Insulin receptor substrate 1 is an effector of sonic hedgehog mitogenic signaling in cerebellar neural precursors

Susana R. Parathath¹,*, Lori Anne Mainwaring¹,²,*, Africa Fernandez-L¹, Dane Ohlosson Campbell¹ and Anna Marie Kenney¹,²,³,†

Sonic hedgehog (SHH) and insulin-like growth factor (IGF) signaling are essential for development of many tissues and are implicated in medulloblastoma, the most common solid pediatric malignancy. Cerebellar granule neuron precursors (CGNPs), proposed cells-of-origin for specific classes of medulloblastomas, require SHH and IGF signaling for proliferation and survival during development of the cerebellum. We asked whether SHH regulates IGF pathway components in proliferating CGNPs. We report that SHH-treated CGNPs showed increased levels of insulin receptor substrate 1 (IRS1) protein, which was also present in the germinal layer of the developing mouse cerebellum and in mouse SHH-induced medulloblastomas. Previous roles for IRS1, an oncoprotein that is essential for IGF-mediated proliferation in other cell types, have not been described in SHH-mediated CGNP proliferation. We found that IRS1 overexpression can maintain CGNP proliferation in the absence of SHH. Furthermore, lentivirus-mediated knock down experiments have shown that IRS1 activity is required for CGNP proliferation in slice explants and dissociated cultures. Contrary to traditional models for SHH signaling that focus on gene transcription, SHH stimulation does not regulate Irs1 transcription but rather stabilizes IRS1 protein by interfering with mTOR-dependent IRS1 turnover and possibly affects Irs1 mRNA translation. Thus, we have identified IRS1 as a novel effector of SHH mitogenic signaling that may serve as a future target for medulloblastoma therapies. Our findings also indicate a previously unreported interaction between the SHH and mTOR pathways, and provide an example of a non-classical means for SHH-mediated protein regulation during development.

KEY WORDS: Sonic hedgehog, Cerebellum, Neural precursor, Insulin-like growth factor, Insulin receptor substrate 1, Proliferation, Mouse

INTRODUCTION

Medulloblastoma is the most common malignant solid tumor in children. These tumors arise in the cerebellum, a region of the brain with important roles in movement, coordination and possibly learning. Traditional treatments for medulloblastomas – radiation, surgery and multi-agent chemotherapy – cause devastating side effects in long-term survivors (Packer et al., 1999), including cognitive decline, psychiatric problems, seizures and movement disorders. The poor understanding of molecular events leading to the formation and maintenance of medulloblastoma has hindered the advancement of treatment options.

CGNPs are proposed cells of origin for some classes of medulloblastoma (Provias and Becker, 1996). After birth (approximately the first 2 weeks in mice), CGNPs undergo a rapid expansion phase in the cerebellar external granule layer (EGL). After this expansion CGNPs migrate through the underlying layer of Purkinje neurons with which they will ultimately form synapses. The mature granule neuron cell bodies localize to the internal granule layer (IGL) (Hatten and Heintz, 1995). Normal CGNP proliferation is dependent upon signaling by both SHH and IGF, which are also implicated in medulloblastomas (Altman and Bayer, 1997; Ho and Scott, 2002; Knoepfle and Kenney, 2006; Marino, 2005; Wetmore, 2003).

SHH is produced by Purkinje neurons and loss of SHH leads to reduced proliferation in the EGL of neonatal mice (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Treatment of CGNPs in culture with SHH increases BrdU incorporation (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999); however, the mechanisms underlying SHH mitogenic signaling in CGNPs continue to be subject to ongoing investigation. Classic mitogens such as epidermal growth factor (EGF) or platelet-derived growth factors (PDGFs) signal through receptor tyrosine kinases. By contrast, SHH activates a non-receptor tyrosine kinase-associated pathway. In the absence of SHH, the transmembrane protein patched (PTCH1) represses smoothened (SMO), a G-protein coupled receptor-resembling protein (Alcedo et al., 1996). When SHH binds to PTCH1, SMO is released from inhibition and the pathway is activated, resulting in activation of target genes including Ptc1 itself, as well as the transcription factors N-myc (Mycn – Mouse Genome Informatics) Gli2 and Gli1, a target of GLI2 (Ho and Scott, 2002). SHH signaling during cerebellar development occurs primarily through the activation of GLI2; mutations in GLI2 result in abnormal CGNP proliferation, as well as foliation defects (Corrales et al., 2006; Corrales et al., 2004).

