Lipocortin V May Function as a Signaling Protein for Vascular Endothelial Growth Factor Receptor-2/Flk-1[1]

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Binding of vascular endothelial growth factor (VEGF) to its receptor, VEGFR-2 (Flk-1/KDR), induces dimerization and activation of the tyrosine kinase domain of the receptor, resulting in autophosphorylation of cytoplasmic tyrosine residues used as docking sites for signaling proteins that relay the signals for cell proliferation, migration, and permeability enhancement. We explored the VEGF/receptor signaling pathway by performing a two-hybrid screen of a rat lung cDNA library in yeast using the intracellular domain of rat VEGFR-2 as bait. Two clones encoding lipocortin V were isolated. Subsequent studies with the yeast two-hybrid assay showed that the complete intracellular domain of VEGFR-2 was required for the interaction. Co-immunoprecipitation of translated proteins confirmed the interaction between the VEGF receptor and lipocortin V. VEGF induced a rapid tyrosine phosphorylation of lipocortin V in human umbilical vein endothelial cells (HUVEC). Pretreatment of HUVEC with antisense oligodeoxyribonucleotide (ODN) for lipocortin V significantly inhibited VEGF-induced cell proliferation, which was accompanied by a decrease in protein synthesis and tyrosine phosphorylation of lipocortin V. Our results indicate that lipocortin V may function as a signaling protein for VEGFR-2 by directly interacting with the intracellular domain of the receptor and appears to be involved in regulation of vascular endothelial cell proliferation mediated by VEGFR-2.   © 1999 Academic Press

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Abbreviations used: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; Flt-1, *fms*-like tyrosine kinase 1; Flk-1, fetal liver kinase 1; KDR, kinase insert domain receptor; PLC-γ1, phospholipase C-γ1; SH2, Src homology 2; MAP, mitogen-activated protein; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor; HGF, hepatocyte growth factor; DNA-BD, DNA binding domain; AD, activation domain; PAGE, polyacrylamide gel electrophoresis; HUVE cell, human umbilical vein endothelial cell; ODN, oligodeoxynucleotide.

Vascular endothelial growth factor (VEGF) is a specific mitogen for vascular endothelial cells and potently increases micro-vessel permeability to macromolecules (1, 2). VEGF is thought to play a major role in physiological and pathological angiogenesis (3, 4). The biological effects of VEGF are mediated primarily via its two high affinity receptors, VEGFR-1 (Flt-1) (5) and VEGFR-2 (Flk-1/KDR) (6, 7). The VEGF receptors are structurally similar, containing seven extracellular immunoglobin-like domains, a juxtamembrane and membrane domain, a carboxyl-terminal tail, and a conserved intracellular tyrosine kinase domain separated by a long kinase insert domain (5, 8, 9).

Recently, splice variants of VEGF receptors have been described. There are two splice variants of VEGFR-1 receptor mRNA: a full-length membrane-spanning receptor and a soluble form, denoted sFlt-1, that is truncated on the C-terminal side of the sixth extracellular Ig-like domain (10). Pure sFlt-1 retains its specific high affinity binding for VEGF (11) and completely inhibits VEGF-stimulated endothelial cell mitogenesis (10). A cDNA clone isolated from rat retina encodes a short form of VEGFR-2 that contains the complete seven N-terminal immunoglobulin-like extracellular ligand-binding domains, a transmembrane region, and the N-terminal half of the intracellular kinase domain, and kinase insert domain (12). However, it does not encode the C-terminal half of the intracellular kinase domain and C-terminal tail region. The absence of the C-terminal kinase domain in the short form of VEGFR-2 may result in differential downstream signaling compared to the full-length receptor.

The kinase domains of VEGFR-1 and VEGFR-2 contain 66 (5) and 70 (7) amino acid residue insert sequences, respectively. At least 4 tyrosine residues in

the VEGFR-2 cytoplasmic domains are subject to either auto- or trans-phosphorylation, two of which are within the kinase insert region (13) and may generate docking sites for downstream signal transduction proteins upon phosphorylation. VEGF stimulation of VEGFR-1-expressing porcine aortic endothelial (PAE) cells results in receptor auto-phosphorylation and phosphorylation of members of the Src family of protein kinases, GAP, phospholipase Cγ (PLCγ), and Shc proteins (14, 15). However, VEGF activation of VEGFR-1 signaling does not effectively induce the proliferation of vascular endothelial cells (14). VEGF stimulation of VEGFR-2-expressing cells results in the phosphorylation of GAP, members of the Src family of protein kinases, PLCγ, and p42 MAP kinase determined by an *in vitro* kinase assay and Western blot analysis. The overexpression of VEGFR-2 in PAE cells induced dramatic changes in cell morphology, actin reorganization, membrane ruffling, proliferation, and chemotaxis (14, 16). VEGFR-2 is able to induce multiple signal transduction events in PAE cells by activating proteins of the Ras pathway and associating with SHP-1 and SHP-2, the SH2 protein-tyrosine phosphatases (17).

To better understand the biochemical signaling pathways of VEGFR-2, we applied the yeast two-hybrid screening to search for proteins that physically interact with the intracellular domain of the receptor. Lipocortin V was identified as VEGFR-2 interacting protein using this method and this was confirmed by immunoprecipitation experiments in *in vitro* translation. Evidence for interaction in a mammalian cell line (HUVEC) expressing both these proteins was obtained by tyrosine phosphorylation experiments. Lipocortin V was tyrosine phosphorylated after VEGFR-2 activation, and vascular endothelial cell proliferation was significantly reduced by antisense treatment that selectively inhibits lipocortin V expression. Our data suggest a role for lipocortin V in VEGFR-2-mediated functional responses in vascular endothelial cells.

