

Proteomic Analysis of Pathogenic and Attenuated Alcelaphine Herpesvirus 1[∇]

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The gammaherpesvirus alcelaphine herpesvirus 1 (AIHV-1) causes malignant catarrhal fever in susceptible ungulates but infects its natural host, wildebeest, without obvious clinical signs. In tissue culture, AIHV-1 is initially predominantly cell associated and virulent but on extended culture becomes cell-free and attenuated. We wanted to determine what changes in protein composition had taken place during the transition from virulent to attenuated virus in culture. Purified virus preparations were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were analyzed by liquid chromatography-electrospray ionization–tandem mass spectrometry. Peptides were identified in serial gel slices by using MASCOT software to interrogate virus-specific and nonredundant sequence databases. Twenty-three AIHV-1-encoded proteins and six cellular proteins were identified in the attenuated and virulent viruses. Two polypeptides were detected in only the virulent virus preparations, while one other protein was found in only the attenuated virus. Two of these virus-specific proteins were identified by a single peptide, suggesting that these may be low-abundance virion proteins rather than markers of attenuation or pathogenesis. The results suggest that attenuation of AIHV-1 is not the result of gross changes in the composition of the virus particle but probably due to altered viral gene expression in the infected cell.

Malignant catarrhal fever (MCF) is an important, sporadic, and usually fatal lymphoproliferative disease of cattle and other susceptible ungulates. MCF is caused by either of two closely related gammaherpesviruses, alcelaphine herpesvirus 1 (AIHV-1) and ovine herpesvirus 2 (OvHV-2), that belong to the genus *Rhadinovirus* (28). These viruses are carried asymptotically by their natural hosts, the wildebeest (*Connochaetes taurinus*) in the case of AIHV-1 and the domestic sheep (*Ovis aries*) in the case of OvHV-2. Transmission of these viruses to MCF-susceptible species can give rise to wildebeest-associated MCF (26) and sheep-associated MCF, respectively (7, 36).

AIHV-1, the causative agent of WA-MCF, can be propagated in cell culture where, in early passage, virulent virus particles can be obtained. The prolonged passage of AIHV-1 in culture, however, causes genomic rearrangements that result in the production of a cell-free form of the virus that is attenuated for disease induction (28).

The published annotations of the genomes of AIHV-1 (11) and OvHV-2 (14) have identified a number of genes that are common only to the MCF-causing viruses, including the AIHV-1 open reading frames (ORFs) A7, A8, and A10 that are predicted to encode glycoproteins that may be found on the virion.

Comparison of restriction enzyme profiles of the genomes of

virulent and attenuated AIHV-1 strains identified a restriction fragment (ATT-1) that was found only in the attenuated virus genome (13). Sequence analysis of ATT-1 showed that it carried the ORF50 gene (homologue of the Epstein-Barr virus [EBV] lytic cycle regulator, RTA), the A6 gene (a predicted transcription factor), and a truncated A7 gene fused to sequences from the right terminal repeat (37). A range of similar fragments cloned in the same study showed a common feature that a central segment of the genome, encoding ORF50, A6, A7, and A8, was brought into proximity with the right terminal region, encoding A10, suggesting that such rearrangements may be a critical feature of the attenuation process. It was concluded that attenuation of the virus could be associated with the disruption of the expression of the putative glycoproteins A7, A8, and A10 or of the transcription factors encoded by A6 or ORF50.

The objectives of the present study were (i) to identify the major components of the AIHV-1 virion, complementing the existing genome annotation, and (ii) to determine whether structural differences existed between the attenuated and virulent forms of AIHV-1. Proteomic analysis of purified virions has proved to be a successful approach to define the protein composition of gammaherpesviruses (5, 15, 23, 38). We report the results of a qualitative proteomic analysis of both virulent and attenuated AIHV-1 to identify differences that might contribute to the altered pathogenicity of the two forms of the virus in vivo.

MATERIALS AND METHODS

Cells and viruses. AIHV-1 C500 (27), originally from an MCF-affected cow, had been isolated and propagated from the spleen of an infected rabbit in accordance with previously published methodology (13). Bovine turbinate (BT) cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) fetal calf serum and 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. AIHV-1 C500 virulent and attenuated strains were propa-

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gated on BT cells cultured in Iscove's modified Dulbecco's medium supplemented with 2% (vol/vol) fetal calf serum and 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. AIHV-1 C500 strain particles were harvested and purified at passage 4 (virulent) or passage 1009 (attenuated). The attenuated and virulent viruses have been tested in vaccination and challenge experiments in cattle which showed that only the virulent virus induced MCF after intranasal or intravenous inoculation (D. Haig, unpublished data).

