CD98hc (SLC3A2) participates in fibronectin matrix assembly by mediating integrin signaling

Chloé C. Féral,1,2 Andries Zijlstra,3,4 Eugene Tkachenko,1 Gerald Prager,1 Margaret L. Gardel,5,6 Marina Slepak,1 and Mark H. Ginsberg1

1Department of Medicine, University of California, San Diego, La Jolla, CA 92093
2Institut National de la Santé et de la Recherche Médicale, U634, Faculté de Médecine, 06107 Nice Cedex 2, France
3Department of Pathology, Vanderbilt University, Nashville, TN 37232
4Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037
5Department of Physics and 6Ben May Cancer Research Institute, University of Chicago, Chicago, IL 60637

Integrin-dependent assembly of the fibronectin (Fn) matrix plays a central role in vertebrate development.

We identify CD98hc, a membrane protein, as an important component of the matrix assembly machinery both in vitro and in vivo. CD98hc was not required for biosynthesis of cellular Fn or the maintenance of the repertoire or affinity of cellular Fn binding integrins, which are important contributors to Fn assembly. Instead, CD98hc was involved in the cell’s ability to exert force on the matrix and did so by dint of its capacity to interact with integrins to support downstream signals that lead to activation of RhoA small GTPase. Thus, we identify CD98hc as a membrane protein that enables matrix assembly and establish that it functions by interacting with integrins to support RhoA-driven contractility. CD98hc expression can vary widely; our data show that these variations in CD98hc expression can control the capacity of cells to assemble an Fn matrix, a process important in development, wound healing, and tumorigenesis.

Introduction

Fibronectin (Fn) is required for mammalian development and for blood vessel formation (George et al., 1993, 1997; Hynes, 1994; Peters and Hynes, 1996; Francis et al., 2002). Fn exists in both a soluble form in the plasma and as insoluble disulfide-bonded multimers in the extracellular matrix. The insoluble matrix form of Fn is essential for most of its biological functions in events such as wound healing, embryogenesis, and formation of the tumor microenvironment (Grinnell, 1984; Mosher, 1984; Ruoslahti, 1984). The soluble form of Fn is converted to the insoluble form by a process termed Fn matrix assembly, an active cellular process in which the soluble, dimeric Fn molecules are assembled into an insoluble, fibrillar pericellular matrix. Thus, an understanding of Fn matrix assembly is of broad biological significance.

Pioneering studies (McKeown-Longo and Mosher, 1983, 1985; Schwarzbauser, 1991; Sottile and Mosher, 1993; Sechler et al., 1996, 2001) have defined the regions of Fn important in the assembly process. The N-terminal 70-kD domain of Fn plays a pivotal role in matrix assembly. It does so by interacting with several other sites within the Fn molecule. These sites, which are generally contained within the type III repeats, are cryptic in soluble Fn and exposed as a consequence of conformational changes in Fn (Wierzbicka-Patynowski and Schwarzbauser, 2003). These conformational changes may involve extension of the Fn or unfolding of particular repeats. Studies from several laboratories show that Fn conformation can be changed by mechanical deformation (Zhong et al., 1998; Krammer et al., 1999; Erickson, 1994, 2002; Ohashi et al., 2002). Indeed, the hypothesis that matrix assembly site exposure is initiated by integrin-dependent mechanical deformation of Fn (Wu et al., 1995c) has been experimentally validated (Zhong et al., 1998). Thus, the assembly of an Fn matrix results from Fn–Fn interactions initiated by mechanical deformation of the Fn molecule.

The binding of Fn to integrins, such as α5β1, initiates the matrix assembly process (Corbett and Schwarzbauser, 1999; Mao and Schwarzbauser, 2005). In particular, forces generated by the cytoskeleton and conveyed to Fn via integrins cause deformation of the Fn molecule (Zhong et al., 1998). For assembly to begin, these integrins must (1) physically associate...
with Fn, usually by binding it with high affinity (operationally defined as an "activated" integrin; Wu et al., 1995c), and (2) physically associate with the cytoskeleton (Wu et al., 1995c). Several signaling enzymes, including Src family kinases, pp125^Fak, Rho GTPases, and PI3 kinase, are also involved in the matrix assembly process (Wierzbicka-Patynowski and Schwarzbauer, 2002). These same signaling enzymes can be regulated by integrin ligation in a process referred to as outside-in integrin signaling (Boudreau and Jones, 1999; Arias-Salgado et al., 2005; Shattil, 2005). Thus, it is possible that integrin signals that regulate kinases such as pp125^Fak could participate in the assembly process.

