Integrin-associated Lyn Kinase Promotes Cell Survival by Suppressing Acid Sphingomyelinase Activity*

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Integrins govern cellular adhesion and transmit signals leading to activation of intracellular signaling pathways aimed to prevent apoptosis. Herein we report that attachment of oligodendrocytes (OLs) to fibronectin via \( \alpha_3 \beta_1 \) integrin receptors rendered the cells more resistant to apoptosis than the cells attached to laminin via \( \alpha_5 \beta_1 \) integrins. Investigations of molecular mechanisms involved in \( \alpha_3 \beta_1 \) integrin-mediated cell survival revealed that ligation of the integrin with fibronectin results in higher expression of activated Lyn kinase. Both in OLs and in the mouse brain, Lyn selectively associates with \( \alpha_3 \beta_1 \) integrin, not with \( \alpha_5 \beta_1 \) integrin, leading to suppression of acid sphingomyelinase activity and preventing ceramide-mediated apoptosis. In OLs, knockdown of Lyn with small interfering RNA resulted in caspase activity and preventing ceramide-mediated apoptosis. In dendrocytes (OLs) to fibronectin via integrin receptors, Lyn selectively associates with \( \alpha_3 \beta_1 \) integrin, leading to suppression of acid sphingomyelinase activity and preventing ceramide-mediated apoptosis. In OLs, knockdown of Lyn with small interfering RNA resulted in OL apoptosis with concomitant accumulation of C16-ceramide. Knocking down ASMase partially protected OLs from apoptosis. In the brain, ischemia/reperfusion (IR) triggered rearrangements in the \( \alpha_3 \beta_1 \) integrin-Lyn kinase complex leading to disruption of Lyn kinase-mediated suppression of ASMase activity. Thus, co-immunoprecipitation studies revealed an increased association of Lyn kinase complex with the C16-ceramide due to activation of acid sphingomyelinase (ASMase) and sphingomyelin hydrolysis. Knocking down ASMase partially protects OLs from apoptosis. In the brain, ischemia/reperfusion (IR) triggered rearrangements in the \( \alpha_3 \beta_1 \) integrin-Lyn kinase complex leading to disruption of Lyn kinase-mediated suppression of ASMase activity. Thus, co-immunoprecipitation studies revealed an increased association of Lyn kinase complex with the C16-ceramide due to activation of acid sphingomyelinase (ASMase) and sphingomyelin hydrolysis. The data suggest a novel mechanism for regulation of ASMase activity during cell adhesion in which Lyn acts as a key upstream kinase that may play a critical role in cerebral IR injury.

Many mammalian cells are dependent on adhesion to the extracellular matrix (ECM) ligands for their continued survival, and anchorage dependence has long been recognized as a requirement for cell viability (1). ECM influences execution of the apoptotic program through actions of adhesion receptors. The principal adhesion receptors that coordinate survival or death responses as a function of ECM composition are integrins (2). Disruption of adhesion causes the cells to undergo apoptosis, a process termed anoikis (1, 3). It has been also suggested that nonligated integrins can actively promote cell death (4).

Integrin receptors are heterodimers of noncovalently associated \( \alpha \) and \( \beta \) subunits. The mammalian system has eight \( \beta \) and 18 \( \alpha \) subunits, which are known to form 24 distinct integrin receptors (5). The binding of ECM ligands to integrin receptors leads to cross-linking or clustering of integrins that result in formation of focal adhesions where integrins link the outside matrix to intracellular cytoskeleton. Focal adhesions are dynamic multiprotein complexes containing protein kinases, cytoskeletal proteins, and signaling molecules that are required for integrin-mediated cellular responses (6). Integrin-mediated adhesion acts as a pluripotent mediator of cell signaling, triggering many pathways that promote proliferation, differentiation, and migration and permit them to resist exogenous proapoptotic insults. Integrin signals are integrated with those originating from growth factor and cytokine receptors, which could physically associate with integrins (7–9).

Integrins and many proteins reported to bind to integrins are adapters with no intrinsic enzymatic activity (10). Several protein kinases and phosphatases have been demonstrated to attach directly to integrins, providing a logical mechanism for initiation of integrin-mediated signaling, including Src family nonreceptor tyrosine kinases (6). Src kinases share a conserved domain structure consisting of Src homology 3, Src homology 2, and tyrosine kinase domains. Crystallographic analysis of essentially intact Src kinase has shown that Src homology 3 and Src homology 2 protein interaction motifs turn inward and lock the kinase in an inactive conformation via intramolecular interactions (11). Src family members require phosphorylation within a segment of the kinase domain termed the activation loop for full catalytic activity (12).

The Src family is composed of nine protein tyrosine kinases (c-Src, Fyn, Yes, Hck, Lck, Blk, Fgr, Yrk, and Lyn), but only in some cell types.

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3 The abbreviations used are: ECM, extracellular matrix; ASMase, acid sphingomyelinase; FB1, fumonisin B1; IR, ischemia/reperfusion; MS, mass spectrometry; OL, oligodendrocyte; siRNA, small interfering RNA; LDH, lactate dehydrogenase; ICA, internal carotid artery; RT, reverse transcription; PLP, proteolipid protein; PKC, protein kinase C; AMPA, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid; RGDfV, cyclo[Arg-Gly-Asp-d-Phe-Val].
c-Src, Yes, and Fyn are ubiquitously expressed (12). Lyn was originally discovered in hematopoietic cells and has been shown to have important positive and negative effects on proliferation, differentiation, immune response, and receptor-mediated signaling (13).

