Multimeric Protein Structures of African Horsesickness Virus and their use as Antigen Delivery Systems

By

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Ek gee nuwe krag aan dié wat moeg is,
Ek maak die moedelose weer vol moed. *Jer. 31:25*

Hy gee die vermoeides krag,
Hy versterk dié wat nie meer kan nie.
Selfs jongmanne word moeg en raak afgemat,
selfs manne in hulle fleur struikel en val,
maar dié wat op die Here vertrou,
kry nuwe krag.
Hulle vlieg met arendsvlerke,
hulle hardloop en word nie moeg nie,
hulle loop en raak nie afgemat nie. *Jes. 40:29-31*

Die Here my God gee vir my krag.
Hy maak my voete soos dié van 'n ribbok
op hoë plekke laat Hy my veilig loop. *Hab. 3:19*

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SUMMARY

African horsesickness virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae*, is the aetiological agent of African horsesickness, a highly infectious non-contagious disease of equines. The AHSV virion is composed of seven structural proteins organised into a double layered capsid, which encloses ten double-stranded RNA segments. The double stranded (ds) RNA genome of AHSV encodes, in addition to the seven structural proteins, at least three non-structural proteins (NS1 to NS3). The assembly of viral proteins in AHSV-infected cells results in at least three characteristic particulate structures. The first of these structures are the complete virions and viral cores. Empty virions or particles that simulate the virion surface can be produced synthetically by the co-expression of various combinations of AHSV structural genes in insect cells. Apart from the core particles and complete virions, there are two additional structures observed in AHSV-infected cells. Unique virus-specified tubular structures, composed of NS1, are observed in the cytoplasm of all orbivirus-infected cells. The second structure, distinctive hexagonal crystals, is unique to AHSV and is composed entirely of VP7, the major core protein. The assembly of all these particles can be produced synthetically when expressed individually in an insect cell expression system. The aim of this investigation was first of all to investigate the structure and assembly of these structures and secondly to evaluate their use as vehicles for foreign immunogens.

The NS1 gene of AHSV-6 was cloned as a complete and full-length cDNA fragment from purified dsRNA genome segment 5 and the complete nucleotide sequence determined. The gene was found to be 1749 bp in length with one major open reading frame (ORF) of 1645 bp, encoding a protein comprising 548 amino acids. The 5' and 3' termini of the gene were found to contain the conserved terminal hexanucleotide sequences of AHSV RNA fragments, followed by inverted heptanucleotide repeats. The deduced amino acid sequence was analysed and found to define a hydrophobic protein of 63 kDa. Antigenic profile analysis indicated a hydrophilic domain with relative high antigenicity in the C-terminus of the protein. This represents a possible insertion site for immunogenic epitopes. The cloned NS1 gene of AHSV-6 was modified at the 5' and 3' terminal ends to facilitate expression of the gene. *In vitro* expression yielded a protein corresponding to the predicted size of NS1. The gene was also expressed in insect cells, using a recombinant baculovirus and yields of approximately 1.0mg NS1 protein/10^6 cells were obtained. Expression of NS1 in insect cells resulted in the intracellular formation of tubular structures with diameters of 23 ±2 nm. Biophysical analysis of the AHSV tubules suggests that they are more fragile and unstable than BTV NS1 tubules.

To gain more insight into the structure, assembly and the biochemical characteristics of AHSV cores and virions, a number of baculovirus multigene expression vectors have been developed and utilised to co-express various combinations of AHSV genes. Cells infected with a dual-recombinant baculovirus, expressing AHSV-9 VP3 and VP7 genes, contained high levels of VP7 and low levels of VP3. The simultaneous expression of the two proteins resulted in the spontaneous intracellular assembly of empty multimeric core-like particles (CLPs) with a diameter of approximately 72 nm. These particles structurally resembled authentic AHSV cores in size and appearance. The yield of CLP production was low as a result of the insolubility of VP7, which aggregates preferably into large hexagonal crystal as well as the low yield of VP3. The interaction of CLPs with either VP2 or VP5 was investigated by co-infection of the VP3 and

VP7 dual recombinant baculovirus with a VP2 or VP5 single recombinant baculovirus. Each of the outer capsid proteins interacted separately with CLPs. Co-expression of all four major structural proteins of AHSV, using two dual recombinant baculoviruses one expressing VP2 and VP3, the other VP5 and VP7, resulted in the spontaneous assembly of empty virus-like particles with a diameter of 82 nm. Although co-expression of the different combinations of AHSV proteins was obtained, the levels of expression were low. This low levels of the AHSV capsid proteins and the aggregation of VP7 downregulated the assembly process.

In order to investigate the possibility of the use of CLPs and VP7 crystals as particulate delivery systems, insertion analysis of VP7 was used to identify certain sequences in the VP7 protein that are not essential for the assembly of CLPs or trimer-trimer interactions in the crystals. Two insertion mutants of VP7 (mt177 and mt200) were constructed. In each case three unique restriction enzyme sites were introduced that coded for six amino acids. In mt177 these amino acids were added to the hydrophilic RGD loop at position 177 - 178 and for mt200 to amino acid 200 - 201. Both regions were located in the top domain of VP7. Insertion mt177 increased the solubility of VP7, but did not abrogate trimerisation and CLP formation with VP3. The yield of mutant CLPs was significantly higher than the normal CLPs, possibly due to the increased solubility and availability of VP7 trimers. Evidence about the size of an insert that can be accommodated by VP7 was provided by the insertion of a 101 amino acid region of VP2, containing a previously identified immunodominant region of VP2. The two chimeric VP7/TrVP2 proteins were investigated for their ability to form crystal structures and CLPs. The chimeric proteins did not produce the typical hexagonal crystal structure, but rather small ball-like structures.