Traditional receptor tyrosine kinase signaling mediated by IGF family members has roles in CGNP proliferation and SHH-associated medulloblastomas. IGF1 and IGF2 are expressed in the developing and mature cerebellum. Activation of the IGF pathway is found in medulloblastomas (Reiss, 2002), and IGF2 in particular is required for SHH-mediated medulloblastoma formation (Hahn et al., 2000) in vivo and medulloblastoma cell proliferation in vitro (Hartmann et al., 2005). IGF1 and IGF2 activate the IGF receptor. One way through which IGF-mediated phosphoinositide-3 kinase (PI-3K) signaling cooperates with SHH signaling is by inhibiting GSK3β (Kenney et al., 2004; Mill et al., 2005), which blocks cell cycle progression in CGNPs by phosphorylating N-myc and targeting it to the proteosome for destruction.
nucleopore track-etched membrane (Fisher Scientific) in serum-free media for 24 hours supplemented with SHH. After 24 hours, indicated sections were infected with shRNA lentiviruses targeting IRS1 for 6 hours. The slices were maintained in serum free media for 48 hours and pulsed with BrdU for an additional 4 hours. Slices were flash frozen, sectioned and stained for BrdU and DAPI.

Protein preparation and immunoblotting
For immunoblot analysis, cells were scraped cells loose into their medium. Cells were washed once in PBS and protein extracts were prepared as previously described (Kenney and Rowitch, 2000). Protein content was determined using the Bio-Rad protein assay. Assays were performed in duplicate for each sample. Each sample (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% polyacrylamide gels and then transferred in 20% methanol buffer at 4°C to Immobilon polyvinylidene difluoride (Millipore). Standard western blot procedures (see Kenney and Rowitch, 2000) were used to determine protein levels. Antibodies used for western blotting were: IGFβ, N-myc and cyclin D2 (Santa Cruz), IRS1, IRS2, phosphorylated IRS1 (S636/639), phosphorylated AKT (S473), AKT, GAB1, phosphorylated ERK, phosphorylated p70S6 kinase (T389), phosphorylated ribosomal protein S6 (S235/236) (Cell Signaling) and β-tubulin (Sigma). Peroxidase activity was detected using Amersham’s ECL reagents and exposing membranes to Kodak Biomax film. Multiple exposures were taken to avoid saturating film. The film was scanned and the digitalized images were quantified by densitometry using Adobe Photoshop 9.0 software.

Immunofluorescence
Frozen sections (10 μm) from SW129 pups were dried and then boiled in 0.01 M citric acid for 15 minutes for antigen retrieval. For paraffin-embedded sections, tissues were first de-waxed and re-hydrated prior to antigen retrieval. After cooling, slides were washed twice with PBS for 10 minutes. Sections were blocked with 10% normal goat serum (Sigma) in 0.25% Triton X-100/PBS for 1 hour at room temperature. Primary antibodies for IRS1 (Cell Signaling), GFAP (Cell Signaling), BrdU (Becton Dickinson) and Ki67 (Vector Laboratories) were added to the blocking solution at a 1:100 dilution and incubated overnight at 4°C. After washing in PBS, slides were incubated with either goat anti-rabbit or goat anti-mouse fluorescently tagged secondary antibody (Invitrogen) at a 1:5000 dilution for 1 hour at room temperature. Sections were mounted using Vectashield mounting media with DAPI (Vector Laboratories).

For detecting BrdU incorporation, dissociated CGNP s were grown on poly-DL-ornithine-coated glass coverslips. Cells were pulsed with 20 μg/ml BrdU for 2 hours. The cells were fixed with 4% paraformaldehyde for 20 minutes followed by two 10-minute washes with PBS. The coverslips were treated with 2 N HCl for 2 minutes followed by two 10-minute washes with PBS. Cells were blocked for 30 minutes then exposed to primary and secondary antibodies according to standard methods (details can be provided on request). All other antibodies, IRS1 (Cell Signaling or Upstate), Ki67 (Vector Laboratories), p27 (BD Pharmingen), PCNA (Calbiochem), ZIC1 (gift from Rosalind Segal, Harvard) and cleaved caspase 3 (Cell Signaling) were used at a 1:100 dilution in blocking solution.

Reverse transcriptase and quantitative PCR
RNA from CGNPs was collected using TRIZOL reagent according to the manufacturer’s instructions. RNA samples were resuspended in 87.5 μL DEPC-treated water. In order to remove fully any residual DNA from the samples, RNA was further purified using the RNasy Mini Kit (Qiagen) according to manufacturer’s instructions. DNase (Qiagen) digestion was performed in solution prior to further RNA purification over the RNasey column.

A 50 μl reaction volume was used for 50 ng RNA of each sample using SuperScriptOne-Step RT-PCR with Platinum Taq (Invitrogen). Samples were run as per manufacturer’s instructions. Absence of genomic DNA was verified by omitting the RT step and using Taq alone. Primer sequences were as follows: β-actin sense, 5'-CACACAGCTCAACAAGAGCCGCTCACC-3'; β-actin antisense, 5'-CACTGATCTCTAAGGTTGTTGTTCTC-3'; cyclin D2 sense, 5'-CCTTCCCTCTCCAAATGGCCCA-3'; cyclin D2 antisense, 5'-CCTGCCGGGCTGCTGACTC-3'; IRS1 sense, 5'-CCCCGGGTTTAA-
GAGGCTCTG-3′; IRS1 antisense, 5′-TGCTGGTGACGGTTGGTTGT-3′; GLI1 sense, 5′-CCAGGGAGGAGGAGGAA-3′; GLI1 antisense, 5′-AGGAGGAGGAGGAGGAA-3′; GLI2 sense, 5′-AGCCCTCTG- CAGGTGAAAGA-3′; GLII antisense, 5′-CTGGGGTGGCAGA- GCCTAAGG-3′; N-myc sense, 5′-GTCCTTCTGTTTCCACAG-3′; N-myc antisense, 5′-GGGTTACACCTTTACAG-3′. PCR products were resolved on a 2.5% agarose-ethidium bromide gel.

