Notch, Epidermal Growth Factor Receptor, and β1-Integrin Pathways Are Coordinated in Neural Stem Cells

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Notch1 and β1-integrins are cell surface receptors involved in the recognition of the niche that surrounds stem cells through cell-cell and cell-extracellular matrix interactions, respectively. Notch1 is also involved in the control of cell fate choices in the developing central nervous system (Lewis, J. (1998) Semin. Cell Dev. Biol. 9, 583–589). Here we report that Notch and β1-integrins are co-expressed and that these proteins cooperate with the epidermal growth factor receptor in neural progenitors. We describe data that suggests that β1-integrins may affect Notch signaling through 1) physical interaction (sequestration) of the Notch intracellular domain fragment by the cytoplasmic tail of the β1-integrin and 2) affecting trafficking of the Notch intracellular domain via caveolin-mediated mechanisms. Our findings suggest that caveolin 1-containing lipid rafts play a role in the coordination and coupling of β1-integrin, Notch1, and tyrosine kinase receptor signaling pathways. We speculate that this will require the presence of the adequate β1-activating extracellular matrix or growth factors in restricted regions of the central nervous system and namely in neurogenic niches.

Integrins and extracellular matrix (ECM) molecules play crucial roles during embryogenesis (2) in mesoderm development, epithelial morphogenesis, neural tube closure, anchorage to the ECM basal lamina, and central nervous system (CNS) development. In particular, β1-integrins (αβ1) are highly expressed in embryonic stem (ES) cells (3) and in neural stem cells (NSC) (4) and are required for cortical development (5).

Notch1 is a cell surface protein involved in the control of cell fate choices in the developing CNS (1). This transmembrane receptor is involved in stem cell maintenance (6) and promotes glial and neural fates in a stepwise manner, first by inhibiting neuronal fate and promoting glial fate and second by promoting astrocyte differentiation (7). Notch1 also plays a role in the control of neurite extension in mammalian cells and in axon growth in Drosophila (8–10). Interestingly, in the immune system Notch1 serves two biologically contrasting functions; it is responsible for the apoptotic cell death of B lymphocytes (11), whereas it promotes the survival of T cells (12). The diverse effects of Notch1 activation observed in multiple cell types and at different stages of development suggest the presence of context-dependent control mechanisms. Growth factors (GF) and ECM molecules (acting through integrins) belong to the complex environment that surrounds NSC during development (13) and that affect Notch signaling. For example, FGF-1 and -2 inhibit neural differentiation by affecting (directly or indirectly) the Notch pathway (14), and EGFR activation leads to Notch signaling during pancreas tumorigenesis (15). Integrins may also be involved in the Notch response during angiogenesis, when Notch4-expressing endothelial cells display β1-integrin in an active, high affinity conformation (16). Furthermore, in zebrafish the boundary cells between developing somites behave differently depending on the levels of Notch activation, and it has been suggested that the extracellular matrix (which differs at the rhombomere boundaries) plays a role in this process (17). Nevertheless, the coordination between β1-integrin, Notch1, and GF pathways is poorly understood.

Lipid rafts are special membrane regions that affect signaling by sorting proteins and lipids into specific membrane domains, where privileged interactions occur. Caveolae are specialized lipid rafts that contain cholesterol, sphingolipids, and caveolins (22–24-kDa membrane proteins, required for the formation of the caveolae) and that serve as scaffolds for signaling molecules.

