

Integrin-associated Protein Stimulates $\alpha 2\beta 1$ -dependent Chemotaxis via Gi-mediated Inhibition of Adenylate Cyclase and Extracellular-regulated Kinases

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Abstract. Integrin-associated protein (IAP/CD47) augments the function of $\alpha 2\beta 1$ integrin in smooth muscle cells (SMC), resulting in enhanced chemotaxis toward soluble collagen (Wang, X-Q., and W.A. Frazier. 1998. *Mol. Biol. Cell.* 9:865). IAP-deficient SMC derived from IAP^{-/-} animals did not migrate in response to 4N1K (KRFYVVMWKK), a peptide agonist of IAP derived from the COOH-terminal domain of thrombospondin-1 (TSP1). When normal SMC were preincubated with 4N1K or an anti- $\alpha 2\beta 1$ function-stimulating antibody, cell migration to soluble collagen was significantly enhanced. 4N1K-induced chemotaxis was blocked by treatment of SMC with pertussis toxin indicating that IAP acts through Gi. In agreement with this, 4N1K evoked a rapid decrease in cAMP levels which was intensified in the presence of collagen, and forskolin and 8-Br-cAMP both inhibited SMC migration stimulated via IAP. 4N1K strongly inhibited extracellular regulated kinase (ERK) activation in SMC attaching to

collagen and reduced basal ERK activity in suspended SMC. Pertussis toxin treatment of SMC significantly activated ERK, suggesting that an inhibitory input was alleviated. Inhibition of ERK activity by (a) the MAP kinase kinase (MEK) inhibitor, PD98059, (b) antisense oligonucleotide depletion of ERK, and (c) expression of mitogen-activated protein (MAP) kinase phosphatase-1 in SMC all led to increased migration to collagen, 4N1K, or 4N1K plus collagen. Thus, IAP stimulates $\alpha 2\beta 1$ integrin-mediated SMC migration via Gi-mediated inhibition of ERK activity and suppression of cyclic AMP levels. Both of these signaling pathways could directly modulate the state of the integrin as well as impact downstream components of the cell motility apparatus.

Key words: integrin-associated protein • chemotaxis • MAP kinase • heterotrimeric G-proteins • cyclic AMP

MIGRATION of vascular smooth muscle cells (SMC)¹ is a major contributor to intimal thickening during the progression of vascular disease (Casscells, 1992; Ross, 1993). Many potential regulators of SMC migration have been identified, including chemotactic cytokines and growth factors, as well as extracellular matrix (ECM) molecules (Dubey et al., 1995; Nelson et al., 1996;

Pickering et al., 1997). Integrins are the major family of cell surface adhesion receptors that have been implicated in cell migration (Hynes, 1992; Huttenlocher et al., 1995). In cultured SMC, migration has been noted to be dependent on $\alpha v\beta 3$ integrin (Yue et al., 1994), but a number of recent studies have also implicated $\alpha 2\beta 1$ integrin in SMC migration. For example, platelet-derived growth factor (PDGF) induced SMC migration on collagen via activation of $\alpha 2\beta 1$ integrin (Skinner et al., 1994). Fibroblast growth factor-2 (FGF-2) upregulated $\alpha 2\beta 1$ expression and potentiated SMC migration to PDGF (Pickering et al., 1997). Further, blockade of $\alpha 2\beta 1$ function inhibited SMC migration to collagen (Yue et al., 1994; Wang and Frazier, 1998). Efficient cell migration requires that integrin-mediated interactions between the cell's surface and the substratum be dynamically regulated to allow a cell to progress over its substratum (Dimilla et al., 1991; Lauffenburger and Horwitz, 1996). Directed cell migration, be it haptotaxis or chemotaxis, requires that another layer of

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1. *Abbreviations used in this paper:* 4N1K, KRFYVVMWKK; 4NGG, KRFYGGMWKK; cAMP, 3'-5' cyclic adenosine monophosphate; ECM, extracellular matrix; ERK, extracellular regulated kinase (MAP kinase p42/p44); FGF-2, fibroblast growth factor-2; IAP, integrin-associated protein or CD47; MAP, mitogen-activated protein; MEK, MAP kinase kinase; MKP-1, MAP kinase phosphatase 1; PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TSP, thrombospondins (TSP1, platelet thrombospondin).

sensory inputs take control of the basic mechanisms that generate cell motility. This regulation of integrin function is particularly difficult to understand in terms of our current views of cell adhesion. Clearly, changes in affinity or avidity of integrins must occur during cell migration, which very likely depend on rapid, controlled modulation of inside out signaling (Hynes, 1992; Shattil et al., 1994; Hughes and Pfaff, 1998).

The classic example of integrin affinity modulation is the nearly instantaneous transition of platelet α IIB β 3 from a low affinity/avidity state to a state in which it can effectively bind soluble fibrinogen resulting in rapid platelet aggregation (Shattil et al., 1994, 1998; Chung et al., 1997). Resting platelets are unable to aggregate because their α IIB β 3 is in an inactive conformation that has a low affinity for soluble fibrinogen. However, they can attach quite well to surfaces coated with fibrinogen (Chung et al., 1997; Shattil et al., 1998), a situation in which ligand valency overcomes the inherent low affinity of each receptor. After platelet activation by specific agonists, such as thrombin and other coactivators that bind to heptahelical or seven transmembrane segment receptors, inside out signaling causes α IIB β 3 to undergo a conformational change that dramatically increases its binding affinity and/or avidity for fibrinogen, resulting in platelet aggregation (Shattil et al., 1998). Unactivated platelets adherent to fibrinogen cannot spread unless α IIB β 3 is activated by inside out signaling initiated by one of these costimulators (Chung et al., 1997; Shattil et al., 1998).

We have recently reported that integrin-associated protein (IAP or CD47) on platelets is also a costimulatory receptor for α IIB β 3 activation (Chung et al., 1997), and that the COOH-terminal domain of thrombospondin-1 (TSP1), a ligand for IAP (Gao et al., 1996a,b), initiates the same signaling pathway leading to α IIB β 3 activation as receptors for other agents such as thrombin, ADP, and epinephrine. IAP forms a physical complex with certain integrins (Brown et al., 1990; Gao et al., 1996a,b; Wang and Frazier, 1998), and blockade of IAP with mAbs inhibits some aspects of integrin signaling (Lindberg et al., 1996). This data has led to the notion that IAP is involved in outside in signaling. However, our recent studies of platelet activation and spreading and chemotaxis of nucleated cells indicate that IAP initiates a signaling pathway to upregulate integrin function, that is, inside out signaling (Blystone et al., 1995; Gao et al., 1996a; Chung et al., 1997, 1999; Wang and Frazier, 1998). In platelets, ligation of IAP with the agonist peptide 4N1K (KRFYVVMWKK), the recombinant COOH-terminal domain of TSP1 or TSP1 itself, initiates a signaling pathway very similar to that of platelet agonists such as thrombin or epinephrine, resulting in platelet activation and affinity modulation of α IIB β 3 to allow binding of soluble fibrinogen and spreading on a fibrinogen substrate (Chung et al., 1997). Endothelial cells (Gao et al., 1996b) and melanoma cells (Gao et al., 1996a) migrating on gelatin-coated surfaces are attracted towards 4N1K, and this appears to involve modulation of α v β 3 which these cells use for traction on this RGD-containing matrix.

