Host and Viral Proteins in the Virion of Kaposi's Sarcoma-Associated Herpesvirus

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Received 15 October 2004/Accepted 1 December 2004

Infection of cultured cells with Kaposi's sarcoma associated herpesvirus (KSHV) typically establishes a latent infection, in which only a few viral genes are expressed. Recently, it has been reported that a subset of lytic genes are transiently expressed very early after viral entry but that this burst of abortive lytic gene expression is terminated with the supervention of latency (H. H. Krishnan, P. P. Naranatt, M. S. Smith, L. Zeng, C. Bloomer, and B. Chandran, *J. Virol.* 78:3601–3620, 2004). To identify molecules imported into cells by KSHV that might influence this gene expression program, we have examined the protein composition of the KSHV particle. Immunoblotting of virus particles demonstrated that RTA, the lytic switch protein, and RAP, a viral protein that is a transcriptional and cell cycle modulator, were both incorporated into virus particles. In a second approach, polypeptides isolated from purified virions were identified by mass-spectrometric analysis of their constituent tryptic peptides. With this approach we were able to identify 18 major virion proteins, including structural, regulatory, and signaling proteins of both viral and cellular origin.

Herpesviruses comprise a family of large DNA viruses that share several structural characteristics. The herpesvirus virion is composed of three distinct parts: an icosahedral capsid containing the double-stranded DNA viral genome, a lipid envelope studded with virally encoded glycoproteins, and an amorphous layer of protein termed the tegument, which resides between the capsid and the envelope. The tegument is composed of both cellular and viral proteins that are encapsidated during viral egress. Upon virus entry into a cell, the tegument proteins are released and are able to affect the cell, preparing it for viral replication. Tegument proteins are thought to play important roles in the virus life cycle and exhibit diverse functions, including cell cycle modulation (30, 43), transcriptional activation of viral genes (14, 32, 42, 73), shutoff of host gene expression (57) and translocation of the viral capsid to the nucleus (59, 74).

Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8) is associated with the endothelial neoplasm Kaposi's sarcoma (KS) as well as with two B-cell lymphoproliferative disorders, primary effusion lymphoma and multicentric Castleman's disease (8–10). Fragments of the KSHV genome were first identified in 1994, and subsequent sequencing of the entire viral genome placed it into the gammaherpesvirus subfamily (10, 56). Like Epstein-Barr virus, the prototype human gammaherpesvirus, KSHV establishes a latent infection upon entry into cultured cells; only a few viral genes are expressed, and no viral progeny are produced (1–3, 12, 21, 35, 50, 65, 68). Latently infected cells display a low level of spontaneous lytic replication, characterized by a temporally

regulated cascade of viral gene expression, replication of the viral DNA, and the release of virus particles. Lytic replication can be induced in latently infected cells by the addition of phorbol esters or butyrate or by overexpression of the viral switch protein, RTA (3, 7, 8, 22, 25, 31, 45, 47, 48, 63, 67). The physiological triggers controlling RTA expression (and thus, the shift between lytic and latent infection) are unknown, however (44). Although de novo infection typically results in latency by 24 h postinfection, a recent study by Chandran and colleagues has shown that immediately after infection there is a burst of viral gene expression that includes several markers traditionally expressed only during lytic replication (34). The full lytic program is not induced, and viral DNA replication is not triggered. Instead, this initial burst of lytic gene expression is followed by a rapid decline, giving way to a more stable state in which mostly latent transcripts are detected (34). The lytic transcripts identified included transcriptional regulators, immunomodulatory and antiapoptotic molecules, and it has been proposed that these may play important roles in establishing KSHV infection (34). How this burst of aberrant lytic expression comes about is unknown, as is the mechanism by which it is extinguished.

One possibility is that virion proteins imported into the cell during infection may influence viral gene expression immediately upon entry. Little is known of KSHV virion proteins, aside from the identities of the four predominant capsid proteins; in particular, very little is known about the KSHV tegument, where many important regulatory activities are likely to reside (52). Zhu and Yuan demonstrated that ORF45, a viral inhibitor of type I interferon induction via IRF7, was present in KSHV virions (most likely in the tegument), but there has been no systematic assessment of the components of the virus particle (75). Accordingly, we employed gel fractionation, mass spectrometry, and immunoblotting to identify components of

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purified KSHV virions. Here we describe the most abundant cellular and viral proteins packaged into the virus particle and discuss their potential implications in the virus life cycle.

MATERIALS AND METHODS

Virus and cells. BCBL-1 cells were grown as previously described (3). KSHV replication was induced by addition of 0.3 mM sodium butyrate. Virus was isolated from the supernatant of BCBL-1 cells 6 to 7 days postinduction as previously described (3). KSHV virions were isolated from crude stocks by gradient centrifugation as described previously (75). In brief, concentrated KSHV was layered onto 9 ml of 20 to 35% Histodenz gradients and centrifuged for 2 h at $71,000 \times g$ in an SW41 rotor, and then fractions were collected from the bottom by puncturing a hole in the tube.

