO<sub>2</sub> environment in minimal essential medium (MEM) with 20% fetal calf serum and identified by immunostaining of  $\alpha$ -actin (Janat and Liau, 1992). Passages 2–10 were used for experiments.

### Cell Adhesion

Assays were performed in 96-well plates as previously described (Kosfeld and Frazier, 1993). Synthetic peptides were solubilized in Tris-buffered saline (TBS) (25 mM Tris, 150 mM NaCl, pH 7.4) and 50  $\mu$ l of solution was added to each well of 96-well plates (Nunc Immuno Plate Maxisorp, Naperville, IL), and incubated at 4°C overnight. Wells were rinsed with TBS and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Cells were harvested from near-confluent cultures by brief treatment with trypsin/EDTA and were immediately washed and resuspended in Ca<sup>2+</sup>-free TBS with 0.4% BSA. Cell suspension (100  $\mu$ l) was added

to each well. After incubation at 37°C for 2 h, the plates were rinsed three times with TBS. Cell attachment was quantified with a colorimetric reaction using endogenous cellular phosphatase activity by adding 100  $\mu$ l of the following substrate/lysis solution to each well: 1% Triton X-100, 6 mg/ml *p*-nitrophenyl phosphate, in 50 mM sodium acetate, pH 5.0. Wells were incubated for 1–2 h at 37°C, after which the reaction was stopped by the addition of 50  $\mu$ l 1 N NaOH and read in an enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Cambridge, MA) with a 410-nm filter. Wells were set up in triplicate, and all experiments were repeated at least three times.

### Cell Migration

Chemotaxis assays were conducted in microBoyden chambers (Neuroprobe, Cabin John, MD) using 8  $\mu$ m PVP-free, polycarbonate filters (Nuclepore, Pleasanton, CA). Filters were precoated by soaking them in 100  $\mu$ g/ml gelatin at 37°C overnight, followed by washing twice in phosphate-buffered saline (PBS). Smooth muscle cells were harvested with trypsin/EDTA and diluted in MEM with 0.1% BSA to a final concentration of  $3\text{--}5 \times 10^5$  cells/ml; chemoattractants were diluted in the same solution. The assembled chamber was incubated for 6 h at 37°C. Filters were fixed, stained, and mounted. Cells were counted in five high-power fields in each of the triplicate wells. Checkerboard assays were used to distinguish between chemotaxis (directed migration) and chemokinesis (random migration) (Zigmond and Hirsch, 1973; Wilkinson and Allan, 1978).

### Fluorescence-activated Cell Sorter (FACS) Analysis

Human smooth muscle cell were harvested by trypsin/EDTA and resuspended in culture medium with 10% fetal calf serum. Primary mAbs (5  $\mu$ g/ml) were added to cell suspensions and incubated for 2 h at 4°C with rocking. After several washes in PBS, the cells were stained with fluorescein isothiocyanate-labeled anti-mouse secondary antibody (Pierce, Rockford, IL) for another 1 h in the cell culture medium, washed again with PBS, and analyzed by flow cytometry.

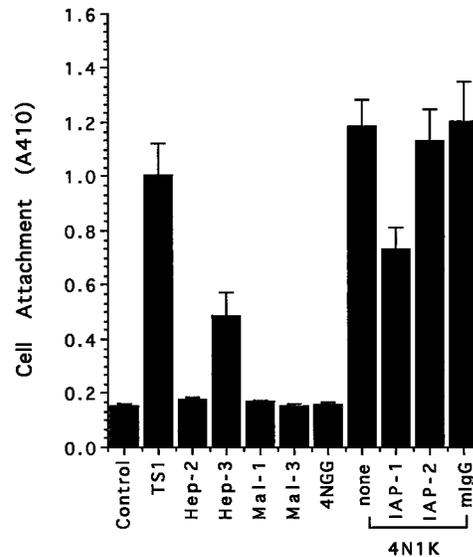
### Immunoprecipitation and Western Blotting

Cells were lysed in 30 mM *n*-octyl- $\beta$ -D-glucopyranoside in TBS with proteinase inhibitors (10  $\mu$ g/ml each of antipain, pepstatin A, chymostatin, leupeptin, soybean trypsin inhibitor, aprotinin, and 1 mM phenylmethylsulfonyl fluoride) by rocking for 30 min at 4°C followed by microcentrifugation at top speed (13,000 rpm) for 30 min. The soluble material from equal amounts of protein was incubated with the specified monoclonal antibody overnight at 4°C and immunoprecipitated with anti-mouse IgG-agarose in the presence of 3% goat serum. The precipitates were extensively washed with lysis buffer, dissolved, and boiled in a small volume of SDS-sample buffer. Proteins were separated by SDS-PAGE on 10% precast Tris-Glycine gels (NOVEX, San Diego, CA) and transferred to nitrocellulose membranes. Blots were blocked with 3% BSA plus 3% dried milk in TBST (0.1% Tween-20 in TBS) for at least 1 h and probed with the indicated antibodies overnight at 4°C, washed, and incubated with 1:25,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG(Fab)<sub>2</sub> for another 2 h. Detection was by chemiluminescence with an enhanced chemiluminescence kit.