For quantitative PCR, total mRNA was extracted from untreated and SHH-treated CGNPs cultures as described above. cDNA was generated with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer’s instructions. TaqMan Gene Expression Assays (Applied Biosystems) using TaqMan custom designed MGB probes for IRS1 (Mm01278327_m1) and β-actin (Mm0119484_m1) were performed in triplicate according to the manufacturer’s protocol on an ABI 7000 Sequence Detection System. Data were analyzed with ABI GeneAmp SDS software. For quantification of BrdU uptake into newly synthesized DNA, TIFF images of four random fields were taken for each experimental group using the 10× objective. The percentage of cells staining positive for BrdU was determined using Image Pro Plus software (MediaCybernetics). Confocal images were visualized with Leica TCS SP2 (Inverted Stand) and images captured with Leica LCS Lite software.

**Statistics**

Statistical analysis was performed using one-way ANOVA followed by Bonferroni-Dunn test for multiple comparisons within a group, or a two-tailed t-test for comparisons between groups, as indicated by the figure legends; *P* < 0.05 was considered significant and is marked by an asterisk.

**RESULTS**

**SHH treatment upregulates IRS1 protein levels in CGNPs**

In order to determine whether expression levels or activity of IGF pathway components are altered in CGNPs induced to proliferate by SHH in comparison with levels in differentiating (vehicle-treated) CGNPs, we established CGNP primary cultures from PN5 SW129 mice and treated them with vehicle (PBS) or added SHH to the media for 24 hours. We then prepared protein lysates and used western blot analysis to assess levels of IGF receptor substrates and AKT phosphorylation, a downstream target of PI-3K (Fig. 1A). We also examined levels of cyclin D2, a well-established marker for SHH-induced CGNP proliferation, and ERK phosphorylation, which is known to be unaffected by SHH treatment in CGNPs (Kenney and Rowitch, 2000). We found that SHH-treated CGNPs showed increased levels of IRS1 protein, and that IGF receptor, IRS2 or GAB1 levels did not change in response to SHH (Fig. 1A). IRS1 upregulation correlates with increased cyclin D2 expression. It has been reported that SHH treatment of a fibroblast cell line results in modestly increased phosphorylation of AKT (Riobo et al., 2006). By contrast, we detected no changes in AKT phosphorylation in SHH-treated CGNPs (Fig. 1A, Fig. 4A, Fig. 5A) suggesting functions for IRS1 in CGNPs in addition to its AKT-activating role. The upregulation of IRS1 is also accompanied by activation of IRS1 as seen by its tyrosine phosphorylation, which occurs when the IGF receptor is bound by ligand (Van Obbergen et al., 2001) (see Fig. S1 in the supplementary material).

We next asked whether maintenance of increased IRS1 levels requires ongoing SHH signaling. We treated CGNPs with two well-characterized SHH pathway inhibitors, forskolin and cyclopamine, for increasing lengths of time. Forskolin serves as a potent inhibitor of SHH signaling by increasing cAMP levels, which in turn activates PKA and leads to CGNP cell cycle exit and differentiation (Cai et al., 1999). Cyclopamine is an antagonist of SMO, the activator of SHH signaling (Chen et al., 2002). Treatment with either inhibitor reduced IRS1 and cyclin D2 protein levels in a time-dependent manner (Fig. 1B), with IRS1 loss apparent 6-9 hours after addition of inhibitors. This delayed, rather than immediate, response of IRS1 to SHH inhibition may be a result of the long half-life of IRS1, which has been reported to be up to 10 hours (Lee et al., 2000) or it may suggest that IRS1 is sensitive to cell cycle exit, as CGNPs remain proliferation competent for 6 hours after SHH withdrawal or inhibition (Kenney and Rowitch, 2000).

Recent evidence suggests that IRS1 may have nuclear as well as cytoplasmic functions (Chen et al., 2005; Morelli et al., 2004). To determine the cellular localization of IRS1, we cultured CGNPs on coverslips with or without SHH for 24 hours. We then carried out immunostaining for IRS1 and p27, a predominantly nuclear protein associated with CGNP differentiation (Uziel et al., 2005). As shown in Fig. 1C, SHH-treated CGNP cultures contained populations of cells expressing either p27 (green) or IRS1 (red), and expression of IRS1 and p27 is mutually exclusive in individual cells. When images were merged with blue DAPI (nuclear) staining, we observed IRS1 expression in the nucleus and cytoplasm, which was confirmed by confocal microscopy (Fig. 1C, right panel). These results suggest that IRS1 may perform signaling functions in the cytoplasm, and may also play roles in regulating transcription or DNA repair, nuclear functions previously ascribed to IRS1 (Reiss, 2006; Chen, 2005). The role played by nuclear versus cytoplasmic IRS1 in proliferating CGNPs remains to be determined.

