Melanoma chondroitin sulphate proteoglycan regulates cell spreading through Cdc42, Ack-1 and p130Cas

Kathryn M. Eisenmann  
*University of Minnesota, Minneapolis, Minnesota*

James B. McCarthy  
*University of Minnesota, Minneapolis, Minnesota*

Melanie A. Simpson  
*University of Nebraska - Lincoln, msimpson2@unl.edu*

Patricia J. Keely  
*University of Wisconsin, Madison, Wisconsin*

Jun-Lin Guan  
*Cornell University, Ithaca, New York*

See next page for additional authors

Follow this and additional works at: [http://digitalcommons.unl.edu/biochemfacpub](http://digitalcommons.unl.edu/biochemfacpub)

Part of the [Biochemistry, Biophysics, and Structural Biology Commons](http://digitalcommons.unl.edu/biochemfacpub)


[http://digitalcommons.unl.edu/biochemfacpub/18](http://digitalcommons.unl.edu/biochemfacpub/18)

This Article is brought to you for free and open access by the Biochemistry, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Biochemistry -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Authors
Kathryn M. Eisenmann, James B. McCarthy, Melanie A. Simpson, Patricia J. Keely, Jun-Lin Guan, Kouichi Tachibana, Louis Lim, Ed Manser, Leo T. Fucht, and Joji Iida
Melanoma chondroitin sulphate proteoglycan regulates cell spreading through Cdc42, Ack-1 and p130^cas


* Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455, USA
† Biomedical Engineering Institute, University of Minnesota, Minneapolis, Minnesota 55455, USA
‡ Cancer Center, University of Minnesota, Minneapolis, Minnesota 55455, USA
§ Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706, USA
¶ Department of Molecular Medicine, Cornell University, Ithaca, New York 14853, USA
** Department of Cancer Immunology and AIDS, Harvard Medical School, Boston, Massachusetts 02115, USA
†† Institute of Neurology, London WC1N 1PJ, UK
‡‡ Institute of Molecular and Cell Biology, National University of Singapore, 15 Lower Kent Ridge Road, 119076 Singapore
§ Corresponding author: James B. McCarthy, email: mccar001@tc.umn.edu

Abstract: Melanoma chondroitin sulphate proteoglycan (MCSP) is a cell-surface antigen that has been implicated in the growth and invasion of melanoma tumors. Although this antigen is expressed early in melanoma progression, its biological function is unknown. MCSP can stimulate the integrin-α,β-mediated adhesion and spreading of melanoma cells. Here we show that stimulated MCSP recruits tyrosine-phosphorylated p130^cas, an adaptor protein important in tumor cell motility and invasion. MCSP stimulation also results in a pronounced activation and recruitment of the Rho-family GTPase Cdc42. MCSP-induced spreading of melanoma cells is dependent upon active Cdc42, a Cdc42-associated tyrosine kinase (Ack-1) and tyrosine phosphorylation of p130^cas. Furthermore, vectors inhibiting Ack-1 or Cdc42 expression and/or function abrogate MCSP-induced tyrosine phosphorylation and recruitment of p130^cas. Our findings indicate that MCSP may modify tumor growth or invasion by a unique signal-transduction pathway that links Cdc42 activation to downstream tyrosine phosphorylation and subsequent cytoskeletal reorganization.

Metastasis of tumor cells requires rapid, dynamic regulation of cell-surface adhesion receptors important for migration, invasion, extravasation and growth. One group of proteins involved in tumor adhesion is the integrins, a family of heterodimeric adhesion receptors fundamentally important to mediating cell–cell and cell–extracellular matrix (ECM) interactions. In particular, integrin α,β has been implicated in tumor cell invasion and metastasis. Although integrin α,β is expressed in a variety of malignancies, such as melanoma, and α,β expression has been linked to melanoma progression, the mechanism by which tumor cells modulate integrin adhesiveness is not fully understood. However, clustering of integrins triggers a cascade of intracellular signalling pathways leading to the phosphorylation of cytoplasmic and cytoskeletal substrates, such as focal adhesion kinase (FAK), paxillin and p130^cas. Furthermore, both p130^cas and FAK play a part in integrin-mediated tumor cell migration, indicating that modifying integrin signalling pathways may stimulate tumor invasion and metastasis.