MATERIALS AND METHODS

Yeast two-hybrid screening for proteins interacting with the intracellular domain of VEGFR-2. The yeast strains Y190 and CG-1945, and the yeast expression vector pAS2-1 were purchased as a MATCHMAKER Two-Hybrid System 2 from Clontech. For construction of the GAL4 DNA-BD-long form VEGFR-2 (pAS2-1/RRFlk-L), the cDNA encoding the entire RRFlkL (12) intracellular domain (amino acids 789-1343) was obtained by PCR with primers RRFlkL2595S/NdeI (5′<u>GGC AGC CAT ATG</u> CGG ACC GTT AAG CGG GCC AAT GAA G 3′) and RRFlkL4255AS/SalI (5′ <u>GCA GGT CGA C</u>TA AAC AGG AGA TGA GCG CAG TGT GG 3′). This was subcloned into the *Nde* I/*Sal* I sites of the yeast expression vector pAS2-1 (with *TRP* marker) in the same frame of GAL4 DNA binding domain (GAL4-DNA-BD) and used as the 'bait'. The *Nde* I or *Sal* I restriction site (underlined sequence) was included in the 5′-end of each primer to facilitate the subcloning of the amplified cDNA into

plasmid vector. A rat lung cDNA MATCHMAKER library (Clontech) fused to the GAL4-activation domain (GAL4-AD) in pGAD10 vector (with *LEU* marker) was used as the "prey." The yeast strain CG-1945, which is deficient for *TRP* and *LEU* and contains two reporter genes *HIS3* and *LacZ,* was co-transformed with the "bait" and "prey" constructs by the lithium acetate protocol (18). The transformants were grown on synthetic, minimal medium lacking Trp, Leu and His, with 3-amino-1,2,4-triazole (3-AT) at the concentration of 5 mM (SD/-Trp/-Leu/-His/+3AT). The colony lift β-galactosidase filter assay (19) was performed after the transformants were grown for 8 days. From approximately 0.75×10^6 co-transformants, 6 clones were isolated exhibiting positive phenotype under both nutritional and color selection. After further cycloheximide selection (20) for colonies that have lost the DNA-BD/target plasmid, the plasmid DNAs from the positive clones were isolated from the yeast by the methods of Hoffman and Winston (21), transformed to *Escherichia coli* HB101, and sequenced with the Sequenase kit (Amersham Life Science, Inc.) or on an Automatic DNA Sequencer (Applied Biosystems, Inc., Foster City, CA). Sequence similarities were sought in GenBank using the network BLAST search.

Yeast two-hybrid assay for verification of positive two-hybrid interactions. To verify the interaction of the intracellular domain of RRFlk-L with the candidate AD/protein (pGAD10/LAD11) identified in the two-hybrid library screen, co-transformation of pAS2-1/RRFlk-L with pGAD10/LAD11 was performed in Y190 strain, using pAS2-1 (GAL4 DNA-BD vector only) and pAS2-1/Lam5′-1 (GAL4 DNA-BD-human lamin C construct, Clontech) as negative controls. To investigate the interaction of different parts of VEGFR-2 intracellular domain with the identified proteins, two more GAL4 DNA-BD fusion plasmids containing different parts of the VEGFR-2 intracellular domain were constructed. For construction of the GAL4 DNA-BD-short form VEGFR-2 (pAS2-1/RRFlk-S), the cDNA encoding the entire RRFlk-S (12) intracellular domain (amino acids 789-991) was obtained by PCR with primers RRFlkL2595S/NdeI (5′<u>GGC AGC CAT ATG</u> CGG ACC GTT AAG CGG GCC AAT GAA G 3′) and RRFlkS3236AS/SalI (5′ <u>GCA GGT CGA C</u>TC CAC CCA GCA AGA AAC CCT GAG TT 3′) and subcloned into the *Nde* I/*Sal* I sites of the pAS2-1 vector in the same frame of GAL4-DNA-BD. For construction of the GAL4 DNA-BD-C terminal half of the tyrosine domain of the long form VEGFR-2 (pAS2-1/cTKD), the cDNA encoding the C terminal half of the tyrosine domain and the C-tail (amino acids 979-1343) of the RRFlk-L (12) was obtained by PCR with primers RRFlkL3183S/NdeI (5′ <u>GGC AGC CAT ATG</u> CTC AGT GAC GTA GAG GAA GAA GAA G 3′) and RRFlkL4255AS/SalI (5′ <u>GCA GGT CGA C</u>TA AAC AGG AGA TGA GCG CAG TGT GG 3′) and subcloned into the *Nde* I/*Sal* I sites of the pAS2-1 vector in the same frame of GAL4-DNA-BD. Pairwise co-transformation of either pAS2-1/RRFlk-S or pAS2-1/RRFlk-cTKD with pGAD10/LAD11 in yeast strain Y190 was performed as described above.

In vitro translation and co-immunoprecipitation. To confirm the two-hybrid results, proteins were produced by *in vitro* transcription/translation with TNT T7 Polymerase Coupled Reticulocyte Lysate System (Promega, Madison, WI) in the presence of [^{35}S] methionine using PCR products as templates (22). The PCR was performed with the following primers: for the intracellular domain of RRFlk-L, RRFlkL2595S/T7 (5′<u>TAA TAC GAC TCA CTA TAG GG</u>C GGA CCG TTA AGC GGG CCA ATG AAG 3′) and RRFlkL4255AS (5′ TAA ACA GGA GAT GAG CGC AGT GTG G 3′); for the intracellular domain of RRFlk-S, RRFlkL2595S/T7 (5′<u>TAA TAC GAC TCA CTA TAG GG</u>C GGA CCG TTA AGC GGG CCA ATG AAG 3′) and RRFlkS3236AS (5′ TCC ACC CAG CAA GAA ACC CTG AGT T 3′); for the C terminal half of the tyrosine kinase domain of RRFlk-L, RRFlkL3183S/T7 (5′<u>TAA TAC GAC TCA CTA TAG GG</u>C TCA GTG ACG TAG AGG AAG AAG AAG 3′) and RRFlk4255AS; and for LAD11, LipV18S/T7 (5′ <u>TAA TAC GAC TCA CTA TAG GG</u>A GCC TGC GCC CTA CCT TCT GC 3′) and MATCHMAKER 3′ AD LD-insert-screening am-

