

# Thrombospondin Modulates $\alpha_v\beta_3$ Function through Integrin-associated Protein

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**Abstract.** Integrin-associated protein (IAP) is a receptor for the carboxyl-terminal "cell-binding domain" (CBD) of thrombospondin 1 (TS1). IAP associates with  $\alpha_v\beta_3$  integrin and mAbs against IAP inhibit certain integrin functions. Here we examine the effects of the TS1 CBD and 4N1K (KRFYVVMWKK), a cell-binding peptide derived from it, on the adhesion and spreading on vitronectin (VN) of C32 human melanoma cells which express IAP,  $\alpha_v\beta_3$ , and  $\alpha_v\beta_5$ . Cells adhere to VN at low surface densities via  $\alpha_v\beta_5$  and spread very slowly while adhesion to higher density VN involves both  $\alpha_v\beta_5$  and  $\alpha_v\beta_3$  and results in rapid spreading. Spreading of the cells, but not adhesion, on sparse VN coatings is markedly enhanced by the presence of soluble TS1, the recombinant CBD and 4N1K, but not the "mutant" peptide 4NGG, KRFYGGMWKK,

which fails to bind IAP. This enhanced spreading is completely blocked by mAb LM609 against  $\alpha_v\beta_3$  and the anti-IAP mAb B6H12. Correlated with this enhanced spreading is increased tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin, and a protein of ca. 90 kD. The enhanced spreading induced by TS1 and 4N1K and the constitutive spreading on higher density VN are both blocked by calphostin C (100 nM), wortmannin (10 nM), and tyrosine kinase inhibitors. In contrast, pertussis toxin specifically blocks only the TS1 stimulated spreading on low density VN, indicating that IAP exerts its effects on signal transduction via a heterotrimeric Gi protein acting upstream of a common cell spreading pathway which includes PI-3 kinase, PKC, and tyrosine kinases.

THE family of thrombospondins has been implicated in acute regulation of a number of diverse physiological processes such as platelet aggregation, inflammation, angiogenesis, cell adhesion and migration, and other developmental processes (5, 6, 16, 61). The prototypic member of this family is platelet thrombospondin or thrombospondin-1 (TS1).<sup>1</sup> Many cell interaction sites and peptides have been identified within the modular TS1 structure and as many as a dozen different receptors, including several  $\beta_1$  and  $\beta_3$  integrins have been proposed to mediate the effects of TS1 on cells (12, 16, 33, 70). Of this plethora of putative receptors, the direct binding of TS1 to molecularly characterized receptors has been demonstrated only for HSPGs (40), sulfatides (49), CD36 (1), and the integrins  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_3$  (33). In the case of the two integrins, binding of native TS1 has been demonstrated for some cell types, while for other cells, the TS1 RGD se-

quence appears to be cryptic (62). A number of peptide epitopes within the complex TS1 structure have been identified with specific functions of the protein. For example, sequences within the procollagen-like domain, and two of the type 1 (properdin- or malaria-like) repeats inhibit angiogenesis in vivo and endothelial cell (EC) motility in vitro (65). Also, a peptide from the amino-terminal heparin-binding domain reverses focal adhesions in fibroblasts and EC (41, 42) and a tripeptide in one of the type 1 repeats is capable of activating latent TGF $\beta$  thus potentially influencing cell behavior via this pathway (53).

In addition to these active sequences in the central protease-resistant stalk-like region of the TS1 subunit, the carboxy terminal domain of TS1 interacts with many cell types and has been designated the cell-binding domain or CBD. The mAb C6.7 directed to the CBD, has been used to implicate this domain of TS1 as a stimulator of motility of a number of cell types (37, 38, 64, 71, 72) and to establish a role for TS1 in secondary phase or secretion-dependent platelet aggregation (14). To better define the structure-function relationships of the CBD, we expressed it in bacteria exclusive of the nearby RGD sequence of TS1 and established that its cell-binding activity was independent of the RGD site (29). Using overlapping peptides we then defined two related active peptide sequences within the 221 residues of the CBD: RFYVVM (the minimal C4

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1. *Abbreviations used in this paper.* CBD, carboxy terminal domain; EC, endothelial cell; FAK, focal adhesion kinase; HUVEC, human umbilical vein endothelial cell; IAP, integrin-associated protein; LM, laminin; TS1, thrombospondin-1; VN, vitronectin.

peptide) and IRVVM (the minimal C7 peptide) (27, 28). The peptides competed with one another in cell binding assays and both affinity labeled a 52-kD membrane glycoprotein CBD receptor candidate (17). We have recently identified this 52-kD protein as the widely distributed integrin-associated protein (IAP or CD47) (7, 18, 35, 36). IAP is found in association with  $\alpha_v\beta_3$  and other integrins, and it has been implicated in modulating integrin functions required for  $\text{Ca}^{++}$  fluxes (54), phagocytosis (4), and cellular migration events such as transendothelial (11) and trans-epithelial (45) migration of PMNs. The function-blocking anti-IAP mAb B6H12 abolishes chemotaxis of human umbilical vein endothelial cells (HUVECs) towards the CBD and C4 peptides (18) under conditions in which the HUVECs employ  $\alpha_v\beta_3$  integrin for locomotory traction. Furthermore, both TS1 and RFYVVMWK peptide stimulate a  $\text{Ca}^{++}$  transient in IMR90 fibroblasts that is inhibited by mAb C6.7 directed against the CBD (in the case of TS1) and by anti-IAP mAb B6H12 (66). Taken together these data suggest that the CBD can stimulate a chemotactic response by a concerted mechanism in which  $\text{Ca}^{++}$  regulated events necessary for cell motility are activated by a  $\text{Ca}^{++}$  flux and at the same time, the function of  $\alpha_v\beta_3$  is modulated.

The most studied example of  $\beta_3$  integrin activation is the platelet integrin  $\alpha_{IIb}\beta_3$  which is maintained in a state of low affinity and avidity on circulating platelets, but is rapidly activated to bind fibrinogen by thrombin, epinephrine, and ADP acting via seven transmembrane-spanning (7TMS or heptahelical) receptors (56). The signal transduction pathway by which these receptors "costimulate" fibrinogen binding by  $\alpha_{IIb}\beta_3$  is incompletely understood and complex, involving proximal heterotrimeric G proteins, lipid mediators derived from arachidonic acid via both cyclooxygenase and lipoxygenase, which lead to the activation of PKC and small G proteins of the rho family (55, 57). Other examples of integrin activation such as the leukocyte CD11a,b,c/CD18 ( $\beta_2$ ) family also involve costimulation from 7TMS chemokine receptors acting through heterotrimeric and rho family G proteins, lipid mediators, and PKC (60). There is also accumulating evidence that integrin-mediated cell spreading on a variety of matrix proteins requires signal transduction events with features common to the pathways of integrin activation and costimulation, suggesting that some integrins are constitutively activated or can "autoactivate" to cause cell spreading. Chemotaxis and spreading of cells are closely allied functions which depend on similar activation states of the integrin and similar downstream signal transduction events (9, 15, 25, 55).

Given the previously established role of IAP in  $\alpha_v\beta_3$  integrin signaling (4, 7, 36, 54, 66, 75), we have investigated the potential role of the CBD-IAP interaction in modulating the  $\beta_3$  integrin-mediated adhesion and spreading of cells on vitronectin-(VN) coated surfaces. We have chosen a cell type that expresses IAP and the VN receptors  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ . Under appropriate conditions of low VN surface density, the initial adhesion event is mediated by  $\alpha_v\beta_5$  while subsequent spreading of the cells requires  $\alpha_v\beta_3$ . Spreading of unstimulated cells occurs only slowly, but addition of TS1, its recombinant CBD or the RFYVVM containing CBD peptides all greatly accelerate spreading

with the concomitant tyrosine phosphorylation of focal adhesion kinase (FAK) and associated proteins. This IAP-dependent response to the CBD requires a pertussis toxin-sensitive, heterotrimeric G protein. In contrast, constitutive spreading on high density VN surfaces is unaffected by pertussis toxin while both TS1-stimulated and constitutive spreading are blocked by inhibitors of PI-3 kinase, PKC, and tyrosine kinases. Thus, ligation of IAP by the TS1 CBD stimulates a Gi-like protein, which then appears to lead to the activation of PI-3 kinase, protein kinase C, and tyrosine kinases, elements common to the activation of integrin-dependent cell spreading in many systems (2, 25, 34, 44, 55, 67, 69).

