Ets-1-dependent Expression of Vascular Endothelial Growth Factor Receptors Is Activated by Latency-associated Nuclear Antigen of Kaposi’s Sarcoma-associated Herpesvirus through Interaction with Daxx

Yuko Murakami, Satoshi Yamagoe, Kohji Noguchi, Yutaka Takebe, Naoko Takahashi, Yoshimasa Uehara, and Hidesuke Fukazawa

Vascular endothelial growth factor (VEGF) and its receptors are highly expressed in Kaposi's sarcoma (KS) lesion and play a key role in angiogenesis. Latency-associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) has multiple functions related to viral latency and KSHV-induced oncogenesis. In this report, we have identified Daxx as a LANA-binding protein by co-immunoprecipitation analysis of HeLa cells stably expressing LANA. LANA associated with Daxx in a PEL cell line infected with KSHV. LANA and Daxx also bound in vitro, suggesting direct interaction. From the results of binding assays, a region containing the Glu/Asp-rich domain within LANA, and a central region including the second paired amphipathic helix within Daxx contributed to the interaction. To address the physiological significance of this interaction, we focused on a Daxx-mediated VEGF receptor gene regulation. We found that Daxx repressed Ets-1-dependent Flt-1/VEGF receptor-1 gene expression, and that LANA inhibited the repression by Daxx in a reporter assay. Analyses of flow cytometry and real-time PCR revealed that expression of VEGF receptor-1 and -2 in LANA-expressing human umbilical vein endothelial cells (HUVECs) significantly increased. Co-immunoprecipitation and immunoblotting experiments suggested that LANA-bound Daxx to inhibit the interaction between Daxx and Ets-1. Chromatin immunoprecipitation assays showed that Daxx associated with VEGF receptor-1 promoter in HUVECs, and that LANA expression reduced this association. These results suggested that LANA contributes to a high expression of VEGF receptors in KS lesion by interfering with the interaction between Daxx and Ets-1.

Kaposi’s sarcoma-associated herpesvirus (KSHV2/HHV8) has been found to be the pathogen of Kaposi’s sarcoma (KS) (1), two B cell malignancies, primary effusion lymphoma (PEL), and multicentric Castleman’s disease (MCD) (3). Among over 80 ORFs of KSHV (4), LANA (latency-associated nuclear antigen) is exceptionally highly expressed in KS lesion, PEL, and also in MCD (5) (3), so that LANA is used as a diagnostic marker of KSHV. LANA is reported to be a multifunctional protein that tethers its own viral episomal DNA to host chromosomes in mitosis to segregate KSHV into progeny cells (6) (7) and also binds many host molecules to regulate expression of cellular genes. LANA inhibits p53-induced apoptosis (8), transforms fibroblast by co-transfection with the Ras oncoprotein (9) and also stabilizes β-catenin to stimulate entry into S phase (10). LANA seems to contribute to pathogenesis of KSHV-associated malignancies through these interactions.

We identified Daxx as a new LANA-interacting host protein. To know the biological significance of the interaction between LANA and Daxx, we focused on Daxx-modulated transcription. Daxx was found initially as a Fas-binding protein to regulate apoptosis (11) and later reported to bind with several nuclear proteins and transcription factors. Daxx was shown to act as a transcriptional repressor of Ets-1 (12), Pax3 (13), Pax5 (14), and p53 (15) through protein-protein interaction. In the case of Ets-1, Daxx repressed Ets-1-dependent expression of matrix metalloproteinase 1 (MMP1) and Bcl-2 (12). Ets-1 belongs to the Ets family of transcriptional factors, and regulates various gene expressions through binding to a unique motif (GGAA) on their promoters. Ets-1 regulates genes related to angiogenesis: Flt-1/VEGF receptor-1, KDR/VEGF receptor-2, and matrix metalloproteinases (MMPs) (16). Ets-1 is specifically expressed in lymphoid tissues, endothelial cells (17), and also in the spindle cells of KS lesion, derived from endothelial origins (18). In KS lesion, angiogenic factors such as VEGF and VEGF receptors were highly expressed (19). Vascular angiogenesis plays an important role in the development and progression of tumors, especially KS (20) (21) (22). We therefore examined the role of LANA in interaction between Daxx...
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and Ets-1, and show here a possible new function of LANA on the expression of VEGF receptors.