In our hands, mixed CGNP cultures contain up to 10% GFAP-positive cells. Previously, it has been shown that the only SHH-responsive cells and BrdU-incorporating cells in mixed cultures are CGNPs, not those that stain positive for glial markers such as GFAP or O4 (Wechselberger-Reya and Scott, 1999). In vivo staining of postnatal day 7 mouse cerebella for GFAP and IRS1 shows that these two
Fig. 1. IRS1 protein is upregulated in proliferating CGNPs. (A) CGNP cultures were prepared from different litters on different dates. Preparations of the cells were treated with SHH or left untreated for 24 hours prior to lysis. The autoradiograph depicts a western blot for several downstream components of IGF signaling pathway and the cell cycle progression marker cyclin D2. Only IRS1 levels are upregulated in SHH-treated samples, which correlates with increases in cyclin D2. β-Tubulin demonstrates equal protein loading. (B) The autoradiographs show western blots for CGNPs treated with two SHH signaling pathway inhibitors, forskolin (10 μM) or cyclopamine (1 μg/ml), for increasing time points. In the absence of continuous SHH signaling, levels of IRS1 decrease, which also correlates with decreased cyclin D2 expression. (C) SHH-treated CGNPs were fixed and immunostained for IRS1 (red) and p27 (green). IRS1 and p27 mark different populations of cells. Confocal imaging (right panel) confirms IRS1 presence in the nucleus (arrowhead) and cytoplasm (arrow). (D) Left: cerebellar section from postnatal day 7 mouse immunostained for IRS1 (red) and GFAP (green). Middle (low power) and right (high power): SHH-treated primary CGNP cultures immunostained for IRS1 (red) and GFAP (green). IRS1 is excluded from glia both in vivo and in vitro. (E) In the left (low power) and middle (high power) panels, PN 7 mice were pulsed with BrdU and stained for BrdU (green) and IRS1 (red). IRS1 colocalizes to proliferating CGNPs in the EGL. In the far right panel, IRS1 (red) is expressed in the cytoplasm of BrdU-positive cells (green) in CGNP cultures.

markers do not colocalize (Fig. 1D, left panel). To confirm that increased IRS1 expression occurs in CGNPs, not glia, cultures were treated with SHH for 24 hours and then immunostained for GFAP and IRS1 (Fig. 1D, middle and right panels) or BrdU and IRS1 (Fig. 1E, right panel). BrdU-incorporating cells were also Zic1 positive, confirming their identity as CGNPs (see Fig. S2 in the supplementary material). Our results demonstrate that increased IRS1 expression occurs in cells that have incorporated BrdU, both in culture and in vivo, and is excluded from GFAP-expressing glial cells (Fig. 1D,E) (Aruga et al., 2002). Thus, IRS1 is upregulated in proliferating CGNPs.

In vivo, by postnatal day 15 IRS1 levels are reduced, although some expression remains in the EGL in conjunction with the proliferation marker PCNA (see Fig. S2 in the supplementary material). We found that IRS2 is restricted to Purkinje neurons and is excluded from the EGL (see Fig. S2 in the supplementary material). Our observation that IRS2 is not found in CGNPs, but rather located in Purkinje neurons, the cells that provide SHH to CGNPs, suggests that a reported requirement for IRS2 in CGNP proliferation (Schubert et al., 2003) may be attributed to Purkinje cell defects (see Fig. S2 in the supplementary material). Indeed we detected reduced SHH signaling, and reduced levels of IRS1 protein in the EGL of IRS2-null neonatal mouse cerebella (see Fig. S3 in the supplementary material).

**SHH signaling stabilizes IRS1 protein levels without altering Irs1 transcripts**

Canonical SHH signaling occurs through the action of GLI and Nmyc transcription factors resulting in the upregulation of target mRNA transcripts. In order to determine whether SHH signaling affects IRS1 protein levels by increasing Irs1 transcripts, we used RT-PCR analysis for Irs1, cyclin D2 and actin (Fig. 2A,B). RNA was collected for RT-PCR or quantitative PCR from CGNPs treated with or without SHH for 24 hours. Levels of cyclin D2, an indirect target of SHH signaling (Kenney and Rowitch, 2000) are increased in SHH-treated CGNPs (Fig. 2A). However, levels of Irs1 are constant regardless of treatment, indicating that SHH does not affect Irs1 transcription (Fig. 2A,B). These results are in agreement with previous work performed in non-neural cell types showing that IRS1 protein levels can change without changes in Irs1 transcription (Nemoto et al., 2006; Renstrom et al., 2005; Rice et al., 1993).

