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CD98hc (SLC3A2) mediates integrin signaling

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Integrins regulate cellular behaviors through signaling pathways, including Rho GTPases and kinases. CD98 heterodimers, comprised of a heavy chain (CD98hc, SLC3A2) and one of several light chains, interact with integrins through CD98hc. CD98hc overexpression leads to anchorage-independent cell growth and tumorigenesis in 3T3 fibroblasts and activates certain integrin-regulated signaling pathways. To establish the biological function of CD98hc, we disrupted the gene and analyzed CD98hc-null cells. Here we report that CD98hc contributes to integrin-dependent cell spreading, cell migration, and protection from apoptosis. Furthermore, CD98hc is required for efficient adhesion-induced activation of Akt and Rac GTPase, major contributors to the integrin-dependent signals involved in cell survival and cell migration. CD98 promotes amino acid transport through its light chains; however, a CD98hc mutant that interacts with β1 integrins, but not CD98 light chains, restored integrin-dependent signaling and protection from apoptosis. β1 integrins are involved in the pathogenesis of certain cancers. CD98hc deletion markedly impaired the ability of embryonic stem cells to form teratocarcinomas in mice; teratocarcinoma formation was reconstituted by reexpression of CD98hc or of the mutant that interacts exclusively with integrins. Thus, CD98hc is an integrin-associated protein that mediates integrin-dependent signals, which promote tumorigenesis.

amino acid transport | signal transduction | cell adhesion | apoptosis | cancer

The β1 integrins regulate numerous cellular functions by signaling through a variety of biochemical pathways (1, 2). CD98 (4F2 antigen) is expressed on a family of heterodimers composed of a common type II transmembrane heavy chain (CD98hc, 4F2hc, SLC3A2) and one of several light chains (3). CD98hc is highly expressed on proliferating lymphocytes and on other rapidly growing cells (3). Overexpression of CD98hc transforms NIH 3T3 cells (4). Furthermore, previous studies suggested two distinct functions of CD98hc: (*i*) It can associate with and regulate the function of selected integrins (5–8), and (*ii*) it can regulate the expression and distribution of the light chains to modulate amino acid transport function (3). These two functions depend on distinct domains within CD98hc: the extracellular domain is required for its interaction with light chains, and the transmembrane and cytoplasmic domains are required for interaction with integrins (9). Finally, overexpression of the portion of CD98hc that interacts with integrin β1A subunit leads to anchorage and serum-independent growth of CHO cells *in vitro* (10). To definitively address the role of CD98hc in integrin function and tumorigenesis, we disrupted the CD98hc gene. Here we report that CD98hc-null cells are markedly defective in integrin-dependent cell spreading and cell migration. Furthermore, these cells manifest increased sensitivity to anchorage deprivation-induced apoptosis (anoikis) (11). A requirement for CD98hc in adhesion-induced activation of Akt and Rac GTPase accounted for the defects in cell survival (12) and cell migration (13). Furthermore, a CD98hc mutant that interacts with β1 integrins, but not CD98 light chains, rescued integrin-dependent signaling and protection from apoptosis. Finally, embryonic stem (ES) cells null for CD98hc lost their tumorigenic potential *in vivo*. Formation of teratocarcinomas in mice was rescued by reexpression of CD98hc or of the mutant that interacts only with integrins. These data establish an important role for CD98hc in integrin-dependent signals that contribute to tumorigenesis.

Methods

Generation of CD98hc$^{-/-}$ ES Cells. A P1 mouse ES cell clone containing the CD98hc gene was isolated from a 129Sv/J mouse library by PCR screening (Genome Systems, St. Louis). The targeting vector, pKO 4F2, consisted of a 1.6-kb 5′ homologous region, a 5-kb 3′ homologous region (Fig. 6*A*, which is published as supporting information on the PNAS web site), and in the region of exon 1, encoding the transmembrane domain of CD98hc, was replaced with a neomycin selection cassette. The linearized targeting construct was electroporated into R1 ES cells. G418- and ganciclovir-resistant colonies were selected for 7 days and screened by Southern blotting. To create double knockout ES cells, clones carrying a single null allele were retargeted with a construct encoding puromycin resistance. Two homozygous CD98hc-deficient clones were obtained (Fig. 6*B*); both lacked CD98hc protein as determined by flow cytometry with anti-CD98 (clone H202-141, Pharmingen) (Fig. 1*A*).

Cell Culture. ES cells were cultured in complete DMEM high glucose (GIBCO/Invitrogen), supplemented with 15% FBS (HyClone)/20 mM Hepes, pH 7.3 (GIBCO/Invitrogen)/0.1 mM nonessential amino acid (GIBCO/Invitrogen)/0.1 mM 2-mercaptoethanol (GIBCO/Invitrogen)/2 mM L-glutamine (GIBCO/Invitrogen)/500 units/ml mouse leukemia inhibitory factor (LIF) (Chemicon). ES cells were maintained on irradiated mouse feeder cells. The reconstituted cells were generated by infecting CD98hc-deficient cells with pMSCV-hygromycin retrovirus (Clontech) encoding human CD98hc or the CD98hc/CD69 chimeras (9). Viruses were generated in EcoPack 293 cells (Clontech), and viral titers ranged from 0.9 to 1.7 $\times$ 10^6 units/ml. After hygromycin selection, CD98hc or chimera expression was confirmed by flow cytometry (9). To produce embryoid bodies (EB), ES cells (10^4) were cultured without LIF or feeder cells; vimentin-positive fibroblasts were derived from outgrowths of these EB and were immortalized by using pBRSV (ATCC) encoding for the SV40-large T antigen.