Cell-surface proteoglycans are a second group of adhesion receptors that mediate both cell–cell and cell–ECM interactions. Many ECM proteins contain closely spaced proteoglycan- and integrin-binding domains, indicating that these two distinct types of adhesion receptor may generally function in concert to stimulate cytoskeletal reorganization, migration and invasion. Of the proteoglycans, MCSP is abundantly and ubiquitously expressed on most human melanoma cells, whereas its expression is lower on normal melanocytes. MCSP may be involved in the spreading, migration and invasion of melanoma cells, as antibodies directed against MCSP inhibit these processes in vitro. We have shown previously that MCSP enhances α,β integrin function, in part by stimulating signalling pathways involving tyrosine kinases.

Members of the Rho family of GTPases, Rho, Rac and Cdc42, regulate cytoskeletal rearrangements, leading to the formation of actin stress fibres, lamellipodia and filopodia, respectively. The cooperative signalling of integrin and syndecan proteoglycan that leads to the assembly of focal adhesions has been shown to be Rho-dependent. Integrin-β1-mediated signalling has also been linked to cytoskeletal rearrangements through functional interactions of Cdc42 with phosphatidylinositol-3-OH kinase (PI(3)K) and potentially FAK23,24, indicating that Rho-family GTPases may be responsible for regulating adhesive signals mediated by both integrins and cell-surface proteoglycans.

Members of the Ack (activated-Cdc42-associated kinase) family of non-receptor tyrosine kinases act as potentially important links between certain activated Rho-family GTPases (such as Cdc42) and signalling pathways. Members of this family interact specifically with the GTP-bound (active) form of Cdc42 (refs 25, 26). Ack-1 is homologous to FAK in its kinase and proline-rich domains, as well as to Src in its kinase and Src homology 3 (SH3) domains, indicating that Ack-1 may interact with, and phosphorylate, effector proteins similar to those that interact with FAK and Src. Ack-2 is a structural variant of Ack-1; it lacks 344 residues within the proline-rich carboxy-terminal tail. Activated Ack-2 coprecipitates with β1 integrin, suggesting a role for this family of kinases in modulating cell adhesion.

We show here that clustering of MCSP activates Cdc42 to a GTP-bound state. Furthermore, activated MCSP recruits a signalling complex that includes activated Cdc42 and Ack-1. Formation of this complex results in recruitment and tyrosine phosphorylation of p130^cas and subsequently enhances α,β-integrin-mediated melanoma cell spreading.
MCSP clustering induces tyrosine phosphorylation of p130 protein. We have shown previously that cooperative stimulation of both MCSP and α4β1 integrin induces cell spreading and focal-contact formation in A375SM human melanoma cells. The cytoskeletal rearrangements delineating this morphology were, in part, dependent upon tyrosine kinases, as shown by an inhibition of melanoma cell spreading and focal-contact formation upon pretreatment with a tyrosine kinase inhibitor, genistein. Hence, we have now evaluated a role for tyrosine phosphorylation in MCSP-induced signalling pathways. Signal transduction was stimulated in A375SM human melanoma cells by addition of magnetic beads coated with 9.2.27, a monoclonal antibody directed towards the MCSP core protein. MCSP clustering induced both the recruitment and the tyrosine phosphorylation of p130 (Figure 1c, d, lanes 2, 4). These results indicate that, under these experimental conditions, recruitment and tyrosine phosphorylation of p130 specifically require MCSP stimulation.