Whereas members of the β 1 family of integrins in general do not depend on inside out signaling for activation, α 4 β 1 is subject to this sort of rapid modulation or activa-

tion in leukocytes, a process with an essential role in the pathogenesis of thrombosis, inflammation, and autoimmune diseases (Hynes, 1992; Carlos and Harlan, 1994). The β 1-integrin-mediated adhesion of leukocytes to ECM proteins can be activated by chemokines (Springer, 1994), divalent cations (Dobrina et al., 1991), phorbol ester (Wilkins et al., 1991), cross-linking of cell surface receptors (Shimizu et al., 1991), and certain antibodies to β 1 integrins (Kovach et al., 1992) without changes in cell surface expression of the integrin. In addition, α 2 β 1 can exist in three distinguishable functional states, depending on the cell type in which it is expressed (Chan and Hemler, 1993; Santoro and Zutter, 1995). When expressed in K562 cells, the α 2 β 1 integrin is present on the cell surface but does not allow the cells to bind to either collagen or laminin, two common ligands for α 2 β 1. When it is expressed on platelets and some types of fibroblasts, α 2 β 1 binds collagen, and when expressed on endothelial and many epithelial cells, it binds both collagen and laminin. We have observed another type of more acute regulation of α 2 β 1 integrin on cultured aortic SMC in which TSP1 and a peptide derived from its COOH-terminal domain (4N1K) can modulate the function of α 2 β 1 such that it more effectively promotes chemotaxis of the cells towards soluble collagen. This effect on α 2 β 1 function is mediated by the cell surface TSP1 receptor IAP (or CD47) (Wang and Frazier, 1998). SMC migrate on gelatin-coated filters using α 2 β 1 for traction and this migration can be directed by gradients of either soluble collagen (Nelson et al., 1996), or the IAP agonist peptide 4N1K (Wang and Frazier, 1998). We noted that, when present together, soluble collagen and 4N1K provoke a highly synergistic chemotactic response (Wang and Frazier, 1998). This enhanced chemotaxis requires both α 2 β 1 and IAP as judged by the effects of function blocking antibodies. In this report, we investigate the mechanism by which IAP modulates α 2 β 1-mediated SMC migration. We find that, just as in platelets (Chung et al., 1999; Frazier et al., 1999), IAP acts via a heterotrimeric Gi protein to decrease intracellular cyclic AMP levels. In addition, ligation of IAP evokes a rapid and substantial inhibition of mitogen-activated protein (MAP) kinase (ERKs). Both of these events have been implicated in modulation of integrins and both also impact the cell's motility apparatus downstream of integrin signaling.

Materials and Methods

Reagents

Peptides were synthesized, purified, and confirmed by mass spectrometry at the Protein and Nucleic Acid Chemistry Laboratory of Washington University School of Medicine as described previously (Kosfeld and Frazier, 1992; Gao et al., 1996a,b). The amino acid sequences of the TSP1 peptides and preparation of human platelet TSP1 were as described (Santoro and Frazier, 1987). Rat tail collagen-I was obtained from Collaborative Biochemical Products. The signal transduction inhibitors and activators were purchased from CalBiochem-Novabiochem. Both anti- α 2 β 1 antibodies (function stimulating, JBS2, and blocking, BHA2.1) were from Chemicon International. Anti-ERK antibody was from Upstate Biotechnology Inc. Anti-active (-phosphorylated) ERK antibody was from Promega. Anti-ERK polyclonal antibody for Western blotting was from Santa Cruz Biotechnology, Inc. cAMP enzyme immunoassay (EIA) system (dual range) kits and γ - 32 P]ATP were from Amersham. Myelin basic

protein and anti-mouse IgG agarose were from Sigma Chemical Co. The plasmids 3CH134 encoding MAP kinase phosphatase 1 (MKP-1) and the catalytically inactive mutant of MKP-1 were generously provided by Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Cell Culture and Transfection

Human and mouse aortic SMC were isolated by the explant method and cultured as described (Ross and Kariya, 1980). Cells were maintained in a humidified 37°C and 5% CO₂ environment in MEM with 20% fetal calf serum, and identified by immunostaining of α -actin (Janat and Liau, 1992). Passages 2–10 were used for experiments. Cell transfection using Lipofectamine was as described (Sun et al., 1993). In brief, cells at 50–60% confluence were transiently transfected with 20 μ g cDNA of each plasmid for 5 h, and recovered in growth medium for 2 d before they were harvested, tested for viability, and counted for use in assays.

Treatment of Cells with Anti-Sense ERK Oligonucleotides

Depletion of ERK from cells was performed according to the procedure of Klemke et al. (1997). In brief, SMC grown to 60% confluence in 100-mm tissue culture dishes were incubated in Opti-MEM medium (GIBCO BRL) containing Lipofectamine (10 μ g/ml) and 1.5 μ M ERK antisense (5'-GCC GCC GCC GCC AT-3') or control (5'-CGC GCG CTC GCG CAC CC-3') phosphorothioate oligonucleotides for 5 h at 37°C. Cells were then rinsed, incubated for 2 d in fresh culture medium containing oligonucleotide, and then tested in migration assays as described above and processed for Western blotting with anti-ERK antibody.

Cell Migration

Chemotaxis assays were conducted in 48-well micro-Boyden chambers (Neuroprobe) using 8- μ m PVP-free, polycarbonate filters (Nucleopore). Filters were precoated by soaking in 100 μ g/ml gelatin at 37°C overnight, followed by washing twice in PBS. SMC were harvested with trypsin/EDTA, washed, and diluted in MEM with 0.1% BSA to a final concentration of 3–5 \times 10⁵ cells/ml. Chemoattractants were diluted in the same solution. Signal transduction inhibitors or activators were added to the cell suspension directly at the concentration indicated. 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 (HPF) in each of the triplicate wells for each condition.

ERK Assays

Immune Complex ERK Assay. Human SMC were harvested by EDTA/trypsin. After challenging with different treatments, either on matrix protein-coated 35-mm dishes or in suspension for indicated times, SMC were lysed directly at 4°C for 30 min in RIPA buffer including phosphatase inhibitors (50 mM Tris, pH 7.4, 0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM Na₃VO₄, and 20 mM β -glycerophosphate) and protease inhibitors (10 μ g/ml each of antipain, pepstatin A, chymostatin, leupeptin, soybean trypsin inhibitor, and aprotinin). The clarified cell lysates were incubated with 3 μ g/tube of anti-MAP kinase antibody overnight at 4°C and then immunoprecipitated with anti-mouse IgG agarose for 1 h at 4°C. The precipitates were extensively washed with 1:3 diluted RIPA buffer three times and then two times with PBS. The immune complexes were resuspended in 60 μ l kinase assay buffer (20 mM Hepes, pH 7.4, 1 mM EGTA, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄, 1 mM dithiothreitol, and 10 mM MgCl₂) including 20 μ g myelin basic protein, 50 μ M ATP and 5 μ Ci γ -[³²P]ATP, and incubated at 30°C for 30 min. The reaction was stopped by adding 20 μ l of 100 mM EDTA (pH 7.0). After centrifugation to pellet the beads, aliquots (20 μ l) of the supernatants were spotted onto P81 filters (Sevetson et al., 1993). The filters were washed 10 times with 1% phosphoric acid and then rinsed with 90% ethanol. The amount of ³²P incorporated into myelin basic protein was determined by scintillation counting.

