Id1, an inhibitory partner of basic-helix-loop-helix transcriptional factors, has recently been recognized as a potent contributor to angiogenesis. However, the molecular mechanism underlying its role in angiogenesis remains essentially unknown. Herein we demonstrate the subcellular localization of Id1 to be altered depending on the cellular context of vascular endothelial cells. Id1 was localized in the nuclei of human umbilical vein endothelial cells (HUVECs) cultured on uncoated plates, whereas it was translocated to the cytoplasm in HUVECs on Matrigel along with the formation of capillary-like structures. Treatment with the nuclear export inhibitor leptomycin B and mutagenesis analysis using green fluorescent protein-fused Id1 revealed CRM1/exportin-dependent nuclear export of Id1 in HUVECs on Matrigel. This nuclear export of Id1 was inhibited by protein kinase A (PKA) activation by dibutyryl cyclic AMP and forskolin but was promoted by PKA inactivation by H-89 and MDL-12,330A. Mutagenesis analysis of Id1 showed the phosphorylation of Ser-5 to possibly mediate the effect of PKA. These results suggest the function of Id1 as a transcriptional factor to be controlled by nuclearcytoplasmic shuttling during angiogenesis and that PKA might be involved in this process. This may serve as a novel mechanism regulating angiogenesis and as a possible target for therapeutic vascular regeneration.

Angiogenesis is a complex process involving various cellular events such as growth, differentiation, migration, and morphogenesis (1). Endothelial cells (ECs)2 play a principal role in angiogenesis, giving rise to vascular extensions from the existing vasculature by sprouting and intussusception (1). ECs receive extracellular signals such as vascular endothelial growth factor (VEGF) and angiopoietin with their receptors, thereby responding via intracellular signaling pathways and leading to changes in cellular behavior appropriate for vascular network formation (1). In the nucleus, various transcriptional factors function to control gene expression, which contributes to the angiogenic property of ECs (1, 2).

Id proteins, a subfamily of helix-loop-helix (HLH) transcriptional factors, have been implicated in regulating a variety of cellular functions including cell growth, differentiation and neoplastic transformation (3, 4). Id lacks a basic DNA binding domain and acts primarily as a transcriptional inhibitor of basic HLH transcriptional factors by inhibiting DNA binding and subsequent activation of transcription via heterodimerization (3). Recent evidence (5–11) suggests that Id1 and Id3 play pivotal roles in regulating developmental and postnatal angiogenesis. Complete loss of Id1 and Id3 genes resulted in vascular malformation in the forebrain and thereby in brain hemorrhage (10). With partial loss of these genes, neo-angiogenesis is blocked in tumor xenografts and spontaneous tumors (6–8, 11). Ectopic expression of Id1 in ECs enhanced angiogenicity in both in vitro and in vivo angiogenesis models (5, 9). To date, α6 and β4 integrins (7), matrix metalloprotease-2 (7), fibroblast growth factor receptor 1 (7), chemokine receptor 4 (11), and angiopoietin-1 (5) have been suggested to be downstream molecules of Id proteins. However, the molecular mechanism underlying the function and control of Ids in angiogenesis remains virtually unknown.

The cellular functions of Id proteins are strictly regulated at the levels of transcription and protein stability (3). In addition, recent studies have found that Id proteins are localized in the cytoplasm rather than the nucleus in various cells such as hemopoietic (12), neural (13–15), muscle (16, 17), and renal cells (18), suggesting that translocation between the nucleus and the cytoplasm (nucleocytoplasmic shuttling) may be involved in the functional regulation of Id proteins. Nucleocytoplasmic shuttling serves to modulate the functions of many signaling molecules and transcriptional proteins (19–21) and is mediated by the nuclear pore complex (22), where importin and the CRM1/exportin family facilitate nuclear import and export by binding cargo via recognition of nuclear import and export sequences (NLS and NES), respectively (22). Recent
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reports have suggested that Id proteins possess both NLS and NES and can be actively transported between the nucleus and the cytoplasm in an importin- and CRM1/exportin-dependent manner (23–25).