Purification and isolation of AIHV-1C500. Both virulent and attenuated AIHV-1 were obtained by a freeze-thaw cycle from infected BT cells, when virus-induced cytopathic effect reached 100% (at approximately 4 days post-infection). Cellular debris was cleared from the lysates by centrifugation at $3,000 \times g$ for 15 min at 4°C. The viral particles were pelleted from the supernatant by centrifugation at $100,000 \times g$ for 3 h at 4°C. The resulting pellet was resuspended in TNE buffer (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA) and loaded onto a 20 to 55% continuous sucrose gradient to further purify the virus. The gradient was subjected to centrifugation at $100,000 \times g$ for 18 h at 4°C. Virus-containing fractions from the gradient were identified by Western blotting using monoclonal antibody 12B5, specific for the main glycoprotein complex of the virus (1). Positive fractions were pooled and diluted approximately 1:3 in TNE buffer. The virus particles were then pelleted by centrifugation at $100,000 \times g$ for 3 h at 4°C, resuspended in TNE buffer, and frozen at -70°C until used. Virus preparations were examined by electron microscopy to obtain an indication of the purity of the virus preparation.

Analysis of virus preparations by electron microscopy. A standardized suspension of 91-nm-mean-diameter polystyrene latex particles (Agar Scientific, Essex, United Kingdom) at 10^{10} particles/ml was used for virus particle counting, based on the method described by Williams and Backus (35). As a precaution against clumping, the stock suspension was sonicated for 1 min before dilution and immediately before use.

Equal volumes of virus suspension and latex particle suspension were thoroughly mixed, loaded onto a carbon-Formvar grid and placed onto a drop of 2.0% phosphotungstic acid (pH 7.0) for 1 min. Stained grids were viewed in the electron microscope at a screen magnification of $\times 45,000$ and scanned vertically along the rows of grid squares. Three complete scans were made (one toward either end and one at the center). All virus particles and latex particles seen separately or in groups of no more than three during each individual scan were counted. The ratio of virus particles to latex particles was then calculated for each scan, and the mean ratio was multiplied by the latex particle concentration to give the virus particle concentration per milliliter.

SDS-PAGE gel electrophoresis. Purified virions (approximately 3.5×10^8 particles) were denatured at 100°C for 10 min in sample buffer (Tris-glycine, sodium dodecyl sulfate [SDS] sample buffer, 1× sample reducing agent; Invitrogen, Paisley, United Kingdom) and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel (Invitrogen). Proteins were visualized by staining with SimplyBlue SafeStain (Invitrogen). A series of gel slices of equal size (2.5 mm), covering the entire sample lane, were excised from the SDS-PAGE gel and analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

LC-ESI-MS/MS. LC-ESI-MS/MS analysis was carried out essentially as described by Batycka et al. (3). Briefly, the proteins within each gel slice excised from the sample lane were subjected to standard in-gel trypsinolysis. LC was then performed on samples of 4 µl from each gel slice, applied by direct injection into a monolithic reversed-phase column (200-µm inner diameter; Dionex), using an Ultimate 3000 Nanoflow HPLC system (Dionex-LC Packings). Bound peptides were eluted in order of increasing hydrophobicity by the application of a 15-min linear gradient of 8 to 45% acetonitrile in 0.1% formic acid and monitored by UV absorbance in a 3-nl UV detector flow cell. LC was interfaced with a three-dimensional high-capacity ion trap mass spectrometer (Esquire HCTplus, Bruker Daltonics) utilizing a low-volume (<50 µl/min) stainless steel nebulizer (Agilent) and ESI. Digests were analyzed for protein content by LC-ESI-MS/MS in ultrascan mode (26,000 amu/s). MS/MS analysis was initiated on a contact closure signal triggered by Chromeleon chromatography software (Dionex).

Proteomic analysis of AIHV-1 derived peptides. Peptide spectra were submitted to MASCOT (version 2.1; Matrixscience [25]) and searched against AIHV-1-specific and NCBI nonredundant protein sequence databases. Generally, proteins were identified by the assignment of at least two peptides with Mascot Mowse scores of >40 ($P < 0.0001$). Any peptide hits with lower scores were inspected manually and included in the assignment only if a series of at least four continuous y or b ions was observed. The identification of any protein based on a single peptide match was considered reliable only if the protein was identified in more than one virus preparation.

The relative abundance of individual proteins in the virulent and attenuated

viruses was estimated by comparing the number of peptides identified for each protein obtained from the different virus preparations.

RESULTS

One of each of the low-pass virulent and high-pass attenuated AIHV-1 virion preparations was analyzed by electron microscopy to assess the quality of the purified virus. Little contaminating cellular material was observed in either preparation. The low-pass AIHV-1 was found to contain 4.43×10^{10} particles per ml with 5% defective nonenveloped particles, while the high-pass preparation contained 7.76×10^9 particles per ml with 10% defective viruses. Thus, the method used yielded high-titer, high-quality virus preparations with relatively low contamination with unenveloped virus particles or nonvirion material.