## Materials and Methods

### Reagents and Antibodies

Human vitronectin, fibronectin, and collagens I and IV were obtained from Collaborative Biomedical Products (Bedford, MA). Fibrinogen was from Sigma Chem. Co. (St. Louis, MO). The rCBD was expressed as a His<sub>6</sub>-tagged protein in *E. coli* using the pQE30 vector (Qiagen, Chatsworth, CA) and purified by Ni-NTA chromatography (Dimitry, J., A. Jurkovich and W. Frazier, manuscript in preparation). 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 (7, 35, 36). mAbs 4G10 (anti-phosphotyrosine) and 2D7 (anti-p125<sup>FAK</sup>, for immunoprecipitation) were from Upstate Biotechnology Inc. (Lake Placid, NY). Western blotting mAb against FAK, phosphotyrosine (PY20), and paxillin were the products of Transduction Laboratories (Lexington, KY). All protein kinase inhibitors were purchased from CalBiochem (La Jolla, CA). Rainbow protein molecular weight markers and ECL Western blotting detection kit were from Amersham Corp. (Arlington Heights, IL). Anti-mouse IgG-agarose and other reagents were from Sigma. All peptides used were synthesized by the Protein and Nucleic Acid Chemistry Laboratory of Washington University School of Medicine as described previously (17, 28). Peptides were evaluated by mass spectrometry before and after purification on high performance liquid chromatography. Human platelet TS1 was purified as described (50). The amino acid sequences of the TS1 peptides used in this study are 4NK, KRFYVVMWKQVTSQSKKY (native sequence 1016-1029); 4N1K, KRFYVVMWKK; 4N7G, KRFYVVMGKK; 4NGG, KRFYGGMWKK; Hep III, TRDLASIALRLRIAKGVNDNF (170-190); MAL III, SPWDIASVTAGGGVQKRSR (481-499). The synthetic peptides GRGDSP and GRGESP were from GIBCO-BRL (Gaithersburg, MD).

### Cell Culture, Adhesion, and Spreading

C32 human melanoma cells (ATCC CRL 1585) were cultured in Gibco's Minimum Essential Medium (MEM) supplemented with 10% FCS. Murine 3T3 fibroblasts (ATCC CRL 1658) were grown in DMEM plus 10% calf serum. Cell adhesion assays were performed as described previously (28). Briefly, 96-well plates were coated with substrates overnight at 4°C. C32 cells were harvested with 0.04% EDTA in PBS and washed twice with PBS. Cells were resuspended in Hepes-buffered saline (HBS; 20 mM Hepes/NaOH, pH 7.4, 0.15 M NaCl, 1 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>) allowed to adhere to the immobilized substrates for 90 min at 37°C in the presence of indicated additions in solution. After washing, the adherent cells were lysed in 1% Triton X-100 and quantified by endogenous phosphatase activity with *p*-nitrophenyl phosphate as a substrate (46). For cell spreading experiments, glass chamber slides (LabTek) or 12-well tissue culture plates were coated with VN overnight at 4°C. C32 cells were harvested for cell adhesion experiments and resuspended in HBS containing calcium and magnesium, both at 1 mM. Indicated additions of stimulators and inhibitors were also added to cell suspensions at this time. Cell spreading at 37°C was followed by washing, fixing, and staining with a LeukoStat stain kit (Fisher Scientific, Pittsburgh, PA). For the inhibition of cell attachment or spreading, C32 cells were pre-incubated with antibodies or inhibitors for 15 min at 0°C before the assay. The required light activation of calphostin C (8, 19, 26) was also performed during this pre-incubation time.

## FACS Analysis of the Expression of Cell Surface Receptors

The expression of integrins and IAP was detected by fluorescent flow cytometry. C32 cells were harvested and washed as described above and resuspended in C32 cell culture medium (with 10% FCS). The mAbs LM609 vs  $\alpha_v\beta_3$ , P1F6 vs  $\alpha_v\beta_5$ , or 2D3 vs IAP were added to aliquots of cell suspensions and incubated for 60 min at 4°C with rocking. After several washes in PBS, the aliquots were stained with FITC-labeled anti-mouse secondary antibody (Pierce, Rockford, IL) for another 60 min in the cell culture medium, washed again with PBS, and then analyzed by flow cytometry. The second antibody and the mouse IgG, instead of primary antibody, were both used as negative controls and mAb W6/32 against HLA was the positive control.

## Immunoprecipitation Studies

C32 cell spreading was carried out as described above. At indicated times, cells were lysed by addition of 6× precooled modified RIPA lysis buffer. 1× RIPA lysis buffer is 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl, 1% (wt/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 1 mM EGTA, 1 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitors consisting of 10  $\mu\text{g/ml}$  each of antipain, pepstatin A, chymostatin, leupeptin, soybean trypsin inhibitor, aprotinin, and 1 mM phenylmethylsulfonylfluoride. Cell lysis was completed by 30 min rocking at 4°C followed by microcentrifugation at top speed (13,000 rpm) for 30 min. The soluble material from equal numbers of cells (or equal amounts of protein) was incubated with the specified monoclonal antibody for 3 h at 4°C and immunoprecipitated with anti-mouse IgG-agarose. The precipitates were extensively washed with 1× RIPA buffer, dissolved, and boiled in a small volume of SDS-sample buffer.

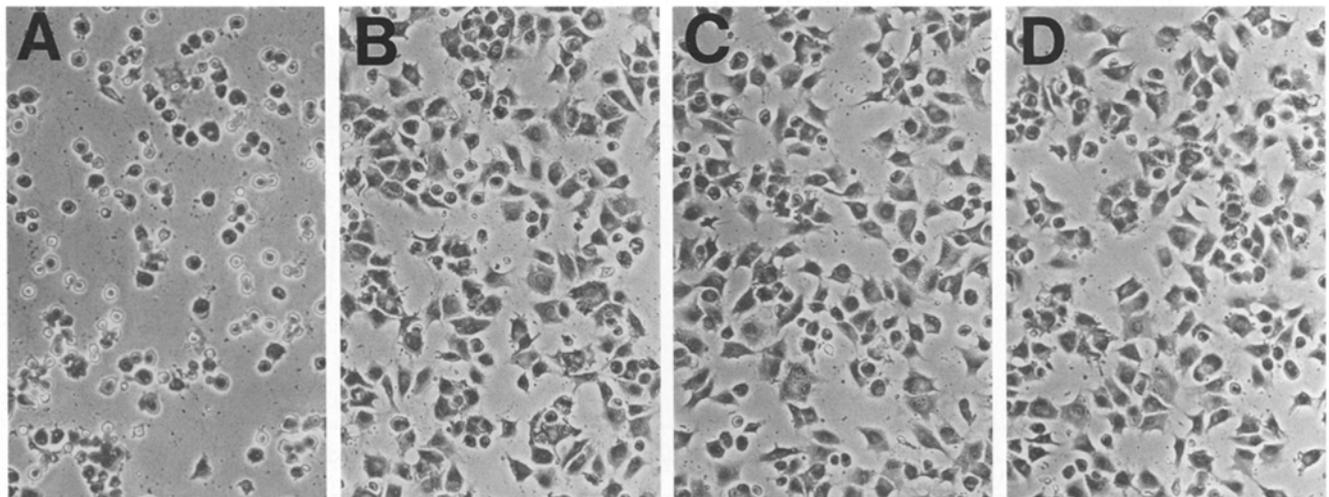
## Identification of Immunoprecipitated Proteins by Western Blot Analysis

Proteins were separated by SDS-PAGE on 4–12% or 8% precast Tris-Glycine gels (NOVEX, San Diego, CA) and transferred to nitrocellulose membranes. Blots were blocked with 3% BSA plus 3% dried milk in TBS-T (0.1% Tween 20, 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl) overnight and probed with the indicated mAb at 1  $\mu\text{g/ml}$  in TBS-T plus 10% blocking buffer for 1–2 h, washed, and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce). Detection was by chemiluminescence. For identification of coimmunoprecipitated protein(s), the same blot was stripped at 68°C (2×, 20 min) in stripping buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 100 mM  $\beta$ -ME). The membrane was reprobed with the indicated mAb and processed as described above.