p130 is required for MCSP-induced αβ4-mediated cell spreading. We next evaluated the requirement for p130 in MCSP-induced melanoma-cell spreading by using a dominant-negative p130 SH3 construct that binds to a proline-rich span in proteins such as FAK. As reported previously, both untransfected and mock-transfected A375SM melanoma cells adhered but did not spread when either α4β1 integrin or MCSP was engaged alone on rIIICS- or 9.2.27-coated plates, respectively (Figure 2a, b, d, e). Only when MCSP and α4β1 integrin were engaged cooperatively was cell spreading observed (Figure 2c, f). Cells transfected with the dominant-negative p130 SH3 construct were unable to spread on a substrate engaging both MCSP and α4β1 integrin (Figure 2g–i). To determine whether tyrosine phosphorylation of p130 was required for MCSP-induced cell spreading, we transfected cells with p130 ΔSD, a mutated p130 that lacks residues 119–420, which include multiple YYXP motifs (single-letter amino-acid code) that function as tyrosine kinase substrates. Cells transfected with p130 ΔSD failed to

MCSP clustering induces p130 recruitment and phosphorylation. We then re-immunoprecipitated MCSP-associated proteins with antibodies directed against various proteins of M130K. A monoclonal antibody recognizing p130 immunoprecipitated a MCSP-associated protein of M130K (Figure 1c, lane 5). Although p130 did not associate with MCSP in the absence of clustering, clustering of MCSP induced both the recruitment and the tyrosine phosphorylation of p130 (Figure 1c, d, lanes 3, 5). Clustering of another cell-surface receptor, the class I major histocompatibility complex (MHC), by an isotype-matched monoclonal antibody did not induce p130 recruitment or tyrosine phosphorylation (Figure 1c, d, lanes 2, 4). These results indicate that, under these experimental conditions, recruitment and tyrosine phosphorylation of p130 specifically require MCSP stimulation.
spread on a substrate engaging both MCSP and α4β1 integrin (data not shown). Collectively, these data indicate that recruitment and tyrosine phosphorylation of p130<sup>cas</sup> function in mediating MCSP-induced signal-transduction pathways leading to α4β1-integrin-dependent spreading of melanoma cells.

**Cdc42 associates with MCSP upon MCSP clustering.** Previous studies have localized MCSP to microspikes and filopodia of cultured melanoma cells, indicating that MCSP may mediate early cell–matrix recognition events important to the process of cell adhesion. As the Rho-family GTPase Cdc42 stimulates cell–matrix recognition events important to the process of cell adhesion, we next determined whether Cdc42 associates with MCSP. We incubated A375SM melanoma cells with magnetic beads coated with monoclonal antibody 9.2.27 or P4C2 (anti-α4-integrin antibody), and analysed associated proteins for the presence of Cdc42 by immunoblotting. In the absence of adhesion-receptor clustering, Cdc42 showed a low level of constitutive association with MCSP but not with α4β1 integrin (Figure 3a, lanes 3, 4). This association was markedly enhanced (about 4.4-fold, as determined by densitometry) upon MCSP clustering (Figure 3a, lane 7; data not shown). MCSP-associated Cdc42 represents ~59% of total cellular Cdc42 (data not shown). Clustering of α4β1 integrin, however, did not induce Cdc42 association with the integrin (Figure 3a, lane 6). We also determined the activation state of Cdc42 upon MCSP stimulation. Using the PAK binding domain (PBD) of PAK3, which binds preferentially to GTP-bound Cdc42 or Rac, we found that MCSP stimulation resulted in a rapid and robust activation of Cdc42 (Figure 3b). Furthermore, MHC stimulation did not induce Cdc42 activation (Figure 3b), indicating that MCSP activation of Cdc42 is specific.

To determine whether Cdc42 influences MCSP-induced signal transduction leading to α4β1-integrin-dependent cell spreading, we transfected cells with expression vector alone or with constructs encoding Cdc42 as a dominant-negative (Cdc42(17N)) or constitutively active (Cdc42(12V)) enzyme. We assayed transfected cells for cell spreading as described above. Unlike both untransfected and mock-transfected cells, cells transfected with Cdc42(17N) adhered but did not spread when both MCSP and α4β1 integrin were engaged (Figure 3c). Furthermore, melanoma cells transfected with Cdc42(12V) fully spread when α4β1 integrin alone was engaged on rIIICS-coated plates, bypassing the requirement for MCSP engagement in inducing α4β1-integrin-mediated cell spreading (Figure 3d). Together, these data implicate Cdc42 in mediating post-adhesion cytoskeletal changes following engagement of MCSP and α4β1 integrin in A375SM human melanoma cells.