Oligodendrocytes (OLs), the myelin-forming cells in the central nervous system, express integrins αβ1, αβ2, and αβ5, which play critical roles in proliferation and differentiation of these cells (14). Integrin expression is regulated during OL differentiation, with expression of αβ1 down-regulated and αβ5 up-regulated (15). Differentiation of OLs involves activation of the Src family kinase Lyn that, in turn, engages Rho family GTPases to promote OL maturation and process extension (16). In Lyn-deficient mice, the myelin sheath is thinner and more irregular than in wild-type mice, suggesting Lyn involvement in myelination (17). Lyn selectively associates with αβ1 and is required to amplify growth factor receptor-mediated cell survival and required for OL differentiation with enhancement of myelin membrane formation (9). Furthermore, muscarinic acetylcholine receptors have been shown to promote OL survival through Lyn-mediated activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (18).

Lyn has been found associated with αβ1 integrin and is required to drive growth factor receptor-dependent OL progenitor proliferation (9). Emerging evidence suggests that the αβ3 integrin signaling pathways coordinate with glutamate receptor- and sphingosine-1-phosphate receptor-initiated signaling in OLs (19, 20). Few studies implicated Lyn in the regulation of cell survival responses. Lyn-mediated activation of phosphatidylinositol 3-kinase/Akt signaling has been shown to determine suppression of chemotherapy-induced apoptosis in colon carcinoma cells (21). Lyn deficiency resulted in lymphoma cells becoming more susceptible to ceramide-induced apoptosis (22). Recent studies suggest that Lyn may play an important role as a negative regulator of genotoxic apoptosis when overexpressed in mammalian cells (23, 24) and of programmed cell death during granulocytic differentiation (25), but little is known of Lyn function in the brain.

The current studies were aimed to delineate the molecular mechanisms of the αβ3-Lyn-initiated signaling pathway and identify its downstream targets in promoting OL survival. Herein, we report that αβ3 integrin-mediated cell attachment to fibronectin enhanced expression and phosphorylation of Lyn kinase, leading to increased cell survival. Investigation of downstream targets of the Lyn-mediated prosurvival signaling pathway revealed a critical role of the αβ3-Lyn signaling pathway in suppression of ceramide-dependent apoptosis in OLs. Thus, knocking down αβ3 integrins or Lyn with siRNA resulted in activation of ASMase, sphingomyelin hydrolysis, and accumulation of C16-ceramide, leading to OL death. The αβ3-Lyn-mediated prosurvival signaling appears to be disrupted after cerebral ischemia/reperfusion (IR), which results in ASMase activation, sphingomyelin hydrolysis, and accumulation of ceramide. Co-immunoprecipitation experiments revealed an increased association of the αβ3 integrin-Lyn kinase complex with ionotropic glutamate receptor subunits, GluR2 and GluR4, concomitantly with ceramide accumulation and sphingomyelin hydrolysis. These studies identify Lyn tyrosine kinase as an important upstream regulator of ASMase activity in the brain.

EXPERIMENTAL PROCEDURES

Animals and Reagents—Male C57BL/6j mice (28 g each; Jackson Laboratory) were acclimated for 1 week prior to experimentation. Cell culture Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Invitrogen. SU6656 was obtained from Calbiochem. Cyclo(Arg-Gly-Asp-d-Phe-Val) was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Complete Mini protease inhibitor mixture was from Roche Applied Science. Caspase-8 inhibitor (benzoxycarbonyl-ETD-fluoromethyl ketone), caspase-9 inhibitor (benzoxycarbonyl-LEHD-fluoromethyl ketone), and pancaspase inhibitor (benzoxycarbonyl-VAD-fluoromethyl ketone) were from B&D Systems (Minneapolis, MN). [Choline-methyl-14C]Sphingomyelin was provided by Dr. Alicja Bielawska (Medical University of South Carolina, Charleston, SC). All other chemicals were purchased from Sigma.

Antibodies—The following antibodies were used: rabbit polyclonal anti-Lyn (sc-15), rabbit polyclonal anti-integrin αv (sc-10719), rabbit polyclonal anti-Fyn (sc-16), and mouse monoclonal anti-Lyn (sc-7274). These antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-β-actin mouse monoclonal antibody (A1978) was purchased from Sigma. Antibodies against αβ2 integrin (monoclonal antibody 1978), αβ3 integrin (monoclonal antibody 1976), GluR2 (monoclonal antibody 397), and GluR4 (antibody 1508) were supplied by Chemicon International (Temecula, CA). Anti-Src (Tyr(P)118) rabbit polyclonal antibody that recognizes the Tyr(P)397 site of Lyn (9) was purchased from BIOSOURCE (Invitrogen). Secondary horseradish peroxidase-conjugated antibodies were supplied by Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

Cell Culture—Dissociated rat neonatal cortices were cultured on poly-L-lysine-coated flasks, as described (20, 26). Briefly, the cerebra of rat pups were dissected and minced to generate a single-cell suspension. Cells were plated into 75-cm2 flasks and grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C and 5% CO2. By day 10, mixed glial cell cultures were obtained, consisting of OLs and microglia. Glial cultures were obtained, consisting of OLs and microglia when overexpressed in mammalian cells (23, 24) and of programmed cell death during granulocytic differentiation (25), but little is known of Lyn function in the brain.