plimer. A T7 RNA polymerase promoter sequence (underlined) was fused to the 5′ end of each sense primer. Five μl of each *in vitro* transcription/translation reaction mixture was mixed with 200 μl of "Colrain" buffer (100 mM KCl, 25 mM Hepes, pH 7.5, 10 mM MgCl$_2$, 10% glycerol, 1 mM DTT, 0.1% Nonidet P-40, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA), incubated at room temperature for 30 min, diluted with 700 μl of "washing" buffer (Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM DDT, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM phenylmethylmethylsulfonyl fluoride), and incubated at room temperature for 30 min. Samples were divided into three tubes and polyclonal antibodies were added: anti-mouse Flk (c1158) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-rat lipocortin V (23); anti-rat LEP503 (rat lens epithelial protein, as a negative control). After a 30-min incubation at room temperature, the immunocomplexes were conjugated with protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 hour to overnight and washed five times with 500 μl of "washing" buffer. The agarose pellet was resuspended in SDS electrophoresis sample buffer and boiled, and the supernatants were analyzed by 17.5% SDS-PAGE and autoradiography.

Northern blot analysis. To examine the expression of lipocortin V in various tissues and cell lines, total RNA was isolated from adult rat tissues, human embryonic kidney cells (HK 293 cells) and human umbilical vein endothelial cells (HUVEC) using the guanidinium thiocyanate acid-phenol method (24). Poly(A$^+$) RNA was prepared using the PolyATtract mRNA Isolation System (Promega, Madison, WI). One μg of poly(A$^+$) RNA from each tissue and cell line was fractionated on 1% agarose-formaldehyde gels (25) and transferred to NYTRAN membranes (Schleicher & Schuell, Keene, NH). The inserts cut by *Eco*R I from pGAD10/LAD11 (for lipocortin V) identified from yeast two-hybrid screening and a ~700-bp fragment generated by RT-PCR with rat β-actin Amplimer set (Clontech, Palo Alto, CA) were used as probes. The probes were radiolabeled with [α-^{32}P]dCTP using a random primer labeling kit (Stratagene, La Jolla, CA) to specific activities of $1\text{-}10 \times 10^8$ cpm/μg. The blot was hybridized (26) with radiolabeled probe overnight at 65°C in a buffer containing 0.45 M sodium phosphate (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), and 20 mM EDTA. The hybridized blot was washed with $0.1 \times$ SSC/0.1% SDS at 65°C and autoradiographed using a Phosphor Imager 445 SI (Molecular Dynamics, Sunnyvale, CA). Hybridization with a rat β-actin cDNA was performed to check the quality and quantity of RNA.

Immunoblotting and immunoprecipitation. For *in vivo* tyrosine phosphorylation, HUVEC (Clonetics, San Diego, CA) were grown to sub-confluence in endothelial cell growth medium with 2% FBS (EGM) (Clonetics), and starved overnight in serum-free endothelial cell basal medium (EBM) (Clonetics). Cells were stimulated with 50 ng/ml recombinant human VEGF$_{165}$ (Sigma) for the indicated times at 37°C. After washing twice with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM Na$_3$VO$_4$, cells were lysed in RIPA buffer ($1\times$ PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM Na$_3$VO$_4$, 1 mM phenylmethylsulfonl fluoride, 1 mg/ml aprotinin) at 4°C for 10 min. The cell lysates were clarified by centrifugation at 10,000g for 15 min. The protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) and the same amount of each sample was used for analysis. For immunoblotting, the cell lysates were separated on an 8.5% SDS-PAGE, transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and blotted with antibody against phosphotyrosine (PY20) (Santa Cruz Biotechnology, Santa Cruz, CA). The signal was visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (American Qualex, San Clemente, CA) and an enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacture's protocol. For immunoprecipitation, the cell lysates were incubated with the primary anti-

bodies (anti-Flk, PLCγ1, or lipocortin V) and protein A-agarose. The immunoprecipitates immobilized on protein A-agarose were washed five times with "washing" buffer, followed by separation on 8.5% SDS-PAGE and immunoblotting with various antibodies. Reprobing of blots was done after incubation of the membrane for 30 min at 50°C in stripping buffer containing 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7, and 2% SDS, followed by incubation with another specific antibody.

Antisense oligodeoxyribonucleotides and HUVEC proliferation. For lipocortin V, a 21-mer oligodeoxyribonucleotide (ODN) complementary to the 5′ region of human lipocortin V mRNA (GenBank Accession Number M21731) coding for amino acids 2-8 and the corresponding sense ODN were synthesized. Sequences of antisense and sense ODNs were 5′ AGT GCC TCT GAG AAC CTG TGC 3′ (ASLipV, complementary to nucleotide 146-166) and 5′ GCA CAG GTT CTC AGA GGC ACT 3′ (SLipV, nucleotide 146-166). The ODNs were added to 2-day serum-starved HUVE cells at 1 μM, 100 nM and 10 nM for 24 h prior to the addition of VEGF at 50 ng/ml. After additional 1-, 2-, 3-, and 4-day incubation [*methyl*-^{3}H]thymidine (Amersham) was added to a final concentration of 1 μCi/ml, and the cells were further incubated for 4 h, washed with PBS, and solubilized with lysis buffer (0.2% Na$_2$CO$_3$, 0.1 M NaOH). [^{3}H] radioactivity was measured by liquid scintillation counting. VEGF-treated HUVEC without ODN served as controls. HUVEC without ODNs and VEGF was used as the negative control. A parallel experiment was conducted under the exactly same condition except that instead of adding [*methyl*-^{3}H] thymidine the cells were resuspended in PBS for staining with 0.4% Trypan blue and counting. The treated (10 nM ODNs) and untreated cells showed 98-100% viability after 5 days growth. The immunoprecipitation and Western blotting after the treatment with ODNs followed by 20-min stimulation with VEGF were used as described above to examine the effect of ASLipV on the tyrosine phosphorylation and protein synthesis of lipocortin V.