Proteins from low-pass and high-pass virion preparations were analyzed by LC-ESI-MS/MS after fractionation by SDS-PAGE to provide a qualitative picture of virion protein composition. The entire analysis procedure was repeated for two independent virus preparations of virulent and attenuated AIHV-1 C500. Twenty-three AIHV-1-encoded proteins and six cellular proteins were identified in the attenuated and virulent particles. Two virus proteins were detected only in the virulent virus preparations, while one virus protein was found only in the attenuated virus (Fig. 1 and Table 1).

Analysis of AIHV-1 virion-associated proteins. (i) Capsid proteins. The gammaherpesvirus viral capsid is comprised of four structural proteins encoded by ORF25 (major capsid protein), ORF62 (triplex-1 protein), ORF26 (triplex-2 protein), and ORF65 (small capsomer-interacting protein [SCIP]) (18). In addition, the scaffolding protein, encoded by the 3' end of ORF17 (termed ORF17.5), is involved in the assembly of the capsid but is lost when the viral genome is packaged. Variable amounts of ORF17.5 have been found associated with immature but not mature forms of the human herpesvirus 8 (HHV-8) virion (21).

In both attenuated and virulent AIHV-1 particles the major capsid protein, ORF25, appeared to be the most abundant protein identified, based on the number of peptides detected (Table 1). The triplex proteins (ORF62 and ORF26) were also detected in both forms of the virus (Table 1). Interestingly, ORF65, encoding the SCIP protein, was only detected as a single peptide in one preparation of attenuated virions but was identified in matrix-assisted laser desorption ionization-time of flight analysis of prominent bands in an attenuated virion preparation fractionated by SDS-PAGE (Mowse score 153 [data not shown]). This may reflect low abundance of the SCIP protein in AIHV-1 capsids or difficulties in fractionation and/or ionization of SCIP peptides in the LC-ESI-MS/MS method.

Peptides corresponding to ORF17 were identified in both attenuated and virulent preparations of AIHV-1. All of the ORF17 peptides detected corresponded to peptides in the C-terminal part of the ORF17 sequence and are therefore probably derived from the scaffold protein ORF17.5. This is supported by the observation that all ORF17 peptides were derived from gel bands nearer the predicted size of ORF17.5 (30 kDa) than that of ORF17 (60 kDa) (Fig. 1).

(ii) Glycoproteins. Analysis of the AIHV-1 sequence predicts that it encodes homologues of the herpesvirus glycopro-