This investigation yielded valuable information regarding the structure and assembly of AHSV tubules, CLPs and VLPs. These findings also have practical value, since the multimeric structures can be utilised as delivery systems for immunogens, like the AHSV VP2 immunodominant epitopes.

LIST OF ABBREVIATIONS

AcNPV	-	*Autographa californica* nuclear polyhedrosis virus
AHS	-	African horsesickness
AHSV	-	African horsesickness virus
AHSV-9	-	African horsesickness virus serotype 9
ATP	-	adenosine-5'-triphosphate
amp	-	ampicillin
BHK	-	Baby hamster kidney cells
bp	-	base pairs
BRDV	-	Broadhaven virus
BSA	-	bovine serum albumin
BT	-	bluetongue
BTV	-	bluetongue virus
°C	-	degrees Celcius
ca.	-	approximately
cDNA	-	complementary DNA
ccc	-	covalently closed circular
CF	-	complement fixation
Ci	-	Curie
CIP	-	calf intestinal alkaline phosphatase
CLP	-	core-like particle
cm^2	-	square centimetre
cpm	-	counts per minute
CsCl	-	cesium chloride
Da	-	dalton
ddH_2O	-	deionized distilled water
DEPC	-	deithylpyrocarbonate
dATP	-	2'-deoxyadenosine-5'-triphosphate
dCTP	-	2'-deoxycytidine-5'-triphosphate
dGTP	-	2'-deoxyguanosine-5'-triphosphate
dTTP	-	2'-deoxythymidine-5'-triphosphate
dNTP	-	deoxyribonucleoside-triphosphate
ddATP	-	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	-	2',3'-dideoxycytidine-5'-triphosphate
ddGTP	-	2',3'-dideoxyguanosine-5'-triphosphate
ddTTP	-	2',3'-dideoxythymidine-5'-triphosphate
DNA	-	deoxyribonucleic acid
DNAse	-	Deoxyribonuclease
ds	-	double stranded
DTT	-	1,4-dithiothreitol
EDTA	-	ethylenediaminetetra-acetic acid
EEV	-	Equine encephalosis virus
e.g.	-	*exempli gratia* (for example)

EHDV	-	epizootic haemorrhagic disease virus
et al.	-	*et alia* (and others)
etc.	-	*et cetera* (and so forth)
EtBr	-	ethidium bromide
FCS	-	fetal calf serum
FESEM	-	Field emission scanning electron microscopy
Fig.	-	figure
g	-	gram / gravitational acceleration
GTP	-	guanosine-5'-triphosphate
GST	-	glutathione S-transferase
h	-	hour
HPRI	-	human placental RNase inhibitor
IgA	-	immunoglobulin class A
IgG	-	immunoglobulin class G
IPTG	-	isopropyl-β-D-thiogalactopyranoside
kb	-	kilobase pairs
kDa/kd	-	kilodalton
l	-	litre
LB	-	Luria-Bertani
log	-	logarithmic
M	-	Molar
mA	-	milliampere
MAb	-	monoclonal antibody
MCS	-	multiple cloning site
mg	-	milligram
min	-	minute
ml	-	millilitre
mM	-	millimolar
mmol	-	millimole
MMOH	-	methylmercuric hdroxide
MOI	-	multiplicity of infection
M_r	-	molecular weight
mRNA	-	messenger ribonucleic acid
m/v	-	mass per volume
N	-	normal
NaAc	-	sodium acetate
nm	-	nanometre
NS	-	non-structural
OD_{550}	-	optical density at 550 nm
OD_{260}	-	optical density at 260 nm
ORF	-	open reading frame
OVI	-	Onderstepoort Veterinary Institute

PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
p.f.u.	-	plaque forming units
p.i.	-	post infection
pmol	-	picomole
PSB	-	protein solvent buffer
PSV	-	perdesiekte virus
RE	-	restriction endonuclease
RNA	-	ribonucleic acid
RNAse	-	ribonuclease
rpm	-	revolutions per minute
RT	-	room temperature
RT-PCR	-	reverse transcriptase PCR
s	-	second
S	-	Svedberg unit
S 1-10	-	segment 1-10
SDS	-	sodium dodecyl sulphate
SEM	-	Scanning electron microscopy
Sf9	-	*Spodoptera frugiperda*
ss	-	single stranded
T_{An}	-	annealing temperatures
TC	-	transcriptase complex
TdT	-	terminal deoxynucleotidyl transferase
TEM	-	Transmission electron microscopy
TEMED	-	N,N,N',N'-tetramethylethylenediamine
tet	-	tetracycline hydrochloride
TFB	-	Transformation buffer
Tris	-	Tris(hydroxymethyl)-aminomethane
TSB	-	Transformation suspension buffer
TSBG	-	Transformation suspension buffer with glucose
TX-100	-	Triton X-100
U	-	units
µCi	-	microcurie
µg	-	microgram
µl	-	microlitre
UV	-	ultraviolet
V	-	volts
v	-	volume
VIB	-	viral inclusion body
VLP	-	virus-like particle
VP	-	viral protein
VT	-	viral tubules

v/v	-	volume per volume
W	-	watt
X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside