

Ribosomal Protein S6 Interacts with the Latency-Associated Nuclear Antigen of Kaposi's Sarcoma-Associated Herpesvirus[▽]

Wuguo Chen and Dirk P. Dittmer*

*Department of Microbiology and Immunology, Lineberger Comprehensive Cancer Center, Center for AIDS Research (CfAR),
University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7290*

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The latency-associated nuclear antigen (LANA) is central to the maintenance of Kaposi's sarcoma-associated herpesvirus (KSHV) and to the survival of KSHV-carrying tumor cells. In an effort to identify interaction partners of LANA, we purified authentic high-molecular-weight complexes of LANA by conventional chromatography followed by immunoprecipitation from the BC-3 cell line. This is the first analysis of LANA-interacting partners that is not based on forced ectopic expression of LANA. Subsequent tandem mass spectrometry (MS/MS) analysis identified many of the known LANA-interacting proteins. We confirmed LANA's interactions with histones. Three classes of proteins survived our stringent four-step purification procedure (size, heparin, anion, and immunoaffinity chromatography): two heat shock proteins (Hsp70 and Hsp96 precursor), signal recognition particle 72 (SRP72), and 10 different ribosomal proteins. These proteins are likely involved in structural interactions within LANA high-molecular-weight complexes. Here, we show that ribosomal protein S6 (RPS6) interacts with LANA. This interaction is mediated by the N-terminal domain of LANA and does not require DNA or RNA. Depletion of RPS6 from primary effusion lymphoma (PEL) cells dramatically decreases the half-life of full-length LANA. The fact that RPS6 has a well-established nuclear function beyond its role in ribosome assembly suggests that RPS6 (and by extension other ribosomal proteins) contributes to the extraordinary stability of LANA.

Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with primary effusion lymphoma (PEL), Kaposi's sarcoma (KS), and the plasmablastic variant of multicentric Castleman disease (MCD) (6, 8, 73). Like other herpesviruses, KSHV exhibits two distinct phases in its life cycle: latent episomal persistence and lytic replication. During latent infection, only a small subset of viral proteins is expressed (7, 12–14, 28, 56, 74, 83). One of these is the KSHV latency-associated nuclear antigen (LANA). It is encoded by open reading frame 73 (ORF73) and consistently expressed in all latently infected cells. LANA is necessary and sufficient for viral episome persistence and faithful partition of the latent KSHV genome. LANA tethers the viral plasmid to cellular chromosomes (1, 2, 24, 25). However, this is not the only function of LANA.

LANA is a multifunctional protein. Many proteins bind to LANA. These can be grouped according to their presumed functions. (i) Barbera et al. demonstrated that the N terminus of LANA docks onto cellular chromosomes by directly binding to the folded regions of histones H2A and H2B to mediate nucleosome attachment (3). Both histones H2A and H2B were necessary for LANA to bind nucleosomes. (ii) Robertson and colleagues found that LANA also binds histone H1 as well as other proteins involved in the structural remodeling of DNA (77). (iii) Cellular replication and replication licensing factors can bind to LANA (45, 75, 78), as well as the chromatin-modifying factors SAP30, mSin3A, CIR, meCP2, and DEK (35, 36). Further binding partners have been described (5, 71).

These include Ku70, Ku80, and PARP-1. (iv) Cellular transcription factors can bind to LANA, such as Sp-1 (33), RBP-jk (also known as CSL) (40), glycogen synthase kinase 3 β (GSK-3 β) (21), CBP/p300 and ATF4/CREB2 (43), Ring3 (48, 53, 60, 79), and KSHV Rta/orf50 (41). (iv) LANA binds to Rb (61) as well as to p53 (18, 80). The LANA-p53 complex can be destroyed by the mdm-2/p53 interaction inhibitor nutlin (9, 58, 67), which leads to p53-dependent apoptosis in PEL. Because of its ability to decorate host chromosomes, LANA can induce chromosome instability phenotypes that are akin to p53 inactivation (54, 70).

Here we find a new binding partner of LANA: the ribosomal protein S6 (RPS6). RPS6 (32 kDa) is a component of the 40S ribosomal subunit and the major phosphoprotein of the ribosome (59). The phosphorylation sites in RPS6 have been mapped to five clustered residues: Ser235, Ser236, Ser240, Ser244, and Ser247 (34, 66). RPS6 phosphorylation and function are highly regulated. RPS6 has been implicated in the regulation of translational initiation and protein synthesis in response to extracellular stimuli such as TRAIL and gamma interferon (IFN- γ), as well as upon activation of the phosphatidylinositol 3-kinase (PI3K)–Akt–mTOR pathway (4, 38, 42). Ribosome biogenesis and translation are regulated at multiple levels and are associated with cell growth and proliferation (65). Several ribosomal proteins are overexpressed in a variety of tumors. It remains to be determined whether this represents a cause or a consequence of tumor formation (66). Phosphorylated RPS6 is a biomarker for mTOR-targeted therapy in sarcoma (26), including KS (unpublished data). RPS6 is consistently phosphorylated in PEL and KS (72). Importantly, and perhaps underappreciated, the RPS6 protein is both cytoplasmic and nuclear localized (57). The RPS6 protein consists of

* Corresponding author. Mailing address: University of North Carolina at Chapel Hill, CB 7290, 715 Mary Ellen Jones Bldg., Chapel Hill, NC 27599-7290. Phone: (919) 966-7960. Fax: (919) 962-8103. E-mail: ddittmer@med.unc.edu.

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three modules: a nuclear localization signal (NLS), a nucleolar binding sequence (Nobis), and the C-terminal serine cluster of phosphorylation sites, which is evolutionarily conserved (38, 44, 68).

We find that RPS6 exists in complex with the KSHV LANA protein in PEL. This complex was stable after multiple chromatographic purification steps and was resistant to DNase and RNase treatment. This interaction suggests a new role for LANA in protein translation and ribosome biogenesis and vice versa a role for RPS6 in LANA function and, as we report here, LANA protein stability.

MATERIALS AND METHODS

Cell culture. BC-3 cells were cultured in RPMI 1640 medium (Cellgro, Inc.) containing 2 mM L-glutamine, 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin sulfate (100 µg/ml) and supplemented with 0.05 mM 2-mercaptoethanol (Sigma, Inc.), 0.075% sodium bicarbonate (Life Technologies, Inc.), and 1 U/ml human interleukin-6 (IL-6) (Roche, Inc.). HeLa cells (American Tissue Culture Collection [ATCC] no. CCL-2) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). All cells were grown at 37°C under 5% CO₂ atmosphere.

Antibodies. Anti-LANA monoclonal antibody (MAb) LN53 was purchased from Advanced Biotechnology, Inc., and used at a dilution of 1:2,000. Anti-LANA polyclonal rabbit antiserum was raised against the LANA repeat region (9). The additional antibodies used were as follows: anti-p53 MAb DO1 (1:2,000) (Santa Cruz, Inc.) and anti-phospho RPS6 rabbit antiserum (1:2,000 Ser 235/236 and 1:2,000 Ser 240/244), as well as total anti-RPS6 antibody (1:2,000) (Cell Signaling), anti-β-actin mouse MAb ((1:3,000) (Sigma, Inc.), and anti-p21 mouse MAb (1:1,000) (Santa Cruz). Mouse anti-RPL11 (clone 3a4a7) was from Invitrogen, Inc., and anti-hemagglutinin (anti-HA; clone HA-7) and anti-FLAG (clone M2) were from Sigma, Inc.