Western Blotting of ERKs. Human SMC were treated as described above and lysed in RIPA buffer. Clarified cell lysates (50 μ l) were added to 2 \times SDS-sample buffer and run on 12% Tris-glycine gels under reducing conditions. The proteins were transferred electrophoretically onto nitrocellulose membranes which were blocked with 3% BSA plus 3% dried milk in TBS buffer with 0.1% Tween-20 for at least 1 h, and subsequently incubated with antibody against ERKs overnight at 4°C. The membranes

were washed extensively and incubated with peroxidase-conjugated secondary antibody for 1 h. Detection was by chemiluminescence with an ECL kit (Amersham).

Measurement of Cyclic AMP

Human SMC were harvested with EDTA/trypsin, resuspended in serum-free MEM at 2.5 \times 10⁵/ml, and incubated with peptides or inhibitors for the indicated times. Cells were pelleted and cAMP was rapidly extracted twice with the addition of 100 μ l of ice-cold ethanol (70%) for 30 min. Supernatants were collected and evaporated in a SpeedVac. The samples were dissolved in 5 ml assay buffer. The levels of cAMP were determined using a cAMP enzyme immunoassay system kit from Amersham.

Results

4N1K Stimulation of Chemotaxis Requires IAP

We previously found that either soluble type I collagen or the TSP1 peptide 4N1K alone are relatively weak chemoattractants of human aortic SMC migrating on a matrix of gelatin. However, in combination they provoke a strong chemotactic response. This response is, in fact, chemotactic (not chemokinetic) and depends on the function of both α 2 β 1 and IAP (Wang and Frazier, 1998). To determine if the α 2 β 1 integrin-dependent migration of SMC stimulated by 4N1K is strictly dependent on IAP, we examined the response of IAP-deficient SMC isolated from aortae of IAP^{-/-} mice (Lindberg et al., 1996). As shown in Fig. 1, both 4N1K and collagen can induce migration of wild-type SMC (IAP^{+/+}), and 4N1K plus soluble collagen together provoke a strong response, ~200% compared with control cells, similar to the response of human SMC (Wang and Frazier, 1998). However, 4N1K does not induce migration of IAP-deficient SMC. When soluble collagen is added along with 4N1K, the level of response is equal to the effect of collagen alone (Fig. 1) which reflects the action of α 2 β 1 in the unstimulated state (Wang and Frazier, 1998). This response to collagen also indicates that there is no significant defect in the ability of the IAP^{-/-} SMC to adhere to collagen. This was confirmed in quantitative cell adhesion assays (not shown). Thus, 4N1K stimulation of chemotaxis with or without soluble collagen is receptor mediated and is strictly dependent on the presence of IAP. This also rules out a number of trivial explanations

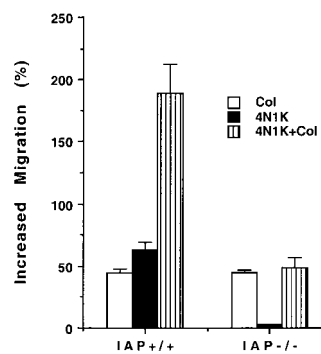


Figure 1. IAP is necessary for stimulation of chemotaxis by 4N1K. SMC from both IAP^{+/+} and IAP^{-/-} mice were tested for chemotactic migration when stimulated with 5 μ g/ml collagen (Col), 100 μ M 4N1K, or 100 μ M 4N1K plus 5 μ g/ml collagen-I (4N1K+Col). SMC were allowed to migrate through gelatin-coated filters toward attractants placed in the bottom wells of the micro-Boyden chamber. After staining, cells were counted in five high power fields in each of triplicate wells. Data is expressed as the percentage increased migration relative to control wells with no attractant to control for random motility. Error bars indicate standard deviation.

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(e.g., aggregation of collagen) for the enhanced chemotaxis.

Pretreatment with 4N1K Activates Chemotaxis

Our previous work suggested the hypothesis that IAP stimulates $\alpha 2\beta 1$ integrin function in SMC by activating the integrin in some way (Chung et al., 1997, 1999; Wang and Frazier, 1998). If this is in fact the case, 4N1K may not need to be present during the chemotaxis assay and pretreatment of SMC with 4N1K might sensitize the cells to respond better to a gradient of soluble collagen. Thus, we compared the effects of pretreatment of SMC with 4N1K and with an anti- $\alpha 2\beta 1$ mAb known to activate the integrin on other cell types (Arroyo et al., 1993; Chan and Hemler, 1993; Stupack et al., 1994). As shown in Fig. 2, cells pretreated with 4N1K for 30 min and washed before placing them into the chemotaxis apparatus displayed significantly increased migration to soluble collagen. Pretreatment with the control peptide 4NGG (KRFYGGMWKK) had no effect. This effect of 4N1K is not due to residual bound peptide since direct binding assays using ^{125}I -4N1K reveal a very fast off rate (Dimitry, J.B., and W.A. Frazier, unpublished data). Similar results to those with 4N1K were observed when the cells were preincubated with the $\alpha 2\beta 1$ function stimulating antibody: the migration to collagen increased more than threefold in three experiments (Fig. 2). An $\alpha 2\beta 1$ functional blocking mAb was tested in the same experiment, and it effectively inhibited cell migration (Wang and Frazier, 1998). These data support the notion that ligation of IAP activates $\alpha 2\beta 1$ for a period of time and it is this activation of $\alpha 2\beta 1$ that is responsible for the enhanced migration of SMC towards soluble collagen (Pickering et al., 1997; Wang and Frazier, 1998).

Effect of IAP Stimulation on Adhesion of SMC

Chemotaxis is complex process in which cell adhesion and deadhesion are regulated in a dynamic way (Lauffenburger and Horwitz, 1996). In an attempt to assess the effects of 4N1K stimulation of IAP on a more direct index of integrin function, we tested the effect of 4N1K on the adhesion of SMC to gelatin and collagen-coated surfaces. As expected, collagen is a much better ligand for $\alpha 2\beta 1$ than gelatin (Fig. 3). These SMC adhere to both collagen and gelatin via $\alpha 2\beta 1$ as determined with a function-blocking mAb (not shown). For both immobilized ligands, 4N1K

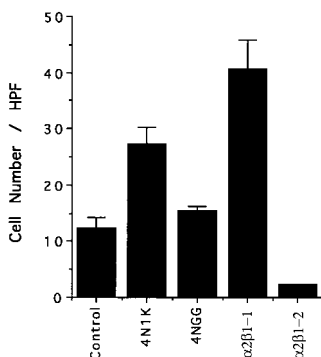


Figure 2. Activation of $\alpha 2\beta 1$ results in enhanced chemotaxis. Human SMC were pretreated with 100 μM 4N1K, 4NGG, or 1:400 diluted anti- $\alpha 2\beta 1$ antibodies for 30 min at 37°C before being added to the wells. 5 $\mu\text{g}/\text{ml}$ of collagen-I was tested as chemoattractant. $\alpha 2\beta 1$ -1 was a function stimulating antibody, and $\alpha 2\beta 1$ -2 was a function blocking antibody. Data are expressed as cells counted per high power \pm SD.

