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Antigenic diversity of Norwalk-like viruses: expression of the capsid protein of a genogroup I virus, distantly related to Norwalk virus

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Summary. The gene encoding the capsid protein of a genogroup I Norwalk-like virus (NLV) (Hu/NLV/Stav/95/Nor) was cloned and expressed in insect cells using a baculovirus vector. The His-tagged recombinant capsid protein (rStav) was antigenic and immunogenic, showed an apparent molecular weight of approximately 68 kD in protein gels, and was only soluble under denaturing conditions. The amino acid sequence of the rStav protein showed 65–88% similarity to capsid protein sequences from other genogroup I NLV and was most closely related to Desert Shield virus. Norwegian recruit sera were tested for antibodies against rStav by Western blotting (rStav WB). The sera had previously been tested for antibodies against a recombinant Norwalk virus capsid protein in an ELISA (rNV ELISA). Several rNV ELISA-negative sera showed a positive response in the rStav WB, indicating that the use of antigens representing different stains may be necessary when screening sera for antibodies against genogroup I NLV.

Introduction

Caliciviruses are major etiological agents of epidemic gastroenteritis in humans [21]. The human caliciviruses (HuCV) are transmitted by the fecal-oral route. Ingestion of contaminated food and water, or contact with infected person, are main modes of transmission.

Norwalk virus (NV) is the prototype of a group of HuCV associated with outbreaks of gastroenteritis [20]. Hence other viruses in this group have been denoted Norwalk-like viruses (NLV) or have been named after the location where they were first discovered. It has not been possible to cultivate these viruses in vitro, which severely restricted knowledge of their genetic and antigenic relationships. However, molecular cloning and characterization of the genome of NV,

and subsequently of related viruses, placed these viruses in the *Caliciviridae* family [4, 9, 13, 15, 23, 25]. After the classification of the NLV, the term human caliciviruses is used for this virus group. The other HuCV subgroup contains the Sapporo-like viruses, which differ from NLV in epidemiology, EM appearence and genomic arrangement [24, 30].

Genotyping based on the nucleotide sequences of the RNA polymerase and capsid genes differentiates the NLV into genogroup I and II (GI and GII) [1, 18, 33, 38, 40]. The NLV genome consists of three open reading frames (ORF). The ORF1 encodes non-structural proteins required for viral replication, the ORF2 codes for the capsid protein, while the function of the ORF3-encoded protein is unknown.

The antigenic diversity of the NLV is an important property of this group of viruses, which is relevant for the epidemiology as well as for serodiagnosis. A commonly used classification system contains four different antigenic types represented by the prototypes Norwalk, Snow Mountain, Hawaii and Taunton viruses [23]. Capsid proteins from several different NLV, mostly GII viruses, have been expressed recombinantly, including those of NV (GI), Desert Shield virus (DSV) (GI), Lordsdale virus (GII), Mexico virus (GII), Toronto virus (GII), Hawaii virus (GII), and Grimsby virus (GII) [4, 7, 8, 14, 16, 26, 27, 34]. No common group specific antigens have been described for NLV. There are, however, indications that serological response after infection with genogroup I viruses can be detected using Norwalk virus antigen alone, while detection of serological response after infection with genogroup II viruses may require antigens from several viruses [7, 29, 33]. The antigenic diversity of NLV thus requires that additional strains be cloned and expressed in order to have tools for detection and characterization of the full range of NLV.

In this study, the ORF2 of a genogroup I NLV, which originated from an outbreak of gastroenteritis in Norway, was cloned, the nucleotide sequence determined and the gene expressed recombinantly in a baculovirus system. The ORF2-encoded antigen was used for detection of antibodies in sera that previously had been tested using recombinant NV antigen.

Materials and methods

Virus

The virus originated from a fecal sample collected from a patient during a hospital outbreak of gastroenteritis in Stavanger, Norway in 1995. The fecal sample was confirmed to contain a NLV (Hu/NLV/Stav/95/Nor) by EM and was kindly provided by the National Institute of Public Health. The fecal sample had been diluted 1:10 in Hank's salt solution with antibiotics, stirred for 15 min and cleared by centrifugation. The supernatant (fecal extract) was kept at $-70\,^{\circ}$C.

RT-PCR

RNA was extracted from 2.5 ml fecal extract using TRIzol reagent (Gibco, N.Y., NY) according to the manufacturer's instructions, and solubilized in DEPC-treated water containing 30 mM dithiothreitol (Gibco) and 3 mM ribonucleoside vanadyl complexes (Sigma, St.

