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ScienceDirect
Virology 358 (2007) 233–247
VIROLOGY
www.elsevier.com/locate/yviro

Protein composition of the vaccinia virus mature virion

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Received 27 July 2006; returned to author for revision 3 August 2006; accepted 18 August 2006
Available online 26 September 2006

Abstract

The protein content of vaccinia virus mature virions, purified by rate zonal and isopycnic centrifugations and solubilized by SDS or a solution of urea and thiourea, was determined by the accurate mass and time tag technology which uses both tandem mass spectrometry and Fourier transform-ion cyclotron resonance mass spectrometry to detect tryptic peptides separated by high-resolution liquid chromatography. Eighty vaccinia virus-encoded proteins representing 37% of the 218 genes annotated in the complete genome sequence were detected in at least three analyses. Ten proteins accounted for approximately 80% of the virion mass. Thirteen identified proteins were not previously reported as components of virions. On the other hand, 8 previously described virion proteins were not detected here, presumably due to technical reasons including small size and hydrophobicity. In addition to vaccinia virus-encoded proteins, 24 host proteins omitting isoforms were detected. The most abundant of these were cytoskeletal proteins, heat shock proteins and proteins involved in translation.
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Keywords: Vaccinia virus; Poxvirus; Mass spectrometry; Proteomics

Introduction

The *Poxviridae* comprise a family of large double-stranded DNA viruses that replicate exclusively in the cytoplasm of vertebrate and invertebrate species (Moss, 2001). The best-known poxviruses are the closely related members of the genus *Orthopoxvirinae*: these include variola virus, the etiologic agent of smallpox; vaccinia virus (VACV), used as the vaccine to prevent smallpox; and monkeypox virus, which is endemic in parts of Africa and can cause a fatal disease in humans. VACV, the most thoroughly studied poxvirus, has a genome containing about 200 open reading frames (ORFs) that have been shown to encode proteins or are thought likely to do so. The ORFs can be divided into early, intermediate and late classes based on the time of their transcription. The early proteins include enzymes and factors needed for DNA replication and intermediate gene transcription as well as modulators of host defense; the intermediate proteins include factors for late gene transcription; and the late proteins include the major membrane and core components of the virus particle. Assembly of VACV particles occurs in specialized cytoplasmic areas known as virus factories and begins with the formation of crescent membranes that engulf electron-dense granular material known as viroplasm and enlarge to form spherical immature virions (reviewed in Condit et al., 2006). The immature virion, which probably contains all of the internal proteins and most of the membrane proteins, undergoes further maturational processes to form the mature virion (MV). MVs, the most abundant of the infectious forms, are released from the cell after lysis. However, a subpopulation of MVs is wrapped by a double membrane derived from virus-modified trans-Golgi or endosomal cisternae and transported via microtubules to the plasma membrane. There, the outermost membrane fuses with the plasma membrane resulting in the exocytosis of the extracellular virion (EV), which has one additional membrane relative to the MV. The MV is the most basic as well as the most abundant infectious particle and has been used for the vast majority of experimental studies.

MVs are barrel-shaped particles of approximately $360 \times 270 \times 250$ nm that lack icosahedral symmetry and have a complex internal structure comprising a core, core wall, lateral bodies and a surface membrane (Cyrklaff et al.,

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0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2006.08.025

2005). The MV contains many structural and enzymatic components, including a large protein complex involved in entry (Senkevich et al., 2005), a multi-subunit RNA polymerase (Kates and McAuslan, 1967; Munyon et al., 1967) and other factors required for the initial stages of virus replication. Previous estimates for the number of proteins in MVs, based on spots in two dimensional polyacrylamide gels, have been as high as 100 (Essani and Dales, 1979; Oie and Ichihashi, 1981). However, only the major 30 proteins resolved by gel electrophoresis were identified as unique by sequence analysis (Jensen et al., 1996; Takahashi et al., 1994). Additional virion proteins were identified by genetic and immunological methods during the course of numerous investigations. In two very recent reports, 63 (Yoder et al., 2006) and 75 (Chung et al., 2006) virus-encoded proteins were identified by mass spectrometry (MS) in MVs. The latter study also reported the presence of 23 cellular proteins associated with purified virions.

The work presented here confirms and extends the list of viral and cellular proteins using a complementary mass spectrometry approach based on peptide accurate mass and time tags (AMTs). In this approach, potential mass and time tags (PMTs) are identified by tandem MS (MS/MS) following separation of a large pool of tryptic peptides by a single step high pressure liquid chromatography (HPLC) using a high-resolution reversed phase C-18 capillary column. After generation of the database of PMTs, samples are analyzed multiple times using a Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer with high mass accuracy and a wide dynamic range. Only peptides from the PMT library that match a given normalized elution time and mass of peptides detected by the FT-ICR mass spectrometer, called AMTs, are reported. Once a PMT library has been generated, samples isolated under different conditions or prepared by different methods from the same organism can be analyzed by the high-resolution FT-ICR alone, without the need for routine MS/MS (Smith et al., 2002a, 2002b). The validation of peptides by the two methods results in higher confidence protein identifications. In addition, the FT-ICR approach is more sensitive than MS/MS alone and provides an abundance value for each peptide determined from the summed ion intensities which proves useful for relative quantitation.

Results and discussion

Identification of VACV-encoded proteins packaged into the MV

VACV MV particles were purified from cells infected with strain Western Reserve (WR) using two successive rate zonal centrifugations in sucrose gradients followed by isopycnic banding in cesium chloride. MV particles were analyzed after rate zonal banding as well as after the isopycnic purification step, but most data presented here are based on the latter preparation. To avoid the loss of sensitivity and poor resolution associated with gel electrophoresis based methods, purified MVs were solubilized directly with either 1% SDS or a mixture of 7 M urea and 2 M thiourea in the presence of a reducing agent. Soluble proteins were then alkylated and digested with trypsin. After desalting, the resulting peptide mixtures were separated and detected by reversed phase C-18 HPLC coupled with an ion trap mass spectrometer for MS/MS analysis. PMTs were identified using a database containing all putative open reading frames (ORFs) with 20 or more amino acids encoded by VACV WR. For each solubilization condition, mass tags were confirmed by matching measured masses and elution times from triplicate FT-ICR MS analyses with the PMT database. The relative abundance of detected proteins was approximated by the mean ion current of the representative peptides in the FT-ICR experiments. The abundance of these peptides between conditions used to solubilize the MV particles was normalized and compared between the six FT-ICR experiments. Clustering analysis showed the proteins that were more abundant in urea-solubilized particles than in pools from SDS-solubilized particles and vice versa (Fig. 1). However, grand average