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siRNA in the cuvette of the Amava electroporation device. Scrambled siRNA was used as a control. After transfection, OLs were plated onto tissue culture plates precoated with the 10 µg/ml fibronectin solutions overnight at 37 °C.

**Western Blotting**—Proteins were analyzed by Western blotting as previously described (20, 27). Briefly, cells were rinsed in PBS, scraped off from dishes, and lysed in a buffer containing 50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, pH 7.4, 1 mM Na3VO4, and 10 mM NaF, supplemented with the protease inhibitor mixture. After 1 h on ice, cell lysates were centrifuged at 15,000 × g for 10 min to remove insoluble material. Protein concentrations were determined by the bicinchoninic acid method (Sigma). Protein samples were prepared by boiling lysates in reducing SDS-sample buffer. Proteins were separated by 8–10% SDS-PAGE, blotted to polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in TBS-T buffer (10 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 8.0) overnight at 4 °C, and subsequently probed with appropriate primary antibody. Immunoreactive bands were visualized using a chemiluminescence kit (ECL-Plus; GE Healthcare).

**Immunoprecipitation**—For immunoprecipitation, cell lysates (500 µg) were precleared in a buffer A (0.15 M NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.05% SDS, 10 mM NaF, and 1 mM Na3VO4), protease inhibitor mixture, 0.05 M Tris, pH 7.5, 0.2% bovine serum albumin) by incubation with appropriate species-specific, IgG-conjugated magnetic beads (Dynabeds, Invitrogen/Dynal, Carlsbad, CA) for 1 h. Antibodies then were added. After incubation at 4 °C overnight with gentle mixing, antibody-antigen complexes were captured with Dynabeads and washed two times with buffer A (without bovine serum albumin) and then washed twice with Tris-buffered saline, pH 7.5. The immunoprecipitates were eluted by boiling in SDS-sample buffer.

**Analysis of Sphingolipids by Tandem Mass Spectrometry (MS)**—Cells or tissues were lysed in buffer containing 10 mM Tris, 1% Triton X-100, pH 7.4, for analysis by reverse-phase high pressure liquid chromatography coupled to electrospray ionization followed by separation by MS. Analysis of ceramides and sphingomyelins was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction-monitoring positive ionization mode, as described (27, 28). Tissue or cell samples, fortified with internal standards, were extracted with ethyl acetate/isopropyl alcohol/water (60:30:10, v/v/v), evaporated to dryness, and reconstituted in 100 µl of methanol. The samples were injected on the HP1100/TSQ 7000 liquid chromatography/MS system and gradient-eluted from the BDS Hypersil C8, 150 × 3.2-mm, 3-µm particle size column, with a 1.0 mM methanol/ammonium formate, 2 mM aqueous ammonium formate mobile phase system. The peaks for the target analytes and internal standards were collected and processed with the Xcalibur software system. Calibration curves were constructed by plotting peak area ratios of synthetic standards, representing each target analyte, to the corresponding internal standard. The target analyte peak area ratios from the samples were similarly normalized to their respective internal standard and compared with the calibration curves using a linear regression model.

**Cell Survival Assay**—Cell death was measured using a lactate dehydrogenase (LDH)-based CytoTox-ONE™ homogeneous membrane integrity assay (Promega, Madison, WI), according to the manufacturer’s recommendations. Cell survival was expressed as the percentage of viable cells based on the measurements of LDH activity associated with the cells versus the LDH activity in the medium. The fluorescence of the sample was measured at 590-nm emission with 560-nm excitation in a microplate reader (FLUOstar Optima; BMG LABTECH Inc., Durham, NC).

**Middle Cerebral Artery Occlusion Surgery and Induction of Ischemia**—Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and followed the National Institutes of Health guidelines for experimental animal use. Under temporary anesthesia, mice were subjected to middle cerebral artery occlusion, as previously described (29). Briefly, the left common carotid artery was exposed through a midline incision in the neck. A microsurgical clip was placed around the origin of the internal carotid artery (ICA). The distal end of the ICA was ligated with 6-0 silk and transected. A 6-0 silk was tied loosely around the ICA stump. The clip was removed, and the fire-polished tip of a 5-0 nylon suture (poly-L-lysine-coated) was gently inserted into the ICA stump. The loop of the 6-0 silk was tightened around the stump, and the nylon suture was advanced ∼11 mm (adjusted for body weight) into and through the ICA after removal of the aneurysm clip until it rested in the anterior cerebral arterial territory, thereby occluding the anterior communicating and middle cerebral arteries. After 1 h of middle cerebral artery occlusion, the suture was removed, blood flow was restored to normal, and the incision was closed.

**Sphingomyelinase Activity Assay**—The ASMase activity assay was performed as previously described (30, 31). Briefly, proteins (100 µg) from OL lysate were added to 100 µl of reaction mixture containing 100 mM sodium acetate (pH 5.0), 10 mM MgCl2, 0.2% Triton X-100, 10 mM dithiothreitol, 100 µM [choline-methyl-14C]sphingomyelin (10 cpm/pmol). The final volume was adjusted to 200 µl with 50 mM sodium acetate (pH 5.0). After 30 min of incubation at 37 °C, the reaction was terminated by the addition of 1.5 ml of chloroform/methanol (2:1); the phases were separated by the addition of 200 µl of water followed by centrifugation at 2000 × g for 5 min. Computation of the amount of released radioactive phosphocholine was determined by subjecting 400 µl of the upper phase mixed with 4 ml of Safety Solve (Research Products International) for liquid scintillation counting.