Table 1. The nucleotide sequences of primers used for the amplification of the Hu/NLV/Stav/95/Nor ORF2. The locations are related to the Norwalk virus genome

Identification	Sequence (5′-3)	Location
SOU1	CCA GGA TGG CAA GCC ATG TTC C	5270–5291
SOUEX1	GGG GAT CCA TGA TGA TGG CGT CTA AGG ACG	5358–5379
SOUEX3	A TTA TGG CTT GGG CCA TTA	6966–6948

Louis, Missouri). A total of 2 μg RNA was reverse transcribed using M-MLV-RT (Gibco) and random hexamers (Pharmacia Biotech, Uppsala, Sweden) as primers for 60 min at 37 °C in a 20 μl reaction. Five μl of the RT-product was used in the first PCR (a total volume of 50 μl) with denaturation at 94 °C for 5 min, 35 cycles of 94 °C (1 min), 54 °C (2 min), 72 °C (1.5 min) and finally 72 °C for 7 min. The PCR product (1 μl) was transferred to a semi-nested PCR (a total volume of 50 μl), with denaturation at 94 °C for 5 min, 30 cycles of 94 °C (1 min), 60 °C (2 min), 72 °C (1.5 min) and finally 72 °C for 7 min. Taq polymerase (2 U) (Gibco) was used in both PCR's. The primers were designed according to the Norwalk and Southampton virus sequences (GenBank accession numbers M87661 and L07418) and are listed in Table 1. The primer pairs SOU1/SOUEX3 and SOUEX1/SOUEX3 were used in the first and the semi-nested reaction, respectively. The SOUEX1 and SOUEX3 primers were complementary to the 5′-ends of the Norwalk virus ORF2 and ORF3. The SOUEX1 primer had a *Bam HI* restriction site in the 5′end. The seminested PCR generated an amplicon of approximately 1640 bp which was purified from a 2% agarose gel using the Qiagen gel extraction kit (Qiagen, Hilden, Germany).

Cloning and sequencing of ORF2

The ORF2 DNA fragment was ligated into the plasmid vector pCR 2.1-TOPO using the TOPO TA cloning system (Invitrogen, Groningen, The Netherlands). Sequencing of one clone was performed using an automated DNA sequencer (ABI Prism 377, Perkin Elmer Biosystems, Foster City, CA).

Expression of ORF2

The cloned ORF2 DNA fragment was digested out from the pCR 2.1-TOPO vector using *Bam HI/Not I*, and subcloned into the pFastBacHTb vector (Bac-to-Bac system, Gibco). This vector encodes a His affinity tag at the N-terminal of the protein encoded by the insert. Competent *Escherichia coli*, strain DH10Bac, containing a baculovirus shuttle vector (bacmid), were transformed with the plasmid. A transposition produced a recombinant bacmid (bac-ORF2/Hu/NLV/Stav/95/Nor) in which the ORF2 DNA segment was inserted downstream from the baculovirus polyhedrin promoter. The recombinant bacmid was purified from the DH10Bac cells and transfected into *Spodoptera frugiperda* cells (Sf9) by the use of Cellfectin transfection reagent (Gibco). The supernatant was passed once on Sf9 cells, and the cells were analysed for the presence of recombinant baculovirus by PCR using primers complementary to sequences on both sides of the ORF2 DNA insert. Recombinant baculovirus was cultured in monolayer Sf9 cells for 5–6 days. The recombinant capsid protein was expressed in Sf9 cells kept in spinner suspensions.

The Sf9 cells were grown in Sf 900 II SFR medium (Gibco) with 50 U/ml penicillin and 50 μg/ml streptomycin. Fetal calf serum was added (10%) to increase the stability of the cell

culture. To check if the recombinant capsid protein was glycosylated, 1 μg/ml tunicamycin was added to a monolayer culture of infected cells [22].

Purification of recombinant protein

The Sf9 cells and the supernatant were analysed for recombinant protein 2, 3, 4, 5 and 6 days after the cells had been infected with recombinant baculovirus. Proteins in the supernatant were concentrated by polyethylene glycol (PEG 8000) precipitation [12]. The Sf9 cells were pelleted and resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-pH 8.0, 300 mM NaCl) and sonicated on ice for 5×5 sec. The recombinant protein and cell debris were pelleted by centrifugation at 4 °C for 15 min at $10\,000 \times \mathbf{g}$. This pellet was resuspended in the lysis buffer supplemented with 6 M guanidinium hydrogenchloride (GuHCl) (Sigma) for denaturation, and 15 mM imidazole (Sigma). After stirring for 60 min at room temperature, the suspension was centrifuged at 4 °C for 15 min at $10\,000 \times \mathbf{g}$. The supernatant was mixed with Ni-NTA resin (Qiagen) for 60 min at room temperature to capture the His-tag on the rStav. The resin was washed three times with lysis buffer containing 8 M urea and 15 mM imidazole. Resin bound protein was eluted with 500 mM imidazole and 8 M urea. Urea was removed from the eluate by dialysis against the lysis buffer overnight at 4 °C.

Proteins were analysed by SDS-PAGE (polyacrylamid gel gradient 10–15) and were transferred to a nitrocellulose membrane (Western blotting-WB). Murine anti-His antibodies (Pharmacia Biotech) or rabbit anti-rNV antibodies were used as primary antibodies. The secondary antibodies were horseradish peroxidase conjugated, and 3-amino-9-ethylcarbazole (AEC) (Sigma) used as substrate. rNV was included as a positive control. Proteins purified from Sf9 cells infected with native baculovirus were included as a negative control. The anti-rNV antibodies and the rNV antigen were kindly provided by Dr. Mary Estes, Baylor College of Medicine, Houston, Texas.