We report herein that the subcellular localization of Id1 in ECs is regulated by nucleocytoplasmic shuttling during angiogenesis. Id1 is translocated from the nucleus into the cytoplasm in vascular ECs forming capillary-like structures in a CRM1/exportin-dependent manner. Interestingly, protein kinase A (PKA) appears to block the nuclear export of Id1, possibly via phosphorylation of Ser-5. These findings indicate that the function of Id1 as a transcription factor may be regulated by nucleocytoplasmic shuttling, which is under the control of PKA activity, during angiogenesis.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-Id1 and Id3 antibodies were purchased from Santa Cruz Biotechnologies. Rabbit anti-GFP antibody was obtained from Medical & Biological Laboratories, rabbit anti-acetylated histone H3 antibody from Upstate Biotechnology, mouse anti-α-tubulin antibody from Sigma, biotinylated antibody against rabbit immunoglobulins from Immunoblotting, mouse anti-Id1, and horseradish peroxidase-conjugated antibodies against mouse immunoglobulins and rabbit immunoglobulins from Dako.

Plasmids and Construction of Id1 Mutants—The mouse Id1 cDNA was cloned as described previously (5). For construction of the N- or C-terminally GFP-tagged Id1, an Id1 cDNA fragment was inserted into the XhoI/EcoRI sites of the pEGFP-C2 expression vector (Clontech) or the XhoI/BamHI sites of the pEGFP-N3 expression vector (Clontech). Mutations in the NES-like region and putative PKA phosphorylation site were created by PCR-based site-directed mutagenesis. All of the constructs were verified by sequencing.

Cell Culture and Transfection—HUVECs were purchased from Clonetics, maintained in EBM medium (Clonetics, Inc.) according to the supplier’s instructions, and used at three to five passages for all experiments. EOMA cells (a cell line obtained from murine hemangioma) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and antibiotics (10 μg/ml streptomycin and 100 units/ml penicillin). Transient transfection was performed with Lipofectamine 2000 (Invitrogen) and the cells were used 17–24 h later for experiments. Cells transfected with plasmids encoding GFP-derivatives were observed by fluorescent microscopy. Transfection efficiency in HUVECs was 30–40% using this method.

Matrigel Tube Formation Assay—Formation of capillary-like structures by HUVECs was assessed on growth factor-reduced Matrigel for 20 to 24 h were treated with 10 nM of a specific inhibitor of CRM1/exportin, leptomycin B (LC Laboratories), for 1 h and used in the following experiments. Preliminary experiments showed the dosage used and the duration of LMB treatment to be optimal for nuclear accumulation of Id1 in HUVECs.

RT-PCR—Gene expression was evaluated by semiquantitative RT-PCR analysis. Total RNA was extracted using ISOGEN (Nippon Gene). After DNase I (Qiagen) treatment, total RNA was reverse-transcribed using Superscript II (Invitrogen) with random hexamer primers. For semiquantitative RT-PCR, the resultant cDNAs were amplified with Taq polymerase (Takara) in a thermocycler. The primer pairs used were as follows: for human Id1, 5′-AACCAGGTTGACAAAGTGCATGCGCT/3′ (218 base pair PCR product for Id1) and 457 base pair PCR product for Id1); for human Id1, 5′-GCTGCTCTACGACATGAAAC/3′ (255-base pair PCR product); and for human glyceraldehyde-3-phosphate dehydrogenase, 5′-TGGGCGCTCCGAACCAGTGA/3′ (318-base pair PCR product). Thermal cycling was performed for 22–30 cycles to maintain PCR conditions within the linear range of amplification before reaching saturation. Each cycle consisted of 30 s of denaturation at 94 °C, 1 min of annealing at 60 °C, and 1 min of extension at 72 °C.