Plasmids. The human RPS6 ORF was PCR amplified from Rps6-FLAG-VRP (a gift from R. Johnston [50]). To construct HA-tagged RPS6, we fused RPS6 with HA-tagged sequence (YPYDVPDYA) into plasmid pcDNA3 (Invitrogen, Inc.) at the N-terminal portion of RPS6 ORF (pDD1902). This procedure used the primers F1 (5'-GCATACCCATACGATGTTCCAGATTACGCTCTGAACATCTCTTCCC; HA tag is underlined) and F2 (5'-CGACCCAAGCTTACCATGGCATACCCATACGATGTTCC), which incorporates a HindIII site (underlined), as well as the common reverse primer R (5'-GTCACGGGATCCCGTTATTTCTGACTGGATTC), which incorporates a BamHI site (underlined). The full-length plasmid of FLAG-LANA was a gift from D. Hayward (20). A series of mutants (amino acids [aa] 1 to 930, 1 to 428, 1 to 329, 330 to 1162, and 930 to 1162) of LANA were individually constructed into the pFLAG-CMV plasmid (Invitrogen, Inc.) using HindIII and EcoRI sites to yield pDD1926, pDD1927, pDD1928, pDD1929, and pDD1931, respectively.

Chromatography and MS/MS. BC-3 cells (5×10^9) were harvested and the nuclear extracts were obtained as described previously (11). The supernatant was loaded onto a Sepharose 6B column (Sigma, Inc.) and washed with 0.1 M KCl in buffer D (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT], 0.5% cocktail protein inhibitor). Sepharose 6B was used not as an affinity resin, but as a sizing matrix for high-molecular-weight complexes. This choice was based on the initial use of Sepharose 6B prior to its discovery as an affinity resin. When using 6B resin to separate large protein complexes such as LANA, multimers (220 kDa \times 4) and multimer-containing complexes elute close to the void volume, monomers, and broken complexes tend to be retained. Column fractions containing LANA were collected and loaded onto Hipep 16/10 heparin FF column (Amersham, Inc.), followed by a wash with 20 column volumes of 0.2 M KCl in buffer D and elution with 0.5 M KCl in buffer D. The fractions were then loaded onto a Mono-Q column (Sigma, Inc.). We collected two peaks at 0.4 M buffer D and 0.7 M KCl in buffer D. Immunoprecipitation with anti-LANA antibody (LN53; ABI, Inc.) was from a the second peak as described previously (9). Proteins were resolved by 8 to 16% gradient SDS-PAGE and stained with Coomassie blue (Bio-Rad, Inc.). Visible bands were cut and further subjected to mass spectrometry at the University of North Carolina—Chapel Hill core facility. They were further digested with trypsin, and the resulting peptides were analyzed on an ABI 4800 matrix-assisted laser desorption/ionization—tandem time of flight (MALDI TOF/TOF) MS analyzer. MS spectra were executed in a reflector positive-ion mode, with a mass range of 700 to 4,000 Da, and with a total number of shots/spectrum of 1,250. MS/MS spectra were executed in a positive-ion mode

at 2 kV and with a total number of shots/spectrum of 1,350 or less. The peptides with signal/noise ratio above 20 at the MS mode were selected for MS/MS run; a maximum of 45 MS/MS was allowed per spot. The precursor mass window was 200 relative resolution (fwhm). All spectra were searched against taxonomy, NCBI nr database, using GPS Explorer software version 3.6 (ABI) and the Mascot (MatrixScience) search engine. Variable modifications included oxidation (M). Mass tolerance was 80 ppm for precursor ions and 0.5 Da for fragment ions; two missed cleavages were allowed.

Immunoprecipitation and Western blot analysis. For immunoprecipitation, the collected transfected cells were lysed with cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5% cocktail protein inhibitor [Sigma, Inc.]) for 20 min. After centrifugation at $12,000 \times g$, the supernatants (or column fractions where indicated) were precleared with 30 µl protein G-agarose beads (Sigma) for 2 h and then incubated first with the appropriate antibodies overnight and then with 40 µl of protein G-agarose beads with gentle rotation for 2 h at 4°C. The beads were collected by centrifugation at $5,000 \times g$ for 1 min, and the pellets were washed four times with ice-cold RIPA buffer.

For immunoprecipitation of FLAG-tagged proteins, the cell lysates were incubated with 30 µl EZview anti-FLAG M2 affinity resin (Sigma, Inc.) for 4 h or overnight at 4°C. FLAG-tagged proteins were eluted by incubation with 50 µl of 150 µg/ml $3 \times$ FLAG peptide (catalog no. F 4799; Sigma, Inc.) in Tris-buffered saline (TBS; 10 mM Tris-HCl, 100 mM NaCl [pH 8.0]) for 1 h at 4°C. Samples were then added to $5 \times$ SDS protein sample buffer (300 mM Tris-HCl [pH 8.0]), 10% SDS, 25% β-mercaptoethanol, 0.1% bromophenol blue, 50% glycerol) and analyzed with 8 to 16% SDS-PAGE Tris-HCl gel, and transferred to Hybond P membranes (GE Life Sciences, Inc.). After blocking with 5% nonfat milk, the membrane was incubated with the corresponding primary antibody in TBS-Tween (TBST; 10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1% Tween 20) plus 5% nonfat dry milk and washed three times for 10 min with TBST. After incubation with a horseradish peroxidase-linked secondary antibody (1:3,000) (VWR, Inc.), an enhanced chemiluminescence system (Thermo Scientific, Inc.) was used for detection via X-ray film (Genesee, Inc., catalog no. 30-101) for visualization.

ChIP assay. For chromatin immunoprecipitation (ChIP), BC-3 cells (1×10^6) were cross-linked with formaldehyde at a final concentration of 1% for 10 min at 37°C. After being washed with phosphate-buffered saline (PBS) twice, cellular chromatin was sheared to approximately 500 bp using the Sonifier 450 sonicator (Branson output, 3.0; duty cycle, 30%) on ice four times for 30 s each. Immunoprecipitation, washing, and recovery of bound DNA were performed according to the manufacturer's protocol (Upstate Biotechnology, Inc.). The antibodies used were mouse monoclonal anti-LANA (LN53; ABI, Inc.) at 1 µg/reaction and anti-RPS6 (54D2; Cell Signaling, Inc.) at 1 µg/reaction. Mouse IgG at 1 µg/reaction was used as a control. Equal amounts of DNA pellets were used for PCR (Promega, Inc., catalog no. M7122). The forward primer for the LANA promoter (LANAp) was 5'-AGATCGCAGACACTGAAACGCTGA-3', and the reverse primer was 5'-GCAAAGCAGACACGCTTCTTCAGT-3'. β-Actin was used for a control with forward primer 5'-GGCATCGTGATGGACTCCG-3' and reverse primer 5'-GCTGGAAGGTGGACAGCGA-3'.

shRNA knockdown of RPS6. A set of pLKO.1 lentiviral vectors for RPS6 (NM_001010) were obtained from Open Biosystems/Thermo, Inc. The reconstructed lentiviruses were produced by the Lenti-shRNA Core Facility of University of North Carolina. BC-3 cells (5×10^5) were grown in six-well plates, infected with either of the respective lentiviruses (titer of 10^6 IU/ml) and Polybrene at a final concentration of 10 µg/ml, and incubated at 37°C for 6 h. After infection for 6 h, BC-3 cell medium was replaced with fresh RPMI 1640 supplemented with 10% fetal bovine serum. After 24 h, puromycin (5 µg/ml) was added to the medium, and cells were harvested 3 or 4 days after transfection. The numbers of viable cells were counted throughout the time course. Lentivirus short hairpin RNA (shRNA) against green fluorescent protein (GFP) or untreated BC-3 cells were used as a control.