The concentration of recombinant protein was estimated by Coomassie staining and by the method described by Bradford [2].

Immunization of rabbit

One rabbit was injected subcutaneously with 100 μg purified rStav in Freund's complete adjuvant. Three booster injections of 70–100 μg purified protein in Freund's incomplete adjuvant were administered at three-week intervals. The animal was bled prior to immunization and 5, 8, 10, 11 and 12 weeks after the first immunization.

Antibodies against the rStav were detected in WB using 100 ng of rStav or rNV. Rabbit sera were diluted 1:5000 in PBS pH 7.4 and incubated with the blots at 4 °C over night. The membranes were subsequently incubated for 2.5 h at room temperature with biotinylated anti-rabbit antibodies (Amersham, Amersham, UK). Streptavidin-biotin horseradish peroxidase complex (Bio-Rad, California) and AEC were used for detection. Similar WBs were also performed with anti-rNV antibodies.

Detection of anti-rStav antibodies

Sera collected from Norwegian military recruits ($n = 60$) in 1994 were tested in a WB for the presence of antibodies against the rStav. The sera had previously been tested in an ELISA for antibodies against the rNV and had been divided into three groups; anti-rNV negative ($n = 30$), indeterminate ($n = 12$) and positive ($n = 18$) [32]. Purified rStav (100 ng) was run in SDS PAGE (gradient 10–15) and blotted onto nitrocellulose membranes. The blots were incubated at 4 °C over night with serum samples diluted 1:100 and then with biotinylated anti-human antibodies (1:1000 dilution) (Organon Teknika, West Chester, PA) for 2.5 h at room temperature, followed by streptavidin-biotin horseradish peroxidase (1:20 dilution) for

20 min prior to addition of AEC substrate. PBS (pH 7.4) with 0.05% Tween-20 and 3% carnation nonfat milk was used as diluent. Between each incubation, the membranes were washed 3×5 min in PBS with 0.05% Tween-20. A serum with high response in the rNV ELISA was used as positive control in the WB. A pool of three sera that were rNV antibody negative was used as negative control. The negative control sera were kindly provided by Dr. U. Desselberger, Addenbrooke's Hospital, Cambridge, UK.

Sequence date

The nucleotide and deduced amino acid sequence of the Hu/NLV/Stav/95/Nor capsid gene has been deposited in the EMBL, GenBank and DDBJ databases under accession no AF145709.

Results

The sequence of the capsid gene and its predicted protein

Nucleotide sequence analyses of the complete vector insert showed a 1654 nucleotide fragment which encoded a polypeptide of 545 amino acids. Alignment studies of the nucleotide and amino acid sequences displayed 67–77% and 65–88% similarity to the capsid genes and proteins, respectively, of the genogroup I viruses. An 88% similarity was found between the amino acid sequences of Hu/NLV/Stav/95/Nor and Desert Shield virus. The nucleotide and amino acid similarities between Hu/NLV/Stav/95/Nor and the genogroup II virus were 49–51% and 43–45%, respectively. The regions that corresponded to the primer sequences were excluded in the comparisons.

The variation in the amino acid sequence of the capsid protein between Hu/NLV/Stav/95/Nor and the genegroup I viruses was most pronounced in the middle of the sequence of the capsid protein (residues 281–404 for NV) (Fig. 1). In this part of the peptide sequence, the rStav showed a similarity of 71.6% to that of Desert Shield virus and 49.2% to the peptide sequence of NV. The divergence between the capsid peptide sequences from Desert Shield virus and Hu/NLV/Stav/95/Nor was solely based on substitutions (except one aa). The difference to the more distantly related NV was, however, also caused by insertions/deletions as well as substitutions. Six cysteine residues are conserved in all three sequences.

Expression, purification and detection of the recombinant capsid protein

A protein band possibly corresponding to the Hu/NLV/Stav/95/Nor capsid protein was detected in WBs from cells infected with recombinant baculovirus, but not from cells infected with the native baculovirus (Fig. 2). Coomassie staining of the eluate after electrophoresis showed high purity of the protein. This protein, with an estimated molecular weight of approximately 68 kDa, could be detected using either anti-His or anti-rNV antibodies (Fig. 2). The rNV appeared as a protein with a lower molecular weight, previously reported to be 58 kD [14]. The expression of rStav in medium containing the glycosylation inhibitor tunicamycin produced no alternation in the molecular weight of rStav (data not shown). A suspension of 500 ml Sf9 cells infected with the recombinant baculovirus yielded an estimate of 100–300 μg purified rStav.