## RESULTS

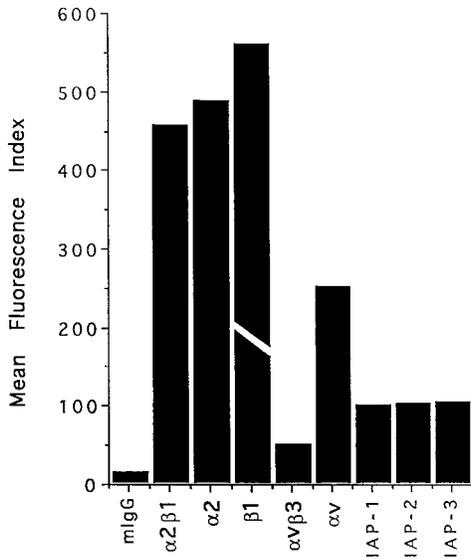
### Smooth Muscle Cells Bind to an IAP-Agonist Peptide from the Carboxyl-terminal Domain of Thrombospondin

To confirm the interaction of smooth muscle cell with the thrombospondin-1 cell-binding domain and to identify other potential regions of smooth muscle cell



**Figure 1.** Direct attachment of human smooth muscle cells to thrombospondin-1 and peptides from different domains of thrombospondin-1. Microtiter plates were coated with 50  $\mu$ g/ml thrombospondin-1 or 50  $\mu$ M peptides at 4°C overnight. Cells were suspended in 0.1% BSA in TBS or pretreated with 100  $\mu$ g/ml antibodies in the same solution for 15 min at 37°C before being added to the wells. After 2 h at 37°C, the attached cells were quantified by the absorbance at 410 nm due to endogenous cellular phosphatase hydrolysis of *p*-nitrophenyl phosphate.

interaction with thrombospondin-1, we screened a number of peptides derived from different domains of thrombospondin-1 for their ability to bind human and rat aortic smooth muscle cells. As seen in Figure 1, peptide 4N1K from the cell-binding domain of thrombospondin-1 bound human smooth muscle cells as well as intact thrombospondin-1. The control peptide 4NGG (kRFYGGMWKk) has a sequence identical with that of 4N1K except for the two glycine residues replacing the VV sequence. Thus, even though the heparin-binding peptide Hep-3 from the N-terminal heparin-binding domain of thrombospondin-1 binds the smooth muscle cell to a limited extent, the positively charged 4NGG binds the smooth muscle cells not at all. Identical results were obtained with rat aortic smooth muscle cells (not shown). The binding of cells to 4N1K was partially inhibited by the function blocking anti-IAP mAb 1F7, but not by 2D3, which binds to IAP but does not inhibit its function in a number of assays (Brown *et al.*, 1990; Gao *et al.*, 1996a,b; Chung *et al.*, 1997). These binding data suggest that the newly identified thrombospondin-1 receptor IAP (Gao *et al.*, 1996b) is at least partially responsible for the interaction of the smooth muscle cells with thrombospondin-1 in this assay. The expression of IAP on the surface of cultured human smooth muscle cells was confirmed by flow cytometry using three anti-IAP mAbs (Figure 2) as well as by specific affinity labeling