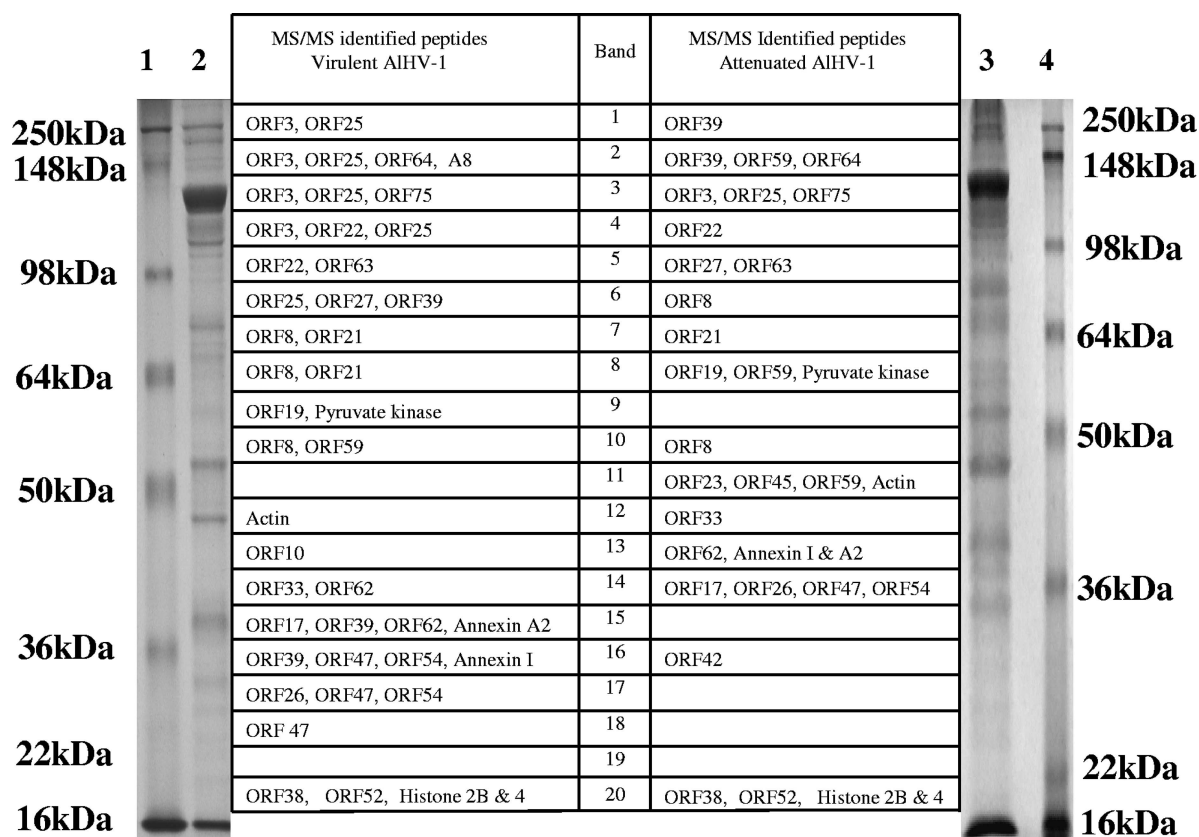


FIG. 1. Protein composition of purified virulent and attenuated forms of the AIHV-1 virus particle. Virulent (lane 2) or attenuated (lane 3) sucrose-gradient purified virions were resolved on SDS-10% PAGE gels alongside lanes containing SeeBlue Plus2 prestained protein standard (lanes 1 and 4; Invitrogen). Protein bands were visualized by staining with SimplyBlue SafeStain (Invitrogen). Each lane of the SDS-PAGE gel was split into 20 equal bands. Proteins in each band were digested in-gel with trypsin and subjected to LC-ESI-MS/MS analysis. MS/MS spectra matching AIHV-1 proteins and cellular proteins are indicated in each band. An estimate of the size of the identified proteins can be obtained from the molecular mass markers (lanes 1 and 4).

teins B (gB, ORF8), gH (ORF22), gL (ORF47), gN (ORF53), and gM (ORF39). In addition, putative glycoproteins are encoded by the gammaherpesvirus conserved gene ORF27 and by the MCF virus-specific genes A7, A8, and A10 (19, 11). AIHV-1 homologues of gB, gM, gH, and gL were identified in both the attenuated and the virulent virus preparations (Table 1). Peptides corresponding to gB were isolated from two distinct gel slices with approximate masses of 80 and 50 kDa, respectively (Fig. 1). Detailed analysis confirmed that the gB-specific peptides identified in the 80-kDa gel slice localized to the N-terminal part of the protein, while peptides in the 50-kDa gel slice came from the C-terminal portion of gB. Analysis of the AIHV-1 gB protein sequence identified a furin cleavage site (RRQR) at the boundary between the two domains (Fig. 2). Supporting evidence for the cleavage of gB was obtained by matrix-assisted laser desorption ionization-time of flight analysis of prominent AIHV-1 virion bands extracted from polyacrylamide gels (data not shown). The absence of a detectable signal for full-length gB, found in association with cellular membranes of other gammaherpesviruses, by MS or Western blotting (data not shown), is further evidence for the quality of the virion preparations used in the present study.

Peptides corresponding to gM (44 kDa), ORF27 (35 kDa), and gL (19 kDa) and the N terminus of gB (50 kDa) were

isolated from gel slices above their predicted masses (Fig. 1). This is in accord with the predicted glycosylation of these proteins. Indeed, gM (ORF39) was identified at multiple positions in both virulent and attenuated virus preparations, at approximate masses of 35, 80, and >100 kDa. The presence of the slowest-migrating form may be a consequence of temperature-dependent aggregation which has been observed for the gM of other herpesviruses (2, 16, 17).

AIHV-1 specific glycoproteins A7, A8, and A10 were among 10 genes in the AIHV-1 genome reported as being "unique" to this virus (11). Based on the presence of transmembrane regions and potential glycosylation sites, these proteins have been described as putative glycoproteins (8, 11). Of these three proteins, only A8 was detected in the present study, as a single peptide, in both preparations of the virulent virus. This peptide was derived from a gel slice with an estimated mass of 150 kDa (Fig. 1). The identity of the peptide DENLYVLAQIPALEGHK (residues 272 to 290; Fig. 3) was confirmed by secondary fragmentation, in which the predicted sequence of the peptide could be inferred from the masses of multiple adjacent fragmentation ions ($n = 4$ and $n = 7$; Mowse scores of 35 and 55). No peptides corresponding to A7, A8, or A10 were identified from preparations of attenuated C500 particles.

TABLE 1. Virus and host proteins associated with attenuated or virulent AIHV-1 virions identified by LC-ESI-MS/MS^a

AIHV-1 ORF	Identified polypeptides Description	Predicted size (kDa)	C500 ^b			
			Attenuated		Virulent	
			% Coverage	No. of peptides	% Coverage	No. of peptides
Glycoproteins						
8	N-terminal gB	50	17	6	15	5
8	C-terminal gB	47	13	5	20	10
22	gH	84	19	9	16	4
27	Glycoprotein	35	5	1	8	2
39	gM	44	16	4	11	2
47	gL	19	17	2	11	2
A8	Putative glycoprotein	75	0 ^c	0	2	1
Capsid proteins						
17	SCAF/PRO	59	19	5	8	2
25	Major capsid protein	154	49	40	41	33
26	TRI-2	35	29	4	21	4
62	TRI-1	37	58	9	21	3
Tegument proteins						
3	FGARAT	154	30	21	32	18
19	Tegument	62	16	3	5	3
33	Tegument	38	47	5	28	2
38	Tegument (role in egress)	7	72	2	69	2
52	Tegument (role in egress)	15	37	2	33	2
63	Tegument	108	14	4	10	5
64	Tegument	292	18	23	16	13
75	FGARAT	148	11	8	16	11
Virion-associated proteins						
10	Unknown function	46	0	0	14	3
21	Thymidine kinase	64	23	8	30	10
45	Unknown function	26	4	1	0	0
54	dUTPase	34	23	8	18	2
59	Processivity factor	46	22	4	12	2
Host proteins						
	Actin		56	11	49	7
	Annexin A2		44	8	42	9
	Annexin I		23	3	28	4
	Histone 2B		35	3	35	3
	Histone 4		42	4	40	4
	Pyruvate kinase		9	3	25	7