**Cdc42(17N) inhibits MCSP-induced p130<sup>cas</sup> recruitment and phosphorylation.** To assess the role of Cdc42 in signal transduction from MCSP, we studied the effects of mutant Cdc42 constructs on MCSP-induced recruitment and tyrosine phosphorylation of p130<sup>cas</sup>. Cells were transfected with either vector alone or Cdc42(17N). Transfected cells were incubated with 9.2.27-coated magnetic beads to induce tyrosine phosphorylation of p130<sup>cas</sup>. Melanoma cells expressing dominant-negative Cdc42 failed to recruit or tyrosine phosphorylate p130<sup>cas</sup> upon MCSP stimulation (as compared with both mock-transfected and untransfected cells) (Figure 4a, b). Cells transfected with a constitutively active Cdc42 construct showed enhanced p130<sup>cas</sup> recruitment and subsequent tyrosine phosphorylation upon clustering of MCSP (data not shown). Hence, the GTPase Cdc42

---

**Figure 3** Activated Cdc42 associates with MCSP and induces spreading of melanoma cells. **a.** Clustering of MCSP specifically recruits Cdc42. **b.** Cdc42 is specifically activated upon stimulation of MCSP, as determined by interaction with PAK binding domain (PBD)-coated beads, which bind preferentially to active Cdc42 or Rac. **c.** Untransfected (UT) or vector-transfected (mock) A375SM cells (c, d), or A375SM cells transfected with dominant-negative Cdc42 (Cdc42(17N)) (c) or constitutively active Cdc42 (Cdc42(12V)) (d) were allowed to adhere and spread on plates coated with rIIICS, monoclonal antibody 9.2.27 or both rIIICS and 9.2.27. *P < 0.001 compared with untransfected cells.

**Figure 4** Cdc42 mediates MCSP-induced p130<sup>cas</sup> recruitment and tyrosine phosphorylation. A375SM cells that were untransfected (UT) or transfected with vector alone (mock) or dominant-negative Cdc42 (Cdc42(17N)) were incubated with beads coated with monoclonal antibody 9.2.27, which induced clustering of MCSP and tyrosine phosphorylation (a) and recruitment (b) of p130<sup>cas</sup>.
is involved in activating MCSP induced tyrosine-kinase pathways that lead to the recruitment and tyrosine phosphorylation of p130cas.

**Ack-1 associates with MCSP.** As association of Cdc42 with MCSP induced tyrosine phosphorylation of p130cas, we proposed that activated Cdc42-associated kinase-1 (Ack-1) might mediate this phosphorylation. We lysed A375SM melanoma cells and immunoprecipitated Ack-1 kinase from cell lysates subsequently ablated the 120–130K MCSP-associated in vitro kinase activity.

![Figure 5](image1.png)

**Figure 5** Ack-1 kinase activity is associated with MCSP. **a**, In vitro kinase activity of a 120K–130K protein is specifically associated with MCSP. **b**, Immunodepleting Ack-1 kinase from cell lysates subsequently ablates the 120–130K MCSP-associated in vitro kinase activity.

To determine whether Ack-1 participates in MCSP-induced signal transduction leading to integrin-mediated cytoskeletal changes, we constructed an Ack-1 antisense expression vector and expressed it in A375SM melanoma cells to inhibit Ack-1 protein expression. To confirm that the antisense vector was expressed, we assayed Ack1 kinase activity using myelin basic protein (MBP) as an exogenous substrate (Figure 6a). This assay confirmed that antisense Ack-1 expression inhibits Ack-1 kinase activity. The Ack-1 kinase activity in mock-transfected and untransfected cells was unaffected (Figure 6b). Melanoma cells expressing the Ack-1 antisense construct adhered, but did not spread, on a substrate engaging both α4β1 integrin and MCSP (Figure 6b). These data are consistent with a role for Ack-1 in MCSP-induced signalling pathways that stimulate cytoskeletal rearrangements.