In this paper, we explore how some of the receptors for ECM and GF (that are present on the surface of the NSC) act together with the Notch1 pathway to control the NSC responses to changes in the microenvironment. We discuss the possibility that lipid rafts may play important roles in directing the changes in signaling and the responses to environmental changes that occur during cortical development and may act as integrators of parallel and simultaneous signals originated from integrins, growth factors, and Notch receptors. We conclude that the GF and ECM composition of biological neural stem cell “niches” may affect NSC maintenance and differentiation by affecting Notch signaling, in a context- and time-dependent manner.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—FGF-2 was obtained from PeProtech, EGF was from Calbiochem, and B27 supplement was from Invitrogen. Antibodies for immunoprecipitation-blocking experiments and Western blots were obtained from Chemicon and Pharmingen (anti-β1 integrins), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (EGFR), Upstate Biotechnology, Inc. (Lake Placid, NY) (EGFR), and Cell Signaling Technology (phosphorylated MAPK and MAPK). Antibodies used for immunohistochemistry included polyclonal Notch1 and caveolin 1 (Santa Cruz Biotechnology), monoclonal anti-Nestin (Pharmingen), and monoclonal anti-β III tubulin (Sigma). All fluorescent secondary antibodies were obtained from Jackson Immunoresearch or Molecular Probes, Inc. (Eugene, OR). The EGF receptor inhibitor AG1478 (Calbiochem) was used at 20 μM. Mixtures of protease and phosphatase...
Western Blots and Immunoprecipitations—For Western blotting, neurospheres or ES cell-derived NSC were lysed (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl buffer, 1% Triton X-100) in the presence of protease and phosphatase inhibitors (5 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 2 mM sodium fluoride, 2 mM sodium vanadate, all from Sigma) or the equivalent Calbiochem mixtures. The supernatant was clarified by centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentrations were determined with a Bio-Rad protein assay with bovine serum albumin as a standard, and equal amounts of protein were loaded in each well. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond-C, Amersham Biosciences). Membranes were blocked in 10% nonfat dry milk in Tris-buffered saline for 1 h at room temperature. Blots were then incubated with the primary antibodies overnight at 4°C in milk/Tris-buffered saline containing 0.1% Tween 20 (TBS-T), followed by a 2-h incubation with the appropriate secondary peroxidase-conjugated antibody (Amersham Biosciences). Blots were developed using ECL reagents (Amersham Biosciences), following the manufacturer’s instructions (Amersham Biosciences). For immunoprecipitations, the samples were lysed as previously described. To remove nonspecifically binding proteins, 150–200 μg of proteins were preclarified by mixing with agarose beads (A/G plus; Santa Cruz) for 30 min at 4°C. The samples were then incubated with the adequate antibody in the presence of fresh agarose A/G beads, either at 4°C overnight or at room temperature for 2 h on a rotating platform. The beads were then washed and boiled for 10 min in Laemmli loading buffer. Equal amounts (as measured by protein assay) were loaded on 10% SDS-polyacrylamide gels and processed for immunoblotting as previously described.

Raft Isolation—Isolation of rafts was performed using sucrose gradients, as previously described (25). Briefly, neurospheres were placed on ice and suspended for 30 min in 0.2 ml of extraction buffer: 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and a mixture of protease and phosphatase inhibitors (Calbiochem). Cell lysates were then adjusted to 40% OptiPrep and overlaid with solutions of 30 and 10% Optiprep in the extraction buffer. These gradients were centrifuged for 16 h at 35,000 rpm at 4°C in an SW50.i rotor (Beckman Instruments). Fractions of equal volume, including the raft (floating fraction) and nonraft (bottom fraction) fractions, were collected and analyzed by SDS-PAGE (10%), followed by immunoblotting. Protein assays were performed on all fractions before immunoblotting to ensure equal loading.

Reverse Transcription-PCR—RNA was extracted using the RNAeasy kit (Qiagen), and 0.1 μg of RNA from each sample was used to generate cDNA using the transcriptor first strand cDNA synthesis kit (Roche Applied Science). Reverse transcription-PCR was done using the published primers for Hes5 and glyceraldehyde-3-phosphate dehydrogenase and following the conditions described by Zine et al. (26)
FIGURE 1. β1-integrins and Notch1 co-localize in the developing VZ and in neurospheres. Notch1 and β1-integrins are detected in the VZ during mouse gestation (4, 32) and both play important roles in neural stem cell control. Ligands for the Notch1 and β1-integrins (delta 1 and laminin, respectively) are available in the VZ, and their expression levels change in time (4, 76). High levels of β1-integrins (red, C) and Notch1 (green, B) are present in the embryonic day 12.5 mouse VZ. Note that Notch1 (green, B) and β1-integrin (red, A) are co-expressed in the VZ (yellow, D). Note the lack of overlap in blood vessels (arrow, C and D). NSC can be cultured from embryonic or postnatal CNS tissue (19, 77) in suspension cultures that give rise to spheroid structures (neurospheres) that contain NSC at the edge (29), where these cells express high levels of Notch1 (29) and lex/SSEA (33). Notch1 is expressed in sectioned neurospheres (E) together with β1-integrin (F), and they partially overlap (yellow, G), predominantly at the edge. The edge of neurospheres contains a nestin-rich cell population, which expresses EGFR (see Fig. 2) and β1-integrins (4). Neural progenitors derived from ES cells express radial glial markers, Notch1, and β1-integrin; ES cells can be driven toward a neural progenitor fate using diverse protocols (22, 53, 55) and also give rise to RGC (21) that express RGC markers. Taking into account that RGCs are now generally accepted to be NSC (34, 78), the ES cell-derived neural progenitors can be considered to be an NSC population that can be readily grown and maintained in large numbers to analyze complex pathway interactions in NSC. The ES-derived neural progenitors express high levels of Notch1 receptor (H) and β1-integrins (I), like the mouse embryonic SVZ and the edge of primary neurosphere cultures. Note that Notch1 and β1-integrins expression patterns overlap in the ES cell-derived NSC (J, yellow). The ES cell-derived NSC cultures are therefore suitable to study, in vitro, the cooperation between β1 and Notch1 pathways, both of which are known to be crucial for NSC and RGC maintenance and development (4, 31, 35). RC2 (K) and GLAST (L) are also expressed by ES cell-derived NSC.