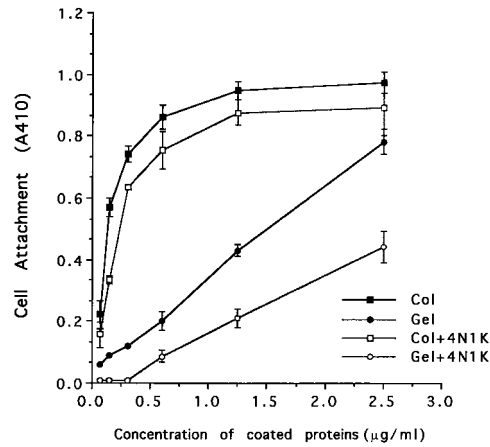


Figure 3. Adhesion of human SMC to collagen and gelatin. Human SMC were harvested as for chemotaxis assays and plated in 96-well plates coated with either collagen or gelatin at the indicated concentrations. After incubation and washing, adherent cells were quantified using a colorimetric assay as described (Wang and Frazier, 1998).

significantly decreases stable adhesion of the SMC. At submaximal densities of collagen and at all densities of gelatin, this inhibition is as much as 50%. Thus, the effect of 4N1K (and TSP1) on SMC chemotaxis, on gelatin, and collagen (Wang and Frazier, 1998), may represent a case in which loosening of tightly adherent cell contacts promotes migration as pointed out by Dimilla et al. (1991) and Lauffenburger and Horwitz (1996). This deadhesion effect of 4N1K and TSP1 has been seen previously in C32 cells adhering to vitronectin (Gao et al., 1996b), even though stimulation of IAP in C32 cells causes enhanced cell spreading which uses much the same cellular apparatus as cell motility. Thus, it is not clear at this point just what the state of the integrin is when it is activated via the IAP pathway. By analogy with the platelet system, perhaps $\alpha 2\beta 1$ is in a state in which it binds soluble collagen better (Shattil et al., 1994, 1998). Why this should result in decreased adhesion of cells to immobilized ligand is not obvious, but may relate to changes in the mobility or clustering of the integrin on the cell surface (Lauffenburger and Horwitz, 1996).

IAP Stimulates Chemotaxis via a Heterotrimeric Gi Protein

In platelets (Chung et al., 1997), C32 melanoma cells (Gao et al., 1996a), and endothelial cells (Gao et al., 1996b), IAP stimulates the activity of $\beta 3$ integrins when ligated with TSP1 or 4N1K peptide. This results in platelet aggregation, C32 cell spreading, and endothelial cell chemotaxis. In all of these cases, the action of IAP is abrogated by pertussis toxin treatment of the cells (Frazier et al., 1999). Therefore, we tested the effect of pertussis toxin treatment on the response of the SMC to 4N1K and collagen. Fig. 4 shows that treatment overnight with 50 ng/ml pertussis toxin inhibited random migration to some extent, but completely eliminated the directional migration in response to 4N1K, collagen, or 4N1K plus collagen. Thus, the effects of

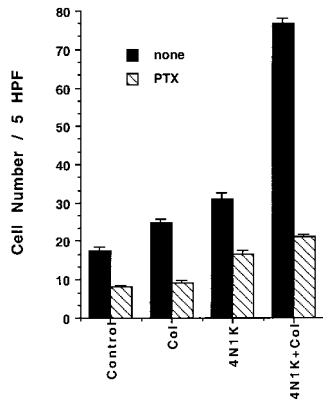


Figure 4. Pertussis toxin inhibits chemotaxis. SMC were treated with pertussis toxin (PTX, 50 ng/ml) overnight in serum-free medium, and harvested with EDTA/trypsin. Cells were resuspended in MEM with 0.1% BSA and 50 ng/ml of pertussis toxin, and added directly to the chemotaxis chamber. Chemoattractants were as described in Fig. 1. Data are expressed as in Fig. 2.

IAP stimulation on $\alpha 2\beta 1$ integrin function, like $\beta 3$ integrins, appear to be mediated via a heterotrimeric G_i protein.

Since IAP signaling is mediated via activation of G_i , the alpha subunit of G_i might inhibit adenylate cyclase activity (Neer, 1995). Thus, we examined the effect of IAP stimulation on intracellular levels of cAMP in SMC. As seen in Fig. 5 A, 4N1K alone evoked a rapid decrease in cAMP levels to less than half their original value in 20 min while the control peptide 4NGG had no significant effect. Interestingly, soluble collagen alone caused a substantial but transient decrease in cAMP levels while 4N1K plus collagen, the most potent chemotactic stimulus, rapidly decreased intracellular cAMP to very low levels that did not rebound by 40 min (Fig. 5 A). As seen in Fig. 5 B, pertussis toxin pretreatment of the SMC increased control (prestimulus) levels of cAMP as expected due to attenuation of inhibitory signals. The toxin also prevented the marked decrease in intracellular cAMP (at 40 min) caused by 4N1K with or without collagen. These results strengthen the conclusion that, in SMC as in other cell types, IAP acts via G_i .

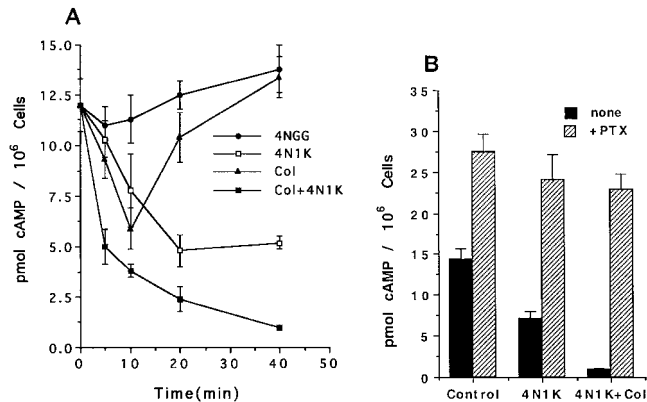


Figure 5. 4N1K decreases intracellular cAMP levels and pertussis toxin prevents the decrease in cAMP. (A) SMC were incubated in suspension with 4N1K (100 μ M), 4NGG (100 μ M), soluble collagen-I (5 μ g/ml), or 4N1K plus collagen-I for the indicated times at 37°C. The cAMP assay was as described in Materials and Methods. (B) Cells were treated with pertussis toxin (50 ng/ml) overnight and then incubated with 4N1K (100 μ M), or 4N1K plus collagen-I (5 μ g/ml) for 40 min in suspension. Data are picomoles of cAMP \pm SD.

They also suggest that a decreased level of intracellular cAMP may be necessary for the response.

To test this idea, SMC were treated with forskolin, a direct activator of adenylate cyclase. As shown in Fig. 6, forskolin strongly inhibits the migration of SMC toward 4N1K or collagen alone and to 4N1K plus collagen. We also tested the cell-permeable cAMP derivative 8-Br-cAMP, and found that it also inhibited SMC chemotaxis to all three stimuli (Fig. 6). Thus, 4N1K/IAP induces SMC migration via activation of G_i , a necessary effect of which is decreased cytoplasmic cyclic AMP levels.

