Mammalian NOTCH-1 Activates β1 Integrins via the Small GTPase R-Ras*

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Notch is a central regulator of important cell fate decisions. Notch activation produces diverse cellular effects suggesting the presence of context-dependent control mechanisms. Genetic studies have demonstrated that Notch and integrin mutations have related phenotypes in key developmental processes such as vascular development and somitogenesis. We show that the intracellular domain of mammalian Notch-1 activates integrins without affecting integrin expression. Integrin activation is dependent on γ-secretase-mediated intramembranous cleavage of membrane-bound Notch releasing intracellular Notch that activates R-Ras, independent of CSL-transcription. Notch also reverses H-Ras and Raf-mediated integrin suppression without affecting ERK phosphorylation. Membrane-bound Notch mutants that are inefficiently cleaved or intracellular Notch mutants lacking the ankyrin repeat sequence do not activate R-Ras or integrins. Co-expression of Msx2-interacting nuclear target (MINT) protein with Notch or expression of intracellular Notch-1 truncation mutants lacking the C-terminal transactivation/PEST domain suppresses Notch transcriptional activity without affecting integrin activation. Notch ligand, Delta-like ligand-4, stimulates R-Ras-dependent α5β1 integrin-mediated adhesion, demonstrating the physiological relevance of this pathway. This new CSL-independent Notch/R-Ras pathway provides a molecular mechanism to explain Notch, integrin, and Ras cross-talk during the development of multicellular organisms.

The diverse biological processes intrinsic to the development of multicellular organisms are coordinated by communication between adjacent cells involving a small number of evolutionarily conserved signaling pathways. The Notch signaling pathway is an important mechanism for mediating these intracellular signaling events to direct cell fate decisions (1). The components of the Notch pathway have been identified in a broad range of metazoans and have been extensively studied in insects, nematodes, and mammals (2–4). Consequently, Notch has been shown to be a key regulator of many developmental processes including somitogenesis, vasculogenesis, and neurogenesis (4–6). In addition, the Notch pathway plays a critical role in mammalian immune development and carcinogenesis (7, 8).

The molecular components of the Notch pathway have been extensively studied and are highly conserved between species. NOTCH encodes a single-pass heterodimeric transmembrane receptor with an extracellular domain that contains epidermal growth factor-like repeats (9). Four NOTCH homologs (NOTCH1–4) and two groups of ligands (Delta-like (Dll-1, -3, and -4) and Serrate-like (Jagged 1 and 2)) have been identified in mammals (10, 11). Notch-ligand interaction triggers two distinct proteolytic cleavage events (S2 and S3) that release the intracellular portion of Notch (NIC) from the plasma membrane (12–14). NIC translocates to the nucleus where it binds to a transcriptional regulator CSL (CBF-1/Su(H)/LAG-1), displacing co-repressors and recruiting co-activators, thus inducing expression of Hairy-Enhancer of Split (HES) and HES-related proteins genes (15–18). Data from several groups suggest that Notch may also signal without cleavage at S3 or CSL-dependent transcription (19–21). The molecular components of this “non-classical” Notch signaling pathway are not yet fully understood.

Recent genetic studies have indicated important parallels between the developmental processes controlled by Notch and integrin-mediated adhesion. Integrins are heterodimeric transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions and have been identified in insects, nematodes, and vertebrates (22). A key feature of integrins is their ability to modulate ligand binding affinity in response to intracellular signals, a process called activation (23). They are essential for embryogenesis and are involved in neurogenesis, myogenesis, and angiogenesis, processes also controlled by Notch (24–26). α6β1 and α5β1 integrins are highly expressed on stem cells and regulate survival, migration, and differentiation (27, 28). Notch has been shown to associate with β1 integrins in neural stem cells and expression of NOTCH 4 in endothelial cells increases adhesion to collagen (29, 30). Additionally, data from zebrafish

4 The abbreviations used are: NIC, intracellular Notch-1; CHO, Chinese hamster ovary; ECM, extracellular matrix; ERK, extracellular-regulated kinase; AI, activation index; MINT, Mxs2-interacting nuclear target; HES, Hairy-Enhancer of Split; TADP, C-terminal transactivation/PEST; ICN1, intracellular Notch-1; MAP kinase, mitogen-activated protein kinase.