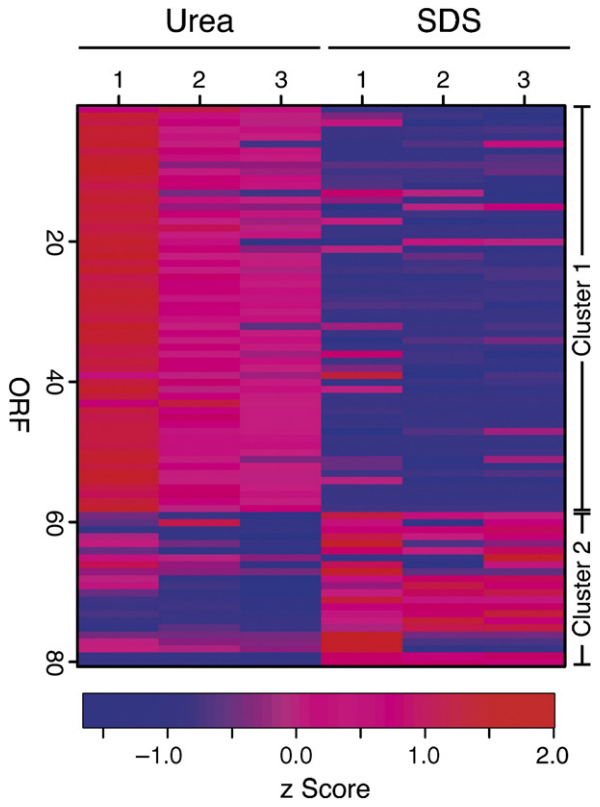

Fig. 1. Effect of protein extraction conditions on measured protein abundance. For each protein, 3 abundance measurements from the SDS peptide pool and 3 abundance measurements from the urea/thiourea pool were available. Each of the 6 values for a protein was standardized by subtracting their mean and dividing by their standard deviation. This results in negative values for measurements below the average of all 6 measurements and positive values for measurements above the average. The standardized abundance values were used to divide the 80 proteins into two clusters using kmeans clustering. Cluster 1 consisted of 58 proteins that were more abundant in the urea/thiourea peptide pools (A3, A5, A6, A10, A12, A13, A15, A18, A19, A2.5, A21, A24, A27, A29, A30, A42, A45, A50, B1, C22, D1, D2, D3, D6, D12, D13, E3, E6, E8, E9, E11, F4, F8, F9, F10, F13, F17, G4, G7, G9, H1, H2, H3, H5, H6, I1, I3, I6, I8, J1, J4, K4, L1, L4, L5, M1, N1, O2). Cluster 2 consisted of 22 proteins that were more abundant in the SDS peptide pool (VACV-WR_148, A4, A7, A9, A11, A14, A17, A26, A28, D8, D11, E1, E4, E10, G1, G5.5, H4, I7, J3, J5, J6, L3).

hydropathy (Kyte and Doolittle, 1982), molecular weight or charge of the proteins was not correlated with the clustering (data not shown). Peptides from some proteins were only detected under one of the specific conditions, highlighting the utility of different sample preparation methods. From here on, abundance will refer to the mean abundance value of all six experiments which represents the summed peak intensity values for all peptides of a given protein, rolled-up into a protein abundance value. Note that different physical properties of individual peptides, such as ionization efficiency and solubility make this abundance value only a rough estimate of absolute protein abundance not well suited for comparison between different proteins. However, the abundance estimates were correlated with the coverage of protein sequence by detected AMTs (Spearman correlation coefficient 0.62; $P=10^{-9}$), a surrogate measure for protein abundance, as well as abundance measurements carried out previously using a different method (Spearman correlation coefficient 0.74; $P=10^{-6}$) (Chung et al., 2006). This suggested that the abundance values might be used to determine overall patterns and trends in the group of MV proteins as a whole.

Neither this mass spectrometry study nor the others (Chung et al., 2006; Yoder et al., 2006) identified any novel ORF that had not been annotated in the published WR genome sequence (***NC_006998***). AMTs for a total of 93 VACV proteins were detected. Of these, 80 had at least one AMT detected in at least three experiments carried out with density equilibrium purified MV (Table 1) and at least 2 unique AMTs in at least three experiments carried out with either rate zonal or density equilibrium purified MV (data not shown). Identified proteins were sorted in order of decreasing estimated abundance. Table 1 shows ORFs encoding packaged proteins by their annotated nomenclature, as well as the more commonly used nomenclature for their orthologs in the Copenhagen strain. For simplicity, the latter notation is used throughout this manuscript (except where there is no corresponding Copenhagen ORF) and the letter indicating directionality of the ORF (L or R) is omitted when referring to proteins.

The majority of proteins identified in this study were involved in forming and maintaining the MV structure, transcription or entry (Fig. 2). The structural proteins had higher estimated abundance values and AMT coverages than other components of the MV ($P=0.0008$), as was expected. Based on rough calculations of protein abundance and molecular weight, the 10 most abundant proteins, which include the known major MV proteins F17, A3, A4, A10 and A17, accounted for approximately 80% of the MV protein mass, similar to data reported previously (Chung et al., 2006; Sarov and Joklik, 1972).

Of the identified proteins, 51 (64%) are known to be expressed late and 11 (14%) are expressed throughout the replication cycle, typical for viral structural proteins. Thirteen proteins (16%) with early or intermediate expression kinetics were identified as well. However, 8 of the 13 proteins were found to have a TAAAT late promoter motif (Davison and Moss, 1989) within 30 base pairs upstream of their translation initiation codon, raising the possibility that there may be previously undetected late expression. The majority of the proteins (51; 64%) were known to be essential and highly conserved proteins were considerably overrepresented in the MV protein complement ($P<10^{-5}$; Fig. 3A). Consistent with this observation, ORFs encoding MV proteins were enriched in the more highly conserved center of the VACV genome (Fig. 3B; $P<10^{-4}$; randomization test). In addition, MV proteins had a higher median isoelectric point by 0.7 ($P=0.008$), and a 7.5 kDa higher median molecular weight ($P=0.002$) than non-MV proteins. Alanine and proline residues were approximately 1.3-fold overrepresented in MV proteins ($P=0.02$ and $P=0.04$, respectively), whereas cysteine residues were 1.5-fold less frequent than expected ($P=0.001$).