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                                Region 1
Hu/NLV/Stav/95/Nor    (1) MMMASKDAPPNMDGTSGAGQLIPEANTAEPISMEPVAGAATAAATAGQVN
     Desert Shield    (1) MMMASKDAPTNMDGTSGAGQLVPEANTAEPISMEPVAGAATAAATAGQVN
           Norwalk    (1) MMMASKDATSSVDGASGAGQLVPEVNASDPLAMDPVAGSSTAVATAGQVN
         Consensus    (1) MMMASKDA    DG SGAGQL PE N   P  M PVAG  TA ATAGQVN

Hu/NLV/Stav/95/Nor   (51) MIDPWIMNNYVQAPQGEFTISPNNTPGDILFDLQLGPHLNPFLSHLAQMY
     Desert Shield   (51) MIDPWIMSNYVQAPQGEFTISPNNTPGDILFDLQLGPHLNPFLSHLAQMY
           Norwalk   (51) PIDPWIINNFVQAPQGEFTISPNNTPGDVLFDLSLGPHLNPFLLHLSQMY
         Consensus   (51)  IDPWI  N VQAPQGEFTISPNNTPGD LFDL LGPHLNPFL HL QMY

Hu/NLV/Stav/95/Nor  (101) NGWVGNMKMKVLLAGNAFTAGKIIISCIPPGFAAQNISIAQATMFPHVIA
     Desert Shield  (101) NGWVGNMRVKVLLAGNAFTAGKIIISCIPPGFAAQNISIAQATMFPHVIA
           Norwalk  (101) NGWVGNMRVRIMLAGNAFTAGKIIVSCIPPGFGSHNLTIAQATLFPHVIA
         Consensus  (101) NGWVGNM     LAGNAFTAGKII SCIPPGF   N  IAQAT FPHVIA

Hu/NLV/Stav/95/Nor  (151) DVRVLEPIEVPLEDVRNVLFHNND-NTPTMRLVCMLYTPLRASGSSSGTD
     Desert Shield  (151) DVRVLEPIEVPLEDVRNVLFHNND-SSPTMRLVCMLYTPLRASGSSSGTD
           Norwalk  (151) DVRTLDPIEVPLEDVRNVLFHNNDRNQQTMRLVCMLYTPLRTGGGTG--D
         Consensus  (151) DVR L PIEVPLEDVRNVLFHNND    TMRLVCMLYTPLR  G     D

Hu/NLV/Stav/95/Nor  (200) PFVIAGRVLTCPSPDFSFLFLVPPNVEQKTKPFSVPNLPLNTLSNSRVPS
     Desert Shield  (200) PFVIAGRVLTCPSPDFSFLFLVPPNVEQKTKPFSVPNLPLNTLSNSRVPS
           Norwalk  (199) SFVVAGRVMTCPSPDFNFLFLVPPTVEQKTRPFTLPNLPLSSLSNSRAPL
         Consensus  (201)  FV AGRV TCPSPDF FLFLVPP VEQKT PF  PNLPL  LSNSR P
                                                          | Region 2
Hu/NLV/Stav/95/Nor  (250) LIKSMMVSRDHGQMVQFQNGRVTLDGQLQGTTPTSASQLCKIRGSVFHAN
     Desert Shield  (250) LINAMMISRDHGQMVQFQNGRVTLDGQLQGTTPTSLSQLCKIRGKVFHAS
           Norwalk  (249) PISSIGISPDNVQSVQFQNGRCTLDGRLVGTTPVSLSHVAKIRG----TS
         Consensus  (251)  I     S D  Q VQFQNGR TLDG L GTTP S S   KIRG

Hu/NLV/Stav/95/Nor  (300) GGNGYNLTALDGSPYHAFESPAPIGFPDLGECDWHMEASPTTQFDTGDVI
     Desert Shield  (300) GGNGLNLTELDGSAYHAFESPAPIGFPDIGDCDWHMSATATNNFTGSSNE
           Norwalk  (295) NGTVINLTELDGTPFHPFEGPAPIGFPDLGGCDWHINMT---QFGHSSQT
         Consensus  (301)  G   NLT LDG   H FE PAPIGFPD G CDWH        F

Hu/NLV/Stav/95/Nor  (350) KQINVKQESAFAPHLGTIQADGLSDVSVNTNMIAKLGWVSPVSDGHRGNV
     Desert Shield  (350) YQILIKQESAFAPHLGHVQADNLS-AGANTDLIVSLSWISPVSDQHRHDV
           Norwalk  (342) QYDVDTTPDTFVPHLGSIQANGIG--SGN--YVGVLSWISPPSHPSGSQV
         Consensus  (351)            F PHLG  QA       N      L W SP S      V
                                          | Region 3
Hu/NLV/Stav/95/Nor  (400) DPWVIPRYGSTLTEAAQLAPPIYPPGFGEAIVFFMSDFSIAHGTNGLSVP
     Desert Shield  (399) DPWVIPRYGSSLTEAAQLAPPIYPPGFGEAIVFFMSDFPVVSGVNGMRIP
           Norwalk  (388) DLWKIPNYGSSITEATHLAPSVYPPGFGEVLVFFMSKMP---GPGAYNLP
         Consensus  (401) D W IP YGS  TEA  LAP  YPPGFGE  VFFMS      G      P