EXPERIMENTAL PROCEDURES

Plasmids—LANA gene was cut from L54 Lambda FIX II vector (NIH AIDS Research & Reference Reagent Program), at the Ehel site (123743 and 127293 of KSU75698) and inserted into the EcoRV site of pFLAG-CMV-2 expression vector (Sigma) to make an N-terminal FLAG-tagged LANA expression vector, pFLAG-LANA. For in vitro translation/transcription system, the LANA gene from pFLAG-LANA was subcloned between the EcoRI and KpnI sites of pBluescript II KS(+) plasmid after correction of the N-terminal 5 bases using synthetic oligonucleotides, 5’-AATTCAATGCATGCGCCTCCCGGAATTCG-3’ and 5’-CAATTCGCCGCTCTCAGATG-3’ (using EcoRI and BsmI sites), to obtain pBluescript-LANA. A series of C-terminal deletion mutants of LANA (L1 to L4) were constructed from pBluescript-LANA using an exonuclease III/mung bean deletion kit (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions. An N-terminal deletion mutant of LANA (L5) was constructed with pBluescript-LANA by cutting the N-terminal region at EcoRI and PstI sites and joining it with synthetic oligonucleotides, 5’-AATTCAATGCATGCGCCTCCCGGAATTCG-3’ and 5’-GGGCTCCATCGATG-3’. PFLAG-LANA deletion mutants (pFLAG-LANA-N1 to pFLAG-LANA-C) were constructed with L1–L5 and pFLAG-CMV-2 vector. Full-length and various deletion mutants of the Daxx gene were generated by PCR amplification from cDNA of HeLa cells, subcloned into pCR-Blunt II-TOPO plasmid (Invitrogen, Carlsbad, CA), and cloned between the EcoRI and the SalI sites of pcDNA3.1 (−) (Invitrogen) (termed pcDNA-Daxx), pCMV-HA (Clontech Laboratories, Inc. Palo Alto, CA), or pGEX-6P-3 (Amersham Biosciences, Piscataway, NJ). A luciferase reporter plasmid, pFlt-1-luc (containing human Flt-1 promoter 1174 to 748, D64016) was kindly provided by Dr. R. Li (12), and cloned into the SalI sites of pcDNA3.1(−) (Invitrogen). The constructed plasmids were purified using affinity matrix glutathione-Sepharose beads (Amersham Biosciences). 35S-labeled LANA was made in vitro translation/transcription system (Promega, Madison, WI). GST-Daxx fusion proteins were bound to glutathione-Sepharose beads and incubated with the translated products containing 35S-labeled LANA in binding buffer (25 mm HEPES, pH 7.6, 50 mm NaCl, 2.5 mm MgCl₂, 1 mm DTT, 0.05% Triton X-100, 1 mm PMSF) at room temperature for 15 min. After washing with washing buffer (25 mm HEPES, pH 7.6, 150 mm NaCl, 2.5 mm MgCl₂, 1 mm DTT, 0.5% Triton X-100, 1 mm PMSF), proteins adsorbed to the beads were resolved and applied to SDS-PAGE. Proteins in gels were transferred to PVDF membrane followed by Western blotting using anti-FLAG antibody (M5, Sigma), anti-Daxx antibody (sc-7152), anti-LANA antibody (Advanced Biotechnologies, Columbia, MD), anti-Ets-1 antibody (sc-350), or anti-HA antibody (Sigma).

GST Pull-down Assay—Glutathione S-transferase (GST)-Daxx fusion proteins were expressed in Escherichia coli, and purified using affinity matrix glutathione-Sepharose beads (Amersham Biosciences). 35S-labeled LANA was made in vitro translation/transcription systems (Promega, Madison, WI). GST-Daxx fusion proteins were bound to glutathione-Sepharose beads and incubated with the translated products containing 35S-labeled LANA in binding buffer (25 mm HEPES, pH 7.6, 50 mm NaCl, 2.5 mm MgCl₂, 1 mm DTT, 0.05% Triton X-100, 1 mm PMSF) at room temperature for 15 min. After washing with washing buffer (25 mm HEPES, pH 7.6, 150 mm NaCl, 2.5 mm MgCl₂, 1 mm DTT, 0.5% Triton X-100, 1 mm PMSF), proteins adsorbed to the beads were resolved and applied to SDS-PAGE. Proteins in the gel were stained with Coomassie Brilliant Blue, and the radioactivity was detected by BAS-1500 (Fuji Film, Tokyo).

Identification of LANA-binding Protein—PFLAG-LANA was transfected into HeLa cells and stable LANA-expressing clones were selected. LANA-expressing cells of six liters were harvested, nuclear extract was prepared as previously described (25), and dialyzed against a buffer containing 20 mm Tris-HCl, pH 7.5, 100 mm NaCl, 0.2 mm EDTA, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride (PMSF), and 10 mm β-mercaptoethanol. The nuclear extract was adjusted to 150 mm NaCl and 0.1% Tween 20 before absorption into anti-FLAG antibody (M2) affinity gel (Sigma). The gel was washed with 150 mm washing buffer (20 mm Tris-HCl, pH 8.0, 5 mm MgCl₂, 150 mm NaCl, 1 mm dithiothreitol (DTT), 10% glycerol, 1 mm PMSF), and eluted with the same buffer containing 200 μg/ml of FLAG peptides. The eluted protein was applied to SDS-PAGE and stained with Coomassie Brilliant Blue or silver stained. The Coomassie-stained band was cut and treated with lysyl endopeptidase. The extracted peptides were purified using HPLC, and analyzed with a Procise 494 HT Protein Sequencing System (Applied Biosystems, Foster City, CA).