VACV proteins previously shown to be present in MVs

The majority of the proteins identified in this study (67 out of 80 total) have been identified in previous publications examining one or a few individual proteins (see references in Table 1), illustrating the utility of the approach in the identification of proteins in a biological system. When consolidating our analysis with two recently reported proteomic studies of MVs purified by sucrose gradient sedimentation only (Chung et al., 2006; Yoder et al., 2006), a consensus MV complement of 73 proteins detected by at least two of the three studies could be identified. This set was expanded to 80 by the addition of 7 proteins (I6, J5, B1, D7, A22, A14.5, G5) that have been previously described as MV proteins and were detected in one or none of the MS studies (Betakova et al., 2000; da Fonseca et al., 2004; DeMasi et al., 2001; Garcia and Moss, 2001; Lin et al., 1992; Quick and Broyles, 1990; Senkevich et al., 2005). Of the 80 VACV proteins, 72 were demonstrated in this study and are highlighted in Table 1. The functions of the other 8 proteins are described in Table 2. Two (A14.5, G5) of the 8 proteins were previously described as MV proteins but were not detected in any of the proteomic studies. Technical reasons, in particular the high hydrophobicity and small size of the A14.5 protein, may explain the inability to detect these proteins. The D7 and A22 proteins were previously described as MV proteins and detected in one of the proteomic studies. The I5, G3, A16 and A46 proteins were detected by both previously published proteomic studies and were also reported as MV proteins in earlier studies (Ojeda et al., 2006; Senkevich et al., 2005; Takahashi et al., 1994) but were absent from the present analysis of virions purified by rate zonal and isopycnic centrifugation. We did detect three of the latter four proteins as well as one other, however, when MVs were purified by two sucrose gradients only and the isopycnic centrifugation step was omitted (Table 2).

VACV proteins not previously reported to be associated with MVs

Five proteins (H5, F8, E3, E6, A6) in the consensus MV protein set of the three proteomic studies were not previously reported as components of VACV virions. Except for A6, these proteins were in the upper 50% with respect to confidence in our analysis (Table 1). The product of the H5R ORF is a major

Table 1
VACV proteins identified by AMTs

WR [a]	Cop ortholog	Description	Unique AMTs						C [b]	Mass [c]	T [d]	E [e]	References
			Urea [f]			SDS							
			1	2	3	1	2	3					
VACV-WR_056	***VACV-CP_F17R***	Putative DNA-binding phosphoprotein in virus core; morphogenesis	5	6	6	5	4	3	42	11.3	L	+	Jensen et al., 1996; Kao and Bauer, 1987; Wittek et al., 1984
VACV-WR_137	***VACV-CP_A17L***	MV membrane protein; morphogenesis	3	2	3	3	2	2	15	23.0	L	+	Jensen et al., 1996; Rodriguez et al., 1993; Takahashi et al., 1994
VACV-WR_122	***VACV-CP_A3L***	P4b [74 kDa] precursor of core protein 4b [62 kDa]; morphogenesis	28	29	28	21	16	13	49	72.6	L	+	Katz and Moss, 1970; Rosel and Moss, 1985; Takahashi et al., 1994
VACV-WR_091	***VACV-CP_L4R***	Core protein VP8; early transcription defect; stimulates I8 helicase [WR_077]	13	13	13	9	8	6	63	28.5	L	+	Jensen et al., 1996; Takahashi et al., 1994; Yang et al., 1988
VACV-WR_123	***VACV-CP_A4L***	Immunodominant 39 kDa core protein; morphogenesis	11	10	10	10	6	8	40	30.9	L	+	Jensen et al., 1996; Maa and Esteban, 1987
VACV-WR_133	***VACV-CP_A14L***	Phosphorylated MV membrane protein; morphogenesis	1	1	1	2	2	1	17	10.0	L	+	Jensen et al., 1996; Rodriguez et al., 1997; Takahashi et al., 1994
VACV-WR_129	***VACV-CP_A10L***	Precursor P4a [103 kDa] of core protein 4a [62 kDa]; morphogenesis	50	49	46	18	14	13	54	102.3	L	+	Jensen et al., 1996; Katz and Moss, 1970; Takahashi et al., 1994; Van Meir and Wittek, 1988
VACV-WR_150	***VACV-CP_A27L***	MV surface protein; required for MV wrapping	5	5	6	9	9	5	76	12.6	L	−	Ichihashi et al., 1984; Jensen et al., 1996; Takahashi et al., 1994
VACV-WR_149	***VACV-CP_A26L***	p4c protein; directs intracellular mature virus particles into a-type inclusions	13	13	12	7	6	6	27	58.0	L	−	McKelvey et al., 2002
VACV-WR_113	***VACV-CP_D8L***	MV membrane protein binds cell surface chondroitin	7	7	7	5	5	4	33	35.4	L	−	Jensen et al., 1996; Niles and Seto, 1988; Takahashi et al., 1994
VACV-WR_132	***VACV-CP_A13L***	MV membrane protein; phosphorylated; morphogenesis	5	5	4	3	3	3	51	7.7	L	+	Jensen et al., 1996; Takahashi et al., 1994; Unger and Traktman, 2004
VACV-WR_131	***VACV-CP_A12L***	Virion core protein	6	6	6	5	5	4	26	20.5			Takahashi et al., 1994
VACV-WR_101	***VACV-CP_H3L***	MV membrane protein; binds heparin; involved in IV maturation	11	12	12	7	6	5	40	37.4	L	−	da Fonseca et al., 2000a, 2000b; Jensen et al., 1996; Takahashi et al., 1994
VACV-WR_103	***VACV-CP_H5R***	Substrate of B1 [WR_183] kinase; late gene transcription factor VLTF-4; morphogenesis	2	2	2	1	1	1	15	22.3	EL	+	Beaud et al., 1995; Murcia-Nicolas et al., 1999
VACV-WR_099	***VACV-CP_H1L***	Tyr/Ser protein phosphatase (VH1); required for early transcription by MV	8	8	8	5	5	5	57	19.7	L	+	Guan et al., 1991; Liu et al., 1995; Paez and Esteban, 1984
VACV-WR_126	***VACV-CP_A7L***	82 kDa large subunit of early gene transcription factor VETF	14	11	10	6	3	4	25	82.3	L	+	Gershon and Moss, 1990; Hu et al., 1996; Li et al., 1994
VACV-WR_093	***VACV-CP_J1R***	Virion core protein; morphogenesis	5	5	5	1	1	1	31	17.9	L	+	Chiu and Chang, 2002; Szajner et al., 2004a
VACV-WR_107	***VACV-CP_D2L***	Virion core protein; morphogenesis	6	6	6	0	0	0	51	16.9	L	+	Dyster and Niles, 1991; Szajner et al., 2004a
VACV-WR_085	***VACV-CP_G7L***	Virion core protein; morphogenesis	11	11	10	7	6	5	32	42.0	L	+	Szajner et al., 2003; Szajner et al., 2004a; Takahashi et al., 1994
VACV-WR_047	***VACV-CP_F8L***	Protein with iActA-like proline repeats; not required for actin tails formation	3	2	2	3	3	2	46	7.9	EL	−	Higley and Way, 1997
VACV-WR_090	***VACV-CP_L3L***	Required for early transcription by cores	5	5	5	5	4	4	15	40.6	L	+	Resch and Moss, 2005
VACV-WR_088	***VACV-CP_L1R***	MV membrane protein; target of neutralizing antibody; S–S bond	2	2	2	1	1	1	9	27.3	L	+	Franke et al., 1990; Jensen et al., 1996;

Table 1 (*continued*)

