The Tetrascapin CD151 Functions as a Negative Regulator in the Adhesion-dependent Activation of Ras*

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Transmembrane proteins of the tetraspanin superfam- 

My family are associated with integrins and are thought to 

regulate adhesion-dependent signaling. The molecular 

mechanisms of this regulation remain unknown. We 

used rat fibroblasts to analyze the contribution of the 

tetraspanin CD151 in the adhesion-dependent signaling. 

Expression of CD151 specifically attenuated adhesion- 

dependent activation of Ras. Furthermore, activation of 
PKB-Akt and ERK1/2, downstream targets in the Ras 
signaling pathway, was also diminished in cells expressing 
CD151. In contrast, adhesion-dependent activation of FAK and c-Src were not affected by CD151. The attenuation of Ras signaling did not correlate with phosphor- 
ylation of Tyr925-FAK, tyrosine phosphorylation of Shc, or with assembly of the p120RasGAP-p62Dok complex. Using mutants of CD151 we established that the cyto- 

plasmic C-terminal portion is critical for activity of 

CD151 toward Ras. Taken together these results identify 

CD151 as a negative regulator of Ras and suggest a novel mechanism of adhesion-dependent regulation of Ras activity.

The four transmembrane domain proteins of the tetraspanin superfam- 

ily are involved in regulation of various biological phenomena (1–3). Although numerous reports have established that tetraspanins are involved in cell migration (1–5), their role in this process remains unclear. Despite the fact that tetraspanins are associated with various integrins, in most cells they are excluded from the focal adhesions (6) and do not affect adhesion of cells to the extracellular matrix (2, 5). Therefore, it has been proposed that tetraspanins may specifically regulate post-ligand binding integrin-dependent signaling (2, 5). Indeed, earlier studies showed that tetraspanins could in-

fluence adhesion-dependent downstream signaling events, 

including tyrosine phosphorylation of cellular proteins (7) and activation of the serine-threonine kinase PKB-Akt (8). However, it is not known how tetraspanins target these signaling pathways or whether these events are connected with cell migration.

A number of integrin-dependent signaling pathways have been linked to cell motility. These include: association with, and activation of, receptor tyrosine kinases (9); activation of Src-family non-receptor tyrosine kinases (10); and activation of focal adhesion kinase (FAK)1 (11). All of these signaling events, in turn, trigger a network of parallel and intersecting down- 

stream signaling reactions (12, 13). Proteins of the Ras family of small GTPases take one of the central positions in this signaling map, by regulating the activities of Erk1/2 and Rac1 (13). It has been demonstrated that several pathways could be responsible for the adhesion-dependent activation of Ras pro- 

teins. Two of them (i.e. the assembly of the phospho-FAK- 

(Tyr925)-Grb2-Sos and phospho-Shc-Grb2-Sos complexes) re- 

quire the upstream activation of Src kinases (14, 15). In addition, it has been reported that CD151 adhesion triggers the assembly of the p120RasGAP-p62Dok complex, an event that suppresses the activity of RasGAP, which, consequently, leads to the increase in active, GTP-bound, Ras in cells (16).

Recently, it has been shown that the tetraspanin CD151 is involved in regulation of migration of neutrophils (17), endothelial cells (18), and various tumor cell lines (19, 20). Furthermore, it appeared that CD151 could manifest its activity only on the FAK-positive cellular background, suggesting a role for 

FAK in CD151-dependent migration (19). However, no bio- 

chemical signaling events have been ascribed to the activity of 

CD151.