Reporter assays. SLK cells (1×10^5 to 5×10^5) were seeded in 12-well plates at 37°C. After 24 h, the cells were transfected with reporter (pDD83) (62) and effector plasmids (pDD104 expressing LANA and pDD1902 expressing RPS6), using 7.5 µl Superfect reagent (Qiagen, Inc.) according to previously published methods (29). pcDNA3 (Invitrogen, Inc.) was used to adjust total DNA concentration, and the medium was changed 24 h after transfection. The cells were harvested 48 h posttransfection, washed twice with ice-cold PBS, and lysed in 200 µl of $1 \times$ reporter lysis buffer, and then the luciferase substrate was added (Promega, Inc.). Luciferase activity was quantified via a luminometer (Fluorstar Optima; BMG, Inc.). The background (pGL3 basic vector) activity was below 100 relative light units (RLU). The transfection efficiencies were normalized by cotransfecting a lacZ expression plasmid (pDD173) and analyzing β-galacto-

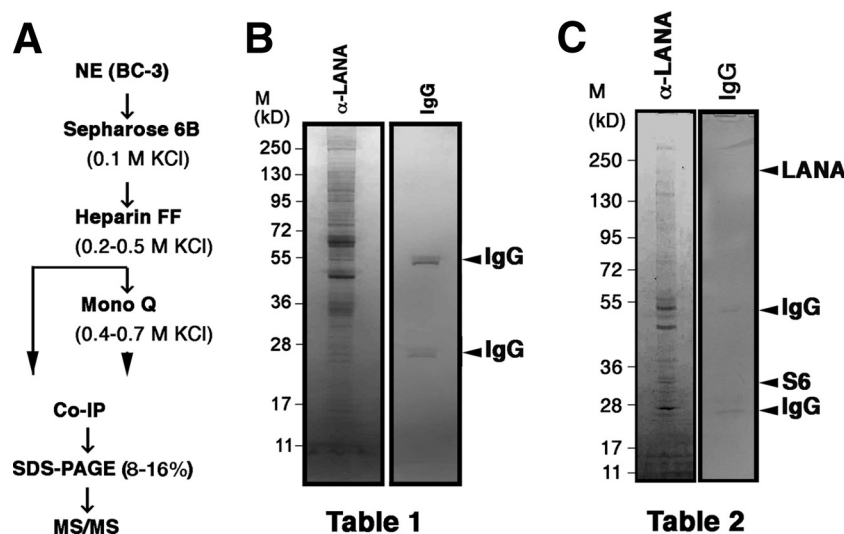


FIG. 1. Purification and identification of LANA binding proteins. (A) A schematic of the procedures used to purify LANA complexes. (B and C) SDS-PAGE of the 8 to 16% gradient gel stained with Coomassie blue. Samples were eluted following immunoprecipitation with rat anti-LANA (α -LANA) antibody from heparin fractions (B) or after additional ion-exchange (MonoQ) purification (C). The isolated protein bands of complexes are denoted with black arrows (LANA, S6, and IgG). Co-IP, coimmunoprecipitation; M, molecular mass markers.

sidase activity using the Galacto-Light plus β -galactosidase assay kit (Applied Biosystems, Inc.). The data series represents one of three transfections. We used robust regression to establish a linear dose response relationship (64).

RESULTS

Identification of nuclear LANA binding proteins under physiological conditions. During viral latency in KSHV-infected PEL cells, LANA is expected to interact with multiple cellular proteins. We hypothesized that there exist multiple distinct complexes of LANA and one or more associated cellular proteins, each for the different physiological functions of LANA. Because of their different enzymatic functions, these complexes are expected to have different biochemical properties. Since these complexes may have different biochemical properties, they are enriched by different biochemical purification procedures. Because LANA has a distinct domain structure with the two terminal globular domains separated by a large variable, and for the most part dispensable repeat region, prior studies (3, 31) used domain-based baits rather than the whole protein to identify interaction partners.

To identify the components of LANA complexes that bound to the whole LANA protein in latently infected cells, we first prepared nuclear extracts from BC-3 cells and then used multiple steps of chromatographic enrichment (Sepharose, heparin FF, and Mono-Q column) prior to immunoprecipitation with a LANA-specific monoclonal antibody. Figure 1A diagrams our purification strategy. This approach requires LANA-interacting proteins to be stably bound to LANA and the interaction to withstand high-salt- and low-protein-concentration conditions. Many transcription factors were initially identified by this approach (11), and we previously used it to discern different LANA-p53 complexes (9). Coimmunoprecipitation was then used to identify the proteins bound to LANA either after chromatography on just a size exclusion column and heparin column (Table 1) or after chromatography that included an additional ion-exchange column (Table 2). The

immunoprecipitates were ultimately analyzed by 8 to 16% gradient SDS-PAGE (Fig. 1B and C), and individual bands were subjected to tandem MS (MS/MS) analysis.

We identified 48 proteins that coimmunoprecipitated with LANA after heparin fractionation (Table 1). Twenty-two (46%) were previously found in MS/MS screens for mutant LANA-associated proteins. These included the previously identified histone H2A. Ten (21%) of the bands corresponded to ribosomal proteins, and the remainder corresponded to novel potential LANA interaction partners and IgG. We did not subject bands of the expected molecular weight of LANA to MS/MS for economic reasons. Twenty-one proteins remained bound to LANA after an additional MonoQ purification step (Table 2). We recovered LANA itself (131,268 kDa), as expected, as well as IgG(κ) light chain (8,496 kDa). We recovered multiple forms of myosin, actin, and tubulin. Although it seems unlikely that these are bound to LANA, another KSHV protein, LANA-2, has been shown to interact with tubulin and to thereby affect paclitaxel resistance of PEL (51).

We recovered heat shock protein 96 (Hsp96) precursor (90,138 kDa) and Hsp70 (70,854 kDa) from LANA coimmunoprecipitates, as well as the signal recognition particle SRP72 (74,560 kDa). The significance of these results is under investigation, although it seems plausible that LANA binds to Hsps analogous to the Epstein-Barr virus (EBV) EBNA5/EBNA-LP (16, 23, 32, 46) and EBV EBNA3A (81) proteins binding Hsps.