WR [a]	Cop ortholog	Description	Unique AMTs						C [b]	Mass [c]	T [d]	E [e]	References
			Urea [f]			SDS							
			1	2	3	1	2	3					
		formation pathway thiol substrate; myristylated; morphogenesis											Ravanello and Hruby, 1994; Senkevich et al., 2000b
VACV-WR_076	***VACV-CP_I7L***	Viral core cysteine proteinase; morphogenesis	8	9	8	3	3	2	18	49.0	L	+	Ansarah-Sobrinho and Moss, 2004a; Byrd et al., 2002; Kane and Shuman, 1993
VACV-WR_095	***VACV-CP_J3R***	Multifunctional: stimulatory poly-A polymerase subunit, cap (nucleoside-O_2)-methyltransferase and transcription elongation factor	12	12	11	7	6	5	42	38.9	E		Brown et al., 1973; Gershon et al., 1991; Latner et al., 2000; Schnierle et al., 1992
VACV-WR_098	***VACV-CP_J6R***	DNA-dependent RNA polymerase subunit rpo147	24	25	25	16	13	10	28	146.8	EL	+	Broyles and Moss, 1986; Ensinger, 1987; Hooda-Dhingra et al., 1989
VACV-WR_121	***VACV-CP_A2.5***	Part of S–S bond formation pathway ; morphogenesis	2	2	2	1	1	1	24	8.9	L	+	Senkevich et al., 2002a; Senkevich et al., 2002b
VACV-WR_064	***VACV-CP_E8R***	Membrane-associated protein may help wrap virosome; associates with IV/MV and cores	9	9	8	3	3	3	44	31.9	E		Doglio et al., 2002
VACV-WR_108	***VACV-CP_D3R***	Virion core protein; morphogenesis	6	6	6	3	1	1	42	28.0	L	+	Dyster and Niles, 1991; Szajner et al., 2004a
VACV-WR_059	***VACV-CP_E3L***	Double-stranded RNA binding protein; inhibits antiviral activities of interferon; host-range determination	3	3	3	4	2	2	21	21.5	E	−	Beattie et al., 1996; Chang et al., 1992; Murcia-Nicolas et al., 1999; Yuwen et al., 1993
VACV-WR_060	***VACV-CP_E4L***	DNA-dependent RNA polymerase subunit rpo30, intermediate-gene transcription factor VITF-1; TFIIS-like	3	3	2	4	5	5	21	29.8	EL	+	Ahn et al., 1990a; Broyles and Pennington, 1990; Rosales et al., 1994
VACV-WR_144	***VACV-CP_A24R***	DNA-dependent RNA polymerase subunit rpo132	13	13	12	7	7	6	16	133.4	EL	+	Amegadzie et al., 1991b
VACV-WR_102	***VACV-CP_H4L***	RAP94; tightly associated with DNA-dependent RNA polymerase; confers specificity for early promoters	10	10	9	8	6	5	13	93.6	L	+	Ahn and Moss, 1992b; Baldick et al., 1994; Kane and Shuman, 1992; Zhang et al., 1994
VACV-WR_062	***VACV-CP_E6R***	Unknown	10	11	9	2	0	0	28	66.7	L		
VACV-WR_069	***VACV-CP_O2L***	Nonessential glutaredoxin; not part of E10R-G4L S–S bond formation pathway	4	4	3	0	1	1	61	12.4	L	−	Ahn and Moss, 1992a; Johnson et al., 1991; Rajagopal et al., 1995
VACV-WR_070	***VACV-CP_I1L***	DNA-binding core protein; morphogenesis	3	3	3	3	1	1	12	35.8	L	+	Klemperer et al., 1997; Shchelkunov et al., 1993b
VACV-WR_116	***VACV-CP_D11L***	ATPase, nucleoside triphosphate phosphohydrolase-I, NPH-I; transcription elongation, termination; interacts with H4 [Rap94, WR_102]	11	10	10	5	3	2	23	72.3	L	+	Kahn and Esteban, 1990; Paoletti and Moss, 1974; Rodriguez et al., 1986
VACV-WR_106	***VACV-CP_D1R***	Large subunit of mRNA capping enzyme; transcription termination factor VTF; required for intermediate transcription	16	17	16	8	4	4	28	96.7	E		Ensinger et al., 1975; Martin et al., 1975; Morgan et al., 1984; Shuman et al., 1987; Vos et al., 1991
VACV-WR_140	***VACV-CP_A21L***	Member of MV entry/fusion protein complex	2	2	2	1	1	1	14	13.6	L	+	Senkevich et al., 2005; Townsley et al., 2005b
VACV-WR_167	***VACV-CP_A42R***	Profilin-like; trace amounts found in MV by western	1	1	1	0	0	0	7	15.1	L	−	Blasco et al., 1991; Machesky et al., 1994
VACV-WR_066	***VACV-CP_E10R***	Part of S–S bond formation pathway; sulfhydryl oxidase; morphogenesis	0	0	0	1	1	1	7	10.9	L	+	Senkevich et al., 2000a,2000b
VACV-WR_092	***VACV-CP_L5R***	Member of MV entry/fusion	1	2	2	0	0	0	13	15.0	L	+	Senkevich et al., 2005;

(continued on next page)

Table 1 (*continued*)

