Smooth muscle cell (SMC) proliferation plays a key role in vascular proliferative disorders. The molecular mechanisms that control cell cycle entry of SMCs in response to vascular injury are not well understood. Id2 (inhibitor of DNA binding) is a member of the helix-loop-helix (HLH) family of transcription regulators that are known to promote cell cycle progression. Thus, we investigated the role of Id2 in SMC growth and cell cycle regulation. The results demonstrated that overexpression of Id2 resulted in a significant enhancement of SMC growth via increased S-phase entry. A possible mechanism of Id2-enhanced SMC growth is via regulation of p21 expression, as overexpression of Id2-inhibited transcriptional activity of a 2.3-kb p21 promoter/luciferase reporter construct as well as p21 protein levels. Id2 enhancement of SMC growth and inhibition of p21 expression were dependent on phosphorylation of Id2 by cyclin E/cdk2, as an Id2 cDNA containing a mutation in the cdk2 phosphorylation site (serine 5) failed to regulate SMC cell cycle progression or p21 promoter activity. The mechanism of cyclin E/cdk2 control of the Id2 effect may in part involve regulation of nuclear transport; unlike wild-type Id2, the Id2 mutant was not transported to the nucleus. Finally, in a rat carotid model of arterial injury, Id2 was expressed in a temporal pattern that parallels the kinetics of cellular proliferation. In summary, these results provide evidence that the Id2 protein is integrated into the cell cycle regulatory cascade that results in SMC proliferation following vascular injury and suggest that this effect is at least in part via a cdk2-dependent inhibition of p21 gene expression.

Smooth muscle cell (SMC) proliferation is a key component of the response to vascular injury in atherosclerosis and restenosis in humans and following experimental angioplasty in animals (1–3). Several recent studies have focused interest on the role of Id proteins in regulation of the cell cycle in the SMC proliferative response to injury. These data provide evidence for a unique link between Id2 and the cell cycle in the SMC proliferative response to injury.
night at 4 °C in PBS containing 4% nonfat milk. Blots were then incubated for 1 h at room temperature with the following antibodies at 1:200 dilution: rabbit polyclonal anti-Id2 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-p33 (Santa Cruz Biotechnology), or mouse monoclonal anti-p21 antibody (BD PharMingen, San Diego, CA). Blots were washed twice and incubated in PBS containing a 1:2000 dilution of horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were visualized using a chemiluminescent detection substrate (Luminol Reagent, Santa Cruz).  

**Plasmid Construction**—A full-length, wild-type Id2 cDNA and an Id2 cDNA (Id2S5A) were generated using RT-PCR on total RNA isolated from cultured rat SMCs. To make Id2S5A, an AG—GC mutation was inserted at nucleotides 76–77 of the forward primer. The PCR products were inserted into the HindIII and XbaI sites of pRSV/Invitrogen to make pRcId2 and pRcId2S5A or the plasmid p3XFLAG14 (Sigma) to make pFId2 and pFId2S5A. The plasmid p21Luc was constructed as follows: a 2.3-kb fragment of the human p21 promoter was obtained from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). This promoter was subcloned into the plasmid pGL3 (Promega Corp., Madison, WI) upstream of the luciferase reporter gene, using the XhoI and MluI sites on pGL3.  

**Transient Transfections**—Transfections were performed using Fu- gene transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol.  

**Adenoviral Infections**—The adenoviral vector AdId2, which contains a full-length Id2 cDNA under the control of a CMV promoter, was a gift from Dr. Mark Israel (Dept. of Neurosurgery, University of California, San Francisco, CA). Rat aortic SMCs were infected at a multiplicity of infection (MOI) value of 100 with AdId2 or AdGal control.  