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somitogenesis suggests that mutations in the Notch pathway and integrin α5 subunit can produce a complementary disruption in somite formation (31). This suggests the possibility that Notch may affect integrin activation, modulating important developmental processes by altering cell-matrix interactions (32). We therefore sought to investigate whether Notch signaling could activate integrins and define the mechanisms of this important interaction using an in vitro model of integrin affinity.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—Mouse Notch-1 constructs: NIC, NΔE, NΔE(V1774K), and NLNG in pCS2 (C-terminal Myc tag) were from R. Kopan (Washington University School of Medicine, St. Louis, MO) (14). Human NOTCH-1 constructs: ICN1, ΔTADP, ΔEN1, and ΔANK in pcDNA3.1, from J. C. Aster (Department of Pathology, Harvard Medical School) (33), were subcloned into pCMV tag 4A (Stratagene) to incorporate a C-terminal affix. Human NOTCH-1 constructs: ICN1, ΔTADP, ΔEN1, and ΔANK in pcDNA3.1, from J. C. Aster (Department of Pathology, Harvard Medical School) (33), were subcloned into pCMV tag 4A (Stratagene) to incorporate a C-terminal tag. Msx2-interacting nuclear target (MINT) (vector pEF-Bos-Neo, N-terminal Myc tag) (T. Honjo, Kyoto University, Japan) (34), Tac-α5, pDCR-H-Ras(G12V) (hemagglutinin-tagged), pSG5 R-Ras(WT), R-Ras(T43N) (Myc tagged), and pSG5 R-Ras(G38V) (Myc tagged) were as previously described (35, 36). pCDNA3-Raf-CAA5, pSG5 R-Ras(WT), R-Ras(T43N) (Myc tagged), and pSG5 R-Ras(G38V) (Myc tagged) were as previously described (35, 36). pCDNA3-Raf-CAA5 (FLAG tagged) was from Dr. C. K. Weber (University of Ulm, Germany). pG2L4 cXSL from A. Israel (Institute Pasteur, France) (37) and pcDNA3.1 LacZ from Invitrogen.

**Cell Lines/Transfection**—CHO(αβ-py) cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) with 10% (v/v) fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate, and 4.5 g/ml glucose, pH 7.4, 1:25 (v/v) anti-mouse IgM-fluorescein isothiocyanate (DAKO) and chemiluminescence (ECL) (Amersham Biosciences).

**CSL-luciferase Activity**—CSL-luciferase activity was assayed as previously described (14). In brief, αβ-py cells transfected with test DNA plus pGL2 4xCSL-luc (0.2 μg) and pcDNA3.1 LacZ, 0.1 μg were lysed after 48 h and luciferase activity was determined using Steady-Glo (Promega) according to the manufacturer’s instructions. In parallel samples, galactosidase activity was determined using Galacto-Plus (Tropix). Luciferase activity for each transfection condition was normalized for galactosidase activity and expressed as a fold-change from that observed with empty vector.

**R-Ras Activation Assay**—R-Ras activity was determined by binding to the Ras-binding domain of Raf as previously described (38). In brief, αβ-py cells were transfected with R-Ras(WT) or R-Ras(G38V) (0.25 μg) and test DNA. Cells were quiesced in serum-free media and lysed 48 h in buffer (200 mM NaCl, 2.5 mM MgCl2, 50 mM Tris-HCl, pH 7.4, 15% glycerol, 1% Nonidet P-40, and Complete protease inhibitor (Roche Applied Science) at 4°C for 20 min. The clarified lysate was incubated with glutathione-S-transferase-Raf-Ras-binding domain coupled to glutathione-agarose beads for 2 h at 4°C. Beads were washed and eluted protein separated by SDS-PAGE. Bound R-Ras was detected by Western blotting for Myc.

**Cell Adhesion Assay**—2 × 105 K562 cells were resuspended in HEPES/NaCl buffer and incubated with 4B4 (Beckman Coulter) (10 μg/ml, 45 min at 37°C), EDTA (5 mM final concentration), or Mn2+ (100 mM final concentration) as indicated. Cells were seeded into plates coated with fibronectin or poly-L-lysine (10 μg/ml in phosphate-buffered saline for 60 min at 37°C) for 2 h with or without MW167 (50 μM) or Me2SO (Vehicle) (Calbiochem) as indicated.

**Flow Cytometry**—CHO(αβ-py) cells were transfected with test DNA together with 0.75 μg of Tac-α5 transfection reporter construct. After 48 h, cells were sequentially labeled with PAC-1 antibody (BD Biosciences) (5 μg/ml) ± 5 mM EDTA or 100 μM MnCl2 in HEPES/NaCl buffer (20 mM HEPES, 140 mM NaCl, 1.8 mM CaCl2, 1 mM MgCl2, and 2 mg/ml glucose, pH 7.4), 1:25 (v/v) anti-mouse IgM-fluorescein isothiocyanate (BIOSOURCE), and 1:50 (v/v) anti-Tac-RPE (ACT-1) (Dako). ToPro3 (Molecular Probes) was added to each sample (1 μM) and integrin affinity was analyzed by three-color flow cytometry. PAC-1 binding was determined by gating for live and highly transfected cells. Integrin activation index (AI) was calculated (AI = (Fα5Fα5 - Fα5Fα5) × 100), where Fα5 is geometric mean (GM) fluorescence intensity (MFI) of PAC-1 binding of the native integrin, Fα5 is mean fluorescence intensity of PAC-1 binding in the presence of 5 mM EDTA, and Fα5 is mean fluorescence intensity of PAC-1 binding in the presence of 100 μM MnCl2. AI was used to calculate percentage of integrin suppression (AIα5/AIα5 x 100). AIα5 is the activation index with the control vector and AI is the activation index with DNA under test. For analysis of β3 integrin surface expression (independent of integrin affinity), anti-CD61(β3) antibody (Sarotec) was used in the above conditions.