Induction of Teratocarcinomas. A suspension of ES cells (1.5 $\times$ 10^6 cells per site) was injected s.c. into either syngeneic 129/Sv or athymic BALB/c WEHI nude mice. The mice were examined every 3 days, and the tumors were quantified by measurement with calipers. After 33 days, tumors were fixed in 10% formaldehyde and paraffin-embedded, sectioned, and stained with hematoxylin and eosin. In addition, some tumors were stained for proliferating cell nuclear antigen by using clone PC10 (Zymed) and counterstained with the nuclear dye YOPRO-1 (Molecular Probes). To ensure similar expression levels in reconstitution experiments, each cell line was supplemented with CD98hc-null ES cells so that a similar number of expressing cells was injected with each clone.

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Abbreviations: EB, embryoid bodies; FAK, focal adhesion kinase; Fn, fibronectin; Ln, laminin-1; PI, propidium iodide; PI3-kinase, phosphatidylinositol 3-kinase.

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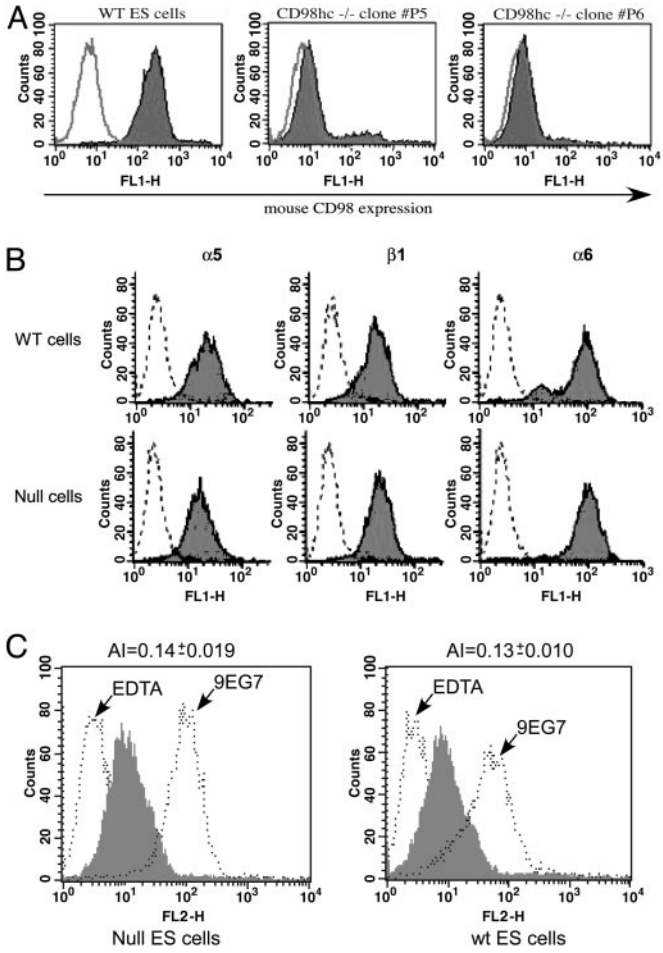

Fig. 1. CD98hc is not required for integrin expression or activation. (*A*) Flow cytometry analysis of CD98hc-deficient ES cells. In *A* and *B*, the filled histograms show specific staining, and the open histograms show staining with irrelevant IgG. (*B*) Flow cytometric analysis of cell surface expression of integrin subunits in ES cells, by flow cytometry. (*C*) Measurements of $\alpha5\beta1$ integrin affinity state by binding to the soluble cell-binding domain of Fn (Fn 9–11). Fn 9–11 binding alone (filled histogram), Fn 9–11 in the presence of EDTA (dotted line), and Fn 9–11 in the presence of the activating anti-$\beta1$ monoclonal antibody 9EG7 (dashed line) are illustrated for CD98hc$^{-/-}$ (*Left*) or WT (wt) (*Right*) ES cells. Activation index, calculated as described in *Methods*, was not significantly different between wt and CD98hc$^{-/-}$ ES cells.

Anoikis Assay. ES cell lines were trypsinized, rinsed, and suspended in complete medium. After depletion of feeder cells, ES cells were counted, and 10^6 cells were kept in suspension at 37°C for either 90 or 180 min in 2-ml complete medium. Then, cells were rapidly rinsed in PBS and stained for 5 min at 4°C with 50 μg/ml propidium iodide (PI) (Roche Diagnostics). Dead cells were defined as PI-positive cells by flow cytometry. Each assay was performed in triplicate.

Spreading Assay. Cell-spreading assays were performed as described in ref. 14 by using coverslips coated with fibronectin (Fn) or laminin-1 (Ln) (Sigma). Cells were fixed with 1% formaldehyde, examined by phase contrast microscopy, or stained for F-actin with rhodamine-phalloidin (Molecular Probes), and spreading was quantified by cell area by using IMAGE PRO PLUS software (Media Cybernetics, Silver Spring, MD).