4N1K Inhibits MAP Kinase/ERKs upon Activation of G_i

In many cell types, integrin-mediated attachment of cells to matrix proteins results in activation of MAP kinase p42/44 or ERK (Chen et al., 1994; Shattil et al., 1994, 1998). Furthermore, it has been reported (Klemke et al., 1997) that ERK can regulate $\alpha \nu \beta 3$ -dependent haptotaxis of FG carcinoma cells on vitronectin. Thus, we asked whether IAP modulation of SMC chemotaxis might involve regulation of ERKs. When SMC adhere to gelatin (or collagen, not shown), they display a transient burst of ERK activity that peaks \sim 10 min after plating and then returns to baseline levels (Fig. 7 A). 4N1K completely inhibited the increase in MAP kinase activity at 10 min and continued to decrease the baseline level of activity over time (Fig. 7 A). The control peptide 4NGG had no effect (Fig. 7 A). Western blotting using an antibody that detects the phosphorylated state of ERK confirmed this time course of activation (not shown). The inhibition of ERK activity appears to be an active process since even the basal level of kinase activity significantly declines over time with 4N1K treatment even under conditions in which cells are actively engaging the matrix (Fig. 7 A). To determine if engagement of the integrin is required for IAP-mediated inhibition of ERK activity, we harvested SMC and treated them with 4N1K in suspension. As seen in Fig. 7 B, the level of ERK activity in suspended SMC is, as expected, somewhat lower than in SMC attaching to collagen. Nonetheless,

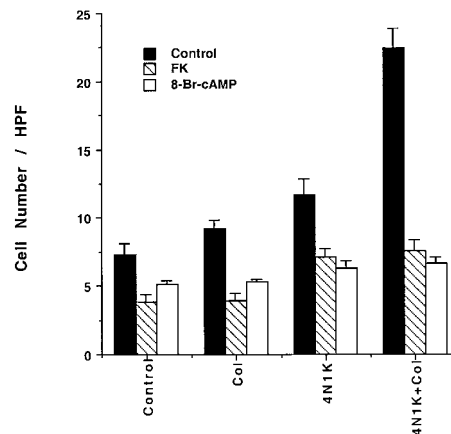


Figure 6. Forskolin and 8-Br-cAMP inhibit SMC chemotaxis. SMC were incubated with forskolin (FK, 24 μ M) or 8-Br-cAMP (100 μ M) for 15 min before being added to the chemotaxis chambers. Data are cell number \pm SD.

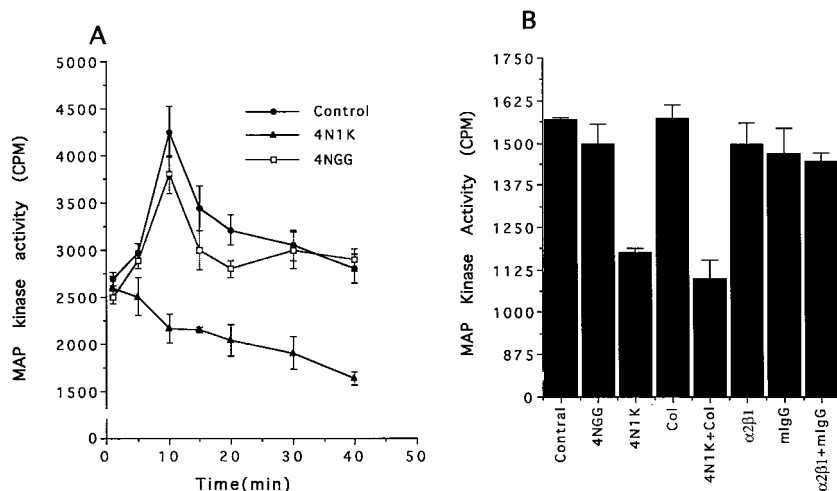


Figure 7. 4N1K inhibits MAP kinase activation. (A) SMC were plated on gelatin- (100 μ g/ml) coated dishes in the presence of 4N1K or 4NGG (100 μ M) for the indicated times, and lysed in RIPA buffer. Cell lysates were subjected to immunoprecipitation with anti-ERK antibody. Kinase assays were performed as described in Materials and Methods. Data are MAP kinase activity \pm SD. (B) SMC harvested as above were suspended in buffered serum free culture medium and treated indicated for 20 min when they were harvested and processed for ERK activity assay as above. Treatments were: control, no additions; 4NGG and 4N1K (100 μ M); Col, collagen at 5 μ g/ml; 4N1K+Col, 100 μ M and 5 μ g/ml; $\alpha 2\beta 1$, function-stimulating anti- $\alpha 2\beta 1$ mAb was used at a 1:50 dilution of the commercial preparation; mIgG, cross-linking anti-mouse IgG was at 20 μ g/ml; $\alpha 2\beta 1$ +mIgG, both at the stated concentrations.

4N1K caused a significant inhibition of ERK activity in suspended SMC while 4NGG had no effect. Significantly, collagen had no effect on ERK activity under these conditions, and collagen did not synergize with 4N1K to further reduce ERK activity (Fig. 7 B). Since it is not clear to what extent soluble collagen can bind to $\alpha 2\beta 1$ under these conditions, we also treated suspended SMC with the $\alpha 2\beta 1$ integrin-activating mAb used above. Neither the mAb alone nor in combination with a cross-linking anti-mouse IgG had any effect on ERK activity. Thus, ligation of IAP alone is sufficient to inhibit ERK activity and ligation and/or cross-linking of $\alpha 2\beta 1$ alone is not.

To test whether ERK in SMC can be regulated by G_i , SMC were treated with pertussis toxin (50 ng/ml) overnight. These same conditions lead to a blockade of IAP-dependent chemotaxis (Fig. 4) and an increase in intracellular cAMP levels (Fig. 5). The activation status of ERK was then determined by MAP kinase immune complex kinase assays. Fig. 8 shows that pertussis toxin treatment significantly activates ERK in SMC, and the activation is concentration dependent. These results were confirmed by Western blot using an antibody that detects the phosphorylated state of ERK (not shown). Thus, blockade of a G_i -dependent pathway stimulates ERK activity indicating that an inhibitory input has been attenuated, just as in the elevation of intracellular cAMP levels by pertussis toxin (Fig. 5 B). However, elevated cAMP does not cause the increase in ERK activity. We find that treating SMC with forskolin does not elevate ERK activity (our unpublished data; Giasson et al., 1997).