**Gel Electrophoresis and Western Blotting**—Cells were lysed in RIPA buffer, protein balanced by BCA protein assay (Pierce) and resuspended in Laemmli sample buffer. Samples were resolved on 8–12% SDS-PAGE gels (10 μg of protein per lane) and transferred onto Hybond C nitrocellulose (Amersham Biosciences). Immunoblotting used anti-hemagglutinin (Y-11), anti-Myc (9E10), anti-ERK2 (C-14), anti-β integrin (N-20) (Santa Cruz Biotechnology, USA), anti-phospho-ERK1/2 (ERK-PT115), and anti-ß-actin (AC-20), anti-FLAG (M2) (Sigma) in 5% nonfat milk, detected with species-specific horseradish peroxidase-conjugated antibodies (DAKO) and chemiluminescence (ECL) (Amersham Biosciences).
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**RESULTS**

**Notch-1 Activates Integrins and Reverses H-Ras/Raf-mediated Integrin Suppression**—To investigate whether Notch signaling could modulate integrin activation, we used a well established model of integrin affinity (39). This utilizes a CHO cell line (αβ-py) that stably expresses an active chimeric integrin (αIIbβ3β1), which has the ligand binding properties of αIIbβ3 but is activated through the α6β1 cytoplasmic domains. We assessed the activation status of the chimeric integrin expressed on the αβ-py cells using flow cytometry to detect binding of a monoclonal antibody specific for the active confirmation of αIIbβ3 (PAC-1). A cell surface marker encoding the extracellular domain of the interleukin-2 receptor, termed Tac, and the intracellular domain of the α5 integrin (Tac-α5) was used as a marker for DNA transfection. Transfection of αβ-py cells was detected by flow cytometry using an antibody against the interleukin-2 receptor, Tac-R-PE (R-phycoerythrin). We routinely observed transfection efficiencies of 70–80%. Using this model we were able to determine integrin activation status (geometric mean fluorescence intensity of fluorescein isothiocyanate-labeled PAC-1 binding) in highly transfected cells (Tac-R-PE positive cells) allowing accurate assessment of the effects of transfected test DNA on integrin affinity. For each DNA transfection we compared PAC-1 binding under “native” conditions to those in the presence of EDTA (maximally inhibited integrins) or manganese (maximally activated integrins) allowing calculation of an integrin activation index as described under “Experimental Procedures.”

**HES-1 Expression**—Total RNA was extracted from 1 × 10^6 K562 cells using RNase kit (Qiagen), contaminating DNA was removed with RNase-free DNase (Promega) and cDNA was generated by reverse transcription of 400 ng of RNA using TaqMan reverse transcription reagents (Applied Biosystems). cDNA quality was verified by PCR amplification of β-actin. Real time quantitative reverse transcriptase-PCR analysis was performed with TaqMan reagents and an ABI 7900HT machine as per the manufacturer’s instructions (Applied Biosystems). The sequences for HES-1 were CATTCTGGAATGTACAGTGAAGCA; HES-1 rev, CAGGCCAGCCGTCATCT; and  HES-1 probe, CTCGGGAACCTGCAGCGGGC Fam-labeled. Triplicate measurements were performed and analyzed with ABI sequence detector software (version 2.1) using the 2^−ΔΔCt method. HES-1 expression for each condition was normalized for 18S expression and represented as a -fold change from control cells.

**Statistical Analysis**—Data were analyzed by one-way analysis of variance and the appropriate post-test analyses were applied. p values < 0.05 were considered to be significant.

**FIGURE 1.** Notch activates integrins and reverses H-Ras-mediated suppression of integrin affinity. a, CHO(αβ-py) cells transfected with Tac-α5 (0.75 μg), NIC (1 μg), and H-Ras(G12V) (0.375 μg). Integrin activation state was analyzed by flow cytometry (n = 7 independent experiments). The representative dot blots display PAC-1-fluorescein isothiocyanate binding (integrin activation status) on the x axis and Tac-R-PE antibody binding (transfection efficiency) on the y axis. The quadrant marker on each dot blot differentiates on the x-axis, cells with high and low integrin affinity status and on the y-axis, highly transfected cells (upper quadrants) against cells transfected to a lesser extent (lower quadrants). The quadrant marker separating highly transfected cells was set for individual experiments to contain 20–25% of Tac-α5 positive cells. The figure in the right upper quadrant of each dot blot represents the percentage of highly transfected cells present in that quadrant (i.e. cells with high integrin affinity). Representative dot blots are shown. H-Ras(G12V) induced a left shift in PAC-1 binding in transfected cells indicating integrin suppression. Co-transfection of NIC increased PAC-1 binding and reversed the left shift, indicating integrin activation. b, mean percentage integrin activation ± S.E. is shown. Inset, dose-dependent effect of NIC on H-Ras(G12V) (0.375 μg)-mediated integrin suppression. c, representative immunoblot of NIC, H-Ras, β3 integrin, ERK2, phospho-ERK, and β-actin expression. d, CHO(αβ-py) cells were transfected with Tac-α5 (0.75 μg) and NIC (1 μg), H-Ras(G12V) (0.375 μg), or R-Ras(G38V) (0.5 μg). Surface expression of β3 integrins was determined by flow cytometry. Representative overlay histograms of anti-β3 integrin (white) or isotype control (black) staining on highly transfected cells are shown. Statistically significant differences between results are indicated by * (p < 0.05) or ** (p < 0.01).
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We found that expression of the intracellular domain of mouse Notch 1 (designated NIC), which constitutively activates the Notch signaling pathway (14), increased integrin activation in αβ-py cells compared with vector control. This was demonstrated by a rightward shift in PAC-1 binding in highly transfected cells (vector 49.8%, NIC 75% cells in right upper quadrant) (Fig. 1a). There was no change in PAC-1 binding in the untransfected cells. Correspondingly, NIC expression increased integrin activation index (88.4 ± 5.7%) compared with expression of vector alone (67.3 ± 2.2%) (Fig. 1b).