E–G (4, 29, 33) and in NSC/radial glial cell cultures, derived from ES cells (Fig. 1, H–J). The observation that both proteins are simultaneously expressed in neural progenitors and that their expression overlaps raises the hypothesis that they may cooperate or act in a coordinated fashion. To further test the hypothesis that β1-integrins and Notch pathways interact in neural progenitors/NSC, we used primary neurospheres and ES cell-derived NSC, the later providing an NSC-enriched population positive for RGC markers (Fig. 1, A and L) (21) (currently accepted to be NSC (34)). Notch1 is highly expressed in the ES cell-derived NSC cultures and co-localizes with the β1-integrin (Fig. 1, H–J). The ES cell-derived NSC cultures are therefore suitable to study, in vitro, the cooperation between β1-integrin, growth factors, and Notch pathways, all of which are known to be crucial for NSC and RGC maintenance and development (4, 31, 35).

β1-Integrin and Growth Factor Receptors Are Required for Secondary Neurosphere Generation—To test the role of β1-integrin in NSC, we treated primary neurospheres with morpholino antisense oligonucleotides against β1-integrin to decrease the β1 subunit protein levels in EGF- or FGF-2-grown cells, prior to secondary neurosphere formation assays (see “Experimental Procedures”). The decrease in β1-integrin was confirmed by Western blot (Fig. 2E). Spheres treated with antisense or missense (control) morpholino oligonucleotides were dissociated and tested for their capacity to form new spheres (secondary neurosphere formation assay; see “Experimental Procedures”). These experiments show that a decrease in β1-integrin is associated with a moderate decrease in secondary neurosphere formation (Fig. 2, A–D). Interestingly, in EGF-grown (A and B) and FGF-2-grown (C and D) cells, the decrease in secondary neurosphere formation is more significant at low EGF levels (2 ng/ml; Fig. 2B) than at higher EGF levels (20 ng/ml; Fig. 2A), suggesting that in the presence of high EGF levels, the cells are less dependent on β1-integrin to maintain adequate levels of proliferation or survival. After morpholino treatment, the decrease in secondary neurosphere formation in FGF-2-grown cells (Fig. 2, C and D) is already apparent at high levels of FGF-2 (20 ng/ml; Fig. 2C) when compared with spheres grown with low levels of FGF-2 (2 ng/ml; Fig. 2D).

EGFR and β1-integrin interactions have been extensively demonstrated in three-dimensional breast culture systems (36), and it is conceivable that the high levels of EGFR found on the nestin-positive FGF-2-grown spheres (Fig. 2, F–H) may be responsible for the more acute response to a decrease in β1-integrin observed in FGF-2-grown cells. To test this hypothesis, spheres grown in both EGF and FGF-2 were used in a secondary neurosphere formation assay in the presence of FGF-2 and an EGFR inhibitor, AG1478 (20 μM). The exposure to AG1478 resulted in a sharp decrease in the number of secondary neurospheres formed, indicating that even for FGF-2-grown spheres, the EGFR is the crucial pathway involved in proliferation (Fig. 2), as indeed suggested by the high levels of EGFR found on the FGF-2-grown cells (Fig. 2, G and H). Consequently, in FGF-2-grown spheres with decreased levels of β1-integrin (EGFR-strongly positive/β1-depleted), a lack of exposure to EGF...
will be severely felt (despite the high levels of EGFR expression) and cannot be compensated by (lacking) integrin activation. In the EGFR-grown spheres (EGFR-positive/β1-depleted), even low levels of EGFR will be enough to respond to the EGFR in the medium. These results suggest that β1-integrin may be important for EGFR activation in neurospheres and point toward a potential cooperation between the two pathways, as already described for epithelial cells and fibroblasts (37, 38). Furthermore, it was recently shown that a decrease in β1-integrin causes a reduction in neurosphere size during secondary neurosphere formation assays, due to reduced progenitor proliferation and increased cell death (39). The loss of β1-integrin in neurospheres also reduces the number of nestin-positive cells in a growth factor-dependent manner, and this phenotype can be rescued by exposing the cells to high growth factor levels (39). This result is consistent with our morpholino experiments, and both may be explained by the signaling confluence of EGFR and β1-integrins toward the MAPK pathway. In fact, in the absence of β1-integrins, the signaling through the MAPK pathway may become more dependent on the presence of EGFR in the medium (4). Interestingly, we have also observed that the addition of EGF and FGF-2 to starved neurospheres leads to an increase of the detectable levels of NICD expression by Western blot (Fig. 2I). Other authors have observed that growth factors, such as ciliary neurotrophic factor, increase NICD expression levels (40) or affect Notch activation (15, 41). Taking into account that β1-integrins modulate the response to GF in neural progenitors (39), we raise the hypothesis that the GF effects on the levels of NICD could be partially dependent or coordinated with the activation of β1-integrins. How the β1-integrins, GF receptors, and Notch signaling pathways are coordinated in neural progenitors remains to be elucidated, and we suggest that special membrane domains may be involved in the coordination of these pathways interactions, cross-talks, and sequential and/or simultaneous effects.