We recently found that CD98hc, a type II transmembrane protein, mediates outside-in integrin signaling (Féral et al., 2005). CD98hc has two distinct functions: (1) it can associate with and regulate the function of selected integrins and (2) it can regulate the expression and distribution of CD98 light chains to modulate amino acid transport function. Each function depends on distinct domains within CD98hc, with the intracellular portion being required for interaction with integrins (Fenczik et al., 2001). Deletion of CD98hc in embryonic stem (ES) cells impaired the formation of teratocarcinomas in vivo and many integrin-dependent functions in vitro because CD98hc is a contributor to integrin-dependent biochemical signals (Féral et al., 2005). The role of CD98hc in integrin signaling suggested that it might participate in the Fn matrix assembly process. Here, we report that CD98hc is required for efficient Fn matrix assembly both in vitro and in vivo. Furthermore, deletion of CD98hc has little effect on Fn biosynthesis or integrin activation; instead, lack of CD98hc impairs outside-in integrin signals that result in RhoA-mediated cellular contractility necessary for Fn matrix assembly. Finally, the portion of CD98hc that interacts with integrins is necessary and sufficient to support cellular contractility and Fn matrix assembly. Thus, we find that CD98hc participates in Fn matrix assembly by mediating the outside-in integrin signals required for the contractile events that initiate and sustain Fn matrix assembly.

**Results**

**CD98hc is involved in Fn assembly in vivo**

To determine whether CD98 regulates matrix assembly in vivo, we examined the distribution of Fn in teratocarcinomas in vivo.
formed in nude mice after subcutaneous injection of ES cells. As previously reported, injection of wild-type (WT) ES cells into nude mice led to the formation of large tumors. In contrast, CD98hc-null cells either did not form tumors or formed very small tumors (Féral et al., 2005). The CD98hc-null tumors that formed exhibited profound reduction in Fn fibrils, as judged by staining with a mAb against Fn (Fig. 1 A). In contrast, a prominent network of Fn was detected in the tumors formed by WT ES cells. Thus, CD98hc is involved in Fn matrix formation in vivo. Importantly, these CD98hc-null teratocarcinomas, like those formed from WT ES cells contain tissues from all three germ layers. Furthermore, absence of CD98hc is compatible with differentiation of multiple cellular lineages (Féral et al., 2005); hence, changes in cell lineages in the tumors are unlikely to account for the observed defect in matrix assembly. Thus, these results strongly suggest that lack of CD98hc resulted in a marked reduction in Fn matrix formation in vivo.

Because Fn is required for vascular development, we examined the blood vessels formed in the teratocarcinomas (Fig. 1 B) by whole-mount staining with endothelial cell–specific fluorescent-labeled lectin. We also visualized pericytes/smooth muscle cells and endothelial cells on these tumors by detection of the specific markers α-smooth muscle actin and platelet/endothelial cell adhesion molecule 1 (PECAM-1), respectively (Fig. 1 C). In the CD98hc-null tumors, endothelial cells were present but failed to form intact blood vessels (Fig. 1 B) or to invest with α-smooth muscle actin–expressing cells (Fig. 1 C). Instead of associating with endothelial cells, α-smooth muscle actin–expressing cells were dispersed throughout the tumors. In sharp contrast, WT tumors exhibited an organized network of blood vessels, including larger vessels with an endothelial lining and smooth muscle cell–containing intima and media (Fig. 1, B and C). Thus, the absence of CD98hc impairs Fn matrix deposition and vascular development, a process known to depend on Fn.

Lack of CD98hc impairs Fn matrix assembly in vitro

To learn whether CD98hc deficiency by itself leads directly to defective cellular Fn matrix formation and to understand the mechanisms by which CD98hc supports Fn matrix formation, we developed conditional CD98hc-null mouse embryonic fibroblasts (MEFs; Fig. 2, A and B). CD98hc conditional–null mice were generated as described in Materials and methods. We deleted CD98hc in vitro by infecting these cells with adenoviruses expressing Cre recombinase, resulting in 95% excision of exons 1 and 2 of CD98hc (Fig. 2 B). These cells were then mass sorted with immunomagnetic beads to select fibroblasts lacking detectable CD98hc (Fig. 2 B). Importantly, the CD98hc-null fibroblasts and parental WT cells expressed similar quantities of integrins α5, β1, α6, and αv, as judged by flow cytometry (Fig. 2 C) and showed defects in integrin signaling similar to those seen in CD98hc-null fibroblasts derived from ES cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200705090/DC1). None of the cells expressed...
a detectable amount of $\alpha_1$, $\alpha_2$, $\alpha_4$, or $\beta_3$ integrins. Thus, these conditional CD98hc MEFs provide a tool to analyze the effect of CD98hc deletion on Fn matrix formation and integrin signaling by fibroblasts.

