

proteins (summarized in Figure 5A). This defined p22 as the most variable protein in human noroviruses [39]. Despite this, two regions within amino acids 50–148 showed clear sequence conservation. The first region was the MAD (Figure 5A, blue box), indicating that this domain is well-conserved amongst homologues of p22 and likely serves a similar function between genogroups. The second was a YXΦESDG motif (Figure 5A, red box), where X is any amino acid and Φ is a bulky, hydrophobic residue (e.g. M, I or L), which was fully conserved in 65 of the 72 (90%) sequences available for examination (summarized in Figure 5A). Unexpectedly, conservation of this motif was limited to p22 homologues of human noroviruses. It was not present in either the MNV or FCV homologues of p22, both of which exhibit very low amino acid identity with NV p22, and was similarly absent in the 3A protein of poliovirus and coxsackievirus B3. Further examination revealed that the YXΦESDG motif resembled the criteria for a di-acidic ER export signal, which typically contains a YXXΦ motif immediately proximal to two acidic residues separated by a single amino acid [e.g. YXXI(E/D)X(E/

D)], all of which are located on the cytosolic side of a TM domain (Figure 5B) [9,10]. Such signals increase the rate of protein export from the ER into COPII vesicles and onward to the Golgi [9,10,40].

To explore if this conserved norovirus motif could play a role in the previously demonstrated inhibition of cellular protein secretion by p22 (Figure 2C), individual alanine mutations were made within each of the conserved residues within the putative ER export signal of p22 and tested as before using the SEAP system. Mutations within the S, D and G residues had no effect on the ability of p22 to inhibit cellular protein secretion (Figure 5C); however, mutation of both the Y and E residues led to intermediate levels of protein secretion. When these two residues were combined into a single AXΦASDG construct, SEAP secretion at 36 hpt was not statistically different from that of GFP alone (p=0.08). Total SEAP expressed by all p22 mutants did not significantly differ from that of wildtype p22 and similar levels of all p22 proteins were expressed, as confirmed by western blot analysis of intracellular fractions at 36 hpt (Figure S2),

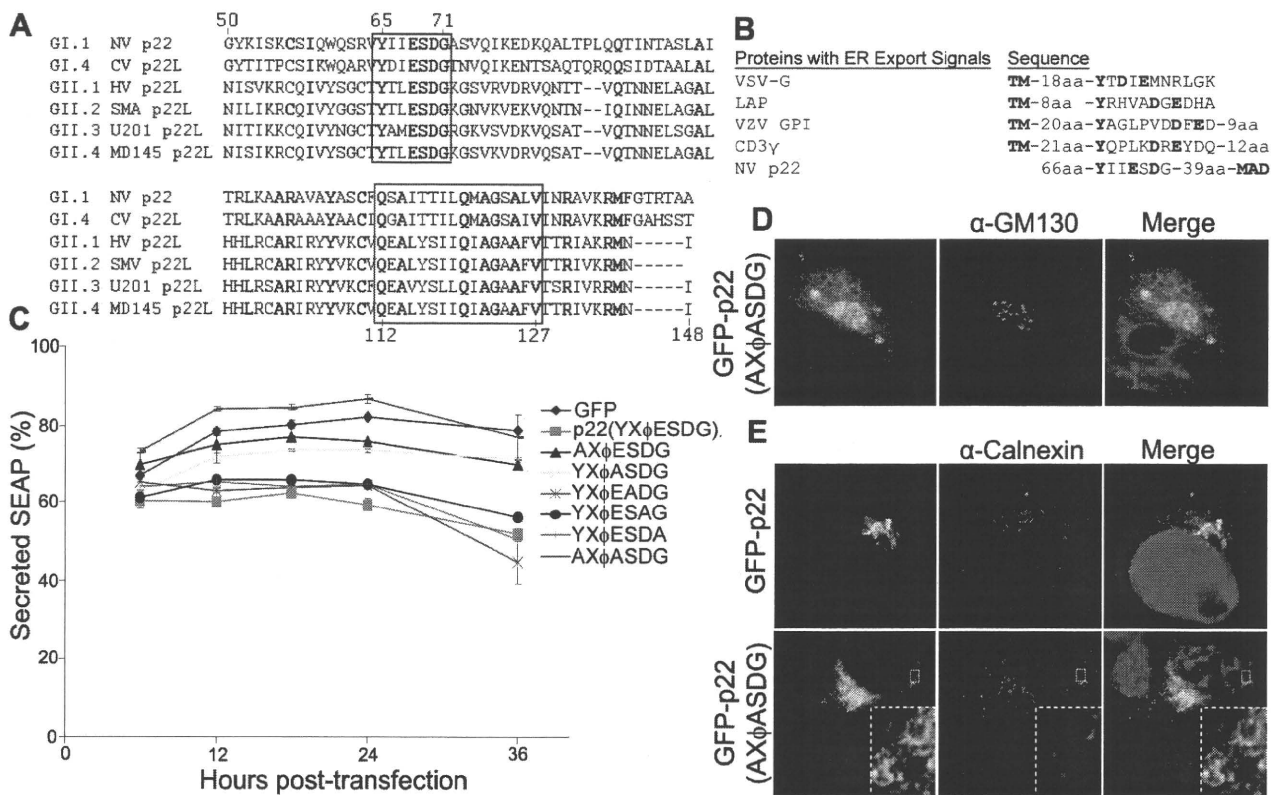


Figure 5. A conserved noroviral ER export signal is necessary for p22 to inhibit protein secretion. (A) Amino acids 50–148 from NV p22 were aligned with homologues [“p22-like (p22L) proteins”] from representative genogroup 1 (GI) and genogroup 2 (GII) human noroviruses of various genotypes. The figure illustrates six of 72 sequences analyzed. Conserved residues are shown in bold; the blue box indicates conservation of the membrane association domain (MAD); the red box indicates conservation of the YXΦESDG motif. NV is Norwalk virus (NC_001959), CV is Chiba virus (AB042808), HV is Hawaii virus (U07611), SMV is Snow Mountain virus (AY134748), U201 is Saitama U201 virus (AB039782), and MD145 is MD145 virus (AY032605). (B) Alignment of NV p22 with various cellular and viral proteins that contain an ER export signal. VSV G is the vesicular stomatitis virus glycoprotein, LAP is lysosomal acid phosphatase, VZV GPI is varicella zoster virus glycoprotein I, and CD3γ is a component of the T cell receptor. Adapted from Nishimura and Balch, 1997 [9]. (C) Cells were transfected with the plasmid pCMV-UTR-SEAP expressing GFP, GFP-tagged NV p22, or the indicated mutants within the ER export signal of p22. Secreted SEAP was quantified (see Materials and Methods) as a representative indicator of cellular protein secretion at the indicated time points and was defined by the equation: Secreted SEAP = (SEAP_{extracellular})/(SEAP_{extracellular} + SEAP_{intracellular}) × 100. Data are representative of three independent experiments (n = 3 for each time point; ±SD). (D and E) p22 with the YXΦESDG motif mutated to AXΦASDG (D; E, bottom panels) or wildtype NV p22 (E, top panels) were expressed as GFP fusion proteins. Cells were fixed at 24 hpt and stained with antibody against the cis Golgi marker protein GM130 (D) or the endoplasmic reticulum marker protein calnexin (E) (Alexa 594-conjugated secondary antibody, red fluorescence). Nuclei were stained with DAPI (blue fluorescence) and cells were imaged by deconvolution microscopy. The inset in E is an 8X magnification of the boxed region. doi:10.1371/journal.pone.0013130.g005

showing that the observed decreases in inhibition of protein secretion were not due to changes in protein expression or stability.

To examine Golgi phenotype in cells expressing the AX Φ ASDG mutant of p22, we next explored the phenotype of the Golgi in cells expressing this construct. By electron microscopy at 48 hpt and after flow sorting, cells expressing p22(AX Φ ASDG) had wildtype Golgi (data not shown) with intact cisternae present in 26 of 56 (46%) cells, similar to the frequency of intact Golgi in the presence of GFP alone. Consistent with this observation and the inability of p22(AX Φ ASDG) to inhibit SEAP secretion, the Golgi was intact in cells expressing p22(AX Φ ASDG) by immunofluorescence at 24 hpt (Figure 5D). Moreover, the mutant was much more diffuse throughout the cytoplasm of cells with few to no puncta, and exhibited a prominent reticular pattern suggestive of ER localization. To confirm this observation, we next stained cells expressing wildtype p22 or p22(AX Φ ASDG) for the ER marker protein calnexin, the use of which was validated by complete co-localization with protein disulfide isomerase (Figure S3), another ER marker protein.

Wildtype p22 did not co-localize with the ER-marker protein calnexin (Figure 5E), as would be expected for a protein that utilizes an ER export signal [40,41]. In contrast, the AX Φ ASDG construct of p22 exhibited a more diffuse and reticular staining pattern, much of which overlapped with the ER (Figure 5E, inset), suggesting that the mutated residues were either re-localizing the protein to the ER or slowing the trafficking of the protein from the ER. Constructs that had individual mutations within the Y and E residues had intermediate ER localization, and a construct that had both the glutamic and aspartic acid residues mutated to alanine exhibited ER localization and SEAP secretion equivalent to that of the YX Φ ASDG construct (data not shown). These data indicate that the Y and E residues within the conserved YX Φ ESDG motif are necessary for the proper localization of p22 and may constitute a unique arrangement of an ER export signal.

Taken together, these data demonstrate that despite altered trafficking within the secretory pathway, p22(AX Φ ASDG) that was not ER-localized appeared to be retained at the Golgi, reflecting the ability of ER-localized proteins to be non-specifically trafficked from the ER to the Golgi in the absence of a specific export signal [9,10,40]. This indicates that the Y and E residues of the YX Φ ESDG motif together are critical for the cellular localization of p22, as well as inhibition of protein secretion and Golgi disassembly.

Although the conserved YX Φ ESDG motif was clearly critical to the cellular effects exhibited by p22, two aspects of this signal remained unclear: 1) if this motif is indeed a functional ER export signal; and 2) if this motif alone is sufficient to antagonize ER/Golgi trafficking. To answer both of these questions, we next directly tested if the conserved motif within p22 could functionally substitute for a *bona fide* ER export signal. To do this, the well-characterized ER export signal within the vesicular stomatitis virus glycoprotein (VSV G) was replaced with either the conserved motif found within p22 (G/p22), the mutated p22 signal that abrogates the downstream effects of p22(G/AX Φ A), or a construct of VSV G that has the entire ER export signal mutated to all alanines (G/6xA) and has a significantly decreased rate of ER export [40] (Figure 6A). The efficiency of ER export of VSV G or the various chimeric or mutant G proteins was examined by a previously described technique [9,10,40,42] in which newly made protein is metabolically labeled with ³⁵S-methionine. At the indicated time points, G is purified and digested with EndoH, which only digests G that has not yet reached the Golgi, resulting

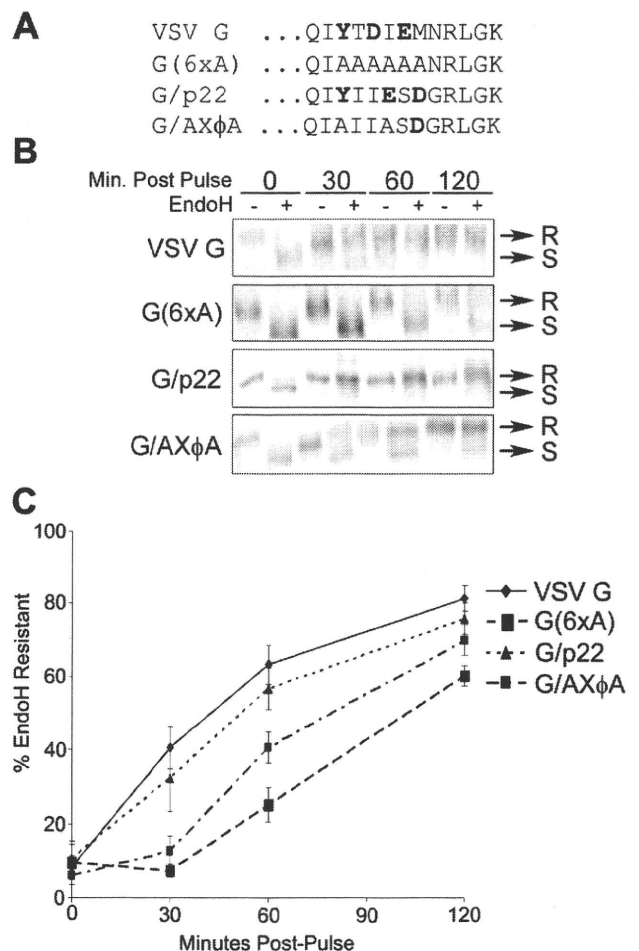


Figure 6. The ER export signal mimic of p22 can substitute for the signal of VSV G. (A) Summary of the sequence of the wildtype, mutant, and chimeric VSV G proteins used. Critical residues of the VSV G ER export signal and homologous regions of p22 are shown in bold. (B) Representative individual samples from EndoH sensitivity assay of VSV G proteins. Wildtype (G), mutant [G(6xA)] and chimeric (G/p22 and G/AX Φ A) VSV G proteins were metabolically labeled with ³⁵S-Methionine at 24 hours post-transfection and incubated for the indicated period of time, harvested in lysis buffer, immuno-precipitated with monoclonal antibody against the luminal domain of VSV G and digested with endoglycosidase H (EndoH). R = EndoH resistant; S = EndoH sensitive. (C) Cells were transfected with plasmids encoding the indicated constructs of VSV G. At 24 hours post-transfection, cells were labeled with ³⁵S-Met and at various times post-pulse cells were harvested, immuno-precipitated with antibody against the luminal domain of VSV G and assayed for their sensitivity to EndoH. Data are composite (mean \pm SD) of six individual samples (n = 6) for each time point from two independent experiments. doi:10.1371/journal.pone.0013130.g006

in a shift in its apparent molecular weight following resolution by SDS-PAGE (Figure 6B). The percent of G that is resistant to EndoH allows quantitation of the amount of protein that is ER-associated compared to protein that had reached the Golgi, thereby yielding a direct measure of ER export efficiency.