**Stable Transfections/Proliferation Assays**—Cultured rat aortic SMCs were transfected with 5 µg of linearized plasmid (pRc/RSV control or pRcId2). Forty-eight hours after transfection, 400 µg/ml G418 (Cellgro, Alexandria, VA) was added to the culture medium. SMCs stably transfected with either pRc/RSV or pRcId2 plasmids were plated onto 96-well plates at a density of 1,000 cells/well. Cells were grown in DM10 for 5 days, then counted using a colorimetric cell proliferation assay (Celltiter Aqueous 1 reagent, Promega). Absorption at 492 nm was converted to cell number using a previously generated standard curve of absorption versus SMC number. Overexpression of Id2 in pRcId2 stables was confirmed by Western blotting.  

**S-phase Analysis**—Rat aortic SMCs were co-transfected with 2 µg of the SV40-encoded neomycin resistance gene and 4 µg of either pRc/ RSV, pRcId2 or pRcId2S5A. Twenty-four hours after the transfection, the medium was changed to serum-deprived medium and the cells were incubated for an additional 72 h. Cells were pulsed with 10 µM bromodeoxyuridine (BrdUrd, Roche Molecular Biochemicals) for 24 h followed by fixation with 4% paraformaldehyde for 1 h. Transfected cells were identified by staining with RedGal reagent (Research Organics, Cleveland, OH) for 4 h at 37 °C. When sufficient gal staining was achieved, cells were incubated in 2 N HCl for 1 h at 37 °C, washed with PBS, and incubated for 1 h with a mouse monoclonal anti-BrdUrd antibody (Sigma) diluted 1:500 in PBS. Following two washes with PBS, cells were incubated with a biotinylated horse anti-mouse secondary antibody and then a peroxidase-labeled DAB reagent substrate. Transfected cells were counted in each well using bright field microscopy. For each sample, the percent transfected cells traversing S-phase was the percent RedGal-positive cells that were also BrdUrd-positive. The experiment was performed in triplicate for a total of three repeats, and the results were combined for statistical analysis.  

**Promoter Reporter Assays**—Rat aortic SMCs were transfected with 1 µg of p21Luc and 6 µg of either pRc/RSV, pRcId2, or pRcId2S5A. Following transfections, cells were incubated in medium containing 10% FBS for 48 h at 37 °C with 5% CO2. Cells were then harvested, and luciferase activity was measured. The transfections and luciferase assays were repeated in triplicate in three separate runs. Luciferase activity for each sample was normalized to total sample protein using a protein assay (Bio-Rad) and reported as a percentage of control promoter activity.  

**Cellular Localization of Id2/Id2S5A**—Rat aortic SMCs were plated on sterile coverslips and transfected with 0.5 µg of an E47 expression vector and 0.5 µg of either pFId2 or pFId2S5A. Thirty-six hours after transfection cells were fixed with methanol/acetone for 1 min, washed, and incubated with 10 µg/ml of a fluorescein isothiocyanate-labeled anti-FLAG antibody (M-2, Sigma) for 1 h at room temperature. Coverslips were then washed, mounted, and examined under fluorescence.  

**Rat Model of Arterial Injury**—Male 300-g Sprague-Dawley rats (Harlan Labs, Indianapolis, IN) were subjected to balloon arterial injury as previously described (13). At 1, 6, 14, 28, and 60 days (n = 5 for each time point) following injury, the rats were given an overdose of intraperitoneal ketamine/xylazine, pressure perfused with 4% paraformaldehyde, and the injured left and control right carotids were removed and postfixed for 2 h, dehydrated in a graded alcohol/xylene series, and paraffin-embedded. Five-micron sections were stained using the Vectastain Elite ABC kit (Vector Laboratories) as follows. Sections were blocked for 30 min in secondary serum followed by incubation for 2 h at room temperature with a 1:100 dilution of a rabbit polyclonal anti-Id2. Slides were washed twice with PBS and incubated with a biotinylated anti-rabbit antibody diluted 1:2000 in 2% goat serum for 1 h at room temperature. Slides were then incubated for 30 min in 0.3% hydrogen peroxide followed by Vectastain Elite ABC Reagent for 30 min. Slides were washed twice and incubated with diaminobenzene peroxidase substrate (Sigma) for 2 min, counterstained with hematoxylin, and mounted with Vectashield mounting medium (Vector Laboratories).  