To examine Fn matrix assembly, we cultured WT or CD98hc-null fibroblasts and assessed matrix deposition by immunofluorescence. 48 h after plating, WT cells exhibited abundant Fn fibrils, whereas CD98hc-null cells (Fig. 3 A, green) were devoid of Fn fibrils (Fig. 3 A). Similar differences were observed regardless of whether the cells were subconfluent (Fig. 3 A) or confluent (not depicted). The lack of matrix was confirmed biochemically, by assaying the formation of deoxycholate (DOC)-insoluble Fn (Fig. 3 B), which was dramatically reduced in the confluent CD98hc-null fibroblasts. Because reduced assembly was observed in confluent CD98hc-null cells, the lack of matrix in these cells cannot be ascribed to reduced cell density (Wierzbicka-Patynowski and Schwarzbauer, 2003). Cell-associated Fn is present in fibroblast cultures in two separate pools, distinguishable on the basis of their solubility in DOC. One pool contains cell-bound DOC-soluble Fn, whereas the other one contains matrix-associated DOC-insoluble Fn. Fn matrix assembly proceeds by the binding of Fn to cells in the DOC-soluble pool with subsequent transfer and accumulation of assembled Fn in the DOC-insoluble pool (McKeown-Longo and Mosher, 1983). Cell-associated Fn is present in fibroblast cultures in two separate pools, distinguishable on the basis of their solubility in DOC. One pool contains cell-bound DOC-soluble Fn, whereas the other one contains matrix-associated DOC-insoluble Fn. Fn matrix assembly proceeds by the binding of Fn to cells in the DOC-soluble pool with subsequent transfer and accumulation of assembled Fn in the DOC-insoluble pool (McKeown-Longo and Mosher, 1983). Although there was at least a 20-fold reduction in the DOC-insoluble Fn with the CD98hc-null cells (Fig. 3 B, left), there was only a modest, approximately twofold reduction in the DOC-soluble cell-bound Fn (Fig. 3 B, middle) and no decrease Fn in the conditioned medium (Fig. 3 B, right) compared with WT cells. Thus, CD98hc deletion leads to defective Fn matrix assembly, and the defect appears to be at the step of transfer from the DOC-soluble to -insoluble pool.

CD98hc-null cells' lack of Fn matrix
is not due to reduced Fn biosynthesis or integrin affinity

Because CD98hc participates in amino acid transport (Deves and Boyd, 2000), we considered the possibility that CD98hc deficiency reduced the biosynthesis of Fn. To assess the availability of endogenous soluble Fn, we cultured the cells in medium containing Fn-depleted FBS (Fig. 4 A). Roughly twofold greater quantities of secreted soluble Fn were detected in the conditioned medium of CD98hc-null cells cultured in Fn-depleted medium (Fig. 4 A, left), and a profound reduction in DOC-insoluble Fn matrix was evident (Fig. 4 A, middle). This result suggests that both cell types produce similar quantities of Fn, but CD98hc-deficient cells cannot assemble the Fn, resulting in the increase in the conditioned medium. Furthermore, when we supplemented the cell cultures with an excess of plasma Fn, the CD98hc-null cells still failed to assemble a matrix (Fig. 4 B). Thus, CD98hc is required for Fn matrix assembly, but not for Fn synthesis or secretion, and the lack of assembly in CD98hc-null cells is not due to reduced Fn availability.

Matrix assembly requires that Fn first binds to high affinity (operationally defined as activated) integrins, such as $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, or $\alpha\beta_3$ (Wu et al., 1995a,b; Sechler et al., 1997, 2000). To assess the effect of CD98hc deletion on the affinity of Fn binding integrins in these MEFs, we examined their binding to a recombinant-soluble cell binding domain of Fn (McKeown-Longo and Mosher, 1983). Although there was at least a 20-fold reduction in the DOC-insoluble Fn with the CD98hc-null cells (Fig. 3 B, left), there was only a modest, approximately twofold reduction in the DOC-soluble cell-bound Fn (Fig. 3 B, middle) and no decrease Fn in the conditioned medium (Fig. 3 B, right) compared with WT cells. Thus, CD98hc deletion leads to defective Fn matrix assembly, and the defect appears to be at the step of transfer from the DOC-soluble to -insoluble pool.

To assess the effect of CD98hc deletion on the affinity of Fn binding integrins in these MEFs, we examined their binding to a recombinant-soluble cell binding domain of Fn, composed of type 3 repeats 9–11 (3Fn[9–11]). The absence of CD98hc had no effect on the ability of the cells to bind to 3Fn(F9–11), a direct measure of the affinity of Fn binding integrins, such as integrin $\alpha_5\beta_1$ (Chou et al., 2003). Importantly, when incubated with an activating anti-β1 integrin antibody, 9EG7, there was a marked increase in 3Fn(9–11) binding to both the WT and the null cells (Fig. 4 C); however, this enforced activation of...
integrins did not rescue Fn assembly by the CD98hc-deficient cells (Fig. 4 D). Thus, the CD98hc-null cells’ failure to assemble matrix is not due to a defect in their ability to synthesize or secrete Fn or to bind to soluble Fn with high affinity.

**CD98hc mediates RhoA-driven traction forces on the extracellular matrix**

Fn must be conformationally altered to initiate assembly into fibrils (Wierzbicka-Patynowski and Schwarzbauer, 2003). Once Fn binds to integrins, and the integrins connect to the cytoskeleton, RhoA-mediated cellular contractility exerts force on the Fn, leading to these conformational changes (Zhang et al., 1994; Zhong et al., 1998). We therefore examined the role of CD98hc in cellular contractility by assaying the contraction of an Fn–fibrin provisional matrix. Fibrin clots formed from blood plasma contain covalently cross-linked Fn that serves as a ligand for integrin α5β1–dependent matrix contraction (Corbett and Schwarzbauer, 1999). Incorporation of WT MEFs in these matrices led to a 52 ± 0.8% contraction after 2 h (Fig. 5, A and B). In contrast, contraction was markedly reduced when CD98hc-null cells were incorporated in the clot (24 ± 0.55% clot contraction). These cells lack β3 integrins, suggesting that the contraction was mediated by Fn binding β1 integrins, such as α5β1. Indeed, clot retraction was strongly inhibited by a function-blocking anti–mouse β1 antibody (clone Ha2/5; 18 ± 0.2 vs. 52 ± 0.8% clot contraction; unpublished data). Thus, CD98hc mediates the β1 integrin–dependent contraction of an Fn–fibrin matrix.