As expected, wildtype VSV G rapidly became resistant to EndoH digestion, whereas G(6xA) exhibited a significant decrease in kinetics of ER export compared to wildtype G [$p < 0.00001$ for all time points except 0 minutes post-pulse (mpp)] (Figure 6C). In contrast, G/p22 demonstrated ER export statistically indistinguishable from wildtype VSV G ($p \geq 0.07$ for all time points except

0 mpp), indicating that the conserved motif found within p22 can substitute for the signal found within G and is therefore a functional ER export signal. In contrast, and similar to G(6xA), G/AXΦA had significantly decreased kinetics of ER export ($p \leq 0.001$ for all time points except 0 mpp). The ability of G/AXΦA to ultimately become resistant to EndoH digestion more efficiently than G(6xA) is likely attributable to the presence of the aspartic acid residue on G/AXΦA, which has previously been shown to improve ER export efficiency compared to alanines alone [9,10,40]. In addition, the Golgi was phenotypically intact in cells expressing both wildtype G and G/p22 (data not shown), suggesting that protein trafficking is not inhibited due solely to the presence of the YXΦESDG motif.

Taken together, these data suggest that the motif found within p22 can function for the well-characterized ER export signal found within VSV G and thus this motif constitutes a true ER export signal. For this reason, we henceforth refer to the YXΦESDG motif within p22 as a mimic of an ER export signal, or MERES, motif. In addition, mutation of two residues within the MERES motif that are critical to the function of p22 is sufficient to decrease ER export efficiency. This observation coupled with the inability of the MERES motif alone to induce Golgi disassembly when expressed on VSV G demonstrated that it alone is insufficient to antagonize ER/Golgi trafficking.

p22 targets COPII, but not COPI, trafficking

We lastly sought to determine, first, what is the fate of the cargo in the secretory pathway? And second, does p22 target the anterograde or retrograde pathway of ER/Golgi trafficking? To answer both of these questions, we utilized the SEAP vector described above to monitor the localization of COPI and COPII vesicle marker proteins with respect to secretory pathway cargo, in this case again monitoring SEAP itself as the cargo, and alterations that occur due to the presence of p22.

The 3A protein from several picornaviruses inhibits protein secretion by deregulating COPI vesicle budding from the *cis* Golgi [12,13,43]. To determine if p22 might utilize a similar mechanism of action, the phenotype of COPI vesicle transport in cells expressing p22 was explored by immunofluorescence of the COPI marker protein β -COP. In the presence of GFP alone, SEAP was present only in an intact Golgi, where COPI puncta are prominently, though not exclusively, localized (Figure 7A). As expected, SEAP was prominently retained in cells expressing both PV 3A (Figure 7B) and NV p22 (Figure 7C), although with different phenotypic localizations. In 3A-expressing cells, COPI puncta were diffuse and unapparent, and SEAP was retained in diffuse, minimally punctate cellular structures reminiscent of Golgi that has been redistributed into the ER due to antagonism of trafficking by 3A, as has been reported previously [12,34,44]. In contrast, COPI puncta in cells expressing p22 were apparent but re-localized widely throughout the cytoplasm, demonstrating a failure of these vesicles to properly localize and/or traffic within cells. SEAP in p22-positive cells was present both in discrete punctate vesicles that did not co-localize with COPI puncta, and also in peri-Golgi clusters that did co-localize with COPI puncta. This suggested that SEAP, and therefore cellular cargo, was being retained in non-COPI puncta and at a *cis* Golgi site due to the expression of p22. This suggested that the retrograde, Golgi-to-ER arm of the secretory pathway was intact, but an aspect of the forward pathway was non-functional in p22-expressing cells. These data demonstrate that, in contrast to 3A, p22 does not specifically target COPI trafficking, but does induce cellular cargo retention between the ER and Golgi.

Due to these observations and because ER export signals promote the rapid and direct uptake of cargo into COPII-coated vesicles, which are necessary for ER-to-Golgi protein trafficking, we hypothesized that p22 is acting on the forward, ER-to-Golgi trafficking pathway to inhibit COPII vesicle budding or trafficking to the Golgi to thereby induce Golgi disassembly and inhibit protein secretion. To test this hypothesis, we again examined the specific sub-cellular localization of the retained SEAP that was present in discrete cytoplasmic puncta. Under the same di-cistronic SEAP expression system, in the presence of GFP alone SEAP was again localized exclusively in a phenotypically intact Golgi with COPII puncta immediately surrounding it, although with a near complete lack of co-localization with COPII puncta (Figure 8A). In contrast, in the presence of p22 SEAP was localized in peri-nuclear puncta (Figure 8B), similar to the phenotype of a disassembled Golgi previously described (Figure 2A). Both SEAP and p22 also co-localized with COPII puncta that were also prominently re-localized to the same presumably peri-Golgi structures, although there was some diffuse, likely ER-localized, COPII staining that did not localize with either p22 or SEAP. Additionally, these discrete p22-positive puncta co-localized with SEAP and COPII vesicles (Figure 8B, inset). This suggested that p22 and SEAP are retained with COPII puncta that have properly budded from the ER, but have been mislocalized and did not properly traffic into the Golgi. Due to the apparent size of the p22/SEAP/COPII puncta observed by immuno-fluorescence, it is tempting to speculate that the cellular vesicles with cargo inside them that were observed by EM (Figure 3B) may be enlarged COPII puncta that were mislocalized within cells. When the AXΦA mutant of p22 was expressed, SEAP and COPII puncta both returned to a wild type distribution (Figure 8C) that was phenotypically indistinguishable from cells expressing GFP alone. This demonstrated that, in the presence of p22 and dependent upon the MERES motif, both COPII puncta and their cargo are mislocalized, suggestive of a failure of vesicles to either traffic to or fuse with the Golgi apparatus.

Discussion

We have described for the first time a novel function for the Norwalk virus nonstructural protein p22. The major new findings of this study can be summarized as follows: 1) independent expression of p22 disassembles the Golgi, which also occurs during NV replication, and inhibits cellular protein secretion; 2) subcellular localization of p22 depends on a motif that mimics a cellular ER export signal in both sequence and function, which we have named a MERES motif; and 3) p22 depends on the MERES motif likely to antagonize COPII vesicle trafficking, resulting in Golgi disassembly and an inhibition of cellular protein secretion. Due to the observed trafficking of p22, we propose that p22 is initially localized to the membranes of the secretory pathway via amino acids 103–148, which contain a membrane association domain. The MERES motif then mediates the uptake of p22 onto COPII vesicles, which mislocalize within cells and inhibit, by an as-of-yet undetermined mechanism, proper trafficking to or fusion with the Golgi. Restricting the proper flow of COPII vesicles then induces disassembly of the Golgi and ultimately results in an inhibition of cellular protein secretion.

The MERES motif found within p22 is somewhat unconventional for an ER export signal in that p22 does not encode a signal sequence to mediate ER import, is not glycosylated, and is not exclusively ER localized at any point during expression (Figure 4D and data not shown). Moreover, it has several unique features compared to other known di-acidic ER export signals. First,

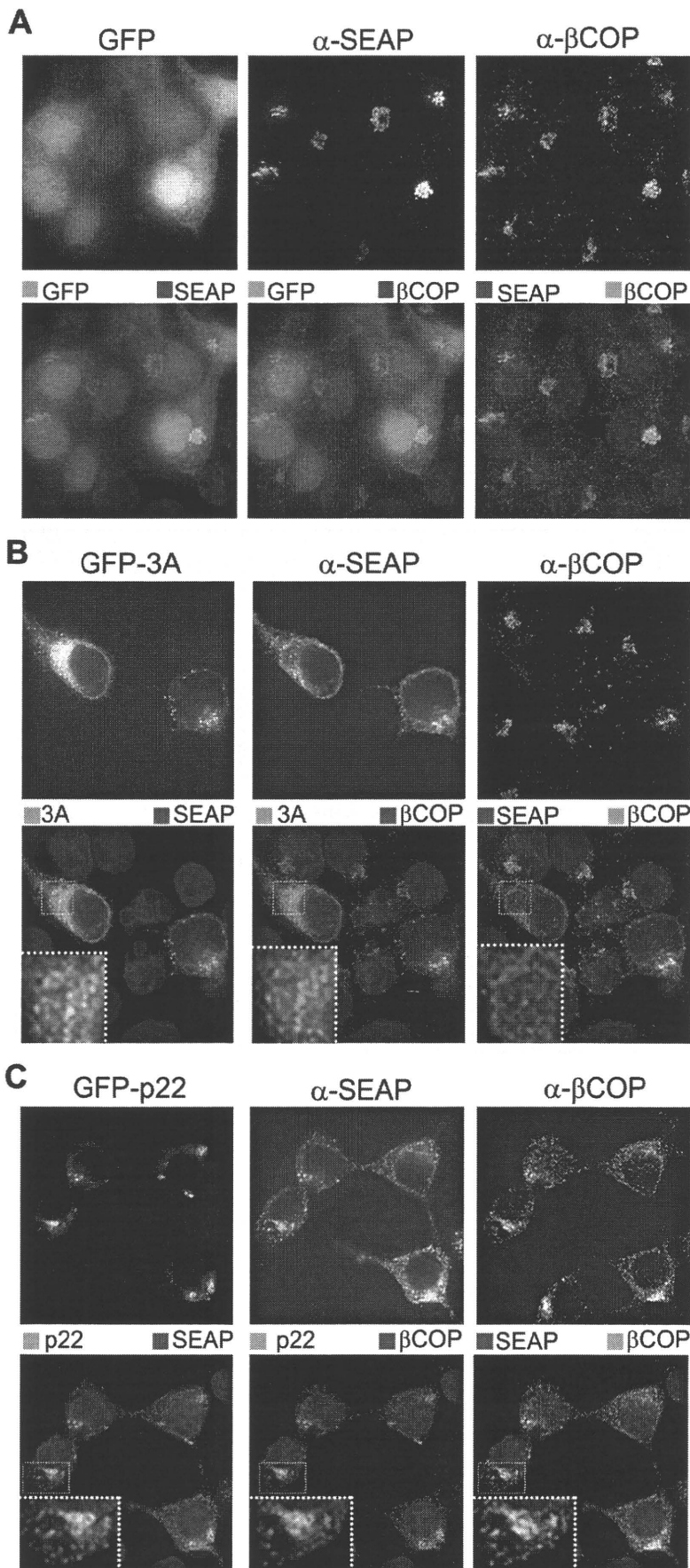


Figure 7. SEAP is differentially retained in the presence of PV 3A and NV p22. Cells expressing GFP alone (A), or GFP-tagged 3A (B) or NV p22 (C) were fixed at 24 hpt and immuno-stained with antibody against SEAP (Alexa 594-conjugated secondary antibody) and the COPI marker protein β -COP (Alexa 647-conjugated secondary antibody), stained with DAPI (blue fluorescence), and imaged by deconvolution microscopy. Channels were pseudo-colored as indicated for merged images. Insets in B and C represent a 6X zoom of the boxed region. doi:10.1371/journal.pone.0013130.g007

instead of the tyrosine residue being located between the MAD and the acidic residues, it is located N terminal to the acidic residues and MAD. Second, instead of encoding two amino acids between the Y and Φ residues, p22 encodes only a single residue. Third, most, though not all, ER export signals are located towards the extreme C terminus of proteins, whereas the signal found within p22 is located in the middle of the protein. The human asialoglycoprotein receptor H1 subunit is the only other protein described to have this arrangement of a di-acidic ER export signal [9]. Additionally, we have no data to support any portion of p22 being present inside the lumen of the ER and instead p22 appears to be a peripheral membrane protein based on sodium carbonate extraction (data not shown). For these reasons, the YX Φ ESDG motif found within p22, though similar in sequence and function to a traditional di-acidic ER export signal, may in reality simply mimic the function of these signals to exploit the cellular machinery responsible for protein secretion in order to recruit p22 to COPII vesicles. This lends support to the nomenclature of this motif being a mimic of an ER export signal, as it shares many of the features and effects of a traditional ER export signal, but ultimately has a unique composition and function.