We recovered multiple ribosomal proteins that coimmunoprecipitated with LANA. This mirrors earlier results by Kaul et al. (31). However, we did not recover exactly the same ribosomal proteins. Our collection of copurified ribosomal proteins is characterized by proteins with predominantly basic isoelectric points (pIs). With the exception of P0, the other 10 ribosomal proteins have predicted pIs of ≥ 9.7 . In contrast, full-length LANA has a predicted pI of 3.9. Hence, it seems unlikely that the ribosomal proteins would copurify over

TABLE 1. LANA binding proteins with anti-LANA immunoprecipitation after purification by heparin column chromatography^a

Lane	Database identification no.	Protein name	Size (no. of aa)	Mol mass (Da)	No. of peptides (hits)	MS/MS score	Source or reference ^b
1	AAP69525	Protein kinase, DNA activated	4,128	468,787.9	49	195	34
2	BAD52438	Nonerythrocytic spectrin α	2,452	282,108	44	155	
3	Q53R99	Hypothetical protein SPTBN1	2,314	268,549	48	494	
4	MYH9_HUMAN	Myosin-9 (myosin heavy chain, IIa)	1,960	226,260.5	58	732	34
5	Q9HBD4_HUMAN	SMARCA4 isoform 2, BRG1	1,679	188,030.8	28	242	74
6	A54854	Ras GTPase-activating protein-related protein	NA ^c	189,133.8	31	183	
7	Q5T5X0	Eukaryotic translation initiation factor 3	1,382	166,468.3	42	120	
8	AAB41498	Alpha II spectrin β	2,477	284,890.5	55	556	34
9	AAB48855	RNA helicase A	1,279	141,979.7	20	158	74
10	Q75M86	Hypothetical protein GTF2I	978	110,211	35	527	
11	Q59FF0	EBNA-2 coactivator variant	964	107,366.3	32	564	
12	ILF3_HUMAN	Interleukin enhancer binding	894	95,279.1	20	370	27
13	A46302	PTB-associated splicing factor	NA	76,101.6	26	441	
14	AAH27713	Heterogeneous nuclear ribonucleoprotein	804	90,235.8	23	159	
	Q86VG2	Splicing factor	707	76,140.7	15	116	27
	Q2VPJ6	Hsp90AA1 protein	585	68,328.8	13	75	
15	AAB48826	BTk-associated protein 135	957	107,812	25	204	
16	CAH10627	HSM803020 NID— <i>Homo sapiens</i>	652	72,496.1	17	151	
17	Q59GX6	DEAD/H (Asp-Glu-Ala-Asp/His) box	674	74,532.7	20	180	74
18	JC1087	RNA helicase, ATP-dependent	NA	69,104.7	19	249	34, 74
19	A25707	U1 snRNP 70K protein	NA	70,039.5	17	121	
20	Q8TBR3	Fusion [involved in t(12;16) in malignant liposarcoma]	526	53,367.8	12	175	74
21	Q59H57	Fusion (involved in t(12;16) in malignant liposarcoma]	300	31,974.3	14	294	74
22	Q8TBR3	Fusion [involved in t(12;16) in malignant liposarcoma]	526	53,367.8	11	155	74
23	AAQ05673	Ig heavy chain variable region, VH3	120	13,308.5	4	77	IgG
24	AAA35750	DNA-binding protein B	364	39,953.8	16	223	
	Q53HR5	Eukaryotic translation EF1 α	426	50,093.1	12	90	27
25	AAH12854	ACTB protein	360	40,194.1	24	899	
26	Q53FG3	Interleukin enhancer binding	390	43,021.2	14	255	
27	CAF06488	AX961958 NID— <i>Homo sapiens</i>	285	30,569.1	12	214	
	AAH04383	Polypyrimidine tract binding protein	531	57,185.6	5	118	
28	Q53RW7	Hypothetical protein HNRPA3	378	39,570.6	14	244	74
29	B34504	Heteronuclear ribonucleoprotein B1	NA	37,406.7	22	603	81
30	Q2HJ60	Heteronuclear ribonucleoproteins A2	341	35,983.9	21	766	74
31	B34504	Heteronuclear ribonucleoprotein A2/B1	NA	37,406.7	18	595	74
32	Q3MIB7	HNRPA1 protein	267	29,368.2	22	781	74
34	AAH12197	Ribosomal protein L8	257	27,993.3	14	69	ribo
35	RL7A_HUMAN	60S ribosomal protein L7a	266	29,846	17	200	ribo
36	Q3KQU0	RPL7 protein	318	36,998.8	24	213	ribo
	Q53HV1	Ribosomal protein S4	263	29,560	12	134	ribo
37	I38049	MUC18 precursor	NA	71,748.7	14	84	
	Q5JR95	Ribosomal protein S8	188	21,866	9	80	ribo
38	CAE93910	AX887985 NID— <i>Homo sapiens</i>	209	25,112.9	16	193	
39	RL10_HUMAN	60S ribosomal protein L10	214	24,429.8	19	261	ribo
	RL13A_HUMAN	60S ribosomal protein L13a	203	23,431.3	15	124	ribo
40	RL18_HUMAN	60S ribosomal protein L18	188	21,490	12	115	ribo
41	RS9_HUMAN	40S ribosomal protein S9b	194	22,446.5	13	177	34
	S55916	Ribosomal protein S5	NA	22,762.9	8	125	ribo
42	MLRM_HUMAN	Myosin regulatory light chain 2	171	19,650.4	7	214	
	AAB94895	60S ribosomal protein L12	129	14,332.8	5	100	ribo
43	MLRM_HUMAN	Myosin regulatory light chain 2	171	19,650.4	7	272	
44	RS13_BOVIN	40S ribosomal protein S13	151	17,080.6	8	150	ribo
	Q9BQ05	Ribosomal protein L27	106	12,007.4	5	109	ribo
	R3RT18	Ribosomal protein S18	NA	17,707.9	13	108	34
45	MOHU6 M	Myosin alkali light chain 6	NA	16,919.1	8	193	
46	H2A1A_HUMAN	Histone H2A type 1-A (H2A/r)	131	14,093.9	5	99	4, 34
47	R3HU12	Ribosomal protein S12, cytosolic	NA	14,516.5	2	86	ribo
48	S55054	Sm protein G—human	NA	8,490.4	5	75	

^a In each case, the highest-scoring hit is shown.^b Previously published in the reference(s) indicated. IgG refers to human IgG, and ribo refers to ribosomal components.^c NA, not available.

TABLE 2. LANA binding proteins with anti-LANA precipitation after purification by heparin and anion-exchange column chromatography

Lane	Database identification no.	Protein name	Size (no. of aa)	Mol mass (Da)	No. of peptides (hits)	MS/MS score	pI
1	Q60FE2	Myosin	1,960	226,391.6	40	488	5.5
2	Q9QR71_HHV8	LANA	1,129	131,267.9	12	264	3.9
3	AAA59888	Myosin	1,337	154,265.7	30	492	5.7
4	AAK74072	Hsp96 precursor	782	90,138.1	18	208	4.73
5	AAC97490	SRP72	671	74,560	12	203	9.31
6	A27077	Hsp70	646	70,854.2	24	662	5.62
7	AAH04949	Tubulin	449	49,863.5	13	464	4.96
8	AAH20946	Tubulin	444	49,639.9	17	715	4.75
9	AAH20946	Tubulin	444	49,639.9	14	208	4.75
10	RL4_HUMAN	Ribosomal L4 (L1)	427	47,536.4	15	91	11.07
11	Q96HG5	Actin	368	40,978.4	22	1,100	5.56
12	AAH01127	Ribosomal P0	317	34,252.7	17	378	5.42
13	Q6NXR8	Ribosomal S3A	264	29,955.8	13	183	9.75
14	AAH09427	Ribosomal S6	247	28,403.8	8	128	10.84
15	RL7A_HUMAN	Ribosomal L7a	266	29,846	12	129	10.61
16	Q53HV1	Ribosomal S4	263	29,560	13	159	10.11
17	S23753	Ribosomal L13	211	24,276.5	8	129	11.69
18	CAC94454	IgG(κ)	78	84,96.1	2	168	5.55
19	RL10_HUMAN	Ribosomal L10	214	24,429.8	13	381	10.11
20	RS13_BOVIN	Ribosomal S13	151	17,080.6	10	358	10.53
21	R3HU16	Ribosomal S16	146	16,435	8	192	10.42

charge-based separation columns (heparin and MonoQ) if they were not tightly bound to the LANA protein.