**Neutral Sphingomyelinase Activity Assay**—The assay was performed as described for the ASMase assay, except that the reaction mixture contained 100 mM Tris (pH 7.4) instead of sodium acetate (32).

**Real Time RT-PCR**—Real-time RT-PCR was performed on a MyiQ single-color real time PCR detection system (Bio-Rad), as described (20). Primers were designed using the Beacon Designer software. The following primers were used for PCR amplification: ASMase (accession number NC_005100; forward 5′-CCCTGTCAGCCGTGTCCTTTCC-3′) and reverse 5′-CCCCAGGCCCCGTCCGTGAGTT-3′), β-actin (accession number NC_005111; forward 5′-TTCTCAAT-
AGCTCTTCTCCAGGGAGGA-3' and reverse (5'-CTCATAGCTTCTCCAGGGAGGA-3'). The β-actin gene was used as an internal reference control to normalize relative levels of gene expression. Real time RT-PCR results were analyzed using Q-Gene software (33), which expresses data as mean normalized expression, which is directly proportional to the amount of RNA of the target gene relative to the amount of RNA of the reference gene.

Statistical Analysis—All experiments and assays were performed three or more times. Typically, there were four to six replicates of each treatment in each assay. Data were collected, and the mean value of the treatment groups and the S.E. were calculated. Data were analyzed for statistically significant differences between groups by one-way analysis of variance with a post hoc Bonferroni’s test, which adjusts for multiple simultaneous comparisons. Statistical significance was ascribed to the data when p was < 0.05.

RESULTS

Effect of OL Adhesion to Different ECM Proteins on Cell Survival—To determine the role of integrin receptors in OL survival, cells were grown on different ECM proteins, including laminin, fibronectin, and collagen, which engage specific integrin receptors. Previous studies have shown that adhesion of OLs to laminin is mediated by an αvβ3 integrin receptor (9), whereas αvβ4 integrin mediates OL adhesion and spreading on fibronectin (19). OLs do not express integrin receptors binding to collagen (34), which was used as a negative control. The cell death was measured after OLs were cultured for 24, 48, or 72 h after plating on the ECM proteins (Fig. 1). The number of viable cells was gradually reduced in OL cultures grown on laminin or collagen, reaching 10–12% by 72 h in culture. Fibronectin provided high OL survival over of 72 h, suggesting that ligation of the αvβ3 integrin receptors triggers an important prosurvival signaling pathway in OLs.

To further investigate the role of αvβ3 integrin receptor in OL survival, the αv subunit of the integrin receptor was knocked down using siRNA. The OLs were transfected with 25 or 50 nM siRNA targeting the αv subunit of the integrin receptor or scrambled siRNA in an Amaza Nucleofector device. After transfection, cells were grown for 48 h on plates precoated with fibronectin, and cell death was determined using an LDH-based assay (Fig. 2). The siRNA transfection of OLs resulted in concentration-dependent knockdown of the αv subunit protein expression up to 50–75% (Fig. 2 A and B) by Western blotting. Knocking down the αv subunit of the integrin receptor enhanced OL death while the cells were grown on fibronectin (Fig. 2C). In contrast, knocking down the αv subunit of the integrin did not affect the survival of OLs while the cells were grown on laminin (Fig. 2C).

To elucidate whether the activity of the αvβ3 integrin receptor is required to promote OL survival, a specific inhibitor of αvβ3 integrin receptor activity, cyclo(Thr-Gly-Asp-D-Phe-Val), a cyclic RGD-containing peptide, was employed (35). The cyclic pentapeptide was developed in a spatial screening approach as a very active and selective ligand of αvβ3 integrin. It has high inhibitory capacity (IC50 = 1 μM) toward cell adhesion mediated by αvβ3 integrin (36). OLs were grown on fibronectin with/without the cyclic RGD-containing peptide for 48 h, and cell death was measured (Fig. 2D). OL survival was significantly decreased in the presence of a 25 μM concentration of the cyclic RGD-containing peptide, consistent with our findings showing the critical role of the αvβ3 integrin receptor in OL survival on fibronectin.
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To examine the mechanism of OL death initiated by the knockdown of the αv subunit of integrin, we employed specific inhibitors of major caspsedes that could be involved in OL responses (37, 38). OLs were transfected with 50 nM αv integrin-specific or scrambled siRNA targeting the αv subunit of the integrin receptor or scrambled siRNA and treated with specific inhibitors of activated caspase-8 or caspase-9 or pancaspase inhibitor, and cell death was measured 48 h later. The pancaspase inhibitor effectively prevented OL death (Fig. 3), indicating that cell death initiated by knockdown of the αv subunit of the integrin receptor is caspase-dependent. Inhibitors of both activated caspase-8 and activated caspase-9 only partially protected OLs from apoptosis, suggesting the involvement of both caspsedes in OL apoptosis. Altogether, the data suggest that the αvβ3 integrin receptor is essential for OL survival on fibronectin, and the engagement of this receptor initiates an important prosurvival signaling pathway in OLs.