Immunoblotting. Protein samples were separated on 7.5 or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked in 5% nonfat dry milk in TBST (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween 20), and all antibody incubations were done in 1% milk in TBST. Primary antibodies were used at the following concentrations: LANA (latency-associated nuclear antigen), 1:10,000; RTA, 1:5,000; RAP, 1:5,000; K8.1, 1:5,000; SOX, 1:3,000; kaposin, 1:5,000; ezrin and moesin (Santa Cruz), 1:250; enolase (Santa Cruz), 1:250; EF-2 (Santa Cruz), 1:250; actin and tubulin (Sigma), 1:20,000; Hsc70 (Stressgen), 1:1,000. Secondary antibodies were conjugated to horseradish peroxidase and used at the following concentrations: mouse (Sigma) and rat (Jackson ImmunoResearch), 1:10,000; rabbit and goat (Santa Cruz), 1:5,000.

Dot blot. A total of 25 μ l of each fraction was lysed by addition of $2 \times$ lysis buffer (20 mM Tris [pH 8], 50 mM EDTA, 200 mM NaCl, 1.2% SDS) containing 200 μ g of proteinase K/ml. Samples were incubated for 2 h at 37°C, extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated by addition of 100% ethanol. The DNA was resuspended in water and then adjusted to 10 mM EDTA and 400 mM NaOH and boiled for 10 min and applied to a nylon membrane (Amersham Pharmacia Biotech) by use of a dot blotter. The DNA was fixed by rinsing wells with 0.4 M NaOH. The nylon was then probed with 32 P-radiolabeled DNA corresponding to the locus of PAN, a polyadenylated nuclear RNA.

Trypsin and Triton X-100 treatment of KSHV virions. Gradient-purified virions (isolated from 5 liters of nonconcentrated induced BCBL-1 cell supernatants) were treated with 170 U of trypsin (Promega) in the presence or absence of 1% Triton X-100 (Sigma) for 30 min at 37°C in 1 mM CaCl_2 –100 mM NaCl–50 mM Tris (pH 7.4). The reaction was quenched by the addition of soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (Sigma). Samples were then mixed with an equal volume of $2 \times$ Laemmli sample buffer containing 20% 2-mercaptoethanol and boiled for 5 min.

Protein separation and Coomassie blue staining. Protein samples were separated on 7.5% SDS-PAGE gels at 70 V for at least 16 h. The gels were then stained in Coomassie blue (0.05% Coomassie brilliant blue, 50% methanol, 10% acetic acid) for 1 h and then destained (50% methanol, 10% acetic acid) for 3 h. Coomassie blue-stained bands were excised from gels rinsed 20 times in distilled water and placed in an Eppendorf tube. The gel slices were dehydrated for 1 to 2 days and then stored at room temperature.

In-gel tryptic digest and MALDI-MS. 45 mM dithiothreitol (Sigma) in 10 mM ammonium bicarbonate (pH 7.8) was added to polyacrylamide gel slices and then incubated for 30 min at 55°C. The solution was changed to 100 mM acrylamide (Bio-Rad) in 10 mM ammonium bicarbonate and incubated 1 h at room temperature. The previous solution was removed, and 0.5 ml of 10 mM ammonium bicarbonate and 50% acetonitrile was added to the gel and incubated for 30 min. The gel slice was then dried to completion. A small volume (2 to 10 μ l) of 10 mM ammonium bicarbonate containing 4 to 20 pmol of trypsin (Promega) was added to the slice. The gel was then saturated with 10 mM ammonium bicarbonate buffer. Buffer was added continuously over 2 h until the gel was swollen and covered with buffer. The gel was then incubated with trypsin overnight at 37°C. Peptides were extracted onto ZipTips (Millipore) from an aliquot of the solution and then washed with 0.1% trifluoroacetic acid (Applied Biosystems) and eluted directly to a matrix-assisted laser desorption/ionization (MALDI) plate with 0.5 μ l of 50% acetonitrile and 0.1% trifluoroacetic acid. The eluate was partially dried, and then 0.5 μ l of α -cyano-4-hydroxycinnamic acid (Agilent) (5 mg/ml) was added. MALDI-mass spectrometry (MS) was performed using the reflector mode to obtain monoisotopic peptide masses and tandem MS. The results were then used to search protein and genomic databases with Mascot software.

FIG. 1. Gradient purification of KSHV virions. Concentrated KSHV virus stocks were separated on a 20 to 35% Histodenz gradient. Fractions were collected from the bottom (fraction 1) and analyzed for viral DNA and glycoprotein content as described in Materials and Methods. (A) Plot of phosphorimager quantitation of KSHV viral DNA isolated from each fraction, spotted onto nylon membranes, and probed for the PAN locus. (B) Immunoblots of proteins isolated from each fraction, separated on 10% SDS-PAGE gels, transferred to PVDF, and probed for K8.1.

RESULTS

Virion purification. The goal of our study was to identify the protein components of the KSHV virion by mass spectrometry and immunoblotting. Since both infectious virions and noninfectious particles are released from infected cells, it was important to separate virions from other particle types. Virions were isolated by separating the released particles on 20 to 35% Histodenz gradients, followed by assaying each fraction for the presence of viral DNA (by blot hybridization) and the KSHV glycoprotein K8.1 (by immunoblotting). DNA extracted from each fraction was spotted onto nylon membranes and then probed for the KSHV gene encoding PAN, a polyadenylated nuclear RNA. Phosphorimager quantitation of the signal from each spot was then plotted, and a peak of viral DNA was observed in fractions 4 and 5 (Fig. 1A). Protein from each

fraction was separated on SDS-PAGE gels, blotted to PVDF, and then probed with a monoclonal antibody specific for K8.1. We found that the K8.1 glycoprotein was also enriched in fractions 4 to 5 (Fig. 1B). These fractions were pooled, diluted with PBS, and re-concentrated by centrifugation. There was also a small peak of viral DNA at fraction 17 that additionally displayed K8.1 staining. We suspect that this fraction contains disrupted, defective, or incomplete particles, and these were not further examined.