Signaling Assays. After overnight serum starvation, cells were detached and kept in suspension in serum-free DMEM containing 0.2% BSA for 1 h. Cells were then plated on dishes coated with Fn. Rac assays were performed on both ES cells and immortalized fibroblasts by using binding to the p21-activated kinase binding domain to quantify GTP-Rac, as described in ref. 15. At the indicated time points, cells were chilled on ice, washed with ice-cold PBS, and lysed in Rac assay buffer [50 mM Tris·HCl, pH 7.0/0.5% Nonidet P-40/500 mM NaCl/1 mM $MgCl_2$/1 mM EGTA/protease inhibitors (Roche)] containing 20 μg of recombinant GST-p21-activated kinase binding domain. Lysates were clarified by centrifugation and then incubated with glutathione-agarose beads (Amersham Pharmacia Biotech) for 30 min at 4°C. Beads were washed with Rac assay buffer and eluted with SDS/PAGE sample buffer. Bound Rac was analyzed by Western blotting by using anti-Rac antibody (23A8, Upstate Biotechnology, Lake Placid, NY).

To measure phospho-Akt, cells were lysed in 20 mM Tris·HCl, pH 7.6/250 mM NaCl/3 mM EGTA/5 mM EDTA supplemented with protease inhibitors (Roche) and protein phosphatase inhibitors (Upstate Biotechnology). Western blotting was performed by using phospho-specific Ser-473 Akt protein or total Akt antibodies (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instructions. To assess phosphorylation of p130^{CAS} and focal adhesion kinase (FAK), cells were lysed in modified radioimmunoprecipitation assay buffer [50 mM Hepes, pH 7.4/150 mM NaCl/1.5 mM $MgCl_2$/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/protease inhibitor mixture (Roche)] and immunoprecipitated with the anti-p130^{CAS} or anti-FAK. Tyrosine phosphorylation was assessed by immunoblotting with anti-phosphotyrosine antibody (PY20) or with anti-phospho FAK (pY576) antibodies (BioSource International, Camarillo, CA).

Cell Migration Assay. Cell migration was assayed in a modified Boyden chamber system by using transwell membranes coated with Fn. Fibroblasts (6×10^4) were added to the top chamber and, after a variable time at 37°C, filters were fixed, stained with crystal violet, and migrated cells in the lower chamber were enumerated.

Flow Cytometry. For integrin profiles, anti-mouse α_1 (clone Ha 31/8), anti-mouse α_2 (clone HMα2), anti-mouse α_4 (PS/2), anti-mouse α_5 (clone HMα5-1), anti-mouse α_6 (clone GoH3), anti-mouse α_v (clone RMV-7), anti-mouse β_1 (clone 9EG7), and anti-mouse β_3 (clone 2C9-G2) were purchased from Pharmingen and used at the recommended concentrations. Goat FITC-conjugated anti-rat IgG and goat FITC-conjugated anti-hamster IgG were obtained from BioSource International and were used as secondary antibodies for α_4, α_6, α_v, and β_1 detection and α_1, α_2, α_5, and β_3 detection, respectively. Fn 9–11 binding was assayed by two-color flow cytometry as described in ref. 16. To obtain numerical estimates of integrin activation, we calculated an activation index, defined as $100(F_o - F_r)/(F_o9EG7 - F_r)$, where F_o is the median fluorescence intensity (MFI) of B9–11 binding, F_r is the MFI of B9–11 binding in the presence of EDTA, and F_o9EG7 is the MFI of B9–11 binding in the presence of activating anti-$\beta1$ mAb 9EG7.

Adhesion Assay. Ninety six-well tissue culture plates were coated with Fn (Sigma) overnight at 4°C, then blocked with a 2% heat-inactivated BSA (Sigma). Cells (5×10^4) were plated in each well and were allowed to adhere for 5, 10, 30, 60, or 120 min at 37°C. Nonadherent cells were washed off, adherent cells were fixed and stained with crystal violet, and the crystal violet was extracted with 10% acetic acid and measured in a microplate reader (Molecular Devices) at 560 nm.

Results and Discussion

Because the loss of CD98hc leads to embryonic lethality (17), we disrupted both alleles of CD98hc in ES cells (Fig. 6 *A* and *B*) to