The finding that CD98hc was required for efficient clot contraction suggests that it mediates the generation of traction force on the extracellular matrix, a critical requirement for Fn assembly (Zhong et al., 1998). To examine this question, we used displacement of embedded fluorescent microbeads in flexible polyacrylamide substrates as a direct measure of traction forces (Beningo and Wang, 2002). Subconfluent MEFs were seeded for 2 h on Fn-coated polyacrylamide substrates in serum-free medium. Deformation of substrate caused by cellular traction forces was detected by tracing the displacement of fluorescent beads, and displacement vector maps, or strain maps, were generated (Fig. 5 C, green). The resulting strain maps demonstrated a marked reduction in traction forces exerted by CD98hc-deficient MEFs compared with WT cells (Fig. 5 C).
Figure 5. **CD98hc mediates cellular traction forces on the extracellular matrix.** (A) WT and CD98hc-deficient MEFs were mixed with 200 μl of Fn-containing platelet-poor plasma, 200 μl of 28 mM CaCl₂, and 5 U/ml human thrombin in Hepes-DME. Tubes were incubated for 2 h at 37°C. Depicted are digital images of WT and CD98hc-null clots. (B) Quantification of the percentage of clot contraction is presented [see Materials and methods]. Values represent the mean ± SEM of triplicate determinations. The assay was repeated three times with similar results. (C) Decreased cellular traction forces in CD98-deficient MEFs. WT and CD98hc-deficient MEFs were plated on a 120-kD fragment of Fn-coated polyacrylamide sheets, in which fluorescent beads were embedded, as described in Materials and methods. Strain maps (green) overlaid with the brightfield images of WT (top) and CD98hc-null (bottom) cells plated on 120-kD Fn-coated polyacrylamide substrate (gray). CD98hc-deficient cells demonstrate reduced traction forces compared with WT cells. Bars, 10 μm.

There was about a threefold reduction in integrated strain for CD98hc-null cells compared with WT (0.77 vs. 2.5 pixels/area [arbitrary units], respectively; see Materials and methods). Thus, CD98hc-null cells are defective in the capacity to exert traction forces on the underlying substrate.

The small GTP binding protein RhoA is a major regulator of cell contractility (Chrzanowska-Wodnicka and Burridge, 1996; Hall, 1998) that provides the traction forces to initiate Fn matrix assembly (Zhong et al., 1998). Matrix assembly within tissues occurs when cells are in contact with surrounding 3D extracellular matrix, leading us to examine the effect of CD98hc deletion on adhesion-mediated alterations in RhoA activity when cells interact with 3D matrix (Cukierman et al., 2001). 30 min after adhesion of WT MEFs to a 3D matrix, RhoA activity decreased, followed by a secondary increase as previously described (Ren et al., 1999). In sharp contrast, the CD98hc-null cells did not show this late increase in RhoA activity (Fig. 6 A). Strikingly, both CD98hc-null and WT MEFs exhibited similar increases in RhoA activity in response to lysophosphatidic acid (LPA), an agonist that activates RhoA via G protein–coupled receptors (van Corven et al., 1999). In sharp contrast, the CD98hc-null cells did not show this late increase in RhoA activity (Fig. 6 A). Strikingly, both CD98hc-null and WT MEFs exhibited similar increases in RhoA activity in response to lysophosphatidic acid (LPA), an agonist that activates RhoA via G protein–coupled receptors (van Corven et al., 1999). Thus, CD98hc is required for RhoA activation in response to LPA, suggesting that matrix-driven RhoA activation is important in the assembly process. Integrins are the principal receptors that lead to matrix-initiated biochemical signals (Ingber, 1991; LaFlamme and Auer, 1996), and we previously found that the physical interaction of CD98hc with integrins is required for efficient integrin signaling (Féral et al., 2005). To examine the role of CD98hc–integrin interaction in matrix assembly, we reconstituted CD98hc-null MEFs with retroviruses encoding chimeras (Féral et al., 2005) formed between CD98hc and another type II transmembrane protein, CD69 (Fig. 7); each was well expressed as judged by flow cytometry (Fig. 7 B). The C98T98E69 (cytoplasmic domain CD98hc, transmembrane domain CD98hc, and extracellular domain CD69) chimera interacts with integrins, whereas C98T98E69 and C98T98E69 do not associate with integrins. Only the integrin binding chimera (C98T98E69) rescued the defect in Fn matrix assembly (Fig. 7 A) and contractility (Fig. 7 C). CD98hc-deficient cells expressing the chimera that binds to integrins (C98T98E69) were able to induce a clot contraction (56 ± 1%) even more efficiently than WT cells (37 ± 0.8%). Thus, the capacity of
CD98hc to mediate contraction of an Fn-fibrin clot depends on its ability to interact with integrins.