Inhibition of ERKs Facilitates SMC Chemotaxis

Since 4N1K had a strong inhibitory effect on ERK activity, the effect on chemotaxis of inhibiting ERK by other means was tested. This was done in several ways. First, SMC were treated with the MAP kinase kinase (MEK) inhibitor PD98059 resulting in a modest but significant stimulation of chemotaxis to 4N1K and 4N1K plus soluble collagen (Fig. 9). In another approach, we downregulated the

amount of ERK protein in SMC using an antisense oligonucleotide based on the ERK mRNA sequence (Klemke et al., 1997). Human SMC were treated with antisense and control (scrambled) oligonucleotides in the presence of lipofectamine for 5 h followed by a 2-d recovery period (Klemke et al., 1997). As shown in Fig. 10 A, treatment with the antisense ERK oligonucleotide resulted in increased migration to collagen, 4N1K, and 4N1K plus collagen compared with cells treated with the scrambled oligonucleotide. The same samples were subjected to Western blot analysis with an anti-ERK antibody. As shown in Fig. 10 B, the ERK protein levels were in fact substantially decreased in cells treated with the antisense ERK oligonucleotide (lane AS). ERK levels were the same in untreated cells (Fig. 10 B, lane C) and in those treated with lipofectamine and the control scrambled oligonucleotide (lane SC).

As seen in Fig. 7 A, 4N1K causes a progressive decrease

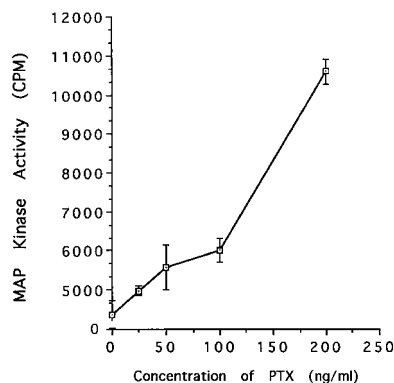


Figure 8. Pertussis toxin stimulates MAP kinase in SMC. SMC were treated with pertussis toxin (PTX) at the concentrations indicated overnight, and lysed directly in RIPA buffer. Cell lysates were subjected to immunoprecipitation using anti-ERK antibody. Kinase assays were performed as described in Materials and Methods. Data are MAP kinase activity \pm SD.

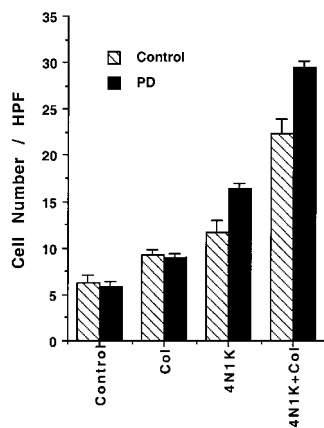


Figure 9. Effect of PD 98059 on SMC chemotaxis. SMC were incubated with PD98059 (PD, 50 nM) for 30 min at 37°C before being added to the chemotaxis chambers. Chemoattractants were as described above. Data are cell number \pm SD.

in ERK activity in SMC suggesting an active suppression of ERK activity. This is normally accomplished in cells by MAP kinase phosphatases which serve to balance and attenuate the activation of MAP kinases (Keyse, 1998). Thus, in a separate approach, we transiently transfected SMC with plasmid 3CH134 encoding MAP kinase phosphatase-1 (MKP-1) which can dephosphorylate MAP kinases activated by serum or v-raf (Sun et al., 1993). To determine if the MKP-1 was active in the transfected SMC, ERK activity was tested in cells replated on gelatin-coated surfaces after the cells were recovered for 48 h. As shown in Fig. 11 A, MAP kinase (primarily p42) phosphorylation was increased when control cells were attached to a gelatin-coated surface for 10 min (control). By 30 min, phosphorylation had returned to near basal levels (as in Fig. 7). Treatment with PMA (Fig. 11 A, right lane) sustained phosphorylation through 30 min. Transfection with the vector control (Fig. 11 A, VT) gave the same result as the control. However, in cells expressing wild-type MKP-1

(WT), the attachment-induced increase in ERK phosphorylation at 10 min was essentially blocked. Interestingly, in SMC transfected with the mutant MKP-1 in which the essential catalytic cysteine is replaced (Sun et al., 1993), phosphorylation of ERK is sustained through 30 min (Fig. 11 A, MT), indicating that MKPs likely play a normal role in effecting the decrease in ERK phosphorylation in SMC. The same transfected cells were also subjected to chemotaxis assays in which they were stimulated with soluble collagen alone, 4N1K alone, or 4N1K plus collagen (Fig. 11 B). In the MKP-1-transfected cells, all three stimuli were about twofold more effective chemoattractants than in the vector control or mutant MKP1 transfected SMC. The mutant MKP-1, which causes a prolonged activation of ERK (Fig. 11 A), has the same chemotactic response as the control. Thus, it appears that the transient nature of ERK phosphorylation may not be essential for the normal level of chemotaxis. The significant result here is that when ERK activation is attenuated, the chemotactic response to all three stimuli is enhanced. Thus, three different methods of decreasing ERK activity all result in enhanced chemotaxis toward collagen and magnify the effect of 4N1K.

Discussion

IAP is a widely distributed membrane protein implicated in modulating integrin functions required for Ca^{2+} fluxes (Schwartz et al., 1993), phagocytosis (Blystone et al., 1995), transendothelial (Cooper et al., 1995), and transepithelial (Parkos et al., 1996) migration of PMNs, endothelial cell migration (Gao et al., 1996a), as well as integrin-mediated C32 cell spreading (Gao et al., 1996a) and platelet spreading and aggregation (Chung et al., 1997, 1999). Many of these functions involve modulation of $\beta 3$ integrins. We have shown that TSP1, through its IAP-binding motif, the 4N1K peptide, is able to modulate the activities associated with the $\alpha 2\beta 1$ integrin such that it can promote chemotaxis of human, rat (Wang and Frazier, 1998), and mouse (this report) arterial SMC toward soluble collagen-I. This effect of TSP1 and 4N1K on $\alpha 2\beta 1$ -mediated chemotaxis requires IAP. Chemotaxis of IAP-deficient SMC toward soluble collagen cannot be stimulated by 4N1K (Fig. 1) even though these cells adhere to gelatin and collagen as well as IAP^{+/+} control cells. These experiments represent the most direct proof to date that TSPs, via the 4N1K peptide, act through IAP to augment $\beta 1$ integrin function.

How does this occur? The effect of 4N1K-IAP on $\alpha 2\beta 1$ -dependent migration on gelatin can be conceptualized in two different ways. First, IAP may alter the affinity state of $\alpha 2\beta 1$, either by direct physical interaction (Wang and Frazier, 1998) or via inside out signaling. Our previous data from platelets support such an affinity modulation model. The ability of 4N1K (or TSP 1 or its recombinant COOH-terminal domain, McDonald, J.F., X.-Q. Wang, and W.A. Frazier, unpublished data) to enhance SMC chemotaxis to soluble collagen is reminiscent of the activation of platelet $\alpha \text{IIb}\beta 3$ by 4N1K/TSP-1 (Chung et al., 1997, 1999). This effect in platelets is also dependent on IAP since platelets from IAP deficient mice do not aggregate in response to 4N1K or TSP 1 (Chung et al., 1999). The fact that 4N1K pretreatment can stimulate migration to solu-