The chimeric integrin in αβ-py cells is relatively activated in the resting state. Therefore, we sought to suppress integrin affinity and determine whether Notch signaling would reverse integrin suppression. The small GTP-binding protein H-Ras has been shown to suppress integrin activation (39). We found that transfection of constitutively active H-Ras(G12V) alone caused marked inhibition of PAC-1 binding; however, co-transfection with NIC completely reversed suppression of integrin activation (Fig. 1a and b). These effects were not produced by alterations in H-Ras(G12V) or NIC expression when the constructs were co-transfected, nor was expression of the chimeric integrin in the αβ-py cells affected (Fig. 1c).

Hughes et al. (39) have previously shown that H-Ras-mediated suppression of the chimeric integrin in αβ-py CHO-K1 cells is not a consequence of a decrease in integrin expression levels. To confirm that Notch expression did not alter surface integrin expression we used flow cytometry to detect surface expression of β3 integrins. Using an anti-β3 integrin antibody whose binding was not dependent on integrin activation status we found that neither H-Ras(G12V), NIC, nor R-Ras(G38V) expression altered surface expression of β3 integrins in αβ-py cells (Fig. 1d). Thus changes in PAC-1 binding are due to alterations in integrin activation not expression.

H-Ras suppresses integrin activation via its downstream effector kinase Raf-1 (39). We therefore tested the ability of active Notch to reverse integrin suppression by an activated membrane-targeted variant of Raf, Raf-CAA. Similar results were observed to those found with H-Ras(G12V) (Fig. 2, a–c). Thus NIC reversed the suppressive effect of activated H-Ras or Raf-1 on integrin affinity, suggesting that Notch signaling can override integrin inactivation caused by the H-Ras-dependent suppression pathway.

Previous work has suggested that suppression of integrin activation by H-Ras and Raf-1 is dependent upon Raf-1/2 function but does not correlate with bulk phosphorylation of ERK (40). However, reversal of integrin suppression by the small death effector domain-containing protein PEA-15 depends upon its capacity to bind ERK1/2 (40, 41). Notch signaling has previously been shown to have differing effects on ERK phosphorylation depending upon cellular context (42, 43). Therefore, we investigated whether Notch signaling could reverse suppression of integrin activation by H-Ras through effects on ERK1/2. We found that transfection with NIC alone did not affect ERK phosphorylation. Furthermore, NIC did not affect phosphorylation of ERK induced by H-Ras(G12V) or Raf CAA (Figs. 1c and 2c). In addition, we were unable to demonstrate a physical association between NIC and ERK1/2 by co-immunoprecipitation (data not shown). This suggests that Notch modulation of integrin affinity is distinct from the Ras/Raf pathway.

Activation of β1 Integrins by Notch-1 Requires Intramembrane Cleavage at S3 to Release the Intracellular Domain—Classically, Notch activation requires ligand-induced cleavage of the Notch receptor ~12 amino acids N-terminal to the transmembrane domain (S2), which allows intramembrane cleavage (S3) by the protease complex γ-secretase (13, 14, 44, 45). However, analysis of the role of Notch in patterning of the Drosophila embryo dorsal epidermis suggest that Notch may signal without membrane cleavage (46). We therefore determined whether activation of β1 integrins by Notch was dependent on intramembrane cleavage.