Discussion

Fn matrix assembly can be conveniently divided into three steps. First, Fn binding integrins must be activated to bind Fn with high affinity (Wu et al., 1995c; Sechler et al., 1997). Recent studies (Tadokoro et al., 2003; Kuo et al., 2006) establish that integrin activation is mediated by talin binding to the integrin β cytoplasmic domain. Second, the integrins must connect to the actin cytoskeleton (Ali et al., 1977; Mautner and Hynes, 1977; Wu et al., 1995c), a connection that can be mediated by talin (Brown et al., 2002; Jiang et al., 2003). Third, cellular contractility exerts force on the integrin-bound Fn, deforming it and leading to the initiation of Fn matrix assembly (Zhang et al., 1994; Zhong et al., 1998). Here, we show that CD98hc participates in Fn matrix assembly and that it does so by physically associating with integrins to mediate matrix-driven activation of RhoA and the resulting cellular contractility that exerts force on the matrix.

The variable expression of CD98hc and its contribution to Fn assembly have implications for the relationship between cell proliferation and formation of the extracellular matrix. CD98hc expression is tightly regulated coordinately with cell proliferation (Diaz et al., 1997), a pivotal event in wound healing, tumorigenesis, and development. Repair of skin wounds depends on precisely controlled formation of a fibrous extracellular matrix. The extracellular matrix deposition correlates with proliferation of fibroblasts and endothelial cells within the damaged area (Midwood et al., 2004). The dramatic up-regulation of CD98hc in proliferating cells could thus serve to promote Fn matrix assembly in healing wounds; conversely, a failure to down-regulate CD98hc could lead to excess matrix deposition and cell proliferation, as seen in fibrosis and keloid scars (Muir, 1990). Similarly, the marked up-regulation of CD98hc in tumors (Deves and Boyd, 2000) could contribute to enhanced formation of Fn extracellular matrix, thus altering the microenvironment of cells during tumorigenesis (Weaver et al., 1996; Lelievre et al., 1998; Schmeichel et al., 1998; Bissell et al., 2002; Weaver and Gilbert, 2004). Moreover, we show that CD98hc mediates cell contractility, an event strongly associated with progression of some tumors (Paszek et al., 2005). Finally, the deposition of Fn along the blastocoele roof is reported to be a critical step in gastrulation (Boucaut et al., 1985) and later for morphogenetic movements driven by convergent extension (Davidson et al., 2006). This spatiotemporally regulated matrix assembly is controlled by the activity of cellular integrins, as manifested by their ability to promote cell spreading (Ramos and DeSimone, 1996; Ramos et al., 1996), a CD98hc-dependent process (Féral et al., 2005).

Thus, the importance of Fn matrix assembly in development and the role of CD98hc in matrix assembly shown here provide a cogent explanation for the early embryonic lethality of CD98hc gene disruption (Tsumura et al., 2003) and suggest that changes in CD98hc expression, or its association with integrins, may influence a wide range of developmental processes through the regulation of Fn matrix assembly.

Materials and methods

Fn staining on tumor sections

A suspension of ES cells [1.5 × 10⁶ cells per site] was injected subcutaneously into athymic BALB/c nudi nude mice. After 33 d, tumors were fixed in 10% formaldehyde, paraffin embedded, sectioned, and stained for Fn with a rabbit polyclonal antibody against human Fn (Sigma-Aldrich). Alexa Fluor 546

Figure 6. CD98hc mediates adhesion-induced RhoA activation and matrix contraction to enable Fn matrix assembly. (A) RhoA activity was measured in an ELISA-based Rho assay in WT and CD98hc-deficient cells after plating on a 3D Fn matrix. The error bars represent SEM. The assay was repeated three times with similar results. Samples were also resolved by SDS-PAGE and immunoblotted with anti-RhoA antibody (total RhoA) to confirm that both WT and CD98hc-null MEFs express similar amounts of total RhoA (not depicted). (B) Activation of RhoA by LPA in WT and CD98hc-null MEFs. Adherent serum-starved CD98hc-null cells were treated with 1 μg/ml LPA or buffer, and RhoA activity was measured after 5 min. Values represent the mean and range of duplicate determinations. The assay was repeated twice with similar results. (C) Activation of RhoA bypasses the defect in matrix contraction in CD98hc-null cells. Clot contraction was measured 1 h after WT, CD98hc-null, and CD98hc-null MEFs were stimulated with LPA [see Materials and methods for details]. Values represent the mean ± SEM of triplicate determinations. Depicted is one of two such experiments with identical results. (D) Activation of RhoA bypasses the defect in Fn matrix assembly in CD98hc-null cells. DOC-insoluble Fn produced by WT, CD98hc-null, and CD98hc-null MEFs treated with LPA was evaluated biochemically as described in Fig. 3. CD98hc-null MEFs stimulated with LPA were able to assemble Fn into fibrils as efficiently as WT cells. Depicted are the means of triplicate measurements. The assay was repeated two times with similar results.

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Figure 7. CD98hc-integrin interaction mediates Fn assembly. (A) CD98hc-deficient MEFs reconstituted with each chimera depicted in B (C98T69E98, C69T98E98, or C98T98E69) were treated as described in Fig. 3A. Fixed cells were stained for Fn (green), actin (red), and nucleus (blue). Only C98T98E69 rescued CD98hc-deficient cells’ ability to assemble Fn fibrils. Bars, 25 μm. (B) Expression and schematic of the chimeras used in A. CD98hc protein is depicted in black and CD69 in gray. Each chimera is defined by its cytoplasmic (C), transmembrane (T), or extracellular (E) domain derived from either CD98hc (98) or CD69 (69). CD98hc extracellular domain is necessary and sufficient for amino acid transport, whereas the intracellular and transmembrane domains are required for interactions with integrins (Fenczik et al., 2001). Flow cytometry analysis (right) of cell surface expression of exogenous chimeras in CD98hc-deficient cells (filled histogram) is shown. Control staining (empty histogram) was performed with irrelevant IgG. (C) CD98–integrin association is required for contraction of the extracellular matrix. WT, CD98hc-deficient, and CD98hc-deficient MEFs reconstituted with C98T98E69 were mixed with 200 μl of Fn-containing platelet-poor plasma, 200 μl of 28 mM CaCl₂, and 5 U/ml human thrombin in Hepes-DME. Tubes were incubated for 1 h at 37°C. Depicted are digital pictures of clots, as well as the calculated percentage of clot contraction (see Materials and methods). Values represent the mean ± SEM of triplicate determinations.
were approved by the University of California, San Diego, Institutional Animal Care and Use Committee.

**Cell culture**

MEFs were derived from CD98hc-conditioned knockout homozygote embryos. MEFs were cultured in complete DMEM high glucose (Invitrogen), supplemented with 10% FBS (HyClone), 20 mM Hepes, pH 7.3 (Invitrogen), 0.1 mM nonessential amino acid (Invitrogen), 0.1 mM b-mercaptoethanol (Invitrogen), and 2 mM L-Glutamine (Invitrogen). The CD98hc-null MEFs were generated by infecting CD98hc-conditional knockout MEFs with adenovirus encoding CRE recombinase. Viral titers ranged from 0.6 to 1.2 × 10^{11} U/ml. CD98hc deletion was detected by PCR (forward primer A, see the previous section; and reverse primer C, 5'-CAGGGTGTTCTGTGATGTTGGCGTG-3') and confirmed by flow cytometry. Cells were stimulated with 2 μg/ml (unless otherwise mentioned) LPA (1-oleyl-2-hydroxy-sn-glycero-3-phosphate, monosodium salt) as described elsewhere (Zhang et al., 1998).

In brief, MEFs were plated at 1 million per 10-cm Petri dish and treated every 4–5 h for 18 h (starting 24 h after plating). The reconstituted cells were generated by infecting CD98hc-null MEFs with pBabe-PuroMyclin retrovirus encoding or CD98hc/CD69 chimera (Fenczik et al., 2001). Viruses were generated in EcoPack 293 cells (CLONTECH Laboratories, Inc.), and viral titers ranged from 0.9 to 1.7 × 10^{10} U/ml. After puromycin selection, chimera expression was confirmed by flow cytometry.

**Visualizing/imaging Fn assembly**

MEFs were seeded in 1% FBS at 10,000 cells/well in 24-well plates onto 12-mm coverslips coated with 4 μg/ml Fn and blocked with 0.5% BSA. When indicated, 25 μg/ml of exogenous plasma Fn or 10 μg/ml of activating anti-β1 mAb, 9EG7, was added to the culture medium. After 48 h in culture, the cells were fixed with 2% PFA in PBS for 20 min and permeabilized for 10 min with 0.05% Triton X-100. Fixed cells were washed with PBS, and nonspecific sites were blocked with 3% BSA before the addition of primary antibody (rabbit anti-Fn [Sigma-Aldrich]; 1:2,000) for 2 h at 37°C. Specific binding was detected using goat anti-rabbit antibody conjugated with Alexa 488 (Invitrogen). The actin cytoskeleton was visualized by incubating with rhodamine-conjugated phalloidin while the nuclei were stained with DAPI (Invitrogen). Individual coverslips were mounted in aqueous mounting media with anti-fade (Gel Mount; Sigma-Aldrich) and visualized by incubating with rhodamine-conjugated phalloidin while the nuclei were stained with DAPI (Invitrogen). Individual coverslips were mounted in aqueous mounting media with anti-fade (Gel Mount; Sigma-Aldrich). Digital images were captured at room temperature with a charge-coupled device camera (Orcar; Hamamatsu) using a standard upright fluorescence microscope (Axioskop 2 [Carl Zeiss Microimaging, Inc.]; Plan-Neofluar 20× objective with 0.50 NA) controlled by the image capture and processing program Openlab (Improvement). Photoshop (Adobe) was used to increase the γ of images.