Caveolin 1, a Lipid Raft-resident Protein, Is Present on Neurospheres, on ES-derived NSC/RGC, and in the Embryonic VZ—Lipid rafts are membrane domains that act as privileged signaling platforms (42), where interactions and cross-talk between different signaling pathways may take place. Recently, lipid rafts were found to be present on neuroepithelial progenitors, where they play a role in signal transduction (43). We reasoned that the embryonic VZ, the ES-derived NSC and primary neurospheres could also contain lipid rafts. Using Western blots and immunofluorescence, we confirmed the existence of caveolin 1 (a component of lipid rafts) on ES-derived NSC, in the embryonic VZ, and on neurospheres (Fig. 3, A–H).

Notch1 is Present on Caveolin 1-enriched Fractions Isolated from Neurospheres, and ES-derived NSC and GF Can Mobilize NICD Out of the Caveolin-containing Fractions—Lipid rafts may compartmentalize and direct signal transduction at the plasma membrane (44–46). The
context-dependent modulation of Notch (or other receptors) may require the simultaneous activation of (or the interaction with) other membrane receptors, like β1-integrins, promoted by specific signaling platforms like the lipid rafts, in a temporally and spatially controlled manner. Rafts can be isolated within detergent-insoluble glycosphingolipid-rich microdomains by density gradient centrifugation at 4 °C (47). We examined the distribution of Notch1 in Optiprep density gradient fractions of Triton X-100 extracts prepared at 4 °C. We reasoned that changes in the protein levels in rafts (membrane compartments of specific lipid composition that are privileged for receptor interactions and act as signaling platforms (42, 48)) are more relevant than the overall changes in expression and allow for subtle changes to be detected in functional fractions. Lipid rafts are insoluble in Triton X-100 and float at the 10–30% interface of the density gradient, and we used the presence of caveolin 1 to confirm that the raft fraction had been correctly identified. Note that we also observed that caveolin 1 (a raft-resistant protein) is present on the cells located at the edge of neurospheres (Fig. 3C) in ES-derived NSC (Fig. 3F) and in the embryonic VZ (Fig. 3B). To test the effect of growth factors on neural progenitors, we starved (overnight) intact EGF- and FGF-2-grown neurospheres and then added EGF or FGF-2, respectively (20 ng/ml), for 24 h. Using intact neurospheres ensured that only the cells located at the edge (enriched in β1-integrins, EGFR, and Notch1 (29)) were exposed to the changing environment.

The lysates from spheres treated in this manner were centrifuged on Percoll gradients, and the resulting fractions were analyzed for the presence or absence of Notch1 protein in caveolin 1-containing membrane compartments, with or without growth factor activation (Fig. 4, A and B). When EGF is added to starved EGF-grown neurospheres (Fig. 4A, EGF panel), an increase in NICD is detected in the nonraft fraction (lane 1) at the expense of the caveolin 1-positive fractions (lanes 2–5 in Fig. 4B). Likewise, FGF-2 produces the same effect when added to starved FGF-2-grown neurospheres (Fig. 4A, FGF-2 panel).

Taking into account that β1-integrins modulate the response to GF in neural progenitors (39), we raised the hypothesis that the GF effects on the levels of NICD could be partially mediated by β1-integrins. If so, activation of β1-integrins should also lead to activation of the Notch pathway.