Engagement of the αvβ3 Integrin Receptor Results in Selective Association with Activated Lyn Kinase—To determine the downstream targets of αvβ3 integrin signaling, the expression of Lyn kinase was measured over 72 h in nonattached cells (NA) and cells grown on fibronectin or collagen. Lyn attachment to fibronectin enhanced Lyn protein expression (about 2-fold) compared with nonattached cells, indicating that αvβ3 integrin engagement stimulated Lyn protein expression. In contrast, there was less Lyn expressed in OLs grown on non-αv integrin substrate collagen compared with nonattached cells.

Numerous studies have shown that Lyn and the other Src family kinases share two tyrosine phosphorylation sites, one (Tyr397) causing the activation of the kinase and the second (Tyr508) causing its inhibition, both through a conformational change of the kinase (12). Autophosphorylation of Tyr397 in Lyn has been demonstrated to reflect the degree of Lyn activation (13). To investigate whether αvβ3 integrin ligation induces phosphorylation at Tyr397, that is required for Lyn activation (9), Lyn was immunoprecipitated with specific anti-Lyn antibody. Then phosphorylation was measured with specific antibody recognizing phospho-Tyr397 (Fig. 4, C and D). Phosphorylated Lyn was increased up to 1.9-fold 6 h after OL attachment to fibronectin, and it was further increased (5-fold) 24 h later. Therefore, the engagement of the αvβ3 integrin receptor provoked an increase in Lyn expression and activation of the kinase. This suggests that Lyn could be an important downstream target of αvβ3 integrin-mediated prosurvival signaling in OLs.

To examine the specificity of Lyn association with the αvβ3 integrin receptor, we performed co-immunoprecipitation experiments in OLs grown on fibronectin using anti-αv or anti-β3 antibodies (Fig. 5A). Analysis of immunoprecipitates revealed that Lyn associated with αvβ3 integrin receptor. These results are consistent with previous reports of Lyn associating with the αvβ3 integrin receptor in OLs in response to growth receptor stimulation (9). Moreover, Lyn was found to be associated specifically with the αvβ3 integrin receptor, not with the αvβ5 integrin receptor (Fig. 5B), in co-immunoprecipitation experiments using antibodies against αvβ3 or αvβ5 integrin. Next, we conducted similar co-immunoprecipitation experiments in mouse brain samples. Fig. 5C shows association of Lyn with αv integrin and, more importantly, selective association with the αvβ3 integrin receptor, not αvβ5 integrin, in the mouse brain. In contrast, Lyn was not found in co-immunoprecipitates with the αvβ3 integrin receptor either in OLs or in the brain. The data suggest selective association of Lyn kinase with the αvβ3 integrin receptor in cultured OLs and in brain neural cells. Taken together, the results of these studies are in line with the model whereby Lyn is primed for activation by direct interaction with integrin receptor, and integrin clustering by its ligand (fibronectin) would increase local Lyn concentration,
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with the receptor, we investigated whether Lyn is an important molecular determinant of the αβ3 integrin receptor prosurvival signaling pathway. To examine the role of Lyn in OL survival, we knocked down Lyn using siRNA. OL transfection with 50 nM siRNA targeting Lyn resulted in down-regulation of Lyn protein expression up to 75% compared with scrambled siRNA (Fig. 6, A and B). There was no interference of siRNA targeting Lyn with Fyn tyrosine kinase expression, which could associate with the αβ1 integrin receptor and mediate OL survival on laminin (9). Knocking down Lyn significantly reduced OL survival on fibronectin (Fig. 6C), indicating Lyn involvement in the αβ3 integrin receptor-mediated prosurvival signaling pathway.

To assess whether Lyn activity is required for promoting OL survival, cells were treated with the Src family kinase activity inhibitor, SU6656. SU6656 was identified in a chemical library screening, and biochemical assays confirmed that it inhibits Src family kinase activity with an IC_{50} value in the range of 0.02–0.2 μM in an in vitro assay (40). Kinetic analysis of the inhibition of SU6656 activity confirmed that it was a competitive inhibitor with respect to ATP. SU6656 is a potent inhibitor of Lyn but a rather poor inhibitor of other Src family kinases (40). Fig. 6D depicts a dose response of OL survival on fibronectin at 48 h post-treatment with the inhibitor. There was a profound decrease in OL survival in the presence of 20 μM SU6656, indicating that Src family kinase activity is required for OL survival mediated by the αβ3 integrin receptor. Because Lyn is the only Src family kinase associated with the receptor, the data suggest that Lyn kinase activity is of importance for αβ3 integrin receptor-initiated, prosurvival signaling in OLs.

αβ3 Integrin Receptor-Lyn Kinase Signaling Promotes OL Survival by Blocking ASMAse Activity—To determine the downstream targets of the αβ3 integrin receptor-Lyn kinase signaling pathway, we elucidated the possibility that ASMAse could be crucial for OL survival. ASMAse has been implicated in apoptotic cell death in response to various stress stimuli, including ligation of death receptors, radiation (UV-C and ionizing radiation), chemotherapeutic agents, and cytokines (reviewed in Refs. 32, 41, and 42). Activation of ASMAse by the stress stimuli could result in hydrolysis of sphingomyelin and generation of sphingosine that is metabolized to ceramide by ceramide synthase, which belongs to a family of proteins termed LASS or CerS (43). Six known mammalian LASS (CerS) family members are important enzymes involved in two major pathways generating ceramide, de novo ceramide synthesis and sphingolipid recycling that involves sphingomyelin hydrolysis (44).