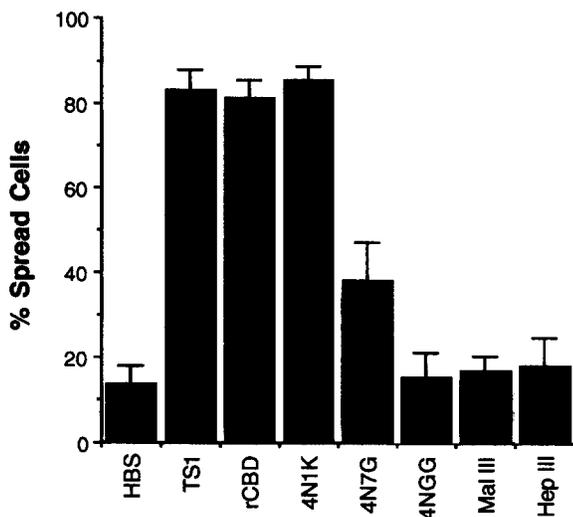
## Results

### Spreading of C32 Cells on VN

The spreading of C32 cells was examined on surfaces coated with a concentration range of VN (0.1–10  $\mu\text{g/ml}$ ). Below ca. 3  $\mu\text{g/ml}$  VN, spreading was very slow, requiring 1.5–2 h at 37°C to develop fully. At 5–10  $\mu\text{g/ml}$  spreading was relatively rapid, being complete within 30 min (not shown). The effect of soluble TS1, its recombinant CBD and peptides on C32 cell spreading on VN was tested. As seen in Fig. 1, all three agents dramatically accelerated spreading on suboptimal 1  $\mu\text{g/ml}$  VN-coated surfaces. Fig. 1 A shows the complete lack of spreading observed on VN (1  $\mu\text{g/ml}$ ) at 30 min, while Fig. 1, B–D show fully spread, polygonal cells at 30 min in the presence of TS1, the rCBD and 4N1K in solution. This cell spreading data is quantified in Fig. 2. When coated on the surface in the absence of VN, TS1 supports only very weak spreading of these cells, while the rCBD and 4N1K (and related peptides from the CBD) support no spreading at all when immobilized (not shown). A VN-coated surface is required for spreading to take place since no spreading occurs on BSA-coated surfaces with or without TS1, the rCBD, and its peptides (not shown). To determine if TS1 and its fragments could stimulate cell spreading on other matrix proteins besides VN, C32 melanoma cells were plated on concentration gradients of fibronectin, collagens I and IV, gelatin, fibrinogen, and human and mouse laminin (LM) (as well as VN as a positive control). Concentrations used for coating were determined in preliminary experiments and covered a range from no visible spreading to fully spread cells during a 1-h incubation. The C32 cells spread well on the higher densities of all these proteins except fibrinogen. The addition of 50  $\mu\text{M}$  4N1K or 50  $\mu\text{g/ml}$  TS1 did not stimulate cell spreading at any density of fibronectin, collagens, or fibrinogen. However, 4N1K and TS1 did stimulate cell spreading on lower densities of gelatin and LM (and VN). Attachment to gelatin, like VN, was inhibited by blockade



**Figure 1.** TS1, rCBD, and 4N1K all accelerate C32 cell spreading on immobilized vitronectin. C32 cells were allowed to spread on 1  $\mu\text{g/ml}$  VN-precoated glass slides for 30 min at 37°C, followed by fixing and staining. The following were included in solution: (A), no addition; (B), TS1 at 50  $\mu\text{g/ml}$ ; (C), recombinant CBD at 50  $\mu\text{g/ml}$ ; (D), 50  $\mu\text{M}$  4N1K peptide. BSA or peptides from other domains of TS1 did not affect cell spreading (not shown).



**Figure 2.** The effect of TS1, rCBD, and peptides on C32 cell spreading. C32 cell spreading assays were performed as in Fig. 1. After washing, fixing, and staining, cells with a diameter at least three times larger than that of round unspread cells were counted as spread cells. Results are expressed as the mean percentage ( $\pm$  SD) of spread cells compared to total attached cells in six high power fields from three separate experiments. The peptide sequences are described in Materials and Methods and all peptides were used at 50  $\mu$ M in solution. Peptides 4NGG (KRFYGGM-WKK) and 4N7G (KRFYVVMGKK) are "mutants" of 4N1K while Mal III and Hep III are cell-binding peptides from two other domains of TS1.

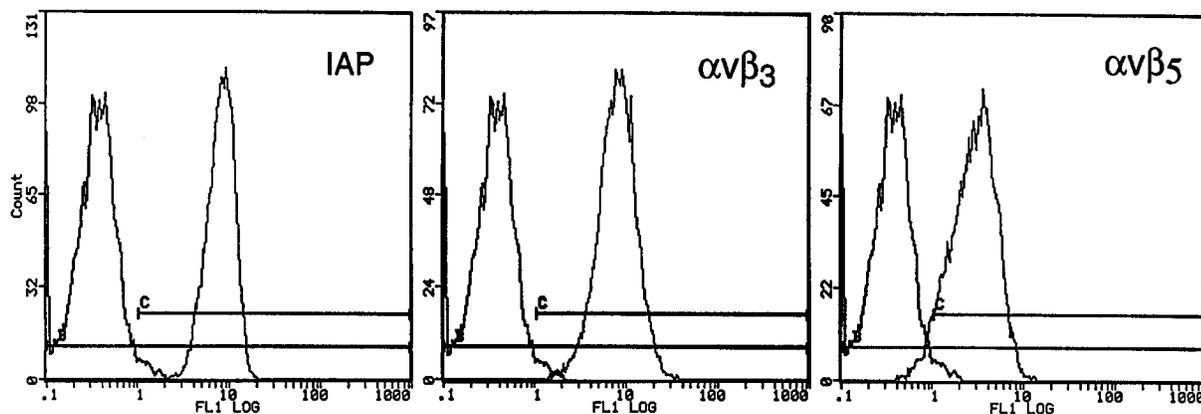
of  $\alpha_v\beta_5$  and spreading was inhibited by anti- $\alpha_v\beta_3$  (LM609) and anti-IAP (B6H12) (not shown). Attachment to LM was blocked by an anti- $\beta_1$  mAb, not by LM609 or P1F6, and stimulated spreading on LM was blocked by B6H12 but not by LM609 (not shown) suggesting that ligation of LM609 is not required for the action of IAP. To determine if TS1 and its CBD fragments could stimulate spreading of other cell types, murine NIH3T3 cells were tested for attachment and spreading on VN and the other matrix proteins above. As with the human C32 cells, TS1 and 4N1K

stimulated spreading on low densities of VN, gelatin, and LM, but not other matrix proteins (not shown).