RESULTS

Identification of Proteins Interacting with the Intracellular Domain of VEGFR-2

A yeast two-hybrid assay (27) was used to identify proteins that interact with the intracellular domain of the rat VEGF receptor. We constructed a set of GAL4 DNA-BD-RRFlk fusion proteins containing either the entire intracellular domain of RRFlk or portions of it (Fig. 1). The construct (pAS2-1/RRFlk-L) containing the entire intracellular domain of the long form RRFlk (RRFlk-L) was used as "bait" to screen a rat lung cDNA library in the yeast two-hybrid system. Approximately 0.75×10^6 co-transformants were screened, resulting in 6 clones that were positive for transcriptional activation of the *HIS3* and *LacZ* reporter genes. The sequences of these double positive clones were compared with existing cDNA sequence databases. Two clones were unidentified sequences, and one clone corresponded to a ribosomal protein that is believed to be a common false positive in this system (28). However, one clone (LAD2) contained an 864-bp insert that was identical to the cDNA sequence of rat PLC-γ1 (nt. 1369-2233) (29), and encodes a protein with a N-terminal SH2 domain of PLC-γ1. Two additional clones (LAD11 and LAD12) were found to contain 1028-bp and 855-bp inserts coding for most of the pep-
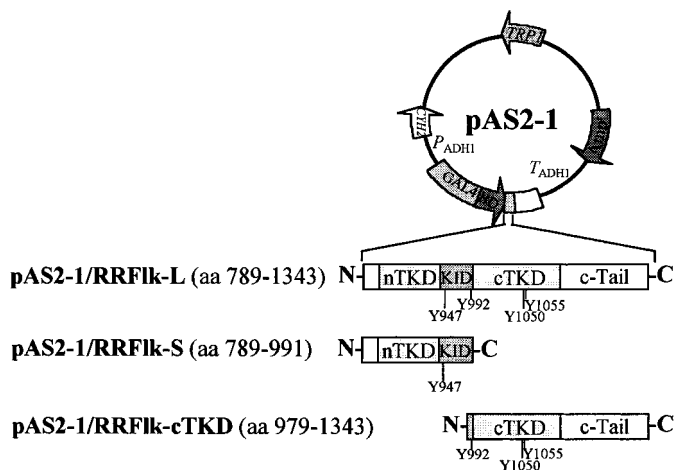
FIG. 1. Yeast expression constructs for the RRFlk intracellular domains in the GAL4 DNA-BD vector. The cDNA fragments encoding the entire intracellular domain (amino acid residues 789-1343) of the long form of RRFlk (RRFlk-L), entire intracellular domain (amino acid residues 789-991) of the short form of RRFlk (RRFlk-S), and the C-terminal half of the tyrosine kinase domain with the C-terminal tail of RRFlk (amino acid residues 979-1343) were inserted into the *Nde* I/*Sal* I sites of the yeast expression vector pAS2-1 (*TRP* marker) in the same frame as the GAL4 DNA binding domain. The construct pAS2-1/RRFlk-L was used to screen a rat lung cDNA library in the yeast two-hybrid system. All constructs were used for the yeast two-hybrid assay. Abbreviations: N-terminal tyrosine kinase domain (nTKD), kinase insert domain (KID), C-terminal tyrosine kinase domain (cTKD).

tide sequence of rat lipocortin V (30). Clone LAD11 has an extra 55-bp sequence (GenBank Accession No. AF051895) upstream to the 5′-uncoding sequence of the rat lipocortin V.

The specificity of the interaction between the intracellular domains of VEGFR-2 and the lipocortin V was further tested in the yeast two-hybrid assay (Fig. 2). To investigate their interaction with different regions within the intracellular domain of RRFlk, the GAL4 DNA-BD constructs, pAS2-1/RRFlk-S and pAS2-1/cTKD, were also included in the yeast two-hybrid assay (Figs. 1 and 2). An interaction was detected between pAS2-1/RRFlk-L and pGAD10/LAD11 (lipocortin V), with numerous yeast colonies growing on the SD/-Leu/-Trp/-His/+3AT plate (Fig. 2B$_1$), and a positive result in the β-galactosidase assay in ~5 hours (Fig. 2B$_2$) which is slower than the reaction with PLC-γ1 (~1 hour, data not shown). In contrast, the GAL4 DNA-BD constructs of the intracellular domain of the short form RRFlk (pAS2-1/RRFlk-S) and the C-terminal half of the tyrosine kinase domain of the long form RRFlk (pAS2-1/Flk-cTKD) did not show the interaction with lipocortin V (Fig. 2C and D). Some yeast colonies were found on the SD/-Leu/-Trp/-His/+3AT plates (Fig. 2C$_1$, and D$_1$), but no positive reaction was detected in the β-galactosidase assay (Fig. 2C$_2$, and D$_2$). No interaction was detected between pAS2-1 (no insert) and pGAD10/

LAD11 (lipocortin V) (Fig. 2A), indicating that there was no autonomous *LacZ* activation, and that the interactions between pAS2-1/RRFlk-L and lipocortin V clone (fused to AD) were dependent on the presence of the intracellular domain of RRFlk-L. Another negative control, pAS2-1/Lam5′-1, did not interact with pGAD10/LAD11 (lipocortin V) (Fig. 2E), eliminating the possibility of non-specific interactions.