^a Virus and host proteins were identified using MS/MS data according to the criteria described for proteomic analysis in Materials and Methods.

^b The percent coverage and number of peptides were derived from MASCOT output.

^c The "0" indicates that no peptides meeting the described criteria were reproducibly identified from this ORF in this virus.

(iii) **Tegument proteins.** The tegument layer is believed to contain proteins that may be involved in the initiation of virus infection or in the assembly and morphogenesis of progeny virus. Based on comparisons with other gammaherpesviruses, 10 AIHV-1 proteins are predicted to belong to the tegument (11, 14). Proteomic analysis of purified virions identified seven of these proteins (the products of ORFs 3, 19, 33, 38, 63, 64, and 75; Table 1) as being present in both the attenuated and the virulent forms of the virus. The other three described tegument proteins (ORFs 23, 35, and 42) were not reproducibly identified in either attenuated or virulent particle preparations.

(iv) **Nonstructural viral proteins.** In the present study, viral nonstructural proteins were considered putative components of the virion if relevant peptides could be identified in the analysis of two independent particle preparations. In both attenuated and virulent particles, LC-ESI-MS/MS data identified the products of ORF21 (thymidine kinase), ORF54 (dUTPase), and ORF59

(processivity factor; Table 1) as being putative virion proteins. In addition, peptides from the ORF10 protein were only found in the virulent virus particles, while ORF45 was identified only in the attenuated virus (Table 1).

(v) **Cellular proteins.** During particle assembly, cellular proteins may be encapsulated into the virion. Peptides from cellular proteins associated with the virus particle were identified by searching all LC-ESI-MS/MS data against the nonredundant NCBI protein sequence database. Cellular proteins specifically associated with virus, i.e., identified in at least two independent particle preparations, included annexins, histones, actin, and pyruvate kinase (Table 1).

DISCUSSION

The aims of this study were twofold: (i) to identify the major components of the AIHV-1 virion, complementing the existing

MAHTGSTVCA FLIFAVLKNV FCQTPTSSE VEDVIPEANT VSDNIIRQQR NNTAKGIHSD
 PSAFPFRVCS **ASNIGDIFRF** QTSHSCPNTK **DKEHNEGILL** **IFKENIVPYV** **FKVRKYRKIV**
TTSTIYNGIY **ADAVTNQHFV** **SKSVPIYETR** RMDTIYQCYN SLDVTVGGNL LVYTDNDGSN
 MTVDLQPVVDG LSNSVRRYHS **QPEIHAEPGW** **LLGGYRRRTT** VNCEVTETDA **RAVPPFRYFI**
TNIGDTIEMS **PFWSKAWNET** EFSGEPDRTL TVAKDYRVVD YKFRGTQPQG HTRIFVDKEE
YTLSSWAQQFR NISYCRWAHW KSFDNAIKTE HGK**SLHFVAN** **DITASFYTPN** **TQTRVFLGKH**
 VCLNNTIESE LKSRLAKVND THSPNGTAQY YLTNGGLLLV WQPLVQQKLL DAKGLLDAVK
 KQQNTTTTTT TTRSRR**QRRS** **VSSGIDDVYT** **AESTILLTQI** **QFAYDTLRAQ** **INNVLEELSR**
 AWCREQHRAS **LMWNELSKIN** PTSVMSSIYG RPVSAKRIGD VISVSHCVV DQDSVSLHRS
 MRVPGRDKTH ECYSRPPVTF KFINDSHLYK GQLGVNNEIL LTTAVEICH ENTEHYFQQG
 NNMYFYKNYR HVKTMPVGDV ATLDTFMVLN LTLVENIDFQ VIELYSREEK **RMSTAFDIET**
MFREYNYTQ RVTGLRRDLT DLATNR**NQFV** **DAFGSLMDDL** **GVVGKTVLNA** VSSVATLFSS
 IVSGIINFIK NPFQGLLFG LIAAVVITVI LLNRKAKRFA **QNPVQMIYPD** **IKTITSQREE**
 LQVDPIISKHE LDRIMLAMHD YHASKQPEK QDEEQGSTTS GPADWLNKAK NVLRRRAGYK
 PLKRTDSFE STGVP