Mutants of mouse Notch-1 that are membrane-bound and show differing efficiencies of processing by γ-secretase were used (14) (summarized in Fig. 3) to assess rescue of H-Ras(G12V)-induced integrin suppression. All subsequent αβ-py assays are represented as levels of integrin suppression.
The Notch mutant NΔE consists of transmembrane and intracellular domains of mouse Notch-1 with a short extracellular segment and is cleaved by the γ-secretase complex to yield active intracellular Notch. Transfection of αβ-py cells with NΔE yielded a fragment of similar molecular weight to NIC and reversed H-Ras-mediated integrin suppression without affecting H-Ras expression or ERK1/2 activation (Fig. 4, a and b). The effect on integrin activity was less pronounced than observed with NIC, presumably as a result of a lower yield of intracellular Notch from NΔE (Fig. 4a). To investigate this, two Notch mutants were used that are inefficiently processed by γ-secretase and do not yield detectable levels of intracellular Notch: an NΔE variant with a point mutation at amino acid 1774, NΔE(V1774K); and NLNG, which has identical transmembrane-intracellular domains to NΔE but an extracellular domain containing LNG repeats. Transfection of αβ-py cells with NΔE(V1774K) or NLNG did not yield detectable intracellular Notch and did not significantly reverse H-Ras suppression of integrins (Fig. 4a). Furthermore, pre-treatment of αβ-py cells with the γ-secretase inhibitor MW167 blocked the processing of NΔE to intracellular Notch and prevented integrin activation without altering H-Ras(G12V) expression or ERK1/2 phosphorylation (Fig. 4b).

We confirmed the transcriptional activity of the Notch mutants by measuring CSL-dependent luciferase activity (14). CSL-luciferase activity was significantly stimulated by NIC and NΔE (Fig. 4c). However, transfection of the Notch mutants NΔE(V1774K) or NLNG, which are inefficiently cleaved, stimulated CSL-luciferase activity significantly less than NIC (Fig. 4c). Furthermore, inhibiting γ-secretase with MW167 significantly reduced the capacity of NΔE to activate CSL (Fig. 4d). Co-transfection of H-Ras(G12V) with the Notch mutants did not affect CSL-luciferase activity (data not shown). Taken together this data indicates that S3 cleavage is essential for both classical Notch activation and Notch-mediated integrin activation.

Activation of β1 Integrins by Notch-1 Is Not Dependent on CSL-mediated Transcription—S3 cleavage releases the intracellular portion of the Notch receptor, which associates with CSL (46). In the resting state CSL binds to DNA acting as a transcriptional repressor, but upon interaction with Notch, co-repressors are displaced and CSL activates transcription leading to up-regulation of downstream target genes (e.g. HES-1) (47, 48). However, Notch may also signal independently from CSL-mediated transcription (49). We therefore sought to determine the role of transcription in Notch activation of integrins using two approaches: inhibition of Notch-CSL interaction and deletion of the transactivation domain of Notch-1.

We used MINT protein, which has been shown to compete with the intracellular region of Notch for binding to CSL, suppressing the transcriptional activity of Notch (34). Transfection of MINT into αβ-py cells did not affect integrin affinity or the ability of H-Ras to suppress integrins/phosphorylate ERK1/2 (Fig. 5, a and b). Available MINT antibodies are ineffective for Western blot analysis, therefore expression of MINT was confirmed by immunofluorescence microscopy (Fig. 5, c–j) (34). Importantly, MINT expression did not block NIC activation of integrins despite significantly reducing CSL-luciferase activity (Fig. 5k). This suggests that Notch activation of integrins is not dependent upon transcriptional activity.
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FIGURE 5. Notch activation of integrins is independent of CSL transactivation. a, the effect of NIC (1 μg) and MINT (2 μg) on H-Ras(G12V) (0.375 μg)-mediated integrin suppression (mean ± S.E., n = 3 independent experiments); b, representative immunoblot of NIC, H-Ras(G12V), phospho-ERK, and ERK2 expression, c–j, expression of MINT (2 μg) and NIC (1 μg) in αβ-py cells shown by immunofluorescence microscopy; c and d, αβ-py cells expressing NIC-GFP (green), and phase contrast; e–f, αβ-py cells expressing Myc-MINT (labeled with Alexa Fluor 568) (red), and phase contrast; g and h, αβ-py cells expressing NIC-GFP (green) and Myc-MINT (Alexa Fluor 568) (red); i, merge of green (NIC) and red (MINT) channels; j, phase contrast of ICN, H-Ras(G12V), phospho-ERK, and ERK2 expression. k–l, representative immunoblot of Notch, H-Ras(G12V), phospho-ERK, and ERK2 expression, m, the effect of ICN1 and ΔTADP (1 μg) on CSL-luciferase activity (mean ± S.E., n = 3 independent experiments). c–j, the effect of Notch mutants, ICN1 and ΔTADP (1 μg), on H-Ras(G12V) (0.375 μg)-mediated integrin suppression (mean ± S.E., n = 3). Representative immunoblot of Notch, H-Ras(G12V), and β-actin expression, m, the effect of ICN1 and ΔTADP (1 μg) on CSL-luciferase activity (mean ± S.E., n = 3 independent experiments). Statistically significant differences between results are indicated by * (p < 0.05).

To confirm our transcriptional activity data we used intracellular Notch-1 truncation mutants (Fig. 3). The C-terminal transactivation/PEST (TADP) domain of Notch-1 is important for Notch transactivation and CSL-dependent transcription in Notch activation of integrins.