In Neurospheres, the Activation of β1-Integrins with Mn2+ Mobilizes NICD out of the Caveolin-containing Fractions—To further characterize the mechanisms involved and to challenge the role of β1-integrins, we stimulated β1-integrins in intact spheres and in ES-derived neural stem cells, using manganese (Mn2+). Mn2+ is a divalent cation known to activate β1-integrins (49) and to induce the redistribution of LFA-1, αβ1 (50), or αβ1 (47) into lipid rafts. Whereas no significant change in Notch1 distribution occurred after Mn2+ treatment in EGF-grown spheres, Mn2+ leads to the redistribution of Notch from caveolin-positive to caveolin-negative fractions in FGF-2-grown neurospheres (Fig. 4A, FGF-2 panel). The specificity of this effect on neurospheres was analyzed by exposing the suspension cultures to a β1-integrin blocking antibody (Ha2/5), prior to Mn2+ exposure (Fig. 4C). We observed that preincubation of the starved neurospheres with the blocking β1-integrin antibody decreases the Notch mobilization induced by the addition of Mn2+ (Fig. 4) in both EGF- and FGF-2-grown NS cultures, with a greater proportion of NICD remaining in the caveolar compartment. When combined with our β1-integrin loss of function (morpholino) data, these results suggest that a decrease in β1-integrin may affect neurosphere formation assays by altering Notch 1 processing/transfer to the nuclear fraction and thus preventing its downstream proliferative actions. Interestingly, integrin-mediated adhesion has been proposed to govern the presence of cholesterol-enriched microdomains or lipid rafts on the plasma membrane by controlling internalization via a caveolin-dependent pathway (51). Together with our results, these experiments raise the possibility that β1-integrins play a role in the growth factor-modulated transfer of NICD between membrane compartments and/or into the nucleus, possibly via lipid rafts.

β1-Integrin Activation with Mn2+ Leads to Movement of NICD into the Nucleus in ES-derived NSC—To test whether the activation of β1-integrins affects Notch internalization, we exposed cells to Mn2+ (a divalent cation known to activate β1-integrins (49)) and evaluated the movement of NICD to the nucleus, by immunocytochemistry. Mn2+ is known to activate β1-integrins and to induce the redistribution of β1-integrins into lipid rafts. Using an antibody specific for NICD (raised against an epitope exposed only after cleavage), we observed that β1-integrin activation with manganese led to a shift of NICD into the nucleus, detectable by immunohistochemistry (Fig. 5). This experiment was technically less demanding to do on monolayers of ES-derived NSC than on three-dimensional neurospheres, where the cell-cell contacts and large amounts of ECM present account for activation of integrins, even when GF levels are low.

β1-Integrin Activation with Mn2+ Is Followed by Changes in HES5 mRNA Expression—To study the effects of β1-integrin activation on the Notch1 downstream pathway, we used Mn2+ to induce a change in the β1-integrin conformation and activation state, as previously described.
FGF-2 is added to FGF-2-starved neurospheres. This suggests that the addition of growth factor stimulates Notch1 transfer between compartments.

The NICD is mobilized from a caveolin-positive fraction (49). Other molecules known to activate integrins were also used, namely ECM molecules (laminin 1, laminin 2, and fibronectin) and EGF. It is noteworthy that fibronectin and laminin can cause EGFR phosphorylation, through β1-integrin activation (37, 38, 52), and EGF may also activate β1-integrin, through cross-talk between the EGFR and β1-integrin. Cells grown in EGF and FGF-2 were starved overnight and then stimulated with Mn²⁺ or EGF, or ECM for 3 h. Changes in HES5 mRNA expression were evaluated by reverse transcription-PCR (Fig. 5). This experiment revealed that Mn²⁺, EGF, EGF + FGF-2, and ECM all lead to HES transcription, whereas FGF-2 alone does not (Fig. 5). The GF response pattern suggests that β1-integrin activation may affect Notch activation and HES transcription through the EGFR, which can be activated through β1-integrin stimulation by ECM ligands or EGF but not by FGF-2 alone. In fact, recent evidence shows that fibronectin and laminin can cause EGFR phosphorylation through β1-integrin activation (52).

DISCUSSION

In this paper, we show that Notch1 and β1-integrins are co-expressed in ES-derived NSC, neurospheres, and in the mouse embryonic VZ. We
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FIGURE 5. β1-integrin activation in ES cell-derived NSC leads to movement of NICD into the nucleus and to changes in HESS mRNA expression. To evaluate if the NICD mobilization leads to movement into the nucleus, ES cell-derived NICD were starved overnight and then exposed to Mn²⁺. NICD was detected using a polyclonal antibody that is specific for the intracellular portion of Notch1, released after the second cleavage. A–D show the control cell expression of β1-integrin (A and I in red), Notch1 (B and J in green), nuclear DAPI (C, blue in K), and a merged image (D and L) when the cells are not exposed to Mn²⁺. Note that Notch1 is detected mainly in the cytoplasm (B and D). E–H show the same stainings in cells that were starved overnight and then exposed to Mn²⁺: β1-integrin (E and M in red), Notch1 (F and N in green), and nuclear DAPI (G and O in blue) and a merged image (H and P). Note that NICD is detected in both the cytoplasm and the nucleus in F and H (compare B and F). Note also that in dividing cells, Notch1 and β1-integrin overlap (yellow; arrow in J). This experiment was technically less demanding to do on monolayers of ES cell-derived NSC than on three-dimensional neurospheres, where the cell-cell contacts and large amounts of ECM present may account for activation of integrins, even when GF levels in the medium are low. Furthermore, these cultures represent faithfully the radial glial cell/NSC population (79). In this paper, we generate a population of neural progenitors/NSC that express radial glial markers, β1-integrins, and Notch1 by exposing ES cells, sequentially, to a variable environment. This culture system can be used to study the underlying mechanisms of neural development. In particular, changes in protein signaling that occur during NSC generation and differentiation can be analyzed in these cells, and the context-dependent effects of proteins such as Notch are more amenable to analysis using the ES-derived NSC cultures than using the heterogeneous neurosphere and VZ explant cultures.