In this report we investigated in detail how expression of 

CD151 affects integrin-dependent signaling in non-transformed fibroblasts. We show that CD151 specifically attenuates adhesion-dependent activation of Ras and the correspond- 
ing downstream activation of ERK1/2 and PKB-Akt. Furthermore, we establish that this attenuation arises from the marked effect of CD151 on the inactivation of Ras proteins upon cell detachment. Finally, detailed analysis of upstream signaling events indicates that CD151 affects the adhesion-de- 

pendent Ras activation via a novel mechanism.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents—Rat-1 cells and various transfect- 

ts of Rat-1 cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The Rat-1/ 

CD151wt cells were described previously (21). Rat-1/CD151ΔC and Rat-1/CD151ΔN cell lines were generated by transfecting pZeoSV/ 

CD151ΔC and pZeoSV/CD151ΔN plasmids, respectively, into Rat-1 

cells. Zeocin-resistant colonies in each transfection experiment were 

pooled together (20–30 individual colonies) and further selected in two 

cycles of cell sorting using a mixture of anti-CD151 mAbs, 5C11 (22) and 

11G1B4 (kindly provided by Dr. L. Ashman). Mouse mAbs against FAK, 

Ras, Rac1, and p120RasGAP were purchased from BD Biosciences. 

Mouse mAb to RhoA and rabbit polyclonal Ab to Cdc42 were from Santa 

Cruz Biotechnology. Rabbit anti-phospho specific Abs to FAK and rab- 

bit anti-Src polyclonal serum were from BioSource International. Rab- 

bit anti-FAK Ab was purchased from Autogen Bioclear. All antibodies to 

PKB, anti-pTyr mAb, and anti-pSrc416 were purchased from Cell Sig-

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naling Technology. Mouse anti-Src mAb, CD11, was purchased from Upstate Biotechnology. All other reagents were purchased from Sigma.

Construction of CD151 Mutants—The CD151ΔN and CD151ΔC mutants were engineered by a standard PCR protocol on the pZeoCD151 template. The N-terminal Gly2-Leu13 and the C-terminal Ser248–Tyr253 regions of human CD151 were substituted for the hemagglutinin tag sequence (YPYDVPDYA) to generate CD151ΔN and CD151ΔC fragments, respectively. The PCR fragments were subcloned (HindIII-EcoRI) into the pZeoSV plasmid.

Small G-protein Pull-down Assays—Cells (2–4 × 106) were washed twice with PBS and then scraped into RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Lysates were subsequently incubated with the appropriate GST-RBD beads for 45 min at 4 °C. After the washes with 50 mM Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl 10 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, the captured material was eluted into Laemmli buffer and resolved in 11% SDS-PAGE. The proteins were transferred to the nitrocellulose membrane and probed with the appropriate Ab. The pGEX-2TK-RhoCtk(RBD), pGEX-2TK-Pak(c/rib), and pGEX-2TK-Raf1(RBD) plasmids were kindly provided by Dr. M. Schwartz, Dr. J. Collard and Dr. R. Marais, respectively. 

Immunoprecipitation—Proteins were solubilized into 1% Triton X-100/BSA supplemented with 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin for 2–16 h at 4 °C. The insoluble material was pelleted at 12,000 rpm for 10 min, and protein lysates were precleared by incubation for 4 h at 4 °C with agarose-conjugated goat anti-mouse antibodies. Immune complexes were collected on the agarose beads prebound with mAbs, followed by four successive washes with the immunoprecipitation buffer. Immune complexes were eluted from the beads with Laemmli sample buffer, and proteins were resolved in 8–10% SDS-PAGE.

Analysis of Activation of FAK, ERK1/2, and PKB/c-Akt—Adhesion-dependent activation of FAK, ERK1/2, and PKB/c-Akt was analyzed as described previously (21).

Analysis of Activation of c-Src—Serum-starved cells plated on the laminin-5 matrix were scraped into Triton X-100-based lysis buffer, and the c-Src complexes were immunoprecipitated using CD11 mAb. Equal aliquots of the immunoprecipitated proteins were resolved in 8% SDS-PAGE, transferred to the nitrocellulose membrane, and probed either with the anti-Tyr416-Src Ab or with the polyclonal Ab recognizing total c-Src.