**Ack-1 mediates MCSP-induced p130cas tyrosine phosphorylation.** We next determined whether p130cas was a target substrate for Ack-1 in MCSP-induced signal-transduction pathways. Cells that were untransfected, mock transfected or transfected with the Ack-1 antisense construct were incubated with magnetic beads coated with monoclonal antibody 9.2.27 to induce p130cas phosphorylation and recruitment of p130cas.

![Figure 6](image2.png)

**Figure 6** Ack-1 kinase induces spreading of melanoma cells and tyrosine phosphorylation of p130cas. **a**, Expression of Ack-1 antisense vector inhibits Ack1 in vitro kinase activity, as determined by phosphorylation of an exogenous substrate, myelin basic protein (MBP). **b**, Untransfected A375SM cells (UT) or A375SM cells transfected with either vector alone (mock) or Ack-1 antisense vector were allowed to adhere and spread on plates coated with rillics, monoclonal antibody 9.2.27, or both. *P < 0.001 compared with untransfected cells. **c**, Untransfected A375SM cells (UT) or A375SM cells transfected with vector alone (mock) or Ack-1 antisense vector were clustered with 9.2.27-coated beads to induce tyrosine phosphorylation of p130cas. **d**, Untransfected A375SM cells (UT) or cells transfected with mock or Ack-1 kinase-dead (kd) vector were clustered with 9.2.27-coated beads to induce tyrosine phosphorylation and recruitment of p130cas.
phorylation. Melanoma cells expressing Ack-1 antisense constructs failed to recruit and tyrosine phosphorylate p130cas upon MCSP clustering (Figure 6c), indicating that p130cas may be a downstream target of Ack-1. To determine whether the kinase activity of Ack-1 is specifically required for signalling from MCSP, we transfected melanoma cells with a kinase-dead Ack-1 construct, Ack1(K163A) (Figure 6d). Cells transfected with this kinase-dead construct failed to either recruit or tyrosine phosphorylate p130cas upon MCSP clustering. Together, these data illustrate the importance of Ack-1 kinase activity in propagating MCSP-induced signal transduction, ultimately leading to αβ1-integrin-mediated spreading of melanoma cells.

Discussion

Our previous studies showed that MCSP stimulates αβ1-integrin-mediated adhesion and spreading of melanoma cells16–19, in part through the involvement of activated tyrosine kinases17. Here, we have shown that signalling through MCSP induces the recruitment and tyrosine phosphorylation of p130cas. We propose a unique MCSP-induced signalling pathway that integrates both activated Cdc42 and Ack-1, thereby stimulating the downstream recruitment and tyrosine phosphorylation of p130cas.

Using dominant-negative p130cas constructs, we showed that p130cas promotes MCSP-induced αβ1-integrin-mediated cytoskeletal rearrangements. Phosphorylation of p130cas has been implicated in integrin-induced signal-transduction pathways; upon integrin engagement, p130cas is tyrosine phosphorylated through interactions of FAK/Src with the SH3 domain of p130cas (refs 28, 30, 31). p130cas has also been localized to focal adhesions52. Furthermore, p130cas is involved both in FAK-mediated migration of CHO cells10 and in promoting integrin-dependent tumour-cell migration and invasion through coupling with Crk111. These results indicate that phosphorylation of p130cas by multiple tyrosine kinases may be important for tumor invasion and metastasis by regulating cell adhesion and motility.