β1-Integrins, ECM, and GF Participate in a Complex Network of Interactions That Affect NSC Behavior—The proteins that constitute the neurogenic niche participate in more than one regulation loop. For example, during development, FGF signaling plays a role in neural and mesodermal cell induction, mediated by Ets and GATA transcription factors (58). FGF also promotes changes in the cell responsiveness to the environment by increasing the expression of β1-integrins, laminin, and EGFR on neural progenitors (4, 23, 24, 59, 60). Furthermore, FGF-2 affects Notch signaling (61) and regulates neuronal differentiation by poorly defined interactions with Notch (14). Taken together, these observations suggest that FGF-2 increases the levels of receptors and ligands and prepares the NSC to become responsive to subsequent waves of growth factors and to a changing ECM. Interestingly, two of

report that the Notch pathway cross-talks with the β1-integrin pathway, as indicated by the interaction detected between the NICD fragment and the β1-integrins. Furthermore, GF (EGF and FGF-2) cause an increase of NICD levels, and activation of β1-integrins with Mn²⁺ induces NICD internalization, via caveolin-1-enriched rafts, with the appearance of the NICD fragment in the nucleus. These results suggest that the GF may play a role by enhancing the level of NICD, whereas the β1-integrins could modulate how much of it reaches the nucleus by regulating the internalization and travel through the endocytic compartments. Therefore, the β1-integrin may act dually, as an NICD buffering/sequestering system and as an internalization control, activated by extracellular cues. The ECM/GF environment could play an important modulatory role on NSC behavior through β1-integrins, which may act by controlling the nuclear availability of NICD in a context-dependent manner (possibly by playing a role in Notch internalization, through caveolin-1-containing membrane domains), making the Notch pathway modifiable by extrinsic factors such as GF and the ECM.

ES-derived Neural Progenitors Express β1-Integrins, Notch1, and Caveolin 1 Like the Progenitors Present in the VZ and in Neurospheres—Previously, several groups have described methods to generate neural progenitors/NSC, glial cells, and differentiated neurons from ES cells (53–57). In this paper, we generate a population of neural progenitors/NSC (that express radial glial markers, β1-integrins, and Notch1) by exposing ES cells, sequentially, to a variable environment. This culture system can be used to study the underlying mechanisms of neural development. In particular, changes in protein signaling that occur during NSC generation and differentiation can be analyzed in these cells, and the context-dependent effects of proteins such as Notch are more amenable to analysis using the ES-derived NSC cultures than using the heterogeneous neurosphere and VZ explant cultures.
the receptors up-regulated by FGF-2 (EGFR and β1-integrins) are
known to cross-talk in epithelial cells (36, 52), and manipulation of
either pathway can overcome deficits in β1-integrin or EGFR signaling
in NSC (39). Furthermore, in Drosophila, a dynamic interplay exists
between Notch and EGFR signaling, and both antagonistic and syner-
gistic/additive effects have been described (62, 63). In vertebrates, cross-