Cell Fractionation and Western Blotting—Collected HUVECs were washed twice with cold PBS and suspended in cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5 mM dithiorthirol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2.5 mg/ml leupeptin, 10 mg/ml phenylmethylsulfonyl fluoride, 1.3 mg/ml pepstatin, 7.2 μg/ml aprotinin, 50 mM NaF). After centrifugation at 17,000 × g at 4 °C for 20 min, the supernatant was used as a total cell extract. For extraction of the nuclear and cytoplasmic fractions, HUVECs were suspended in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, 26% glycerol, 2.5 mg/ml leupeptin, 10 mg/ml phenylmethylsulfonyl fluoride, 1.3 mg/ml pepstatin, 7.2 μg/ml aprotinin, 50 mM NaF), and the suspension was vortexed and then centrifuged at 17,000 × g at 4 °C for 20 min. The soluble fractions were used as the cytoplasmic extracts. The pellets were washed twice with lysis buffer, resuspended in radioimmunoprecipitation assay buffer, and centrifuged at 17,000 × g at 4 °C for 20 min. The soluble fractions were used as the nuclear extracts. Similar quantities of the soluble fractions were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Millipore), and immunoblotted with an appropriate first antibody. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody, and the signals were visualized using ECL or ECL plus reagent (Amersham Biosciences). After detection of Id1 protein, the β-actin protein levels were assayed using the same membrane. For cell fractionation analysis, acetylated histone H3 and
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Exogenous Id1 was primarily cytoplasmic in vascular endothelial cells during angiogenesis. To examine molecular mechanisms underlying the role of Id1 in angiogenesis, we used an in vitro angiogenesis model: HUVECs on Matrigel (5). Previous studies (5, 28) have shown overexpression of Id1 to augment angiogenesis while its knockdown inhibited angiogenesis in an in vitro angiogenesis model. With our assay system, following attachment to the Matrigel, ECs start to gradually form networks with each other, resulting in capillary-like structures (data not shown). The expression of TAL-1/SCL, another HLH factor involved in angiogenesis, was reported to be up-regulated during the formation of these capillary-like structures (29). Thus, we initially evaluated the expression levels of Id1 in HUVECs on Matrigel. RT-PCR analysis with a primer pair spanning exons 1 and 2 of Id1 showed Id1 mRNA levels to also be comparable among different culture conditions, i.e. on uncoated slides and on Matrigel, both 3 and 24 h after plating (Fig. 1A). In addition to the expected 218-bp band for Id1 transcripts, RT-PCR with its specific primers also confirmed the expression of Id1, a splice variant form. Id1 RNA and protein contents as well as Id1 mRNA levels were comparable among different conditions.

RESULTS

Id1 Localization Is Predominantly Cytoplasmic in Vascular Endothelial Cells during Angiogenesis—To examine molecular mechanisms underlying the role of Id1 in angiogenesis, we used an in vitro angiogenesis model: HUVECs on Matrigel (5). Previous studies (5, 28) have shown overexpression of Id1 to augment angiogenesis while its knockdown inhibited angiogenesis in an in vitro angiogenesis model. With our assay system, following attachment to the Matrigel, ECs start to gradually form networks with each other, resulting in capillary-like structures (data not shown). The expression of TAL-1/SCL, another HLH factor involved in angiogenesis, was reported to be up-regulated during the formation of these capillary-like structures (29). Thus, we initially evaluated the expression levels of Id1 in HUVECs on Matrigel. RT-PCR analysis with a primer pair spanning exons 1 and 2 of Id1 showed Id1 mRNA levels to be comparable among different culture conditions, i.e. on uncoated slides and on Matrigel, both 3 and 24 h after plating (Fig. 1A). In addition to the expected 218-bp band for Id1 transcript, RT-PCR with this primer pair amplified another 457-bp band corresponding to Id1, a splice variant form. Id1 RNA and protein contents as well as Id1 mRNA levels were comparable among different conditions.

Immunocytochemistry and Microscopic Analysis—Cultured HUVECs on Matrigel were fixed with 4% paraformaldehyde/PBS for 30 min at room temperature. HUVECs and COS7 cells on culture slides were fixed with 4% paraformaldehyde/PBS for 10 min at 4 °C. After extensive washing with PBS, the cells were incubated in 2% skim milk/0.05% Triton X-100/PBS including 20 pg/ml RNase A for 30 min at room temperature. Cells were then incubated with the appropriate first antibody for 2 h at room temperature. After extensive washing with 0.05% Triton X-100/PBS, the cells were incubated with the appropriate secondary antibody conjugated with biotin, and the signal was subsequently detected with FITC-conjugated streptavidin (Dako). Nuclei were stained with propidium iodide (PI) (Sigma) and then observed using a computer-assisted confocal microscope (Nikon D-ECLIPSE C1). Photomicrographs were obtained at 1-μm intervals and reconstituted using EZ-C1 software (Nikon). The dominant subcellular localization of Id1 was determined based on differences in fluorescent intensity between the nucleus and the cytoplasm. For quantitative analysis of subcellular localization of exogenous Id1, percentages of cells with nuclear localization of transfection were calculated. For analysis of dynamic time-lapse imaging of Id1, HUVECs transfected with Id1-EGFP were treated with 10 nM LMB and then imaged every 10 min on a TE300 microscope (Nikon) using a 20× Nikon objective lens (NA = 0.45) and an ORCA 100 cooled CCD camera (Hamamatsu) and then analyzed using AquaCosmos imaging software (Hamamatsu).