Analysis of candidate KSHV regulatory proteins. To determine the localization of individual proteins within virions, we examined the sensitivity of those proteins to proteolysis under a variety of conditions, each chosen to digest one or another virion compartment. KSHV virions are composed of three distinct compartments: the capsid, the tegument, and the envelope. Trypsin treatment of intact virions results in the removal of contaminating proteins that adhere to the outside of the particles (as well as degrading the external domains of the viral glycoproteins), while the tegument and capsid proteins are protected from digestion by the lipid bilayer. Addition of the detergent Triton X-100 to the trypsin digestion solubilizes the lipid bilayer and thus exposes all of the virion proteins to trypsin digestion; this should leave only proteins that are intrinsically resistant to trypsin digestion (and, of course, trypsin-resistant limit-digested products).

We first examined virions for the presence of candidate proteins with likely roles in the regulation of viral and host gene expression, since one of our goals was to look for factors that might shed light on the burst of aberrant lytic gene expression that accompanies viral entry (34). We focused on regulatory proteins whose synthesis is upregulated during lytic replication (when virions are formed) and for which we had available antisera for detection. This list included RTA, RAP (K-bZIP), SOX (ORF37, alkaline exonuclease), kaposin B, and LANA (latency-associated nuclear antigen). RTA is the major viral transcription factor controlling the switch to lytic gene expression; as such, it is an obvious candidate for regulation of early lytic gene expression (44, 63). RAP, which functions importantly in viral DNA synthesis, has recently been shown to bind to RTA and downregulate RTA action on selected lytic promoters (29, 41). SOX is a protein that promotes global degradation of host mRNA (24); in herpes simplex virus (HSV), the vhs protein, which is structurally unrelated to SOX but has an analogous function, was one of the first regulatory functions assigned to the tegument (57). Kaposin B, a latent protein whose expression is strongly upregulated by RTA during the lytic cycle, has recently been found to be an activator of the p38/MK2 mitogen-activated protein kinase pathway (46a). This pathway regulates the degradation of transcripts that contain AU-rich elements (AREs) in their 3' noncoding regions; such elements are often found in cytokine and growth factor mRNAs and in other regulators of signaling and gene expression. In the ground state, such transcripts are rapidly degraded but are strongly stabilized by activators of MK2, including kaposin B. LANA, which is responsible for maintenance of the viral genome in latency, is also a transcriptional modulator and can affect both gene expression and cell proliferation by interacting with p53, phosphorylated Rb, and glycogen synthase kinase 3 β (17, 18, 19, 23). LANA also antagonizes the positive-feedback loop of RTA on the RTA promoter, causing levels of

FIG. 2. Immunoblots for viral components. Induced BCBL-1 cells and mock-treated (V), trypsin-treated (VT), and trypsin- and Triton X-100-treated (VTT) gradient-purified virions were separated on 10% SDS-PAGE gels, transferred to PVDF, and then probed for various KSHV proteins.

RTA to diminish and thereby potentially impacting its ability to cause a switch to lytic replication (37). As such, if delivered in virions it could play a role in assuring that any early RTA action is aborted before full lytic reactivation can proceed.

Gradient-purified virions were either mock treated or trypsin treated in the presence or absence of Triton X-100; products were then separated on 10% SDS-PAGE gels, transferred to PVDF, and probed for the aforementioned KSHV proteins. The blots were also probed for the K8.1 glycoprotein as a control for the trypsin treatment. (The monoclonal antibody against K8.1 recognizes an epitope in the external domain of the protein, exposed on the virion surface. Upon trypsin treatment of the particles, the epitope should be degraded and monoclonal antibody binding should be abolished.) As expected, K8.1 was present on virions but not on virions that were treated with trypsin (Fig. 2; K8.1 panel), confirming that the trypsin treatment had successfully removed the proteins external to the particle. We found that both RTA and RAP proteins are present in the virions, as they are found in both intact and trypsin-treated virions but not in particles treated with trypsin plus Triton X-100 (Fig. 2; RTA and RAP panels). Note that RTA and RAP exist in several different isoforms in infected cells (Fig. 2; RAP and RTA panels, BCBL-1 lane). Both RAP and RTA isoforms were incorporated into KSHV virions, but we observed that the higher-mobility isoform of RTA was present in significantly higher levels than the slower-mobility form, even though it is less abundant at late times of infection (Fig. 2; RAP and RTA panels). This suggests speci-

FIG. 3. Coomassie blue staining of treated virions. Mock-treated (V), trypsin-treated (VT), and trypsin- and Triton X-100-treated (VTT) gradient-purified virions were separated on a SDS-7.5% PAGE gel and then stained with Coomassie blue. The destained gel is shown. Each lane represents material from ~5 liters of induced BCBL-1 cells. Protein identifications to the right of the gel are the identifications resulting from mass spectrometric analysis of the designated bands. Bands designated as fragments represent the tryptic fragments of the protein.

ficity in packaging, although the biological meaning of the preferential incorporation of the lower RTA isoform is unclear. Such preferential incorporation is not without precedent, however; in human cytomegalovirus (HCMV), only one of the phosphorylated isoforms of UL69 protein produced during infection is incorporated in the virus particle (72).