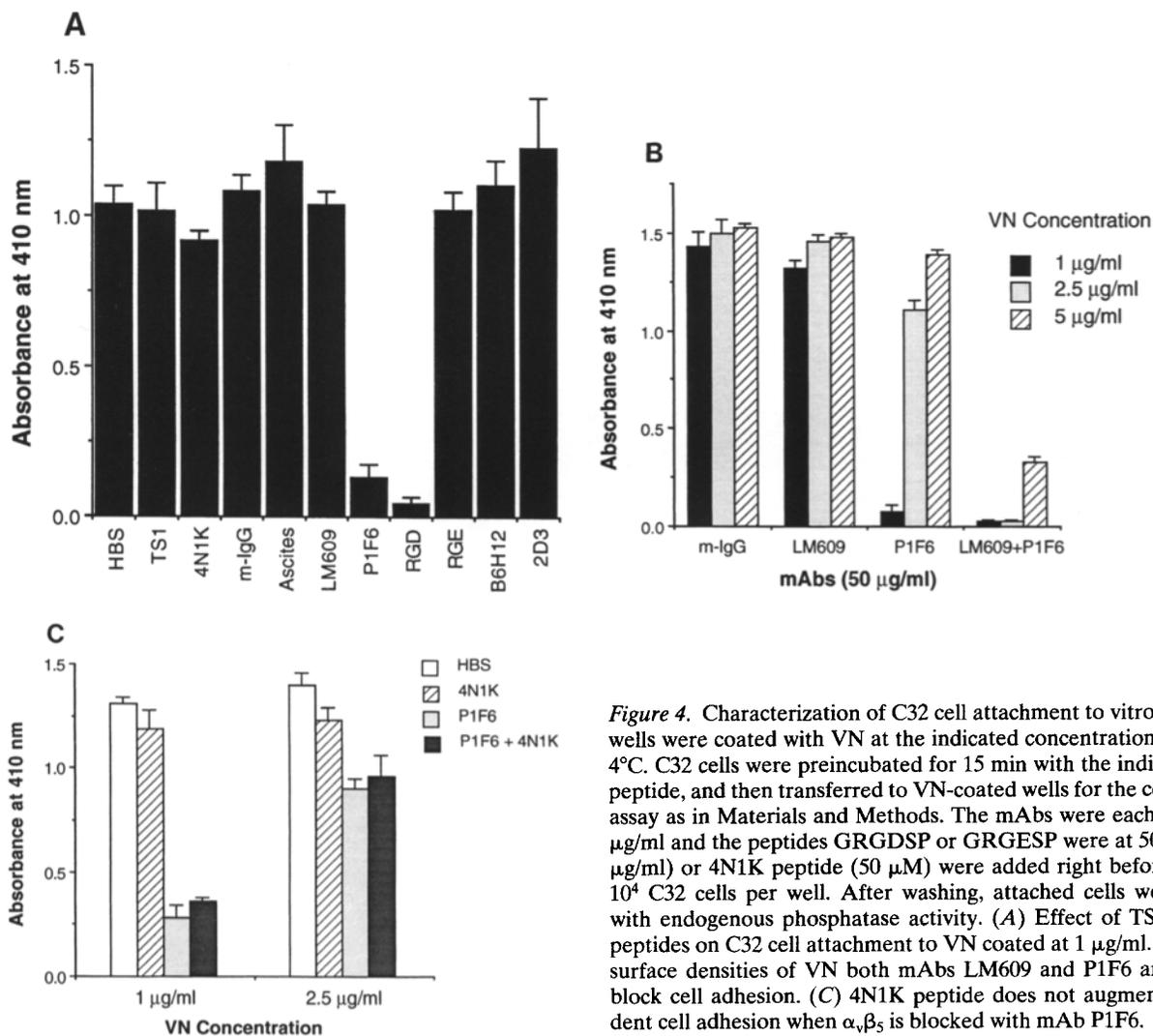
The possibility that contaminants in the platelet TS1 preparation were responsible for the stimulation of cell spreading is ruled out by the potent stimulatory activity of the rCBD produced in bacteria and purified on Ni-NTA resin (as opposed to heparin-agarose for TS1) and the activity of the synthetic peptide 4N1K. Other versions of the CBD peptide containing the RFYVVM sequence in the context of more TS1 native sequence (17, 28) were all active in stimulating cell spreading, while peptides from other domains of TS1, including other cell-binding peptides such as Mal III and Hep III, were without effect (Fig. 2, see Materials and Methods for peptide sequences). The other VVM containing peptide from the CBD, FIRVV-MYEGKK (17, 28), was also active in stimulating cell spreading.

Our previous studies of cell attachment to the CBD peptides indicated that the VVM sequence is important for recognition of IAP (17, 18, 28). Thus "mutant" 4N1K peptides were synthesized in which residues between the KR and KK termini of 4N1K were replaced with G, and one in which the VV sequence was replaced with GG (4NGG). These peptides were tested in a C32 cell adhesion assay which reflects IAP-dependent binding of cells to the immobilized peptide (18), and in the cell spreading assay (Fig. 2). Peptides 4N3G (KRFVVMWKK) and 4N7G (KRFYVVMGKK) had partial activity in both assays while peptide 4NGG (KRFYGGMWKK) was completely inactive in cell spreading (Fig. 2) and in binding cells (not shown). These results are important since the mutant peptides retain the precise charge distribution of the parent peptide.

In summary, these data are important in several respects: they demonstrate that the accelerated spreading is not due to adsorption of the TS1, the rCBD, or peptides to the surface in a nonspecific manner, since the rCBD and peptides support no spreading at all when directly coated on the surface, and only spreading on VN, LM, and gelatin exhibits the effect (and all surfaces are blocked with BSA). Second, only spreading on certain proteins, and thus mediated via certain integrins, is affected by the ligation of IAP



**Figure 3.** Expression of IAP and integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  on C32 cells. C32 cells were stained with mAbs 2D3 (anti-IAP, left), LM609 (anti-integrin  $\alpha_v\beta_3$ , middle), and P1F6 (anti-integrin  $\alpha_v\beta_5$ , right) followed by fluorescein-conjugated goat anti-mouse IgG. The controls were stained with mouse IgG as the primary antibody, the distribution of which is plotted with each indicated mAb for comparison.



**Figure 4.** Characterization of C32 cell attachment to vitronectin. Plastic wells were coated with VN at the indicated concentrations overnight at 4°C. C32 cells were preincubated for 15 min with the indicated mAb or peptide, and then transferred to VN-coated wells for the cell attachment assay as in Materials and Methods. The mAbs were each present at 50 µg/ml and the peptides GRGDSP or GRGESP were at 50 µM. TS1 (50 µg/ml) or 4N1K peptide (50 µM) were added right before plating  $5 \times 10^4$  C32 cells per well. After washing, attached cells were quantified with endogenous phosphatase activity. (A) Effect of TS1, mAbs, and peptides on C32 cell attachment to VN coated at 1 µg/ml. (B) At higher surface densities of VN both mAbs LM609 and P1F6 are required to block cell adhesion. (C) 4N1K peptide does not augment  $\alpha_v\beta_3$ -dependent cell adhesion when  $\alpha_v\beta_5$  is blocked with mAb P1F6.

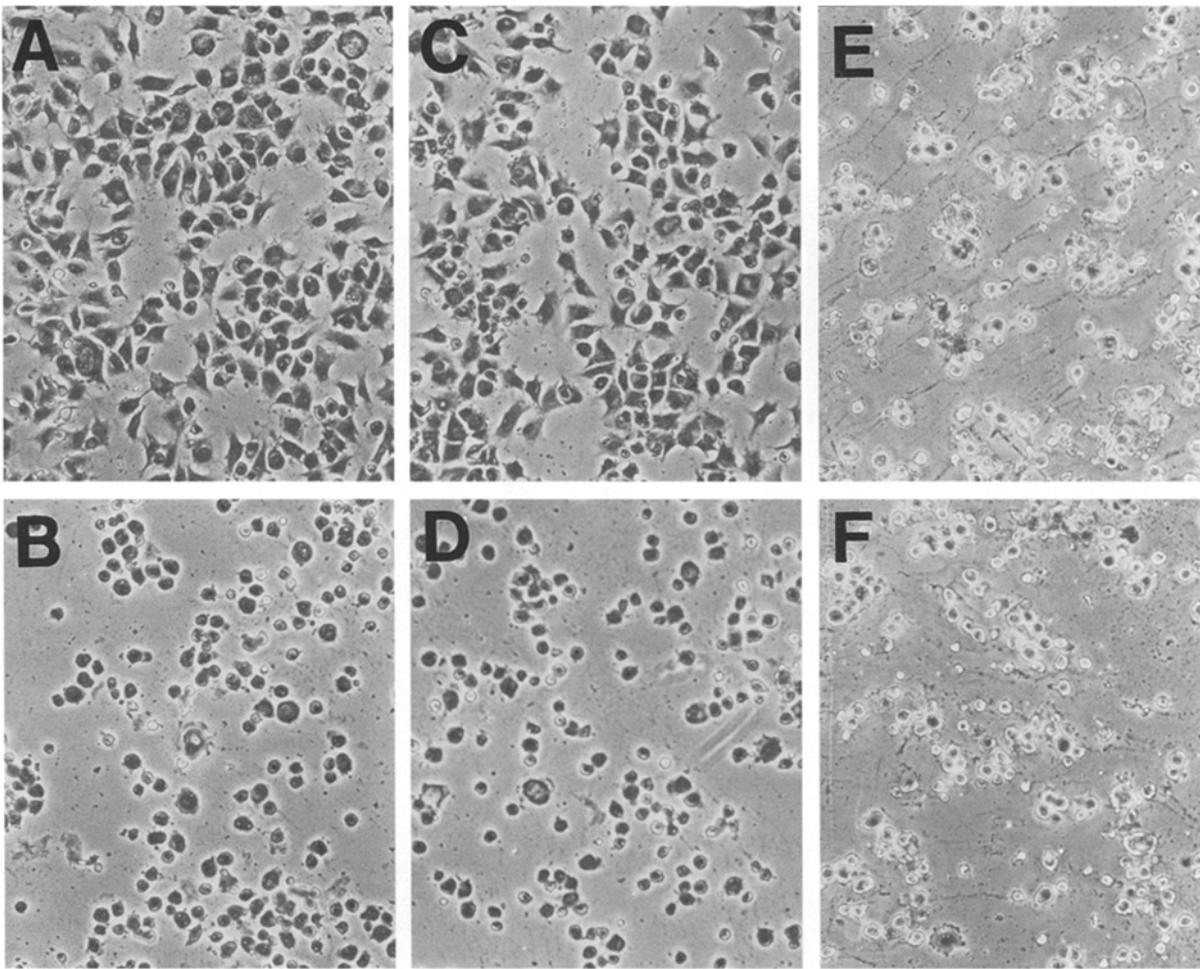
with the TS1 rCBD or peptides. Third, the effect is seen in another species of cell which is not transformed. Fourth, the VV sequence of 4N1K is critical for both binding to IAP and stimulation of cell spreading.