Stimulation and Inhibition of Protein Kinase A—To stimulate the PKA pathway, HUVECs were treated with an analogue of cAMP N6, 2′-O-dibutyryladenosine-3′, 5′-cyclic monophosphate sodium salt (Bt2cAMP, 500 μM) (Nacalai Tesque) or forskolin, a stimulator of adenylyl cyclase, (100 μM) (Sigma) (26, 27) for 2 h, 20–24 h after cultivation on Matrigel. To inhibit the PKA pathway, HUVECs cultured on culture slides were treated with H-89, a selective inhibitor of PKA (1 μM) (Sigma), or MDL 12,330A, an inhibitor of adenylyl cyclase (30, 31) (CHX, 100 ng/ml) (Wako), and a proteasome inhibitor, MG132 (20 μM) (Biomer) (23), for 30 min prior to PKA stimulation or inhibition.

Statistical Analysis—Each assay experiment was carried out at least two times. Data are expressed as means ± S.D. Student’s t test was used to compare continuous values between two groups. Values of p < 0.05 were considered to represent statistically significant differences.
comparable among the different conditions (Fig. 1B). In the following experiments, we focused on the behavior of Id1.

To explore other Id1 regulatory mechanisms during angiogenesis, we next evaluated the subcellular localization of Id1 in HUVECs on Matrigel. Immunocytochemical analyses showed Id1 to be localized predominantly in the nucleus with seeding onto Matrigel (data not shown). Id1 subsequently showed gradual translocation into the cytoplasm of HUVECs along with capillary-like structure formation (Fig. 2, C–F), whereas in HUVECs cultured on uncoated slides Id1 remained in the nucleus throughout the assay (Fig. 2, A and B). These observations indicate that cytoplasmic translocation of Id1 is characteristic of HUVECs forming capillary-like structures. In contrast to Id1, Id3 remained in the nucleus during capillary-like structure formation (Fig. 2, G and H). Findings were similar in EOMA cells, a murine EC line (data not shown).

We further biochemically evaluated the subcellular localization of Id1 in HUVECs during capillary-like structure formation. Nuclear and cytoplasmic fractions were prepared and qualified using specific antibodies for acetylated histone H3 and /H9251-tubulin, respectively (Fig. 2I). Consistent with the immunostaining, Id1 was found mainly in the nuclei of HUVECs cultured on uncoated slides but had shifted to the cytoplasmic fraction in HUVECs forming capillary-like structures (I). Acetylated histone H3 and α-tubulin were probed as nuclear and cytoplasmic markers, respectively.

**FIGURE 2. Subcellular localization of Id1 in vascular ECs during angiogenesis.** HUVECs were plated on Matrigel or on culture plates and cultured in medium-199 with 5% FCS and VEGF (10 ng/ml) for 24 h. Then, Id1 and Id3 were immunocytochemically detected with specific antibodies. Id1 remained localized in the nuclei of control HUVECs 24 h after plating on an uncoated culture slide (A, B). In contrast, Id1 was gradually translocated to the cytoplasm in HUVECs forming capillary-like structures, at 8 h (C, D) and 24 h (E, F) after plating. Id3 remained localized predominantly in the nuclei of HUVECs throughout the angiogenic process (G, H). PI detected nuclei. Scale bars indicate 25 μm. Consistent with the immunostaining, Id1 was found mainly in the nuclei of HUVECs cultured on uncoated slides but had shifted to the cytoplasmic fraction in HUVECs forming capillary-like structures (I). Acetylated histone H3 and α-tubulin were probed as nuclear and cytoplasmic markers, respectively.

**FIGURE 3. Inhibition of CRM1/exportin promotes nuclear accumulation of Id1 during capillary-like structure formation.** HUVECs were cultured for 24 h on Matrigel and then immunostained for Id1 after treatment with 10 nm LMB for 1 h. LMB treatment caused Id1 to accumulate in the nucleus in most of the cells that formed capillary-like structures. PI detected nuclei. Scale bar indicates 25 μm.

**Id1 Is Exported from the Nucleus to the Cytoplasm in a CRM1/Exportin-dependent Manner**—Recent studies have suggested nucleocytoplasmic shuttling of Id proteins to be involved in the regulation of cellular functions (12–17). Therefore, we speculated that the same mechanism might operate during angiogenesis. To test our hypothesis, we first treated HUVECs with a CRM1/exportin inhibitor LMB for 1 h during capillary-like structure formation and analyzed the subcellular localization of Id1 by immunostaining. As expected, with LMB treatment, Id1 accumulated in the nucleus in most HUVECs forming capillary-like structures (Fig. 3). This result suggests that Id1 is exported from the nucleus to the cytoplasm in a CRM1/exportin-dependent manner during capillary-like structure formation.