WR [a]	Cop ortholog	Description	Unique AMTs						C [b]	Mass [c]	T [d]	E [e]	References
			Urea [f]			SDS							
			1	2	3	1	2	3					
		protein complex											Townsley et al., 2005a
VACV-WR_078	***VACV-CP_G1L***	Predicted metalloprotease with HxxEH motif required for formation of normal MV but not viral protein processing	9	7	8	3	2	2	17	68.0	L	+	Ansarah-Sobrinho and Moss, 2004b; Hedengren-Olcott et al., 2004
VACV-WR_124	***VACV-CP_A5R***	DNA-dependent RNA polymerase subunit rpo19	3	3	3	0	0	0	23	19.0	EL	+	Ahn et al., 1992
VACV-WR_117	***VACV-CP_D12L***	Small subunit of mRNA capping enzyme; transcription termination factor VTF; required for intermediate transcription	5	6	5	1	0	0	33	33.4	E	+	Ensinger et al., 1975; Martin et al., 1975; Niles et al., 1989; Shuman et al., 1987; Vos et al., 1991
VACV-WR_048	***VACV-CP_F9L***	S–S bond formation pathway substrate	1	1	1	0	0	0	6	23.8	L	+	Senkevich et al., 2002a; White et al., 2002
VACV-WR_171	***VACV-CP_A45R***	Inactive Cu–Zn superoxide dismutase-like in virion	3	3	3	1	0	0	26	13.7	L	−	Almazan et al., 2001
VACV-WR_057	***VACV-CP_E1L***	Poly-A polymerase catalytic subunit VP55	9	8	9	3	3	2	23	55.5	E		Gershon et al., 1991; Moss et al., 1973
VACV-WR_077	***VACV-CP_I8R***	DExH RNA/DNA-helicase stimulated by L4R (NPH-II); required for early transcription by MV cores	7	7	7	6	3	2	14	77.6	I	+	Bayliss and Smith, 1996; Fathi and Condit, 1991; Paoletti and Moss, 1974
VACV-WR_111	***VACV-CP_D6R***	70 kDa small subunit of early gene transcription factor VETF; ATPase	6	7	7	2	2	1	20	73.8	L	+	Broyles and Fesler, 1990; Gershon and Moss, 1990; Li et al., 1994
VACV-WR_083	***VACV-CP_G5.5***	DNA-dependent RNA polymerase subunit rpo7	1	1	1	2	1	1	32	7.3	EL	+	Amegadzie et al., 1992
VACV-WR_100	***VACV-CP_H2R***	Member of MV entry/fusion protein complex	2	2	2	1	0	1	12	21.5	L	+	Senkevich and Moss, 2005; Senkevich et al., 2005
VACV-WR_096	***VACV-CP_J4R***	DNA-dependent RNA polymerase subunit rpo22	4	4	4	0	0	0	31	21.3	EL	+	Broyles and Moss, 1986; Hooda-Dhingra et al., 1989
VACV-WR_067	***VACV-CP_E11L***	Virion core protein; required for MV infectivity	2	2	2	1	1	1	26	14.9	L	+	Wang and Shuman, 1996
VACV-WR_081	***VACV-CP_G4L***	Part of S–S bond formation pathway; thioredoxin-like; morphogenesis	1	1	1	1	1	1	7	14.0	L	+	Gvakharia et al., 1996; Jensen et al., 1996; White et al., 2002; White et al., 2000
VACV-WR_087	***VACV-CP_G9R***	Myristylprotein; member of MV entry/fusion protein complex	2	2	2	0	1	0	8	38.8	L		Martin et al., 1997; Senkevich et al., 2005
VACV-WR_151	***VACV-CP_A28L***	Member of MV entry/fusion protein complex	0	0	0	2	2	2	15	16.3	L	+	Senkevich et al., 2004, 2005
VACV-WR_049	***VACV-CP_F10L***	Ser/Thr kinase; Vaccinia protein kinase 2; phosphorylates A14, A17; morphogenesis	7	8	8	1	1	1	23	52.1	L	+	Lin and Broyles, 1994; Szajner et al., 2004b, 2004c; Traktman et al., 1995
VACV-WR_028	VACV-CP_N1L	Virokine; host defense modulator	2	2	2	1	1	0	21	14.0	EL	−	Bartlett et al., 2002; Kotwal et al., 1989
VACV-WR_135	***VACV-CP_A15L***	Virion core protein; morphogenesis	2	2	2	0	1	1	34	11.0	L	+	Szajner et al., 2004a
VACV-WR_153	***VACV-CP_A30L***	Virion core protein; morphogenesis	2	2	2	2	0	0	51	8.7	L	+	Mercer and Traktman, 2005; Szajner et al., 2004a; Szajner et al., 2001
VACV-WR_125	***VACV-CP_A6L***	Unknown; interacts with A21 [WR_140]	4	4	4	0	0	0	14	43.2			McCraith et al., 2000
VACV-WR_130	VACV-CP_A11R	Morphogenesis; viral membrane formation	1	1	1	3	2	2	8	36.1	L	+	Resch et al., 2005
VACV-WR_030	VACV-CP_M1L	Ankyrin-like	4	3	3	2	0	1	8	54.3		−	Shchelkunov et al., 1993a
VACV-WR_152	***VACV-CP_A29L***	DNA-dependent RNA polymerase rpo35	2	1	1	0	0	1	9	35.4	EL	+	Amegadzie et al., 1991a

Table 1 (*continued*)

WR [a]	Cop ortholog	Description	Unique AMTs						C [b]	Mass [c]	T [d]	E [e]	References
			Urea [f]			SDS							
			1	2	3	1	2	3					
VACV-WR_035	***VACV-CP_K4L***	Phospholipase-D-like; F13 (WR_052) paralog; nicking/joining activity	4	3	3	1	1	1	10	48.9	L	−	Eckert et al., 2005; Lakritz et al., 1985
VACV-WR_176	VACV-CP_A50R	DNA ligase	2	2	3	0	0	0	9	63.4	E	−	Colinas et al., 1990; Kerr and Smith, 1989; Sambrook and Shatkin, 1969
VACV-WR_052	***VACV-CP_F13L***	Palmytilated EEV membrane protein; phospholipase motif, required for IEV formation	1	1	3	0	0	0	13	41.8	L	−	Blasco and Moss, 1991
VACV-WR_097	***VACV-CP_J5L***	Member of MV entry/fusion protein complex	1	1	1	1	0	0	8	15.2			Senkevich et al., 2005
VACV-WR_004	VACV-CP_C22L	[ITR] gene fragment, TNF-alpha-receptor-like; contains 2 cyctein rich domains homologous to crmB of cowpox virus	2	2	2	0	0	0	14	13.6	E	−	Alcami et al., 1999
VACV-WR_118	***VACV-CP_D13L***	Rifampicin target; forms hexagonal lattice ("spicules") on surface of IV membrane; morphogenesis	3	3	2	1	0	1	6	61.9	L	+	Dales and Siminovitch, 1961; Szajner et al., 2005; Weinrich et al., 1985; Zhang and Moss, 1992
VACV-WR_104	***VACV-CP_H6R***	Topoisomerase type IB; important for early transcription	3	3	3	0	0	0	16	36.7	L	−	Bauer et al., 1977; da Fonseca and Moss, 2003; Shaffer and Traktman, 1987; Shuman and Moss, 1987
VACV-WR_075	***VACV-CP_I6L***	Telomer binding protein; morphogenesis	1	1	1	0	0	0	4	43.5	L	+	DeMasi et al., 2001; Grubisha and Traktman, 2003
VACV-WR_065	VACV-CP_E9L	DNA polymerase	2	2	2	1	0	0	2	116.9	E	+	Jones and Moss, 1985; Magee, 1962; Moss and Cooper, 1982; Traktman et al., 1984
VACV-WR_148		Gene fragment, cowpox A-type inclusion protein; MV-specific in vaccinia	2	2	2	4	1	1	7	84.3	L	−	Funahashi et al., 1988; Ulaeto et al., 1996
VACV-WR_072	***VACV-CP_I3L***	ssDNA-binding phosphoprotein (SSB) in MV core; stimulates ribonucleotide reductase	2	1	1	0	0	0	11	30.0	EI		Davis and Mathews, 1993; Rochester and Traktman, 1998
VACV-WR_139	VACV-CP_A19L	Unknown	1	1	1	0	0	0	13	8.3			
VACV-WR_043	VACV-CP_F4L	Ribonucleotide reductase small subunit	2	2	2	0	0	0	13	37.0	E	−	Slabaugh et al., 1988; Slabaugh and Mathews, 1984
VACV-WR_138	***VACV-CP_A18R***	DNA helicase; effects elongation and termination of postreplicative viral transcription	6	5	3	0	0	0	13	56.7	EL	+	Pacha and Condit, 1985; Pacha et al., 1990; Simpson and Condit, 1994
VACV-WR_183	***VACV-CP_B1R***	Ser/Thr kinase essential for viral DNA replication	1	1	1	0	0	0	4	34.3	E	+	Lin et al., 1992; Rempel and Traktman, 1992
VACV-WR_128	***VACV-CP_A9L***	MV membrane protein; morphogenesis	0	0	0	1	2	1	31	12.1	L	+	Yeh et al., 2000