#### Adhesion of C32 Cells

We next examined the receptors used by the melanoma cells for interaction with surfaces coated with VN over a range of concentrations (0.1–10 µg/ml). Flow cytometry was used to determine the levels of IAP and two common VN receptors  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ , expressed on the surface of C32 cells. As seen in Fig. 3, IAP,  $\alpha_v\beta_3$ , and  $\alpha_v\beta_5$  are all relatively abundant. Antibodies to these receptors were used to determine which participated in cell adhesion to VN-coated surfaces. Fig. 4 A shows that an mAb vs  $\alpha_v\beta_5$  (P1F6) effectively blocked adhesion to VN coated at a low density (1 µg/ml) while anti- $\alpha_v\beta_3$  mAb (LM609) had no effect. As expected, RGD (but not RGE) peptide also blocked adhesion to VN. Thus,  $\alpha_v\beta_3$  is expressed on C32 cells (Fig. 3), but did not mediate binding at the low concentration of VN used. We then examined the attachment of cells to higher surface densities of VN, and found that as

the amount of VN adsorbed on the surface is increased, a larger fraction of adhesion is mediated by  $\alpha_v\beta_3$  as judged by the fact that inhibition requires mAbs against both  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  (Fig. 4 B).

To determine if TS1 or 4N1K could increase the apparent affinity or avidity of  $\alpha_v\beta_3$ , adhesion to VN was performed in the presence of TS1 and the peptide. Addition of TS1 or 4N1K peptide to the soluble phase had no effect on the number of cells adhering to VN (Fig. 4 A), even though TS1 and an active CBD cell-binding peptide 4N1K, bound cells quite well when they were coated directly on the plastic, while several control TS1 peptides and BSA did not bind cells to an appreciable extent (not shown). Two anti-IAP mAbs (B6H12 and 2D3) also had no effect on cell attachment to VN (Fig. 4 A), consistent with the lack of effect of peptide 4N1K, an IAP ligand, on the number of cells adhering. If  $\alpha_v\beta_3$  were activated by 4N1K, the proportion of adhesion mediated by  $\alpha_v\beta_3$  would be greater in the presence of the peptide. However, the degree of inhibition by LM609 was not increased at any VN surface density tested (not shown). Also, when  $\alpha_v\beta_5$  was blocked by P1F6 mAb, the addition of 4N1K did not increase cell adhesion to VN, indicating a lack of effect on adhesion via



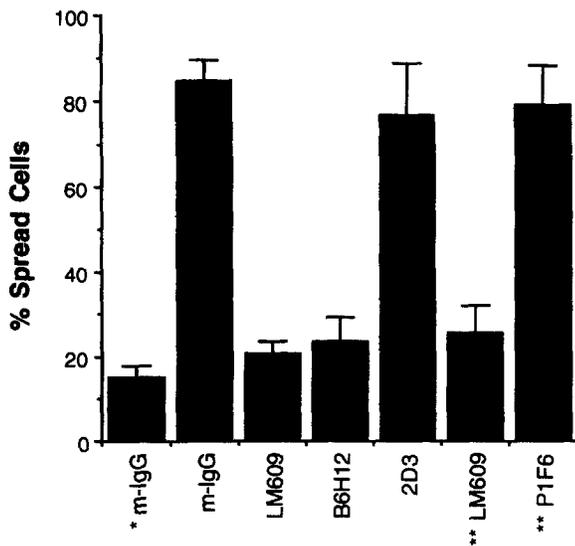
**Figure 5.** Effect of mAbs and kinase inhibitors on C32 cell spreading on vitronectin. After 15 min pre-incubation with mAbs or inhibitors (see Materials and Methods for details), cell spreading was allowed to proceed for 30 min on 1  $\mu\text{g/ml}$  VN-coated glass slides. (A) 50  $\mu\text{M}$  soluble 4N1K stimulated C32 cell spreading as before; (B) mAb LM609 at 20  $\mu\text{g/ml}$  completely abolished spreading stimulated by 4N1K. mAb 2D3, a nonfunction-blocking mAb against IAP, had little effect at 100  $\mu\text{g/ml}$  (C) while B6H12 (D), an anti-IAP function-blocking mAb, completely prevented spreading at 100  $\mu\text{g/ml}$ . The PKC inhibitor calphostin c (at 100 nM) (E) and wortmannin (at 10 nM) (F), a PI-3 kinase inhibitor, both abolished spreading stimulated by 4N1K as well as the spreading which occurred on higher density VN without 4N1K (not shown).

$\alpha_v\beta_3$  or any other receptor (Fig. 4 C). Thus, it appears that TS1 and 4N1K peptide do not increase the affinity/avidity of  $\alpha_v\beta_3$  even though TS1 and its CBD derived fragments have dramatic effects on cell spreading on VN (Fig. 1). This suggests that effects on cell spreading are mediated by stimulation of an IAP-dependent signaling pathway by TS1.

#### Characterization of TS1-stimulated Spreading

We first determined the effect of mAbs on the 4N1K stimulated spreading of C32 cells. Fig. 5 A shows the stimulation (at 30 min) by 50  $\mu\text{M}$  4N1K of cells attached to 1  $\mu\text{g/ml}$  VN. Figs. 5 B and 6 show that mAb LM609 directed against the VN receptor  $\alpha_v\beta_3$ , results in complete inhibition of cell spreading, even though, as seen in Fig. 4 A, initial attachment of the cells to VN is mediated solely by  $\alpha_v\beta_5$ . Thus, it appears that the stimulation of spreading is mediated by  $\alpha_v\beta_3$ . At higher coating concentrations of VN, where spreading is more rapid and stimulation by TS1 is not as easy to discern, spreading is also blocked by anti-

body to  $\alpha_v\beta_3$  and anti- $\alpha_v\beta_5$  has no effect (not shown). This suggests that higher surface densities of VN alleviate the need for an activating stimulus, perhaps by cross-linking the integrin (55). To determine if  $\alpha_v\beta_5$  might become activated and contribute to cell spreading, cells were allowed to attach to VN for 15 min in the absence of an mAb or peptide, which they cannot do in the presence of mAb P1F6 (Fig. 4 B). Then, either mAb LM609 or P1F6 was added along with a stimulatory peptide (4N1K). After an additional 30 min, cells in control mAb or P1F6 had spread equally well while mAb LM609 completely blocked spreading as before (Fig. 6). That the stimulation of spreading by TS1 requires IAP is seen in Fig. 5, C and D. The function-blocking anti-IAP mAb B6H12 inhibits the stimulated spreading while the nonfunction blocking anti-IAP mAb 2D3 has no effect. These results are quantified in Fig. 6. TS1, the rCBD and 4N1K all stimulate cell spreading on VN to the same extent:  $\sim 80\%$  of total cells spread in 30 min vs ca. 15% spread with no stimulation. As seen in Fig. 6, both mAbs LM609 and B6H12 block the



**Figure 6.** Quantitative analysis of C32 cell spreading on immobilized vitronectin. C32 cell spreading assays were performed as in Fig. 5 and quantified as Fig. 2. "\*" indicates spreading in the absence of 4N1K (negative control). 50  $\mu$ M 4N1K peptide was present in all other cases plus: mouse IgG, 100  $\mu$ g/ml; LM609, 20  $\mu$ g/ml; B6H12, 100  $\mu$ g/ml; 2D3, 100  $\mu$ g/ml. In separate experiments, C32 cells were allowed to attach to VN for 15 min in the absence of a mAb or 4N1K peptide. Then, either mAb LM609 or P1F6 was added along with peptide 4N1K followed by an additional 30 min spreading incubation. Data from these experiments are indicated with "\*\*\*".

stimulation of spreading to about the same extent, nearly the unstimulated level. The same results were obtained when spreading was stimulated by TS1 at 50  $\mu$ g/ml (not shown). These data clearly indicate that both  $\alpha_v\beta_3$  and IAP are required for the stimulation of cell spreading on VN by the CBD of TS1. One possibility is that IAP ligation causes an increase in the affinity or avidity (by clustering) of  $\alpha_v\beta_3$ . If this occurs, then 4N1K and TS1 should enhance the adhesion of cells to low density immobilized VN. As seen in Fig. 4, A and C, this does not occur. Over a wide concentration range of VN coating, 4N1K peptide never increases adhesion, only spreading. These results further suggest that  $\alpha_v\beta_3$  can form a signal transducing complex with IAP, as observed in other systems (4, 7, 36, 54, 66, 75), which can lead to the stimulation of cell spreading.