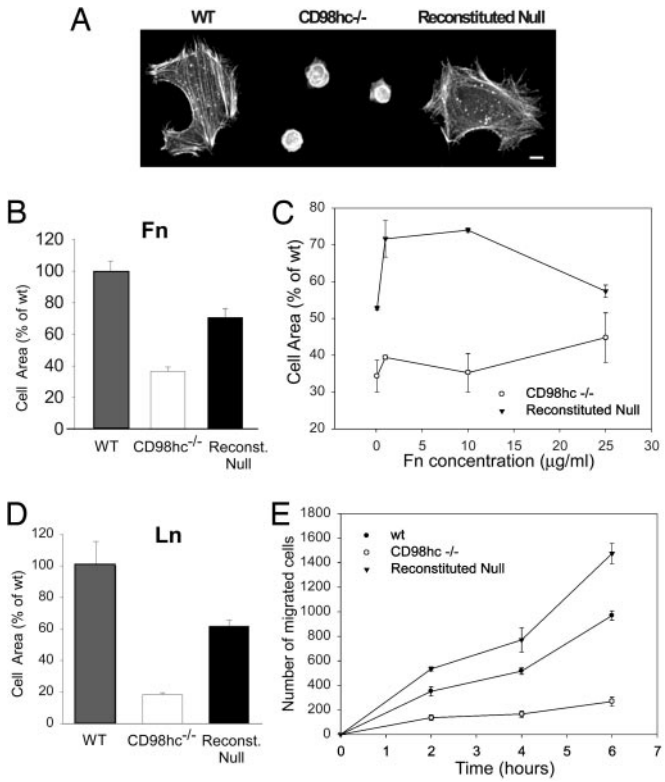

Fig. 2. CD98hc promotes cell spreading and cell migration *in vitro*. (*A*) Cell spreading of CD98hc$^{-/-}$ vs. reconstituted null ES cells. Cells were plated onto 10 μg/ml Fn-coated glass coverslips for 180 min. After fixation, actin filaments were revealed by rhodamine-phalloidin staining (Scale bar, 1 μm.) (*B*) Quantification of cell spreading by cell area measurements. One hundred eighty minutes after adhesion to 10 μg/ml Fn, the surface area of the CD98hc$^{-/-}$ ES cells (open bar) was significantly reduced as compared with either the WT (gray bar) or the human CD98hc-reconstituted null (filled bar) ES cells. (*C*) Cell spreading of variable concentrations of Fn. CD98hc$^{-/-}$ and reconstituted null ES cells were plated for 180 min onto glass coverslips coated with the indicated concentrations of Fn. Cell spreading was quantified by cell area measurements. (*D*) Spreading on Ln. Cell-spreading assays were performed as described in *A* and *B* by using coverslips coated with 4 μg/ml Ln. (*E*) Time course of fibroblast migration on Fn. CD98hc$^{-/-}$, WT (wt), or reconstituted null fibroblasts (3×10^4) were added to the top chamber of a Fn-coated transwell and allowed to migrate for indicated time points. After fixation, cells were stained with crystal violet, and the total number of migrated cells (bottom chamber) was enumerated.

examine the role of CD98hc in cellular behaviors. Two CD98hc-deficient clones were obtained that were null for CD98hc protein (Fig. 1*A*) and had similar properties in the experiments to be described below. WT CD98hc$^{-/-}$ and CD98hc-reconstituted null cells expressed similar quantities of integrins α5, α6, and β1 as judged by flow cytometry (Fig. 1*B*). None of the cells expressed detectable α1, α2, α4, αv, or β3 integrins. The absence of CD98hc had no effect on the ability of the cells to bind to the soluble cell-binding domain of Fn (Fig. 1*C*), a direct measure of the affinity of integrin $\alpha5\beta1$ (18). We also measured the static adhesion of these cells to Fn and, as expected from the lack of effect on integrin repertoires and integrin affinity, the lack of CD98hc did not impair static adhesion (Fig. 6*C*). There was a similar time-dependent static adhesion by 60 min, 26.6 $\pm$ 0.56% of WT cells, 29 $\pm$ 3.2% of CD98hc$^{-/-}$, and 27 $\pm$ 4.7% of reconstituted CD98hc$^{-/-}$ cells were adherent (Fig. 6*C Left*). When we examined adhesion to varying coating concentrations of Fn (Fig. 6*C Right*), a similar dose–response curve was obtained for the three cell types. Thus, CD98hc is not required for integrin expression, integrin affinity, or integrin-mediated adhesion.

The CD98hc$^{-/-}$ cells showed a dramatic reduction in spreading on Fn (FN) (Fig. 2 *A* and *B*) and Ln (Fig. 2*D*), strongly suggesting a defect in integrin signaling. We quantified cell spreading by cell area measurements (Fig. 2*B*). One hundred eighty minutes after adhesion to Fn or Ln, the surface area of the CD98hc$^{-/-}$ cells was 38% and 18% that of the WT cells, respectively. CD98hc reconstitution resulted in more than doubling of the surface area. With different concentrations of Fn (Fig. 2*C*) or Ln (data not shown), CD98hc$^{-/-}$ cells spread significantly less than WT cells at all coating concentrations tested; the defect was complemented by reexpression of CD98hc. To exclude the possibility that this result was ES cell-specific, we derived primary fibroblasts and SV40 large T antigen-immortalized fibroblasts from the CD98hc$^{-/-}$ ES cells. All of the fibroblastic cells (WT, CD98hc$^{-/-}$, or reconstituted CD98hc$^{-/-}$ cells) expressed integrins α5, α6, and β1 at similar abundance, and WT and CD98hc$^{-/-}$ fibroblasts adhered to and bound to Fn to the same extent (data not shown). Similar to ES cells, these CD98hc$^{-/-}$ fibroblasts exhibited impaired spreading that could be rescued by CD98hc reconstitution (data not shown). Because cell spreading and cell migration use many of the same signaling pathways (13), we examined integrin-dependent haptotaxis. The CD98hc$^{-/-}$ fibroblasts showed a 5-fold reduction in migration relative to the WT or reconstituted cells (Fig. 2*E*). Thus, CD98hc is required for efficient integrin-dependent cell spreading and cell migration.

Adhesion-dependent cell migration and spreading involves activation of the small GTPase, Rac, and phosphatidylinositol 3-kinase (PI3-kinase) (13). To ascertain whether CD98hc participates in biochemical signaling initiated by integrins, we assessed adhesion-induced activation of Rac and phosphorylation of a PI3-kinase downstream target, Akt. There was a profound suppression in adhesion-dependent Akt phosphorylation and Rac activation in the CD98hc$^{-/-}$ cells, and this defect was reversed by CD98hc reconstitution (Fig. 3 *A* and *B*). Furthermore, CD98hc was not required for Akt phosphorylation in general because growth factor-induced (Fig. 3*B*) phosphorylation was not impaired. Finally, a constitutively active form of Rac (RacV12) restored spreading in CD98hc-deficient cells (Fig. 3*C*), proving that the spreading defect was ascribable to the defect in Rac activation.