FIGURE 6. β1-integrins co-immunoprecipitate with NICD. A, Western blot with anti-Notch1 of
lysates prepared from 10 days in vitro neurospheres grown in FGF-2 (lane 1) or EGF (lane 2). Co-immu-
noprecipitation of β1-integrin and Notch1 lysates obtained from EGF- or FGF-2-grown neuro-
spheres; lysates from EGF- or FGF-2-grown neurospheres were used to immunoprecipitate (IP)
β1-integrin with a polyclonal rabbit anti-β1 antibody. The proteins were resolved on SDS gels,
transferred to membranes, and blotted for the presence of Notch1 using a goat polyclonal anti-
body. This experiment indicates Notch1 co-immunoprecipitates with β1-integrins. A band
with the size of 110 (cleaved Notch 1) is detected in lanes 4 and 5 (FGF-2-grown neurospheres) and in
lanes 7 and 8 (EGF-grown neurospheres). Note that it is the same band as the one detected in the
Western blot (prepared from aliquots of the same lysates; lanes 1 and 2). A larger band is also observ-
able on the blot, possibly a multiprotein complex. Lanes 3 and 6 are preclear controls, where the
lysate is incubated with beads only, to check the specificity of the Notch-β1 interaction, the reverse immunoprecipitation was done using a GST-NICD fusion protein (or GST protein alone, as a control) to pull down β1-integrins in lysates from
EGF-grown (B) or FGF-2-grown (C) neurospheres. The pool down with GST-NICD was followed by
blotting with a polyclonal antibody against α6β1 and reveals the adequate α6 band (B and C, top
arrowhead) and β1 band (B and C, lower arrowhead). EGF, EGF-grown spheres, FGF-2, FGF-2-
grown spheres, IP, immunoprecipitation. Role for a direct NICD-β1 interaction in the ventricular zone.
β1-Integrins are very abundant in dividing VZ cells. The arrows in D show cells that express high
levels of integrins, and arrows in E point at the dividing cells. Note the condensation of laminin
(laminin α2-rich) in the VZ (F, arrows), indicating that the adequate ligand for β1-integrins is avail-
able in the VZ. Furthermore, in the VZ, β1-integrins, and Notch1 expression overlap (see Fig. 1D).
We propose that in the presence of the adequate ECM or GF, β1-integrins in the VZ restrict the
movement of the cleaved NICD by tethering it to the membrane during symmetric (G) or asymmet-
ic (H) divisions. In both cases (G and H), this ensures that only cells anchored to the ECM pro-
cede to retain NSC characteristics (sustained self-
renewal, blockage of differentiation, and survival).
 Whereas in symmetric divisions (G) both cells are
anchored to the ECM or GF, in asymmetric divisions (H) the anchorage to the ECM is not equal, and the
retention of β1-integrins in the most apical cell conditions the relative availability of NICD in the
two daughter cells, ultimately affecting cell fate. Our co-immunoprecipitation data indicate that
this tethering occurs and that β1-integrins inter-
act with NICD (A–C). The overlap between β1-inte-
grins and actin markers like phalloidin (13) in the
VZ reinforces the hypothesis that β1-integrins help to anchor some crucial molecules and signaling
complexes in a polarized manner in the VZ. Laminin in G and H is depicted in orange and pre-
dominates along the ependymal ventricular sur-
face (as seen in F), whereas fibronectin is predom-
inantly expressed outside the VZ and toward the
pial surface of the developing neural tube. NICD,
Notch1 intracellular domain; ++ + NICD, high
levels of NICD; + NICD, lower levels of NICD; VZ, ven-
tricular zone.
Notch and β1-Integrin Interactions in Neural Stem Cells

talk between transforming growth factor β and Notch occurs (64), and EGFR activation leads to Notch signaling during pancreas tumorigenesis (15). These examples indicate that some functions of Notch may be context-dependent and could require complex interaction loops with other signaling pathways.

Direct interactions can also explain context-dependent signaling effects; our co-immunoprecipitation data (Fig. 6, A–C) suggest that β1-integrins (which are very highly expressed in the VZ, together with laminin 2; Fig. 6, D–F) may sequester the NICD fragment. This raises the possibility that the β1-integrin-associated Notch1 mobilization to the nucleus could be a context-dependent event, partially regulated by the extracellular environment. In fact, integrins can regulate Rac targeting by internalization of membrane domains, such as lipid rafts (65), and in Drosophila selective endocytic pathways are required for the Delta/Serrate/LAG-2 family to activate Notch (66, 67). Therefore, the role of lipid rafts and NSC. Lipid rafts may play a crucial role in controlling signaling in a spatial and temporal manner (68). Therefore, the role of lipid rafts and their biological significance in NSC could be to bring together in the same domain two proteins (Notch and β1-integrin) at defined developmental time points, allowing for interactions to occur between multiple signaling pathways or, alternatively, implementing direct physical interactions between the two proteins (Fig. 6, G and H). The integrative role of lipid rafts may help to explain the context dependence of receptor signaling. For example, whereas Notch1 and β1-integrins are both expressed in ES cells and ES-derived NSC, caveolin 1 is more abundant in the latter (data not shown), perhaps facilitating direct or indirect interactions between β1-integrin and Notch1 pathways, preferentially in neural progenitors.