Furthermore, p22 seems to depend on the MERES motif to function as a secretory pathway inhibitor; however this motif alone is not responsible for antagonism, as replacement of the ER export signal from VSV G with the MERES motif functionally restored ER export efficiency without any obvious impediment. In support of this, none of the deletion mutants of p22 induced Golgi disassembly, although amino acids 50–148 were sufficient to mediate Golgi localization. Similarly, these same residues were sufficient to mediate COPII localization, but do not lead to the mislocalization of COPII puncta (data not shown) that was observed for full length p22. Taken together, these data suggest that the MERES motif of p22 alone is necessary but not sufficient to inhibit the secretory pathway. A second as-of-yet uncharacterized factor, contained within or dependent on the N and/or C termini based on deletion mutant experimentation, is required as a subsequent step in secretory pathway antagonism. This idea is substantiated by chimeric G/p22 properly trafficking to the Golgi and becoming resistant to Endo H digestion with kinetics equivalent to wildtype G. Possibilities for the function of this second factor are many, including binding to and/or disrupting a cellular tethering protein [45,46] or a member of the p24 family that covers COPII vesicles [47], or antagonizing SNARE-mediated vesicle fusion by interaction with a regulatory protein such as VAP-A/B [48], all of which are critical components of ER/Golgi trafficking. This latter possibility is especially attractive, as VAP-B has previously been demonstrated to be a binding partner for another Norwalk virus nonstructural protein [49], and thus may be an over-arching target of the secretory pathway by noroviruses. Both of these possibilities are currently under investigation as potential targets for p22 and as scaffolding factors upon which noroviruses may anchor their genome replication.

The data presented in this study support, but do not prove, that p22 targets COPII vesicle trafficking; this is further complicated by the examination of cells by immunofluorescence primarily at 24 hpt, which may not reflect steady-state localization. Proving this mechanism will require demonstrating a specific binding to or inhibition of the trafficking of COPII vesicles using *in vitro* assays.

Unfortunately, these studies have not been possible due to the inability of producing purified p22 for such studies as well as the lack of an antibody to p22 that allows for immunoprecipitation assays. Therefore, until a direct interaction is demonstrated, it remains possible that p22 could target a non-COPII aspect of the secretory pathway to mediate inhibition. p22 does not activate Arf1 or Sar1 (data not shown), making targeting of the formation of COPI vesicles in the same manner as PV and CVB3 3A, or COPII vesicles by a unique approach, unlikely. A *trans* Golgi mechanism of action seems similarly unlikely based on lack of localization of wildtype p22 with the *trans* Golgi marker protein golgin 97 (Figure S2); the same is also true for possible targeting of endosomes by p22 (data not shown). Thus, although there are many possible alternative explanations for the observed effects of p22, specific targeting and mislocalization of COPII vesicles is at present the most likely explanation.

Although no other cellular or microbial protein to date has been described to use the arrangement of a MERES motif that p22 employs to inhibit the secretory pathway, several previously characterized secretory pathway antagonists have potential ER export signals or mimics thereof. The *Escherichia coli* protein NleA inhibits COPII-dependent export from the ER by direct interaction with Sec24 [50], and the cellular proteins STAM-1 and -2, which are involved in the signaling of growth factors and cytokines, regulate Golgi architecture by interaction the Sec13/31 COPII cage components [51]. Examination of the primary amino acid sequence of NleA and STAM-1/2 revealed motifs similar to a di-acidic ER export signal (unpublished observation). If further studies determine these motifs directly contribute to either the cellular localization or, for STAM-1/2, proper ER export, this would provide support for the idea that ER export signals or their mimics can be used not only to facilitate ER export, but also to promote interaction with COPII vesicles to mediate specific antagonism of the secretory pathway.

Both similarities and differences were noted between p22 and the picornavirus 3A protein. Both proteins localize to membranes via an amphipathic alpha helix, and both inhibit ER-to-Golgi trafficking to decrease cellular protein secretion. However, the mechanism of this shutoff appears to be quite distinct between Norwalk virus and picornaviruses. Inhibition of protein secretion and Golgi disassembly are in some cases separable and distinct, as is the case for PV infection [44], whereas in other cases one will follow the other, for example during cell division [31,52]. There were also clear ultrastructural similarities between cells expressing p22 and 3A in inducing the accumulation of free membranes, double-membrane vesicles and vacuoles [12,34], although cells expressing p22 did not exhibit the swelling of the ER reported for PV 3A [34] or the crystalloid ER patterns seen after expression of the hepatitis A virus 2C and 2BC proteins that also induce significant membrane rearrangements [53]. This further supports the similarity of p22 and 3A in secretory pathway antagonism, but through different arms of this pathway. NV p22 therefore may be more similar in the cellular effects of the hepatitis C virus NS4A/B protein, which, though less studied than PV 3A, antagonizes ER-to-Golgi trafficking, and induces the accumulation of “membranous webs,” vacuoles and double-membrane vesicles, but not ER swelling [54].

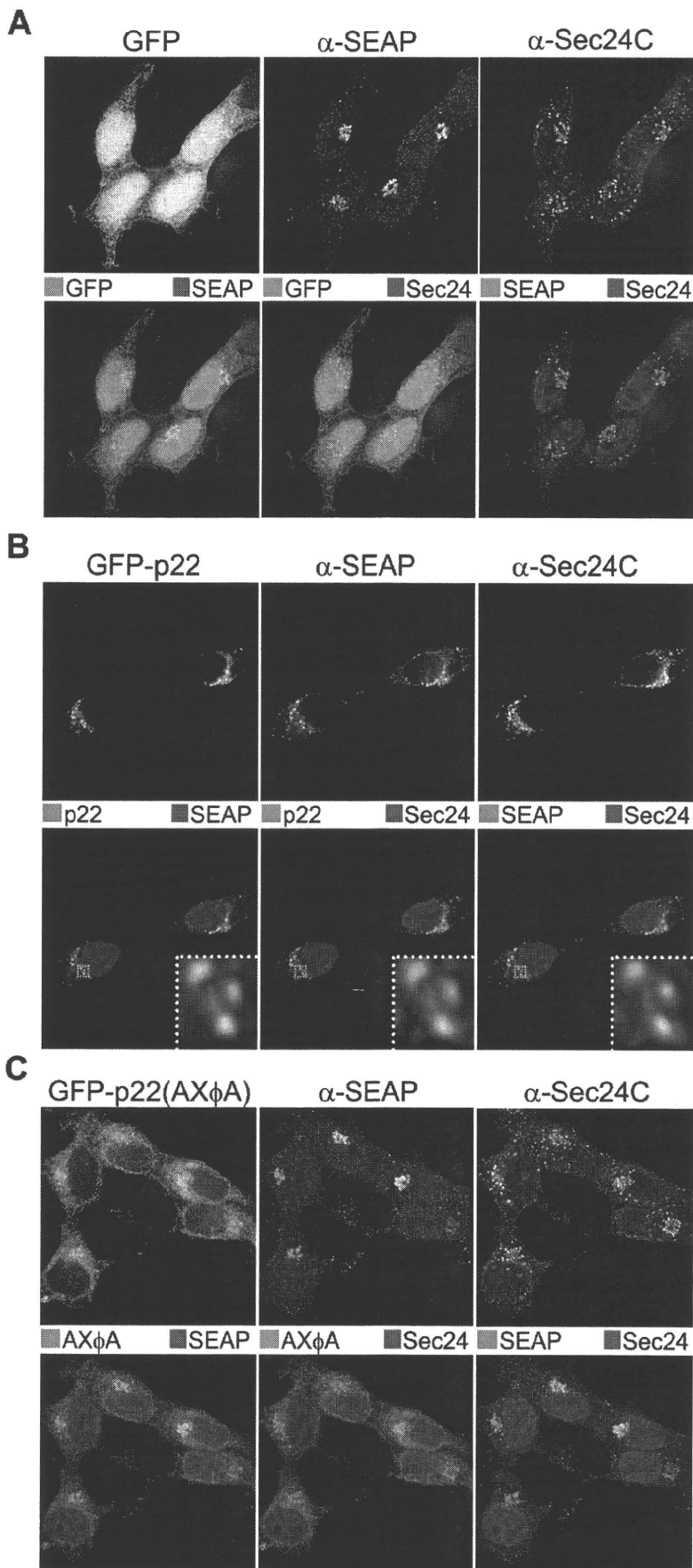


Figure 8. Secretory pathway cargo is retained in COPII vesicles in the presence of p22, but not p22(AXΦA). Cells expressing GFP alone (A), or GFP-tagged NV p22 (B) or p22(AXΦA) (C) were fixed at 24 hpt and immuno-stained with antibody against SEAP (Alexa 594-conjugated secondary antibody) and the COPII marker protein Sec24C (Alexa 647-conjugated secondary antibody), stained with DAPI (blue fluorescence), and imaged by deconvolution microscopy. Channels were pseudo-colored as indicated for merged images. Inset in B represents an 8X zoom of the boxed region.

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Although all the effects of NV infection on the secretory pathway have not yet been explored, the results presented here demonstrate that, like PV 3A, direct antagonism of the secretory pathway is a cause, not an effect, of Golgi disassembly by p22. However, whereas at least two picornavirus 3A proteins interact with the cellular protein GBF-1 to inhibit COPI vesicle budding [13], NV p22 instead appears to target COPII vesicles with dependence upon a motif that is absent from picornavirus 3A proteins, suggesting that these two proteins take different approaches to the same ultimate outcome. It therefore appears that, like several picornaviruses [28,29], noroviruses encode two proteins, p22 and p48 (this study and [27]), with redundant functions of antagonizing the secretory pathway.

FCV p30, a homologue of p22, lacks an ER export signal and localizes exclusively to the ER when independently expressed ([26] and our unpublished observation). This is in line with the inability of BFA to inhibit FCV replication [24], as it does several picornaviruses, and further supports the notion that caliciviruses utilize a different architecture of cellular machinery for replication than do picornaviruses. A recent study has shown that MNV is also resistant to the cellular effects of BFA and does not have an effect on gross Golgi morphology [55]. It will therefore be interesting to determine the roles of p22 homologues from MNV and FCV in possible antagonism of ER/Golgi trafficking, as this would shed much light on similarities and differences between animal and human caliciviruses.

The biological significance of antagonism of the secretory pathway by p22 remains to be understood. This may facilitate viral pathogenesis rather than replication in a manner similar to the effect of the picornavirus 3A proteins that inhibit the immune response to virus-infected cells [14–16], ultimately leading to a more pathogenic infection [12]. Future study of the immune response to norovirus infection should consider secretory pathway antagonism by p22, as this protein may be key in deactivating interferon (IFN) and/or cytokine signaling following infection. Additionally, analysis of the cellular response to NV infection has demonstrated that NV is sensitive to IFN when exogenously added to cells replicating the NV genome [56,57]; however, NV does not induce the IFN pathway or IRF3 activation in Huh7 cells that support a single round of virus replication [58]. Although p22 may be contributing to a reduction in IFN release from cells, the possibility that p22 has additional inhibitory effects on the IFN pathway remains to be explored.

Since viruses utilize cellular processes and machinery for replication, understanding the mechanisms by which viruses parasitize the cell will both increase our understanding of these pathways and aid in the design of effective anti-viral countermeasures. The human norovirus nonstructural protein p22 encodes a novel and well-conserved motif that mimics a traditional di-acidic ER export signal. Instead of increasing the rate of protein trafficking in the secretory pathway, as is the normal function of these signals, the ER export signal mimic allows p22 to gain access to the secretory pathway, induce Golgi disassembly and inhibit cellular protein secretion. This is the first instance in which a pathogen has been described to use a motif similar to an ER export signal to ultimately inhibit cellular protein secretion. This motif constitutes a new target for the design of anti-viral drugs

against noroviruses, as it is necessary for the antagonistic activity of p22 and is highly conserved in human noroviruses.

Materials and Methods

Vectors and cell lines

All genes were cloned using Gateway technology (Invitrogen) and expressed in pcDNA-DEST53 after sequence verification. Mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). HEK-293T and Huh7 cells were maintained in DMEM supplemented with 10% fetal bovine serum. 293T cells were grown on plastic or coverslips coated with 10 µg/ml poly-D-lysine (Sigma). Transfection was carried out using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. cDNA encoding poliovirus 3A protein was obtained from Richard Lloyd of Baylor College of Medicine.