To explore the mechanism by which CD98hc contributes to adhesion-induced signaling, we examined early steps in the pathways leading from integrins to Rac and PI3-kinase activation (13, 19). The phosphorylation of p130^{CAS} is the initiating step in assembly of the CAS/Crk/DOCK180 complex that mediates integrin-dependent Rac activation. The phosphorylation of FAK results in activation of PI3-kinase. Furthermore, phosphorylated FAK functions as a scaffold by bringing active Src family kinases into proximity with p130^{CAS}, thereby promoting p130^{CAS} phosphorylation. Adhesion-dependent p130^{CAS} and FAK phosphorylation (Fig. 4 *A* and *B*) were dramatically impaired in CD98hc$^{-/-}$ cells. Thus, CD98hc functions at critical early steps in integrin signaling pathways that lead to activation of Rac and phosphorylation of Akt.

Previous studies show that integrin-dependent survival signals depend on PI3-kinase-induced activation of Akt (12), thus we tested the susceptibility of the CD98hc$^{-/-}$ cells to cell death after anoikis (11). When ES cells were placed in suspension, the CD98hc$^{-/-}$ cells exhibited increased cell death relative to WT or reconstituted null cells as assessed by PI uptake (Fig. 4*C*). After 90 min in suspension, 32.5 $\pm$ 2.4% (SEM, n = 4 experiments) of CD98hc$^{-/-}$ cells were dead, in comparison with 22.8 $\pm$ 1.8% of the WT and 21.5 $\pm$ 1.7% of the reconstituted CD98hc$^{-/-}$ cells. This difference was also pronounced at 180 min (CD98hc$^{-/-}$ cells = 62.7 $\pm$ 3% vs. WT cells = 44.4 $\pm$ 1.6% and reconstituted CD98hc$^{-/-}$ cells = 41.2 $\pm$ 3%). Thus, CD98hc provides protection from anoikis and promotes efficient cell spreading and migration; it also mediates integrin-dependent biochemical signals such as phosphorylation of Akt and activation of Rac.

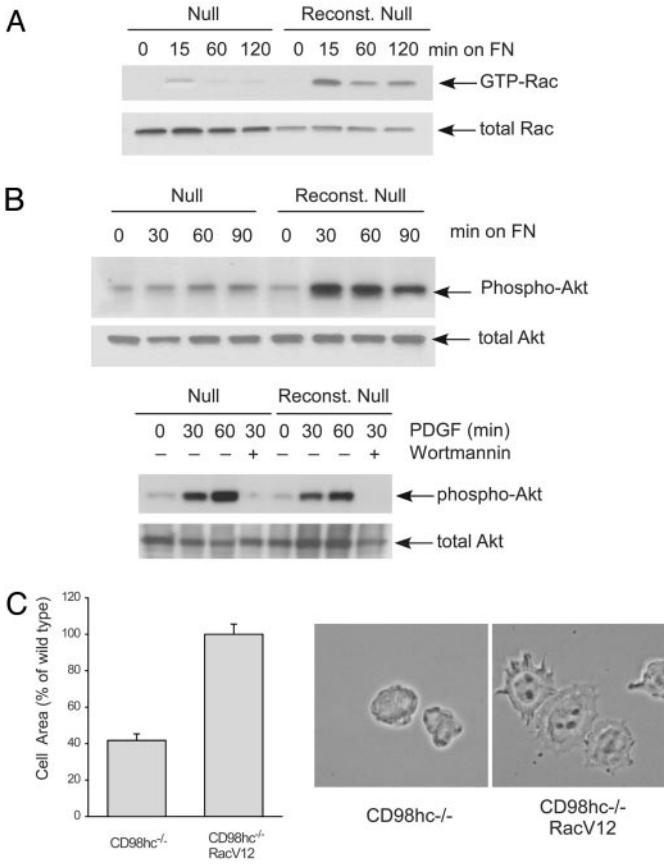

Fig. 3. CD98hc is a major contributor to the integrin-dependent activation of Rac and PI3-kinase. (*A*) Adhesion-dependent activation of Rac. CD98hc$^{-/-}$ and CD98hc-reconstituted ES cells were permitted to adhere to Fn. The Rac bound to GST-p21-activated kinase binding domain (GTP-Rac) was compared with the total Rac as detected by immunoblotting. (*B Upper*) Adhesion-dependent phosphorylation of Akt. CD98hc$^{-/-}$ and human CD98hc-reconstituted ES cells were permitted to adhere to Fn-coated plates as described in *A*. Akt activation was analyzed by Western blotting with anti-phospho-Akt (Ser-473) antibody. Cell lysates were also analyzed for total Akt. (*B Lower*) Growth factor activation of Akt. CD98hc$^{-/-}$ and reconstituted null ES cells were serum-starved overnight and preincubated for 30 min with or without PI3-kinase inhibitor Wortmannin. The cells were then stimulated with platelet-derived growth factor (PDGF) for indicated times. Akt phosphorylation was analyzed by Western blotting with anti-phospho Akt (Ser-473) antibody. Cell lysates were also analyzed for total Akt. (*C*) The spreading defect in CD98hc$^{-/-}$ cells is due to the reduced Rac activation. CD98hc$^{-/-}$ ES cells, Rac V12-expressing CD98hc$^{-/-}$ ES cells, or WT ES cells were plated onto 10 μg/ml Fn-coated glass coverslips for 180 min, and cell spreading was quantified as described in Fig. 2*B*. The data are expressed as the cell area as a percentage of the area of the WT cells. *Right* depicts a representative field of the CD98hc$^{-/-}$ cells and the Rac V12-expressing cells.