FIG. 2. Glycoprotein B-specific peptides from both attenuated and virulent particles were identified in two distinct bands that corresponded to N- and the C-terminal fragments of gB. Tryptic peptides identified from the 80-kDa band are shown in boldface, while peptides identified from the 50-kDa band are shown in boldface and underlined. A furin cleavage site, RQRR (shaded in gray), marks the likely boundary between the N- and the C-terminal domains of the protein.

genome annotation, and (ii) to determine whether structural differences existed between the attenuated and virulent forms of AIHV-1. Sucrose-gradient purified virus preparations were fractionated by SDS-PAGE, and peptides from serial gel slices were analyzed by LC-ESI-MS/MS. This qualitative analysis identified five herpesvirus-conserved envelope glycoproteins, one AIHV-1 “unique” glycoprotein, eight tegument protein candidates, four capsid components, and five nonstructural virus proteins.

Similarities with other rhadinoviruses. Previous analysis of the AIHV-1 genome had identified strong similarities between the genomic organization of AIHV-1 and that of other members of the *Rhadinovirus* genus of the gammaherpesvirus subfamily (6, 11). With the exception of ORF28, which has no homologue in AIHV-1 or OvHV-2 (11, 14), only gN (ORF53) was not found in either attenuated or virulent AIHV-1, but it has been consistently identified in published studies as a component of the gammaherpesvirus virion (23, 38).

ORF53 encodes a type 1 membrane protein (gN) that is one of five glycoproteins (gB, gH, gL, and gM being the others) that are conserved throughout the herpesvirus family. Studies on HHV-8 and EBV have shown that gN is dependent on the expression of gM for its processing and is found in the virion in a complex with gM (16, 17). Since mature gM was identified in both attenuated and virulent AIHV-1 particles, it is likely that gN is also a component of this virus. The failure to detect peptides corresponding to gN may relate to its size. Based on the molecular mass markers, the SDS-PAGE separation reported here resolved virion proteins between 250 and 16 kDa (Fig. 1). Without posttranslational modification, the predicted molecular mass of mature AIHV-1 gN is 8.8 kDa, whereas the native EBV gN has a mass of 15 kDa (17), suggesting that the AIHV-1 gN may have been lost during electrophoresis.

Alternatively, failure to detect peptides derived from gN may indicate that peptides from gN did not ionize efficiently by ESI. Supporting evidence for this is provided by the observa-

MD**NY**TLALNC NHTTHGYAHY VYSTLSYLVF IGNAPGYPCNC ENCIYDITF**N** STSML**N**ISNN
 YFHIANATLG APEYPEQPVT LSVIGEESPW VAWLAVHTPD **KNY**TEDANTA RAVMRVLDPF
 NISWCAVYNV QSVDPDTQD **ISN**CTEVSEE LPVNNQKSSI HLNTYFLNST FTVSVSLNAS
NISSQCSGNV SLE**N**MTSNVH IPCPTLPLTV SVKASTDNMQ TGAAV**N**ASID IGWLL**Q**NLTD
 ASLTVSSSPG NLTQKNCTTY KNISKTRHV **R**DENLYVLAQ **I**PALEGHKDS FTLTRSPILL
 NFSRNSSEFQ LVLYDRNSSC VWANPANGTD TILVSMSCPH ITATISPRGE IKV**N**VTGNFS
 RNANLSLAFLL SSKGKEYAGV LLQAFEPSTR PPPGT**V**APGI **L**STANFETS **T**NKSS**P**TYTP
TPAKLSTPPG LTNTLLLTAG EHNSGIGSTL EPLTTVSVQV **L**QTPSSPTRD **T**STLVIKL**T**T
 VPQDHKT**V**SP SLV**T**PGRST LPIVSM**T**HFS REGSSPK**P**QT **T**AAK**T**SSEAS LPPL**L**TT**T**PT
PT**N**TEKS**Q**ST FAS**S**TVSVD**T** **T**FTGDDV**N**TV **G**T**M**SPS**I**TQ**T** LP**I**TP**T**SGRQ Y**I**VVGC**T**LN
 RRS**G**N**L**FFF**F** LFVA**A**HRES**G** HNT**S**MP**N**PH**H** NSV**K**PE**D**HP**H** H**P**EG**D**HP**D**A**D** H**H**ER**F**Q**I**W**L**L
 P**I**AG**T**I**F**AL**V** AL**V**IV**N**I**A**LR M**T**E

FIG. 3. A single peptide from A8, DENLYVLAQIPALEGHK, with a Mowse score of 55, was identified by MASCOT from the virulent C500 virion preparation. The tryptic peptide DENLYVLAQIPALEGHK (residues 272 to 290), shaded in gray, is located centrally in the predicted A8 polypeptide sequence. Potential N-glycosylation sites are shown in boldface italics. Potential O-glycosylation sites are shown underlined.

tion that gN in the HHV-8 and EBV particles was identified by one C-terminal peptide (38, 15). Furthermore, identification of gN within the EBV particle required the virion to be treated prior to analysis by deglycosylation enzymes (15). In the present study, deglycosylation was not carried out. Analysis of the predicted amino acid sequence of mature AIHV-1 gN identified only four tryptic peptides with masses from 547 to 3,560 mass units. One peptide is predicted to contain both N-linked and O-linked glycosylation sites. It is therefore possible that a combination of posttranslational modification and the small number of tryptic peptides available could also prevent the detection of AIHV-1 gN.