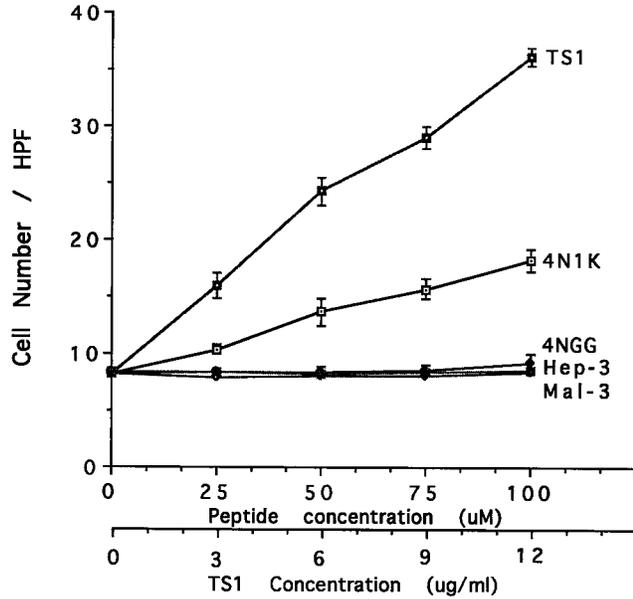


**Figure 2.** Expression of IAP and integrins on human smooth muscle cells. The bars represent the mean fluorescence index observed for each of the antibodies indicated. Mouse IgG was used as a control. IAP-1 is 1F7; IAP-2 is 2D3; IAP-3 is B6H12. The experiment was repeated twice. The actual value of the mean fluorescence index for the anti-β1 mAb P4C10 was 1298. The bar is truncated in the figure to emphasize differences among the values for the other mAbs.

with <sup>125</sup>I-4N1K, which revealed the expected 52-kDa labeled protein (our unpublished results).

**The IAP Agonist Peptide Is a Chemoattractant of Smooth Muscle Cells**

Intact thrombospondin-1 has previously been shown to mediate the chemotactic migration of calf pulmonary artery smooth muscle cells (Yabkowitz *et al.*, 1993). We thus tested intact thrombospondin-1 as a chemoattractant of human and rat aortic smooth muscle cells (Figure 3), and found it to be a relatively potent attractant of both cell types. Since Yabkowitz *et al.* (1993) reported that the chemotaxis of calf pulmonary artery smooth muscle cells toward thrombospondin-1 was blocked by mAb C6.7 against the thrombospondin-1 cell-binding domain, we tested the 4N1K peptide from this domain as a chemoattractant of human and rat smooth muscle cells in the Boyden chamber assay using gelatin-coated filters as for whole thrombospondin-1. For both human (Figure 3) and rat (not shown) cells, 4N1K is an attractant while 4NGG, the control peptide, is devoid of activity. Other peptides from the heparin-binding domain and the type 1 repeats of thrombospondin-1 are also inactive (Figure 3), including Hep-3, which had a low level of cell binding activity (Figure 1). It should be noted that on an M basis, thrombospondin-1 is much more potent



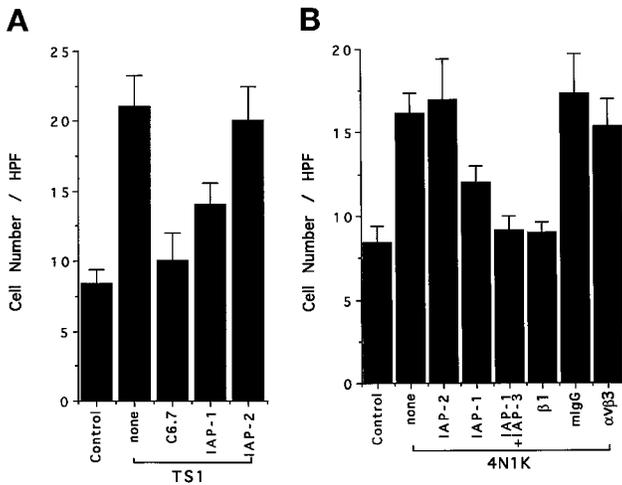
**Figure 3.** Thrombospondin-1 and its peptides stimulate human smooth muscle cell chemotaxis on gelatin-coated filters. The indicated concentrations of thrombospondin-1, 4N1K, 4NGG, Hep-3, and Mal-3 peptides were added to the lower compartments of the Boyden chamber. Data are mean ± SE of the number of migrated cells counted per high-power field determined over five fields for each of triplicate wells.

than 4N1K. This could be due to the fact that in thrombospondin-1 the 4N1K peptide is presumably in its native conformation as well as being trimeric. A checkerboard analysis of the migration of human smooth muscle cells in the Boyden chamber assay with 4N1K is shown in Table 1. This indicates that no matter what the absolute concentration of 4N1K peptide above or below the filter, the cells always respond with migration up the gradient of peptide, the hallmark of a true chemotactic, as opposed to chemokinetic or random response. These results indicate that

**Table 1.** Checkerboard analysis of the chemotactic activity of 4N1K

4N1K in lower chamber (μM)	4N1K in upper chamber (μM)			
	0	25	50	10
0	22 ± 3	18 ± 2	16 ± 3	11 ± 3
25	30 ± 4	24 ± 2	20 ± 3	18 ± 4
50	40 ± 5	32 ± 3	26 ± 4	16 ± 3
100	54 ± 7	39 ± 5	32 ± 4	23 ± 4

Different gradient conditions were created by adding various concentrations of 4N1K to the upper and lower compartment of the chamber as indicated. The motility response is expressed as mean ± SE cells per high-power field (five fields for each of triplicate wells were counted).