Activation of β1 Integrins by Notch Is Mediated by R-Ras—The CSL-independent effectors of Notch are not fully defined. However, our data suggests that Notch may regulate integrin

(33). Transfection of human intracellular Notch-1 (ICN1) into αβ-py cells reversed H-Ras-mediated integrin suppression in a similar manner to mouse NIC (Fig. 5l). Furthermore, transfection of an ICN1 truncation mutant, lacking the TADP domain (ΔTADP) also reversed H-Ras-mediated integrin suppression to a similar degree as ICN1 (Fig. 5l). We confirmed that removal of the TADP region from the intracellular domain of Notch-1 significantly reduced CSL-dependent transcription (Fig. 5m). This data confirms that integrin activation by Notch is not dependent on CSL-dependent transcription.

Activation of β1 Integrins by Notch-1 Requires the Ankyrin Repeat Domain—The intracellular domain of the Notch receptor consists of the RAM domain, two nuclear localization sequences, TAD, PEST, and ankyrin repeat regions (48, 50). We sought to investigate whether the integrin activating function of Notch could be mapped to one of these regions using deletion mutants (Fig. 3) (33). We used a membrane-bound Notch mutant (ΔEN1) that consists of a short extracellular segment, the transmembrane region, and intracellular domain of human Notch-1 (Fig. 3), which is processed by γ-secretase and is constitutively active. We found that this mutant reversed H-Ras suppression of integrins in αβ-py cells (Fig. 6a). In addition, a ΔEN1 mutant (ΔRAM) lacking the 23RAM domain and the ΔTADP mutant (lacking TAD, PEST, or NLS domains) were able to activate integrins (Fig. 6, a and b). However, a mutant of ΔEN1 (ΔANK), which was lacking the ankyrin repeats, was unable to reverse H-Ras suppression of integrins (Fig. 6a). We confirmed the previously described transcriptional activity of these mutants in αβ-py cells (Fig. 6c) (33). This data specifically implicates the ankyrin repeats...
activity by activating effectors that antagonize H-Ras signaling to integrins. R-Ras is a small GTP-binding protein homologous to H-Ras that can activate integrins and reverse H-Ras/Raf-initiated integrin suppression without affecting bulk ERK phosphorylation (36, 51). We found that transfection of low doses of NIC (0.25 μg) had a minimal effect on integrin suppression mediated by H-Ras(G12V) (Fig. 7a, left). Importantly, whereas transfection of wild type R-Ras alone did not affect integrin affinity, co-transfection of wild type R-Ras with 0.25 μg of NIC reversed H-Ras-mediated integrin suppression, without affecting H-Ras expression or ERK1/2 phosphorylation (Fig. 7a, left and right). This suggests that activation of integrins by Notch is potentiated by R-Ras.

To demonstrate a specific role for R-Ras in Notch-mediated integrin activation we used a dominant-negative R-Ras mutant, R-Ras(T43N) (52). Transfection of R-Ras(T43N) alone had a minimal suppressive effect on integrin affinity; however, co-transfection of R-Ras(T43N) with NIC blocked the ability of active Notch to increase integrin affinity (Fig. 7b, left). Furthermore, R-Ras(T43N) prevented NIC from reversing H-Ras(G12V)-mediated integrin suppression but did not affect integrin suppression when co-transfected with H-Ras(G12V) alone (Fig. 7b, left). These effects occurred without alterations in expression of H-Ras, Notch, or dominant-negative R-Ras (Fig. 7b, right). In addition, transfection of R-Ras(T43N) did not alter integrin expression in the αβ-β-py cells or H-Ras stimulation of ERK1/2 phosphorylation (Fig. 7b, right).

To determine whether Notch signaling can directly activate R-Ras, we used the Ras-binding domain of Raf, linked to glutathione S-transferase, to pulldown GTP-bound (active) R-Ras from CHO cell lysates (38). We found that transfection of NIC or NAE stimulated an increase in active GTP-bound R-Ras in αβ-β-py cells, in comparison to wild type R-Ras alone, without affecting R-Ras expression levels (Fig. 7c, left). Furthermore, ΔTADP, which did not activate transcription, was also able to activate R-Ras (Fig. 7c, right). However, the Notch mutant NAE(V1746K), which did not yield detectable intracellular Notch, or ΔANK, which lacked the ankyrin repeats, were not able to stimulate an increase in active R-Ras (Fig. 7c, left and right). These results indicate that the intracellular domain of Notch can specifically activate R-Ras in CHO cells and this requires the ankyrin repeats but not CSL-dependent transcription. These results demonstrate that the ability of Notch signaling to regulate integrin affinity is mediated through R-Ras.

