An investigation into nonstructural proteins NS3 and NS3A of African Horsesickness virus

By

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Dedicated to my parents, Jean and Piet Meiring

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SUMMARY

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For the degree MSc

The aims of this investigation were to compare the nonstructural proteins, NS3 and NS3A, of African horsesickness virus (AHSV) and to further characterise the cytotoxic properties of these proteins.

NS3 and NS3A are encoded from two in-phase overlapping reading frames on the smallest double-stranded (ds) RNA genome segment, segment 10 (S10) of AHSV. The proteins differ only with respect to the presence of an additional 10 or 11 amino acids, depending on the serotype, at the N-terminal of NS3. All known orbiviruses have been shown to encode two closely related proteins from S10. Sequence analysis of the N-terminal region of the NS3 proteins of the different serotypes of AHSV and various orbiviruses revealed that this region is not highly conserved.

Both AHSV NS3 and NS3A are membrane-associated and cytotoxic to insect cells causing membrane permeabilisation and eventual cell death when expressed individually (Van Staden *et al.,* 1995; Van Staden *et al.,* 1998; Van Niekerk *et al.,* 2001a). AHSV infection of Vero cells results in the synthesis of both proteins in equimolar amounts (Van Staden, 1993). The effect on the cytotoxic properties of these proteins when expressed together in insect cells was therefore investigated here.

Whether co-expressed or expressed individually NS3 and NS3A caused a dramatic decrease in the viability of insect cells. The NS3 protein is therefore representative of the NS3 and NS3A proteins together in terms of its cytotoxic effect.

The effect of the exogenous addition of NS3 on the membrane permeability of Vero cells was also investigated. The NS3 protein was found to cause a rapid increase in the membrane permeability of Vero cells. The cytotoxic properties of NS3 appear therefore not to be limited to their endogenous effects on insect cells.

The AHSV-3 NS3 and NS3A proteins were expressed as histidine tagged recombinants in the baculovirus expression system, to allow for the purification of large quantities of protein for functional and comparative studies. The resulting NS3 histidine fusion product, however, displayed a decrease in solubility, probably as a result of incorrect folding due to the presence of the histidine tag extension at the N-terminus of the protein.

To produce antibodies that detect NS3, and not NS3A, in AHSV infected cells, the N-terminal region unique to AHSV-3 NS3 was displayed on the surface of the AHSV core protein, VP7. The chimeric protein VP7-NS3 displayed the same structural characteristics as the wildtype VP7 protein, aggregating into highly insoluble crystals. Antiserum was prepared against purified VP7-NS3 and analysed in terms of its ability to recognise denatured and non-denatured AHSV-3 NS3. Although the antiserum was shown to contain antibodies directed against VP7 epitopes no immune reaction with NS3 was observed. The use of alternate sites on the surface region of VP7 for the display of such a small peptide needs to be investigated.

Although no functional differences between NS3 and NS3A were identified in this investigation, the finding that NS3 causes membrane permeability or damage to Vero cells represents the first indication that this AHSV protein causes extracellular membrane damage in mammalian cells. Many viral membrane damaging proteins or viroporins are thought to contribute significantly to the severity of virus-induced pathogenesis. The mechanism of membrane damage and the contribution of the membrane damaging properties of NS3 to AHSV-induced pathogenesis needs to be investigated.

OPSOMMING

'n Studie van nie-strukturele proteïene NS3 en NS3A van Perdesiekte virus

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Die doel van hierdie studie was om die nie-strukturele proteïene, NS3 en NS3A van perdesiekte virus (PSV), te vergelyk en om die sitotoksiese eienskappe van hierdie proteïene verder te karakteriseer.

NS3 en NS3A word gekodeer vanaf twee oorvleuelende in-fase oop leesrame op die kleinste dubbeldraad (dd) RNA genoomsegment, S10 van PSV. Hierdie twee proteïene verskil slegs van mekaar ten opsigte van 'n addisionele 10 of 11 aminosure, afhangend van die serotipe, aan die N-terminus van die NS3 proteïen. Alle bekende orbivirusse kodeer vir twee byna identiesse proteïene vanaf S10. Aminosuur volgorde analise van die N-terminale gebiede van die NS3 proteïene van die verskillende PSV serotipes en verskeinde orbivirusse, het bewys dat hierdie gebied nie hoogs gekonserveerd is nie.

Beide NS3 en NS3A is sitotoksies vir *Sf9* selle wanneer hulle alleen uitgedruk is in die baculovirus ekspressie sisteem (Van Staden *et al.,* 1995; Van Staden *et al.,* 1998; Van Niekerk *et al.*, 2001a). PSV infeksie van Vero selle lei tot die ekspressie van beide NS3 en NS3A in omtrent dieselfde hoeveelhede (Van Staden,1993). Om die sitotoksiese effek van ko-ekspressie van NS3 en NS3A in insekselle te bestudeer, is

insekselle geïnfekteer met rekombinante baculovirusse wat NS3 en NS3A uitdruk. Dit is bewys dat ko-ekspressie van NS3 en NS3A nie die sitotoksiese effek van hierdie proteïene verander nie.

Die effek van eksogeniese NS3 op die selmembraan permeabiliteit van Vero selle is ook bestudeer. NS3 veroorsaak 'n vinnige toename in die permeabiliteit van Vero selmembrane. Die sitotoksiese eienskappe van NS3 is daarom nie beperk tot hulle endogeniese effek op insekselle nie.