To determine whether SHH stabilizes IRS1 protein levels by inhibiting its degradation, we asked how co-treatment of CGNPs with the SHH inhibitor cyclopamine and the proteasome inhibitor lactacystin affected IRS1 protein levels. In the presence of the SHH inhibitor cyclopamine, IRS1 levels declined as expected (Fig. 2C). Reduction in IRS1 protein levels was prevented when lactacystin was also present, suggesting that inhibition of SHH causes IRS1 to be targeted for degradation (Fig. 2C). Moreover, in addition to preventing IRS1 turnover, SHH may also affect Irs1 mRNA translation, as IRS1 transcripts are present in non-SHH-treated cells but lactacystin treatment did not induce IRS1 protein accumulation, indicating that the Irs1 mRNA is not being translated in CGNPs that have not been exposed to SHH. However, this remains to be conclusively determined, as current methodologies for examining SHH-responsive mRNA translation have not yet been refined for use with such limited starting material as primary CGNP cultures.

Although SHH signaling does not activate IRS1 transcription, it is possible that the SHH transcriptional target GLI1 can regulate IRS1 protein. To determine whether GLI activity can promote accumulation of IRS1 in CGNPs, we infected CGNP cultures with a GLI1 retrovirus. After the 2 hour infection period, the viral supernatent was withdrawn and replaced with fresh CGNP medium. Detection of reduced SHH signaling, and reduced levels of IRS1 protein in the EGL of IRS2-null neonatal mouse cerebella (see Fig. S3 in the supplementary material).
GLI-mediated mechanisms that promote IRS1 protein accumulation.

exogenous SHH, suggesting the existence of GLI-mediated and non-
CGNPs infected with GFP-expressing retroviruses and treated with
reported (Oliver et al., 2003). IRS1 was present but at lower levels than
sustain proliferation as indicated by cyclin D2 levels and as previously
subsequently cultured without SHH for 36 hours. GLI1 infection can
protein levels in CGNPs infected with GLI1 retroviruses and
were treated with SHH, cyclopamine or lactacystin (10 μM) for
indicated times. Levels of IRS1 protein are stabilized in the presence of
the lactacystin and cyclopamine. (D) Western blot analysis of IRS1
protein levels in CGNPs infected with GLI1 retroviruses and
subsequently cultured without SHH for 36 hours. GLI1 infection can sustain proliferation as indicated by cyclin D2 levels and as previously
reported (Oliver et al., 2003). IRS1 was present but at lower levels than in
CGNPs infected with GFP-expressing retroviruses and treated with exogenous SHH, suggesting the existence of GLI-mediated and non-
GLI-mediated mechanisms that promote IRS1 protein accumulation.

that synergize with GLI1 to achieve the full IRS1 accumulation response to exogenous SHH signaling. Future studies will determine whether GLI affects IRS1 stability and/or mRNA translation, and will identify non-GLI mediators of IRS1 accumulation.

SHH signaling stabilizes IRS1 by downregulating S6 kinase

Several signaling proteins have been shown to play roles in IRS1 degradation, including suppressor of cytokine signaling (SOCS) signaling, retinoic-acid mediated protein kinase C activation, and mTOR, which mediates IRS1 phosphorylation on Ser636/639, to promote its degradation (del Rincon et al., 2004; Haruta et al., 2000; Ishizuka et al., 2007; Shah and Hunter, 2006). As CGNPs are not cultured in the presence of cytokines or retinoic acid, we asked whether SHH signaling affected mTOR-regulated IRS1 turnover by reducing IRS1 phosphorylation and/or activity of S6 kinase, the mTOR substrate shown to directly phosphorylate IRS1 (Shah and Hunter, 2006). We treated CGNPs with SHH and cyclopamine for increasing periods of time, and then carried out western blot analysis for total IRS1 and Ser636/639-phosphorylated IRS1. As expected, IRS1 protein levels declined over time in the presence of cyclopamine (Fig. 3A). However, the ratio of Ser636/639-IRS1 to total IRS1 increases, indicating SHH inhibition increases IRS1 phosphorylation on this destabilization-associated site.

IRS1 phosphorylation on Ser636/639 occurs downstream of mTOR in 293HEK cells (Tzatsos and Kandror, 2006). In order to determine whether mTOR signaling has an affect on IRS1 protein levels in response to SHH, we treated CGNPs with rapamycin, a compound that inhibits the mTOR:Raptor complex, in the presence of or after the withdrawal of SHH. Consistent with previous reports (Hartley and Cooper, 2002), we observed that in the presence of rapamycin, IRS1 levels accumulated (Fig. 3B,C) without any affect on cell survival based on activated caspase 3 levels (data not shown). Interestingly, levels of IRS1 were stabilized after treatment with rapamycin, even when SHH was removed at the time of rapamycin addition (Fig. 3B, lane 4). This suggests that inhibiting mTOR can promote IRS1 stabilization in CGNPs. To further investigate the relationship between SHH signaling and the mTOR
pathway, we treated CGNPs with cyclopamine and rapamycin, and examined IRS1 levels. Although treatment with rapamycin increased IRS levels compared with SHH alone, we observed only partial recovery of IRS1 protein when CGNPs were treated with cyclopamine and rapamycin (Fig. 3D). This result suggests that inhibition of mTOR is not the sole mechanism through which SHH mediates IRS1 accumulation. For example, SHH may also regulate Irs1 mRNA translation in an mTOR-independent manner. The results may also indicate that mTOR is regulated in part by signaling through SMO.