It may at first seem counterintuitive that ribosomal subunit proteins would bind to a nuclear transcription factor, such as LANA. However, ribosomal proteins are not exclusively cytoplasmic: some such as RPS6 shuttle in and out of the nucleus and accumulate in nuclear and nucleolar bodies (76). We focused our initial efforts on validating the interaction between LANA and RPS6 because of its known nuclear localization, because of the availability of highly specific reagents, and because the distribution of MS/MS peptide coverage made it unlikely that RPS6 was incorrectly identified (Fig. 2).

LANA interacts with RPS6. To validate the LANA-RPS6 interaction, we used nuclear extract of BC-3 cells and conducted the reciprocal immunoprecipitation using an anti-RPS6 antibody followed by Western blotting with anti-LANA antibody. LANA was associated with RPS6 in the nuclear extract prior to any purification steps (Fig. 2A, lanes 1 and 2).

Since ribosomal proteins like RPS6 are associated with rRNA (19), and since LANA may have nonspecific, as well as sequence-specific nucleic acid-binding capabilities, we examined the hypothesis that the LANA-RPS6 interaction was dependent on copurifying nucleic acids. We used RNase A and DNase I to digest nucleic acids in the partially purified LANA-positive heparin fractions prior to immunoprecipitation with anti-RPS6 antibody. Despite nuclease digestion, LANA still interacted with RPS6 in this assay (Fig. 2A, lanes 3 to 6).

To further investigate the specificity of the LANA-RPS6 interaction, we conducted immunoprecipitation with anti-RPL11 antibody (i.e., a random ribosomal protein that was not found in our MS/MS profiling) (Fig. 2B). We did not observe any interaction between LANA and RPL11, which supports the notion that the LANA-RPS6 interaction was specific and not the result of whole ribosomes binding to LANA multimers or the nonspecific anti-LANA antibody.

Next, we used the FLAG-tagged fusion protein of LANA to

detect the interaction with a HA-tagged protein of RPS6 in cotransfected cells. This experiment confirms specificity and also demonstrates that the LANA-RPS6 interaction is independent of other KSHV proteins. First we generated a full-length HA-tagged version of RPS6 and cotransfected it with a FLAG-tagged version of full-length LANA. Using a FLAG-tagged LANA, rather than anti-LANA antibody, avoids the potential for nonspecific activity of the anti-LANA monoclonal antibody LN53. Using tag-specific antibodies for immunoprecipitation and Western blotting, we found that LANA coimmunoprecipitated with RPS6 and that RPS6 coimmunoprecipitated with LANA after both proteins were transiently transfected into HeLa cells (Fig. 3A). This demonstrates that LANA and RPS6 interact in the absence of other KSHV viral proteins.

To determine which domain of LANA interacts with RPS6, a series of LANA mutants were constructed with a FLAG tag fused to the N terminus of LANA. We also constructed RPS6 with an HA tag fused to the N terminus of RPS6 (Fig. 3B). Cotransfection of HA-tagged RPS6 and different FLAG-tagged LANA mutants into HeLa cells indicated that the N-terminal portion of LANA (aa 1 to 329), excluding the DE repeats, represents the shortest fragment that could still interact with RPS6 (Fig. 3B). Neither the C terminus nor the repeat-rich central domain of LANA interacted with RPS6.

In sum, we have validated the LANA-RPS6 interaction by multiple independent means: (i) immunoprecipitation with anti-LANA monoclonal antibody coprecipitates RPS6 at physiological levels in BC-3 cells, (ii) immunoprecipitation with anti-RPS6 antibody coprecipitates LANA at physiological levels in BC-3 cells, (iii) cotransfection and coimmunoprecipitation with different artificial epitopes (tags) show a complex of both proteins in the absence of other viral proteins, and (iv) the LANA N terminus (but not the central region or C terminus) is sufficient to bind to RPS6.

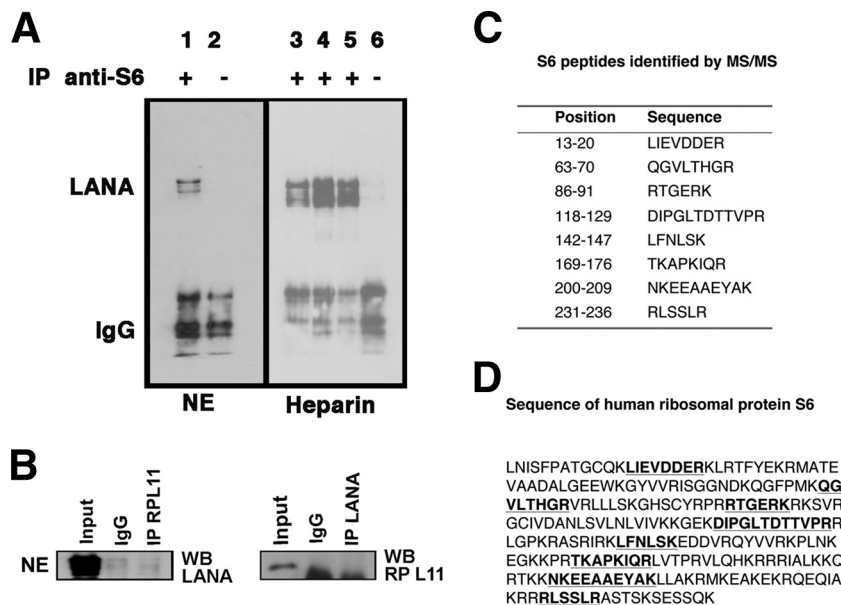


FIG. 2. Analysis of the interaction between LANA and RPS6 in BC-3 cells. (A) Lanes 1 and 2, nuclear extracts (NE) of BC-3 cells; lanes 3 to 6, samples purified by heparin FF column. Treatments for each lane are as follows: lane 1, 37°C for 30 min and treatment with 500 U/ml DNase I and 100 µg/ml RNase A; lane 2, 37°C for 30 min without nuclease treatment; lane 3, 25°C for 20 min without nuclease treatment; lane 4, 35°C for 20 min with DNase I and RNase; lane 5, 37°C for 20 min with 5 U/µl DNase I and 10 U/µl RNase; lane 6, 4°C for 30 min without nuclease treatment. All of the above buffers contain no EDTA. For the immunoprecipitation (IP) assay, lanes 1, 3, 4, and 5 were supplemented with anti-S6 antibodies and lanes 2 and 6 were used as negative controls (IgG only). (B) Immunoprecipitation and Western blot of an irrelevant ribosomal protein L11 from nuclear extract of BC-3 cells. Shown is input and immunoprecipitation with a control antibody (IgG), with anti-LANA mouse monoclonal antibody (IP LANA), or anti-RPL11 antibody (IP RPL11) followed by Western blotting with anti-LANA antibody (left) or anti-RPL11 antibody (right). (C) RPS6 protein and the sequences of its corresponding peptides identified by mass spectrometry analysis, shown in a table of peptides. (D) Alignment of peptides (underlined) with the S6 sequence (GenBank accession no. NP_001001; gene identification [ID] no. 6194 RPS6).

RPS6 associated with LANA on LANAp. We had shown earlier that LANA could bind to a LANA-responsive DNA element within its promoter and thereby autoactivate the LANA promoter (LANAp) (30). We had found that the binding interaction between the LANA protein and this single DNA element is considerably weaker than the interaction between LANA and the multimerized, canonical LANA binding sites in the viral terminal repeats (TRs). Thus, chromatin immunoprecipitation analysis (ChIP) analysis of the LANA promoter represents a more stringent assay for the purpose of demonstrating a trimeric LANA-RBS6-DNA complex than the TR. The LANA promoter also provides for a better PCR target for ChIP, whereas we were unable to PCR across the LANA binding sites within the GC-rich terminal repeat regions (data not shown). Using ChIP, we asked whether RPS6 was present in the LANA-LANA promoter complex *in vivo*. After cross-linking with 1% formaldehyde, the sonicated samples were immunoprecipitated with anti-LANA or anti-RPS6 monoclonal antibodies, respectively, and we PCR amplified the coprecipitated DNA (Fig. 4A, upper panel). We could amplify a band of the expected molecular weight for the specific fragment of the LANA promoter from immune precipitates using anti-LANA as well as anti-RPS6 antibodies, but not the IgG-negative control. We could not PCR amplify any product using primers specific for β-actin, which served as a negative control for nonspecific DNA binding (Fig. 5A, lower panel). We conclude, by another independent assay, that RPS6 is in a complex with KSHV LANA and that at least some of the RPS6-LANA complexes are present at the LANA promoter.

By no means do we want to imply that RPS6 is a transcription factor. Rather, we use the phenotype of LANA to bind its cognate DNA as a biochemical assay to verify the RPS6-LANA interaction. As an alternative readout for the LANAp-LANA-RPS6 interaction, we modified our established transient transfection assay, using a LANA promoter-luciferase construct as the reporter. This represents a variant of a mammalian two-hybrid assay using the LANA DNA binding domain and cognate *cis* element. Instead of fusing RPS6 to a strong artificial transactivation domain, we used a statistical measure (dose dependence under limiting conditions) to validate the LANA-RPS6 interaction. The LANA promoter is constitutively active. Cells were cotransfected with the reporter and increasing amounts of RPS6. We used empty vector to adjust for the total DNA concentration. The background (pGL3 vector) activity was below 100 RLU. In the absence of LANA, RPS6 did not affect promoter activity (Fig. 4B, left panel). This would be expected since RPS6 is not a transcription factor. Transient transfection of LANA itself transactivates this LANA promoter report construct, albeit weakly, and we used a suboptimal amount of LANA in order to measure the effect of RPS6. In the presence of cotransfected LANA, RPS6 increased promoter activity (Fig. 4B, right panel). We observed a linear dose-response curve. Albeit not dramatic, the slope was significant at $P \leq 0.027$ using robust linear regression (64). We interpret the linear dose dependence rather than the overall fold change as evidence for the interaction between LANA and RPS6. Clearly RPS6 is not a transcription factor. If we increase the amount of transfected LANA, the RPS6 becomes negligi-

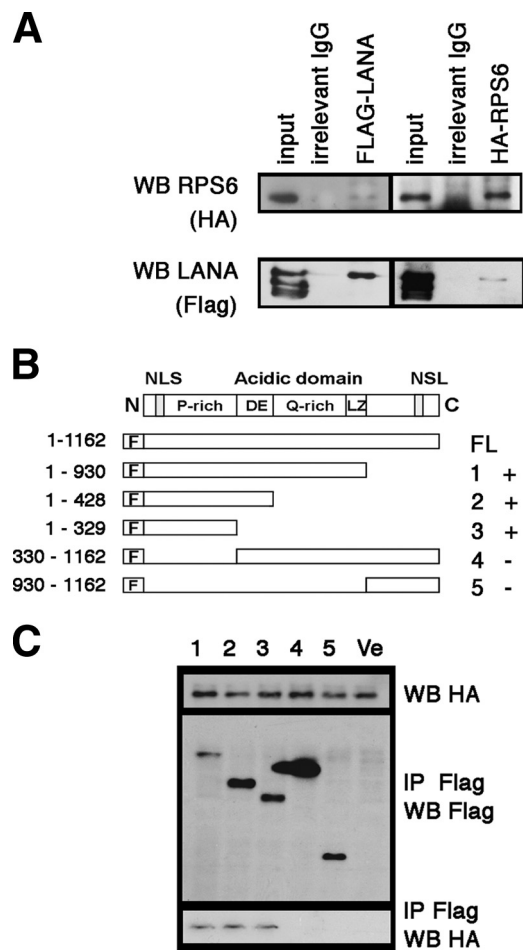


FIG. 3. Analysis of the interaction of LANA and RPS6 in HeLa cells after transfection. (A) Coimmunoprecipitation of full-length FLAG-tagged LANA and HA-tagged S6 after cotransfection into HeLa cells. After cotransfection, protein extract was immunoprecipitated with the indicated reagents. The lanes represent input, IgG only, anti-FLAG, and anti-HA (HA). Anti-FLAG antibodies and anti-HA antibodies were used for Western blot (WB) analysis, as indicated on the left. (B) Schematic diagram of mutant LANA proteins used. All segments were fused with FLAG tag at the N terminus of LANA. The numbers on the right indicate the lane numbers in panel C and the “+” or “-” symbols summarize whether or not an interaction was detected. (C) Coimmunoprecipitation assay of HA-RPS6 and different mutants of FLAG-tagged LANA. Anti-FLAG and anti-HA antibodies were used for immunoprecipitation (IP) or Western blotting (WB) as indicated. Ve, vector.

ble since LANA protein is no longer rate limiting within the cell.

One possible outcome was that RPS6 would alter overall LANA protein levels. To test this hypothesis, the experiment was repeated and LANA and RPS6 levels were determined by Western blot (Fig. 4C). We observed the expected dose-dependent increase of RPS6 protein levels with increased amounts of RPS6 expression vector. In contrast, the level of LANA did not change appreciably. Using LANA’s DNA binding ability as a novel, independent assay, Fig. 5 verifies that LANA binds to RPS6.

Reduction of RPS6 protein reduces LANA protein expression. Since RPS6 was in a complex with LANA, we hypothe-