By contrast, we found that LANA was not present in KSHV virions, even though we could detect significant levels of the protein in lysates prepared at late times of infection (Fig. 2; LANA panel). We also probed mock- and trypsin-treated virions for SOX and kaposin proteins. We found that although both of these proteins were found in induced BCBL-1 lysates and at low levels in gradient-purified virions, neither was detected in trypsin-treated virions, indicating that both proteins were merely contaminants adhering to the outside of the virus particle (Fig. 2; SOX and Kaposin panels). These results again reveal specificity in KSHV packaging, since not all proteins abundantly expressed at late times of infection get incorporated into virus particles.

Mass spectrometric analysis of virion polypeptides. Next, we used gel fractionation followed by mass spectrometric analysis to perform a more systematic analysis of the virion protein composition. Gradient-purified virions (approximately 5 liters of induced BCBL-1 cell supernatants per condition) were mock treated or treated with trypsin in the presence or absence of Triton X-100 and then separated on large, SDS-7.5% polyacrylamide gels and stained with Coomassie blue. As shown in Fig. 3, a series of discrete bands is observed in the mock-treated samples (Fig. 3; V lane). The addition of trypsin affected the migration of several bands (Fig. 3; VT lane), suggesting that these proteins (or domains of them) were external to the virus particle. Several bands disappeared (including a very prominent band of ca. 150 kDa), and at least two new, very prominent, protein bands appeared at around 60 and 80 kDa, likely representing proteolytic cleavage products of the missing 150-kDa band. The majority of virion proteins, however, were unaffected by the trypsin treatment, demonstrating their presence on the inside of the lipid bilayer. We observed

TABLE 1. Distinct peptides identified by mass spectrometry of KSHV virions

Gene product	Accession no.	Size (aa)	Distinct peptides identified
ORF8 (gB)	AAC83368	845	470-481/DGINQVLEELSR 482-485/AWCR 606-611/DYAYLR 650-662/LASSVFDLETMFR 663-670/EYNYTTHR 692-705/DLSEIVADLGGIGK 775-884/APPSGGAPTR 775-888/APPSGGAPTREEIK 789-802/NILLGMMQLQQEER 811-818/STPSVFQR
ORF21 (TK)	gil18845987; NP_572073	580	90-97/FPPRPLIR 126-132/FAFQSPR 195-205/LROGGFAFSPR 197-205/DGGFAFSPR 244-251/TPVTOYR 267-281/STLVNAVGCILPQER 282-294/VTSFPEPMVYWTR 295-301/AFTDCYK 328-333/FSLPFR 334-341/TNATAILR 396-414/ATEGOVVAILTLSSAESLR 422-433/KNOGTVEQNYIR 423-433/NOGTVEQNYIR 547-557/QILSNPAIKPR
ORF22 (gH)	gil1136819; AABO8395	730	234-245/GHATYDELTFAR 341-349/QYAEFLR 391-405/SSQETVLAMVOLGAR 487-498/FSKPOSINIYR 498-507/AFSPCFLGLA
ORF24	gil10140946	752	155-166/VSWTLMQIISR 331-339/TALCMAVAK 331-343/TALCMAVAKHICR 355-367/QQLSLARALVANFEK 446-453/LVNNFVIK 566-573/IHGSOLTK 666-672/VCNSIPK 698-706/GAPHMINTK 721-729/SYHFAGCAK 721-731/SYHFAGCAKAK
ORF25 (MCP)	gil2246481; AAB62606	1,376	95-106/ISMPTIAHGDGR 158-172/TTTSALQFGMOALER 185-193/HAPPVILK 194-203/TLGOPVYSER 260-284/GVSTYTTASGQQVAGVLETTDSVMR 286-309/LMNLLGQVESAMSGPAAYASYVVR 310-321/GANLVTAVSYGR 325-332/NFEQFMAR 333-347/IVDHPNALPSVEGDK 348-359/AALAOGHDEIQR 360-381/IDGKFVAIESLQR 373-381/FVAIESLQR 413-420/YSTSVSVR 421-437/GVESPAIQSTETWVVNK 458-484/NHNPTQSAQALNQALNQAFDPDPDGGHGYGLR 485-495/YEQTPNMNLF 496-504/TFHQYYMGK 516-530/ALVTTEDLLHPTSHR 560-572/TMVGNIPOPLAPR 648-658/LAFVNSYHMVR 672-679/EAHGHYRK 694-702/LAGHETVGR 742-753/IGDQNDNPQNR 754-760/ATFINLR 761-777/GRMEDLVNHVNIYQTR 763-777/MEDLVNNVNIYQTR 861-876/DILQAGDIRPTVOMIR 898-911/RDPAQSFATHEYGK 912-930/DVAQTVLVNGFGAFVADR