Immunoprecipitation and Western Blotting—Cells were harvested and lysed with low salt buffer (10 mm HEPES, pH 7.9, 10 mm KCl, 1.5 mm MgCl₂, 1 mm DTT containing 0.5% Nonidet P-40, 1 mm PMSF, 25 μg/ml each of antipain, pepstatin, and leupeptin), then centrifuged to collect the nuclei. The nuclear pellet was lysed with nuclear extract buffer (20 mm Tris-HCl, pH 7.9, 5 mm EDTA, 300 mm NaCl, 1 mm PMSF), and the same volume of distilled water was added. Nuclear extract was subjected to immunoprecipitation either with anti-FLAG antibody (M2), anti-Daxx antibody (sc-7152) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-Ets-1 antibody (Santa Cruz Biotechnology, sc-350). The immune complex was washed with 150 mm washing buffer, resolved in Laemmli’s sample buffer and applied to SDS-PAGE. Proteins in gels were transferred to PVDF membrane followed by Western blotting using anti-FLAG antibody (M5, Sigma), anti-Daxx antibody (sc-7152), anti-LANA antibody (Advanced Biotechnologies, Columbia, MD), anti-Ets-1 antibody (sc-350), or anti-HA antibody (Sigma).

Cell Culture and Transfection—HeLa cells and human embryonic kidney 293T cells were cultured in Dulbecco’s modified medium supplemented with 10% bovine fetal serum. A KSHV-infected PEL cell line, BCBL-1 cells (kindly provided by Dr. H. Katano) were cultured in RPMI 1640 with 10% bovine fetal serum. Human umbilical vein endothelial cells (HUVEC) (Clonetics, San Diego, CA) were cultured in EGM-2 medium (Clonetics). Transfection was performed with FuGENE6 (Roche Diagnostics, Indianapolis, IN) for HeLa and 293T cells or by Nucleofector system (amaxa GmbH, Cologne, Germany) for HUVEC.
cells/assay) were treated with 1% formaldehyde for 5 min for scanning microscope using a Carl Zeiss LSM510 system (Carl Zeiss, Oberkochen, Germany).

Transcriptional Reporter Assay—293T cells (2 × 10⁵ cells/well) grown in 24-well plates were transfected with pFt-1-luc, pRSV-β-Gal (for transfection efficiency), and the combination of pcDNA-p51Ets-1, pcDNA-p42Ets-1, pcDNA-Daxx, or pFLAG-LANA, with 2 μl of FuGENE6. Total DNA was adjusted to a constant amount (800 ng). Two days after transfection, the cells were lysed and applied to luciferase assay (Promega). Assays were performed in triplicate, and the experiments were repeated three times.

Flow Cytometric Analysis—HUVECs transfected with either pIRES2-LANA-EGFP or pIREAS2-EGFP as control, were subjected to FACS Vantage (Becton Dickinson, Franklin Lakes, NJ) to collect GFP-expressing cells 2 days after transfection. The GFP-expressing cells cultured for 10 days were incubated with anti-Flt-1 or anti-KDR antibody (V4262, V9134, respectively, Sigma) and PE-labeled secondary antibody (R0439, Dako Cytomation, Carpinteria, CA). The cells were analyzed by a FACCalibur flow cytometer (Becton Dickinson).

Quantitative Real-time RT-PCR—HUVECs were transfected with either pIRES2-LANA-EGFP or pIREAS2-EGFP using Nucleofector system, and sorted with FACS Aria (Becton Dickinson) to collect GFP-expressing cells 2 days after transfection. Total RNA was extracted with RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), and reverse-transcribed to cDNA with oligo(dT) by Superscript First-strand synthesis system according to the manufacturer’s instructions (Invitrogen). The cDNA was applied to Real-Time PCR using SYBR Premix Ex Taq (Takara Bio Co.) with ABI PRISM7000 (Applied Biosystems). PCR was performed at 95 °C for 10 s, followed by 30 cycles of 90 to 95 °C for 5 s and 60 °C for 34 s. The primers were designed using software Primer Express (Applied Biosystems).