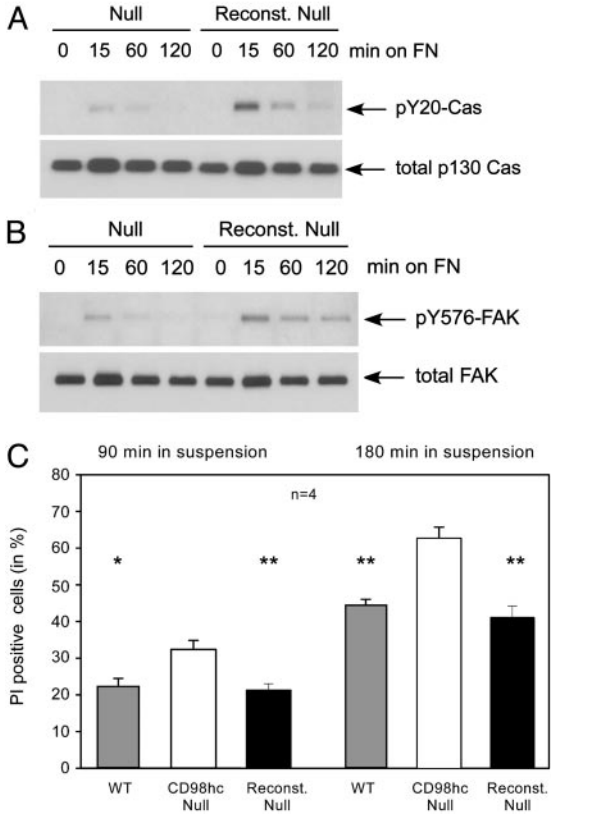

Fig. 4. CD98hc functions at early steps in integrin signaling pathways that activate Rac and PI3-kinase. (*A*) Adhesion-dependent phosphorylation of p130^{CAS}. CD98hc$^{-/-}$ and CD98hc-reconstituted ES cells were permitted to adhere to Fn. Cells were lysed and p130^{CAS} was immunoprecipitated. Immunoprecipitates were resolved by SDS/PAGE and immunoblotted with PY20 to detect phosphotyrosine (*Upper*) or anti-p130^{CAS}(*Lower*). (*B*) Adhesion-dependent phosphorylation of FAK. CD98hc$^{-/-}$ and CD98hc-reconstituted ES cells 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 (*Upper*) or anti-FAK (*Lower*). (*C*) Anchorage dependence of cell survival in CD98hc$^{-/-}$, WT, and reconstituted null ES cells. One million cells were kept in suspension for either 90 or 180 min and then stained with PI. Dead cells were identified as PI-positive cells by flow cytometry. At 180 min, the WT and cells reconstituted with CD98hc and C98T98E69 mutants were significantly protected from apoptosis (***, $P < 0.001$ vs. CD98hc$^{-/-}$ cells by two-tailed *t* test).

CD98hc is a type II transmembrane protein composed of a cytoplasmic, a transmembrane, and an extracellular domain. By constructing chimeras with CD98hc and another type II transmembrane protein (CD69), we previously found that the intracellular domain of CD98hc, comprised of the transmembrane and cytoplasmic domains, is necessary and sufficient for interactions with β1 integrins. In contrast, the CD98hc extracellular domain is required for light chain association and amino acid transport (9). Thus, we asked whether CD98hc contributions to anchorage-dependent survival signals could be ascribed to the domain that binds β1 integrins. We reconstituted CD98hc$^{-/-}$ ES cells and CD98hc$^{-/-}$ fibroblasts with retroviruses encoding the previously characterized (9) chimeras (Fig. 5*A*); each was well expressed as judged by flow cytometry (data not shown). The $C_{98}T_{98}E_{69}$ chimera, which associates with integrins but not light chains, completely rescued the defect in Rac activation. In sharp contrast, neither the $C_{98}T_{69}E_{98}$ nor the $C_{69}T_{98}E_{98}$ chimeras, which do not associate with integrins, had any effect (Fig. 5*B*). These data show that the intracellular and transmembrane domains of CD98hc are necessary and sufficient for reconstitution of adhesion-dependent Rac activation. Because this same domain is involved in the interaction of CD98hc with the integrins (9), these results suggest that the interaction of CD98hc with integrins mediates its effects on integrin signaling.