In Vitro Binding of the Intracellular Domains of RRFlk and Lipocortin V

To confirm the interaction between the intracellular domain of RRFlk and lipocortin V identified from the yeast two-hybrid studies, we assayed the interaction of the corresponding proteins by *in vitro* binding. The protein fragments representing the intracellular domain of RRFlk-L (residues 789-1343) and RRFlk-S (residues 789-991), the C-terminal tyrosine kinase domain of RRFlk-L (residues 979-1343), and lipocortin V were produced by *in vitro* transcription/translation. The appropriate *in vitro* translation products were mixed, co-immunoprecipitated with polyclonal antibodies against Flk, or lipocortin V, followed by SDS-PAGE analysis. Lipocortin V (~35-kD band) was found in immunocomplexes with the intracellular domain of RRFlk-L (~60-kD band) precipitated with the anti-Flk antibody (Fig. 3, lane 3). Similarly, the intracellular domain of RRFlk-L was retained with lipocortin V precipitated by anti-lipocortin V antibody (Fig. 3, lane 4). This suggests an interaction between these two proteins. The intracellular domain of RRFlk-S (~24-kD band) was not co-immunoprecipitated with lipocortin V by anti-lipocortin V antibodies and there was no interaction between the C-terminal half of the tyrosine kinase domain of RRFlk (~42-kD) and lipocortin V (data not shown). An antibody against lens epithelial protein LEP503 was used as a negative control and as expected showed no interactions (Fig. 3, lane 5). The results with co-immunoprecipitation confirm those of the two-hybrid assay and show that only the full-length intracellular domain of VEGFR-2 binds lipocortin V.

RNA Expression of Lipocortin V in Rat Tissues and Cell Lines

Northern blot analysis was performed to examine the distribution pattern of lipocortin V in different tissues and cell lines. Lipocortin V (~1.5 kb) was expressed in all tissues and cell lines examined (Fig. 4). However, the expression of this gene is relatively high in HUVEC. Since HUVEC express lipocortin V and can proliferate in response to VEGF, we used this cell line to study the functional role of lipocortin V in VEGF receptor signaling.
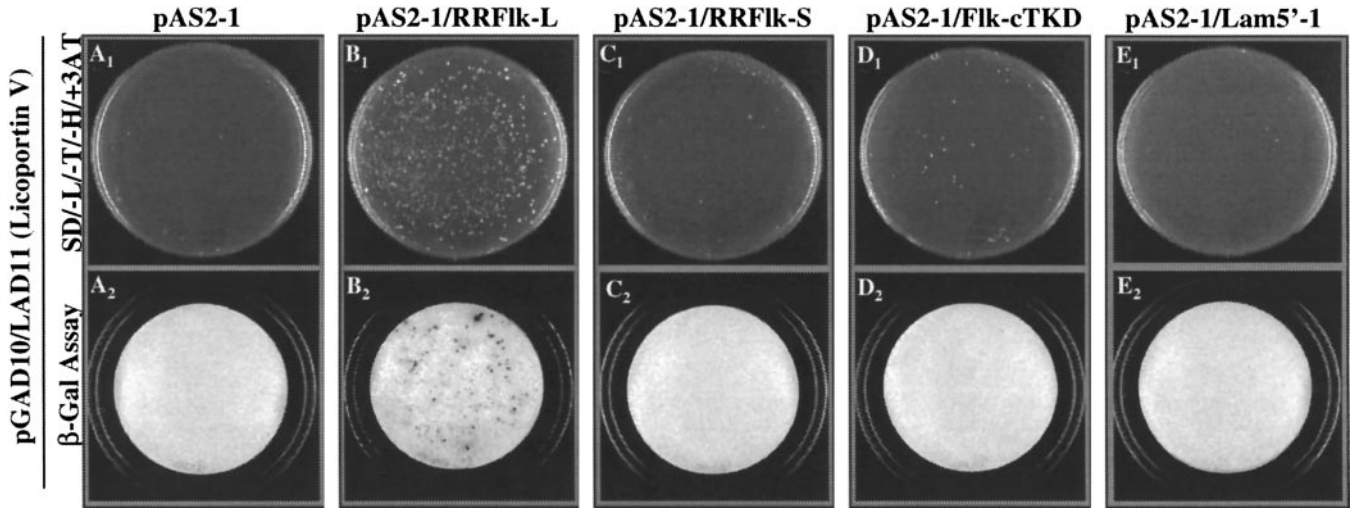
FIG. 2. The specificity of the interaction between the clone LAD 11 (lipocortin V) and different portions of the intracellular domain of VEGFR-2/Flk-1 was determined using the yeast two-hybrid system. Isolated clone pGAD10/LAD11 (lipocortin V) was re-transformed into yeast strain Y190 on SD/-Trp/-Leu/-His/+3AT plates with different GAL4 DNA-BD fusion baits: pAS2-1 alone (A), pAS2-1/RRFlk-L (B), pAS2-1/RRFlk-S (C), pAS2-1/RRFlk-cTKD (D), and pAS2-1/Lam5′-1 (E). Interactions between the baits and LAD11 (lipocortin V) were detected by yeast colony growth on SD/-Trp/-Leu/-His/+3AT plates (A_1 to E_1) and colony lift β-galactosidase filter assay (A_2 to E_2).

Tyrosine Phosphorylation of Lipocortin V in Response to VEGF

To determine whether lipocortin V is tyrosine phosphorylated in response to VEGF, serum-starved HUVEC were treated with VEGF and cell lysates were prepared at times up to 20 min after stimulation. The tyrosine-phosphorylated proteins in whole cell lysates were analyzed by Western blotting using an anti-phosphotyrosine antibody (PY20). Stimulation of HUVEC with VEGF resulted in tyrosine phosphorylation of several proteins with molecular masses of $\sim$210, $\sim$200, $\sim$150, $\sim$100, and $\sim$35-kD (Fig. 5). Three of the bands show similar size to the proteins of
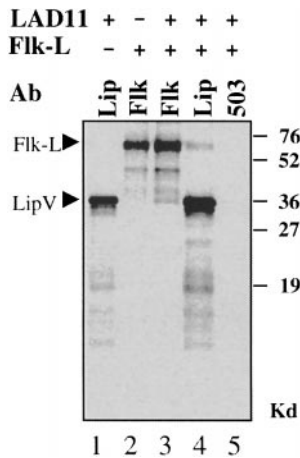