RESULTS

Identification of Daxx as a LANA-binding Protein—To identify host proteins that associate with LANA, we constructed a plasmid expressing FLAG-LANA (N terminus-tagged) to transfect into HeLa cells and established several stable LANA-cross-linking, lysed with 400 μl of lysis buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, 25 μg/ml each of antipain, pepstatin, and leupeptin), and centrifuged to collect the nuclei. The nuclear pellet was lysed with 200 μl of SDS lysis buffer (50 mM Tris-Hcl, pH 8.1, 10 mM EDTA, 1% SDS), and sonicated 6 times for 30 s each time. Centrifuged supernatants were then diluted with 1.8 ml of CHPl dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tri-HCl, pH 8.0), and precleared with protein A agarose/salmon sperm DNA. Anti-Ets-1 (Santa Cruz Biotechnology, sc-350), anti-Daxx antibody (Santa Cruz Biotechnology, sc-7152), or rabbit IgG (Sigma) as the negative control was added respectively to the supernatant, and rotated overnight at 4 °C. The mixture was then incubated with protein A-agarose/salmon sperm DNA for 1 h at 4 °C. The protein A-agarose-conjugated complex was washed, and DNA fragments were eluted and prepared according to the manufacturer’s instructions. The prepared DNA was resolved in 20 μl of H₂O and 2 μl was used for PCR. Primers used were 5′-GGGACGCCCTTGCAATCAAACTGCCCAACCATCATTAGAAGCAAGGA-3′. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used with primers 5′-ACGGATTTGGTCGTA-3′ and 5′-CTGCCAGCTGCACTGCTGACATGTA-3′, respectively, and primers for KDR were 5′-CACCACTAAAACGCTGACATGTA-3′ and 5′-CACAACGGCCAATCGATGGAT-3′. Primers for Daxx were 5′-GAGAGCTGCTTTAGAAGA-3′ and 5′-TGGGATTTCCCTAGT-GACAAG-3′. Obtained data were analyzed according to the sequence detector program (Applied Biosystems).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed basically using a kit from Upstate Biotechnology (Lake Placid, NY) with some modifications. Cells (5 × 10⁶ cells/assay) were treated with 1% formaldehyde for 5 min for...
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expressing clones. We cultured one clone of the LANA-expressing cells and prepared nuclear extract. This extract was incubated with anti-FLAG affinity gel (M2-agarose), followed by elution with FLAG peptides. Eluate was subjected to SDS-PAGE to detect a prominent 120-kDa band (Fig. 1A). Although there was a 75-kDa band, which was a nonspecific binding protein commonly found with the antibody. We determined the sequences of the N-terminal 10 residues of the 120-kDa protein, which revealed the protein to be Daxx. To confirm the identification, the nuclear extract (each 500 μg of protein) was immunoprecipitated with anti-FLAG antibody to apply to immunoblotting with anti-Daxx antibody. As shown in Fig. 1B, anti-Daxx antibody recognized a band of 120 kDa. These results indicated that Daxx is a cellular binding protein of exogenously expressed LANA in the HeLa cell. To confirm LANA-Daxx interaction in a physiological context, we immunoprecipitated with anti-Daxx antibody from nuclear extracts of BCBL-1 cells, a PEL cell line infected with KSHV. LANA was co-immunoprecipitated with Daxx as well (Fig. 1C). This result suggested that LANA formed a complex with Daxx in KSHV-infected cells.

Colocalization of LANA and Daxx in the Nuclei of KSHV-infected Cell Line BCBL-1—Next we examined the localization of LANA and Daxx in BCBL-1 by immunofluorescence microscopic assay (Fig. 2A). LANA gave a characteristic speckled staining pattern in nuclei of the cells (Fig. 2A, panel b), Daxx also showed some speckles in the nuclei (Fig. 2A, panel c). The merged image indicated that LANA considerably co-localized with Daxx in the nuclear dots (Fig. 2A, panel d). We also investigated the localization of Daxx using HeLa cells (Fig. 2B). LANA gave fine patchy staining in the nucleus (Fig. 2B, panel f), which is a typical observation in the absence of KSHV genome (Fig. 2B, panel g). The parental HeLa cells showed diffused staining of Daxx throughout the cell (Fig. 2B, panel c). In contrast, Daxx appeared to accumulate in the nuclei of the LANA-expressing cells (used in Fig. 1) (Fig. 2B, panel g). LANA and Daxx largely localized in the nucleus of the HeLa cells (Fig. 2B, panel h). We performed biochemical fractionation using three independent clones of LANA-expressing HeLa cells and examined cellular localization of Daxx by Western blotting. The results indicated that the amount of Daxx in the nuclear fraction increased as LANA expression increased, although total amounts of Daxx were comparable in these HeLa clone cells (data not shown).

A Region Containing the Acidic-rich Domain in LANA Is Required for Binding with Daxx—To determine the interacting domain of LANA with Daxx, we constructed a series of LANA deletion mutants (Fig. 3A), which were translated in vitro and subjected to pull-down assay with GST-Daxx. As shown in Fig. 3B, full-length LANA was pulled down with GST-fused full-length Daxx, indicating direct interaction between LANA and Daxx. Three N-terminal mutants of LANA (L1–L3) bound with GST-Daxx, but the shortest N-terminal LANA (aa 1–261) (L4), and C-terminal LANA (aa 496–740) (L5) failed (Fig. 3B). We constructed mammalian expression plasmids, LANA-N (aa 1–564), LANA-C (aa 496–1162), LANA-N1 (aa 1–260), LANA-N2 (aa 1–320), LANA-N3 (aa 1–344), and LANA-ΔAD (with aa 322–493 deleted) (Fig. 3A). These plasmids were cotransfected with pcDNA-Daxx into 293T cells, and the nuclear extracts were analyzed. Immunoprecipitation with anti-Daxx antibody and Western blotting with anti-FLAG antibody indicated that Daxx formed a complex with full-length LANA and LANA-N, and weakly with LANA-N3, but not with the other LANA fragments (Fig. 3C). Taken together, these results suggested that aa 320–344 of LANA, which contains many aspartic acids and glutamic acids, were required for binding with Daxx.