**Figure 4.** (A) Human smooth muscle cell migration to thrombospondin-1 is inhibited by anti-IAP and thrombospondin-1 antibodies. Smooth muscle cells were preincubated with 100  $\mu\text{g}/\text{ml}$  C6.7 or 1F7, 2D3 at 37°C for 15 min before being added to the upper compartment of the Boyden chamber. 4N1K (100  $\mu\text{M}$ ) was present in the lower compartment, and TBS was used as control. (B) Human smooth muscle cell migration to 4N1K is inhibited by antibodies against IAP and  $\beta 1$  integrin. Smooth muscle cells were preincubated in the presence or absence of  $\beta 1$  mAb (1:1500) or other mAbs as indicated (100  $\mu\text{g}/\text{ml}$ ) at 37°C for 15 min before being added to the upper compartment of the Boyden chamber.

the chemotaxis of smooth muscle cells toward thrombospondin-1 reported by Yabkowitz *et al.* (1993) is probably due to the activity of the cell-binding domain, specifically the IAP agonist sequence contained within peptide 4N1K.

To determine the receptors responsible for the migration of the smooth muscle cells, they were tested in the Boyden chamber assay with thrombospondin-1 or 4N1K as the attractant and challenged with mAbs. Figure 4A shows that mAb C6.7, which binds to the cell-binding domain of thrombospondin-1, virtually eliminated migration toward thrombospondin-1, and mAb 1F7, a function blocking anti-IAP mAb, substantially reduced the migration of the cells toward thrombospondin-1 while the nonfunction-blocking anti-IAP mAb 2D3 had no effect. Thus, the cell-binding domain region of thrombospondin-1 is responsible for its chemotactic activity, which is mediated via IAP. We next characterized the chemotactic response of human smooth muscle cells to peptide 4N1K (Figure 4B). As with whole thrombospondin-1, mAb 2D3 had no effect while mAb 1F7 significantly inhibited migration. The addition of a second function blocking anti-IAP mAb B6H12 along with 1F7 resulted in total inhibition of directed migration.

#### $\alpha 2\beta 1$ Is Required for Chemotaxis to 4N1K Peptide

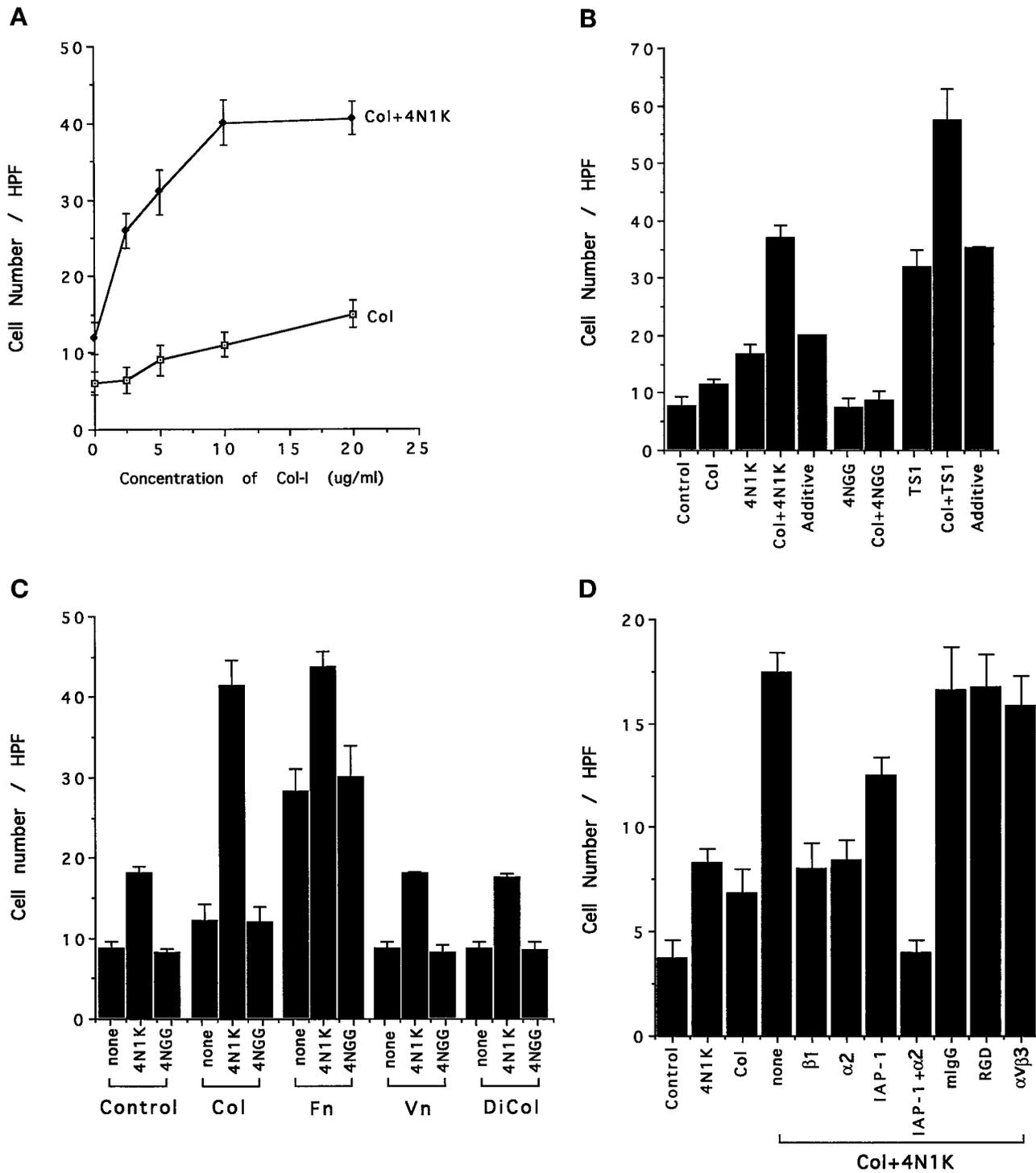
In previous studies with endothelial cells we found that chemotaxis to 4N1K on gelatin-coated filters re-