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TABLE 1—*Continued*

Gene product	Accession no.	Size (aa)	Distinct peptides identified
			968-972/LPNQR 1030-1042/HRMHPGFAMTVVR 1032-1042/MHPGFAMTVVR 1043-1056/TDEVLAEHILYCSR 1057-1070/ASTSMFVGLPSVVR 1057-1071/ASTSMFVGLPSVVR 1138-1150/MKAGVQTGSPGNR 1140-1150/AGVQTGSPGNR 1151-1162/MOHVGYTAGVPR 1201-1217/AACVVSCDAYSNEAER 1218-1232/LLTDHSIPOPAYECR 1233-1242/STNNPWASQR 1243-1255/GSLGDVLYNITFR 1268-1277/QFFHKEDIMR 1281-1292/GLYTVNEYSAR 1346-1365/QTHAPVHMGQYLJEEVAPMK
ORF26 (TRI-2)	gil11086047; A55371	305	6-14/SIVVNFTSR 15-26/LFADELAALQSK 27-38/IGSVLPLGDCHR 39-53/LQNIQALGLGCVCSR 69-83/CTAVLEEVRPDSL 225-231/LLTALVR 323-251/HDRHPLTEVFEGVVPDEVTR 235-251/HPITEVFEGVVPDEVTR
ORF33	gil2246501; AAB62626	334	10-14/NFLNX 117-126/FPYIAPPPSR 135-145/QELVHTSQVVVR 197-203/VAALLTR 217-203/GHVNVR 257-270/NFLGLLFDPIVQSR 276-303/ITSHPIPTHVENVLTGVLDDGTLVPSSK 304-310/APWVLLR 318-324/LLIYECK
ORF62 (TR1-1)	gil16846032; NP_572118	331	14-25/QVLGLLPPPTHR 45-61/YAASTRPTVGSLEALR 235-250/VNQRPSMALTFQSGK 251-260/GFAEVVAMIK 261-268/DHFTDVIR 271-275/YIQLR 276-282/HELYINA
ORF63	gil18846033; NP_572119	928	26-33/LLMEFQLR 69-74/VFALVR 75-92/AAVFLEPPSSIOPLEAAR 129-141/LLAHYADQIAGFK 219-231/TLVTEHHLEFVSR 248-253/ALAIYR 306-312/LESFLSR 430-439/HPGISDIPLR 445-460/ALAFFVPPAPINTLQR 461-471/VYAALPSQLMR 480-492/TTWGGAVPANLAR 523-534/VGDTEYDEDIVR 535-544/SPLFADAFTK 562-569/NRALFQIR 564-569/ALFQIR 592-603/AYFHIMDILEER 683-695/LEFEYDSEGDFVR 715-724/TIQTTEQATR 725-743/EATVAMTTIAKPIYPAYIR 748-753/LEYLNR 754-760/LNHHILR 761-781/IPFPQDALSELQETYLAFAAR 800-817/YFGVLFQHQLVPTAIVKK 818-844/LLHIDEAXDTTEAFLQSLAQPVVQGOR

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TABLE 1—*Continued*

Gene product	Accession no.	Size (aa)	Distinct peptides identified
ORF75	gil5669897; AAD46504	1,296	826-844/DTTEAFLOSLAQPVVQGR 865-882/INPQFTDAQANIPPSIKA 888-902/YDVPEVSVDWETYSR 903-913/SAFEAPDDELRL 914-923/FVPLTLAAGLR
			63-84/DVEIQTVLAVLSPLLGYPHVIR 120-128/ELGLQEWAR 139-153/ITQTLLEPHPPQFIR 216-222/YFVIPGR 235-252/QAFGIHGAYTHVHSSVQR 256-289/GLGNLLFHSTLFPGGQTQGALTGLYATE 301-321/GVQQAEMLOGAGVPTLGGFLK 365-379/FEPTOGPTYPNLYR 401-409/GPIFSGLNR 419-426/HLQALAPR 510-517/TSEQPGIR 518-528/IVDDLTGETTR 529-551/VFSVDQPSSTPPSPWLALSDGVR 552-575/VSGHPEDVDWGLFATGSTIHLQLLR 655-667/ALPQELLVPVPAWR 741-760/ELGVALSISAAASSPTLSER 792-799/YRVTPDVK 991-999/LEHHLGSLR 1009-1017/LFSCPTSPR 1055-1069/QSLSGFSGLITCLR 1127-1141/SESPYTYGTPPPQR 1157-1161/SVFLR 1193-1203/YEQDALEYILR 1204-1224/QRGEITLTYHGNAADETLPAR 1229-1245/NPTGNSTVAGLTSSDGR
Actin (beta)	gil14250401; AAH08633	368	12-21/AGFAGDDAPR 22-32/AVFPSIVGRPR 89-106/VAPEEHPVLLTEAPLNPK 171-176/LALAGR 185-189/ILTER 190-199/GYSFTTTAER 232-247/SYELPDGQVITIGNER 278-283/COVDIR 322-328/IIAPPER 322-329/IIAPPERK 353-365/QEYDESGPSIVHR
α -Enolase	gil4503571; NP_001419	434	1-9/MSILKIHAR 10-15/EIFFDSR 16-28/GNPTVEVDLFTSK 33-50/AAVPSGASTGIYEALST 82-92/LNVTEQEKIDK 93-103/LMIEMDGTENK 127-132/GVPLVR 163-179/LAMQEFMILPVGAANFR 184-193/IGAENVYHNLK 203-221/DATNVGDEGGFAPNILENK 240-253/VVIGMDVAASEFFR 270-281/YISPDQLADLYK 307-326/FTASAGIQVVGGDLTVTNPK 407-412/YNQLLR
EF-2B	AAH24689	517	51-59/GPLMMYISK 69-74/FYAFGR 75-85/VFSGLVSTGLK 98-108/KEDLYLKPIQR 99-108/EDLYLKPIQR 158-165/FSVSPVVR 179-185/LVEGLKA 219-231/DLEEDHACIPIKK