A Central Domain of Daxx Is Required to Interact with LANA—To determine the critical region of Daxx for binding with LANA, a series of GST-fused deletion mutants of Daxx (Fig. 4A)
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Figure 3. A region containing an acidic-rich domain in LANA is required for binding with Daxx in vitro and in vivo. A, domain structure of LANA and its deletion mutants. LANA is constituted of domains of proline-rich, acidic-rich, glutamine-rich, and basic leucine-zipper. A series of deletion mutants of LANA and the binding activity in vitro and in vivo are shown. B, result of pull-down assay with GST-fused full-length Daxx of 35S-labeled LANA deletion mutants (L1–L5). C, co-immunoprecipitation of Daxx and LANA deletion mutants in 293T cells. PFLAG-CMV-2 vector (4.0 g), pFLAG-LANA-N3 (2.0 g), pFLAG-LANA-N (2.0 g), pFLAG-LANA-C (4.0 g), pFLAG-LANA-ΔA (4.0 g), pFLAG-LANA-ΔA (4.0 g) were individually co-transfected with pCDNA-Daxx (1.0 μg) in 60-mm dishes with adjustment of total DNA amount (5.0 μg). The immunoprecipitates (IP) with anti-Daxx antibody were followed by immunoblotting (WB) with anti-FLAG antibody (MS).

Figure 4. A central region containing PAH 2 and acidic-rich domain in Daxx is required to interact with LANA. A, domain structure of Daxx and various deletion mutants. Daxx is composed of two PAH and acidic-rich and Ser/Pro/Thr-rich domains. A series of mutants of Daxx and the binding activity in vitro and in vivo are shown. B, purified GST-Daxx variants (G1–G9) were applied in in vitro pull-down assay with full-length 35S-LANA. C, mammalian expression plasmids, pCMV-HA-Daxx-H1 (full-length) (2.0 μg), pCMV-HA-Daxx-H2 (aa 1–500) (2.0 μg), pCMV-HA-Daxx-H3 (aa 1–440) (1.0 μg), pCMV-HA-Daxx-H4 (aa 110–500)(1.0 μg), pCMV-HA-Daxx-H5 (aa 500–740) (1.0 μg) were co-transfected with pFLAG-LANA (1.0 μg) left two panels. pCDNA-Daxx-D1 (full-length) (1.0 μg), pCDNA-Daxx-D2 (deleted aa 271–509) (3.0 μg), pCDNA-Daxx-D3 (aa 63–740)(3.0 μg), pCDNA-Daxx-D4 (aa 111–740)(3.0 μg), and pCDNA-Daxx-D5 (aa 243–740) (2.0 μg) were individually co-transfected with pFLAG-LANA (1.0 μg) right two panels. Immunoprecipitates (IP) with anti-FLAG antibody (M2) were followed by Western blotting (WB) with anti-HA antibody (left panels) or anti-Daxx antibody (right panels).
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**LANA Inhibited Daxx-mediated repression on Ets-1-dependent VEGF receptor 1 (Flt-1) gene expression.**

*Panel A* represents the relative luciferase activity (RLU) of Flt-1-luciferase reporter in 293T cells transfected with Flt-1-luciferase reporter, pcDNA-Daxx (200 ng), and pFLAG-LANA (0, 25, 50 ng) in inverse proportion to the amount of LANA. The relative luciferase activity (RLU) was normalized by β-galactosidase activity. Assays were performed in triplicate, and error bars indicate S.D.

**Panel B** indicates the relative luciferase activity (RLU) of Flt-1-luciferase reporter in 293T cells transfected with Flt-1-luciferase reporter, pcDNA-Daxx, and pFLAG-LANA (0, 25, 50 ng) in inverse proportion to the amount of LANA. The relative luciferase activity (RLU) was normalized by β-galactosidase activity. Assays were performed in triplicate, and error bars indicate S.D.