Hu/NLV/Stav/95/Nor  (450) CTIPQEFVTHFVNEQAPTRGEAALLHYLDPDTHRNLGEFKLYPEGFMTCV
     Desert Shield  (449) CTLPQEYVAHFVNEQAPTRGEAALLHYVDPDTHRNLGEFKMYPEGFMTCV
           Norwalk  (435) CLLPQEYISHLASEQAPTVGEAALLHYVDPDTGRNLGEFKAYPDGFLTCV
         Consensus  (451) C  PQE   H   EQAPT GEAALLHY DPDT RNLGEFK YP GF TCV

Hu/NLV/Stav/95/Nor  (500) PNSSGTGPQTLPINGVFVFVSWVSRFYQLKPVGTAGPAR-RLGIRRS
     Desert Shield  (499) PNSSGSGPQTLPINGVFTFVSWVSRFYQLKPVGTAGPAR-RLGIRRS
           Norwalk  (485) PNGASSGPQQLPINGVFVFVSWVSRFYQLKPVGTASSARGRLGLRR-
         Consensus  (501) PN   SGPQ LPINGVF FVSWVSRFYQLKPVGTA  AR RLG RR
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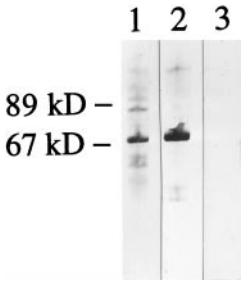

Fig. 2. Western blotting of purified rStav proteins from recombinant baculovirus (RB). Native baculovirus (NB) were used as negative control. Anti-His or anti-rNV antibodies were used as primary antibodies. *1* RB and anti-His antibodies; *2* RB and anti-rNV antibodies; *3* NB and anti-rNV antibodies. Molecular weights are indicated at the left

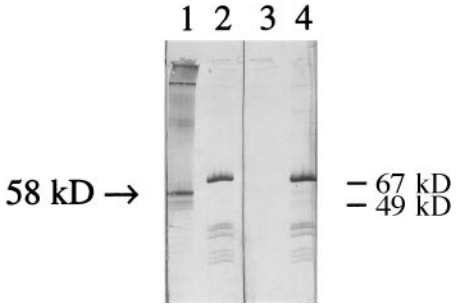

Fig. 3. Western blotting using sera from rabbits immunized with rStav and rNV. *1* rNV and anti-rNV antibodies; *2* rStav anti-rNV antibodies; *3* rNV and anti-rStav antibodies; *4* rStav and anti-rStav antibodies. Molecular weights are indicated at the right

Monitoring of rStav expression in Sf9 cells showed that the rStav appeared at day 2 post infection (p.i.) and was detectable at least until day 6 p.i. No peak in the expression was noticed and the Sf9 cells were regularly harvested at day 3. When cellular and extracellular proteins were tested for the presence of rStav by WB, the rStav was found to locate strictly intracellularly and was not found in the culture medium. The rStav could only be dissolved in a buffer containing 6 M GuHCl, a strong denaturant. The use of 8 M urea could not solubilize the expressed rStav, but could keep the protein in solution after denaturation, and facilitated recovery of the purified protein. Removel of GuHCl was necessary due to interference with SDS during SDS-PAGE electrophoresis.

Immunogenicity of the rStav

Rabbit blood samples were collected at day 0 and 5, 8, 10, 11 and 12 weeks p.i. Antibodies to rStav were detected by WB in all samples collected after immunization. The sample collected at day 0 did not contain rStav-antibodies.

Rabbit anti-rNV antibodies showed a strong reponse against the rStav, while the rabbit immunized with rStav only showed a weak antibody reponse against the rNV (Fig. 3).

Fig. 1. Alignment of the capsid protein sequence of three genogroup I NLV (Hu/NLV/Stav/95/Nor, Desert Shield virus and Norwalk virus). The consenus sequences consist of identified amino acids of all three sequences. Bold letters denote differences between Hu/NLV/Stav/95/Nor and Desert Shield virus sequences. Region 2 is most variable. Six cystein residues (positions nos. 127, 184, 209, 326, 435, 483, for NV) are conserved for all sequences

Table 2. Sixty recruit sera tested for anti-rStav antibodies in a WB. The sera had previously been tested for antibodies against rNV in an ELISA

		rStav WB positive sera
rNV ELISA-positive sera	(n = 18)	18 (100%)
rNV ELISA-indeterminate sera	(n = 12)	11 (91.7%)
rNV ELISA-negative sera	(n = 30)	17 (56.7%)
Total number of sera tested	(n = 60)	46 (76.7%)

Detection of anti-rStav antibodies

The sera collected from Norwegian military recruits were tested in WB using rStav as antigen. The results are presented in Table 2. Seventeen of the 30 rNV ELISA-negative sera were positive in the rStav WB. Eleven of the 12 rNV ELISA-indeterminate sera, and all or the 18 rNV ELISA-positive sera, were positive in the rStav WB.