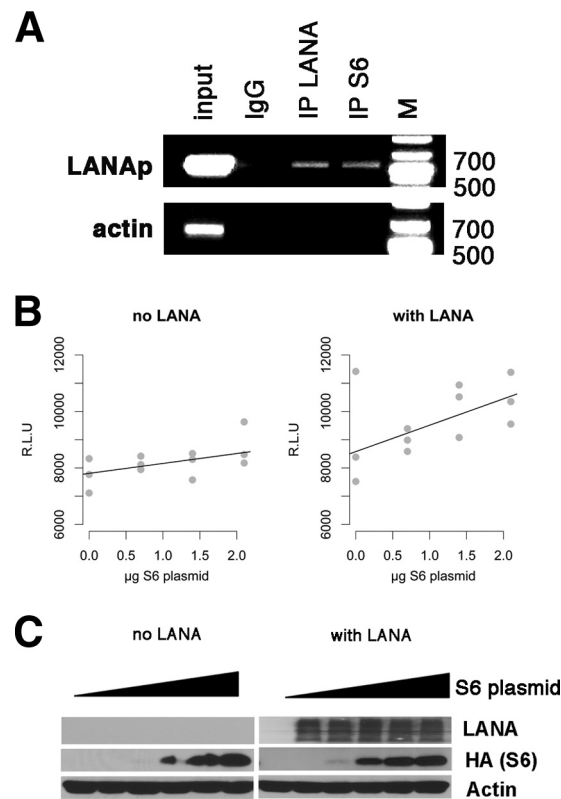


FIG. 4. Complex of LANA and S6 binds to LANA promoter. (A) BC-3 cells were cross-linked and sonicated, and chromatin immunoprecipitation (IP) was performed with mouse anti-LANA (LN53) and mouse anti-RPS6 (54D2) monoclonal antibodies. IgG was used as a control. Inputs were 5% of lysates of each sample. The DNA fragments immunoprecipitated were amplified with the respective primers for the LANA promoter (LANAp) and β -actin. (B) Cotransfection of LANA and RPS6 into SLK cells increases the activity of a LANA promoter-luciferase reporter. Relative light units (RLU) are shown on the y axis, and the amount of S6 plasmid per transfection mixture is shown on the horizontal axis. All measurements were conducted in triplicate, and each is shown. The background (pGL3 vector) activity was below 100 RLU. Also shown are the regression lines (black), which demonstrate a linear dose dependence of LANA promoter activity on RPS6. (C) Western blot analysis of SLK cells cotransfected with increasing amounts of HA-tagged RPS6 expression vector in the absence or presence of LANA. A Western blot for actin is shown as a loading control.

sized that RPS6 positively supports the translation of LANA (and perhaps contributes to the extraordinary stability of LANA). To test this hypothesis under physiological conditions, BC-3 cells were infected with a series of recombinant shRNA lentiviruses that target the RPS6 mRNA. One vector (Sh-4) resulted in marked reduction of RPS6 levels at day 4 after infection (Fig. 5A) and was selected for further study. Transduction of BC-3 cells with this validated shRNA lentiviral vector against RPS6, but not an irrelevant (GFP) shRNA lentiviral vector led to a reduction of LANA protein steady-state levels as early as day 3 post-shRNA lentiviral vector transduction (Fig. 5B). Levels of an unrelated, equally stable protein (β -actin) were not affected (Fig. 5B). Transduction of BC-3 cells with this validated shRNA lentiviral vector against RPS6, but not an irrelevant (GFP) shRNA lentiviral vector, induced

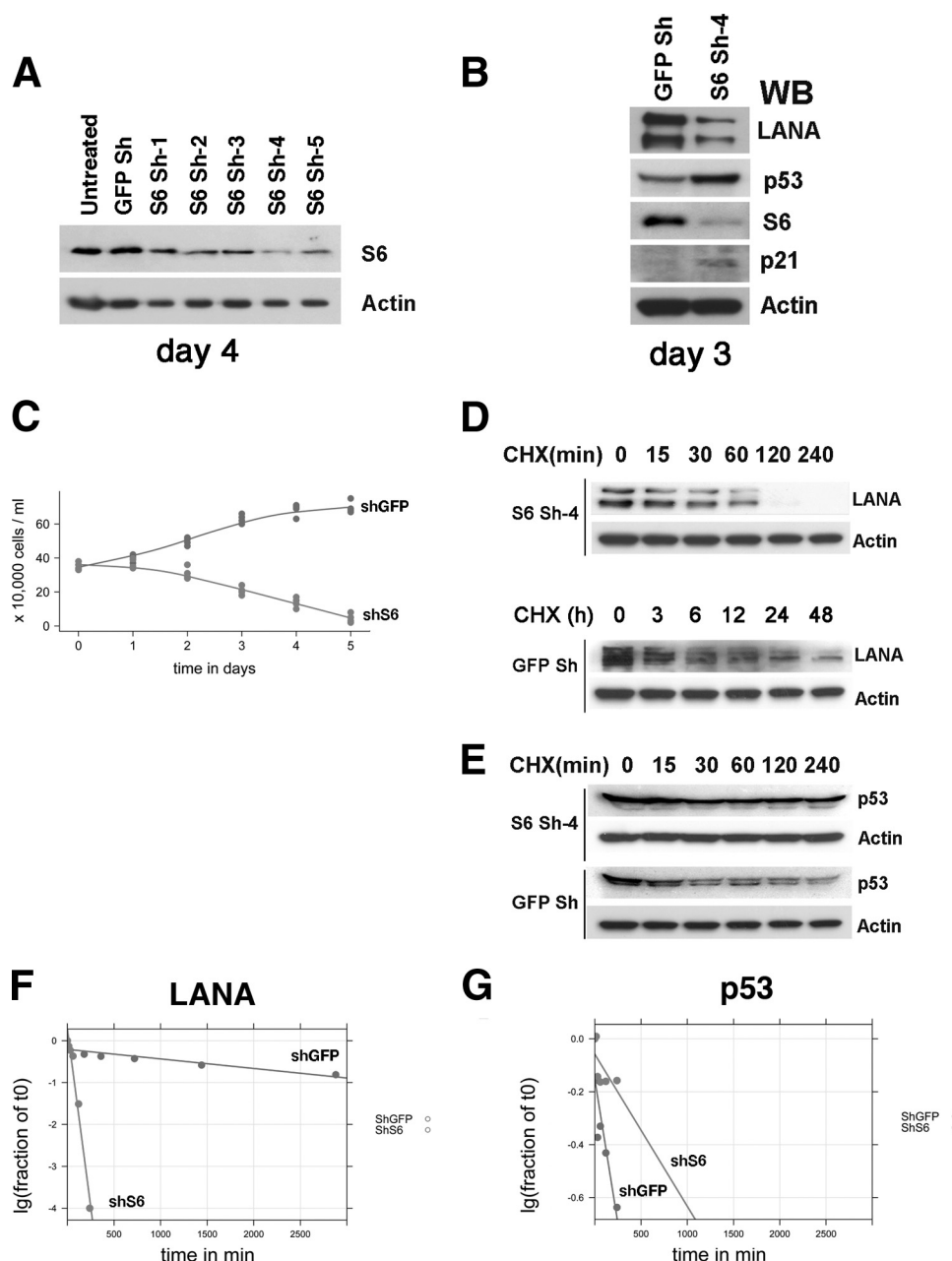


FIG. 5. Analysis after knockout of RPS6. (A) BC-3 cells were transduced with five recombinant lentiviruses (S6 Sh-1 to Sh-5) encoding different shRNAs directed against RPS6 in six-well plates. shRNAs against GFP-encoding lentivirus and untreated BC-3 cells were used as controls. Western blot analysis was performed 4 days posttransduction using anti-RPS6 (S6) and anti- β -actin (Actin) antibodies. (B) Western blots were performed 3 days after knockdown of RPS6 with shRNA no. 4, probing with anti-LANA, anti-p53, anti-p21, and anti- β -actin antibodies, respectively, at 3 days posttransduction. (C) Proliferation of BC-3 cells was measured at different time points following shRNA-mediated RPS6, as described above. The number of cells is shown on the y axis, and the time after transduction is shown on the x axis. The cells were not refed in between time points. (D) Protein stability analysis of LANA. BC-3 cells were transduced with anti-RPS6 (S6 Sh-4) shRNA vector or control shRNA vector (GFP Sh) and exposed to cycloheximide (CHX) starting at day 3 posttransduction, and LANA and actin protein levels were determined by Western blot analysis at the indicated times (in hours). (E) Protein stability analysis of p53. BC-3 cells were transduced with anti-RPS6 (S6 Sh-4) shRNA vector or control shRNA vector (GFP Sh) and exposed to cycloheximide and LANA, and actin protein levels were determined by Western blot analysis at the indicated times (in minutes). (F) Protein stability determination for LANA and p53 (G). The protein bands in panels D and E were scanned, and band intensities were calculated. The \log_{10} band intensity of the fraction at time zero is shown on the y axis, and time is shown on the x axis. Lines represent the robust fit of the data.

levels of p21 and p53 (Fig. 5B). We used p53 as a positive control to investigate the hypothesis that ablation of RPS6 does not abrogate protein translation in general but results in a specific cellular signaling event. Disturbing ribosome assem-

bly by ablating RPS6 expression is known to activate p53 (22), which in turn induces transcription and translation of p21. This piece of data is consistent with p53 activation, not nonspecific translation inhibition.