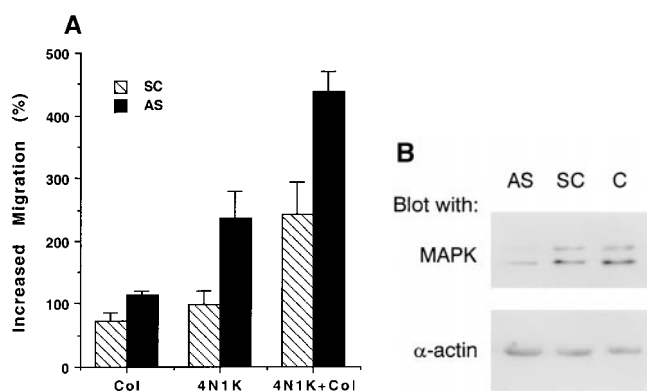


Figure 10. Anti-sense downregulation of ERK enhances SMC chemotaxis. (A) Cell migration assay. Cell migration was tested after ERK was depleted with anti-sense ERK oligonucleotide (AS). Data are expressed as the percentage of increased cell migration above that of the scrambled oligonucleotide-treated cells (SC). (B) Western blotting. The same cells tested in the migration assay in A were lysed in RIPA buffer, and after SDS PAGE, blots were probed with anti-ERK antibody. Lane AS, treated with anti-sense oligonucleotide; lane SC, treated with control oligonucleotide; lane C, no treatment. Blots were stripped and probed with an anti-actin antibody as a loading control.

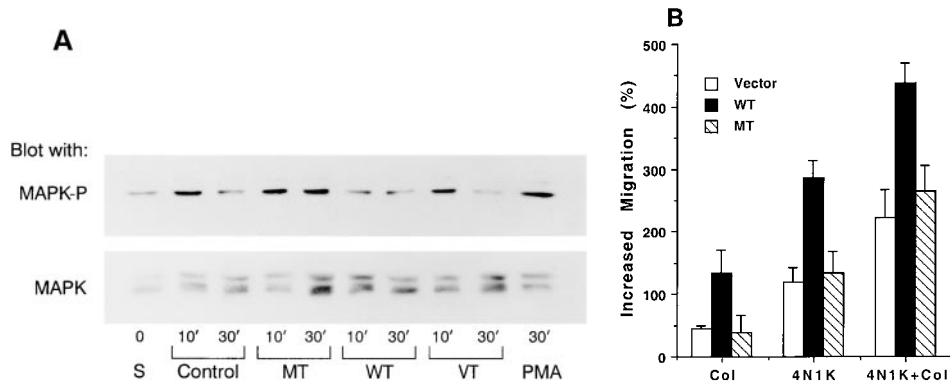


Figure 11. Expression of MKP-1 in SMC enhances chemotaxis. (A). Western blotting. After transfection and recovery, cells were plated on 100 μ g/ml gelatin-coated dishes for the indicated times after which cells were lysed in RIPA buffer, processed for SDS PAGE, and blotted. The activation of MAP kinase was detected through Western blotting with anti-activated MAP kinase antibody. S, cells kept in suspension; Control, untransfected cells attaching to collagen; MT, cells

transfected with inactive mutant MKP1; WT, cells transfected with wild-type MKP1; VT, vector control transfectants. PMA, cells in suspension treated with 100 ng/ml PMA for 30 min. (B) Chemotaxis assay. The same cells were tested in the chemotaxis assay as above. Data are expressed as the percentage of increased cell migration over no stimulus.

ble collagen is suggestive of a change in the activation status of $\alpha 2\beta 1$ that lasts long enough to be manifested during the several hours required for cells to migrate through the filters. An activating mAb against $\alpha 2\beta 1$ gives the same result, i.e., enhanced chemotaxis to soluble collagen, which is a poor ligand for the basal state of the integrin (Santoro and Zutter, 1995) just as soluble fibrinogen is a terrible ligand for $\alpha \text{IIb}\beta 3$ on quiescent platelets (Shattil et al., 1994). Thus, the enhanced chemotaxis toward soluble collagen by 4N1K may be analogous to the stimulation of soluble fibrinogen binding to platelet $\alpha \text{IIb}\beta 3$ by 4N1K (Chung et al., 1997, 1999) which leads to platelet aggregation. In platelets, the binding of the ligand mimetic mAb PAC-1 can be used as an index of $\alpha \text{IIb}\beta 3$ affinity. In the case of $\alpha 2\beta 1$, there is no such convenient marker for a higher affinity/avidity state of the unliganded integrin. The effect of 4N1K to decrease cell adhesion as measured in a static adhesion assay (Fig. 3) appears to argue against a simple activation of ligand binding by the integrin, since this would normally be expected to enhance not inhibit cell adhesion. This result speaks to some change in the state of the integrin's valency or affinity, but the nature of this change is unclear at the present time.

The second hypothesis, which need not be exclusive with affinity modulation, is that IAP signaling is integrated with $\alpha 2\beta 1$ signaling at one or more levels inside the cell. Both collagen and 4N1K lead to a drop in intracellular cAMP levels. With collagen alone, this effect is transient, whereas 4N1K leads to the sustained suppression of cAMP which is enhanced by coadministration of collagen. This effect is G_i dependent, as shown by experiments with pertussis toxin, and independent of adhesion and integrin engagement, as shown by experiments with suspended cells. The decrease in cAMP levels and the inhibition of ERK activity can both have profound effects on the cell's motility apparatus (Tan et al., 1992; Klemke et al., 1997), and perhaps on other events upstream that connect integrin engagement to cell motility.

The precipitous drop of cAMP levels in the SMC upon treatment with 4N1K is likely to be due to a direct action of $G_i\alpha_{[GTP]}$ on adenylate cyclase. In platelets and in leukocytes, high levels of cAMP appear to block the transition to a more active state of the integrins (Shattil et al., 1994,

1998; Laudanna et al., 1997). Thus, our observation that a prominent effect of IAP stimulation in SMC is a profound drop in intracellular cAMP levels may well be related to removing a brake on $\alpha 2\beta 1$ function. This effect is pertussis toxin sensitive and thus clearly requires G_i function (Fig. 5 B). We have found an identical, IAP-dependent, pertussis toxin-sensitive depletion of cAMP in platelets (Chung et al., 1997; Frazier et al., 1999) where other costimulators of integrin activation such as thrombin, epinephrine, and ADP act via G proteins to lower cAMP levels (Shattil et al., 1994). Soluble collagen alone also causes a transient fall in cAMP in SMC. Together, 4N1K and collagen synergize, resulting in a more profound and prolonged decrease of cAMP levels, in parallel with the more pronounced chemotactic response elicited by the combined stimuli. This suggests that the integrin and IAP work together to activate G_i . In fact, we have recently found that 4N1K or TSP1 ligation of IAP on platelets synergizes with collagen ligation of $\alpha 2\beta 1$ to induce platelet activation and aggregation (Chung et al., 1999). That a heterotrimeric complex of integrin and IAP is the functional signaling unit is further indicated by the existence of a detergent-stable integrin/IAP complex in SMC (Wang and Frazier, 1998), in platelets (Chung et al., 1999), and in melanoma cells which is physically associated with G_i (Frazier et al., 1999). In addition, G_i can be copurified with IAP and its integrin partner by affinity chromatography on immobilized 4N1K, and by immunoprecipitation with anti-IAP mAbs. Furthermore, 4N1K stimulates the binding of $\gamma\text{-}^{35}\text{S}$ GTP to cell membranes and this stimulation requires IAP since it is not observed in membranes from cells of IAP knockout mice (Frazier et al., 1999). This result with isolated membranes makes it unlikely that IAP signals the synthesis of an autocrine factor which then indirectly activates G_i through its own G_i coupled serpentine receptor. If ligation of IAP is required to activate G_i , how then does collagen alone cause a decrease in cAMP levels (Fig. 5 A)? Our preliminary data suggests that cross-linking of the integrin-IAP complex by collagen may be responsible.