Discussion

Expression of the ORF2 Hu/NLV/Stav/95/Nor in the Sf9 cells produced a protein with an apparent molecular size of approximately 68 kD, as revealed by SDS-PAGE. The protein was thus larger than rNV, the rDSV, the rLordsdale, the rMX, the rToronto and the rGrimsby capsid proteins which are 58 kD, 59 kD, 58 kD, 57 kD, 58 kD and 58 kD proteins, respectively. However, the molecular size of the peptide sequence of rStav was calculated to be 58.6 kD, and the His tag and TEV protease cleavage site, encoded by the vector, add approximately 2,8 kD to the size of the protein. Sf9 insect cells, like mammalian cells, can introduce post-translational modifications to expressed proteins. The results indicate that rStav is not glycosylated, however, modifications such as phosphorylation may also contribute to an abnormal migration of a protein in SDS-PAGE, and thereby to a discrepancy between the calculated and observed molecular size.

The apparent yield of rStav expression was markedly lower than that reported for rNV, rMX and rLordsdale, which were expressed using recombinant baculoviruses containing ORF3 in addition to ORF2. However, rHV, rToronto and rGrimsby capsid proteins were expressed in insect cells with vectors containing ORF2 only, and no influence of ORF3 was found on the expression of rHV in mammalian cells. The His-tag may have contributed to the low expression and to the cellular location of rStav. In contrast to rStav, all or most of the rNV, rLordsdale, rMX, rTV, rHV and rGrimsby proteins were found in the culture fluid. Capsid assembly was also inhibited when recombinant protein from a Sapporo-like virus was expressed with a His-tag in insect cells [19]. A strong denaturant was needed to solubilize the rStav and indicated that the protein produces inclusion bodies that are difficult to dissolve. GuHCl denaturates the protein completely, without breaking any disulfide bonds. Removal of the denaturing agent may result in renat-

uration of the protein, i. e. the protein regains its native structure [31]. Renaturation of Stav was registered in the absence of a denaturant, as recovery of the protein from the resin was inhibited with the use of an elution buffer devoid of urea. This was probably due to aggregation and entrapment of the rStav in the resin column.

A number of studies have investigated homotypic and heterotypic serologic responses to different recombinant antigens from genogroup I and II NLV [3, 7, 8, 17, 26, 27, 33, 37]. These studies have indicated that the antigenic diversity of these viruses impedes the usefulness of immunoassays. The viruses in genogroup I are antigenically distanct from viruses in genogroup II, and sero-responses to viral antigens from these genogroups thus only display minor cross-reactions. Even within genogroup II, there exist related, but separate, antigenic entities, and therefore several different antigens are required in serological tests to ensure the detection of an immune response to NLV genogroup II viruses. The NLV genogroup I viruses appear to be more antigenically homogenous. Infection with DSV, a genogroup I virus with 32% aa divergence to NV over the entire capsid region, resulted in a serologic response against rNV [27]. However, hyperimmune sera specific for NV did not react with DSV stool antigen in an ELISA, indicating that there may be unique antigenic sites not shared between DSV and NV. There has been a limited expression and use of different antigens from genogroup I NLV, and the extent of heterologic serologic responses to viruses within genogroup I could be clarified by the expression and use of genogroup I capsid proteins other than rNV.

One of the purposes of the present study was to express the capsid protein from a “local” genogroup I NLV. The peptide sequence of rStav showed an overall 35% divergence from that of NV capsid protein (Fig. 1). In region 2 (aa 281–404), which is the most variable part, rStav shows as much as 50.8% divergence to NV. This is in accordance with the general finding that the diversity of capsid protein sequences of NLV is most pronounced in the mid part of the protein [5, 28]. The importance of this region for antigenic diversity has not been fully elucidated. However, it is predicted to be part of the protruding arches of recombinant capsid proteins, and antigenic mapping of the rNV has indicated that dominant epitopes for the humoral immune response are located here [10, 35, 36]. A monoclonal antibody which blocked binding or rNV to human and animal cell lines was localized to the 300–384 residues of the capsid protein, indicating these residues to be involved in binding to Caco-2 cells [39]. The high variability in amino acids in this region could indicate a selection pressure, e.g. the host immune response, on this part of capsid protein, and thus it may be important to the antigenic differences between NLV. In region 2, the rStav showed 27.5%, 49.2%, and 71.6% amino acid sequence identity to Snow Mountain virus (G II), NV and DSV, respectively. The amino acid sequence alignment of the complete capsid revealed that Hu/NLV/Stav/95/Nor is closely related to DSV, with 88% sequence homology. Several studies on the prevalence and age-related acquisition of antibodies against NLV have indicated that children aquire infection with NLV early in life. In England and Sweden, approximately 50% of the children had been infected by NV at 2 and 5 years age, respectively [6, 11]. The English study showed an