[a] ORFs shown in bold italics were identified in at least two proteomic analyses or in this analysis and a reference cited in the table.
[b] Coverage [%].
[c] Mass in kDa.
[d] Timing of expression: E, early; I, intermediate; L, late; and combinations of the three letters.

component of viral factories and has been reported to be involved in late transcription and early morphogenesis (DeMasi and Traktman, 2000; Kovacs and Moss, 1996; Murcia-Nicolas et al., 1999). In a yeast two hybrid assay, H5 was found to interact with the virion component B1 (McCraith et al., 2000). F8 is a non-essential protein containing iActA-like repeats

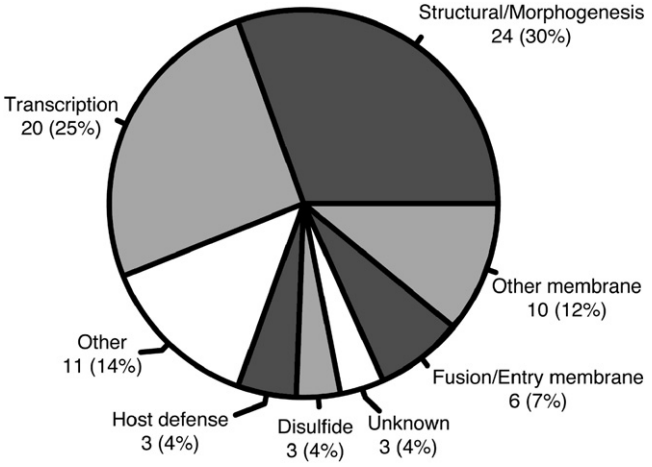

Fig. 2. Functional characterization of MV proteins. MV components can be divided into non-membrane proteins involved in MV structure and morphogenesis (A10, A11, A12L, A15, A30, A3, A4, D13, D2, D3, E11, E8, F10, F17, G1, G7, H5, I1, I3, I6, I7, J1, L4, VACV-WR_148), proteins involved in transcription (A18, A24, A29, A5, A7, D11, D12, D1, D6, E1, E4, G5.5, H1, H4, H6, I8, J3, J4, J6, L3), membrane proteins (A13, A14, A17, A26, A27, A9, D8, F9, H3, L1), membrane proteins involved in fusion/entry (A21, A28, G9, H2, J5, L5), components of the disulfide bond formation pathway (A2.5, E10, G4), host defense proteins (C22, E3, N1), proteins with other functions (A42, A45, A50, B1, E9, F13, F4, F8, K4, M1, O2) and proteins with unknown function (A6, A19, E6).

(Higley and Way, 1997; Kreft et al., 1995) and has been reported to interact with the MV core protein L4 (McCraith et al., 2000). The significance of the association of F8 with virions is unclear. E3 is another major component of virus factories (Murcia-Nicolas et al., 1999) and functions as a double stranded RNA binding protein preventing the induction of the interferon response by overlapping complementary late viral transcripts (Beattie et al., 1996; Chang et al., 1992). In addition, E3 has an N-terminal Z-DNA binding domain (Kim et al., 2003) required for nuclear localization and transactivation of cellular genes (Kwon and Rich, 2005; Yuwen et al., 1993). The VACV genome was predicted to contain several sites with Z-DNA propensity by the Z-Hunt algorithm (Ho et al., 1986), which could explain the presence of E3 in the MV particles. The A6 and E6 proteins are highly conserved but have no known function and the significance of their association with MV particles remains to be determined. The previously reported interaction of A6 with the MV membrane protein A21 (McCraith et al., 2000) was consistent with its detection in VACV virions.

In addition to the set of consensus proteins found in the other mass spectrometry studies, 8 proteins were only identified in this study. The latter include two host defense proteins (N1, C22), one protein involved in morphogenesis (A11), two proteins with unknown function (A19, M1) and three proteins involved in DNA metabolism and transcription (A50, E9, F4). Three proteins, the DNA polymerase E9, the DNA ligase A50 (Sambrook and Shatkin, 1969) and the morphogenesis protein A11 (Resch and Moss, 2005), have previously been reported as being absent from the MV or were unlikely MV components based on their function. Our detection of the proteins may be due to the high sensitivity of the MS technique used here, though this does not differentiate between a low level of specific or non-specific packaging or contamination. One of the ribonucleotide reductase subunits (F4) was identified, consistent with the presence of ribonucleotide reductase in cytomegalovirus particles (Kattenhorn et al., 2004). The A19 protein was previously shown to interact with the MV core protein A12 (McCraith et al., 2000), suggesting that this was not a false positive identification. The significance, if any, of the presence of host defense modulators in MV is unclear.

The isopycnic purification step was used in part to separate MVs from EVs, which have a lower buoyant density due to the additional membrane. Nevertheless, the detection of one EV membrane protein (F13) raised the possibility that the MV preparation could have been contaminated with a small amount of EV, since this is the most abundant of the EV-specific membrane proteins. Alternatively, the F13 protein could be interacting with an MV surface protein resulting in the presence of F13 on a small subset of MV particles derived from disrupted