Another immediate result of IAP stimulation with 4N1K is a suppression of ERK activity and phosphorylation (Fig. 7). That this suppression of ERK activity is causal for chemotaxis is indicated by the fact that three

different methods of inhibiting ERKs all result in enhanced chemotaxis (Figs. 9–11). A mechanism of integrin modulation that involves ERKs is suggested by experiments of Hughes et al. (1997) in which a chimeric integrin expressed in CHO cells was engineered to be in an activated state as determined by the binding of the ligand-mimetic mAb, PAC-1. Then cDNAs were expressed in the CHO cells and screened for inhibition of PAC-1 binding. Several inhibitory or integrin deactivating cDNAs were identified and all encoded activators of the ERK pathway such as activated H-ras and raf-1. These results suggest that MAP kinase, specifically ERKs, inhibit integrin activation of the sort required for binding of soluble ligands. Interestingly, it has been reported that activation of α IIb β 3 and binding of soluble fibrinogen to platelets coincides with inhibition of ERKs (Nadal et al., 1997). Whether ERK inhibition might cause α IIb β 3 activation was not determined. We used three very different ways of decreasing ERK activity and all three led to an increased chemotactic response of SMC. The modulation of α 2 β 1 activity in SMC by IAP suggested by our data may be the first physiological example of the ERK regulation of integrin affinity/avidity.

Klemke et al. (1997) have reported that α 2 β 1-dependent migration of FG carcinoma cells on a collagen substrate required activation of MAPK/ERK. While their assay used a Boyden chamber apparatus like our chemotaxis assays, the filters on which the cells migrated were coated with fibronectin only on the bottom side. Their assay was thus a haptotaxis assay which measures increased adhesion to the matrix protein coated on the underside of the filter. Thus, their result is that activation of ERKs results in increased integrin-mediated adhesion to matrix, not increased chemotaxis as observed here for SMC. In fact, we observe (Fig. 3) that activation of IAP with 4N1K results in decreased adhesion concomitant with increased chemotactic migration. Importantly, Klemke et al. (1997) found that inhibition of ERKs did not prevent spreading of the FG cells, a cellular function closely allied to cell migration. Thus, our results are, in fact, consistent with those of Hughes et al. (1997) and Klemke et al. (1997), and reinforce the idea that activation of MAPK/ERKs inhibits binding of soluble ligands to integrins and also stimulates integrin-mediated adhesion which can inhibit cell migration. The finding that ERKs can phosphorylate MLCK (Klemke et al., 1997) may also support the second model for IAP action to regulate cell motility at downstream sites.

The mechanism by which IAP inhibits ERK activity is not clear. We find that ERK activity is stimulated by disabling Gi with pertussis toxin (Fig. 8) suggesting that under normal growth conditions, some tonic input acts through Gi to exert a negative influence on ERK activity in SMC. This negative regulation may be due to $\beta\gamma$ since expression in SMC of the β -ARK COOH-terminal domain that sequesters $\beta\gamma$ also stimulates ERK activity in randomly cycling, proliferating cultures of SMC (Wang, X.-Q., and W.A. Frazier, unpublished data). This result is in contrast to recently published data on the role of $\beta\gamma$ as a positive effector of ERK activation in serum-starved SMC stimulated with serum or growth factors (Iaccarino et al., 1999). However, the effects of $\beta\gamma$ sequestration are poten-

tially quite complex, impacting not only downstream targets of $\beta\gamma$, but also receptor desensitization and down regulation (Lefkowitz, 1998). Thus, it is conceivable that $\beta\gamma$ sequestration may differ in its net result when tested in proliferating vs synchronized cells stimulated to enter the cell cycle.

Whatever the mechanism, the essential role of a Gi protein in the stimulation of chemotaxis by 4N1K is clearly demonstrated by the effect of pertussis toxin treatment of the SMC. The downstream events that lead to the effects on cell motility that we observe could be mediated by the Gi α subunit or the $\beta\gamma$ heterodimer released upon activation and dissociation of Gi (Hawes et al., 1995; Neer, 1995). The inhibition of chemotaxis by forskolin and 8-Br-cAMP implies that decreased cAMP (and lower PKA activity) is necessary to allow directed migration (Fig. 6). This observation is consistent with other studies of SMC chemotaxis (Dubey et al., 1995; Palmer et al., 1998) and with the inhibitory effect of high cAMP levels on activation of leukocyte integrins (Laudanna et al., 1997) and the long known inhibitory effect of cAMP on activation of the platelet integrin α IIb β 3 (Shattil et al., 1994). Another well known effect of cAMP is the PKA-mediated inhibition of cell motility via phosphorylation of myosin light chain (Tan et al., 1992) which would also put a brake on chemotaxis until cAMP levels are reduced.

It has been reported (Graves et al., 1996) that adenylate cyclase is downstream of MAP kinase in SMC, since activated ERKs can increase cAMP levels. This occurs via ERK-mediated phosphorylation and activation of PLA2 leading to prostaglandin production, and autocrine adenylate cyclase stimulation via Gs (Graves et al., 1996). In addition to 4N1K, PD98059 also decreases cAMP levels in SMC (our unpublished data). Thus, when ERK is inhibited, cAMP levels follow. However, manipulation of cAMP levels does not appear to affect ERK (our unpublished data; Giasson et al., 1997), consistent with the idea that ERK regulation is upstream of adenylate cyclase in SMC (Graves et al., 1996). Thus, activation of Gi can lower cAMP levels in two ways: direct inhibition of adenylate cyclase by Gi α and inhibition of ERKs leading to decreased stimulation of cyclase via the prostaglandin pathway. It appears that both decreased cyclic AMP levels and lower ERK activity are necessary for IAP modulation of SMC motility.

The data reported here indicate a novel role for TSP family members (all of which contain the IAP binding sequence) and IAP in the regulation of SMC migration. This may occur via modulation of a β 1 integrin mediated by inhibition of MAP kinase, and a concomitant decrease in intracellular cyclic AMP levels. Both of these signaling events are tremendously pleiotropic, and both PKA and ERKs have been shown to impact regulation of cell motility via direct phosphorylation of myosin light chain kinase and/or myosin light chain (Tan et al., 1992; Klemke et al., 1997). Thus, IAP may act at multiple levels to modulate integrin function as well as facilitating signaling downstream of integrin ligation. There is ample literature implicating TSP1 in the proliferation and chemotaxis of cultured SMC (Majack et al., 1986, 1988; Yabkowitz et al., 1993; Patel et al., 1997; Wang and Frazier, 1998) and in atherogenesis (Wight et al., 1985) and restenosis (Chen et al.,

1999). Our data suggest the possibility that manipulation of TSP1 signaling through IAP may be of therapeutic benefit.

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