**Biocomoactional assessment of Fn assembly**

To assess the Fn assembly biochemically, the DOC-soluble and insoluble portions of the cell matrix were analyzed as described previously (Zijlstra et al., 1999). In brief, MEFs were plated at 3 million cells per 10-cm Petri dish in complete medium (Fn+) or Fn-depleted medium (Fn−). Fn-depleted medium was generated by depleting Fn from the FBS using gelatin Sephadex. MEFs were derived from CD98hc-conditional knockout homozygote embryos (SLC3A2) AND FIBRONECTIN MATRIX • FÉRAL ET AL. 709

**Flow cytometry**

Anti- mouse α1c (clone Ha 31/8), anti- mouse α2c (clone HMw2), anti-mouse α5 (PS/2), anti-mouse α6c (clone HMw5-1), anti-mouse α6c (clone GoH3), anti- mouse α1c (clone RMW7), anti-mouse β1 (clone 9EG7) integrin, and anti-mouse CD98 (clone H202-141) were purchased from BD Biosciences and used at the recommended concentrations. Goat-anti-rat IgG and goat-anti-conjugated anti-hamster IgG were obtained from Biotech International and were used as secondary antibodies for α1c, α2c, α5, and β1; murine CD98; and α1, α2, and α6 detection, respectively. Fn 9–11 binding was assayed by two-color flow cytometry as previously described (Hughes et al., 2002) in the presence or absence of 10 μg/ml of activating anti-β1 mAb 9EG7.

**Fibrin clot retraction assay**

This assay was performed by modification of a published method (Ylanne et al., 1993). In brief, WT or CD98hc-deficient MEFs were harvested with trypsin-EDTA (Invitrogen), quenched with complete medium, washed twice with PBS one time, and resuspended at 8.5 million cells per milliliter in serum-free DME (Invitrogen) buffered with 25 mM Heps. 350 μl of this cell suspension was added to 7 × 45 mm siliconized glass cuvettes (Sienko, Inc.). Then, 200 μl of human platelet-poor plasma anticoagulated with ACD (85 mM sodium citrate, 65 mM citric acid, and 104 mM glucose) was added, followed by 200 μl Heps-buffereD MME containing 28 mM CaCl₂ and 5 U/ml human thrombin (Sigma-Aldrich). Cuvettes were incubated at 37°C with 5% CO₂. Images were acquired with a digital camera at 1 or 2 h, and subsequently the 2D area of the clot was measured using ImageJ software. The percentage of clot contraction was calculated according to the following equation: percentage of clot contraction (t = 1 h) = 100 [area at t = 1 h/area at t = 0] × 100.

**Cellular tension measurements**

Polyacrylamide gels with embedded fluorescent beads on coverslips were prepared using previously described protocol (Wang and Pelham, 1998) with some modifications (see the supplemental text, available at http://www.jcb.org/cgi/content/full/jcb.200705090/DC1). Cell culturing, image acquisition, and strain map construction were performed as follows. WT and CD98hc-deficient MEFs were harvested with trypsin-EDTA (Invitrogen), quenched with complete medium, washed twice with 1× PBS, and resuspended at 0.1 million cells per milliliter in serum-free DME (Invitrogen) buffered with 25 mM Heps. Cells were kept in suspension for 1 h at room temperature, and 20,000 cells were plated on a polyacrylamide sheet. After culturing on a polyacrylamide sheet for 2 h, images of both cell types were acquired within 10 min from each other. A microscope (TE2000, Nikon) equipped with environmentally controlled enclosure (37°C and 5% CO₂), 60× 1.2 NA water objective (Olympus), motorized stage (Ludl), e-excitation filter wheel (Ludl), filter set (Semrock), and camera (CoolSnap HQ; Roper Scientific) was used to acquire images of fluorescent beads embedded in polyacrylamide and brightfield images of cells in six different fields. After addition of 200 μl RIPA buffer to 2 ml of media, cells detached and new images of the same fields were taken to collect reference data on beads' location in nonstressed gel. Images of fluorescent beads were analyzed using image analysis software written in Matlab (courtesy of G. Danuser's and G. Waterman-Storer's groups, The Scripps Research Institute). Images were divided into 1-μm² areas, and displacement vectors between were calculated (Ji and Danuser, 2005). Vectors (Fig. 5 C, green) on the strain map were increased five times for visualization purposes. Brightfield and strain map images were superimposed using Photoshop (Adobe). Integrated strains were calculated by summing up the magnitudes (WT, 7,400 pixels; and CD99hc null, 1,444 pixels) of the strain vectors inside the cell mask and dividing them by the cell area (WT, 2,945 arbitrary units; and CD98hc null, 1,873 arbitrary units).