Proteins encoded by ORF23, ORF35, and ORF42 were reported to be tegument proteins in rhesus monkey rhadinovirus (RRV [23]). Single spectra corresponding to ORF23 and ORF42 were identified in only one attenuated virus preparation, so these proteins were not considered to be definite virion components. It is possible that the ORF23-, ORF35-, and ORF42-encoded proteins are not abundant in the tegument or that peptides derived from these proteins did not ionize efficiently by ESI. Indeed, these proteins were not identified in analyses of EBV, HHV-8, or murine gammaherpesvirus 68 (MHV-68) virions (5, 15, 38). The analyses of HHV-8 and MHV-68 virions were, however, based on excision of prominent bands from polyacrylamide gels and therefore have the potential to miss less-abundant proteins. The full chromatographic separation of RRV (23) and the systematic analysis of entire gel lanes described here may be more suited to the detection of such proteins.

Six cellular proteins were identified in association with attenuated and virulent AIHV-1 virions. Of these, actin, annexin (I/A2) and pyruvate kinase have previously been identified in association with rhadinovirus virions (5, 38). Bovine protein sequence entries are not as well represented as other species, so it is possible that some virus-associated host proteins may not have been identified from MS data. Equivalent amounts of the annexins and histones, based on the number of peptides detected, were observed in the attenuated and virulent AIHV-1 virions. However, differences were observed in the number of peptides detected for actin and pyruvate kinase between the two forms of the AIHV-1 virions (Table 1). This may reflect differences in the assembly of the two AIHV-1 particle types or suggest that these molecules are nonspecifically included in assembled virions.

Only two proteins were detected in AIHV-1 that were not found in proteomic analyses of other gammaherpesviruses, A8 and ORF10. Interestingly, spectra for these two proteins were observed in the virulent AIHV-1 preparations but not in the attenuated virus. A8 is in a region that is potentially affected by rearrangements leading to attenuation (37), while there is no evidence to suggest these genomic changes involve the ORF10 gene.

A7, A8, and A10. Analysis of the genome of tissue culture-attenuated AIHV-1 C500 predicted that genomic rearrangements might dysregulate or abrogate the expression of putative glycoproteins A7, A8, and A10 (34). Recent sequencing of other livestock gammaherpesviruses has shown that all three genes have homologues in the MCF virus OvHV-2 (14), while homologues of A7 and A8 are found in the sequenced segment of porcine lymphotropic herpesvirus-1 (12).

A7 has sequence similarity to its positional analogue in EBV, BZLF2. This gene encodes gp42, which forms a complex with gH and gL and interacts with HLA-DR, facilitating infection of B cells (30). EBV gp42 is, however, dispensable for infection of epithelial cells (34). Stoichiometric analysis of EBV particles has shown gH and gL to be more abundant than gp42, indicating that a proportion of particles produced during EBV infection naturally lack or contain low levels of gp42 (34). Analysis of the composition of EBV virions using LC-ESI-MS/MS analysis identified the presence of a significant number of peptides corresponding to gp42 within the virus particle. Furthermore, identification of gp42 was not dependent on deglycosylation of the EBV virion prior to analysis (15). In contrast, peptides corresponding to A7 were not identified in either attenuated or virulent AIHV-1 virions. It is feasible that A7 was not detected because its peptides were unsuitable for detection by LC-ESI-MS/MS due to their size, amino acid composition, or posttranslational modification. Alternatively, the lack of A7 in AIHV-1 virion preparations may reflect low levels of A7 expression within the cell line used to propagate AIHV-1. Further investigations, dependent on the development of suitable reagents, are required to determine whether A7 is indeed a virion component and what role it plays, if any, in the attenuation of AIHV-1.

A8 is a positional homologue of genes encoding EBV gp220/350, HHV-8 K8.1, and MHV-68 gp150. These glycoproteins have been implicated in the binding of host cell receptors (31). A8 was identified by the same, single peptide in both preparations of purified virulent virions, derived from a gel slice with an estimated mass of 150 kDa (Fig. 1). A8 is predicted to be highly glycosylated (Fig. 3), so the difference between the predicted mass (75 kDa) and the mass observed here (150 kDa; Fig. 1) is likely to be a consequence of posttranslational modification or, like gM, protein aggregation. A high level of glycosylation may also explain the identification of only a single peptide from A8. Only about half of the 30 predicted tryptic cleavage products of A8 are free of predicted N-linked or O-linked glycosylation, with the nonglycosylated peptides being restricted to two areas: one central area of about 120 residues including the detected peptide, DENLYVLAQIPAL EGHK, and a second area encompassing the C-terminal 90 residues. The size and amino acid composition of the individual peptides may further restrict which peptides can be detected.