**LANA Inhibited Daxx-mediated repression on Ets-1-dependent VEGF Receptor 1 (Flt-1) Gene Expression**—To examine the role of Daxx in Kaposi’s sarcoma, we focused on Ets-1 transcription factor. It was reported that Daxx interacts with Ets-1 to repress Ets-1-dependent transcriptional activity of MMP-1 and Bcl-2 (12). On the other hand, as a characteristic feature of KS, it is known that VEGF and its receptors, Flt-1 and KDR (VEGF-receptor-1 and -2, respectively), are highly expressed in KS (20). There are several Ets-1 motifs in Flt-1 and KDR promoters to regulate the expression (26) (27). We examined the effect of Daxx on Ets-1-dependent Flt-1 expression. We co-transfected a luciferase reporter plasmid pFlt-1-luc driven by Flt-1 promoter, an Ets-1 expression vector, and a Daxx expression vector into 293T cells, to perform luciferase assay. Transcriptional activity on Flt-1 increased depending on the amount of Ets-1 plasmid, although the effect of p51-Ets-1 was quite weak. Daxx evidently repressed Ets-1-dependent activation (Fig. 5A). p51 and p42 are two human variants of the Ets-1 molecule. It is reasonable that the activity of p51-Ets-1 is lower than p42-Ets-1 because p42-Ets-1 lacks exon VII, the internal transcriptional regulatory domain (24). This result is similar to the case of MMP-1 and Bcl-2 expression (28). As we observed the repressive activity of Daxx on Ets-1-dependent Flt-1 expression, we examined the effect of LANA on the Daxx-mediated repression with p42Ets-1. Co-transfection with a LANA expression plasmid dose-dependently reactivated the transcriptional activity repressed by exogenous Daxx (Fig. 5B, 4 and 5), although LANA slightly activated it in the absence of exogenous Daxx (Fig. 5B, 7). These results suggested that LANA inhibited the repression via interaction with Daxx.

**LANA Activated Expression of VEGF Receptors in Vascular Endothelial Cells**—To investigate the possibility that LANA induces Flt-1 in Kaposi’s sarcoma lesion, we tried to express LANA in HUVEC, because endothelial cells (ECs) are regarded as the origins of KS lesions. We constructed a plasmid, pIRES2-LANA-GFP, which contains an internal ribosomal entry site (IRES) to express both LANA and GFP from a single mRNA. We transfected pIRES2-LANA-GFP or pIRES2-GFP as control into HUVEC and Flt-1 and KDR expression in GFP-positive cells were analyzed by flow cytometry. Flt-1 of GFP-positive cells in pIRES2-LANA-GFP-transfected cells was significantly increased as compared with that in control cells (Fig. 6A, left). The number of cells expressing Flt-1 over log intensity 1 (M1) was about 1.9× higher (Fig. 6A, upper, right graph) than that of control. M1 of KDR also increased 1.4× (Fig. 6A, lower, right graph). Furthermore, to examine the level of mRNA of the two receptors, we performed real-time PCR with total RNA prepared from the GFP-expressing HUVEC. LANA expression in pIRES2-LANA-GFP-transfected cells was confirmed by using PCR with primers of LANA (data not shown). The relative expressions of Flt-1 and KDR in LANA-expressing cells were 1.4 and 2.0× higher than that of control cells, respectively (Fig. 6B). Although there was discrepancy between rise of protein and mRNA, results of both FACs and real-time PCR indicated that LANA induced the two receptors in human endothelial cells. The expression of Ets-1 and Daxx was not altered between LANA-expressing cells and control cells (Fig. 6B).

**LANA Sequesters Daxx from Ets-1**—To resolve the mechanism of the activation of VEGF receptors expression by LANA, we examined the relation of the three molecules, LANA, Daxx, and Ets-1. 293T cells were co-transfected with a constant amount of pcDNA-Daxx and pcDNA-Ets-1, and a variable amount of pcDNA-p51Ets-1 or pcDNA-p42Ets-1 (+; 25 ng, + + ; 50 ng) were co-transfected with pcDNA-Daxx (+; 200 ng, + + ; 500 ng) and pFlt-1-luc (100 ng). The relative luciferase activity (RLU) was normalized by β-galactosidase activity. Assays were performed in triplicate, and error bars indicate S.D.

**LANA Sequesters Daxx from Ets-1**

LANA and Daxx competitively bind to Ets-1, so we expected that LANA sequesters Daxx from Ets-1. 293T cells were co-transfected with a constant amount of pcDNA-Daxx or pcDNA-Ets-1, and a variable amount of pcDNA-p51Ets-1 + (+; 25 ng, + + ; 50 ng) or pcDNA-p42Ets-1 (+; 200 ng, + + ; 500 ng) and pFlt-1-luc (100 ng). The relative luciferase activity (RLU) was normalized by β-galactosidase activity. Assays were performed in triplicate, and error bars indicate S.D.