Transfection of NV RNA

NV RNA was purified from volunteer stool samples and transformed into Huh7 cells as described previously [30]. Briefly, virus was purified from volunteer stool samples by sucrose cushion and cesium chloride gradient extraction, following which viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. Five hundred nanograms of purified viral RNA were then transfected into cells, which were then cultured for the indicated period of time.

Immunofluorescence and antibodies

Cells were grown on cover slips and, following the indicated treatments, washed in PBS and subsequently fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M PBS and permeabilized with 0.5% Triton X-100 in 0.1 M PBS. Cells were then washed in PBS and blocked with 1% bovine serum albumin (Sigma, St. Louis, MO) for 1 hour at 37°C, incubated overnight at 4°C with primary antibody in 0.1 M PBS, 1% BSA. Anti-human golgin-97 mouse monoclonal antibody was obtained from Molecular Probes (Invitrogen). GM130 mouse monoclonal antibody was obtained from BD Transduction Laboratories (San Jose, CA). Calnexin mouse monoclonal antibody and rabbit polyclonal antibody against β-COP were obtained from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody against Sec24C was the generous gift of Bill Balch of Scripps Research Institute and has been previously described [59]. Rabbit polyclonal antibody against VP1 was also previously described [60]. Mouse monoclonal antibody 8B6 against placental alkaline phosphatase was obtained from AbCam (Cambridge, MA). Rabbit polyclonal antibody against protein disulfide isomerase (H-160) was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). After incubation with primary antibody, cover slips were washed and incubated at room temperature for one hour with the corresponding AlexaFluor 488-, 594-, or 647-conjugated secondary antibodies (Invitrogen). Nuclei were stained with 300 nM DAPI (Invitrogen) at room temperature for 5 minutes. Cells were then washed and mounted using the ProLong Gold antifade reagent (Invitrogen). All images shown are representative of at least three independent experiments and the direct observation of

no less than 50 cells with GFP expression levels in approximately the middle 70th percentile.

Fluorescence deconvolution microscopy

Deconvolution microscopy was performed with a Zeiss AxioVert S100 TV microscope and a DeltaVision restoration microscopy system (Applied Precision, Inc.) and imaged using either a 63X objective lens (1.40 NA). A *z* series of focal planes was digitally imaged and deconvolved with the DeltaVision constrained iterative algorithm to generate high-resolution images, from which Quick Projections were obtained. Brightness and contrast levels were adjusted appropriately in Adobe Photoshop version CS2.

Flow-coupled electron microscopy

293T cells were transfected with the indicated plasmids and at 24 hpt were harvested in 0.1 M PBS containing 1% BSA. Cells were then flow sorted for GFP on a BD SORP FACS Aria II flow cytometric cell sorter with elution into 10% DMEM. Cells were then centrifuged at 500×g for 10 minutes, suspended in 10% DMEM and plated onto plastic dishes coated with 10 μg/ml poly-D lysine. Twenty-four hours later, and therefore 48 hpt, all cells were validated to be GFP positive under an epifluorescence microscope. Cells were then rinsed once in 0.1 M PBS and fixed in Karnovsky's Fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer +2 mM CaCl₂, pH 7.4) for 1 hour on ice. After being held overnight at 4°C in weak fix (1 part Karnovsky's Fixative: 10 parts 0.1 M cacodylate buffer +2 mM CaCl₂), the cells were rinsed 3 times in 0.1 M cacodylate buffer +2 mM CaCl₂, then post-fixed in 1% OsO₄ in 0.1 M cacodylate at 4°C. After 3 rinses in cacodylate buffer, cells were dehydrated in a gradient series of ethanols from 30–50%, en bloc stained with saturated uranyl acetate in 50% ethanol for 1 hour, carried through to 100% ethanol, then infiltrated in 1 part 100% ethanol: 1 part Spurr's Low Viscosity Resin overnight at room temperature. The rest of the infiltration was performed the next day, through 3 changes of pure resin. A small amount of pure resin was placed in the bottom of thoroughly drained plates, and the plated cells and resin were cured at 60°C for 3 days. Sections were cut at 70–80 nm using a Diatome diamond knife and an RMC MT6000-XL ultramicrotome. Sections were then collected on 100–150 mesh copper grids, counter-stained with Reynold's lead citrate and viewed on a Hitachi H-7500 transmission electron microscope. Image brightness and contrast were adjusted appropriately in Adobe Photoshop version CS2.

Cytosolic and membrane fractionation

Transfected 293T cells were suspended in 0.5 ml of homogenization buffer [200 mM HEPES (pH 7.5), 5 mM sodium pyrophosphate, 5 mM EGTA, 1 mM MgCl₂, 1 mM sodium orthovanadate, 50 μM leupeptin, 200 μM PMSF, 1 μM pepstatin A], followed by sonication and centrifugation at 100,000×g for one hour at 4°C, and the resulting supernatant was the cytosolic fraction. Pellets were resuspended in 0.5 ml extraction buffer [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 100 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM NaF, 1 mM sodium orthovanadate, 50 μM leupeptin, 200 μM PMSF, 1 μM pepstatin A], followed by incubation with rotation at 4°C for one hour and centrifuged at 100,000×g for 1 hour and the supernatant was collected as the membrane fraction. Fractions were then precipitated by addition of trichloroacetic acid to 10% and centrifugation at 16,000×g for 5 minutes. The pellet was then washed in acetone, pelleted, and resuspended in 50 μl of 5X SDS-PAGE sample buffer. Lysate aliquots were boiled for 3

minutes and analyzed by electrophoresis on 12.5% SDS-PAGE gels. Separated proteins were transferred from the gel to nitrocellulose membrane (Amersham Biosciences) and membranes were blocked in 5% Blotto (5% fat-free Carnation milk in 0.01 M PBS) and incubated with a mouse anti-GFP monoclonal antibody (Clontech, Mountain View, CA) in 0.5% Blotto overnight at RT. Primary antibody was removed and membranes were washed three times with 0.5% Blotto. Horseradish peroxidase-conjugated secondary goat anti-mouse immunoglobulin G antibody (Sigma-Aldrich) was incubated with the membranes for ~2 h at RT and subsequently washed three times with 0.5% Blotto. Membranes were developed with SuperSignal sensitivity substrate (Pierce).

SEAP assay

The plasmid pCMV-UTR-SEAP was obtained from J. Lindsay Whitton (Scripps Research Institute) [28] and was made into a Gateway-competent vector using the Gateway Vector Conversion System (Invitrogen) at the *NotI* site of the vector. 293T cells were grown to ~60% confluence in 24 well plates and transfected. Two hours before the indicated time points, cells were washed 2 times in 0.01 M PBS and the media was replaced with 400 μl of fresh media. At the indicated time points, the media containing "extracellular SEAP" was removed. At this same time point, "intracellular SEAP" values were obtained by washing cells once in cold 0.01 M PBS and adding 400 μl of media containing 0.5% Triton X-100 to lyse cells and solubilize any SEAP that had been retained in cells, and then incubating cells at room temperature for ~5 minutes. Lysed cells were then collected and spun at 13,000×g for one minute to remove cellular debris. Enzymatic SEAP activity in "intracellular" and "extracellular" fractions was assayed with the PhosphaLight™ System (Applied Biosystems), and overall Secreted SEAP was calculated with the equation: Secreted SEAP = (SEAP_{extracellular} / (SEAP_{extracellular} + SEAP_{intracellular})) × 100 [28].

EndoH Sensitivity Assay

Sensitivity of VSV G and chimeras to digestion with endoglycosidase H was carried out as previously described [9,40,42] with minor modifications. VSV G or chimeras were expressed in 293T cells from the plasmid pMD2.G. Twenty-four hours post-transfection, cells were washed 3X in Cys/Met-free DMEM and incubated at 37°C for 15 minutes. Cells were then labeled with 200 μCi/ml ³⁵S-Met (Amersham BioSciences) for 15 minutes at 37°C, washed 3X in DMEM containing 10% FBS, and 400 μl of DMEM containing 10% FBS was added to cells, which were then incubated at 37°C. At the indicated time post-pulse, cells were washed 1X in cold PBS and harvested in VSV G Lysis Buffer (50 mM Tris pH 8, 62.5 mM EDTA, 1% NP-40, 0.4% deoxycholate, 50 μM leupeptin, 200 μM PMSF, 1 μM pepstatin A). Supernatants were cleared with 5 μl of Protein A Magnetic Beads (Invitrogen) and incubated overnight with monoclonal antibody against the luminal domain of VSV G (clone 8G5) [42], generously provided by Bill Balch, Scripps Research Institute. Immuno-complexes were collected by magnetic capture and washed 2X in VSV G Lysis Buffer, resuspended in 40 μl of 2.5X SDS-PAGE Sample Buffer and boiled for 5 minutes. Half of the IP was digested with 1 μl of endoglycosidase H (EndoH) (1 U/200 μl, Roche) at 37°C for 1 hour. EndoH treated and non-treated samples were then run on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and bands were quantitated following scanning on a Typhoon Trio Variable Mode Imager (GE Healthcare) and ImageQuant 5.1 to quantitate band intensity.

Statistical analysis

Two-tailed, unpaired Student's *t*-tests assuming unequal variance were used to determine statistical significance; *p* values are indicated where appropriate.

Supporting Information

Figure S1 NV p22 initially localizes to and induces disruption of the trans Golgi. Cells expressing GFP or GFP-tagged Norwalk virus (NV) p22 were immuno-stained for the trans Golgi marker protein golgin-97 (Alexa 594-conjugated secondary antibody, red fluorescence) at the indicated times post-transfection. Nuclei were stained with DAPI (blue fluorescence) and cells were imaged by deconvolution microscopy.

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Figure S2 Equivalent amounts of protein are made during SEAP analysis of p22 constructs containing alanine mutations within the predicted ER export signal. Proteins in lysates from the 36 hpt intracellular fraction of cells utilized in the indicated SEAP assay were run on a 4–20% SDS-PAGE gel and detected with monoclonal antibody against either GFP or actin by western blot.

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Figure S3 Calnexin is an appropriate marker of the endoplasmic reticulum in 293T cells. Non-transfected 293T cells were fixed and immuno-stained for the ER marker proteins calnexin and protein disulfide isomerase with mono- (red fluorescence, Alexa594-conjugated secondary antibody) and poly-clonal antibody (green fluorescence, Alexa488-conjugated secondary antibody), respectively. Nuclei were stained with DAPI (blue fluorescence) and cells were imaged by deconvolution microscopy.

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Author Contributions

Conceived and designed the experiments: TMS SG KK SEC MKE. Performed the experiments: TMS SG. Analyzed the data: TMS SEC MKE. Contributed reagents/materials/analysis tools: KK. Wrote the paper: TMS MKE.

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Binding of Norovirus Virus-Like Particles (VLPs) to Human Intestinal Caco-2 Cells and the Suppressive Effect of Pasteurized Bovine Colostrum on This VLP Binding

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Noroviruses (NoVs), which cannot be grown in cell culture, are a major infectious agent of gastroenteritis. An *in vitro* assay system was established for the evaluation of NoV binding to enterocytes using virus-like particles (VLPs) produced in a baculovirus system expressing a NoV VP1 capsid protein. After confirmation of the purity by MS analysis, VLPs were incubated with human intestinal Caco-2 cells. NoV VLPs were detected clearly by confocal laser microscopy only on a certain population of Caco-2 cells, and were semi-quantified by immunoblotting of cell lysates. Then the suppressive effect of pasteurized bovine colostrum was analyzed on the VLP binding to Caco-2 cells by immunoblotting. The colostrum reduced VLP binding in a dose-dependent manner, at about 50% suppression with 12.5 µg of the colostrum proteins. Furthermore, the colostrum contained IgG antibodies reacting to VLPs, suggesting that cross-reactive antibodies in the bovine colostrums block human NoV binding to intestinal cells.

Key words: food intoxication (poisoning); nonbacterial gastroenteritis; colostrum immunoglobulin; natural antibody

Noroviruses (NoVs) are major etiological agents of human nonbacterial infectious gastroenteritis, including food intoxication.¹⁾ Human NoVs are non-cultivable so far in that the viruses cannot be grown and replicated in cell culture systems,²⁾ resulting in limited information on the infection and replication mechanisms of human NoVs. However, development of recombinant virus-like particles (VLPs) has supplied a new experimental tool for studies of interactions between human NoVs and host cells.³⁾ An approximately 60-kDa capsid protein, VP1, expressed in recombinant baculovirus systems is believed to self-assemble spontaneously into VLPs 38 nm in diameter.⁴⁾ These VLPs have been reported to be morphologically and antigenically similar to the

native virions,⁵⁾ and are regarded as empty virus particles without a virus genome.