**Rho GTPase assay**

WT and CD98hc-null MEFs were grown in 0.5% serum for 24 h and then in serum-free medium for another 17 h. Cells were detached and kept in suspension at room temperature for 1 h in serum-free medium. Serum-starved cells were then plated on 3D Fn plates (3 million cells/plate), and RhoA activity was assayed at the indicated time points. For IPA treatment, cells were grown and serum starved as described, 1 μg/ml IPA was added to each plate, and RhoA activity was measured after a 5-min incubation. 3D Fn matrix was prepared as described previously (Cukierman et al., 2001) using NIH3T3 cells cultured in a 10-cm Petri dish. RhoA activity was measured using a commercially available EUSA-based assay (G-LISA; Cytoskeleton, Inc.) according to the manufacturer's protocol. Lysates were also resolved by SDS-PAGE and immunoblotted with rabbit anti-RhoA (67B9) antibody (Cell Signaling), followed by a IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences), and bands were visualized using blots imaged using an infrared imaging system (Odyssey; LI-COR Biosciences).

**Online supplemental material**

Fig. S1 shows that CD98hc contributes to the integrin-dependent activation of FAK and p130^Cas via its integrin binding domain by reconstitution.


Supplemental materials
Feral et al., http://www.jcb.org/cgi/content/full/jcb.200705090/DC1

Supplemental materials and methods

Cellular tension measurements
Polyacrylamide gels (2.8 kPa stiffness) with embedded fluorescent beads on coverslips were prepared using previously described protocol [Wang and Pelham, 1998] with some modifications. First, 22-mm #1 square coverslips (Fisher Scientific) were activated by incubation in 20% 3-aminopropyltrimethoxysilane (Sigma-Aldrich) for 5 min. Coverslips were rinsed in water, spin-dried, and incubated in 0.5% glutaraldehyde for 30 min. Second, polyacrylamide sheets were prepared by mixing 0.5 ml of solution containing 7.5% acrylamide (Bio-Rad Laboratories), 0.1% bis-acrylamide (Bio-Rad Laboratories), and 10 μl of fluorescently labeled 0.1-μm beads (Invitrogen). This solution was degassed for 20 min. Then, 0.75 μl of TEMED (BioRad) and 1.25 μl of 20% ammonium persulfate (Sigma-Aldrich) were added to the degassed mixture. 30-μl drops were pipetted to glass slides. Activated coverslips were placed on the drop and let polymerize for 20 min. Coverslips with gel sheet were detached from slides using blade and store in PBS, pH 7.4, for up to 2 wk.

Coating of polyacrylamide sheets with Fn was performed by chemical linking. To do so, 1 mg of Sulfo-SANPAH (Pierce Chemical Co.) was dissolved first in 40 μl of DMSO and then in 1 ml of water. 30 μl of Sulfo-SANPAH was pipetted to polyacrylamide sheets and exposed to 365 nm wavelength illumination in Stratalinker 1800 (Stratagene) for 2 min. Coverslips were then rinsed in PBS, pH 7.4, and placed on a 30 μl drop containing 1 mg/ml of a 120-kD fragment of Fn that contains the cell binding domain (Ginsberg et al., 1985). After incubation for 30 min, the coverslips were rinsed in PBS and blocked in 1% BSA/PBS, pH 7.4, for 1 h at room temperature.

Assay of adhesion-dependent tyrosine phosphorylation
After overnight serum starvation, cells were detached and kept in suspension in serum-free DME containing 0.2% BSA for 1 h. Cells were then plated on dishes coated with Fn. To assess phosphorylation of p130Cas and FAK, cells were lysed in modified radioimmuno-precipitation assay (RIPA) buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM NaF, and protease inhibitor mixture (Roche)], and p130Cas was immunoprecipitated. Immunoprecipitates were separated on SDS-PAGE gel and resolved with anti-phosphotyrosine antibody (PY20; Biosource International) to detect tyrosine phosphorylation. For FAK, lysates were run on SDS-PAGE and immunoblotted with anti–phospho-FAK (pY576) antibodies (Biosource International).

References

Figure S1. CD98hc contributes to the integrin-dependent activation of FAK and p130Cas via its integrin binding domain. (A) Model of chimeras of CD98hc and another type II transmembrane protein (CD69), and their interactions with amino acid transporters (a.a. Transport) or with integrins (Integrin Function). CD98hc protein is depicted in black and CD69 in grey. Each chimera is defined by its cytoplasmic-transmembrane-extracellular domain derived from either CD98hc (98) or CD69 (69). CD98hc extracellular domain is necessary and sufficient for amino acid transport, whereas the intracellular and transmembrane domains are required for interactions with integrins (Fenczik et al., 2001). (B) Quantification of adhesion-dependent activation of FAK. WT, CD98hc-deﬁcient MEFs, and CD98hc-null MEFs reconstituted with each chimera were permitted to adhere to Fn. Cells were lysed, and lysates were resolved by SDS-PAGE and immunoblotted with anti-FAK (pY576) to detect FAK phosphorylation or anti-FAK (total FAK). (C) Quantification of adhesion-dependent activation of p130Cas. WT, CD98hc-null, and CD98hc-null cells reconstituted with each of the chimeras were permitted to adhere to Fn. Cells were lysed, p130Cas was immunoprecipitated from the lysates. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with PY20 to detect phosphotyrosine or anti p130Cas [total p130Cas].