To confirm the results for endogenous Id1, we examined the subcellular localization of exogenous Id1 tagged with EGFP in
HUVECs. For this purpose, we generated two constructs of Id1: one in which EGFP was fused to the N terminus (EGFP-Id1) and the other to the C terminus (Id1-EGFP) (Fig. 4A). When expression vectors encoding EGFP-Id1 or Id1-EGFP were transfected into HUVECs, intense EGFP signals tended to be detected in the cytoplasm in cells with high expression level even on uncoated plates, possibly due to saturated expression. Therefore, we analyzed the localization of exogenous Id1 in low expressing cells using both EGFP fluorescent signals and immunostaining with anti-EGFP antibody, and obtained essentially the same results.

In accordance with the subcellular localization of endogenous Id1, Id1-EGFP was recognized mainly in the nuclei of transfected HUVECs on uncoated slides (94%). However, less EGFP-Id1 was localized in the nucleus (46%) (Fig. 4A). In HUVECs on Matrigel, Id1-EGFP was translocated to the cytoplasm, as was the case with endogenous Id1 (Fig. 4B). Quantitative analysis showed the percentage of cells with predominantly nuclear localization of Id1-EGFP to be significantly greater in the transfected HUVECs on uncoated slides than in those on Matrigel (Fig. 4C). The subcellular distribution pattern of EGFP expressed in HUVECs was not altered at any point in the assay. These results indicate that Id1-EGFP dynamics likely recapitulate those of endogenous Id1 in HUVECs during angiogenesis.

Next, we analyzed the nuclear export of Id1 using EGFP-Id1, which tends to be localized in the cytoplasm even on uncoated slides. When treated with LMB, EGFP-Id1 accumulated in the nucleus in nearly all (99%) transfected HUVECs (Fig. 5B). Because Id1 contains a putative NES-like region (92–102) (Fig. 5A), we tested whether this sequence mediated the nuclear export. For this purpose, we generated Id1-EGFP and EGFP-Id1 fusion plasmids containing mutations in the NES-like region (Id1-L100R and Id1-L100R/E101S/L102S; Fig. 5A). As a result, EGFP-Id1 mutants with these substitutions were retained in the nuclei of transfected HUVECs (Fig. 5B), suggesting that this region is required for the nuclear export of Id1.

Then, we transfected HUVECs with wild-type and mutant Id1-EGFP, seeded onto Matrigel and evaluated subcellular localization during capillary-like structure formation in the absence and presence of LMB. Similar to endogenous Id1, wild-type Id1-EGFP was localized mainly in the cytoplasm of cells on Matrigel without LMB (Fig. 6A). LMB treatment resulted in translocation of Id1-EGFP to the nucleus (Fig. 6B). Rapid accumulation of Id1-EGFP in response to LMB was clearly demonstrated by time-lapse microscopy (data not shown). Quantitative analysis revealed that, with LMB treatment, the percentage of cells with a predominantly nuclear localization of Id1-EGFP was significantly increased (31% versus 75%, p < 0.01) (Fig. 6B). Mutation in the NES-like sequence (L100R/E101S/L02S) also caused nuclear accumulation, regardless of whether or not LMB was present (Fig. 6, A and B). Taken together, these results

**FIGURE 4.** Subcellular localization of EGFP-tagged id1 in vascular ECs during angiogenesis. A, EGFP was fused to the C terminus (Id1-EGFP) or to the N terminus (EGFP-id1) of Id1 in an expression vector and transfected into HUVECs. Endogenous Id1 was detected by immunostaining, as a reference. An expression vector coding EGFP was also transfected into HUVECs as a control. Endogenous Id1 was localized mainly in the nuclei of HUVECs (left panel). Id1-EGFP was localized mainly in the nucleus of HUVECs (94%), like endogenous Id1, whereas less EGFP-id1 was seen in the nucleus (46%). B, HUVECs transfected with Id1-EGFP or EGFP were seeded on Matrigel and 24 h later fixed and immunostained with anti-EGFP antibody. B, representative photomicrographs of the subcellular localization of Id1-EGFP in HUVECs cultured on Matrigel. C, quantitative analysis. The number of cells with nuclear localization of Id1-EGFP in HUVECs transfected with Id1-EGFP was determined. Id1-EGFP was localized in the nucleus in most of the HUVECs cultured on culture slides but in only a small fraction of the HUVECs forming capillary-like structures on Matrigel. Subcellular localization of EGFP in HUVECs was not changed by culture conditions. *, p < 0.01 versus culture slide. PI detected nuclei. Scale bars indicate 25 μm.
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FIGURE 5. CRM1/exportin-dependent nuclear export of EGFP-tagged Id1 in vascular ECs. A, alignment of the NES-like sequence in Id1. Hydrophobic residues matching the NES consensus sequence (shown at the bottom) are indicated by an asterisk. The mutated amino acid residues are indicated below. B, HUVECs were transfected with EGFP-tagged Id1 (wild-type (WT) and the NES mutants L100R and L100R/E101S/L102S). If necessary, the HUVECs were treated with 10 nM LMB for 1 h. LMB treatment resulted in accumulation of Id1 tagged with EGFP into the nucleus in wild type (percentages of cells with nuclear localization of Id1 tagged with EGFP, 46–99%). Mutation(s) in NES also produced accumulation of Id1 tagged with EGFP in the nucleus with L100R (46–89%) or L100R/E101S/L102S (46–89%). PI detected nuclei. Scale bars indicate 20 μm.