Our results (Fig. 6, A–C) suggest that the cytoplasmic tail of the β1-integrin interacts (directly or as part of a protein complex) with the NICD fragment and alters the biological availability of the NICD fragment, modulating the amount that can reach the nucleus. The interaction between the cytoplasmic domains of Notch1 and β1-integrins suggests a speculative model, whereby direct linkage of the two proteins could be affected by the ligation state of the integrin. We propose that β1-integrins “mop up” excess free NICD (Fig. 6, G and H) under specific conditions (e.g. during cell division) in the presence of the adequate ECM-like laminin α2-containing laminins (which abounds in the ependymal surface of the VZ and may “anchor” the progenitor/NSC cells) or through cross-talk with tyrosine kinase receptors like the EGFR. This mechanism could be important to modulate the level of transcriptional regulation activity of NICD and to control effects on survival, proliferation, and differentiation. In turn, the “release” of the NICD fragment allows it to reach the nucleus to promote the maintenance of an undifferentiated fate (Fig. 6, G and H).

A model that relies on external factors to alter the equilibrium between bound and free intracellular signaling molecules has also been suggested for the regulation of β-catenin signaling, where a balance between bound and free levels of β-catenin is partially controlled by multiple interactions between cadherins and receptor tyrosine kinases (69). Likewise, proteins like Notch that depend on regulated intramembrane proteolysis for signaling (70) require effective mechanisms to control downstream signaling. We suggest that spatial control can be achieved by a “buffering system” that keeps levels of NICD balanced in the presence of the right ECM. The model we propose predicts that an adequate balance of free and bound NICD is maintained only in the cells that are in contact with the adequate matrix or in the presence of specific growth factors (Fig. 6, G and H), further highlighting the important role played by niches during neural development. The model predicts that changes in β1-integrin levels could lead to an imbalance in NICD levels. Interestingly, NICD overexpression in a chondrogenic cell line inhibits differentiation and decreases proliferation (71), and, likewise, a decrease in β1-integrin in chondrocytes causes diminished proliferation and changes in the G1/S transition and cytokinesis (72). Both of these observations may be due to an imbalance (increase) in free NICD, provoked by the NICD overexpression or by the decrease in β1-integrins, respectively. If changes in β1-integrin expression cause an imbalance in NICD levels, the increase in NICD availability that we observe when GF are added to the neural progenitor cultures may explain the proliferation rescue of the β1-integrin null cells by GF (39). Interestingly, the addition of GF to β1-integrin null cells also increases the number of nestin-expressing cells and decreases differentiation, an effect that could also be explained by activation of the Notch pathway.

An additional role for β1-integrin could be to provide survival signals (in a context-dependent manner that depends on the ECM composition) to counteract the potentially deleterious effect of excessive Notch activation. For example, it is known that in B lymphocytes, Notch1 induces cell cycle arrest and apoptosis (11), whereas in T cells Notch1 has an antiapoptotic function (12). Inhibition of Notch by Numb in the Drosophila serotonin lineage causes cells to differentiate, whereas cells that retain Notch signaling initiate apoptosis (73). Interestingly, the onset of mammary apoptosis in the mouse mammary gland coincides with a change of conformation between ligand-bound and unbound β1-integrin (74). We speculate that the equilibrium between apoptosis and survival in neural progenitors could be due to a balance between the levels of β1-integrins and the levels of NICD and therefore highly context-dependent. During cortical development, once the cells abandon the laminin-rich “VZ niche” (4) (Fig. 6, G and H), they encounter a different matrix and/or growth factor environment, which may favor noncanonical Notch biological roles in differentiation or cell death. This proposed model could also account for the context-dependent effects of Notch1 described for different malignant cells (75).

In summary, Notch1 is a cell surface protein involved in the control of cell fate choices in the developing CNS (1). Our data suggest that the Notch1 pathway is partially dependent on the integrin/ECM/GF environment. β1-integrins may act on Notch signaling through 1) physical interaction (sequestration) of the NICD fragment by the cytoplasmic tail of the β1-integrin, 2) by affecting trafficking of the NICD. The ligation state of β1-integrins could therefore “fine tune” Notch activation processing in a changing ECM and growth factor environment, resulting in differential effects on cell fate according to the microenvironment present at a given moment in time. The different roles attributed to Notch during cortical development, such as the role in the sequential generation of radial glia and of neurons (30, 31) or the later role of Notch in neurite extension (8), could be explained by a context-dependent modulation of the pathway, dependent on the ECM composition and GF availability (13). We conclude that, through a β1-dependent Notch1 pathway modulation, the ECM and GF in the immediate vicinity of NSC may participate in neural stem cell fate determination in neurogenic niches. Furthermore, our findings suggest that caveolin1-containing lipid rafts play a role in the coordination and coupling of β1-integrin, Notch 1, and TKR signaling pathways. We speculate that this will require the presence of the adequate β1-activating ECM or GF in restricted regions of the CNS and namely in neurogenic niches.