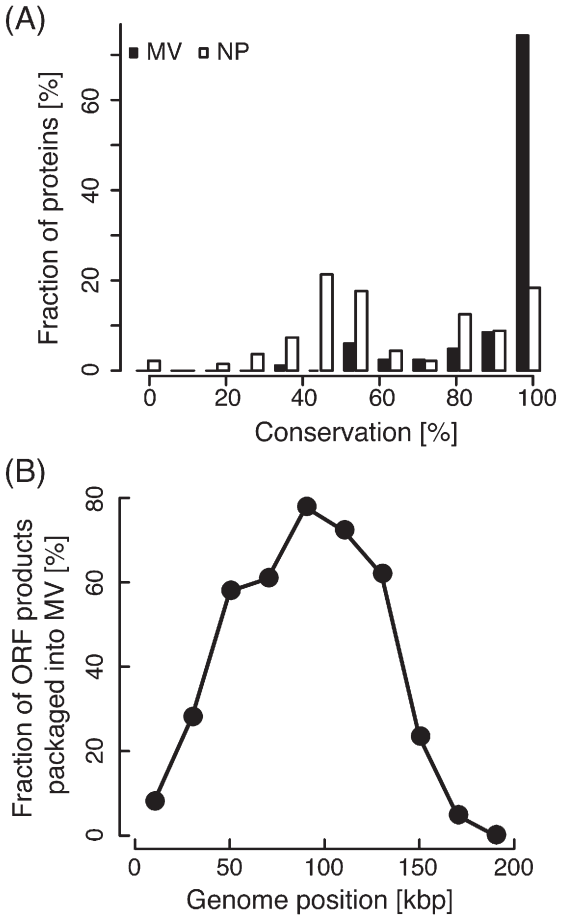

Fig. 3. (A) Distribution of the conservation of packaged (MV) and non-packaged (NP) proteins. The conservation of a protein was calculated as the percentage of sequenced poxvirus genomes encoding orthologs of the protein. The median conservation of MV proteins (55 out of 57 sequenced poxvirus genomes) is significantly different from non-MV proteins (30 out of 57; $P<10^{-5}$). (B) Distribution of ORFs encoding MV proteins. The fraction of ORFs encoding MV proteins was calculated for 10 segments of the genome by dividing the number of ORFs encoding MV proteins by the total number of ORFs.

Table 2
Known MV proteins not detected by LC-FT-ICR

WR	Cop ortholog	Description	Mass [a]	T [b]	E [c]	Sucrose [d]	References
VACV-WR_074	***VACV-CP_I5L***	IMV membrane protein VP13	8.7			1 (18%)	Ichihashi et al., 1984; Takahashi et al., 1994
VACV-WR_079	***VACV-CP_G3L***	Member of MV entry/fusion protein complex	12.8		+	3 (26%)	Senkevich et al., 2005
VACV-WR_082	***VACV-CP_G5R***	Early block in morphogenesis, prior to crescent formation	49.8	E	+	−	da Fonseca et al., 2004
VACV-WR_112	***VACV-CP_D7R***	DNA-dependent RNA polymerase subunit rpo18	17.9	EL	+	1 (7%)	Ahn et al., 1990a, 1990b; Quick and Broyles, 1990
VACV-WR_134	***VACV-CP_A14.5***	Nonessential hydrophobic IV and IMV membrane protein; deletion attenuates virus virulence	6.2	L	−	−	Betakova et al., 2000
VACV-WR_136	***VACV-CP_A16L***	Myristylprotein; member of MV entry/fusion protein complex	43.4	L	+	5 (12%)	Martin et al., 1997; Ojeda et al., 2006; Senkevich et al., 2005
VACV-WR_142	***VACV-CP_A22R***	Palmitylprotein; Holliday junction endonuclease; resolves viral DNA concatemers into unit length genomes	21.9	L	+	−	Garcia et al., 2000; Garcia and Moss, 2001; Grosenbach et al., 2000
VACV-WR_172	***VACV-CP_A46R***	Toll/IL1-recepto; suppresses TIR-dependent signal transduction; host defense modulator	27.6	E	−	−	Bowie et al., 2000; Stack et al., 2005

[a] Mass in kDa.
[b] Timing of expression: E, early; L, late.
[c] Essential (+) or non-essential (−) ORFs.
[d] Number of AMTs detected reproducibly in sucrose gradient purified material; coverage shown in parenthesis.

EV, consistent with the requirement for F13 expression for wrapping of the MV (Blasco and Moss, 1991).

Identification of host-encoded proteins associated with MVs

In addition to the VACV-encoded proteins, 55 host-encoded proteins were identified by reproducibly detecting 2 or more AMTs in three or more experiments. Considerably more cellular proteins were detected in MV preparations prior to the isopycnic purification step (data not shown). Table 3 lists 24 host proteins omitting isoforms of the same proteins, such as different tubulin alpha chains. In general, cytoskeletal proteins, heat shock proteins and proteins involved in translation appeared to have higher abundance values than metabolic proteins or proteins involved in signaling, though differences were not statistically significant. In addition, several mitochondrial proteins were detected at intermediate to low abundance values, possibly reflecting a low level of contamination of the MV preparations with mitochondria (data not shown). The majority of the host proteins identified here were previously reported to be associated with VACV or other viruses (see references in Table 3). One of the newly reported proteins, a putative DEAD box helicase, was reported to interact with the hepatitis C capsid protein and this interaction was shown to result in reduced translation of capped but not uncapped mRNA in vitro (Mamiya and Worman, 1999). The functional implications, if any, of the packaging of these and other host proteins remain to be determined.

Concluding remarks

The complexity of poxvirus MVs is due in part to their need to provide a complete transcription system for initiation of viral gene expression in the cytoplasm of the host cell, in addition to structural proteins and proteins for membrane fusion and entry. Eighty VACV-encoded proteins were detected in at least three analyses of MVs that had been purified by two rate zonal and one isopycnic centrifugation. This number represents 37% of the 218 genes annotated in the complete genome sequence of the WR strain of VACV. Ten proteins made up the bulk of the MV mass, while most proteins appeared to be present in small amounts only. Further experiments are needed to localize the proteins to specific virion structures visualized by electron microscopy.

Material and methods

Statistical analysis

All calculations were carried out with the *R* statistical language package (R Development Core Team, 2005). Unless stated otherwise, *P* values were calculated using the non-parametric Wilcoxon rank sum test.

MV purification

MV was purified by two sequential rate zonal centrifugation steps as described previously (Earl et al., 1998). The resulting material was loaded onto a pre-formed cesium chloride gradient (1.23 g/ml to 1.29 g/ml) and centrifuged for 4 h at 180,000×*g*. An opaque band at a density of 1.27 g/ml was aspirated and diluted 3-fold, followed by the collection of MV particles by centrifugation. Material isolated after the rate zonal purification steps as well as material from the isopycnic gradient was analyzed by MS, but data are not shown for the former due to a higher level of contamination with cellular components and wrapped virions.