Using a metastatic melanoma cell line, we found that MCSP-induced tyrosine phosphorylation of p130cas is dependent on the recruitment and activation of the Rho-family GTPase Cdc42. Although Cdc42 is important for stimulating filopodia formation and maintaining cell polarity during cell migration21,33, emerging evidence also implicates activation of Cdc42 in cellular transformation and tumor progression. For instance, Cdc42 activation confers anchorage-independent growth of rat fibroblasts and is required for Ras-mediated transformation44. Furthermore, activation of Cdc42 promotes integrin-mediated motility and invasion of breast cancer cells23. On the basis of our results, we propose that MCSP enhances tumor cell invasion and metastasis by recruiting a signal-transduction complex that includes active Cdc42.

Our data also show that MCSP-induced sequestration of Cdc42 and tyrosine-phosphorylated p130cas depends upon the activation of the kinase Ack-1. Ack-1 and Ack-2 associate with the GTP-bound form of Cdc42 (refs 25–27), and GTP-bound Cdc42 activates auto-phosphorylation of Ack-2 within cells28. It has, therefore, been proposed that GTP-bound Cdc42 mediates the activation of Ack kinases by directing their proper cellular localization26. The subcellular localization of Ack kinases by interactions with GTP-bound Cdc42 may enhance the ability of these kinases to recruit and/or phosphorylate target proteins, further propagating signal transduction.

Although MCSP can induce signals independently of integrin engagement, our data also indicate that the signals stimulated by MCSP are closely related and/or intersect integrin-mediated signal-transduction pathways. For example, tyrosine phosphorylation of p130cas is also associated with ligation of β1 integrin28,30,31,35,56, indicating that this adaptor protein may be involved in integrin-mediated adhesion and motility. Furthermore, Ack-2 co-precipitates with β1 integrin, and activation of Ack-2 is required for its association with this integrin27; however, a direct relationship between Ack-2 and downstream integrin targets such as p130cas or FAK has not been established. Nevertheless, it is possible that MCSP-and αβ1-integrin-induced signalling pathways may intersect at multiple points. Such intersections may help to amplify these signalling pathways at cell–ECM attachment sites, which in turn act to accelerate cytoskeletal reorganization, leading to cell spreading, focal-contact formation and firm adhesion. Studies in which MCSP-induced signals are evaluated in the absence of functional integrin signalling (for example, using dominant-negative FAK) will be important in defining specific intersection points between MCSP and integrin signalling pathways. Present studies are also focusing on evaluating the potential involvement of other Rho-family GTPases (such as Rac1 and RhoA) in MCSP-induced signalling, as the activity of Rho-family GTPases can be coordinated in certain cell types21.

The structural features of MCSP that stimulate signal transduction are also being studied. Addition of chondroitin sulphate to the MCSP core protein confers on the proteoglycan the ability to interact with the C-terminal heparin-binding domain of fibronectin (and perhaps other matrix proteins). αβ1 integrin subunits also bind to chondroitin sulphate glycosaminoglycan (CSGAG) through a unique site on the integrin subunit, termed SG-1, and this interaction may enhance the activation of certain integrins19. CSGAG does not seem to be involved in MCSP-induced signal transduction, as both cell spreading and signalling are observed following CSGAG removal with chondroitinase ABC (J.I., unpublished observations). Instead, the data support a direct role for the MCSP core protein in signal transduction. Although the MCSP core protein has no apparent catalytic domains, the cytoplasmic tail of MCSP contains three potential threonine phosphorylation sites57. The importance of these sites has not yet been established; however, inhibitors of serine/threonine kinases (such as chelerythrine) inhibit MCSP-induced cell spreading (J.I., unpublished observations). Collectively, these results indicate that distinct structural features of MCSP may function to enhance adhesion of melanoma cells by both activating integrins and stimulating signalling pathways that lead to cytoskeletal rearrangement.

MCSP expression in melanoma cells is increased quite early in tumor progression13,38, indicating that it may also function in melanoma biology in ways that are related to cell growth and survival. For example, antibodies directed against MCSP inhibit anchorage-independent growth of human melanoma cells and suppress melanoma tumor growth in vivo39,40. Expression of NG2, the rat homologue of MCSP, may also increase both the in vivo tumorigenicity of melanoma cells in experimental metastasis models and the proliferation of B16 melanoma cells in vitro41. Cdc42 and Ack-2 have been linked to the activation of a subfamily of mitogen-activated protein kinases, the c-Jun amino-terminal kinases (JNKs), and thereby may stimulate gene expression27,42,43. The sequestration/activation of signalling complexes by MCSP may, therefore, stimulate critical signalling pathways important for controlling growth and programmed cell death.