quired functional  $\alpha v\beta 3$  integrin. Thus, we challenged the smooth muscle cell response to 4N1K with an anti- $\alpha v\beta 3$  function-blocking mAb 4C1 and found no effect (Figure 4B). FACS analysis revealed relatively little  $\alpha v\beta 3$  expressed on these cells (Figure 2). In view of the huge amount of  $\beta 1$  present on smooth muscle cells and previous reports of  $\beta 1$  integrin expression on them (Skinner *et al.*, 1994; Liaw *et al.*, 1995), we tested the anti- $\beta 1$  mAb P4C10 and found that it completely blocked chemotaxis (Figure 4B). Since  $\alpha 2\beta 1$  integrin has previously been shown to be the major collagen-binding integrin on cultured smooth muscle cells (Davis, 1992; Coso *et al.*, 1995; Liaw *et al.*, 1995), we determined the expression of  $\alpha 2$  (mAb P1E6) and  $\alpha 2\beta 1$  (mAb BHA2.1) on the human aortic smooth muscle cells. These two mAbs gave virtually identical staining and indicate robust expression of  $\alpha 2\beta 1$  on the cells. In addition, mAb BHA2.1 directed against  $\alpha 2\beta 1$  completely inhibited adhesion of the cells to immobilized collagen I and gelatin (our unpublished results).

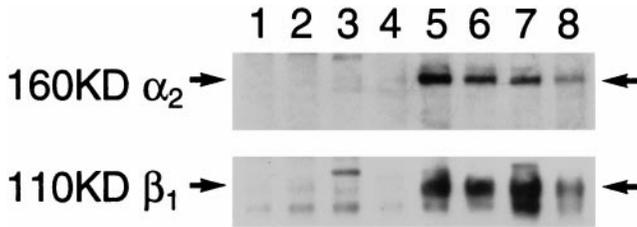
#### 4N1K and Soluble Collagen Stimulate Chemotaxis Synergistically

We next tested the ability of the aortic smooth muscle cells to migrate toward soluble, native collagen type I (Nelson *et al.*, 1996) in the Boyden chamber assay and found that these cells displayed a relatively weak but significant chemotactic response (Figure 5A) similar to that with 4N1K (Figures 3 and 4). However, when soluble collagen I was tested in combination with 4N1K peptide (Figure 5A), the stimulation of cell migration was synergistic (compare the Col + 4N1K with the calculated "additive" response in Figure 5B). As before, the control peptide 4NNGG was completely inactive and was unable to synergize with soluble collagen to give an increased response. Intact thrombospondin-1 gave the expected strong response and was also synergistic with native collagen (Figure 5B). To determine whether 4N1K could stimulate the response of smooth muscle cells to other matrix proteins, soluble collagen type I (Col), fibronectin (Fn), vitronectin (Vn), and collagenase-digested collagen-I (DiCol) were each tested as chemoattractants in the presence of no additives, 4N1K, or 4NNGG (both at 100  $\mu\text{M}$ ). As seen in Figure 5C, native collagen-I and 4N1K together gave a much stronger response than 4NNGG plus native collagen-I, while in the cases of fibronectin, vitronectin, and digested collagen, the amount of additional migration in the presence of 4N1K is only that expected from the action of 4N1K alone. Thus the 4N1K synergy response seems to be limited to native soluble collagen-I.