Table 3
Human proteins identified in VACV MV

Category and protein	Accession number	Mass [kDa]	AMTs	Coverage [%]	Association with other virus particles or virus proteins
Chaperone					
Heat shock 70 kDa protein 8 isoform 2	IPI00037070	53.9	9	25	VACV (Chung et al., 2006)
Heat shock 70 kDa protein 1B	IPI00033946	70	8	15	HCMV (Varnum et al., 2004) KSHV (Zhu et al., 2005) HIV (Gurer et al., 2002)
Cytosceleton					
Tubulin alpha-1 chain	IPI 00387144	50.1	10	33	HCMV (Varnum et al., 2004) VACV (Chung et al., 2006) ASVF (Esteves et al., 1986)
Tubulin beta-2 chain	IPI00007752	49.8	6	21	HCMV (Varnum et al., 2004) VACV (Chung et al., 2006) ASVF (Esteves et al., 1986)
Actin gamma-2	IPI00025416	41.9	5	17	MCMV (Kattenhorn et al., 2004) VACV (Chung et al., 2006) VACV (Jensen et al., 1996) ASVF (Esteves et al., 1986) HIV (Ott et al., 1996)
Keratin 7	IPI00306959	51.4	4	10	
Cofilin 1	IPI00012011	18.5	3	30	MCMV (Kattenhorn et al., 2004) HCMV (Varnum et al., 2004)
Vimentin	IPI00216312	53.7	2	5	HCMV (Varnum et al., 2004)
Translation					
Eukaryotic translation elongation factor 1 alpha 2	IPI00014424	50.4	4	8	MCMV (Kattenhorn et al., 2004) HCMV (Varnum et al., 2004) VACV (Chung et al., 2006)
60S acidic ribosomal protein P0	IPI00008530	34.25	2	8	VACV (Chung et al., 2006)
Signal transduction					
Prohibitin	IPI00017334	29.8	3	17	
Ras-related protein Rab-5C	IPI00016339	23.5	2	11	
Ras-related protein Rab-7	IPI00016342	23.5	2	10	KSHV (Zhu et al., 2005) VACV (Chung et al., 2006)
Metabolism					
Phosphoglycerate kinase 1	IPI00295540	44.6	5	18	KSHV (Zhu et al., 2005)
Pyruvate kinase	IPI00328347	57.8	3	7	KSHV (Zhu et al., 2005)
Other					
DEAD (Asp–Glu–Ala–Asp) box polypeptide 3, X-linked (RNA helicase)	IPI00215637	73.2	4	9	Hepatitis C (Mamiya and Worman, 1999)
Peroxiredoxin 2	IPI00375400	12.9	2	9	VACV (Chung et al., 2006)
Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	38.7	2	8	
Unknown					
Similar to RIKEN cDNA 4732495G21 gene	IPI00003269	42	3	9	
Similar to polyubiquitin C	IPI00220286	43.6	4	50	HCMV (Varnum et al., 2004)
Transgelin 2 (unknown function)	IPI00024057	22.4	4	28	
Similar to ubiquitin and ribosomal protein S27a precursor	IPI00397808	22.2	3	11	
Similar to high mobility group protein 1	IPI00018755	24.2	2	12	
Hypothetical protein LOC388524	IPI00397704	32.9	2	10	

Abbreviations: HCMV, human cytomegalovirus; KSHV, Kaposi sarcoma-associated herpesvirus; HIV, human immunodeficiency virus; ASVF, African swine fever virus; MCMV, murine cytomegalovirus.

Preparation of peptide pools

MV particles containing 0.5 mg of protein were collected by centrifugation and resuspended in either 1% SDS or 7 M urea plus 2 M thiourea prepared in 50 mM ammonium bicarbonate (pH 7.8). After sonication, solutions were adjusted to 10 mM dithiothreitol and incubated at 37 °C for 1 h. Iodoacetamide was added to a final concentration of 40 mM and incubated for 30 min at room temperature. Excess dithiothreitol was used to quench the remaining iodoacetamide for 1 h at room temperature. Salt and denaturant concentrations were reduced by 10-fold dilution with 50 mM ammonium bicarbonate (pH 7.8), calcium chloride was added to a final concentration of 1 mM and proteins were digested with 20 μg of trypsin (Trypsin Gold, Promega, Madison, WI) at 37 °C for 14 h.

Purification of the SDS peptide pool

A 3 ml Discovery SCX strong cation exchange solid phase extraction column (Supleco, Bellefonte, PA) was used to purify peptides from the SDS pool. A SCX column was conditioned in succession with methanol, 10 mM ammonium formate pH 3, 500 mM ammonium formate pH 8 and 10 mM ammonium formate pH 3, all in 25% acetonitrile. Following a water wash, the column was equilibrated in 10 mM ammonium formate in 25% acetonitrile pH 3 and the acidified sample (pH 3.5) was applied. The column was washed with 30 ml of 10 mM ammonium formate in 25% acetonitrile, pH 3 and the peptides were eluted twice with 0.5 ml of 500 mM ammonium formate in 25% acetonitrile, pH 8 and once with 0.5 ml of acetonitrile. Peptides were concentrated to approximately 0.2 mg/ml under vacuum.

Purification of the urea peptide pool

A 3 ml Discovery C18 reverse phase solid phase extraction column (Supleco, Bellefonte, PA) was conditioned with 3 ml of methanol and equilibrated with 6 ml of 0.1% formic acid. The peptide sample was applied and the column was washed with 15 ml of 5% acetonitrile. Peptides were eluted twice with 80% acetonitrile and once with 100% acetonitrile and concentrated to approximately 0.8 mg/ml under vacuum.

MS analysis of peptide pools

Peptide pools were separated by an automated in-house designed HPLC system as described elsewhere (Masselon et al., 2005; Shen et al., 2001). Eluate from the HPLC was directly transferred into an ion trap MS (LTQ, ThermoFinnigan, San Jose, CA) using electrospray ionization (ESI). The mass spectrometer operated in a data-dependent MS/MS mode over a series of seven smaller segmented *m*/*z* ranges (400–700, 700–900, 900–1100, 1100–1300, 1300–1500, 1500–1700, 1700–2000). The details for PMT generation are described elsewhere (Smith et al., 2002b).

MS/MS spectra were interpreted automatically by the peptide identification software SEQUEST (Eng et al., 1994) using a fasta database containing all potential ORFs with 20 or more amino acids in the genome of VACV strain WR (accession number: ***NC_006998***) or the Human International Protein Index database. PMT identifications were based on a minimum cross correlation of 1.9, 2.2, and 3.75 for charge states of 1+, 2+ and 3+, respectively. Additionally a discriminant score of 0.2 or better was applied to all MS/MS data (Strittmatter et al., 2004). These peptides made up the potential mass and time tags (PMTs).

Intact peptide masses measured by an LTQ-FT MS (ThermoFinnigan, San Jose, CA) using electrospray ionization and the same LC system as the MS/MS analysis was then used to verify PMTs as accurate mass and time tags based on mass and normalized elution time with tolerances of ±4.4 and ±0.025 ppm, respectively. Abundance values of each peptide were calculated from the peak intensity of the most abundant charge state form of the peptide in all scans containing ions matching the peptide. The abundance value of each protein was then computed as the average abundance of all peptides within 33% of the most abundant peptide for each protein. This abundance value reflects the approximate number of protein molecules present. The mass of each protein was used to calculate the estimated percentage of virion mass contributed by the protein.

Acknowledgments

We thank N. Cooper for assistance in virus purification. This research was supported in part by the intramural research program of the NIAID, NIH. Mass spectrometry was performed in the Environmental Molecular Sciences Laboratory (a national scientific user facility sponsored by the U.S. DOE Office of Biological and Environmental Research) located at Pacific Northwest National Laboratory, operated by Battelle for the DOE under Contract DE-AC06-76RLO (PNNL-SA-49565).

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