**LANA Activated Expression of VEGF Receptors in Vascular Endothelial Cells**—To investigate the possibility that LANA induces Flt-1 in Kaposi’s sarcoma lesion, we tried to express LANA in HUVEC, because endothelial cells (ECs) are regarded as the origins of KS lesions. We constructed a plasmid, pIRES2-LANA-GFP, which contains an internal ribosomal entry site (IRES) to express both LANA and GFP from a single mRNA. We transfected pIRES2-LANA-GFP or pIRES2-GFP as control into HUVEC and Flt-1 and KDR expression in GFP-positive cells were analyzed by flow cytometry. Flt-1 of GFP-positive cells in pIRES2-LANA-GFP-transfected cells was significantly increased as compared with that in control cells (Fig. 6A, left). The number of cells expressing Flt-1 over log intensity 1 (M1) was about 1.9× higher (Fig. 6A, upper, right graph) than that of control. M1 of KDR also increased 1.4× (Fig. 6A, lower, right graph). Furthermore, to examine the level of mRNA of the two receptors, we performed real-time PCR with total RNA prepared from the GFP-expressing HUVEC. LANA expression in pIRES2-LANA-GFP-transfected cells was confirmed by using PCR with primers of LANA (data not shown). The relative expressions of Flt-1 and KDR in LANA-expressing cells were 1.4 and 2.0× higher than that of control cells, respectively (Fig. 6B). Although there was discrepancy between rise of protein and mRNA, results of both FACs and real-time PCR indicated that LANA induced the two receptors in human endothelial cells. The expression of Ets-1 and Daxx was not altered between LANA-expressing cells and control cells (Fig. 6B).

LANA Inhibited Daxx-mediated repression on Ets-1-dependent VEGF Receptor 1 (Flt-1) Gene Expression—To examine the role of Daxx in Kaposi’s sarcoma, we focused on Ets-1 transcription factor. It was reported that Daxx interacts with Ets-1 to repress Ets-1-dependent transcriptional activity of MMP-1 and Bcl-2 (12). On the other hand, as a characteristic feature of KS, it is known that VEGF and its receptors, Flt-1 and KDR (VEGF-receptor-1 and -2, respectively), are highly expressed in KS (20). There are several Ets-1 motifs in Flt-1 and KDR promoters to regulate the expression (26) (27). We examined the effect of Daxx on Ets-1-dependent Flt-1 expression. We co-transfected a luciferase reporter plasmid pFlt-1-luc driven by Flt-1 promoter, an Ets-1 expression vector, and a Daxx expression vector into 293T cells, to perform luciferase assay. Transcriptional activity on Flt-1 increased depending on the amount of Ets-1 plasmid, although the effect of p51-Ets-1 was quite weak. Daxx evidently repressed Ets-1-dependent activation (Fig. 5A). p51 and p42 are two human variants of the Ets-1
proportion to LANA expression (Fig. 7A, row c, right panel). Consistently, Daxx was detected in the immune complex with anti-Ets-1 antibody in inverse proportion to LANA expression (Fig. 7A, row d, middle panel). LANA was not detected in the immune complex with the anti-Ets-1 antibody (Fig. 7A, row d, left panel), which implies that increasing LANA caused increase of Daxx-LANA interaction, and reduction of Daxx-Ets-1 interaction. These results suggested that LANA sequesters Daxx from Ets-1, which results in inhibition of the interaction between Daxx and Ets-1.

In the experiments above we used transiently transfected 293T cells (Fig. 7A). To address whether the transient expression system for LANA-Daxx interaction is physiologically relevant or not, we analyzed relative expression levels of LANA and Daxx proteins using BCBL-1 and the transfected 293T cells. As shown in Fig. 7B, the expression level of exogenous LANA protein in 293T cells in the same condition of Fig. 7A was similar to that of endogenous LANA in BCBL-1 cells. In contrast, endogenous Daxx expression level is much lower in BCBL-1 cells than in the 293T cells. These data indicated that relative expression ratio of endogenous LANA to Daxx in BCBL-1 cells was much higher than that of LANA-transfected 293T cells.

Daxx associated with Flt-1 promoter and LANA reduced its association in HUVEC. To investigate the possibility that Daxx interacts with Flt-1 promoter in HUVEC, bands indicate PCR products targeting Flt-1 promoter in HUVECs. LANA interacted with Daxx to sequester from Ets-1.
LANA Up-regulates VEGF Receptors through Daxx

antibody, and subjected to PCR to amplify a 98-bp fragment. The PCR product is designed to span the fourth ets motif (−54 to −51) that is thought to be indispensable for Flt-1 promoter activity (26). The anti-Daxx antibody precipitated the Flt-1 promoter as well as anti-Ets-1 antibody (Fig. 7C, lanes 6 and 7). The result indicated that Daxx as well as Ets-1 associated with Flt-1 promoter in ECs. Furthermore, HuVECs transfected with pIRES2-LANA-GFP or pIRES2-GFP as the control were sorted from Ets-1 (Fig. 7D), based on the results of co-immunoprecipitation (Fig. 7A) and ChIP assay (Fig. 7C). LANA slightly activated Ets-1 dependent Flt-1 expression without exogenous Daxx in the reporter assay (Fig. 5B). It is thought that LANA sequestered endogenous Daxx. However it is possible that LANA activates Flt-1 expression through an unidentified mechanism(s). At least LANA did not activate Flt-1 expression through up-regulation of Ets-1 expression (Fig. 6B). In human Flt-1 promoter, there are five Ets motifs and a CRE (cAMP response element). It is reported that co-existence of the fourth Ets motif, and the CRE is necessary for Flt-1 expression (26). LANA is reported to modulate the expression of a reporter plasmid with CRE, but the effect of LANA on CRE is repression (29). There is no CRE in the promoter of KDR.