![Fig. 1. Enhancement of SMC growth by overexpression of Id2.](http://www.jbc.org/)

**RESULTS**  

**Overexpression of Id2 Enhances SMC Proliferation**—To examine whether Id2 has direct effects on SMC growth, cultured SMCs transfected with pRcId2 or pRc/RSV control were selected for neomycin resistance to obtain a stably transfected population of cells. Following selection, resistant cells (~150,000 in each transfection) were pooled to avoid clonal variation and assayed for cell number. Overexpression of Id2 in stably transfected cells was confirmed by Western blot (Fig. 1, inset). Compared with control stable transfectants, SMCs overexpressing Id2 demonstrated a 41% increase in cell number 5 days after plating (Fig. 1: 4625 versus 6513; p < 0.001), demonstrating that Id2 expression enhances SMC proliferation.  

**Enhancement of SMC Proliferation by Id2 Is due to an Increase in S-phase Entry; the cdk2 Phosphorylation Site at Ser-5 Is Essential for This Effect**—To confirm that the increase in the SMC number seen with Id2 overexpressing cells reflected enhanced cell cycle activity, the effect of Id2 overexpression on BrdUrd uptake was assayed. Results suggested that the enhanced SMC number noted with stable wild-type Id2 overexpression may in part be due to the enhancement of cells entering S-phase, as Id2-transfected cells demonstrated an 81% increase in BrdUrd uptake versus control cells (Fig. 2, 24 versus 16% for control, p = 0.01).
Id2 Regulation of Smooth Muscle Cell Proliferation

Previous studies have demonstrated that Id2 contains a consensus cdk2 phosphorylation site (Ser-5), which is phosphorylated by cdk2 in late G1 (14). The N terminus of Id2 contains a consensus cdk2 phosphorylation site at serine 5. Prior results suggested that cyclin E/cdk2 phosphorylates Ser-5 in late G1, an event that was essential for Id2 enhancement of U2OS carcinoma cells. To determine whether the cdk2 site at Ser-5 is important for Id2 enhancement of SMC proliferation, an Id2 cDNA containing a serine to alanine mutation at Ser-5 (pRcId2S5A) was created. This mutation has previously been shown to be incapable of being phosphorylated by cyclin E/cdk2 (14). The effect of pRcId2S5A on S-phase entry of SMCs was compared with wild-type pRcId2-transfected SMCs using the BrdUrd assay. In contrast to SMCs overexpressing wild-type Id2, cells overexpressing Id2S5A did not differ from control (Fig. 2; 14 versus 13%; *p = 0.01). In contrast, transfection of SMCs with Id2S5A did not result in altered S-phase entry (13 versus 14%; **p = 0.535). The graph represents the mean of three repeats. Inset, Western blot demonstrating levels of Id2 protein in SMCs transfected with Id2 and Id2S5A.

Mechanism dependent on its cdk2 phosphorylation site.

To confirm that the effect of Id2 on p21 promoter activity results in an alteration of p21 protein levels, SMCs were infected with an adenoviral vector containing a full-length Id2 cDNA (AdId2) or a control AdLacZ virus. Forty-eight hours after infections, Western blotting of cell lysates was performed. Results demonstrated a decrease in p21 protein levels in AdId2-infected cells. In addition, p53 levels were not affected by AdId2 infection, confirming that the control of p21 by Id2 is via a p53-independent mechanism (Fig. 3b).