### FIGURE 5. Lyn kinase is selectively associated with integrin αβ3 in OLs and in the mouse brain. OLs were harvested after 24 h in culture on fibronectin. A, complexing with Lyn was detected by immunoprecipitation using antibodies against αv, integrin or β3 integrin subunit. B, complexing with Lyn was detected by immunoprecipitation using anti-αv, integrin or αβ3 integrin antibodies. The membranes were stripped and probed using anti-Fyn antibodies. C, samples were prepared from the brain of an adult mouse. Complexing with Lyn was detected by immunoprecipitation using antibodies against α3 integrin subunit or integrin αβ3 or integrin αβ5. The membranes were stripped and probed using anti-Fyn antibodies.
To investigate the role of sphingolipids in αβ3 integrin receptor signaling, OLs were transfected with siRNA targeting the αc subunit of the integrin receptor (Fig. 7A), and the sphingolipid profile was analyzed. The sphingolipid content of OLs grown on fibronectin is shown in Table 1. Transfection of 50 nm specific siRNA sequence of the αc subunit resulted in a substantial decrease in C16:0-sphingomyelin content (34%) and increases in C16-ceramide (1.82-fold) and sphingosine (1.67-fold) content in OLs. The data suggest that αcβ3 integrin receptor-mediated signaling blocks sphingomyelin hydrolysis to promote cell survival. Similarly, transfection of 50 nm specific siRNA targeting Lyn kinase enhanced the content of C16-ceramide about 2.1-fold (Fig. 7B).

To investigate whether ceramide accumulation occurs via sphingolipid recycling, cells were treated with the de novo ceramide biosynthesis inhibitor (45), myriocin, or the LASS (CerS) inhibitor, fumonisin B1 (FB1), after transfection with siRNA targeting Lyn. Myriocin did not affect the C16-ceramide accumulation, indicating that de novo ceramide biosynthesis is not involved. In contrast, FB1 almost completely prevented C16-ceramide accumulation (Fig. 7B). This suggests that ceramide accumulation is due to activation of sphingolipid recycling via the salvage pathway, whereby complex sphingolipids are broken down to ceramide and then to sphingosine, which is reacylated to ceramide (46).

To determine what sphingomyelinase is regulated by αcβ3 integrin-Lyn kinase signaling, we focused first on ASMase, which is a major sphingomyelinase enzyme in OLs (47). The activity of ASMase was measured after OL transfection with siRNA targeting the αc subunit of the integrin receptor or Lyn kinase (Fig. 8, A and B). Knocking down either αc subunit or Lyn kinase triggered activation of ASMase about 2- or 1.7-fold, respectively. There was no change in neutral sphingomyelinase activity, which was 5.64 ± 0.39 pmol/μg/h, which constitutes less than 10% of total sphingomyelinase activity in these cells (47). The results of these studies suggest that αcβ3 integrin receptor-Lyn-mediated signaling blocks ASMase activity in OLs grown on fibronectin. To examine whether αcβ3 integrin-Lyn kinase signaling-induced suppression of ASMase is of importance for cell survival, we used an RNA interference approach. OLs were transfected with 50 nm ASMase-specific siRNA or scrambled siRNA and cultured for 48 h on fibronectin. Then cells were treated with 20 μM Lyn kinase inhibitor SU6656 for 24 h. Fig. 8C shows that ASMase siRNA significantly down-regulated the expression of ASMase mRNA by real time RT-PCR. The ASMase knockdown partially protected OLs from cell death induced by inhibiting Lyn activity (Fig. 8D). Similarly, the inhibitor of ceramide synthase activity, FB1, enhanced OL survival (Fig. 8E), whereas myriocin did not have an effect. This supports the notion that Lyn regulates ASMase involved in sphingolipid recycling. Altogether, these results indicate that αcβ3 integrin receptor-Lyn-mediated signaling suppresses ASMase activity to promote OL survival on fibronectin.

Cerebral Ischemia/Reperfusion Disrupts αcβ3 Integrin Receptor-Lyn-mediated Signaling—It has been demonstrated that the αcβ3 integrin receptor associates with myelin proteolipid protein (PLP) in cultured OLs and in the brain (26). OLs express several AMPA-specific glutamate receptor subunits: GluR1, GluR2, GluR3, and GluR4 (48, 49). Agonist activation of AMPA receptors causes Ca2+ influx in OLs via Ca2+-permeable channels, which consist of GluR3 and GluR4 subunits. About 30% of AMPA receptors contain the GluR2 subunit, which is edited at the Gln/Arg site, rendering the channel impermeable to Ca2+ (48). In OLs, stimulation of AMPA-specific glutamate receptors triggers the formation of a multiprotein complex centered at the αc integrin receptor and PLP, which includes the glutamate receptor subunits GluR2 and GluR4. Formation of this complex changes integrin activity and function in cultured OLs (19).

In cerebral IR, excessive glutamate accumulation in the extracellular space has been proven to overstimulate glutamate receptors, promoting cytosolic Ca2+ overload and producing gross perturbations in intracellular signaling pathways that contribute to neural cell injury or death (52). To determine if cerebral IR could alter the αcβ3 integrin receptor-mediated signaling, we performed co-immunoprecipitation experiments and assessed the composition of the αcβ3 integrin receptor signaling complex in OLs in vivo. Because PLP is selectively expressed in OLs, monoclonal anti-PLP antibody has been very instrumental for selective immunoprecipitation of the αcβ3 integrin or Lyn kinase.
integrin receptor-PLP complex and its associated proteins from OLs in the brain (26).