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FIGURE 6. Notch activation of integrins requires the ankyrin repeats. a, the effect of Notch mutants ΔEN1 and ΔANK (1 μg) on H-Ras(G12V) (0.375 μg)-mediated integrin suppression (mean ± S.E., n = 4 independent experiments). Representative immunoblot of Notch, H-Ras(G12V), and β actin expression. b, the effect of Notch mutants ICN1, ΔTADP, and ΔRAM (1 μg) on H-Ras(G12V) (0.375 μg)-mediated integrin suppression (mean ± S.E., n = 5 independent experiments). Representative immunoblot of Notch, H-Ras(G12V), and β actin expression. c, the effect of ICN1, ΔEN1, ΔRAM, ΔTADP, and ΔANK (1 μg) on CSL-luciferase activity (mean-fold increase compared with empty vector ± S.E., n = 3 independent experiments). Statistically significant differences between results are indicated by * (p < 0.05).
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**FIGURE 7.** Notch activation of integrins is mediated by R-Ras. a, left, the effect of NIC (0.25 µg) and R-Ras(WT) (0.5 µg) on H-Ras(G12V) (0.375 µg)-mediated integrin suppression in αβ-py cells (mean ± S.E.). Right, representative immunoblot of NIC, H-Ras(G12V), R-Ras(WT), β3 integrin, ERK2, phospho-ERK (p-ERK), and β actin expression (n = 4 independent experiments). b, left, the effect of R-Ras(T43N) (1 µg) on NIC (1 µg) reversal of H-Ras(G12V) (0.375 µg)-mediated integrin suppression in αβ-py cells (mean ± S.E.). Right, representative immunoblot of NIC, H-Ras(G12V), R-Ras(T43N), β3 integrin, ERK2, phospho-ERK (p-ERK), and β actin expression (n = 4 independent experiments). c, left, the effect of Notch mutants, NIC, NΔE, and NΔE(V-K) (1 µg), on R-Ras activation in αβ-py cells. Right, the effect of Notch mutants, ΔTADP and ΔANK (1 µg), on R-Ras activation in αβ-py cells. Representative immunoblots of Myc-tagged active R-Ras (from Ras binding domain of Raf), total R-Ras (from whole cell lysates), and Notch (n = 4 independent experiments). Statistically significant differences between results are indicated by * (p < 0.05). NS indicates no significant difference.

of K562 cells with NIC significantly increased adhesion to fibronectin in comparison to empty vector (Fig. 8a). Similar increases in cellular adhesion were observed with ICN1 and ΔTADP (results not shown). Adhesion of K562 cells transfected with either Notch or vector was significantly blocked by prior incubation with the β1 integrin-blocking antibody 4B4 (Fig. 8a). Furthermore, co-expression of dominant-negative R-Ras blocked the effect of NIC on adhesion of K562 cells to fibronectin (Fig. 8b). This occurred without alterations in Notch or β1 integrin expression. We confirmed that NIC activated Notch signaling in K562 by real time PCR measurement of HES-1 expression and that HES-1 expression was unaffected by co-transfection of dominant-negative R-Ras (Fig. 8c). These results indicate that active Notch signaling can regulate natively expressed integrin affinity through R-Ras activation.

K562 cells express Notch-1 receptors in the undifferentiated state and previous data has shown that Notch signaling can be activated in vitro by recombinant Notch ligand coated to tissue culture plastic (54, 55). Human recombinant Dll-4 significantly increased adhesion of K562 cells to fibronectin (Fig. 7d, right). This adhesion was blocked by EDTA and increased by Mn²⁺ (Fig. 8d). Dll-4 increased HES-1 expression in K562 cells and this was prevented by MW167 (Fig. 8e). Crucially, pretreatment of K562 cells with MW167, or preincubation with the β1 integrin blocking antibody 4B4, prevented Dll-4 from increasing adhesion to fibronectin (Fig. 8f). Furthermore, Dll-4 induced activation of α5β1 integrins was blocked by transfection of dominant-negative R-Ras (Fig. 8g). These data indicate that ligation of Notch receptors by naturally occurring Notch ligands can activate β1 integrins increasing cellular adhesion to ECM through R-Ras.

**DISCUSSION**

We show here that Notch-1 signaling in mammalian cells activates β1 integrins. Furthermore, we demonstrate the physiological relevance of our findings by showing that ligation of native Notch-1 receptors by Notch ligand increases cellular adhesion to fibronectin through α5β1 integrins. Crucially our data provides a mechanism for previous observations demonstrating key interactions between Notch and integrins in somitogenesis and vascular development.

Somitogenesis is the process whereby segmented precursors of the skeletal muscle and vertebral column are generated during vertebrate embryogenesis (32). Mutations in zebrafish integrin α5 disrupt anterior somite formation, giving a complementary phenotype to the posterior defects seen in Notch pathway mutants (31). Analysis of integrin/notch double mutants revealed redundancy between integrin and Notch signaling in K562 by real time PCR measurement of HES-1 expression and that HES-1 expression was unaffected by co-transfection of dominant-negative R-Ras (Fig. 8c). These results indicate that active Notch signaling can regulate natively expressed integrin affinity through R-Ras activation.