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TABLE 1—*Continued*

Gene product	Accession no.	Size (aa)	Distinct peptides identified
Ezrin (villin 2)	gil21614499; NP_003370	586	265-284/ARFPDPLAEDIDKGEVSAR 307-335/KIWCFGPOGTGPNILTDITK 327-335/GVQYLNEIK 376-385/GGGQIIPTAR 427-444/GHVFEESQVAGTPMFVVK 445-460/AYLPVNESFGFTADLR 505-517/EGIPALDNFLDKL
			152-162/LIPQVMDQHK 238-246/IGFPWSEIR 263-273/KAPDFVIFYAPR 264-273/APDFVIFYAPR 361-371/AERELSEQIQR 364-371/ELSEQIQR 372-379/ALQLEER 382-393/AQEEAERLEADR 413-427/SQEQLAAELAEYTAK 428-436/LALLEEAR 530-542/QLLTLSELSQLAR 578-586/QRIDEFEAL
Hsc70	S11456; a27077	350	26-36/VEIANDQGNR 37-49/TTPSYVAFTDTER 138-155/VTNAVVTVPAYFNDSQR 160-171/DAGTIAGLNVLRL 221-236/STAGDTHLGGEDFDNR 300-311/ARFEELNADLFR 302-311/FEELNAOLFR 424-447/QTQFTTYSONQPGVLIQVYEGER 459-469/FELTGIPPAPR
Hsp90	HS9A_HUMAN; P07900	732	41-45/EIFLR 74-86/ELHINLIPNKQDR 185-200/VILHLKEDQTEYLEER 292-298/TKIPWTR 327-337/HFSVEGQLEFR 338-344/ALLFVPR 345-354/RAPFDLFENR 346-355/APFDLFENR 346-355/APFDLFENRK 386-399/GVVDSEDLPLNISR 458-463/LSELLR 489-498/HIYYITGETX 567-575/FENLCKIMK 632-646/HLEINPOHSIIETLR
Moesin	AAH01112	329	28-35/QLFDQVVR 41-53/EVWFFGLQYQDTK 54-60/GFSTWLK 54-63/GFSTWLKLNK 72-79/KESPLLFK 73-79/ESPLLFK 84-100/FYPEDVSEELIQDITOR 101-107/LFFLQVK 181-193/GMLREDAVLEYLK 185-193/EDALEYLK 238-246/IGFPWSEIR 255-262/FVIKPIDK 263-273/KAPDFVIFYAPR 264-273/APDFVIFYAPR
Pyruvate kinase	gil20178296; P14618	531	32-42/LDIDSPITAR 33-43/LDIDSPITAR 44-56/NTGIICTIGPASR 93-115/TATESFASDPILYRPVAVALDTK 174-186/IYDOGLISLQVK 231-246/FGVEQDVDMFASFIR 271-278/IENHEGVR 279-294/RFDEILEASDGIMVAR 280-294/FDEILEASDGIMVAR 295-311/GDLGIEIPAEKVFLAQK 368-376/GDYPLEAVR

Continued on following page

TABLE 1—Continued

Gene product	Accession no.	Size (aa)	Distinct peptides identified
			377-383/MQHLLAR 384-400/EAEAAIYHLQLFEELPR 401-422/LAPITSOPTEATAVGAVEASFK 448-455/API LAVTR 462-467/QAHLRY
Tubulin	1TUBA	440	65-79/AVFVDLEPTVIDEVR 85-96/QLFHPEQLITGK 113-121/EIIDLVLDR 216-229/NLDIERPTYTNLVPYPR 244-264/FD GALNVDLTFQTNLVPYPR

that the intensity of staining for some of these bands was decreased in the trypsin-treated lane versus the mock-treated lane. We hypothesize that some of the virions may have been damaged during the isolation and purification procedure, rendering a fraction of the internal proteins now subject to a low level of cleavage. The addition of trypsin and Triton X-100 to the virions results in the degradation of most of the proteins, although a significant number of trypsin-resistant bands were present, especially at the lower molecular weights (Fig. 3; VTT lane). We interpret these bands as displaying intrinsic trypsin resistance.

Individual protein bands were excised from lanes corresponding to either virions or trypsin-treated virions, eluted, and subjected to proteolysis with trypsin. The resulting proteolytic fragments were then analyzed by mass spectrometry. We were able to positively identify 18 of the proteins composing the KSHV virions by aligning peptide sequences with those in the database of known gene products. These proteins were of both cellular and viral origin (Table 1). As expected, we identified several known KSHV structural proteins. Three of the four capsid proteins, ORF25 (MCP), ORF26 (TRI-2), and

ORF62 (TRI-1), as well as two glycoproteins, ORF8 (gB) and ORF22 (gH), were identified in this fashion. ORF25 encodes the KSHV major capsid protein, and we observed both the full-length protein and two tryptic fragments in the virions treated with trypsin (Fig. 3; VT and VTT lanes). The significant level of the ORF25 fragments present in the samples treated with trypsin alone suggested that this internal component of the virion was subject to digestion even without the detergent, supporting our hypothesis that the virions were slightly damaged during purification. The mass spectrometric analysis also identified several viral polypeptides not previously annotated as being structural proteins, including the products of ORF21, ORF24, ORF33, ORF63, and ORF75 (Fig. 3 and Table 1). With the exception of ORF21 protein, the viral thymidine kinase (TK), none of the other proteins have been ascribed catalytic, regulatory, or structural functions.