To determine the nature of this response, the effect of mAbs against integrins and IAP was examined. As seen in Figure 5D, 4N1K again synergizes with soluble native type I collagen (none = no inhibitor added).



**Figure 5.** (A) The synergistic effect of 4N1K and soluble collagen-I on rat smooth muscle cell chemotaxis. 4N1K (100  $\mu$ M) and increasing concentrations of soluble collagen-I were used as chemoattractant. (B) Both 4N1K and thrombospondin-1 are synergistic with collagen-I. Collagen-I was present at 5  $\mu$ g/ml along with 100  $\mu$ M 4N1K or 4NGG, or 6  $\mu$ g/ml thrombospondin-1 as chemoattractants. The experimentally observed values for the concerted effect of 4N1K, thrombospondin-1, and collagen (4N1K $\pm$ Col, TS1 $\pm$ Col) were significantly greater than the calculated additive effect (additive). (C) Comparison of the effect of soluble collagen-I (Col), fibronectin (Fn), vitronectin (Vn), and digested collagen-I (DiCol) on 4N1K-induced rat smooth muscle cell chemotaxis. Collagen-I (5  $\mu$ g/ml) was digested with 0.4 U/ml collagenase type I at 37°C overnight. 4N1K or 4NGG (100  $\mu$ M) and 5  $\mu$ g/ml of the different matrix proteins in MEM with 0.1% BSA were



**Figure 6.** Association of IAP and  $\alpha 2\beta 1$  integrin. Human smooth muscle cells were harvested by trypsin/EDTA and lysed in 30 mM *n*-octyl- $\beta$ -D-glucopyranoside as described in MATERIALS AND METHODS. Soluble material from equal numbers of cells was immunoprecipitated with the following mAbs: mouse IgG from Sigma (lane 1), anti-HLA (lane 2), anti-TNF receptor (lane 3), mouse IgG from Pierce (lane 4), anti- $\alpha 2\beta 1$  BHA2.1 (lane 5), anti-IAP 2D3 and 1F7 (lanes 6 and 7). Lane 8 is a total cell lysate.  $\alpha 2$  and  $\beta 1$  subunits were identified by Western blotting with polyclonal antibodies and ran at the expected sizes of 160 and 110 kDa, as indicated by the arrows.

MABs specific for both the  $\beta 1$  (P4C10) and  $\alpha 2$  (P1E6) integrin subunits reduce the migration to that seen for 4N1K alone. MAb 1F7 against IAP partially reduces migration, but in combination with mAb P1E6 against the  $\alpha 2$  integrin subunit, it reduces migration to background levels. Mouse IgG, RGD peptide, and mAb 4C1, a function-blocking mAb against  $\alpha \nu \beta 3$ , were all without effect on smooth muscle cell migration stimulated by collagen plus 4N1K.

#### IAP and $\alpha 2\beta 1$ Form a Stable Complex

When  $\alpha \nu \beta 3$  or  $\alpha IIb\beta 3$  functions are modulated by 4N1K/IAP, the IAP is found in a detergent-stable complex with the integrins (Gao *et al.*, 1996a,b; Chung *et al.*, 1997). Thus we asked whether IAP could associate with  $\alpha 2\beta 1$  in these smooth muscle cells. Human smooth muscle cells were lysed in *n*-octyl- $\beta$ -D-glucopyranoside, and the clarified lysate immunoprecipitated with a number of control antibodies as well as mAbs against IAP. The immunoprecipitates were then run on SDS gels, blotted onto nitrocellulose, and probed with antibodies specific for the  $\alpha 2$  and  $\beta 1$  integrin chains. Figure 6 shows that both  $\alpha 2$  and  $\beta 1$  integrin subunits are recovered in IAP immunoprecipitates performed with two different anti-IAP mAbs, but not in those using control antibodies from two different suppliers (lanes 1 and 4) or anti-HLA (lane 2)