To elucidate the effect of cerebral IR on the αvβ3 integrin receptor-Lyn signaling pathway, the mouse model of transient ischemia has been employed. The middle cerebral artery of a mouse was occluded for 1 h, cerebral flow was restored, and the brain was removed after 48 h of reperfusion. Tissue lysates were prepared from ipsilateral (ischemic) or contralateral (control) hemispheres of the mouse brain 48 h later. Data are means ± S.E. (n = 9). *p < 0.05 compared with SCR. C and D, OLs were transfected with SCR or 50 nM ASMase-specific siRNA and cultured for 48 h on fibronectin. Then cells were harvested and, and total RNA was isolated and analyzed by RT-PCR. C, OLs were exposed to 20 μM Lyn inhibitor SU6656, and cell death was quantified 24 h later. D, data are means ± S.E. *p < 0.05 (n = 16). E, OLs were exposed to 20 μM Lyn inhibitor SU6656 with or without 20 μM FB1 or 1 μM myriocin, and cell death was quantified 24 h later. Data are means ± S.E. *p < 0.05 (n = 12).

With previously reported sphingomyelin hydrolysis and ceramide accumulation after severe cerebral IR (53–55). The results of our studies indicate that cerebral IR also could trigger the activation of sphingolipid recycling via the salvage pathway, whereby degradation of complex sphingolipids (sphingomyelin, glycosphingolipids) produces ceramide and then sphingosine, which is reacylated to ceramide (46). Altogether, the results of these studies suggest that cerebral IR disrupts αvβ3 integrin receptor-Lyn-mediated signaling, leading to activation of ASMase, sphingomyelin hydrolysis, accumulation of ceramide, and sphingosine, eventually leading to cell death.

**DISCUSSION**

The present studies are unique in describing a novel prosurvival signaling pathway mediated by the integrin-associated Src family tyrosine kinase, Lyn, in OLs. We have shown that engagement of the αvβ3 integrin receptor triggers increased expression of activated Lyn kinase, which results in suppression of ASMase activity, in such a way promoting OL survival. This is the first demonstration of a critical role of the αvβ3 integrin-Lyn signaling pathway in OL survival.

OLs, like many other mammalian cells, are dependent on adhesion for their continued survival. One important function of integrin signaling is to regulate anchorage dependence. Integrin receptor interaction with the appropriate ECM initiates an assembly of an adhesion-dependent signaling scaffold containing a number of adaptor proteins and kinases, leading to activation of signaling pathways aimed to prevent apoptosis. Cells
deprived of the correct attachment to the ECM undergo classical programmed cell death, anoikis (1). Anoikis is a mechanism to ensure that cells displaced from their natural environment are eliminated. To suppress anoikis, different integrin receptors activate distinct signaling cascades, including phosphatidylinositol 3-kinase signaling, the extracellular signal-regulated kinase pathway, and stress-activated mitogen-activated protein kinases, such as c-Jun N-terminal kinase (3).

Our studies identify Lyn tyrosine kinase as a novel determinant of the αβ3 integrin-mediated survival signaling cascade in OLs. Here, we show that αβ3 integrin engagement enhances Lyn protein expression and activates Lyn, leading to suppression of OL death. In contrast, engagement of αβ1 integrin results in decreased OL survival. Co-immunoprecipitation studies demonstrated selective Lyn association with the αβ3 integrin receptor and not with αβ1 integrin. Consistent with previous reports, the Src family kinase Fyn did not associate with the αβ1 integrin receptor (9). Moreover, knocking down αβ3 integrin with siRNA or blocking its ECM binding activity with a specific inhibitor induced OL death. Similarly, knocking down Lyn using siRNA or inhibiting Lyn activity resulted in decreased OL survival. Further investigation revealed that OLs underwent apoptosis through activation of both caspase-8 and caspase-9. Anoikis has been proposed to be regulated via the intrinsic pathway, and it requires outer mitochondrial membrane permeabilization, since it can be blocked by overexpression of antiapoptotic Bcl-2 family proteins (1, 3, 56). In the intrinsic pathway, effector caspase activation occurs as a consequence of mitochondrial outer membrane permeabilization and activation of the initiator caspase-9. This is regulated by the proapoptotic Bcl-2 family of proteins, which control the formation of pores in the outer mitochondrial membrane, releasing proapoptotic factors, such as cytochrome c, which is required for caspase-9 activation. The data are consistent with the notion that activation of the intrinsic apoptotic pathway occurs through activation of caspase-8, which cleaves BH3-only protein Bid, which then permeabilizes the outer mitochondrial membrane (57) and releases cytochrome c, leading to activation of caspase-9 and OL death.

In the present study, we explored the mechanism of αβ3 integrin-Lyn-mediated suppression of OL apoptosis by examining downstream effectors of Lyn. It appears that different cell types may regulate activated Lyn-dependent signaling based on the cell functions required. In colon carcinoma cells, activated Lyn triggered chemoresistance via a pathway shown to involve activation of phosphatidylinositol 3-kinase and Akt (21). In contrast, activation of Lyn resulted in its binding to neutral sphingomyelinase and is requisite for its stimulation and the induction of apoptosis in myeloid leukemia cells (58). It was shown previously that endothelial apoptosis is induced by inhibition of integrins αβ3 and αβ1 involves ceramide metabolic pathways (59). Using a nonselective inhibitor of αβ1 integrins, RGDFV, the report indicated increased ceramide and decreased sphingomyelin in human brain microvascular endothelial cells grown on vitronectin, suggesting that sphingomyelin hydrolysis contributes to RGDFV-induced ceramide increase and apoptosis, suggesting that ASMase activity was required for human brain microvascular endothelial cell apoptosis.