Because of the important role of Akt phosphorylation in integrin regulation of apoptosis (12), we tested the role of the CD98hc–integrin interaction in protection from anoikis. The integrin-coupled $C_{98}T_{98}E_{69}$-protected cells from anoikis to a similar extent as full-length CD98hc after 90 min in suspension (cell death: CD98hc$^{-/-}$ ES cells, 33.6 ± 1.86%; $C_{98}T_{98}E_{69}$ reconstituted cells, 26.7 ± 2.6%; WT reconstituted cells, 23.3 ± 1.6%) and after 180 min (cell death: CD98hc$^{-/-}$ cells, 58.5 ± 2%; $C_{98}T_{98}E_{69}$ reconstituted cells, 38.7 ± 1.3%; WT reconstituted cells, 40.3 ± 1.7%) (Fig. 5*C*). These results show that the portion of CD98hc that interacts

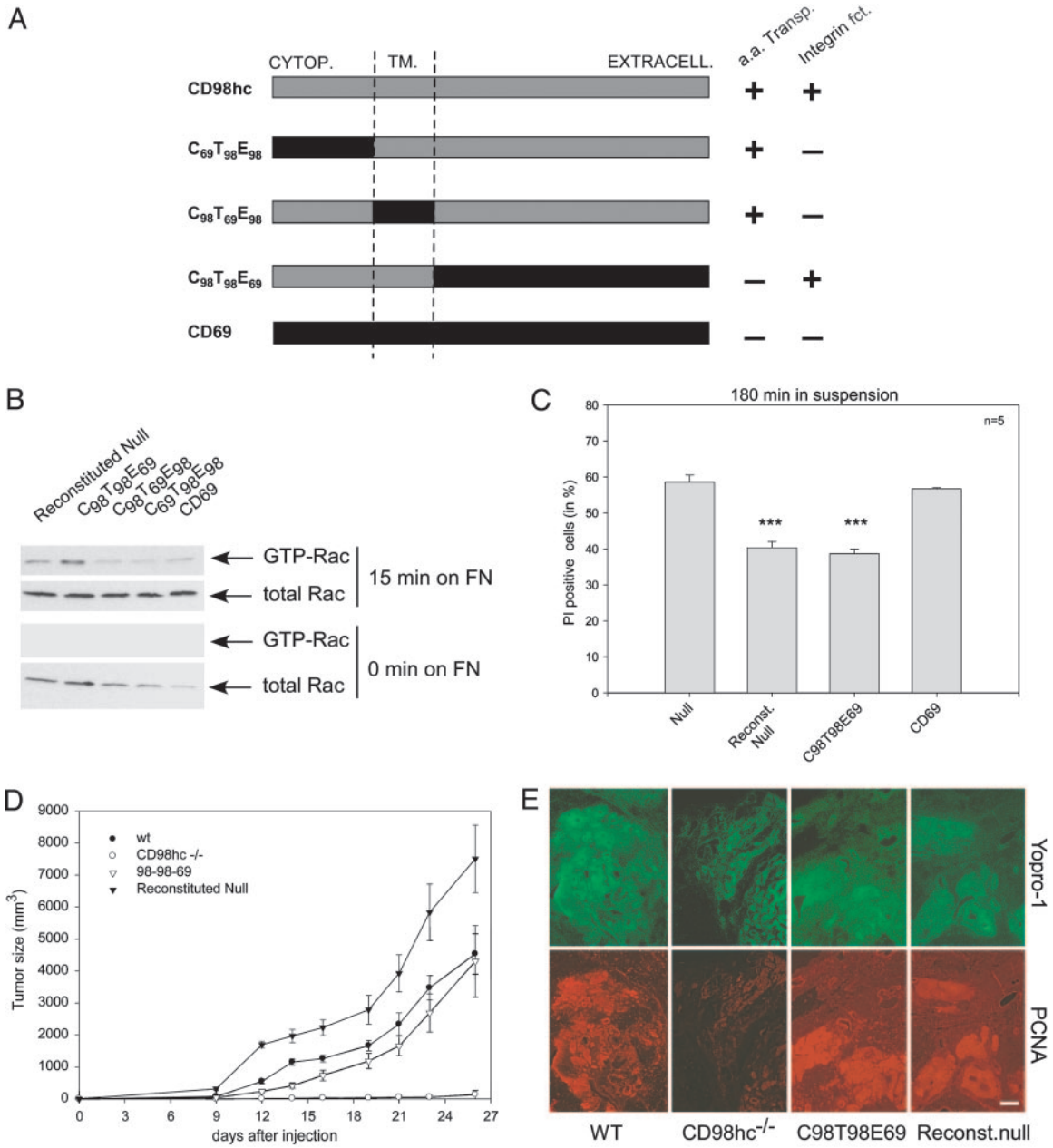

Fig. 5. CD98hc promotes tumor growth and cell proliferation *in vivo* through its integrin binding domain. (*A*) Model of chimeras of CD98hc, another type II transmembrane protein (CD69), and their interactions with integrins or with amino acid transporters (a.a. Transp.). CD98hc protein is depicted in gray, and CD69 is depicted in black. Each chimera is defined by its cytoplasmic (C), transmembrane (T), and 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 (Integrin fct.) (9). (*B*) Adhesion-dependent activation of Rac. CD98hc$^{-/-}$ fibroblasts reconstituted with human CD98hc or each of the chimeras were tested for Rac activation as described in Fig. 3. (*C*) Analysis of $C_{98}T_{98}E_{69}$ chimera-reconstituted ES cells' ability to rescue anoikis. Depicted are histograms representing the percentage of PI-positive cells after 180 min in suspension. $C_{98}T_{98}E_{69}$ chimera ($P < 0.001$, $n = 6$) and full-length CD98hc ($P < 0.001$, $n = 6$) reduced anoikis in comparison with CD98hc$^{-/-}$ cells. Cells reconstituted with full-length CD69 were not significantly different from CD98hc$^{-/-}$ cells (Null). (*D*) Teratocarcinoma development in mice. Mice were injected with 1.5×10^6 cells per site and analyzed for 26 days. Depicted is the quantification of tumor volume (mean values ± SEM). Tumor volumes of WT (wt) (●), CD98hc$^{-/-}$ (○), and CD98$^{-/-}$ ES cells reconstituted with either $C_{98}T_{98}E_{69}$ chimera (△) or full length CD98hc (▲) were determined at various time points by using the formula: $(\text{width})^2 \times \text{length} \times 0.52$. CD98hc$^{-/-}$ cells transfected with full-length CD69 were indistinguishable from untransfected cells (data not shown). (*E*) Immunohistochemical analysis of proliferation by staining for proliferative cell nuclear antigen (PCNA). After 26 days of tumor formation, sections of teratocarcinoma were stained for PCNA and counterstained by using green fluorescent nuclear dye YOPRO-1. Higher-power images revealed that PCNA staining was restricted to the nuclei. (Scale bar, 300 μm.)