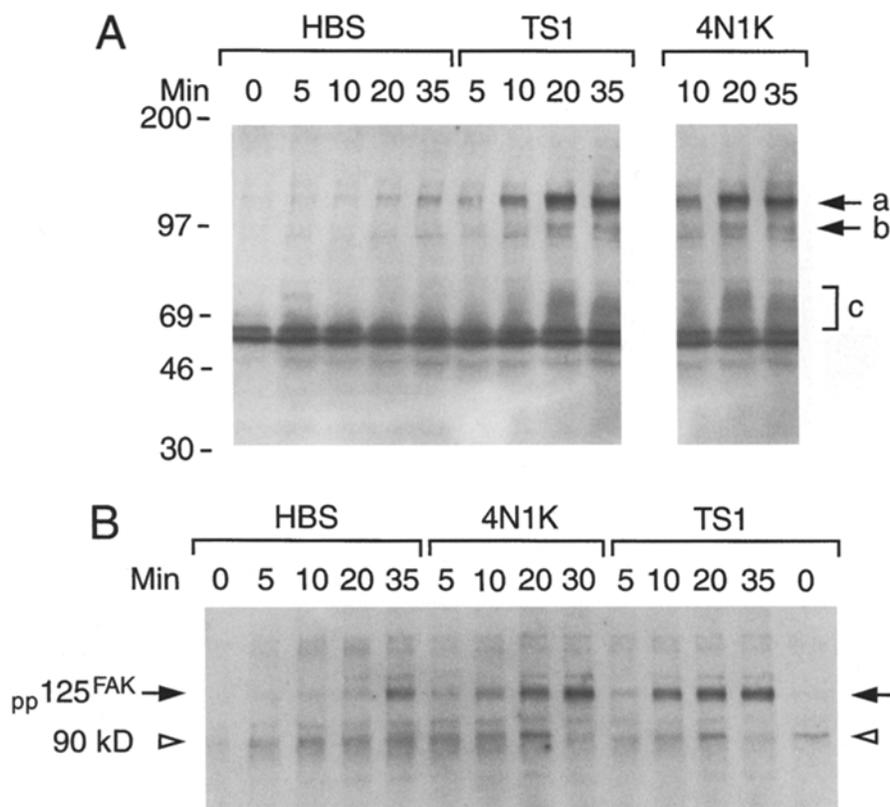
#### **Effect of TS1 and 4N1K on Tyrosine Phosphorylation**

Integrin-mediated spreading of many cell types is accompanied by the phosphorylation on tyrosine residues of a set of cellular proteins involved in focal adhesion assembly and turnover (9, 21, 23, 25, 39, 52). These include focal adhesion kinase (pp125, FAK) and paxillin (pp60-70 triplet). We thus wanted to determine if the stimulated cell spreading observed with TS1 and its CBD led to tyrosine phosphorylation of any such proteins. To do this, C32 cells were allowed to spread on VN-coated surfaces (1  $\mu$ g/ml) in the absence (HBS) or presence of TS1 or 4N1K for various times when the cells were rapidly lysed, immunoprecipitated with an anti-phosphotyrosine (PY) mAb, the precipitated proteins run on SDS-PAGE and Western

blotted with the anti-PY mAb 4G10. As seen in Fig. 7 A, TS1 and 4N1K stimulate the tyrosine phosphorylation of several proteins with  $M_r$  of ca. 125 kD (a), ca. 90 kD (b), and a cluster at 60–70 kD (c) running just above the mAb heavy chain. In an attempt to identify these phosphorylated proteins, specific antibodies to known signal transduction components were employed. Fig. 7 B shows the results of an experiment in which cell lysates were treated like those in Fig. 7 A except that the precipitating antibody was anti-FAK mAb 2D7 (51). Here it is seen that phosphorylated FAK detected with mAb 4G10, appears much earlier in the time course and accumulates to a greater extent in cells spreading on VN in the presence of either 4N1K or TS1. In addition, other phosphoproteins are seen presumably as a result of their association with FAK, with  $M_r$  of ca. 140, 95, and 90 kD. This latter 90-kD protein is of interest because it becomes phosphorylated earlier than FAK itself, and in the TS1- and 4N1K-treated cells it either dissociates from FAK or becomes dephosphorylated (or both) by the last time point. When C32 cells are maintained in suspension so that focal adhesion formation is prevented, the addition of 4N1K causes rapid and intense tyrosine phosphorylation of this 90-kD protein, but phosphorylation of FAK and other proteins is not observed. This also suggests that phosphorylation of this 90-kD protein is an early event following IAP ligation. Preliminary experiments indicate that this pp90 species is not phosphatidylinositol-3 kinase.

#### **Pertussis Toxin Specifically Inhibits Cell Spreading Stimulated by 4N1K and TS1**

To begin defining a mechanism for the stimulation of  $\alpha_v\beta_3$ -dependent cell spreading by ligation of IAP, we tested a number of well characterized inhibitors of signal transduction enzymes. Even though IAP can associate with  $\alpha_v\beta_3$ , previous studies have indicated that signal transduction involving PKC is required for modulation of integrin-mediated functions (75). As noted above, PKC activation is also a common element in integrin-mediated cell spreading (31, 69). We thus tested a number of protein kinase inhibitors for their effect on the rapid spreading elicited by 4N1K and TS1. These included six ser/thr kinase inhibitors of varying specificities (HA1004, chelerythrine, H7, K252b, Ro-31-8220, and calphostin C, see Materials and Methods for details). Of these, the specific protein kinase C inhibitor calphostin C completely inhibited the stimulated cell spreading (Fig. 8) at a concentration of 100 nM which is only twice the reported  $IC_{50}$  (26). Chelerythrine, also a PKC inhibitor, was effective at higher concentrations (Fig. 8). Moreover, PMA treatment of C32 cells adherent to sparse VN coatings rapidly induced cell spreading, demonstrating that direct PKC activation could induce the same behavior as TS1. However, the PKC inhibitors (above) also blocked the rapid constitutive spreading on high densities of VN in the absence of TS1 or its peptides. This along with previous reports from others (31, 69) indicates that PKC plays a general role in spreading, and is not specific to the action of IAP. Wortmannin, genestein, and herbimycin A also abolished cell spreading at appropriate concentrations (Fig. 8). However, as with PKC inhibitors (above), none of these inhibitors were specific for TS1-induced cell spreading.



**Figure 7.** TS1 and peptide 4N1K stimulate protein tyrosine phosphorylation. C32 cells were allowed to spread on 1  $\mu\text{g/ml}$  VN-coated 12-well tissue culture plates with or without 50  $\mu\text{g/ml}$  TS1 or 50  $\mu\text{M}$  4N1K peptide in solution. At the indicated times (min) cells were lysed in RIPA buffer (see Materials and Methods). Soluble material from equal numbers of cells (equal amounts of protein) was incubated with anti-phosphotyrosine mAb PY20 (A) or anti-p125<sup>FAK</sup> mAb 2D7 (B) and precipitated with anti-mouse IgG agarose. SDS-PAGE on 4–12% (A) or 8% (B) acrylamide gels was followed by Western blotting with anti-phosphotyrosine mAb 4G10 with ECL detection.