Id2 Overexpression in SMC Results in Decreased p21 Protein Levels via Inhibition of p21 Gene Transcription—Previous studies have identified a role for Id2 in regulating Rb function. This function is not shared by other members of the Id family (Id1 or Id3) (10); however, Id1 is known to regulate p21 gene expression (12). To determine whether Id2 also inhibits p21 gene expression and whether the cdk2 phosphorylation site is essential for this effect, cultured SMCs were transiently co-transfected with a luciferase reporter plasmid driven by a 2.3-kb fragment of the human p21 promoter (p21Luc) and either pRc/RSV, pRcId2, or pRcId2S5A. The results demonstrated that pRcId2 resulted in a 62% decrease in luciferase activity versus control (p = 0.001) whereas pRcId2S5A resulted in luciferase activity, which was not statistically different from control (p = 0.580, Fig. 3a). Thus, Id2 inhibits p21 gene expression via a mechanism dependent on its cdk2 phosphorylation site.

Id2 Is Expressed in the Vessel Wall following Balloon Vascular Injury—Given that SMC proliferation plays a key role in...
neointimal formation following experimental vascular injury, we sought to examine the expression of Id2 in the vessel wall following balloon injury of the rat carotid. Results demonstrated that the Id2 protein was not detected in the normal rat carotid (Fig. 5A) or 24 h following balloon injury (Fig. 5B). Six days after injury, the Id2 protein was detected diffusely throughout the medium and in scattered neointimal vascular SMCs (Fig. 5B). Fourteen days after balloon endothelial denudation, Id2 staining was noted throughout the neointimal layer with continued expression within the medium (Fig. 5D). Twenty-eight days after injury, Id2 was still expressed in the neointima although in a lower percentage of cells (Fig. 5E). Sixty days after injury, no Id2 protein was detected (Fig. 5F).

Thus, balloon endothelial denudation induces Id2 protein expression in medial and neointimal cells during vascular lesion formation. The pattern of Id2 expression following balloon injury in this model closely parallels the spatial and temporal pattern of SMC proliferation in this model (3), suggesting that Id2 is induced during periods of peak SMC growth in vivo, similar to that seen in cultured vascular SMCs.

**DISCUSSION**

Understanding the molecular mechanisms of cell cycle regulation in SMCs is critical to understanding and intervening in SMC growth, which is the hallmark of vascular proliferative disorders. The present study provides evidence that the HLH protein Id2 is an important factor in regulating cell cycle progression in SMCs. Results demonstrated that overexpression of Id2 enhances S-phase entry and proliferation of SMCs, possibly through regulation of levels of the p21 cdk inhibitor. Furthermore, the consensus cdk2 phosphorylation site (Ser-5) of Id2 is essential for p21 inhibition and growth enhancement. These data are the first to implicate Id2, a member of the HLH family of transcription factors, in the regulation of growth and cell cycle activity in SMCs.

Recent studies provide evidence that Id2 regulates cell cycle progression through mechanisms that do not involve the classic paradigm of bHLH antagonism. In U2OS human osteosarcoma cells, Id2 directly interacted with Rb and Rb-related pocket proteins (10, 16) and functioned as an antagonist to their growth suppressive effects. This novel mechanism was not seen with Id1 or Id3, suggesting that the Id proteins do not regulate cell growth via entirely redundant mechanisms. Recent studies demonstrated that Id1 inhibited p21 expression in HeLa cells and NIH 3T3 fibroblasts (12). The results of the present study are the first to demonstrate that Id2 also inhibits the expression of the p21 gene. Whether Id2 has effects on Rb function in SMCs is unknown. Nonetheless, these data provide evidence that Id2 regulates cell cycle progression through at least two different mechanisms.