Supporting evidence for this view was found during analysis of the A8 homologue, Ov8 from OvHV-2, expressed as a soluble recombinant protein in mammalian cells. Proteomic analysis of a gel band containing microgram quantities of purified Ov8 identified only three Ov8-specific spectra. The corresponding three peptides were derived from the central area of the Ov8 protein sequence that is equivalent to the position of the identified peptide from A8 (J. P. Stewart, unpublished data).

The modification of envelope glycoprotein genes in attenuation has been observed in the alphaherpesvirus equine herpesvirus 1 (EHV-1). Studies on culture-adapted forms of EHV-1 have shown that attenuation results from the expression of a truncated form of a single glycoprotein, gp2 (29). However, truncation of A8 was not observed in the attenuation of AIHV-1 (37). The observed rearrangements in AIHV-1 involve the adjacent A7 gene and suggest that A8 may be dys-

regulated by rearrangements involving its promoter (within A7) or that it may be deleted.

EBV gp350/220 has been demonstrated to mediate attachment to and infection of B lymphocytes (22, 32, 33). Given that the position of A8 in the AIHV-1 genome is equivalent to genes encoding EBV gp350/220, HHV-8 K8.1, and MHV-68 gp150, A8 may act in the same way as these glycoproteins to mediate the attachment of AIHV-1 to lymphocytes. Dysregulation or abrogation of A8 should therefore be accompanied by a concomitant loss of binding of the virus to lymphocytes. Initial studies of virus-cell interactions indicate that binding of attenuated AIHV-1 to peripheral blood mononuclear cells is significantly less efficient than binding of virulent virus, suggesting that the attenuated virus may have a defect in binding to lymphocytes (I. Dry, G. C. Russell, D. M. Haig, and J. P. Stewart, unpublished data).

In contrast to A7 and A8, no clear sequence or positional glycoprotein homolog to A10 has been identified in other gammaherpesviruses. Peptides from the predicted glycoprotein A10 were not identified in any of the AIHV-1 virion preparations, suggesting that A10 is not an abundant virion-associated glycoprotein. Further research is needed to determine whether A10 is, in fact, a component of the infectious virus. Recent analysis of the genome of OvHV-2 has identified multiple nuclear localization signals in addition to a transmembrane anchor in Ov10, the homologue of A10 (14). This may mean that Ov10 and A10 play a nonstructural role in MCF virus infection and are not viral components.

ORF10. Three peptides from ORF10 were identified in two independent preparations of virulent AIHV-1, suggesting either that its expression was high enough for incorporation into the virion in only the virulent virus or that it is a genuine virion component that was not detected in the attenuated virion. ORF10 is a member of a family of herpesvirus genes that encode dUTPase-related proteins (9). In gammaherpesviruses this family also includes the ORF11 gene, whose product appeared to be incorporated into the particles of HHV-8 (38). ORF10 was previously detected in a rhesus monkey rhadinovirus particle (23) but was found in only one analysis; ORF10 was not designated as a component of this virus. No evidence has been presented to suggest that the orthologs of ORF10 are present in the virions of HHV-8 and MHV-68 (4, 38).

The region of the genome in which the ORF10 gene is located was not reported to be affected by the genomic rearrangements associated with the attenuation of AIHV-1 (37). The presence of this dUTPase-related protein within only the virulent form of the AIHV-1 particle suggests that the ORF10-encoded protein is included in the AIHV-1 virion via interactions with other protein(s) whose expression is abrogated or dysregulated by the rearrangements. Research on other gammaherpesviruses has shown that deletion of the region of the genome encompassing ORF10 and ORF11 has no effect on the ability of these viruses to grow in culture (20, 24), although ORF11 deletion viruses were found to have reduced lytic replication *in vivo* (4).

The recent development of a BAC of AIHV-1 (10) will enable the potential roles of genes such as A8 and ORF10 in the pathogenesis and attenuation of AIHV-1 to be studied *in vitro* and *in vivo*. Development of reagents, including recombinant viral antigens and antibodies to the viral proteins iden-

tified in the present study, may also provide important information about the role of the viral proteins in both host infection and the development of MCF in susceptible species.

Proteins that constitute the herpesvirus virion play functional roles both in the assembly and egress of the virus and in the initiation of infection. The qualitative methods used in the present study may not be sufficiently sensitive to detect subtle changes in the composition or assembly of the virion. However, few differences between attenuated and virulent particles were observed, suggesting that AIHV-1 attenuation may be mediated through changes in the expression of proteins that are not virion components, e.g., the transcription factors A6 or ORF50. Construction of a detailed map of the attenuated AIHV-1 C500 virus, based on complete DNA sequencing, may provide further clues to the mechanism of AIHV-1 attenuation.

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