The molecules responsible for NoV binding to host cells at the initial infection step have been searched for using NoV VLPs. Some natural and synthesized carbohydrate chains forming human histo-blood group antigens have been found to bind to NoV VLPs *in vitro*, e.g., by ELISA using anti-VLPs antibodies.⁶⁾ On the other hand, VLP binding to host cells has been investigated using metabolically radio-labeled NoV VLPs, and human intestinal Caco-2 cells were reported to be the most remarkable in the VLP binding among several cell lines, including human intestinal epithelial and embryonic kidney cells and insect fibroblastic cells,³⁾ but no microscopic images showing VLP binding to Caco-2 cells have been reported. Heparan sulfate and an unidentified 105-kDa protein of Caco-2 cells were found to be possible receptors for NoV VLPs in studies using radio-labeled NoV VLPs.^{7,8)} The human intestinal Caco-2 cell line is known to differentiate morphologically and functionally in confluent culture and resembles epithelial cells of small intestine⁹⁾ despite its colon-cancer origin.¹⁰⁾

Breast milk plays important roles in supplying not only nutrients but also immunity against environmental pathogens. Especially, a special milk, termed colostrum, produced by the mammary glands in late pregnancy, is believed to be critical for neonates to grow healthy until their development of own immune systems.¹¹⁾ Colostrum from ruminant animals, including bovine ones, is different from that of humans in that bovine colostrum contains a large amount of IgG antibodies,¹²⁾ which are absorbed by neonates in intact form across the intestinal epithelium, because the mother's serum IgGs are not delivered to the fetal circulation *via* the placenta and umbilical cord.¹³⁾ On the other hand, human breast milk, including colostrum, contains secretory IgA antibodies, which play important roles in preventing the coloniza-

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Abbreviations: NoV, norovirus; SaV, sapovirus; VLP, virus-like particle; P-Col, pasteurized colostrum; R-milk, raw milk; R-Col, raw colostrum; MALDI-TOF, matrix-assisted laser-desorption ionization-time of flight

tion of pathogenic bacteria at the intestinal mucus epithelium.¹⁴) In general, the biological definition of colostrum is breast milk that is synthesized and accumulated in mammary glands during the last stage of pregnancy and secreted within 1 d of giving birth. However, in Japan, bovine milk obtained within 5 d of delivery is regarded to be colostrum as a matter of regulation without definite scientific basis, and may not be used as a food.

The first aim of the present study was to establish *in vitro* assay systems to estimate NoV binding to the intestinal epithelium using non-radio-labeled VLPs and cultured Caco-2 cells, and the second was to evaluate the potential suppressive effects of bovine colostrum on NoV binding to the intestinal epithelium using established assay methods. Immunofluorescence staining of Caco-2 cells and immunoblotting analysis of the cell lysates clearly indicated that NoV VLPs bound to Caco-2 cells, and that the detection sensitivity in the established assay was comparable to that of previously reported methods using radio-labeled VLPs. Moreover, bovine colostrum obtained from healthy lactating cows at 6 or 7 d after parturition suppressed VLP binding to Caco-2 cells and contained IgGs cross-reactive to human NoV VLPs.

Materials and Methods

Bovine colostrums. Pasteurized and dried bovine colostrums were prepared at an industrial level in the facility of Kobayashi Pharmaceuticals (Osaka, Japan). For preparation of the pasteurized colostrum (P-Col), colostrums were collected from healthy lactating cows (Holstein) at 6 or 7 d after parturition and pooled. The pooled colostrums were defatted by centrifugation, pasteurized by heating at 72 °C for 15 s (an HTST condition), and concentrated by ultrafiltration, followed by spray-drying under low heating conditions. The powdered P-Col consisted of 49.8% protein, 36.4% lactose, 1.8% fat, 7.8% minerals, and 4.2% moisture. On the other hand, in the preparation of raw milk and colostrum samples (R-Milk and R-Col respectively), milk and colostrums were collected from mid-lactation (2–4 months after parturition) and early-lactation (36–48 h after parturition) healthy Holstein cows respectively bred at a local dairy farm (the Shimizu farm, Kariya, Japan). The R-Milk and R-Col samples were defatted by centrifugation and kept frozen at –20 °C until use.

Preparation of VLPs. VLPs of NoV (Ueno-7k strain) were produced by means of a baculovirus expression system, in which a recombinant VP1 capsid protein of NoV was overexpressed in an insect cell line, High Five™ (Invitrogen), the VLPs secreted into the cell medium were collected by ultracentrifugation at 30,000 rpm using an SW32 rotor (Beckman, Fullerton, CA), and the 38-nm VLPs of native virion size were purified by ultracentrifugation in CsCl containing solution.¹⁵ The size and purity of the VLP preparation were confirmed by electron microscopy. Sapovirus (SaV) VLPs (NK24 strain) were prepared as described previously.¹⁶

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli.¹⁷ The protein samples were boiled for 3 min in SDS-PAGE sample buffer in the presence of 2-mercaptoethanol. Protein bands in gels were detected with Coomassie Brilliant Blue R-250 (CBB) staining. In immunoblotting, the separated proteins were blotted onto a poly(vinylidene difluoride) (PVDF) membrane (Immobilon; Millipore, Bedford, MA), and were visualized using anti-NoV VLP antibody¹⁸ using an enhanced chemiluminescence (ECL) system, as described previously.¹⁹ The relative intensity of the immune-stained band for VP1 was analyzed semi-quantitatively by densitometric measurement using the Light Capture system and CS analyzer software version 3 (Atto Instruments, Tokyo).

Protein identification by MS analysis. The protein bands of the VLP preparation detected by CBB staining of SDS-polyacrylamide gel were excised, cut into small pieces, and subjected to the in-gel digestion. After they were destained with a 50% acetonitrile/25 mM NH₄HCO₃ mixture, the gel pieces were dehydrated with 100 µl of acetonitrile, dried by vacuum centrifugation, and then rehydrated in buffer containing 25 mM NH₄HCO₃ and 1.1 units of trypsin (Trypsin Gold, MS Grade, Promega). Following digestion for 17 h at 37 °C, the peptides were extracted from the gel stepwise with 0.1% trifluoroacetic acid, a 0.1% trifluoroacetic acid/50% acetonitrile mixture, and 100% acetonitrile. The pooled extract was concentrated by vacuum centrifugation and desalted by ZipTip µ-C18 (Millipore). The extracted peptides were subjected to MALDI (matrix-assisted laser-desorption ionization) TOF (time of flight) mass spectrometry using a 4700 Proteomics Analyzer (Applied Biosystems), as described previously.²⁰ The MS and MS/MS data were analyzed using Mascot software (Matrix Science, London).

Cell culture and VLP binding assay. Caco-2 cells from the American Type Culture Collection (Rockville, MD) were maintained in DMEM supplemented with 10% heat inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO), 1% nonessential amino acids solution (Sigma), 100 U/ml of penicillin, and 100 µg/ml of streptomycin, and grown under humidified 5% CO₂ and 95% air at 37 °C. For the immunoblotting analysis, Caco-2 cells were seeded at 1.0 × 10⁵ cells/cm² in 48-well culture plates (Corning, NY) and cultured for 3 d in DMEM supplemented with 10% FCS at 37 °C in a humidified atmosphere with 5% CO₂. Then the cells in each well were washed with cold DMEM and incubated with various concentrations of VLPs in DMEM at 4 °C for 1 h. After washing with cold DMEM 3 times, the cells were lysed with Laemmli buffer. After centrifugation, the supernatants were collected as cell lysates and subjected to immunoblotting analysis, as described above. African green monkey kidney cells, COS7, were cultured and treated similarly with VLPs as a negative control.

Immunofluorescence microscopy. Caco-2 cells were seeded at 1.0 × 10⁵ cells/cm² onto type-I-collagen-coated coverslips in the 6-well culture plate and cultured for 3 d in DMEM supplemented with 10% FCS at 37 °C under a humidified atmosphere with 5% CO₂. Then the cells on coverslips were washed with cold PBS and incubated with various concentrations of VLPs in DMEM at 4 °C for 1 h. After washing with cold DMEM 3 times, the cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice, washed with DMEM, and blocked with NETG (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.05% Triton X-100, and 0.25% gelatin). The cells were incubated with NETG containing the rabbit anti-NoV VLP for VLPs¹⁸ or mouse anti-ZOI (Zymed) for a cell-tight junction, and subsequently with NETG containing Alexa Fluor® 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) for 1 h at room temperature under darkness. The cells were stained with TOTO-3 (Molecular Probes) too for nuclear staining. After washing with PBS, the cells on coverslips were mounted onto glass slides containing a drop of 50% glycerol in PBS. Imaging was performed on a Zeiss Axioplan2 microscope equipped with a LSM5 PASCAL laser scanning confocal optics (Carl Zeiss, Thornwood, NY) in multi-track mode.

ELISA. IgG antibodies specific for NoV and SaV VLPs in bovine colostrums were measured by ELISA, as described previously.²¹ Briefly, 96-well plates (Nunc, Roskilde) were coated with 1 µg per well of VLPs in PBS. After washing and blocking, the plates were incubated with colostrums diluted to a 1–20 µg protein concentration with PBS/Tween/BSA, washed with PBS/Tween, and incubated with POD-conjugated anti-bovine IgG (H and L chains) antibody (Abcam, Cambridge).

Results

NoV VLPs specifically bound to a certain population of Caco-2 cells

The NoV VLPs used in the cell-binding assay were concentrated and purified by ultracentrifugation from culture supernatants of the infected insect cells. The

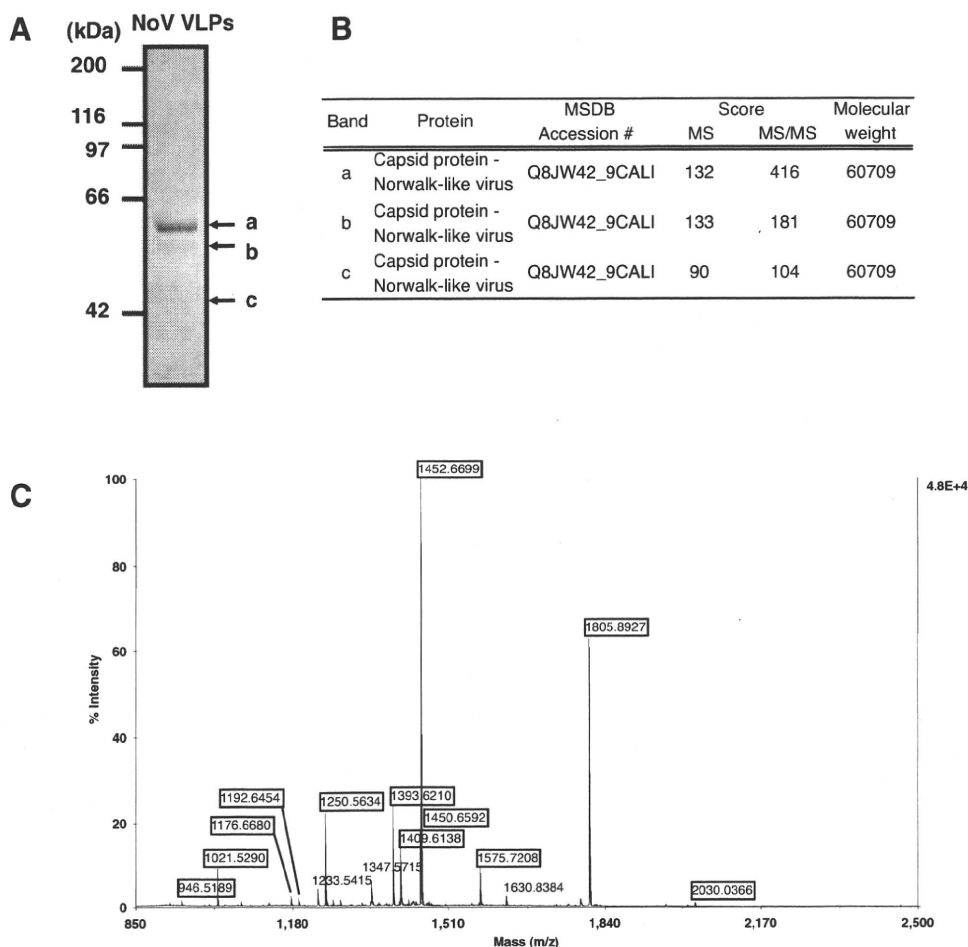


Fig. 1. SDS-PAGE and MS Analysis of NoV VLPs.

NoV VLPs (1 μ g) were subjected to SDS-PAGE, followed by CBB staining (A). Each protein band (a, b, and c, indicated by arrows) was excised, in-gel digested with trypsin, and subjected to MALDI-TOF/MS analysis. All protein bands were identified as the capsid protein, VP1, of NoVs, previously termed Norwalk viruses (B) based on MS data analyzed using the Mascot software. The MS of band c is shown as a representative (C), in which mass peaks identified as VP1 are marked with open squares.

purity of the VLP preparation was examined by SDS-PAGE and subsequent MS analysis. The NoV VLP preparation gave a single major band of 60 kDa, a faint band of 57 kDa, and a nearly invisible 45-kDa band (Fig. 1A). All three protein bands were identified as intact VP1 and fragments of it by MS analysis (Fig. 1B) and their estimated molecular mass by SDS-PAGE was determined. As found in a representative mass spectrum of the 45-kDa band, most of the tryptic peptide signals were assigned to theoretical tryptic peptides of the NoV capsid protein, VP1 (Fig. 1C).