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pathways in promoting somite border morphogenesis, suggests a regulatory relationship between Notch and integrins (32). Our data demonstrates that Notch regulates integrin affinity and provides a mechanism for this interaction that is crucial to vertebrate development.

Genetic studies in mice have suggested that Notch signaling is important for formation of the vasculature. Mutations in Jagged-1, Notch-1, and Notch-1/Notch-4 in mice result in embryonic lethality with severe vascular defects (56). Genetic analyses also indicate a central role for integrins in vascular development, antagonists of β1 integrins inhibit angiogenesis in the chick chorioallantoic membrane, and Notch 4 increases endothelial cell adhesion preventing endothelial sprouting (30). Notch may therefore regulate vascular development through interaction with integrins. Interestingly, R-Ras knockout mice demonstrate defects in angiogenesis in response to vascular injury and tumor growth (57). We provide direct evidence of a link between Notch and β1 integrins involving R-Ras activation that may represent a central mechanism for regulation of vascular development and angiogenesis. Notch-1 and DLL-4 expression on vascular endothelial cells is regulated by vascular endothelial growth factor and thus our findings of integrin activation by DLL-4 may be particularly relevant (5).

We showed that Notch-1 overrider H-Ras-mediated integrin suppression via R-Ras activation. Studies of vulval development in Caenorhabditis elegans and the Drosophila eye have indicated important interactions between Notch and Ras (58, 59). The Notch pathway can antagonize Ras signaling (60, 61). Inhibitory cross-talk between Notch and H-Ras regulates Drosophila bristle patterning, C. elegans vulva formation, and may be important in T-cell positive selection (62–64). These interactions occur in part because each pathway can affect the expression or activi-
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ties of components of the other pathway (65). Our data provides a new mechanism for Notch antagonism of H-Ras through activation of R-Ras. This does not require CSL/transcription and may therefore allow for rapid changes in cellular signaling in response to interaction with Notch ligands expressed on adjacent cells. The importance of this signaling cascade in worms and insects remains to be demonstrated as these organisms have a simpler repertoire of small GTP-binding proteins and therefore may lack the essential signaling components (i.e. R-Ras) for this interaction (66).

The mechanisms by which R-Ras might antagonize H-Ras functions have not yet been defined. Previous work has demonstrated that reversal of H-Ras-mediated integrin suppression by R-Ras is not due to competition for downstream effectors or Raf-induced MAP kinase activation (36). Furthermore, specific mutations in the effector binding loop of R-Ras impaired the ability of R-Ras to reverse H-Ras-mediated integrin suppression but did not correlate with their ability to activate known R-Ras effectors (67). Recent data has indicated that targeting of R-Ras to focal adhesions at the cell surface is critical for its ability to regulate integrin activation (68). Interestingly H-Ras has also been found to be associated with focal adhesions and data has suggested that Notch can interact with β1 integrins involving lipid rafts (29, 69). Therefore, a better understanding of the interaction between Notch, the small GTPases, and integrins at the cell surface may help to explain crucial processes in integrin activation and Notch antagonism of H-Ras.

Our data indicates that Notch-1-mediated integrin activation requires cleavage of the Notch receptor at S3 to release the intracellular domain but is independent of CSL transcription. Importantly we showed that integrin activation by Notch is not inhibited by MINT, which prevents Notch binding to CSL (34). CSL-independent Notch signaling has been suggested by previous analysis of Notch mutant phenotypes in Drosophila, two gain of function classes of notch alleles were defined that prevent development of sensory bristles, one of which is not rescued by removing Su(H) (19). In vivo evidence for CSL-independent signaling in vertebrates was provided by experiments examining Delta-1-activated signals in the developing avian neural crest (70). In vitro studies have shown that Notch can prevent differentiation of C2C12 mouse myoblasts into myotubes. Myoblast differentiation is not blocked by a dominant negative CSL protein and can be mimicked by truncated forms of Notch, which cannot activate a CBF-1-dependent promoter (48); Notch mutants consisting of the TAD/PEST region were unable to activate transcription but this could be rescued by co-expression of the ankyrin repeat domain. Our data concurs with these genetic and in vitro studies. In addition we show that the ankyrin repeats have a previously undefined function not related to CSL binding. Therefore analysis of ankyrin repeat point mutations should enable dissection of Notch CSL-dependent and -independent signaling pathways.

In conclusion, the data presented in this paper demonstrates clear evidence for a new Notch signaling pathway. We show that ligand-induced cleavage of the Notch-1 receptor at S3 releases the Notch intracellular domain. This activates R-Ras, in a CSL-independent manner involving the ankyrin repeats, antagonizes H-Ras-mediated integrin suppression, and increases integrin affinity. Activation of Notch could enable a cell to respond to its environmental context, which may be particularly important in the control of stem cell fate decisions, as these cells express high levels of Notch-1, β1 integrins, and are frequently found in an ECM-rich niche (28). Thus, our data defining an interaction between Notch, the Ras family of GTPases, and β1 integrins adds a new level of complexity to the Notch signaling pathway and has important implications for the future understanding of development and disease.

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