clearly show that Id1 is actively exported from the nucleus to the cytoplasm in a CRM1/exportin dependent manner, resulting in the predominantly cytoplasmic localization of Id1 in ECs during capillary-like structure formation.

Protein Kinase A Inhibits Nuclear Export of Id1—Phosphorylation and mono-ubiquitination have been shown to be important modifications of transcriptional factors regulating nucleocytoplasmic shuttling (20, 32). Id1 possesses consensus sequences for phosphorylation motifs of some kinases, such as PKA and protein kinase C (33). Because PKA activity potently contributes to capillary-like morphogenesis of ECs (34), we speculated that PKA activity might regulate nucleocytoplasmic shuttling of Id1. To test this possibility, we stimulated HUVECs during capillary-like structure formation on Matrigel with dbcAMP for 2 h and evaluated the subcellular localization of Id1 with immunostaining. We found that PKA stimulation by Bt2cAMP resulted in Id1 accumulation in the nucleus (Fig. 7A). To further investigate whether PKA activity contributes to Id1 translocation, we blocked de novo protein synthesis and proteasome-mediated protein degradation by CHX and MG132, respectively, and then treated HUVECs with Bt2cAMP or forskolin. We again observed that PKA induction resulted in nuclear accumulation of Id1 (Fig. 7B), suggesting that PKA-induced nuclear accumulation of Id1 is likely to be due to substitution of which might seriously affect functions other than nuclear export. We generated two mutants by replacing Ser-5 with either alanine (S5A) or aspartate (S5D) in EGFP-fused Id1 (Fig. 8A) and examined the subcellular localization of these mutants on uncoated slides and on Matrigel. On uncoated slides, where Id1-EGFP is localized in the nucleus, the S5A mutant was diffusely distributed in the cytoplasm (Fig. 8B). In contrast, the S5D mutant, mimicking a phosphorylated form, remained in the nucleus (Fig. 8B). Quantitative analysis revealed the number of HUVECs with a predominantly nuclear localization of Id1-EGFP to be significantly reduced by the alanine substitution of Ser-5 (79% to 51%) but not by aspartate substitution (79% to 78%) as shown in Fig. 8C.

To examine how PKA regulates the nucleocytoplasmic shuttling of Id1, we treated HUVECs on uncoated slides with H-89 in the presence of CHX and MG132. In contrast to the nuclear localization in the absence of H-89, Id1 was largely translocated to the cytoplasm in the presence of H-89 (Fig. 7C). This cytoplasmic translocation was suppressed by an additional treatment with LMB (Fig. 7C). Similar findings were obtained when cAMP synthesis was inhibited by MDL-12,330A (Fig. 7C). Taken together, these results suggest that PKA induces nuclear accumulation of Id1 by inhibiting its CRM1/exportin-dependent nuclear export in ECs.

Phosphorylation of Ser-5 May Be Involved in the Nuclear Localization of Id1—Id1 contains two amino acid sequences conserved among several species, which match the consensus sequence for PKA phosphorylation (3). In the present study, we focused on the N-terminal sequence (Fig. 8A), because the other is in the HLH region and also overlaps with a sequence matching the NLS (23), changes in nucleocytoplasmic shuttling rather than net changes in Id protein amounts.
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**FIGURE 6. CRM1/exportin-dependent nuclear export of EGFP-tagged Id1 during capillary-like structure formation.** HUVECs transfected with EGFP-tagged Id1 (wild-type (WT) and the NES mutant L100R/E101S/L102S) were cultured on Matrigel for 24 h and then incubated with or without 10 nM LMB for 1 h. LMB treatment resulted in the accumulation of Id1-tagged EGFP (Id1-EGFP) in the nuclei of HUVECs during capillary-like structure formation in wild type (A). NES mutants (L100R/E101S/L102S) also produced Id1-EGFP accumulation in the nuclei of cells regardless of LMB treatment (A). B, quantitative analysis. Cells with nuclear localization of Id1-EGFP were counted. *, p < 0.01; **, p < 0.05 versus WT without LMB treatment. PI detected nuclei. Scale bar indicates 25 μm.