To elucidate the binding of NoV VLPs to human intestinal Caco-2 cells, an immunofluorescence microscopic approach was applied to cells incubated with the VLPs of NoV and of SaV for comparison. As shown in Fig. 2A and B, fluorescent signals of NoV VLPs were clearly detected by confocal laser scanning microscopy in several restricted cells, which formed colonies in the cell culture wells, whereas no signals of SaV VLPs were detected under the same conditions. The difference in the fluorescent signal was clear between VLP-binding positive and negative cells. Non-specific binding of the antibodies to NoV and SaV VLPs was not observed in the cells without VLP treatment. A representative three-dimensional image of a magnified field clearly indicated that NoV VLPs bound to almost the whole area of the

apical cell surface of the positive cells (X/Y and Y/Z planes of Fig. 2C), whereas none did to any area of the negative cells (X/Y and X/Z planes of Fig. 2C).

The binding of NoV VLPs to Caco-2 cells was analyzed in more detail by immunoblotting of total cell lysates. Caco-2 cells and COS7 cells as a negative control were incubated with the VLPs (100 ng) for various periods of time up to 180 min. Immunostained VP1 band of Caco-2 cell lysates became detectable after 5 min of incubation, and the band intensity increased with incubation time, whereas that of COS7 lysates was faintly detected only in the sample of 180 min of incubation (Fig. 3A). Based on the band intensity obtained by densitometric analysis, the NoV VLPs bound to the Caco-2 cells in a well at 180 min of incubation was estimated to be about 16 ng (data not shown). To estimate the binding affinity of VLPs to Caco-2 cells as well as the detection sensitivity of bound VLPs by immunoblotting, various amounts (5 to 150 ng per well) of VLPs were incubated with Caco-2 cells, and were subjected to immunoblotting analysis. The VP1 band was clearly detected at levels of 75 ng or more in a dose-dependent manner, and it was still detectable when only 5 ng of VLPs was incubated with Caco-2 cells (Fig. 3B).

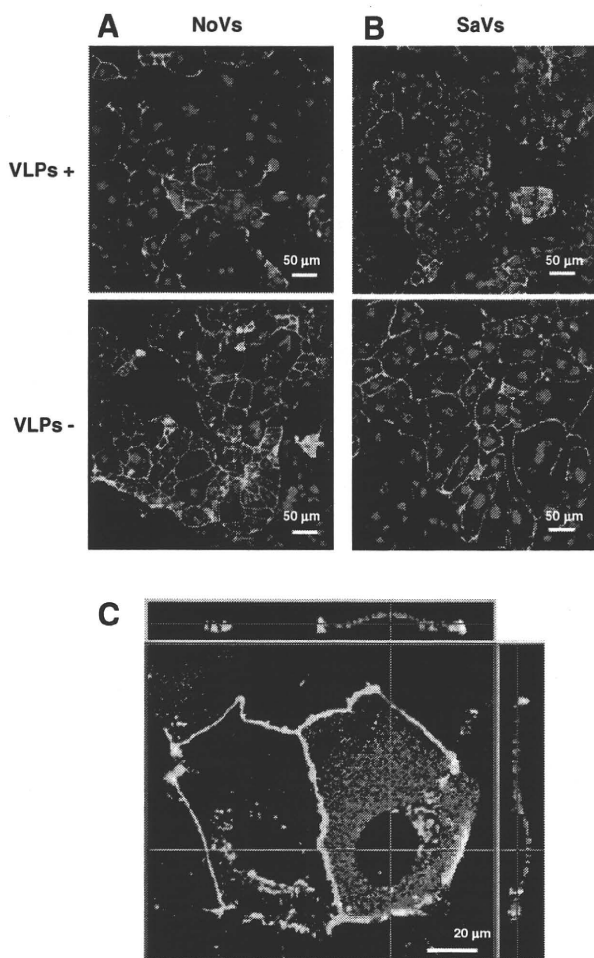


Fig. 2. Confocal Laser Scanning Microscopy of Caco-2 Cells Incubated with NoV and SaV VLPs.

Caco-2 cells were cultured for 3 d on type-I-collagen-coated coverslips in a 6-well culture plate and incubated for 1 h at 4 °C with (+) and without (–) 0.5 μg of NoV VLPs (A and C) or SaV VLPs (B). After washing and paraformaldehyde fixation, the cells were stained with rabbit anti-NoV VLP, rabbit anti-SaV VLP, and mouse anti-ZO-1 (a tight junction marker) antibodies, and subsequently with Alexa Fluor 568 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-mouse IgG. Nuclei were stained with TOTO-3. Typical Z-axis images of Caco-2 cells incubated with NoV VLPs were constructed digitally in the XZ and YZ planes, shown as horizontal and vertical lines in the XY plane (C). Red, NoV VLPs; green, ZO-1; blue, nuclear. Bars, 50 μm in panels A and B; 20 μm in panel C.

Pasteurized bovine colostrums suppressed VLP-binding to Caco-2 cells and contained anti-NoV IgG

Utilizing the above described assay system, the suppressive effect of P-Col on NoV VLP-binding to Caco-2 cells was examined semi-quantitatively by immunoblotting. As shown in Fig. 4A, pre-incubation of the VLPs with an excess amount (25 μg protein against 0.1 μg of VLPs) of P-Col markedly decreased the band intensity of the VLPs in the Caco-2 cell lysate. Non-specific staining was not observed in either lysate of the cells incubated with the various protein concentrations of the colostrums without VLPs. The VP1 band intensity in each cell lysate was quantified by densitometric analysis (Fig. 4B), indicating that the VLPs binding to Caco-2 cells decreased gradually with increases in the amount of P-Col used in pre-incubation with VLPs, and 50% suppression was achieved with 12.5 μg of P-Col.

Bovine colostrums are known to contain large amounts of IgG. The content of the intact form of IgG consisting of two heavy and two light chains in P-Col and R-Col was estimated by SDS-PAGE under non-reduced conditions, to which various amounts (equivalent to 15–50 μg protein) of each sample were subjected (Fig. 5A). The 200-kDa band was identified as IgG by MS and immunoblotting analysis (data not shown), and it was the most dominant protein component in both R-Col and P-Col. The IgG content estimated from the band intensity was higher in P-Col than in R-Col. This higher IgG proportion of P-Col might be ascribed to the preferential concentration of IgG and other proteins during the heat pasteurization process, including the ultrafiltration described in “Materials and Methods.”

Bovine IgG antibodies with reactivity to human NoV VLPs were examined by ELISA on P-Col as well as R-Col and R-Milk for comparison (Fig. 5B). Anti-NoV VLP IgG was detected remarkably not only in R-Col but also in P-Col, while R-Milk showed almost no IgG-binding activity to the VLPs. The IgG reactivity to NoV VLPs of P-Col was a little higher than that of R-Col. VLP binding activity in P-Col was analyzed more in detail at various protein-concentrations using VLPs of SaV in addition to NoV, which belong to the same Calicivirus family. As shown in Fig. 5C, the IgG antibodies in P-Col were reactive to VLPs not only of NoV but also of SaV, and the ELISA value increased in a manner dependent on the sample protein concentration. The rabbit antibody raised by immunizing with NoV VLPs did not cross-react with SaV VLPs under either ELISA or immunoblotting analysis (data not shown).

Discussion

A single VLP is believed to be constructed by spontaneous association of 180 molecules of a single capsid protein, VP1, and the structural or architectural similarity to virus virions has been demonstrated by X-ray crystallography.²²⁾ Moreover, antibodies prepared by immunizing animals with VLPs react well with virus virions in patients’ specimens, and thus immunochemical detection of etiologic agents became possible in diagnosis for NoV food poisoning. Hence, the morphological and immunochemical properties of VLPs have been investigated, while the components of VLPs have not been well characterized biochemically. In the present study, the purity and intactness of VLPs were analyzed by SDS-PAGE and MS-based protein identification, in which all of one major and two minor proteins were identified to be VP1 (Fig. 1). This indicates that the VLPs prepared by the baculovirus expression system are constructed from a single capsid protein, VP1, as expected, without contaminating proteins from the expression system, but a trace amount of degraded VP1 was also present in the VLPs. Nevertheless, such a faint proteolysis of VP1 is expected not to affect the VLPs-cells binding assay, and therefore the VLP preparation was used in the binding assay. It is unlikely that the degraded VP1 or partially degraded VLPs inhibited the binding of intact VLPs to the cells.

A recent study on NoV infection using biopsy specimens from the duodenum of patients with NoV

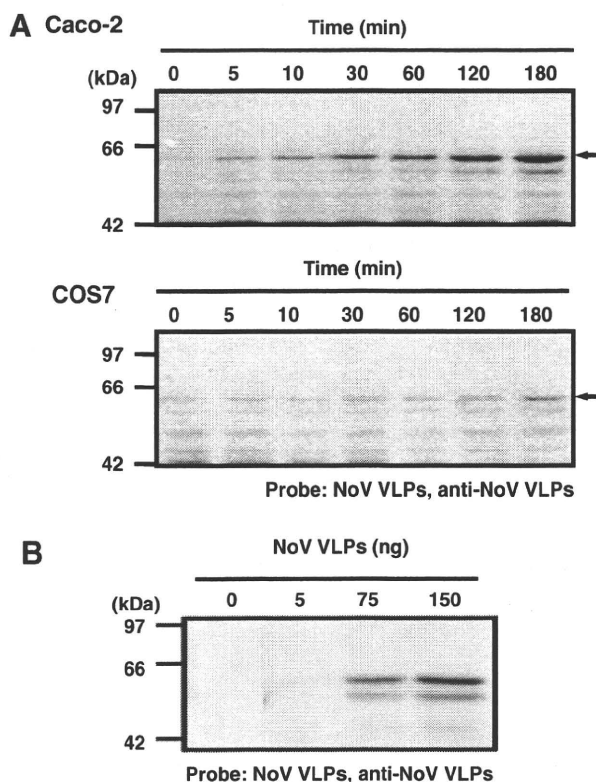


Fig. 3. Immunoblotting Analysis of NoV VLPs Bound to Caco-2 Cells.

Caco-2 cells were cultured in 48-well culture plates for 3 d and then incubated with NoV VLPs (0.1 μ g per well) at 4°C for various periods (5–180 min). Total cell lysates were subjected to SDS-PAGE, followed by immunoblotting for NoV VP1 (A). The migration position of NoV VP1 is indicated by arrows. The cells were incubated with various amounts of NoV VLPs (5, 75, and 150 ng per well) for 60 min, and NoV VLPs were analyzed by immunoblotting as above (B).

infection indicated that NoV infected and replicated in the epithelial cells of the small intestine.²³ NoV infection at the distal small intestine and colon remains to be investigated. Caco-2 is a cell line established from human colon cancer cells.¹⁰ Nevertheless, Caco-2 cells in confluent culture express several hydrolytic enzymes and nutrient transporters, typical markers of absorptive epithelial cells of the small intestine.⁹ Therefore, the observation, in the present and previous studies,^{3,7} that NoV VLPs bound to Caco-2 cells, especially differentiated ones, is reasonable. A recent reverse genetic study of human NoV indicated that native NoV could be produced in Caco-2 cells into which NoV genomic RNA had been transfected.²⁴ Thus it is likely that the NoV-binding assay system established in the present study at least in part reflects or mimics the initial step of *in vivo* NoV infection in the human intestine. This does not necessarily rule out the possibility of NoV infection in human colon epithelia.