Methods

Cell culture.

Highly metastatic A375SM human melanoma cells were selected by in vivo experimental metastasis assays of parent A375P cells in nude mice and were provided by J.I. Fidler46. Cells were maintained in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal calf serum, 50μg/ml gentamycin, minimal essential medium vitamin solution and 1mM sodium pyruvate. These cells were routinely used before 15 cell passages.

Antibodies.

Monoclonal antibody 9.2.27, directed towards the MCSP core protein, was a gift...
from R. Reisfeld. The anti-α₅-integrin monoclonal antibody P4C2 was provided by E. Wayner. Anti-p130Cas monoclonal antibody was purchased from Transduction Laboratories. Anti-Ack-1 polyclonal antibody sc-323 (Santa Cruz Laboratories) was raised against a C-terminal peptide fragment, NLEQAGCHLLGSWG-PAHHKR (amino acids 1,072–1,091). Anti-Cdc42 monoclonal antibodies were obtained from Santa Cruz Laboratories. Monoclonal antibody against MHC class I molecules was obtained from Pharmingen.

Plasmids.

The full-length Ack-1 sequence was excised from the plasmid pHueSscript SK-Ack-1 by digestion with EcoRI. The purified fragment was then ligated into pBKR5S (Stratagene) digested with EcoRI. The ligated plasmid was then used to transform Escherichia coli strain DH5α. The purified plasmid was sequenced at the Microfacs at the University of Minnesota to verify antisense orientation and was subsequently designated pBKR5S-Ack1-1AS. The pXHIA-Ack1-K163A kinase-inactive Ack-1 construct was a gift from E. Manser. Plasmids expressing glutathione-S-transferase (GST)-conjugated PBD were used to estimate the levels of Cdc42 activation as described 45. Dominant-negative Cdc42 (Cdc42(17N)) and constitutively active Cdc42 (Cdc42(12V)) in pZip were gifts from C. Der. The pKHSH3 construct was generated as described 10. pCDL-SRα-p130Cas(AD) was generated as described 15. The p130Cas(AD) insert was liberated from the pCDL-SRα vector by SacI/EcoRI digestion and was ligated into SacI/EcoRI-digested pBKR5S (Stratagene). The reporter plasmid pHook-1 was purchased from In Vitrogen.

Transfections and selection.

Cells were grown in six-well plates to about 60% confluency. Cells were then transfected with 2μg of the vector of interest along with 1μg pHook selection vector using the clonfectin transfection reagent (Clontech). Briefly, transfection media containing 3μg clonfectin and plasmids were mixed in serum-free EMEM for 30 min, after which the transfection media were applied to the cells. Transfection media were removed 3 h later and replaced with standard growth media. 36 h later, melanoma cells were collected with PBS/1mM EDTA and washed several times in serum-free EMEM. Cells were then mixed with CaptureFec selection beads (In Vitrogen) for 30 min at 37°C; the transfected cells were removed through magnetic separation. Selected cells were washed twice in serum-free media and used immediately in experiments.

Preparation of recombinant CS1 (rIIICS).

Recombinant CS1 (rIIICS), representing the alternatively spliced type IIICS domain of human fibronectin, was prepared as described below. A plasmid encoding the C-terminal portion of cellular fibronectin, provided by M.-L. Chu, served as a template for amplification by the polymerase chain reaction (PCR). Synthetic oligonucleotides complementary to the 3′-terminal domains of INCAM-110/VCAM-1.


ACKNOWLEDGEMENTS
We thank Y. Shimizu for valuable discussions. This work was supported by grant CA21463 from the NIH (to L.T.F.). L.T.F. is a recipient of an Allen-Pardee professorship in cancer biology.