The present results show the subcellular localization of Id1 to be altered in ECs in parallel with morphological changes related to angiogenesis. Most notably, its cytoplasmic localization during capillary-like structure formation is due mainly to CRM1/exportin-dependent nuclear export. Although the amounts of Ids are known to be regulated by the ubiquitin-proteasome degradation system in the nucleus (23, 35), the total amount of Id1 protein was not significantly altered during capillary-like structure formation, showing the balance between protein synthesis and degradation to have no effect on the subcellular localization.

Mutagenesis analysis shows that the leucine-rich region spanning residues 92–102 in Id1 is, at least partly, responsible for the nuclear export of Id1 during capillary-like structure formation. Recently, Makita et al. (25) identified the same sequence as a functional NES. Our data confirmed this sequence in ECs and revealed its possible role in angiogenesis. However, experiments with a CRM1/exportin inhibitor and NES mutation did not result in Id1 accumulating in the nuclei of all the ECs forming capillary-like structures. This implies that other factors, such as inhibition of the nuclear import system and cytoplasmic sequestration, may also contribute to cytoplasmic retention. Indeed, recent reports have demonstrated that nuclear import of Id can be prevented through cytoplasmic sequestration via certain cytoplasmic retention factors such as cytoskeleton-associated PDZ-LIM protein enigma homolog (ENH) (15) and polycystin-2 (18).

In contrast to Id1, Id3 remained in the EC nucleus throughout capillary-like structure formation. This may be explained by the absence of the NES-like sequence from Id3.

### Role of Protein Kinase A in the Regulation of Id1 Shuttling

The present results also suggest PKA to be among the regulators of Id1 nucleocytoplasmic shuttling and to operate a CRM1/exportin-dependent manner in ECs. PKA activation between nuclear import and export. As for Id proteins, Id2 was first shown to localize predominantly in the nuclei of proliferating precursor cells and to translocate into the cytoplasm with a link to oligodendrocyte differentiation (14). Similarly altered Id localization along with changes in cellular behavior has been documented in several types of cells by other investigators (12, 13, 15–18). However, the mechanism controlling this subcellular localization remains essentially unknown.

The present results show the subcellular localization of Id1 to be altered in ECs in parallel with morphological changes related to angiogenesis. Most notably, its cytoplasmic localization during capillary-like structure formation is due mainly to CRM1/exportin-dependent nuclear export. Although the amounts of Ids are known to be regulated by the ubiquitin-proteasome degradation system in the nucleus (23, 35), the total amount of Id1 protein was not significantly altered during capillary-like structure formation, showing the balance between protein synthesis and degradation to have no effect on the subcellular localization.

**DISCUSSION**

In the present study, we have demonstrated that the subcellular localization of Id1 in ECs is regulated by nucleocytoplasmic shuttling during angiogenesis. Id1, but not Id3, was gradually translocated from the nucleus into the cytoplasm of HUVECs during capillary-like structure formation on Matrigel. The translocation of Id1 was inhibited by the CRM1/exportin inhibitor LMB. Furthermore, PKA appeared to affect the nucleocytoplasmic shuttling of Id1 by inhibiting nuclear export. Mutagenesis analysis of Id1 suggested the phosphorylation of Ser-5 to mediate the effect of PKA. Taken together, these findings raise the possibility of the function of Id1 as a transcriptional factor being regulated by nucleocytoplasmic shuttling, in which PKA activity is involved, during angiogenesis.

**Translocation of Id1 from the Nucleus into the Cytoplasm during Capillary-like Structure Formation of ECs—** Subcellular localization of transcriptional factors is important for the regulation of their functions and is determined by a balance of PKA-mediated phosphorylation of Ser-5 by PKA may be involved in the nuclear localization of Id1.
resulted in Id1 accumulation in the nuclei of ECs forming capillary-like structures, whereas PKA led to Id1 translocation into the cytoplasm in ECs growing on uncoated plates. The effect of PKA activation was abolished by alanine substitution of Ser-5, suggesting this residue to be involved in the inhibition of Id1 nuclear export by PKA. This speculation was further supported by aspartate substitution of the same residue, which mimicked the effect of PKA activation in ECs on Matrigel.