Caco-2 cells might consist of heterogeneous cell populations, because they have not necessarily been cloned. The observation that only a small population of colonized Caco-2 cells was positive to NoV VLP binding (Fig. 2) might be explained by this authentic heterogeneity of Caco-2 cells. The frequency of the VLP-binding positive cells in cultured Caco-2 cells increased when the cells were cultured for longer

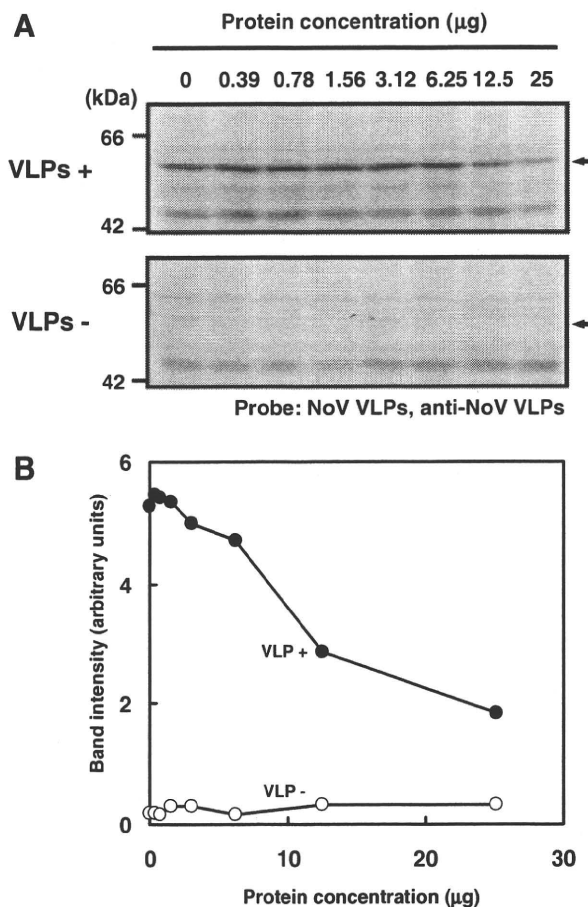


Fig. 4. Suppression of NoV VLP Binding to Caco-2 Cells by Pasteurized Bovine Colostrum.

NoV VLPs (50 ng) and a buffer control without the VLPs were pre-incubated for 15 h at 4°C with various amounts (0.39–25 μ g) of pasteurized bovine colostrum (P-Col), and then incubated with Caco-2 cells for 1 h at 4°C, followed by immunoblotting analysis for NoV VLPs, as described in the legend to Fig. 3A. The migration position of NoV VP1 is indicated by arrows. The VP1 band intensities were digitized and semi-quantified by densitometric analysis (B). Closed and open circles represent NoV VLPs and the buffer control respectively.

periods (10 d), but the positive cells were still only a minor population (Murakami and Matsuda, unpublished results). NoV VLPs might bind preferentially to differentiated cells in a certain population of Caco-2 cells. In fact, differentiated Caco-2 cells have been reported to be 2–3 times more active in binding with radio-labeled NoV VLPs than undifferentiated cells.³ From a practical viewpoint, however, the preparation of well-differentiated Caco-2 cells is time consuming and costly. The results of the present study indicate that Caco-2 cells incubated for 3 d were usable in VLP-binding assay based on sensitive immunodetection systems. Thus the VLP-binding assay using undifferentiated Caco-2 cells, *e.g.*, cultured for only 3 d, should have an advantage in high throughput screening of antiviral factors in food.

NoV VLP-binding to some restricted cells in an all-or-none manner in addition to no binding of SaV VLPs (Fig. 2) indicates that the binding of NoV Ueno-7k VLPs to Caco-2 cells is specific and significant (physiologically or pathologically relevant). Furthermore, almost no binding of NoV VLPs to COS7 cells

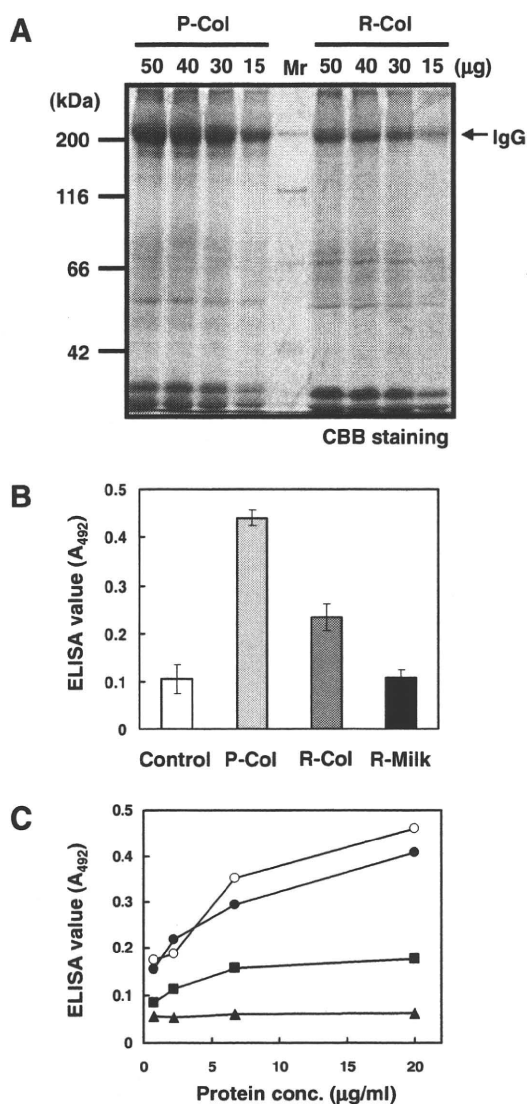


Fig. 5. Bovine Colostrum IgG with Reactivity to NoV- and SaV-VLPs.

Proteins in pasteurized and raw colostrums (P-Col and R-Col respectively) were analyzed comparatively by SDS-PAGE (10% acrylamide gel under non-reducing conditions) (A). ELISA plates were coated with NoV VLPs (0.1 µg/ml) and incubated with 10 µg of protein/ml of P-Col, R-Col, raw milk (R-Milk), and egg albumin as a control. Bovine IgGs bound to the coated VLPs were detected with POD-conjugated anti-bovine IgG (B). ELISA plates were coated with 0.1 µg/ml of NoV VLPs (solid circles and solid triangles), SaV VLPs (open circles), and gelatin (solid squares) as a control and incubated with 10 µg of protein/ml of P-Col. Bovine IgGs bound to the coated VLPs were detected with POD-conjugated anti-bovine IgG. A control assay using POD-conjugated anti-rabbit IgG was also done to test the non-specific binding of the secondary antibody (solid triangles) (C).

(Fig. 3A) suggests that NoV VLP-binding is specific to human intestinal cells. VLP-binding was detected even when only a trace amount of VLPs (5–150 ng) was incubated with Caco-2 cells (Fig. 3B). Based on these results, the binding affinity or avidity of NoV VLPs to Caco-2 cells might not be low. On the other hand, the VP1 band intensity increased almost linearly through an incubation period of 180 min, suggesting that the rate of VLP-binding is low, probably due to the low dispersion rate of VLPs much larger in size than single protein molecules. In the present study, the amounts of VLPs added or bound to the Caco-2 cells were at

10–100 nanogram levels, smaller than previously reported ones (1–10 micrograms), indicating that immunoblotting detection of VLPs using ECL systems has detection-sensitivity higher than the scintillation counting method using VLPs metabolically labeled with ³⁵S-methionine.³⁾

Because maternal IgG is not transmitted to the embryo through the placenta in ruminant species of mammals, bovine colostrums contain a large amount of IgG,¹³⁾ which is important or critical for newborn calves to gain immunity against environmental pathogens until the development of their own immune systems. The pooled colostrums from healthy and non-immunized cows contained IgG with reactivity to human NoV VLPs, suggesting that cows' colostrums contain naturally occurring antibodies with a large repertoire of antigen specificity, including human pathogens. Furthermore, colostrums are known to be rich also in antimicrobial components other than immunoglobulins, including lactoferrin, lactoperoxidase, and lysozyme.²⁵⁾ Therefore, in addition to colostrum IgG, unidentified anti-NoV factors might be present in the colostrums. Hence, colostrums have been expected to contain various immunological and non-immunological defensive factors against enteric pathogens in animals, including humans, but the practical application of bovine colostrums to food as ingredients has been restricted by a Japanese regulatory issue, where bovine colostrums from day 1 to day 5 lactating cows may not be used in food, and moreover milk has to be pasteurized for the market. In the present study, bovine colostrums for P-Col were collected from lactating cows 6–7 d after parturition and heated for pasteurization under mild conditions to prevent or reduce the inactivation of antiviral factors, including antibodies and some other proteins, in the colostrums. In fact, anti-NoV VLPs IgG as well as anti-SaV VLPs IgG was detected in P-Col. Hence, the regulatory and food-safety issues would be resolved at least technically. Bovine colostrums collected at 6 d after parturition or later might be usable in food and food supplements, in which an anti-NoV effect is expected.

Another issue to be discussed is whether such active IgGs escape degradation by digestive enzymes and reach the intestine, where NoV infects the enterocytes. Secretory IgA in human milk and saliva is known to be stabilized by association with a secretory component, and to be resistant against digestive enzymes.^{12,26)} In ruminant animals, including cows, secretory antibodies in colostrums are IgG class,²⁷⁾ though the secretion mechanisms across the mammary gland epithelium and any structural changes or modifications during secretion remain to be investigated. Some food proteins have been reported to remain intact, at least at immunologically detectable levels, in the lumen of the mouse small intestine.^{21,28)} It would be of interest to investigate the digestibility or resistance of bovine colostrum IgG *in vitro* and *in vivo* using animal models.

In conclusion, *in vitro* binding-assay using NoV VLPs with Caco-2 cells can be a useful tool in primary screening for anti-NoV in food and natural products, and bovine colostrums have some potential as an infection-protective factor against enteroviruses, including NoV and SaV.

Acknowledgments

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Structure of Hepatitis E Virion-sized Particle Reveals an RNA-dependent Viral Assembly Pathway^{*[5]}

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Hepatitis E virus (HEV) induces acute hepatitis in humans with a high fatality rate in pregnant women. There is a need for anti-HEV research to understand the assembly process of HEV native capsid. Here, we produced a large virion-sized and a small $T=1$ capsid by expressing the HEV capsid protein in insect cells with and without the N-terminal 111 residues, respectively, for comparative structural analysis. The virion-sized capsid demonstrates a $T=3$ icosahedral lattice and contains RNA fragment in contrast to the RNA-free $T=1$ capsid. However, both capsids shared common decameric organization. The *in vitro* assembly further demonstrated that HEV capsid protein had the intrinsic ability to form decameric intermediate. Our data suggest that RNA binding is the extrinsic factor essential for the assembly of HEV native capsids.

Hepatitis E virus (HEV),³ the causative agent of acute hepatitis in humans, is primarily transmitted through contaminated water and generally results in epidemic outbreaks in many developing countries. Sporadic cases have also been reported between outbreaks in HEV-endemic regions as well as in non-endemic areas, and these cases are transmitted through zoonotic route. The overall mortality rates of HEV during outbreaks range from 1 to 15% in general, and the highest mortality occurs in pregnant women, with fatality rates of up to 30% (1).

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The atomic coordinates and structure factors (codes 2ZZQ and 3IYO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

The EM data reported in this paper have been submitted to the Electron Microscopy Data Bank (Electron Microscopy Data Bank) with accession number EMD-5173.

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–4 and supplemental Table 1.

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³ The abbreviations used are: HEV, hepatitis E virus; VLP, virus-like particle; TEM, transmission electron microscopy; STEM, scanning TEM; TMV, tobacco mosaic virus; DIG, digoxigenin.

HEV consists of a non-enveloped icosahedral capsid and a single-stranded, positive-strand RNA genome of ~7.2 kb that encodes three open reading frames (ORFs) (2). The capsid protein, encoded by the ORF2, is composed of 660 amino acids and responsible for most capsid-related functions, such as virion assembly, host interaction, and immunogenicity. Like other hepatitis viruses, HEV is unable to propagate in currently available cell culture systems, and the research of HEV relies largely on the recombinant HEV capsid proteins (3–6). Virus-like particle (VLP) was obtained when the truncated HEV capsid protein was expressed in insect Tn5 cells with deletion of 52 residues from the C terminus and 111 residues from the N terminus (PORF2) (7). Our previous structural analysis of this HEV-VLP by cryo-electron microscopy (cryo-EM) provided a basic understanding of the quaternary arrangement of PORF2, where the reconstructed VLP displayed a $T=1$ icosahedral particle composed of 60 copies of PORF2 (8). The essential element of PORF2 protein for $T=1$ VLP assembly includes amino acids 125–600 (9). Recently, the structural information was further refined by the crystal structures of genotype-3 $T=1$ VLP (10) and genotype-4 $T=1$ VLP (11), which revealed the tertiary structure of PORF2 to the level of amino acids. However, the $T=1$ VLPs used in these experiments were much smaller than that of the native virion, which has a diameter of 320–340 Å, as determined by immuno-EM (12). There is still a need to investigate the assembly pathway of HEV capsid.

HEV virion is hypothesized to be made of 180 copies of the capsid protein (8, 11). To test this hypothesis, we screened for HEV genotype expression and successfully produced a virion-sized VLP from the HEV genotype-3 ORF2 protein after deleting 52 residues from the C terminus. This VLP allowed us to investigate the molecular interactions that govern HEV virion assembly.