**Figure 5 (cont).** added to the lower compartment of Boyden chamber. (D) The effect of mAbs on collagen- and 4N1K- induced human smooth muscle cell chemotaxis. Smooth muscle cells were preincubated with mAbs at 37°C for 15 min before being added to the upper compartment of the Boyden chamber. The concentrations of mAbs were: anti- $\beta 1$  mAb P4C10 (1:1500 dilution), anti- $\alpha 2$  mAb P1E6 (1:3000 dilution), anti-IAP-1 mAb 1F7 100  $\mu$ g/ml, mIgG 100  $\mu$ g/ml, RGD peptide 50  $\mu$ M, anti- $\alpha \nu \beta 3$  mAb 4C1 100  $\mu$ g/ml.

or anti-TNF receptor (lane 3) mAbs. The same experiment was performed with Triton X-100 detergent lysates of human smooth muscle cell with comparable results, indicating that the complex of the  $\alpha 2\beta 1$  integrin with IAP is not detergent-dependent and probably does not require the integrity of "detergent resistant" domains (Brown and Rose 1992; Hanada *et al.*, 1995).

#### DISCUSSION

The present data indicate that thrombospondin-1, through its IAP-binding motif, the 4N1K peptide, is able to modulate the activity of the  $\alpha 2\beta 1$  integrin such that it can promote chemotaxis of arterial smooth muscle cells to soluble collagen-I. The enhanced ability of integrins to bind soluble ligands is often associated with "affinity modulation" or "inside-out" signaling (Shattil *et al.*, 1994; Chung *et al.*, 1997). In cells in which IAP activates a  $\beta 3$  integrin such as  $\alpha \nu \beta 3$  in C32 cells (Gao *et al.*, 1996a) and  $\alpha IIb\beta 3$  in platelets (Chung *et al.*, 1997), protein kinase C (PKC) and PI-3 kinase activation are required. Our preliminary data indicate that both PKC and PI-3 kinase are also involved in the stimulation of  $\alpha 2\beta 1$ -dependent chemotaxis in smooth muscle cells. These results are in agreement with the  $\beta 3$  systems and suggest that IAP is able to activate the  $\alpha 2\beta 1$  integrin in such a way that it can bind soluble collagen and transduce signals leading to directional migration. The  $\alpha IIb\beta 3$  integrin is maintained in a low-affinity/avidity state in circulating platelets such that it cannot bind its primary ligand, soluble fibrinogen, which is present at a high concentration in plasma. Upon activation by "inside-out" signaling originating with IAP or other costimulatory receptors, i.e., thrombin, ADP, or epinephrine receptors, the integrin is able to bind the soluble ligand, and platelet aggregation ensues (Shattil *et al.*, 1994; Chung *et al.*, 1997). The fact that chemotaxis toward soluble collagen is augmented by the 4N1K-IAP interaction suggests that it is the affinity of  $\alpha 2\beta 1$  for a soluble ligand that is being modulated here. It is well known that  $\alpha 2\beta 1$  can exist in three states depending upon the cell type in which the integrin is expressed (Santoro and Zutter, 1995); perhaps these states are determined by the expression levels of IAP and/or thrombospondin-1, or the competition for available IAP by the integrin population expressed on the different cells.

The human smooth muscle cells used in these studies express relatively little  $\alpha \nu \beta 3$  (as determined by FACS with an anti- $\alpha \nu \beta 3$  mAb, Figure 2). Their interaction with both native and denatured collagen-I is mediated by  $\alpha 2\beta 1$  (as determined in cell adhesion assays, our unpublished results). This is consistent with reports from other laboratories which show that, although apparently not present on normal aortic smooth muscle cell in situ,  $\alpha 2\beta 1$  is expressed abun-

dantly on cultured smooth muscle cells (Skinner *et al.*, 1994; Liaw *et al.*, 1995). While the cells used in our studies probably express lower levels of other collagen-binding  $\beta 1$  integrins such as  $\alpha 3\beta 1$  (Skinner *et al.*, 1994), the fact that the anti- $\alpha 2$  mAb reduces the chemotactic response to the same low level as the anti- $\beta 1$  mAb (Figure 6D) indicates that the response to collagen, which is amplified by 4N1K, is mediated entirely by  $\alpha 2\beta 1$ . Thus it appears that the previously reported chemotactic activity of thrombospondin-1 for smooth muscle cells (Yabkowitz *et al.*, 1993) can be explained by the activity of the 4N1K peptide, which resides in the cell-binding domain. This conclusion is strengthened by the observation of Yabkowitz *et al.* (1993) that, of our panel of domain-specific anti-thrombospondin-1 mAbs, only mAb C6.7 against the cell-binding domain could inhibit the stimulation of chemotaxis. However, their study used bovine pulmonary artery smooth muscle cells, and found that migration of those cells on gelatin-coated polycarbonate filters was dependent on  $\alpha v\beta 3$  integrin (Yabkowitz *et al.*, 1993), as we have found for human umbilical vein endothelial cells, and not  $\alpha 2\beta 1$  as seen here for human aortic smooth muscle cells. The types and levels of integrins expressed by the bovine smooth muscle cells used in that study were not investigated (Yabkowitz *et al.*, 1993). Thus, while the integrin may be different, the mechanism of the stimulation by thrombospondin-1 is probably the same, i.e., modulation of the integrin's affinity/avidity by IAP.