Om groot hoeveelheide van die PSV-3 NS3 en NS3A proteïene te bekom, is die S10 geen en 'n verkorte vorm van die S10 geen uitgedruk as histidien fusieproteïene in die baculovirus ekspressie sisteem. Die NS3 fusieproteïen het 'n sterk afname in solubiliteit getoon in vergelyking met die wildetipe NS3 proteïen en is vir hierdie rede nie gebruik vir die suiwering van NS3 nie.

Om tussen NS3 en NS3A te onderskei in PSV geïnfekteerde selle is antiserum teen die N-terminale gebied van NS3 berei deur gebruik te maak van die PSV strukturele proteïen, VP7. Die eerste 12 aminosure van NS3 is op die oppervlakte van VP7 vertoon. Dit is bewys dat die VP7-NS3 chimera dieselfde strukturele eienskappe as die wildetipe VP7 proteïen vertoon. Antiserum teen die VP7-NS3 proteïen is in hase berei, maar dit is aangetoon dat geen immuun reaksie met NS3 voorkom nie. Altenatiewe posisies op die oppervlakte van VP7 moet ondersoek word.

Die bevinding dat NS3 membraan permeabilisering in Vero selle veroorsaak is die eerste aanduiding dat die PSV proteïen eksogeniese membraan beskadiging veroorsaak. Baie sitotoksiese proteïene of viroporins dra by tot virus geïnduseerde patogenese. Die bydra van NS3 tot PSV geïnduseerde patogenese moet bepaal word.

ABBREVIATIONS

aa	amino acids
AHS	African horsesickness
AHSV	African horsesickness virus
Amp	ampicilin
Amps	amperes
ATCC	American type culture collection
bp	base pairs
BRD	Broadhaven virus
BTV	bluetongue virus
°C	degrees Celcius
cDNA	complementary deoxyribonucleic acid
CHV	Chuzan virus
Ci	Curie
CLP	core-like particle
cm^3	centimeter cubed
CPE	cytopathic effect
cys	cysteine
Da	Dalton
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
EC	endothelial cells
EDTA	ethylenediaminetetra-acetic acid
EHDV	Epizootic hemorrhagic disease virus
EM	electron microscope
ER	endoplasmic reticulum
et al.	and others
EtBr	ethidium bromide
fcs	foetal calf serum
Fig	figure
FMDV	Foot-and-mouth disease virus
g	gravitational force
G	gauge
gent	gentamycin
GTP	guanosine triphosphate
h	hour/s
HBsAgs	HBV surface antigens
HBV	hepatitis B virus
HD	hydrophobic domain
His	histidine
HIV	Human Immunodeficiency virus
h.p.i	hours post infection
Hyg B	hygromycin B
i.e.	that is
IM	intramuscular
IP3	inosytol triphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
IRES	internal ribosome entry site
ISA50	incomplete seppic adjuvant
k	kilo
kan	kanamycin

L1,L2,L3	large segments 1, 2 or 3
LB	Luria broth
LLP1	lentivirus lytic peptide 1
M	molar
M4,M5,M6, M7	medium segments 4,5,6 or 7
MEM	minimal essential medium
min	minute/s
ml	millilitre
mM	millimolar
MMOH	methyl mercuric hydroxide
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
ng	nanograms
nm	nanometers
NS1, NS2,NS3	nonstructural proteins 1, 2 or 3 (refers to *orbivirus*)
NSP4	nonstructural protein 4 (refers to *rotavirus*)
NTA	nitrilo-tri-acetic acid
OIE	Office international des Epizooties
ORF	open reading frame
OVI	Onderstepoort Veterinary Institute
P	particulate
PAGE	polyacrylamide gel electrophoresis
pBS	bluescribe plasmid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
p.i.	post infection
pmol	picomolar
PSB	protein solvent buffer
PSV	perdesiekte virus
RNA	ribonucleic acid
rNTP	ribonucleic acid triphosphate
rpm	revolutions per minute
RRL	rabbit reticulolysate
RT	reverse transcriptase
S	supernatant
S1 – S10	segments 1 to 10 (refers to *orbiviruses*)
^{35}S	radioactive Sulphur 35
SDS	sodium dodecyl sulphate
sec	second/s
Sf9	*Spodoptera frugiperda* insect cells
SIV	Simian immunodeficiency virus
SSP	single shelled particle
ss	single stranded
TE	Tris EDTA
tet	tetracyclin
TM	transmembrane
U	units
UHQ	ultra high quality water
µg	micrograms
µl	microlitres
µm	micrometers
UP	University of Pretoria
V	volts

VIB	virus inclusion body
VLP	virus-like particle
VMP	viral membrane protein
VP1 – 7	virus protein 1 to 7
v/v	volume per volume
v/w	volume per weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
3-D	three dimensional

LIST OF BUFFERS

Elution buffer:

20 mM Tris-HCl pH 8.5, 100 mM KCl, 100 mM imidazole, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol

Lysis buffer without detergent:

50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 mM PMSF, 1 mM 2-mercaptoethanol

Na-K-P buffer:

0.075 M KH_2PO_4/NaH_2PO_4, pH7.4

PBS:

137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4; pH7.3

Protein solvent buffer (PSB)(2x):

0.125 M Tris-HCl pH8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol

STE buffer:

0.01 M NaCl, 0.01 M Tris-HCl pH 7.6, 0.0001 M EDTA

TAE buffer:

0.04 M Tris, 0.002 M EDTA; pH8.5

TBS buffer:

500 mM NaCl, 25 mM Tris, pH7.6

TE buffer:

0.01 M Tris-HCl pH7.6, 0.001 M EDTA

TGS buffer:

0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1% SDS

Wash buffer:

20 mM Tris-HCl pH8.5, 500 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol

CONTENTS