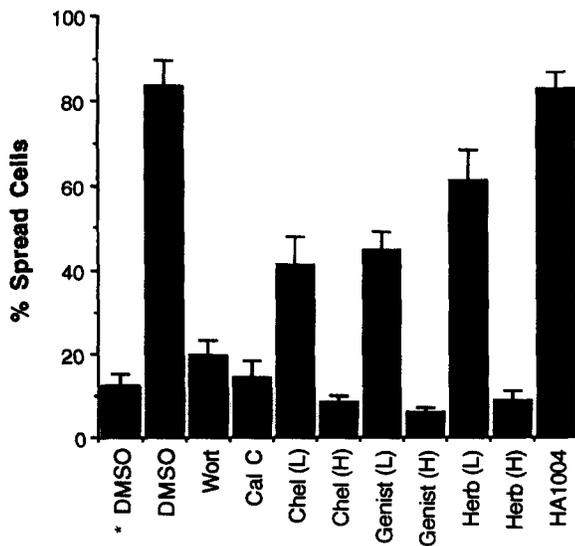
The possible involvement of the Gi/Go class of heterotrimeric G proteins in the stimulation of cell spreading on VN was tested by incubating the C32 cells overnight in *B. pertussis* toxin (20–300 ng/ml). The cells were then plated on 1  $\mu\text{g/ml}$  VN-coated slides in the presence or absence of TS1 and 4N1K peptide as above. Interestingly, the *pertussis* toxin pretreatment (all concentrations) strongly inhibited the ability of TS1 and 4N1K to stimulate cell spreading (Fig. 9, A–D, and G). Unlike inhibition of tyrosine kinases, PKC and PI-3 kinase, the blockade of Gi-like proteins with *pertussis* toxin did not affect unstimulated, rapid spreading on high density VN suggesting that the G protein(s) act more proximally to IAP and upstream of PKC, tyrosine kinases, and/or PI-3 kinase. The carbohydrate binding B oligomer portion of *pertussis* toxin has been reported to be responsible for some effects of the toxin (24). We thus tested the isolated B oligomer (30–300 ng/ml) and found that at all concentrations tested, the B oligomer of *pertussis* toxin was without effect on cell spreading (Fig. 9 G). To ensure that the failure of the whole toxin-treated cells to spread in response to TS1 and 4N1K was due to the specific blockade of a heterotrimeric Gi protein and not a general cytotoxic effect, toxin-treated cells were plated on VN and stimulated with PMA. Spreading of the cells ensued immediately and proceeded to the same extent noted when C32 cells were treated with PMA alone (Fig. 9, E and G). In contrast, cells treated with wortmannin could not be stimulated to spread with short-term PMA treatment (Fig. 9, F and G). Thus, IAP appears to use a Gi protein pathway to access the common pathway described by others in which activation of PKC, PI-3 kinase, and ty-

rosine kinases leads to cell spreading mediated by a number of integrins in several cell types (3, 13, 20, 56).

## Discussion

The data presented here provide insight on a novel mechanism by which TS1 can affect integrin-mediated cellular processes involved in normal development, homeostasis, and pathogenesis. It is thus appropriate to view TS1 and other TS family members which contain the sequences that bind IAP as acute modulators of integrin function. In fact, this *modus operandi* fits well with accumulating knowledge about the expression and biological roles of TS1. For example, TS1 has an acute function in platelet aggregation (see below), and it influences the motility of a number of migratory cell types, particularly during the inflammatory response (37, 38, 63, 64, 71, 72). Its expression in endothelial cells is tightly regulated where it can inhibit angiogenesis if expressed early in the process, but may be important in endothelial cell differentiation into capillaries (58, 65). TS1 expression is acutely regulated by cytokines and growth factors in several cell types (6, 10, 32) as exemplified in vivo during wound healing (47, 48) and in development (22). This acute regulation of TS1 synthesis and secretion is characteristic of agents which regulate underlying processes of adhesion and motility.

In this report we first establish that the effect of TS1/IAP ligation is on the spreading of cells and not on their adhesion. Interestingly, the interaction of the C32 cells with VN is a two step process at low densities of VN, with  $\alpha_v\beta_5$  sup-



**Figure 8.** Effect of inhibitors on cell spreading stimulated by 4N1K. Peptide 4N1K was present in all cases (except the DMSO negative control marked with “\*”). (Left to right) DMSO, (control vehicle); wortmannin 10 nM; calphostin C, 100 nM; chelerythrine 7  $\mu$ M (L) and 21  $\mu$ M (H); genistein 25  $\mu$ M (L) and 75  $\mu$ M (H); herbimycin 9  $\mu$ M (L) and 27  $\mu$ M (H); HA1004 390  $\mu$ M. See Materials and Methods for protocols.

plying the initial binding interaction and  $\alpha_v\beta_3$  leading to spreading. At high densities of VN, both integrins participate in cell adhesion as indicated by mAb inhibition profiles (Fig. 4). We could find no evidence that  $\alpha_v\beta_5$  participates in spreading in these cells. In FB carcinoma cells,  $\alpha_v\beta_5$  can be stimulated by PMA treatment to support cell spreading on VN (25), but this effect requires transcriptional activation via NF- $\kappa$ B and synthesis of new proteins, a process that takes several hours (73). The enhancement of spreading by TS1 reagents occurs very rapidly, within minutes, and is specific for the native RFYVVM sequence as shown with control and mutant peptides. The activity of the recombinant CBD and whole TS1 further indicates that this effect is a property of the native TS1 molecule. The stimulation of spreading via IAP is seen with murine 3T3 cells as well as the transformed human melanoma line C32 employed in most of our experiments. Hence, this phenomenon is not unique to a single species of cell or to transformed cells. Spreading on other matrix proteins such as collagens, fibrinogen, and fibronectin mediated by integrins other than  $\alpha_v\beta_3$  is not augmented by TS1 or 4N1K in either C32 or 3T3 cells. Interestingly, cell spreading on LM is stimulated by the TS reagents, an effect currently under investigation.

The properties of the TS1-enhanced spreading on VN are consistent with previous information about IAP function obtained with the function-blocking mAb B6H12 in leukocyte systems where IAP was seen to be required for signal transduction resulting in effects on the function of  $\alpha_5\beta_1$  integrin in phagocytosis (4). In both endothelial cells (54) and fibroblasts (66), IAP inhibition blocks a calcium transient which may augment PKC activation. B6H12 treatment of either the PMNs or the HUVECs blocks the transmigration of PMNs across HUVEC monolayers in vitro

(11). We have shown that the 4N1K peptide and TS1 stimulate HUVEC chemotaxis on gelatin-coated filters, an  $\alpha_v\beta_3$ -dependent process, and this chemotaxis is blocked by B6H12 (18). Others have noted the similarities between integrin-dependent cell migration and cell spreading (15, 55), and our results with the HUVEC chemotaxis system indicate that the inhibitors which we have found to block the stimulation of C32 cell spreading also block IAP-dependent HUVEC chemotaxis (Tolsma, S., N. Bouck, M.B. Finn, and W. Frazier, manuscript in preparation).

While the use of inhibitors cannot provide definitive proof of the essential role of a given signal transduction component in a pathway, we have used a panel of inhibitors here as a starting point to begin assessing the types of systems which TS1-IAP ligation might affect. In fact our results agree with recent data from other groups who have found that PKC activation may be a universal intermediate step in cell spreading (2, 25, 34, 44, 67, 69) whether “constitutive” or “stimulated” as seen here. Accumulating data using wortmannin also implicate PI-3 kinase as an important component of integrin-mediated cell activation (30, 59, 74). Our data indicate that TS1 and IAP represent a previously unknown way of modulating these constitutive signaling pathways that lead to cell spreading. More interesting from a mechanistic standpoint is the finding that Gi-like proteins are required for the IAP-mediated stimulation, and that pertussis toxin does not block the constitutive spreading response as seen on high density VN. Of all inhibitors used in signal transduction research, the cholera and pertussis toxins can claim specificity due to their recognition of their substrates via the enzymatic subunits of the toxins. We have shown here that pertussis toxin specifically blocks the TS1/IAP-mediated stimulation of spreading on VN. The carbohydrate binding B oligomer has no effect, and the blockade is bypassed by direct PKC stimulation with phorbol ester ruling out a general cytotoxic effect (Fig. 9). The constitutive spreading on high density VN is not affected by pertussis toxin, again ruling out a toxic effect, and, more importantly, indicating that the G protein activated step is proximal to IAP and upstream of PKC activation. Mansfield et al. (1990) reported that the migration of leukocytes toward TS1 is sensitive to pertussis toxin and mAb C6.7 directed to the TS1 CBD (37, 38). Our preliminary data indicate that chemotaxis of several cell types, including monocytes, toward 4N1K is pertussis toxin sensitive (W.A. Frazier, unpublished data). This toxin is known to cause the ADP-ribosylation of the  $\alpha$  subunit of  $G_i$  isoforms 1, 2, 3, and  $G_o$ , as well as the  $G\alpha$  subunits of transducins 1 and 2 (43). It is unlikely (but not impossible) that the transducins or  $G_o$  are expressed in C32 cells, thus narrowing the field of likely candidates to the  $G\alpha_{i1-3}$ . We are currently investigating the expression of  $G\alpha$  subunits in C32 cells and whether there is a direct interaction between IAP and a  $G_i$  protein.