An important aspect of these data is that it extends the biological relevance of the thrombospondin-IAP interaction to another subset of integrins, the  $\beta 1$  family. Not only does ligation of IAP with the agonist peptide 4N1K augment the function of  $\alpha 2\beta 1$  in sensing a gradient of soluble collagen, but we have demonstrated for the first time, the existence of a physical complex that includes IAP and  $\alpha 2\beta 1$  integrin (Figure 6). In addition to the coimmunoprecipitation of  $\alpha 2\beta 1$  and IAP, our preliminary data indicate that  $\alpha 2$  and  $\beta 1$  integrin subunits coelute with IAP from a 4N1K affinity column (Wang and Frazier, unpublished). Neither of these methods can of course distinguish between a direct or indirect association. It has been previously reported that IAP can physically associate with  $\alpha v\beta 3$  (Brown *et al.*, 1990), and we have found that IAP copurifies and coimmunoprecipitates with  $\alpha IIb\beta 3$  from platelet lysates (Chung *et al.*, 1997). In the case of both of these  $\beta 3$  integrins, it appears that IAP associates with the integrin that it modulates, even though these signaling pathways require activation of PKC (Gao *et al.*, 1996a; Chung *et al.*, 1997), and hence would not, a priori, seem to require association. In the one case in which IAP had been shown to modulate a  $\beta 1$  integrin (Blystone *et al.*, 1994, 1995), signaling emanating from a complex of  $\alpha v\beta 3$  and IAP inhibited a high-affinity state of  $\alpha 5\beta 1$ . These observations suggest that

association of IAP with the integrin may be necessary for a positive modulatory effect. The signaling pathways by which this occurs are currently under study.

The modulation of  $\alpha 2\beta 1$  integrin in smooth muscle cells by the IAP-binding domain of thrombospondin-1 may have physiological implications even though expression of  $\alpha 2\beta 1$  on smooth muscle cells in vivo has not been reported (Skinner *et al.*, 1994; Gotwals *et al.*, 1996). Freshly isolated smooth muscle cells from the same sources as those used in our study express large amounts of  $\alpha 1\beta 1$  and  $\alpha 3\beta 1$  (Skinner *et al.*, 1994). If the mechanism of integrin modulation found for  $\alpha 2\beta 1$  in vitro is in place in smooth muscle cells in vivo, the regulated synthesis and/or secretion of thrombospondin-1 at sites of wounding, atherosclerosis and restenosis may affect the function of these  $\beta 1$  integrins as well. Not only is thrombospondin-1 deposited at sites of vessel injury due to platelet discharge, either chronic or acute, but thrombospondin-1 is a major biosynthetic product of endothelial cells, fibroblasts, macrophages, and PDGF-stimulated smooth muscle cells themselves (Adams *et al.*, 1995). Thus the local deposition or biosynthesis of thrombospondin-1 could generate a positively reinforced loop, resulting in the continued attraction of smooth muscle cells into injured sites. The recent finding that  $\alpha 2\beta 1$  is involved in collagen-dependent cell cycle regulation in cultured smooth muscle cells (Koyama *et al.*, 1996) suggests a role for  $\beta 1$  integrins in control of smooth muscle cell proliferation. It also opens the possibility that thrombospondin-IAP interactions may regulate smooth muscle cell proliferation by modulating the ability of  $\beta 1$  integrins to signal to the proteins that regulate the cell cycle (Mechtersheimer *et al.*, 1994). Finally, the physiological role of thrombospondin is underscored by our recent data using a rat carotid artery balloon injury model in which mAb C6.7 against the thrombospondin-1 cell binding domain significantly inhibited both neointimal thickening and smooth muscle cell proliferative index at the site of injury (Chen *et al.*, 1997). Whether this effect is due to integrin modulation via IAP remains to be determined.

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