Given that LANA induces VEGF receptors in KS lesion, we propose this hypothesis: Daxx binds Ets-1 to repress expression of VEGF receptors in normal ECs, while in KSHV-infected cells, LANA binds to Daxx to inhibit Daxx-Ets-1 interaction, resulting in the activation of Ets-1-dependent VEGF receptors. Furthermore, LANA-Daxx interaction might contribute to not only VEGF receptor gene expression but also to other Daxx-mediated gene regulation related to the pathogenesis of KS, PEL, and MCD malignancy.

DISCUSSION

LANA is reported to have multiple functions in KS lesion. It interacts with many host cellular molecules: p53 (8), pRb (9), ATF4/CREB2 (29), CBP (30), c-Jun (31), RING3 (32), mSin3A (33), HP-1 (33), Dek (34), GSK-3b (10) and so on. In the present study, we identified Daxx as a new member of LANA-binding proteins. Daxx was prominently detected in our immunofluorescence system, but this system also detected previously reported LANA-interacting protein such as RING3 by Western blotting (data not shown). We showed the interaction between the two proteins in vivo (Fig. 1) and in vitro (Figs. 3 and 4), which indicates that Daxx and LANA directly bound to each other. Fluorescent immunostaining assay showed co-localization of LANA and Daxx in BCBL-1 cells, supporting LANA-Daxx interaction in cells (Fig. 2).

Daxx is reported to bind many cellular molecules, indicating its involvement in multiple cellular processes. Although Daxx could interact with proteins of cytoplasm or membrane, it also interacted with some transcription factors and localized sometimes in the nuclear matrix structure, PML NBs (promyelocytic leukemia nuclear bodies). PML NBs are thought to provide platforms for transcriptional regulation, DNA repair, apoptosis, DNA replication, RNA transport, and many viruses target PML NBs to pirate host functions (reviewed by Everett, Ref. 35).

Ets-1 associates with a PML NBs protein, Sp100 (36). Therefore, it might be a strategy of KSHV that LANA targets Daxx of Ets-1 for repression. LANA might be an example of constructive interference by fused GST protein. PAH is a characteristic domain that is involved in transcriptional co-repressors such as mSin3 (38). It is interesting that mSin3A binds to aa 1–340 of LANA (28). There is a report that acetylated histone H4 interacts through PAH1 within Daxx, but no report that any other host molecule binds through this region of Daxx. As Daxx interacts with Ets-1 through the C-terminal region of Daxx (12), there may be no direct competition for Daxx between LANA and Ets-1.

Based on the interaction between LANA and Daxx (Figs. 1–4), we found that LANA induced VEGF receptors in ECs (Fig. 6) in accordance with the results of reporter assays (Fig. 5). Although expression level changes were not consistent for Flt-1 and KDR in protein (Fig. 6A) and mRNA (Fig. 6B), it may be caused by time point difference. This is the first report of the function of LANA in angiogenesis. It is reported that KSHV ORF74 (viral G-protein coupled receptor, v-GPCR) contributes to expression of VEGF receptors (39). Because ORF74 is expressed in the viral lytic infection cycle, it is unlikely that ORF74 is the only gene of KHSV that induces angiogenesis in KS. It is likely that some other factors such as VEGF and hypoxia-inducible factor (HIF) additionally affect on expression of these receptors in KS (40) (41).

As to the mechanism of activation of the receptor expression by LANA, we propose a hypothesis that LANA sequesters Daxx from Ets-1 (Fig. 7D), based on the results of co-immunoprecipitation (Fig. 7A) and ChIP assay (Fig. 7C). LANA slightly activated Ets-1 dependent Flt-1 expression without exogenous Daxx in the reporter assay (Fig. 5B). It is thought that LANA sequestered endogenous Daxx. However it is possible that LANA activates Flt-1 expression through an unidentified mechanism(s). At least LANA did not activate Flt-1 expression through up-regulation of Ets-1 expression (Fig. 6B). In human Flt-1 promoter, there are five Ets motifs and a CRE (cAMP response element). It is reported that co-existence of the fourth Ets motif, and the CRE is necessary for Flt-1 expression (26). LANA is proposed to modulate the expression of a reporter plasmid with CRE, but the effect of LANA on CRE is repression (29). There is no CRE in the promoter of KDR.

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