The signaling pathway proposed here for IAP costimulation of  $\alpha_v\beta_3$ -mediated cell spreading is remarkably similar to the pathway reported by Shattil and coworkers (57) to explain the costimulation of  $\alpha_{IIb}\beta_3$ -mediated platelet spreading on fibrinogen-coated surfaces. In that system, unstimulated platelets adhere to fibrinogen but do not spread in the presence of apyrase, an ADP scavenger, unless a costimulatory signal is provided by epinephrine or

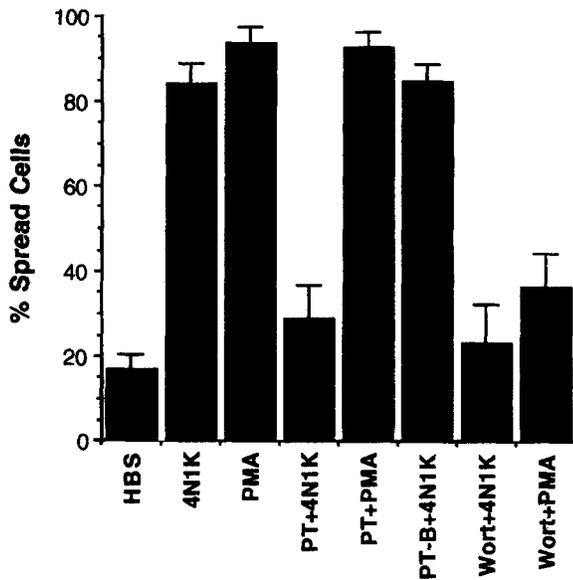
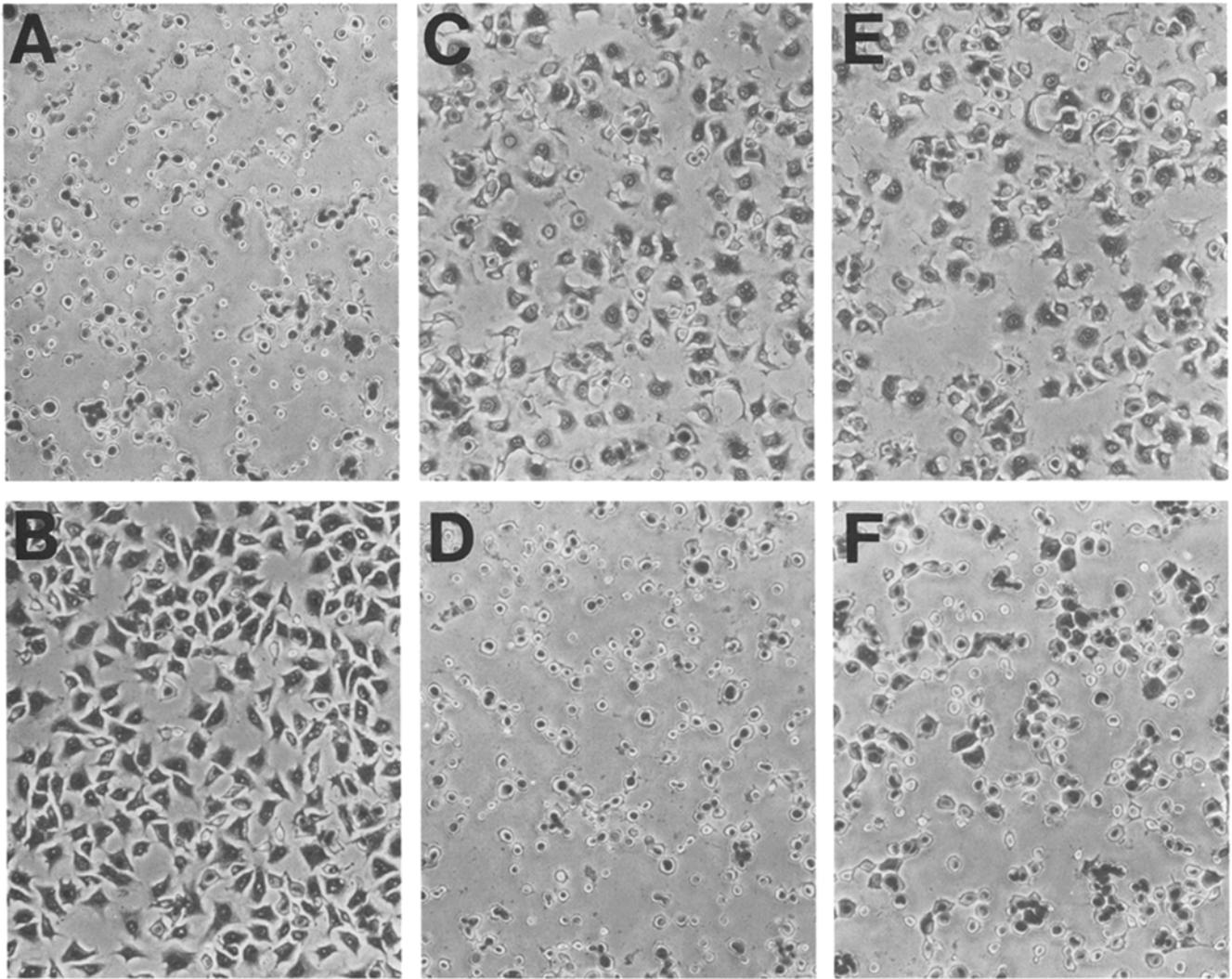


Figure 9. Effects of *pertussis* toxin and phorbol ester on C32 cell spreading on vitronectin. C32 cells were allowed to spread on 1 μg/ml VN in the absence (A, C, and E) or presence (B, D, and F) of 50 μM 4N1K peptide. The phorbol ester PMA alone at 10 ng/ml (C) caused extensive spreading of the cells. After overnight treatment with *pertussis* toxin (see Materials and Methods), 4N1K was no longer able to stimulate cell spreading (D), however, a 10-min pretreatment with 10 ng/ml PMA could cause spreading of the *pertussis* toxin-treated cells (E). PMA (10 min, 10 ng/ml) was not able to reverse the block to spreading imposed by wortmannin (10 nM) (F). (G) Quantification of effects of these inhibitors and controls. PT-B refers to the B oligomer of *pertussis* toxin present at the same concentration used for *pertussis* toxin in D above.

thrombin binding to a heptahelical receptor. These receptors are coupled to heterotrimeric G<sub>i</sub> proteins which lead to the activation of PKC via lipid mediators including arachidonate, thromboxane A<sub>2</sub> (which itself binds to another heptahelical receptor), and diacylglycerol. In this context it is noteworthy that the first reported function for the CBD of TS1 was that of stimulation of secondary phase or secretion dependent platelet aggregation. This was based on inhibition by the mAb C6.7 directed against the CBD (14). Several TS1 receptors have been identified on platelets and several explanations for the role of TS1 in platelet aggregation have been proposed (68). None of these satisfactorily explain the inhibition by the anti-CBD mAb C6.7. IAP is present on platelets in abundance and associates with α<sub>IIb</sub>β<sub>3</sub> (7). Based on the similarities between the IAP signal transduction pathway in C32 cells presented here and the costimulatory pathway in platelets (57), we suggest that a role of TS1 in platelets is that of an integrin costimulator acting through IAP. Experiments to test this hypothesis are currently in progress.

In conclusion, we have identified previously unknown functions for both TS1 and IAP in costimulating α<sub>v</sub>β<sub>3</sub> integrin-mediated cell spreading. The involvement of PKC and PI-3 kinase in cell spreading (2, 25, 34, 44, 55, 67, 69) appears to be a general constitutive mechanism. TS1 binding to IAP can modulate or augment this pathway by activating a G<sub>i</sub> type heterotrimeric G protein. This suggests that TS1 acting through IAP may have a general role in modulating cell motility and other matrix interactions where integrins are the primary mediators. This model for TS action provides a new perspective on the role of TS1 in many cases where it is transiently synthesized and/or secreted at sites of tissue development or remodeling, wound healing, inflammation, and angiogenesis.

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