RESULTS AND DISCUSSION

We examined whether CD151 modifies adhesion-dependent signaling using Rat-1 fibroblasts. This model system provided two main advantages for our study. First, these cells express low levels of the endogenous CD151. Second, adhesion-dependent signaling in Rat-1 cells is well characterized (23). Rat-1 cells were transfected with human CD151 cDNA and further selected by bulk sorting to obtain a population of cells expressing the tetraspanin at a level similar to that found in some cancer cell lines (e.g. HT1080, MDA-MB-231). Initially, adhesion-dependent signaling was analyzed in cells plated on laminin-5-containing matrix (LN5M), an ECM ligand for α5β1, which is a principal integrin partner for CD151 in Rat-1/CD151 cells. The ectopic expression of CD151 in Rat-1 cells did not affect adhesion to, and spreading on, LN5M (Fig. 1, upper panels). Furthermore, the number and distribution of vinculin-containing adhesion complexes were similar in Rat-1 and Rat-1/CD151 cells (Fig. 1, lower panels). As illustrated in Fig. 2, the expression of CD151 had a negative effect on the kinetics and amplitude of adhesion-dependent activation of ERK1/2 and PKB/c-Akt (Fig. 2, A and B, lower panels, compare lanes 2–4 with 6–8). Specifically, we found that, in control Rat-1 cells, the level of ERK phosphorylation reached the plateau at 10 min (the earliest time point analyzed). By contrast it required 40 min for the pERK1/2 to reach their maximum in Rat-1/CD151 cells (Fig. 2A, lower panel, compare lanes 2–5 with 7–10). On the other hand, adhesion of Rat-1 and Rat-1/CD151 cells to LN5M induced comparable increases in tyrosine phosphorylation of Tyr(397)-FAK and Tyr(416)-c-Src (Fig. 2, A and B, top panels). The differences in the adhesion-dependent activation of ERK1/2 were observed when the cells were plated on other ECM ligands (e.g. fibronectin, laminin-10/11) (Fig. 2C, compare...
4 served between Rat-1 and Rat-1/CD151 cells (Fig. 3). Reduced upon cell adhesion, no apparent differences were observed in the detached Rat-1/CD151 cells (Fig. 3). Activation of Ras is reflective of the lower level of the GTP-Ras complex (14). Although phosphorylation of Tyr925 followed by assembly of the FAK-Grb2-Sos1 signaling complex (14). Although phosphorylation of Tyr925-FAK was induced upon cell adhesion, no apparent differences were observed between Rat-1 and Rat-1/CD151 cells (Fig. 3B, lower panel). Furthermore, we were unable to detect adhesion-induced phosphorylation of Shc above the existing baseline in both cell lines (Fig. 3B, upper panel). Therefore, it is highly unlikely that CD151-induced differences in the adhesion-dependent activation of Ras linked to signaling through Fak or Shc. Rather, we propose that CD151 affects Ras activation through a novel pathway.

We propose that the attenuation of the adhesion-dependent activation of Ras is reflective of the lower level of the GTP-Ras complexes in the detached Rat-1/CD151 cells (Fig. 3A, lanes 1 and 4). Indeed, in separate experiments we found that detachment of serum-starved Rat-1/CD151 cells induced a greater decrease in the amount of the precipitated GTP-Ras complexes than detachment of Rat-1 cells (Fig. 4A, lanes 2 and 4). The dissociation of the p120RasGAP/p62Dok complex (Fig. 4A, lanes 2 and 4). The dissociation of the p120RasGAP/p62Dok complex is thought to be a critical event in the detachment-induced inactivation of Ras proteins (16). However, while the p120RasGAP/p62Dok complex dissociated upon detachment of both Rat-1 and Rat-1/CD151 cells, no quantitative differences were observed between the cell lines (Fig. 4B, lanes 2 and 4). These results suggest that the effect of CD151 on inactivation of Ras involves an alternative mechanism(s).

To determine which region(s) of CD151 is responsible for the adhesion-dependent regulation of Ras activity, we carried out

![Fig. 3](image1.png)

**Fig. 3.** CD151 attenuates adhesion-dependent activation of Ras. A, cells were prepared for the analysis as described in the legend to Fig. 2A. GTP-loaded Ras proteins were precipitated from cellular lysates using immobilized GST-Raf1-RBD. Precipitated proteins and total lysates were resolved in the 12% SDS-PAGE and transferred to the nitrocellulose membrane. The blots were developed with the anti-Ras mAb. The graph bars represent the average fold of changes in Ras-GTP ± S.E. relative to that in detached (SUSP) Rat-1 cells (1.0 (four experiments).). B, activation of Ras in Rat-1 cells is independent of phosphorylation of FAK and Shc. Cells were prepared as described in the legend to Fig. 2B. The immunoprecipitation was carried out using rabbit anti-Shc polyclonal Ab and mouse anti-FAK mAb. Proteins were resolved in 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with either anti-pTyr mAb or anti-pTyr925-FAK polyclonal Ab.