S6K1, a major target of mTOR, is required for IRS1 phosphorylation in cells and can also directly phosphorylate IRS1 (Easton et al., 2006; Um et al., 2004). Stabilization of IRS1 protein in CGNPs after treatment with rapamycin could occur directly by the inhibition of mTOR or indirectly by inhibiting S6K1, the downstream target of mTOR. As both mTOR and S6K1 can phosphorylate IRS1, we wanted to determine the affects of SHH on the activation of S6K. Western blot analysis (Fig. 3E) shows that S6K phosphorylation, an indicator of its activation by mTOR, is reduced in SHH-treated CGNPs. Consistent with reduced S6K activity in SHH-treated CGNPs, we also observed reduced phosphorylation of the S6K substrate ribosomal protein S6 in SHH-treated CGNPs (Fig. 3B,D).

S6K de-phosphorylation is mediated by protein phosphatase 2A (Peterson et al., 1999; Petritsch et al., 2000), a positive regulator of N-myc stability (Sjostrom et al., 2005) and we can inhibit PP2A by the addition of okadaic acid (OA). As expected, PP2A inhibition destabilized N-Myc (Fig. 3E) (Sjostrom et al., 2005). OA treatment also rescued S6K phosphorylation in the presence of SHH (Fig. 3E, final lane). In addition, OA treatment not only rescues S6K activity, as determined by phosphorylation of its substrate ribosomal protein S6, but it also blocks SHH-mediated IRS1 stabilization (Fig. 3F). These results suggest that SHH signaling inhibits S6K1 activity, thereby promoting stabilization of IRS1 protein.

Alteration of IRS1 levels modulates CGNP proliferation in vitro

To investigate a role for IRS1 in CGNP proliferation, we used lentiviruses expressing small hairpin RNAs (shRNAs) targeting IRS1. We found that of six shRNAs tested by transfection into a murine cell line, all six effectively knocked down IRS1 (data not shown). SHH-treated CGNPs infected with pooled lentiviruses expressing shRNA against IRS1 had reduced IRS1 protein levels (Fig. 4A). We did not observe compensatory upregulation of IRS2, nor did we detect effects of IRS1 knock down on other members of the IGF pathway (Fig. 4A). Consistent with results shown in Fig. 1, neither SHH treatment nor IRS1 knockdown affected AKT phosphorylation. However, levels of cyclin D2 are decreased in response to IRS1 knockdown, suggesting that reduction of IRS1 protein affects cell cycle progression (Fig. 4A).

To determine whether shRNA-mediated IRS1 knock down impairs CGNP proliferation, we first assayed these viruses on PN5 cerebellar slices. We infected 300 μm cerebellar sections with IRS1 shRNA lentiviruses, then treated the slices with medium containing SHH or SHH vehicle (‘SHH–’). After 48 hours, the sections were pulsed with BrdU for 4 hours, fixed, sectioned and stained for BrdU incorporation. Treatment with exogenous SHH increases levels of BrdU incorporation (Fig. 4B, right panels) as well as EGL thickness as previously reported (Wechslar-Reya and Scott, 1998). Infection of the slice cultures with shRNA lentiviruses in conjunction with SHH leads to reduced BrdU staining (Fig. 4B, bottom right panel).

Importantly, changes in proliferation in response to SHH and/or shRNA lentivirus occurred in the EGL where CGNPs reside during their proliferation phase.

To quantify the effects of shRNA treatment on proliferation, we measured BrdU incorporation in control or shRNA lentivirus-infected dissociated CGNPs. Forty-eight hours after infection, the CGNPs were pulsed with BrdU for 2 hours prior to fixation and immunofluorescent stained for BrdU incorporation or the proliferation marker Ki67 (see Fig. S4 in the supplementary material). We found a significant reduction in BrdU-positive cells
in SHH-treated CGNPs infected with shRNA lentiviruses targeting IRS1 compared with SHH-treated alone (Fig. 4C). To confirm that effects of IRS1 knock down are specifically attributed to CGNPs, Percoll purified cultures comprising 98% CGNPs were treated with shRNAs with or without SHH. As in the mixed culture system, there was a significant decrease in proliferation after exposure to IRS1-specific shRNAs (Fig. 4D). CGNPs infected with lentiviruses targeting GFP did not show reduced proliferation (see Fig. S4D in the supplementary material). Vehicle-treated cells did not proliferate under any conditions. These results demonstrate that IRS1 is a crucial mediator of SHH-mediated CGNP proliferation.