increase in the seroprevalence to 81.4% in the age range 20–29 years. In Sweden, the prevalence was more than 80% for individuals older than 10 years. In Norway, the prevalence of antibodies to NV among military recruits (18–22 years old) was found to be only 29.5%, indicating that infection with this virus is not as common in Norway as in other countries [32]. However, 21.5% of the sera in the Norwegian study were found to be indeterminate, and could represent NLV infections producing weak heterologous anti-rNV responses. Retesting of some of the recruit sera was performed in a rStav WB because a rStav ELISA was difficult to establish due to low amount of antigen. The results indicate that infections with other genogroup I NLV could result in no or only a weak heterologous humoral response against rNV. The recombinant proteins may share some epitopes and rStav may have unique epitopes not found on rNV. The finding of only 49.2% aa similarity between rStav and rNV in a 123 aa region of the capsid which is assumed to be immunological important, makes the existence of unique epitopes likely. The results could also be due to differences in sensitivity and specificity between the rNV ELISA and rStav WB detection systems. The existence of unique epitopes on rStav was also indicated by differences in the specificity of the humoral response between the rNV and rStav immunized rabbits, as shown in WB (Fig. 3). Immunization of the rabbit with denatured rStav could partly explain this result, although removal of urea by dialyzation, prior to immunization, most probably resulted in refolding of the protein. A subsequent study in our lab on immunomagnetic separation of Hu/NLV/Stav/95/Nor using rabbit polyclonal antibodies against rStav showed that the antibodies recognized the authentic virus (data not shown).

The use of an antigen (rStav) derived from a "local NLV", indicated a higher prevalence of anti-NLV antibodies (76.7%) than the use of antigen (rNV) derived from a geographically distant origin (29.5% excluding, and 51% including the rNV ELISA indeterminate sera). Although the study included only a small number of sera ($n = 60$), it indicated that variants of genogroup I NLV other than NV have been more prevalent in Norway. It could therefore be concluded that the prevalence of antibodies against genogroup I NLV may be underestimated if rNV is used as the sole NLV genogroup I antigen. Several different genogroup I antigens may be required, as for genogroup II antigens, to ensure that most of the antigenic range of genogroup I viruses are present, if antibodies to genogroup I NLV are to be detected.

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References

1. Berke T, Golding B, Jiang X, Cubitt DW, Wolfaardt M, Smith AW, Matson DO (1997) Phylogenetic analysis of the caliciviruses. J Med Virol 52: 419–424

2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. Cubitt WD, Green KY, Payment P (1998) Prevalence of antibodies to the Hawaii strain of human calicivirus as measured by a recombinant protein based immunoassay. J Med Virol 54: 135–139
4. Dingle KE, Lambden PR, Caul EO, Clarke IN (1995) Human enteric Caliciviridae: the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J Gen Virol 76: 2 349–2 355
5. Estes MK, Hardy ME (1995) Norwalk virus and other enteric caliciviruses. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant L (eds) Infections of the gastrointestinal tract. Raven Press, New York, pp 1 009–1 034
6. Gray JJ, Jiang X, Morgan-Capner P, Desselberger U, Estes MK (1993) Prevalence of antibodies to Norwalk virus in England: detection by enzyme-linked immunosorbent assay using Baculovirus-expressed Norwalk virus capsid antigen. J Clin Microbiol 31: 1 022–1 025
7. Green KY, Kapikian AZ, Valdesuso J, Sosnovtsev S, Treanor JJ, Lew JF (1997) Expression and self-assembly of recombinant capsid protein from the antigenically distinct Hawaii human calicivirus. J Clin Microbiol 35: 1 909–1 914
8. Hale AD, Grawford SE, Ciarlet M, Green J, Gallimore C, Brown DWG, Jiang X, Estes MK (1999) Expression and self-assembly of Grimsby virus: antigenic distiction from Norwalk and Mexico viruses. Clin Diagn Lab Immunol 6: 142–145
9. Hardy ME, Estes MK (1996) Completion of the Norwalk virus genome sequence. Virus Genes 12: 287–290
10. Hardy ME, Tanaka TN, Kitamoto N, White LJ, Ball JM, Jiang X, Estes MK (1996) Antigenic mapping of the recombinant Norwalk virus capsid protein using monoclonal antibodies. Virology 217: 252–261
11. Hinkula J, Ball JM, Löfgren S, Estes MK, Svensson L (1995) Antibody prevalence and immunoglobulin IgG subclass pattern to Norwalk virus in Sweden. J Med Virol 47: 52–57
12. Ingham KC (1990) Precipitation of proteins with polyethylene glycol. Methods Enzymol 182: 301–306
13. Jiang X, Graham DY, Wang K, Estes MK (1990) Norwalk virus genome cloning and characterization. Science 250: 1 580–1 583
14. Jiang X, Wang M, Graham DY, Estes MK (1992) Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 66: 6 527–6 532
15. Jiang X, Wang M, Wang K, Estes MK (1993) Sequence and genomic organization of Norwalk virus. Virology 195: 51–61
16. Jiang X, Matson DO, Ruiz-Palacios GM, Hu J, Treanor J, Pickering LK (1995) Expression, self-assembly, and antigenicity of a Snow Mountain agent-like calicivirus capsid protein. J Clin Microbiol 33: 1 452–1 455
17. Jiang X, Cubbit D, Hu J, Dai X, Treanor J, Matson DO, Pickering LK (1995) Development of an ELISA to detect MX virus, a human calicivirus in the Snow Mountain agent genogroup. J Gen Virol 76: 2 739–2 747
18. Jiang X, Matson DO, Cubitt WD, Estes MK (1996) Genetic and antigenic diversity of human calicivirus (HuCVs) using RT-PCR and new EIAs. Arch Virol [Suppl] 12: 251–262
19. Jiang X, Zhong W, Kaplan M, Pickering LK, Matson DO (1999) Expression and characterization of Sapporo-like human calicivirus proteins in baculovirus. J Virol Methods 78: 81–91

20. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM (1972) Visualization by immune election microscopy of a 27-nm particle associated with acute infectious nonbacterial gestroenteritis. J Virol 10: 1 075–1 081
21. Kapikian AZ, Estes MK, Chanock RM (1996) Norwalk group of viruses. In: Fields BN, Knipe DM, Howley DM, (eds) Fields Virology. Lippincott-Raven, Philadelphia, pp 783–810
22. King LA, Posse RD (1992) The baculovirus expression system. A labarotory guide. Chapman & Hall, London
23. Lambden PR, Caul EO, Ashley CR, Clarke IN (1993) Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259: 516–519
24. Lambden PR, Caul EO, Ashley CR, Clarke IN (1994) Human enteric caliciviruses are genetically distinct from small round structured viruses. Lancet 343: 666–667
25. Lambden PR, Liu B, Clarke IN (1995) A conserved sequence motif at the 5′ terminus of the Southampton virus genome is characteristic of the Caliciviridae. Virus Genes 10: 149–152
26. Leite JPG, Ando T, Noel JS, Jiang B, Humphrey CD, Lew JF, Green KY, Glass RI, Monroe SS (1996). Characterization of Toronto virus capsid protein expressed in baculovirus. Arch Virol 141: 865–875
27. Lew JF, Kapikian AZ, Jiang X, Estes MK, Green KY (1994) Molecular characterization and expression of the capsid protein of a Norwalk-like virus recovered from a Desert Shield troop with gastroenteritis. Virology 200: 319–325
28. Lew JF, Kapikian AZ, Valdesuso J, Green KY (1994) Molecular characterization of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses. J Infect Dis 170: 535–542
29. Lew JF, Valdesuso J, Vesikari T, Kapikian AZ, Jiang X, Estes MK, Green KY (1994) Detection of Norwalk virus or Norwalk-like virus infections in Finnish infants and young children. J Infect Dis 169: 1 364–1 367
30. Liu BL, Clarke IN, Caul EO, Lambden PR (1995) Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. Arch Virol 140: 1 345–1 356
31. Mazzone HM (1998) Viruses as macromolecules. In: CRC handbook of viruses: mass-molecular weight values and related properties. CRC Press, Florida, pp 15–44
32. Myrmel M, Rimstad E, Estes MK, Skjerve E, Wasteson Y (1996) Prevalence of serum antibodies to Norwalk virus among Norwegian military recruits. Int J Food Microbiol 29: 233–240
33. Noel JS, Ando T, Leite JP, Green KY, Dingle KE, Estes MK, Seto Y, Monroe SS, Glass RI (1997) Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gestroenteritis in the United States, 1990–1995. J Med Virol 53: 372–283
34. Pletneva MA, Sosnovtsev SV, Sosnovtseva SA, Green KY (1998) Characterization of a recombinant human calicivirus capsid protein expressed in mammalian cells. Virus Res 55: 129–141
35. Prasad BVV, Rothnagel R, Jiang X, Estes MK (1994) Three-dimensional structure of Baculovirus-expressed Norwalk virus capsids. J Virol 68: 5 117–5 125
36. Prasad BVV, Hardy ME, Jiang X, Estes MK (1996) Structure of Norwalk virus. Arch Virol 12: 237–242
37. Treanor JJ, Jiang X, Madore HP, Estes MK (1993) Subclass-specific serum antibody responses to recombinant Norwalk virus capsid antigen (rNV) in adults infected with Norwalk, Snow Mountain, or Hawaii virus. J Clin Microbiol 31: 1 630–1 634

38. Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, Estes MK (1994) Sequence diversity of small, round-structured viruses in the Norwalk virus group. J Virol 68: 5 982–5 990
39. White LJ, Ball JM, Hardy ME, Tanaka TN, Kitamoto N, Estes MK (1996) Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. J Virol 70: 6 589–6 597
40. Wright PJ, Gunesekere I, Doultree JC, Marshall JA (1998) Small round-structured (Norwalk-like) viruses and classical human caliciviruses in southeastern Australia, 1980–1969. J Med Virol 55: 312–320

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