![Fig. 4](image2.png)

**Fig. 4.** CD151 potentiates detachment-induced inactivation of Ras. The involvement of the p120RasGAP/p62Dok complex. Serum-starved cells were either left attached to the tissue culture plastic (ATTACH) or detached and kept in suspension for 60 min (DETACH). The Ras “pull-down” assay (A) and immunoprecipitation (B) were carried out as described in the legend to Fig. 3. The graph bars in A were calculated as described in the legend to Fig. 3A (five experiments).

![Fig. 5](image3.png)

**Fig. 5.** The role of cytoplasmic domains and the large extracellular loop in the Ras-inactivating function of CD151. Serum-starved cells were either left attached to the tissue culture plastic (ATTACH) or detached and kept in suspension for 60 min (DETACH). The assays and calculations (three to five experiments) were carried out as described in the legend to Fig. 3A.
N-terminal cytoplasmic portion of CD151 did not affect significantly the "Ras-inactivating function" of the tetraspanin. On the other hand, deletion of the C-terminal cytoplasmic portion completely abolished the detachment-dependent activity of CD151 toward Ras (Fig. 5B, lanes 2, 4, and 6). These data indicate that the C-terminal end is essential for the Ras-inactivating function of CD151.

Activation of Ras proteins is one of the central events in the attachment-dependent signaling mediated by integrins (12, 26). Here we demonstrate that the tetraspanin CD151 plays an important role in this process. Specifically, we identified CD151 as a new negative regulator of Ras proteins, which potentiates the decrease in the amount of the GTP-Ras complex in cells upon detachment. Consequently, adhesion-dependent accumulation of GTP-Ras and subsequent activation of ERK1/2 and PKB is attenuated in cells expressing CD151. Importantly, the effect of CD151 on the adhesion-dependent signaling is specific. Indeed, activation of FAK and c-Src and modulation of PKB is attenuated in cells expressing CD151 as a new negative regulator of Ras proteins, which may play an important role in this process.

The molecular mechanisms that link integrin disengagement with the "Ras-inactivating" function of CD151 remain to be established. CD151 is directly associated with αβ1 and αβ3 integrins through its large extracellular loop (27, 28). Furthermore, the conformation of LECL CD151 can be influenced by these interactions (29). In addition, CD151 is indirectly associated with other integrins within the tetraspanin-enriched microdomains (30). Thus, it is possible that the initial events in signal transduction involve lateral transmission of conformational changes from the disengaged integrins to CD151. Subsequently, the signal may be transmitted allosterically to the cell interior and further involve the C-terminal cytoplasmic part of CD151.

Our results indicate that detachment-induced disassembly of the RasGAP-Dok complex, the only known pathway that regulates decrease in the amount of the GTP-Ras complex in suspended cells, is not affected by CD151. This suggests that the tetraspanin operates through a novel mechanism. This may involve CD151-dependent membrane recruitment and/or compartmentalization of direct regulators of Ras proteins (e.g. RasGAP proteins of the GAPI family, Ras guanine nucleotide exchange factors (31, 32)). In this regard, palmitoylation of CD151 and its association with phosphatidylinositol 4-kinase may play an important role in this process.

Future study should establish whether function of other "pro-" or "anti-migratory" tetraspanins (e.g. CD82 and CO-029) also linked to the Ras pathway. CD151 is associated with various other tetraspanins, each of which may recruit a unique subset of regulatory proteins into integrin complexes (2, 5). Hence, by changing a tetraspanin balance in the integrin/CD151 microdomains it may be possible to amplify, negate, or even reverse the effect of CD151 in the adhesion-dependent activation of Ras.

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