FIG. 3. *In vitro* binding of the intracellular domain of RRFlk with lipocortin V. The protein fragments representing the intracellular domain of RRFlk-L (residues 789-1343) and lipocortin V were *in vitro* translated in the presence of [^{35}S] methionine. After mixing lipocortin V with intracellular domain of RRFlk-L and immunoprecipitating with indicated polyclonal antibodies, the proteins were analyzed by 17.5% SDS-PAGE and autoradiography. An antibody against lens epithelial protein was used to eliminate nonspecific interactions (lane 5). Protein fragment lipocortin V (lane 1) and intracellular domain of RRFlk-L (lane 2) alone precipitated by their antibodies were used as negative controls.
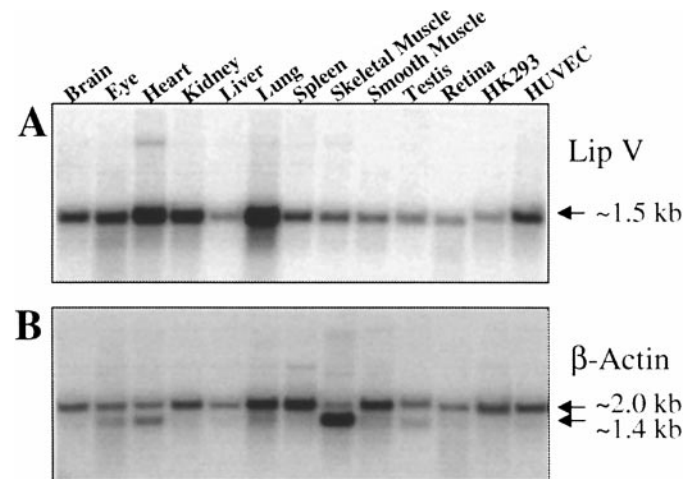

FIG. 4. Expression of lipocortin V in rat tissues and human cell lines by Northern blot analysis. One μg of poly(A$^+$) RNA from adult rat brain, whole eye, heart, kidney, liver, lung, spleen skeletal muscle, smooth muscle, testis, retina, human embryonic kidney cells (HK-293) and HUVE cell lines was hybridized to (A) lipocortin V cDNA fragment or (B) a β-actin cDNA probe. The expected size of the target mRNA is shown.
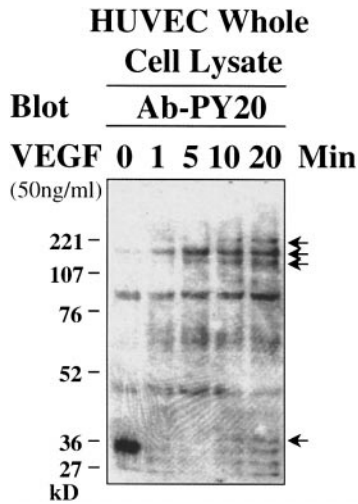
FIG. 5. Induction of tyrosine phosphorylation in response to VEGF in HUVEC. Whole cell lysates from HUVEC either untreated or stimulated for the indicated time with 50 ng/ml VEGF, were separated by 8.5% SDS-PAGE, transferred to a nitrocellular membrane and probed with anti-phosphotyrosine antibody (PY20). Tyrosine-phosphorylated proteins were visualized by enhanced chemiluminescence. Molecular weight standards and the position of tyrosine-phosphorylated proteins are indicated on the left and right, respectively.

interest identified by the yeast two hybrid system: VEGFR-2 (~210-kD), PLC-γ1 (~150-kD) and lipocortin V (~35-kD).

To confirm that lipocortin V is tyrosine-phosphorylated in response to VEGF stimulation, HUVEC lysates were immunoprecipitated with anti-Flk and anti-lipocortin V antibodies, immunoblotted, and probed with the PY-20 antibody (Fig. 6A and B, left panels). The 220-kD tyrosine-phosphorylated proteins was shown to be identical to VEGFR-2, with tyrosine-phosphorylation seen after 1 min exposure to VEGF, and maximal levels detected within 5 min (Fig. 6A). The lipocortin V tyrosine-phosphorylated band of 35-kD was detected within 10-min after stimulation, with an increase up to 20-min (Fig. 6B). These anti-phosphotyrosine blots (Flk, and lipocortin V) were then stripped and blotted with the same antibodies used for immunoprecipitation to show that similar amounts of protein were added to each lane (Fig. 6A and B, right panels). Similar results were obtained for PLC-γ (data not shown).

Effects of Antisense Oligodeoxyribonucleotides to Lipocortin V on VEGF-Induced HUVEC Proliferation and Phosphorylation

We next tested whether lipocortin V is involved in VEGF-induced proliferation of endothelial cells. For this, serum-starved HUVEC were treated with the antisense or sense ODNs ASLipV, or SLipV at 1 μM,

100 nM and 10 nM, 24 hrs before the addition of VEGF (50 ng/ml). The effect of ODN treatment on [^{3}H] thymidine incorporation is shown in Fig. 7A. At a concentration of the antisense ODNs of 10 nM, a significant inhibition of DNA synthesis was observed only in the VEGF-stimulated HUVEC cells treated with ASLipV (by 50~56% at days 1 through 4) (Fig. 7A), whereas the SLipV had little affect on DNA synthesis. However, both antisense (ASLipV) and sense (SLipV) ODNs for lipocortin V significantly decreased DNA synthesis in HUVEC at 1 μM and 100 nM concentrations (data not shown), showing a general cytotoxicity of the ODN's at higher concentrations.