Inhibition of RPS6 expression by shRNA inhibited cell proliferation compared to control shRNA lentiviral transduction, as shown in Fig. 5C. BC-3 cells were transduced on day 0 and then observed over time without refeeding. These experiments were conducted at high cell density so as to maximize the viability of the cells after shRNA transduction. In this experiment, we did add fresh medium after transduction, which would activate the mTOR pathway and may have changed the steady-state levels and function of RPS6. At day 1, there was no statistical difference between RPS6-specific shRNA-treated and control shRNA-treated cells. On subsequent days, RPS6-specific shRNA-infected cells exhibited cell death, whereas control shRNA-treated cells proliferated and remained viable. In sum, we have shown that RPS6 contributes to LANA protein levels, but not to actin protein levels, and that this results in the induction of p53 protein levels. Hence, it is unlikely that our knockdown of RPS6 resulted in widespread, indiscriminate translational arrest of protein synthesis. Rather, the impact of reduced RPS6 levels is most significant upon proteins that are associated with or deeply dependent upon the presence of RPS6.

Next, we tested the hypothesis that RPS6 affects the half-life of LANA. BC-3 cells were transduced with shRNA to RPS6 (S6 Sh-4) or an irrelevant shRNA lentiviral vector (shGFP) and, starting at day 3 postransduction, treated with cycloheximide to block novel protein synthesis. After the indicated times, we probed for LANA levels using Western blotting; actin was used as a control. In BC-3 cells transduced with the RPS6 shRNA vector, LANA showed a substantially decreased half-life (0.60 h) compared to BC-3 cells treated with control shRNA (28 h) (Fig. 5D and G). This complex requires the N terminus, since the LANA C terminus alone has a short half-life—on the order of hours (data not shown). This result is consistent with a model in which RPS6 is part of a complex that is responsible for the well-established, unusually long half-life (on the order of days) of the LANA protein. At the same time p53 was stabilized (Fig. 5E and G). In BC-3 cells transduced with the RPS6 shRNA vector, p53 exhibited a slightly increased half-life (8.8 h) compared to BC-3 cells treated with control shRNA (2.3 h). This is consistent with the activation of p53 and increased steady-state levels and the induction of p21 (Fig. 5B). In sum, these experiments demonstrate that RPS6 contributes to the stability and overall level of LANA in PEL cells.

DISCUSSION

LANA is a multifunctional protein, as is RPS6. Here, we find that both proteins exist in a complex together in KSHV latently infected PEL cells. Both LANA and RPS6 bind to each other in cotransfection experiments in the absence of other viral proteins. Our data reveal that the LANA N-terminal domain mediates this interaction and that this interaction is resistant to nuclease digestion and therefore independent of rRNA. Furthermore, we found that RPS6 is present in RPS6-LANA-DNA complexes on the LANA promoter and that shRNA-mediated knockdown of RPS6 is associated with a reduction in LANA protein levels and in LANA protein half-life. This implicates RPS6 in the translation and/or stability of KSHV LANA.

LANA is an extraordinarily stable protein with an estimated

half-life of several days (39). The biochemical mechanism for its stability is unknown. Therefore, it is conceivable that RPS6 can contribute to LANA stability. RPS6 is a very dynamic protein. It is synthesized in the cytoplasm and then imported back into the nucleolus, where it assembles into the pre-40S ribosomal subunit. These complexes are then extruded into the nucleus proper and then into the cytoplasm. The nuclear import of RPS6 is mediated by three NLSs, and the nuclear export of RPS6 is dependent on a nuclear export signal (NES) and CRM1 (38, 44, 49, 68). CRM1-dependent nuclear export can be specifically inhibited by the pharmacological compound leptomycin B (LMB). LMB interacts with CRM1 and blocks leucine-rich, NES-mediated protein export (15, 37, 52). Similarly to normal cells, export of human RPS6 was controlled by the CRM1-mediated export pathway in PEL cells and in the KSHV-infected TIVE L1 endothelial cells (data not shown). This verifies that RPS6 is localized to the nucleus as part of its normal maturation pathway. RPS6 is phosphorylated by p70S6 kinase, which in turn is mTOR dependent. However, RPS6 can also be phosphorylated by other kinases such as p90S6 kinase.

Surprisingly, RPS6 phosphorylation does not affect overall translation efficiency or the rate of RPS6 incorporation into polyribosomes (66). Chemical inhibition of RPS6 phosphorylation (e.g., by mTOR inhibitors) does not result in widespread, indiscriminate translational arrest or cell death. Whether RPS6 phosphorylation plays a role in ribosome selectivity for specific mRNAs (27) is subject to further study.

We predict that the significance of the LANA-RPS6 and LANA-ribosomal protein interactions will reveal itself in due time, analogous to the Hdm2-ribosome interactions. The first demonstration that Hdm2 bound a ribosomal protein was published in 1994 (47). Subsequently, it was shown that Hdm2 interacts with L11 and/or L23 (10, 82), establishing a tight linkage between Hdm2, p53, and ribosome biogenesis. Recently, it has become well established that Hdm2 and p53 are sensors for ribosomal stress and ribosomal function (22). As a fraction of LANA is associated with both p53 and Hdm2 (9, 67), this provides a mechanism for KSHV to manipulate cellular ribosomal biogenesis and the stress signals that may originate from it for the purpose of latent viral persistence.

What could be the function of the LANA-RPS6 interaction, and what is its significance? We noticed that phosphorylated RPS6 in the nucleus of KSHV-infected cells was shielded from the effect of rapamycin in L1 TIVE cells (unpublished observation). Rapamycin treatment effectively inhibits phosphorylation of RPS6 by p70S6K1 as well as p70S6K1 (42, 55, 69); however, phosphorylation by p90S6K is not inhibited. Furthermore, p70S6K2 is cytoplasmic as well as nuclear and can even be centrosome associated in mammalian cells (63). It is thus conceivable that LANA may promote the phosphorylation of RPS6 by centromeric p70S6K2, which in turn increases the nuclear functionality of RPS6, perhaps during mitosis. It is during mitosis, when the nuclear envelope breaks down, that LANA decorates host chromosomes and that its function is most crucial for viral episome segregation. The discovery of the mTOR/S6K/S6 signaling arm taught us that the ribosomal proteins are not mere building blocks of ribosomes, but may have multiple functions in themselves and may be independent of steady-state protein translation.

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