The results from our study implicate Lyn as an upstream regulator of ASMase activity in the cell response to disruption of αβ1 integrin survival signaling in OLs. Thus, siRNA targeting αβ integrins or Lyn significantly increased sphingomyelin hydrolysis and C16-ceramide accumulation, suggesting activation of sphingomyelinase in OLs. The activity studies confirmed the activation of ASMase, and not neutral sphingomyelinase, in response to either αβ integrin or Lyn knockdown.

ASMase is a well characterized lipid phospholipase involved in sphingomyelin turnover and in regulation of sphingolipid recycling via the salvage pathway (31, 46). In the latter process, complex sphingolipids are broken down to ceramide and then to sphingosine, which is then used by ceramide synthase yielding ceramide. Ceramide accumulation via the salvage pathway requires ceramide synthase (60), which plays a key role in de novo synthesis of ceramide (44). The inhibitory analysis demonstrated that ceramide response to Lyn knockdown requires ceramide synthase (inhibited by fumonisin B1) but not de novo synthesis (not inhibited by myriocin). This suggests that Lyn-dependent suppression of ASMase in the salvage pathway of sphingolipid recycling contributes to αβ3 integrin survival signaling. The data suggest a novel role of Lyn in regulating the sphingolipid recycling via the salvage pathway, which is an important route for controlling cellular ceramide.

This study also highlights the complexity of sphingolipid signaling and the interconnectivity of the sphingolipid metabolism (44). The cellular levels of bioactive sphingolipids, such as ceramide or sphingosine, which are involved in intracellular signaling pathways, are tightly regulated by its utilization pathways. Thus, knockdown of αβ integrin resulted in a net change of sphingomyelin content (341 pmol/mg protein) much greater than combined changes in C16-ceramide (70 pmol/mg protein) and sphingosine (19 pmol/mg protein). This suggests activation of ceramide conversion into complex glycosphingolipids and/or sphingosine conversion into sphingosine 1-phosphate, followed by its degradation by sphingosine 1-phosphate lyase into nonsphingolipid products. It also supports the notion that activation of ASMase could occur concomitantly with activation of other enzymes of sphingolipid metabolism (44, 60).

Numerous reports have accumulated supporting a role for sphingolipids as pleotropic second messengers in intracellular signaling pathways (44, 61). Mounting evidence indicates that ceramide is important in intracellular signaling involved in controlling neural cell death in cerebral IR (27, 62). The signaling cascades activated by cerebral IR that may promote neuronal death are not well understood. It has been demonstrated that mild transient cerebral ischemia (0.5 h) triggers ceramide accumulation due to JNK3 (c-Jun N-terminal kinase 3)-mediated activation of de novo ceramide synthesis after 24 h of reperfusion. In contrast, severe cerebral ischemia (1 h) resulted in ceramide accumulation via activation of ASMase and sphingomyelin hydrolysis (54). Consistent with these data, the extent of brain tissue damage was decreased in mice lacking ASMase (55).

Our studies draw attention to possible impairment of integrin survival signaling as a result of glutamate receptor over-
stimulation that could cause ASMase activation in cerebral IR. The results of co-immunoprecipitation studies revealed major changes in the composition of the α,βγ integrin signaling complex, including increased Lyn association, concomitantly with sphingomyelin hydrolysis, suggesting activation of ASMase. One can only speculate about how Lyn kinase regulates ASMase activity; however, recent data suggest that the novel PKC, PKC δ, might be involved. PKC δ is a key upstream kinase activating ASMase by phosphorylation of serine 508 (31). In several model systems, tyrosine phosphorylation is involved in regulating PKC δ activity with a resultant increase or decrease in catalytic activity, depending on the cell type and the phosphorylation site (63). In keratinocytes, Lyn-mediated post-translational modification of PKC δ on tyrosine 565 in the kinase domain contributed to inactivation of PKC δ (50). This suggests that Lyn-mediated phosphorylation of PKC δ could prevent activation of ASMase. Although determining the molecular mechanisms by which Lyn kinase suppresses ASMase activity requires further investigation, it is clear that this kinase is fundamentally important for regulation of ASMase function in OLs and in the brain, at least in response to IR.

Emerging data suggest that interaction of integrin receptors with ECM could play a critical role in preventing tissue injury after cerebral IR. It has been shown that IR-induced deposition of plasma fibronectin in the brain protected brain tissue following transient focal IR (51). Plasma fibronectin deposits occur throughout an infarct in wild-type mice, but fibronectin was absent in the infarcts of plasma fibronectin-null mice. The absence of plasma fibronectin deposits resulted in up-regulation of caspase-3 and in brain infarcts that were 35% larger than in the wild-type mice. The data imply that fibronectin binding to cell surface integrin receptors, including α,βγ integrin, triggers intracellular signaling, which is essential for the neural cell survival in cerebral IR.

In summary, this study provides evidence that interaction of the α,βγ integrin receptor with its ECM ligand engages Lyn tyrosine kinase to suppress ASMase activity and prevent ceramide-mediated apoptosis in OLs, and our data emphasize the critical role of integrin receptor signaling in neural cell survival.

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