To show that the antisense ODNs to lipocortin V selectively inhibit the protein synthesis and phosphorylation of these gene products, serum-starved HUVEC were treated with the ODNs ASLipV or SLipV at 10 nM, 24 hrs before the addition of VEGF (50 ng/ml for 20 min). HUVEC lysates were immunoprecipitated with anti-lipocortin V antibody, immunoblotted, and probed with PY-20 antibody (Fig. 7B). Tyrosine phosphorylation of V was significantly reduced with ASLipV (Fig. 7B, lane 3 in left panel). The decrease in phosphorylation of lipocortin V was accompanied by a decrease in protein synthesis after antisense treatment (Fig. 7B, lane 3 in right panel). In contrast, the phosphorylation of lipocortin V was similar to control level in HUVEC treated with the sense ODN, SLipV
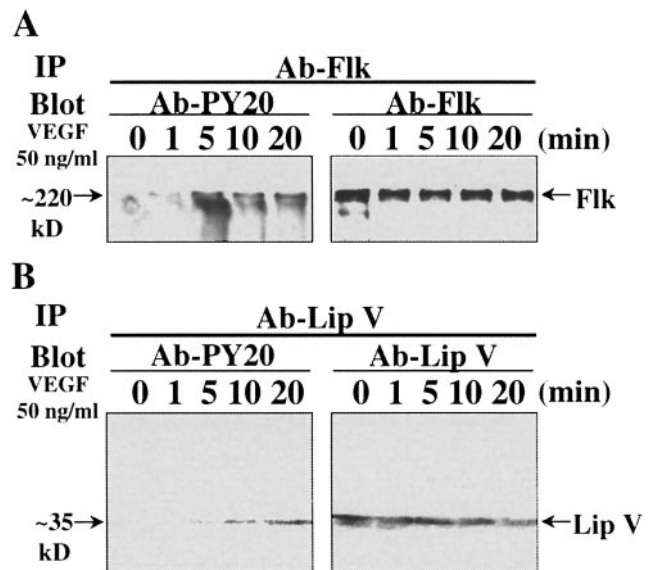

FIG. 6. VEGFR-2, PLC-γ1 and lipocortin V are rapidly and transiently tyrosine phosphorylated in response to VEGF in HUVEC. Serum-starved HUVEC were stimulated with VEGF for the indicated times at 37°C. Cell lysates were immunoprecipitated with anti-Flk (A) or anti-lipocortin V (B) antibodies. Immunoprecipitates were separated on an 8.5% SDS-PAGE, blotted and probed with anti-phosphotyrosine antibody (PY20) (left panels) or antibodies to Flk or lipocortin V (right panels).
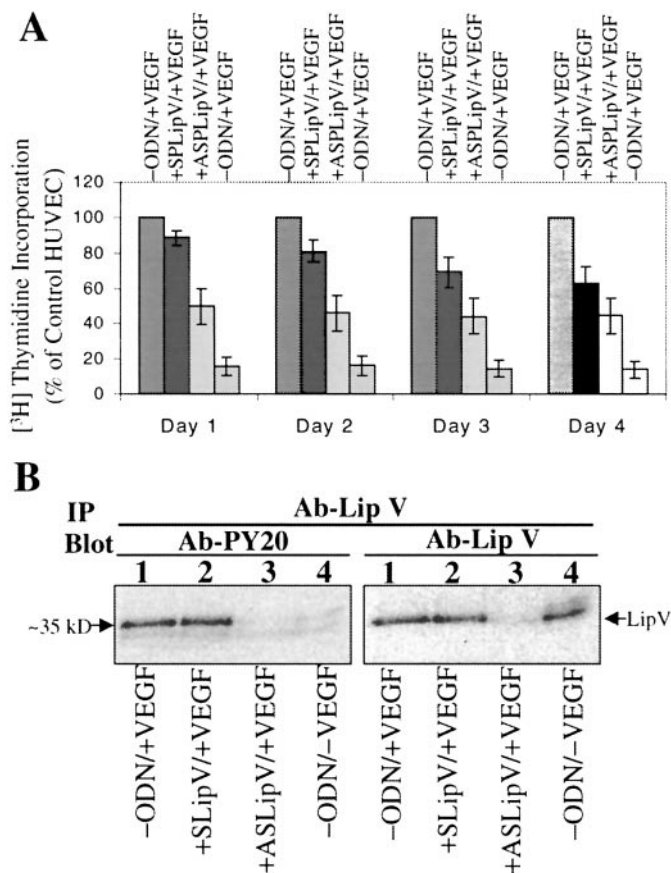
FIG. 7. Effect of antisense lipocortin V oligodeoxyribonucleotide on VEGF-induced DNA synthesis and tyrosine phosphorylation of lipocortin V in HUVE cells. (A) The antisense (ASLipV) or sense (SLipV) oligodeoxyribonucleotide (ODN) for lipocortin V was added to 2-day serum-starved HUVEC at 10 nM for 24 h prior to the addition of VEGF (50 ng/ml). At days 1-, 2-, 3-, or 4 after VEGF addition, [³H] thymidine was added to a final concentration of 1 μCi/ml, and the cells were further incubated for 4 h, washed with PBS, and solubilized with lysis buffer (0.2% Na_2CO_3, 0.1 M NaOH). [³H] radioactivity was measured by liquid scintillation counting. VEGF-treated HUVEC without ODNs were used as controls ($-$ODN/$+$VEGF). HUVEC without ODNs and VEGF were used as negative controls ($-$ODN/$-$VEGF). [³H] thymidine incorporation is expressed as the percentage of control, VEGF-treated cells. The data represent the mean $\pm$ S.E. in triplicate from at least two independent experiments. (B) After 20 min stimulation with VEGF, HUVEC lysates were immunoprecipitated with anti-lipocortin V antibody. The immunoprecipitates were separated by 8.5% SDS-PAGE, blotted and probed with PY20 (left panel) or anti-lipocortin V antibody (right panel).

(Fig. 8B, lane 2 in left panel). These results show that selective inhibition of lipocortin V protein synthesis significantly reduces HUVEC proliferation mediated by VEGFR-2 activation, and suggests a role for lipocortin V in endothelial cell proliferation.