We also identified several cellular proteins that were specifically incorporated into the virus particle. These include actin and tubulin, major components of the cytoskeleton, several housekeeping metabolic proteins (e.g., enolase and pyruvate kinase), two cellular chaperones (Hsp90 and Hsc70), and a translational repressor (EF-2b). In addition, ezrin and moesin, two members of the ERM (for "ezrin-radixin-moesin") family of proteins, which are involved in cytoskeletal rearrangements, were identified (Fig. 3 and Table 1). Ezrin has previously been suggested to perform important functions early during KSHV infection (58). The implications of the inclusion of these proteins in the virus particle will be discussed below.

Immunoblot confirmation of cellular proteins in virions. To confirm the presence of cellular proteins in the KSHV virions, we performed immunoblotting experiments analogous to those of Fig. 2. Gradient-purified virions, either mock treated or trypsin treated in the presence or absence of 1% Triton X-100, were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with the appropriate antibodies. As shown in Fig. 4, EF-2, α -tubulin, ezrin, moesin, and hsc70 were all found in the mock-treated and trypsin-treated virions but not in virions treated with trypsin plus Triton X-100—confirming their presence within the virus particle. Ezrin and moesin proteins migrate at approximately 75 kDa. We detected several bands around 75 kDa, consistent with the fact that the antibody recognizes ezrin, moesin, and radixin. Moesin and radixin are 74 and 77% identical to ezrin, respectively (20, 38).

We detected the α -enolase protein in trypsin-treated virus regardless of the presence or absence of Triton X-100, sug-

FIG. 4. Immunoblots of cellular virion components. Induced BCBL-1 cells, mock-treated virions (V), trypsin-treated virions (VT), and trypsin- and Triton X-100-treated virions (VTT) were separated on 10% SDS-PAGE gels, transferred to PVDF, and then probed with antibodies for the indicated cellular proteins.

gesting the protein is both incorporated into virions and intrinsically resistant to the trypsin digestion. Upon review of our Coomassie blue-stained gel (Fig. 3), it is similarly apparent the 48-kDa band identified by mass spectrometric analysis as α -enolase is resistant to trypsin digestion in that experiment as well, since it is present in all three lanes (Fig. 3; ~48 kDa). Analysis of the protein sequence for α -enolase revealed many potential sites for trypsin digestion (a fact confirmed by the trypsin digestion of the excised, denatured protein described in Table 1). This indicates that either these sites are inaccessible to the enzyme when the protein is folded or that they are shielded from the enzyme by interactions with other proteins in the particle.

DISCUSSION

Here we have used a combination of (i) immunoblotting for candidate proteins and (ii) protein isolation followed by proteolytic fragmentation and mass spectrometry to identify components of the KSHV virion. The immunoblotting approach identified both RTA and RAP in the virus tegument. RTA, the product of the ORF50 locus, plays a critical role in the KSHV life cycle. RTA is composed of an N-terminal DNA binding domain and a C-terminal transactivation domain (44). RTA transactivates various viral promoters, either directly (60–62) or by recruitment of cofactors like CSL (39, 40) or C/EBP (69, 70). It is required for the switch between lytic and latent replication: ectopic expression of wild-type RTA in latently infected cells induces the lytic cycle, while expression of dominant-negative mutant RTA suppresses this switch (44, 45). Our finding of RTA in the virion is surprising, both in light of earlier negative results of attempts of others to detect it there (75) and in view of the fact that *de novo* KSHV infection generally results in latency (3, 21, 35, 67, 68). (RTA is a minor component of the KSHV virion and we suspect the previous attempt [75] to probe for RTA in KSHV virions may have failed because not enough virions were utilized for the assay.) However, this finding may shed light on the recent observations of Chandran and colleagues (34), who reported that immediately following viral entry, a burst of aberrant lytic-cycle gene expression can transiently occur. The delivery of RTA with the tegument could certainly jumpstart such expression. But this raises another question: why does such expression not trigger the full program of lytic gene expression?

Here, we can suggest at least two possibilities, neither of which is exclusive of the other. The first model derives from the fact that RTA undergoes extensive posttranslational modification during infection, especially by phosphorylation (44). But Fig. 2 shows that it is the undermodified isoforms of RTA that appear to be preferentially encapsidated (alternatively, RTA may be actively dephosphorylated following encapsidation). Perhaps these underphosphorylated isoforms of RTA are incapable of activating key promoters required for lytic progression. A second possibility is suggested by the incorporation of RAP into the particles. Recent work of Kung and colleagues (29, 41) demonstrated that RAP can bind to RTA, repressing transactivation of several RTA-responsive viral promoters. This suggests a model in which incoming RTA from the virus tegument may drive initial lytic gene expression, while RAP released into the cell would temper this transactivation and blunt progression of the lytic cycle.

In any case, it is likely that additional mechanisms are also at work to modulate this very-early-expression program. Certainly, virus binding to cell surface integrin receptors can trigger signaling cascades that may affect expression of viral genes. Recent work of Sharma-Walia and colleagues, who have shown that recombinant gB can activate FAK kinase (via integrin-mediated signaling), leading to activation and relocalization of ezrin, is interesting in this connection (58). Our finding that ezrin is present in substantial quantities in virions raises the possibility that some of this substrate ezrin may be supplied by incoming virions, to amplify such signaling. For example, virion-delivered ezrin may traffic preferentially to sites of activation. Clearly, further experiments are required to examine these possibilities.