IGF-induced PI-3K and AKT activation are essential for neuronal survival (Dudek et al., 1997). Although we do not see changes in the activation of AKT in response to SHH (Fig. 1A) or with treatment with IRS1 shRNA (Fig. 4A), knock down of IRS1 may still impact CGNP survival. To determine whether proliferation decreases in IRS1 knock down CGNPs reflect compromised survival, we performed immunostaining for cleaved caspase 3. Knock down of IRS1 in the presence or absence of SHH did not affect cell survival in mixed or Percoll purified cultures (Fig. 4E,F; see Fig. S4 in the supplementary material), suggesting that the function of IRS1 in CGNPs promotes proliferation and not cell survival.

In order to determine whether IRS1 overexpression can maintain CGNP proliferation in the absence of SHH, we infected CGNPs with a retrovirus expressing IRS1. Ectopic expression of IRS1 in CGNPs did not result in alteration of other members of the IGF pathway (Fig. 5A). Ectopic IRS1 expression in SHH-treated cells did not result in increased cyclin D2 levels. However, we observed that overexpression of IRS1 maintained cyclin D2 expression when SHH was removed (Fig. 5A). To determine whether IRS1-driven cyclin D2 expression in the absence of exogenous SHH is associated with activation of intracellular SHH pathway components, we assayed expression levels of Gli1 and Gli2. As shown in Fig. 5B, RT-PCR analysis of these transcription factors demonstrates that IRS1 does not induce their expression. However, expression of N-myc, a well-characterized SHH signaling target (Kenney et al., 2003), is increased in response to IRS1 overexpression. These results suggest that SHH-mediated activation of N-myc may be a result of IRS1 stabilization, and that IRS1 does not act upstream of Gli1.

We next determined the effect of IRS1 expression on CGNP proliferation by staining BrdU-pulsed CGNPs infected with IRS1-expressing retroviruses. We see an increase in BrdU staining as well as increased Ki67, a proliferation marker, in SHH-treated CGNPs compared with untreated cells as expected (see Fig. S5A in the supplementary material). Ki67 expression is maintained in the absence of SHH when cells are infected with IRS1-expressing retrovirus before SHH withdrawal. To confirm these results and to quantify the effects of IRS1 expression on proliferation, CGNPs were pulsed with BrdU as described. CGNPs from which SHH was removed after infection with IRS1 have significantly more BrdU incorporation compared with untreated alone (Fig. 5C). Similar results were obtained with IRS1-infected SHH-treated cells exposed to cycloamine, indicating that IRS1 effects on CGNP proliferation are Smoothened independent (data not shown). However, in comparison with SHH-treated, non-IRS1-infected CGNPs, BrdU incorporation is reduced, indicating that other components of the SHH signaling pathway are necessary to maintain full CGNP proliferation in vitro. Treatment of IRS1-infected CGNPs with cycloamine yielded similar results (data not shown), indicating that sustained proliferation in IRS1-infected, SHH withdrawn CGNPs is not a result of residual SHH in the medium.

The increase in proliferation levels in IRS1 overexpressing, non-SHH-treated cells does not appear to result from a cell survival advantage as levels of activated caspase 3 remain the same in all treatment groups (Fig. 5D, see Fig. S5B). Overexpression of IRS1 followed by ongoing SHH treatment did not promote increased proliferation. We speculate that this is because IRS1 is a large scaffolding protein and inducing supranormal levels may lead to formation of non-functional complexes owing to limiting levels of other components. Taken together, our results suggest that through IRS1 upregulation, the SHH signaling pathway may in effect be hijacking mitogenic effectors of IGF signaling. It is also possible that IRS1 in CGNPs may have additional, IGF-independent functions contributing to proliferation.

**IRS1 in SHH-mediated mouse medulloblastoma**

Aberrant IRS1 expression has been associated with several types of cancer, including medulloblastoma (Del Valle et al., 2002; Waters et al., 1993). As we see a role for IRS1 in mediating CGNP proliferation, we looked at IRS1 protein in two mouse models of...
medulloblastoma. Both the \textit{Ptch1} +/- and Neuro-D2-SmoA1 mice form spontaneous medulloblastoma as a result of aberrant activation of the SHH signaling pathway (Berman et al., 2002; Goodrich et al., 1997; Hallahan et al., 2004). We found that tumors in both mice strains showed elevated IRS1 levels compared with adjacent normal brain tissue (Fig. 6 and data not shown). Tumor lysates from these mice also show increased IRS1 levels compared with non-tumor cerebellar tissue, which correlates with increased cyclin D2 expression (Fig. 6E).

DISCUSSION

Normal CGNP proliferation is dependent upon SHH and IGF signaling, and both signaling pathways are implicated in medulloblastoma, a brain tumor for which CGNPs are a proposed cell of origin. We found that SHH specifically upregulates the IGF receptor-interacting scaffolding protein IRS1 without altering levels of other members of the IGF pathway, including IRS2, GAB1 or activated AKT. Furthermore, IRS1 protein levels depend upon constant SHH signaling in vitro and IRS1 is found in proliferating CGNPs in vivo, suggesting that IRS1 appears at the right time and place to play roles in SHH-dependent CGNP proliferation. IRS1 protein is also seen in mouse medulloblastoma in conjunction with high levels of cyclin D2 expression, suggesting that IRS1 may also be playing a role in disease. Such a role for IRS1 is supported by our experiments showing that IRS1 overexpression in CGNPs is capable of sustaining proliferation even in the absence of SHH, whereas knock down of IRS1 blocks proliferation in the presence of SHH.