DISCUSSION

The two-hybrid assay is a sensitive *in vivo* method for identifying proteins that interact with a protein of interest and is well-suited for detecting weak or transient interactions (31). The two-hybrid system was used to identify proteins interacting with the intracellular domain of the rat VEGFR-2. The activation of phosphoinositide-specific phospholipase C-γ (PLC-γ is one of several known early cellular responses to various growth factors and mitogens (32). In the current study, we indeed found that the intracellular domain of RRFlk-L can physically interact with the N-terminal SH2 domain of PLC-γ1 (a novel demonstration of direct interaction), and in HUVEC PLC-γ1 was rapidly and transiently phosphorylated on tyrosine residues with similar kinetics to that of VEGFR-2 auto-phosphorylation in response to VEGF (data not shown). These results are consistent with previous studies (33, 34), and again suggest that PLC-γ1 is one of the major signaling proteins for the VEGF receptor. In addition to this known interacting protein, a new interacting protein, lipocortin V was found to interact with the intracellular domain of VEGFR-2.

Lipocortins (annexins) are a family of at least 18 structurally related cytoplasmic proteins in mammals, which exhibit Ca^{2+}-dependent binding to phospholipids (35, 36). These proteins are widely distributed in eukaryotic cells and are characterized by a similar structure, with four or eight repeats of a 70-amino acid conserved core, and differ by a variable N-terminal domain. Lipocortin cores share 40-60% amino acid sequence identity and contain the Ca^{2+} and phospholipid binding sites. The N-terminal domain contains phosphorylation sites for different protein kinases such as protein kinase C (PKC) and tyrosine kinases, as well as protein-protein interaction domains. It is speculated that these unique N-terminal domains are specific and responsible for the different functions of lipocortins.

Previous studies suggest that lipocortins play a role in receptor tyrosine kinase signaling. For example, lipocortin I and II are major substrates of EGFR and pp60[v-src], respectively (37–39), and have been shown to be substrates of the tyrosine kinases associated with insulin receptors (40, 41). Tyrosine phosphorylation of lipocortin II was reported in fibroblasts after stimulation with PDGF (42). More recently, hepatocyte growth factor (HGF) receptor tyrosine kinase-mediated phosphorylation of lipocortin I was shown to transduce the proliferating signal of HGF in an A549 lung carcinoma cell line (43).

In the current study, yeast two-hybrid screening identified two cDNA clones coding for lipocortin V interacting with the intracellular domain of RRFlk-L. Both the yeast two-hybrid assay and *in vitro* co-immunoprecipitation confirmed this interaction between the intracellular domain of RRFlk-L and lipocortin V. That interaction was occurring in mammalian cells was shown by rapidly tyrosine phosphorylation of lipocortin V (within 10 min) after VEGF stimulation in

HUVEC (Figs. 5 and 6). This is the first report of tyrosine phosphorylation of lipocortin V by the VEGF receptor.

The phosphorylation process begins with at least four tyrosine (Y) autophosphorylation sites in the intracellular domain of VEGFR-2 (13). Two sites, Y951 and Y996 (Y947 and Y992 in RRFlk), are located in the kinase insert domain (KID) that splits the functional tyrosine kinase domain of the receptor and is thought to be the site of interaction with cellular signal transduction proteins (44). The other two sites, Y1054 and Y1059 (Y1050 and Y1055 in RRFlk-L), are located in the C-terminal half of the tyrosine kinase domain. Both the yeast two-hybrid assay and *in vitro* binding assay show that the entire intracellular domain of RRFlk-L containing four tyrosine phosphorylation sites (Y947, Y992, Y1050 and Y1055) can interact with lipocortin V, whereas no significant interaction was detected for either the intracellular domain of RRFlk-S containing only Y947 or the C-terminal half of the tyrosine kinase domain of RRFlk-L containing Y992, Y1050 and Y1055 (Figs. 2 and 3). This suggests that the interaction of the intracellular domain of RRFlk with lipocortin V depends on the activity of the intrinsic tyrosine kinase. This interaction may require the presence of both Y947 and Y992 in the kinase insert domain, since RRFlk-S is missing Y992 and the C-terminal half of the tyrosine kinase domain of RRFlk-L is missing Y947.

Lipocortin V is the most abundant of the lipocortins and is present in almost all mammalian cells. Its cDNA encodes an unglycosylated protein of 320 residues with a molecular mass of 35kD (45). Although a specific function for lipocortin V has not been determined, it is implicated in Ca^{2+} channel regulation (46), as an inhibitor of protein kinase C (47), and as an inhibitor of phospholipase A_2 (48). Lipocortin V is located in the cytosol and is also associated with the plasma membrane in a variety of cell types (35, 49). Recently, a nuclear location of lipocortin V has been reported (50), and the nuclear location of lipocortin V appears to be controlled by signaling pathways involving serum factors and tyrosine kinases (51). Taken together, these studies suggest that lipocortin V is implicated in cellular signaling pathways due to activation of the VEGF receptor-2.

The *in vivo* association of lipocortin V with the intracellular domain of VEGFR-2 in the yeast two-hybrid system and the tyrosine phosphorylation in response to VEGF in HUVEC indicates that lipocortin V is involved in VEGFR-2 signaling. Further evidence for this was obtained by showing that the lipocortin V antisense 21-mer ODN inhibited VEGF-induced HUVEC proliferation by ~50%, decreased lipocortin V protein synthesis, and significantly inhibited lipocortin V tyrosine phosphorylation. Lipocortin V appears to be a regulator of vascular endothelial cell proliferation mediated by the VEGFR-2. Previous studies have shown that lipocortins may be involved in the regulation of membrane trafficking, exocytosis, and cytoskeletal-plasma membrane interactions (35). It is tempting to speculate that lipocortin V may play an important role in the VEGFR-2-mediated permeability increase and the migration of vascular endothelial cells.

ACKNOWLEDGMENTS

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