with integrins is sufficient to protect cells from anoikis to the same extent as the full-length protein.

β1 integrins play an important role in cell proliferation and in some cancers (20, 21), and we report here that CD98hc enhances β1 activation of signaling pathways such as FAK, Rac, and Akt. These signaling pathways lead to *in vitro* phenotypes such as cell spreading, and these pathways also contribute to tumorigenesis (22). To test the requirement of CD98hc for tumorigenesis *in vivo*, we exploited the observation that ES cells form teratocarcinomas after s.c. injection in mice (23). Injection of WT (wt) ES cells into

nude mice led to the formation of large tumors. In contrast, CD98hc$^{-/-}$ cells either did not form tumors or formed very small tumors (Fig. 5*D*). For WT ES cells, 17 of 20 injections yielded tumors within 33 days. In contrast, only 7 of 28 ES cell injections led to small tumors. The few small CD98hc$^{-/-}$ tumors that formed exhibited profound reduction in cell proliferation as judged by staining with proliferating cell nuclear antigen (Fig. 5*E*). However, the resulting CD98hc$^{-/-}$ tumors were clearly teratocarcinomas because they contained structures derived from all three germ layers (Fig. 7, which is published as supporting information on the PNAS web site). In these tumors, we identified keratin whorls, neural tissue, cartilage, muscles, and secretory epithelia. Quantification of tumor volume (Fig. 5*D*) revealed that there was a delay in appearance of CD98hc$^{-/-}$ tumors (appearance on day 18 vs. day 6) and a marked reduction in tumor volume at all time points. Reconstitution of the CD98hc$^{-/-}$ cells with human CD98hc restored tumor growth (Fig. 4*D*) and cell proliferation (Fig. 5*E*). These experiments were also performed on syngeneic mice, and we obtained similar results to those seen in athymic mice (data not shown). The integrin binding (C98T98E69) chimera also reconstituted tumorigenesis and proliferation (Fig. 5 *D* and *E*). Thus, the portion of CD98hc that binds β1 integrins and enhances integrin signaling promotes tumorigenesis and cell proliferation *in vivo*.

The presence of tissues derived from all three germ layers in the few CD98hc$^{-/-}$ teratocarcinomas that formed suggested that several differentiated lineages can develop in the absence of CD98hc. To test this possibility directly, we examined the *in vitro* differentiation of these ES cells into EB. Although the null cells formed smaller EB, a variety of cell types, including beating cardiomyocytes, differentiated from the EB (data not shown). Furthermore, keratinocyte differentiation was unimpaired in CD98$^{-/-}$ ES cells, as judged by expression of keratin 14 (S. Broad and F. Watt, personal communication). Likewise, *in vitro* megakaryocyte differentiation from these ES cells (24) occurred, as evaluated by both morphology and the presence of CD41 and CD42b (K. Eto and S. Shattil, personal communication). Thus, absence of CD98hc is compatible with differentiation of multiple cellular lineages but leads to profound suppression of tumor development.

The pathogenesis of cancer often includes tumor cell escape from anoikis, i.e., they become anchorage-independent for survival (25). CD98hc binds β1 integrins, and β1 integrins play an important role in the transformation of certain epithelial cells (20, 21). Regulation of the activity of Rac and RhoA, through β1 integrins, contributes to tumor cell motility and invasion of cancer cells (26). Similarly, expression of β1 integrins in β1-deficient cell lines stimulates cell scattering through Rho GTPases (27). In addition, FAK and Akt signaling pathways are involved in tumor formation (22). We now find that CD98hc mediates β1 integrin-mediated activation of FAK, Rac, and Akt. CD98 is highly expressed in many tumors and transformed cells (28, 29). Increased expression is irrespective of tissue of origin, possibly because the first intron of CD98hc gene contains a transcriptional enhancer element that is active in most malignant human cells (30). Furthermore, forced expression of full-length CD98hc can transform mouse fibroblasts (4, 31). Our finding that the domain of CD98hc that binds to β1 integrins stimulates teratocarcinoma growth and mediates integrin signaling indicates that the β1 integrin–CD98hc complex contributes to cellular behaviors leading to tumorigenesis. The genetic instability of cancer cells leads to wide variation in their dependence on signaling pathways that control cell growth and survival (32). Here we show that CD98hc contributes to cell growth and survival by regulating integrin signaling. Future studies with conditional CD98hc gene deletions will be required to test its role in the pathogenesis of specific cancers.

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