In addition to the effects of Id2 on cell cycle protein expression and function, Id2 is integrated into the cell cycle via another mechanism. The N terminus of Id2 contains a conserved, functional site for cyclin E/cdk2 and cyclin A/cdk2 phosphorylation. Previous studies have demonstrated that both cyclin E and cyclin A/cdk2 enzymes can phosphorylate at the Ser-5 residue of Id2 in vitro. In vivo Id2 is phosphorylated on Ser-5 late in G1, coincident with peak cyclin E activity, suggesting that Ser-5 is a substrate for cyclin E/cdk2 phosphorylation in vivo (14). Results of the present study demonstrate that the cdk2 phosphorylation site on Id2 is essential for Id2 enhancement of SMC growth and Id2 inhibition of p21 expression. Our data suggest that the mechanism of this effect may in part involve regulation of nuclear transport of Id2, as the cdk2 phosphorylation site mutant was excluded from the nucleus. A possible explanation for the difference in nuclear transport of wild-type Id2 versus the cdk2 phosphorylation site mutant is that bHLH partnering is regulated by this site. However, we were unable to define a bHLH factor that demonstrated differential binding characteristics between wild-type Id2 and the Id2 mutant.

The expression pattern of Id2 in the rat carotid following arterial injury is consistent with its role as a cdk2-dependent

**FIG. 4. Cellular localization of Id2 and Id2S5A.** Cultured rat aortic SMCs were transfected with FLAG-tagged Id2 or Id2S5A, followed by staining with an fluorescein isothiocyanate-labeled anti-FLAG antibody. A, wild-type Id2-transfected cells demonstrated primarily nuclear anti-FLAG staining. B, Id2S5A-transfected cells demonstrated diffuse, non-nuclear anti-FLAG staining.

**FIG. 5. Expression of Id2 following balloon arterial injury.** An anti-Id2 antibody was used to detect Id2 in the rat carotid after injury. Id2 staining is brown DAB staining, hematoxylin counterstain is blue. No Id2 is detected in the uninjured vessel (A) or at 24 h after injury (B). Id2 staining is noted in the medium at 6 days after injury (C, arrow indicates the internal elastic lamina) and throughout the medium and neointima at 14 days (D). Id2 staining is decreased at 28 days (E, neointima only depicted) and is undetectable at 60 days (F).
regulator of p21. Cyclin E and cdk2 are not detected in the medium of uninjured rat carotid arteries but are abundantly expressed 36 h after injury. Medial expression then declines, and by 10 days, protein expression peaks and is localized to the neointima (17). It is interesting to note that like cyclin E/cdk2, Id2 is initially expressed in the media. At this time p21 levels remain low (18) and proliferation is high (3). Id2 protein is subsequently abundantly expressed in the neointima when cyclin E and cdk2 are also abundant, and p21 expression is inhibited. Interestingly, Id2 is also expressed in the neointima of the injured rat carotid 14 and 28 days after injury, a time when the proliferative index is declining (3). This apparent contradiction could be explained by the fall in cyclin E/cdk2 levels and our data demonstrating that the cyclin E/cdk2 phosphorylation site of Id2 is essential for Id2-induced SMC proliferation. Although Id2 protein is detected within the neointima as late as 28 days after injury, it is likely that the majority of Id2 after 10–14 days is present in the unphosphorylated form, rendering it inactive as an inhibitor of p21 expression and an enhancer of SMC proliferation. The role of Id2 at these late time points is unknown. As the Id class of HLH factors is known to also regulate differentiation in a variety of cell types, it is interesting to speculate that Id2 may have additional but currently unknown roles in regulating the SMC phenotypic modulation seen after vascular injury.

In summary, the results of the present study provide evidence that the helix-loop-helix factor Id2 enhances SMC proliferation at least in part via a cdk2-dependent inhibition of p21 gene expression. Further understanding of the factors that regulate Id2 expression and function in response to vascular injury and additional targets for Id2-induced effects on SMC proliferation will not only enhance our understanding of the molecular mechanisms of vascular lesion formation, but may also provide potential targets for limiting the SMC proliferative response to vascular injury.

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Contribution of the Helix-Loop-Helix Factor Id2 to Regulation of Vascular Smooth Muscle Cell Proliferation

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