In cell lines, it has been shown that IRS1 stability can be regulated by a negative-feedback loop wherein S6K1 phosphorylates IRS1, targeting it for degradation by the proteasome (Easton et al., 2006). We observed that this feedback loop exists in primary CGNP cultures, and that SHH interferes with this process by suppressing S6 kinase activity, thereby stabilizing IRS1. Our results indicate that SHH suppresses S6 kinase activity by inhibiting its upstream regulator mTOR. Two previous studies have indicated interactions between the mTOR pathway and the hedgehog pathway, in that mTOR can regulate Indian hedgehog levels in chondrocytes and that mTOR inhibition impairs survival in epitheloid cells overexpressing GLI1 (Phornphutkul et al., 2008; Louro et al., 1999). These studies did not investigate how SHH signaling affects mTOR activity in primary neurons. Our data indicate that SHH-mediated mTOR inhibition is to some extent dependent upon Smoothened signaling, but the inability of cyclopamine to completely rescue S6K activity indicates existence of additional mechanisms. IRS1 regulation by stabilization instead of increased transcription has been reported in other cell types (Lee et al., 2003; Nemoto et al., 2006; Renstrom et al., 2005), but not in the setting of SHH signaling. Our study indicates a role for SHH-mediated IRS1 mRNA translation in addition to its stabilization in proliferating CGNPs.

In addition to regulating IRS1 stability, SHH may also affect IRS1 mRNA translation, as the mRNA for IRS1 is present in untreated CGNPs but the protein is not detectable, even upon the addition of lactacystin. The determine whether SHH influences loading of IRS1 mRNA onto polysomes in CGNPs will be of future interest when techniques have evolved to make this experiment feasible. Currently, our results are consistent with a role for IGF signaling through AKT to promote survival (Dudek et al., 1997; Miller et al., 1997), coincident with stabilization of N-myc through GSK3β (Kenney et al., 2004). In the absence of SHH signaling, IGF signaling activates the PI-3K pathway, leading to neuronal survival (Fig. 7). As levels of the IGF effectors IRS2 and GAB1 are...
unchanged in response to SHH, IGF survival signals may proceed through these signaling molecules and not IRS1. In the presence of SHH, IGF signaling continues to send survival signals but can now also exert mitogenic effects through newly translated, stabilized IRS1, along with other factors important in mediating CGNP proliferation, such as N-myc (Fig. 7) (Kenney et al., 2003).

Our results demonstrate a role for IRS1 in mediating CGNP proliferation, and thus IRS1 may have a role in cell cycle progression in medulloblastoma. It has been shown that overexpression of IRS1 is sufficient to mediate transformation of mouse fibroblasts (D’Ambrosio et al., 1995). IRS1 has been found to have a role in several types of cancer, including breast cancer, while the IGF pathway has been strongly linked to medulloblastoma (Dearth et al., 2007; Dearth et al., 2006; Del Valle et al., 2002; Rao et al., 2004). One group has reported IRS1 expression in a JC-virus-induced mouse medulloblastoma (Khalili et al., 2003), but a relationship between IRS1 and SHH-mediated medulloblastoma has not been reported. We report overexpression of IRS1 in two models of mouse SHH-induced medulloblastoma. This makes IRS1 an attractive candidate as a potential target for cancer therapies.

How IRS1 mediates CGNP proliferation remains unclear. Our data suggest that the effects of IRS1 do not occur through the activity of PI-3K. One possibility is that IRS1 increases CGNP survival by interacting with Bcl2 (Ueno et al., 2000); however, modulation of IRS1 levels in vitro do not alter cell survival, making this scenario unlikely. Recent studies in mammary tumors suggest that IRS1 interacts with proteins with known roles in proliferation, such as β-catenin (Dearth et al., 2006). It remains to be determined whether this occurs in CGNPs and SHH-derived medulloblastomas. It is also possible that IRS1, a large scaffolding protein, has unknown interactors in SHH-stimulated CGNPs. Future studies exploring the specific mechanism through which IRS1 promotes SHH-stimulated CGNP proliferation may also identify novel targets for development of new treatments for medulloblastoma and other cancers.

We thank the Sontag Foundation and Alex’s Lemonade Stand Foundation for supporting these studies. This work was also supported by NIH 1R01NS061070-01 (A.M.K.) and NRSA fellowship F32AG030888 (S.P.). A.F.-L receives fellowship support from the Spanish Ministry of Education. The contents of this manuscript are the sole responsibility of the authors and do not necessarily represent the official views of the NINDS, the NIA, the Sontag Foundation or Alex’s Lemonade Stand Foundation. We thank Tim Gershon for assistance with generating IRS1 retroviruses, and we thank William Hahn for advice concerning use of shRNA lentiviruses.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/9/3291/DC1

References