In addition, newly released virion DNA may not be chromatinized at early times postinfection, and this may release certain lytic genes from negative regulation by chromatin—which presumably must be reassembled upon circularization of the incoming viral DNA as latency is established. Moreover, we have recently discovered that some of the RNAs found by Krishnan et al. to be present in cells immediately after KSHV entry are also encapsidated into KSHV virions, and so are most likely passively transferred to the cytoplasm upon infection (J. T. Bechtel and D. Ganem, unpublished data). Finally, LANA protein produced early after infection would also affect RTA's activities by limiting its ability to transactivate its own promoter. LANA transcripts are detected within 30 min of infection and peak at 24 h postinfection, so there is potential for LANA to play a critical role in the latent-lytic switch even though it is not deposited in the cell with the virus particle. This is the first report of a herpesvirus TK being localized to the virus tegument. In other herpesviruses, TK is involved in DNA replication, phosphorylating deoxynucleoside triphosphates for incorporation into replicating DNA (55). Given the limited role TK plays during virus infection, it is surprising to find it specifically incorporated into the virus tegument. It is possible that the KSHV TK has evolved a new function that is important during the initial stages of virus infection. Further work will be required to examine this possibility, but this postulated evolution would not be without precedent in KSHV. Another KSHV protein, SOX (ORF37 gene product), is the homolog of the herpesvirus alkaline exonuclease, a protein conserved in all herpesvirus that is involved in the resolution of replicating DNA structures prior to packaging (46). In KSHV, SOX is also responsible for host shutoff, a novel function for this conserved gene (24).

ORF24 and ORF75 have been identified as a virion components for murine gammaherpesvirus 68, another gammaherpesvirus (5). ORF75 is the homolog of the FGARAT gene involved in purine biosynthesis, though why this function should be encapsidated is obscure (53, 56). ORF33 is homologous to HSV UL16 and HCMV UL94. HSV UL16 is a tegument component thought to be involved in capsid maturation, while HCMV UL94 has been identified as a tegument component but has not been ascribed a function (51, 66, 71). ORF63 is conserved among all of the herpesviruses and has been characterized in HSV, pseudorabies virus, and HCMV. Its homologs in HSV and pseudorabies virus (UL37 proteins) are involved in egress and reenvolvement, while a HCMV mutant

lacking the ORF63 homolog (UL47) exhibits an entry defect potentially at the step of capsid disassembly (4, 13, 33).

Several chaperones have been found in the tegument of HCMV (66), another herpesviruses, and play critical roles in both cellular and viral processes. In the cell, they are involved in assisting protein folding and preventing aggregation of improperly folded chains. Heat shock cognate protein 70, or hsc70, is a cellular chaperone that has been found in association with virion components of several different virus families. Hsc70 is specifically incorporated into HIV virions (26). Hsc70 binds posttranslationally to simian virus 40 VP1, colocalizing with it to the nucleus, where it is thought to prevent premature capsid assembly (11). During papillomavirus infection, hsc70 is required for the nuclear translocation of the L2 protein (16). Hsc70 also is responsible for the posttranslational translocation of the hepatitis B large envelope protein into the endoplasmic reticulum (36) and is found in virions and subviral particles of avian hepatitis B virus (64). The HSV regulatory protein ICP0 causes hsc70 relocalization to distinct nuclear domains (6). Proteosomal machinery is also localized to these sites, suggesting a role in quality control, perhaps specifically acting during the formation of the capsid portal, a dodecamer of the HSV UL6 protein.

In addition to being directly incorporated into the virions of several viruses, the chaperones are also intimately involved in diverse events of virus replication. Heat shock protein 90, hsp90, is considered a specialized chaperone, binding proteins at a near-native state and controlling their activities (54). Known targets of hsp90 are involved in signal transduction, cell cycle modulation, and transcriptional regulation. The protein has diverse roles in the replication of different viruses. During influenza virus infection, hsp90 is relocalized to the nucleus and binds to PB2 to stimulate polymerase activity (49). Hsp90 is required for reverse transcription in duck hepatitis B virus, where it assists in the formation of a ribonucleoprotein complex and in protein priming of replication (27). Inhibition of hsp90 by geldanamycin reduces vaccinia virus titers 1 to 3 logs (28). The chaperone is not packaged into the virus, although it binds to the vaccinia virus core protein, and it is speculated to function during viral uncoating. So it is conceivable that delivery of hsp90 by KSHV virions may play a role in influencing biochemical processes on the next round of infection. In KSHV, the v-FLIP protein was found in a complex with IKK γ and hsp90, an interaction that is crucial for v-FLIP's ability to activate NF κ B (15).

However, it is equally possible that some of these cellular proteins are present in virions merely because of their high abundance in the cytosol at the time of envelopment and may play no active role in KSHV biology. Certainly, actin and tubulin are likely to fall into this category; the chaperones and ERM proteins are also highly abundant. Clearly, additional work will be required to address both the mechanisms by which such proteins are incorporated into particles and the biological consequences of that incorporation.

ACKNOWLEDGMENTS

We thank Chris Sullivan, Adam Grundhoff, and Craig McCormick for critical reading of the manuscript.

J.T.B. was supported by a training grant from the National Cancer Institute (T32CA09043).

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