PKA has been shown to exert not only negative but also positive effects on angiogenesis. Suppression of PKA via certain integrins negatively regulates actin polymerization and EC assembly into capillary-like structures in vitro (26, 36, 37) and also inhibits EC migration and survival (27, 38). In addition, PKA stimulation inhibits cytokine-induced proliferation (37), thereby suppressing angiogenesis in vitro and in vivo (27, 39). Moreover, PKA activation reportedly explains the anti-angiogenic effects of molecules such as parathyroid hormone-related peptide (39) and prostaglandin E1 (37). In contrast to these negative effects on angiogenesis, activation of the cAMP-PKA signaling pathway via stimulation of the β-adrenergic receptor or inhibition of phosphodiesterase was reported to promote angiogenesis via endothelial nitric-oxide synthase activation and VEGF up-regulation (40–42). These findings suggest PKA to be involved in various steps controlling angiogenesis and that the regulation of Id1 nucleocytoplasmic shuttling may serve as a novel regulatory machinery explaining a portion of the effects of PKA.

Phosphorylation Status of Ser-5 and Nuclear Export of Id1—The Ser-5 residue is conserved among Id proteins of different...
species. Previous studies have suggested that phosphorylation of Ser-5 is important for cell function (17, 43, 44). Ser-5 in Id2 and Id3, but not Id1, is within a conserved and functional phosphorylation site for cyclin-dependent kinase-2, a cell cycle control gene (43, 44). Phosphorylation of Ser-5 is recognized late in the G1 phase and contributes to the regulation of the late G1 to S transition (43, 44). Intriguingly, alanine substitution of Ser-5 shifted the localization of Id2 from the nucleus to the cytoplasm and suppressed cell proliferation in cultured vascular smooth muscle cells, although the mechanism underlying this difference in localization was not clarified (17). Therefore, phosphorylation of Ser-5 in Id proteins may play a key role in modulating their functions in different cellular contexts.

How can the phosphorylated form of Ser-5 mediate nuclear export of Id1? One possible explanation may be intramolecular masking of NES by phosphorylation. A typical example is direct masking of NES by phosphorylation of Hog1, a MAP kinase in yeast (45). Although the N-terminal position of Ser-5 is apart from the NES region within the Id1 molecule, the phosphorylation status of Ser-5 might affect the accessibility of NES to CRM1/exportin. Another possibility is intermolecular masking as described with NF-AT4 (46), in which the NES is masked by binding of calcineurin through calcium signaling. Id proteins form dimers with the other HLH or non-HLH factor via the HLH region (47). Because NES of Id1 overlaps the C-terminal second helix of the HLH domain, the NES may be masked with various partner(s) bound to phosphorylated Id1. Indeed, previous studies have suggested that the phosphorylation status of Ser-5 in Id proteins could determine the specificity of binding partners (43, 44). Further investigation is required to elucidate the mechanism underlying the relationship between the phosphorylation status of Ser-5 and nuclear export.

In the present study, we did not examine the behavior of Id1 protein. However, this splicing variant form is identical to Id1 protein except for the C-terminal 13 amino acids, sharing Ser-5 and the NES. Therefore, the subcellular localization of Id1 protein may play a key role in modulating their functions in different cellular contexts.

Involvement of Id1 Nucleocytoplasmic Shuttling in Angiogenesis—Angiogenesis is a complex phenomenon composed of multiple processes that include proliferation, migration, morphogenesis, and maturation of ECs. Previous studies using knock-out mice have demonstrated that complete loss of Id1 and Id3 leads to enlarged and dilated vessels and absence of branching and sprouting, whereas one copy of Id1 can reverse this fatal vascular defect (6, 10). Sakurai et al. (28) and our group have also shown that Id1 is a molecule promoting EC prolifer-
Nucleocytoplasmic Shuttling of Id1 during Angiogenesis

... and migration, resulting in the enhancement of in vitro and in vivo angiogenic processes. Considering the present data showing Id1 to be localized predominantly in the cytoplasm of EC forming capillary-like structures but in the nuclei of ECs growing on culture plates, Id1 shuttling may be involved in the regulation of angiogenesis in parallel with the phenotypic alteration of ECs.

In conclusion, Id1 was translocated from the nucleus into the cytoplasm of EC forming capillary-like structures in a CRM1/exportin-dependent manner. Furthermore, PKA appeared to block the nuclear export of Id1, possibly via phosphorylation of Ser-5. We propose that nuclear-cytoplasmic shuttling of Id1 is a novel mechanism regulating angiogenesis and thus a possible target for vascular regeneration therapy.

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