EXPERIMENTAL PROCEDURES

Production of HEV-VLPs and *In Vitro* Disassembly and Reassembly—HEV-VLPs were produced and purified according to the protocol described previously (7, 13). Briefly, the recombinant baculovirus Ac(G3n13ORF2), which harbored the genome of the N-terminal 13 amino-acid-deleted genotype-3 HEV ORF2, was infected into Tn5 cells with recombinant baculovirus at a multiplicity of infection of 10. The recombinant baculovirus-infected Tn5 cells were harvested at 7 days after infection. The medium and cells were separated by centrifuga-

Assembly of HEV $T=3$ Virion-sized Particle

tion at 3,000 rpm for 15 min at 4 °C. The cells were treated with a denaturation buffer containing 50 mM sodium borate, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 5% 2-mercaptoethanol and gently rocked at room temperature for 2 h. The lysate was diluted with EX-CELL 405, and centrifuged at 32,000 rpm for 3 h in a Beckman SW32Ti rotor, and the pellet was resuspended in EX-CELL 405. The VLP was purified by multiple ultracentrifugations followed by separation on a CsCl density gradient. The final pellet was resuspended in 10 mM potassium MES buffer, pH 6.2. A homemade dialysis device was used in the disassembly and reassembly experiments because it allowed dialysis with a small amount of sample (20–40 μ l). Purified VLP was disrupted by dialysis against buffer containing EDTA (10 mM) and DTT (20 mM) at different pHs. After VLP dissociation, 150 mM NaCl in Tris-HCl buffer (pH 7.5) was added, and sample was examined under the electron microscope after a 1-h incubation in the presence of the divalent ion Ca^{2+} (20 mM).

Scanning Transmission Electron Microscopy Analysis of HEV-VLPs—Scanning TEM (STEM) was performed at the Brookhaven National Laboratory STEM facility, with tobacco mosaic virus (TMV) as an internal control. The mixture of VLP and TMV was quickly frozen in liquid nitrogen and then maintained at -150 °C during data collection to eliminate contamination and reduce mass loss. The specimen was scanned by a 40-keV electron beam of 0.25 nm in size, and images were collected with a preamp gain of 10 for both large and small angle detectors (14). The image was recorded with a pixel size of 10 Å and was analyzed with the PCMass29 program. After normalizing the background, the mass of the VLPs was selected with the murine sarcoma virus (MSV) shell model provided by the program. Mass measurements for TMV and HEV-VLPs were always performed from the same image. The HEV-VLP mass was measured in MDa (mass per particle), and the TMV mass was measured in KDa/Å (mass per unit length) (15).

Cryo-electron Microscopic Structure Determination of HEV $T=3$ VLP—The collection of cryo-EM data for image reconstruction was performed on a JEOL JEM-2100F TEM operating at 200 kV according to the procedure described in detail previously (8). Briefly, a 3- μ l solution containing VLP or reassembled ORF2 complex was placed on holey carbon film-coated copper grids and then quickly plunged into liquid ethane after the removal of excess solution. The VLPs were embedded into a thin layer of vitrified ice and transferred into the EM using a Gatan 626 cryo-transferring system. The specimen was observed under 50,000 \times magnification, and the area of interest was recorded on a TVIPS CCD camera (TemCam-F415). The micrographs were recorded with a pixel size of 2.0 Å at a specimen space and defocus level of 0.7–3.5 Å (supplemental Fig. 1A). Digital images with no stigmatism or drift were selected for later image processing. Images of individual HEV $T=3$ VLPs were then boxed out and processed with an established software package for icosahedral particles (16, 17). In total, 7,720 individual images were included in the process, and their defocus levels were distributed mainly within 1.0–2.5 Å.

To correct contrast transfer function effect, we applied phase flipping on each image with an in-house program. The density maps were initially reconstructed by combining 1,812 individ-

ual images to an effective resolution of 14 Å. Next, amplitude correction was applied during map reconstruction while new data were added. The final density map was reconstructed by combining images of 4,348 individual particles, and the final resolution was assessed as 10.6 Å by Fourier shell correlation with a cutoff of 0.5 (supplemental Fig. 1B).

Docking of the $T=1$ crystal structure into the $T=3$ cryo-EM density map was first done manually with the program O (18) and then refined with the Situs software package (19). The PORF2 monomer was treated as a rigid body during the initial fitting and refinement processes.

X-ray Crystallographic Structure Determination of $T=1$ HEV-VLP—Crystallization of the VLPs was performed according to a previously described method (20). Crystals were directly flash-frozen in liquid nitrogen, and x-ray diffraction experiments were performed. All x-ray experiments of the HEV-VLP crystals were performed at SPring-8 in Hyogo, Japan. Particle orientation in the unit cell was determined with a self-rotation function (21), and the particle position was determined by a translation search with the cryo-EM structure as the model. The asymmetric crystal unit contains one particle; as a result, 60-fold non-crystallographic symmetry averaging was enforced. The cryo-EM structure (8) was used to obtain the initial phases of Data I (supplemental Table 1) and generated the envelope (mask) used for non-crystallographic symmetry averaging. The phases were refined by real space electron density averaging with icosahedral symmetry elements and solvent flattening. The resolution was gradually extended to 8.3 Å (R -factor = 0.21, correlation coefficient = 0.92). This structure was used for the phasing of Data II (supplemental Table 1), and the phases were refined and extended to a 3.8 Å resolution (the overall R -factor and correlation coefficient were 0.18 and 0.97, respectively). The electron density map revealed a clear main chain structure. Thus, we built an atomic model into this electron density map using the program O (18) without difficulty.

Sixty icosahedrally related S-subunits were treated as identical, and strict non-crystallographic symmetry constraints were applied during refinement. The data with the resolution range of 20–3.8 Å were included in the refinement (supplemental Table 1 and supplemental Fig. 2) using the CNS program (22). The subunit position and orientation were again clarified by rigid body refinement (R -factor of 0.391 ($R_{\text{free}} = 0.395$)). After the first cycle of simulated annealing refinement, positional and B -factor refinement followed, and the model was enhanced to an R -factor of 0.261 ($R_{\text{free}} = 0.264$). Further positional and B -factor refinement, followed by manual revision of the model, resulted in an R -factor of 0.242 ($R_{\text{free}} = 0.245$) with reasonable stereochemistry (root mean square deviations in bond lengths and bond angles were 0.010 Å and 1.68°, respectively). Because of the high non-crystallographic symmetry, the R -factor and R_{free} factor were almost identical. After refinement, the stereochemistry of the structure was checked with Procheck (23). 98.1% of the non-glycine residues were within the most favored and the additional allowed regions of the Ramachandran plot, and none of the residues were in the additional regions. Atomic structure representations were generated using MolScript (24) and Raster3D (25).

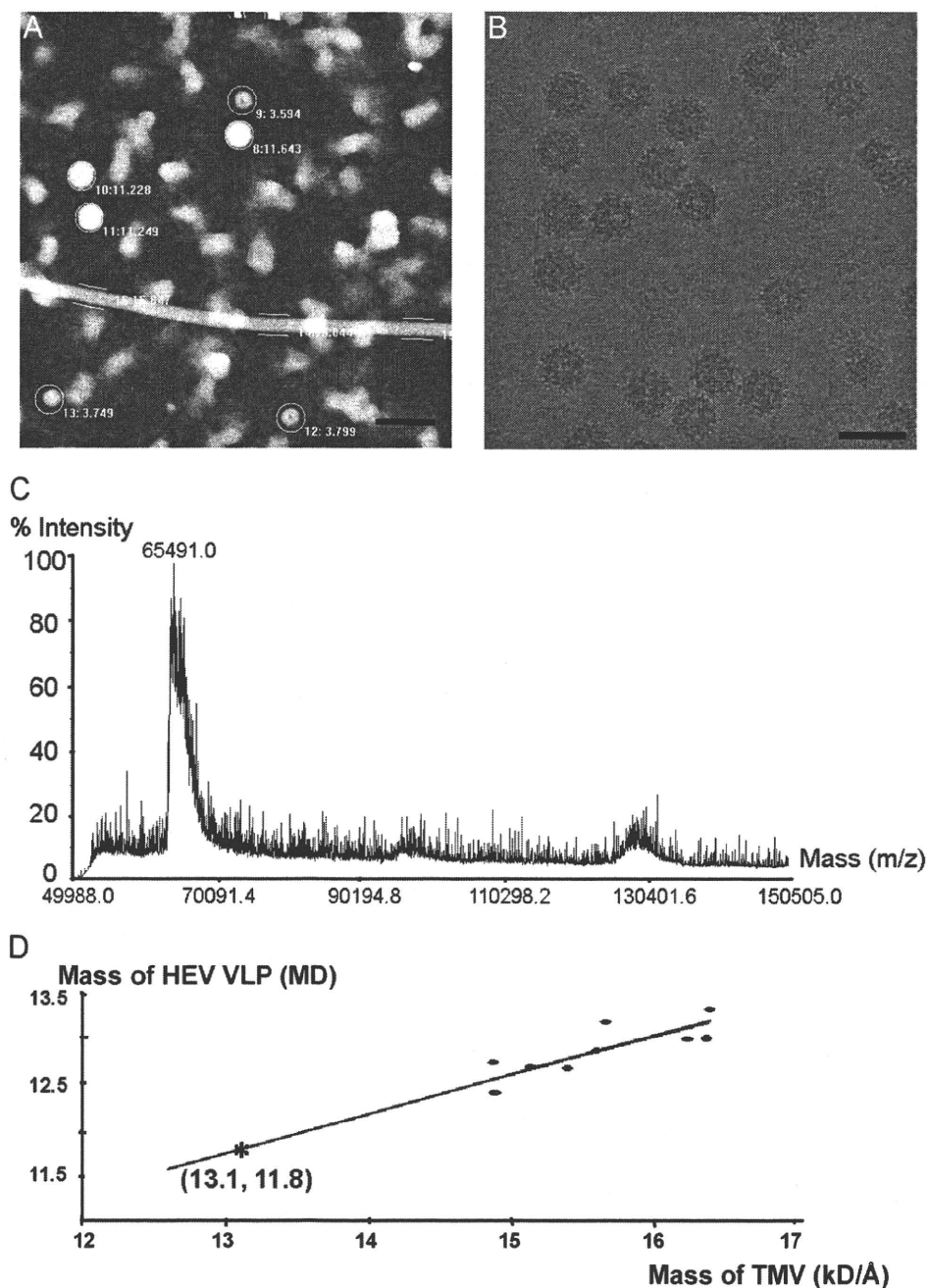


FIGURE 1. The large HEV-VLP is composed of 180 copies of ORF2 protein. *A*, STEM micrograph of HEV-VLPs. Both large and small $T=1$ HEV-VLPs are projected as spherical images, and their corresponding particle mass was calculated. The long straight rod is TMV, which was added as an internal mass standard. (*Bar* = 1,000 Å.) *B*, the large HEV-VLP appeared as an intact particle decorated with a dot-like pattern on the surface on cryo-EM of the large HEV-VLP. (*Bar* = 500 Å.) *C*, mass spectrum of the large HEV-VLP showing that the molecular mass of the ORF2 protein is 65.5 kDa. *D*, plot showing the observed mass/length of TMV against the observed mass of the large HEV-VLP from different image conditions. For a known TMV mass/length of 13.1 kDa/Å, the mass of the large HEV-VLP was calculated as 11.8 MDa (asterisk). MD, megadalton.

Nucleic Acid Extraction and Characterization—100 μ g of purified large and small VLPs were treated with DNase I (final concentration; 0.01 mg/ml) (Sigma) and RNase A (final concentration; 0.5 μ g/ml) at 37 °C for 1 h and then followed by centrifugation at 50,000 rpm for 2 h at 4 °C in a Beckman TLA55Ti rotor. After removal of the supernatant, the pellets were resuspended in 200 μ l of PBS minus buffer. The nucleic acids were extracted with RNazol-LS reagent (Tel-test, Inc., Friendswood, TX) and analyzed on 1% agarose gels.

The norovirus-like particles that were produced by the recombinant baculovirus were used as a control, and the same procedure was used with the HEV-VLPs.

RESULTS

STEM—The virion-sized HEV-VLP was recovered when the genotype-3 ORF2 sequence was expressed in insect cells. This VLP projected as a spherical image with a diameter of \sim 40 nm, larger than the $T=1$ VLP (27 nm in diameter) (Fig. 1A). In

For detection of HEV RNA in VLPs, the extracted RNAs from VLPs were mixed with sample buffer (final concentration: 2% formaldehyde, 50% formamide in MOPS buffer containing 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0), Sigma-Aldrich, Tokyo, Japan). The solution was denatured at 65 °C for 10 min and cooled immediately in ice-cold water and then mixed with 6 \times loading buffer (80% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, 6 mM EDTA, Sigma-Aldrich). After separation on the formaldehyde-denatured agarose gel (18% formaldehyde, 1% agarose, Sigma-Aldrich) in 1 \times MOPS buffer, the gel was washed twice for 15 min with 20 \times SSC (3 M NaCl, 0.3 M trisodium citrate dehydrate (pH 7.0), Roche Applied Science, Tokyo, Japan) and transferred onto a Hybond N⁺ membrane filter (GE Healthcare, Tokyo, Japan) by the capillary method. RNAs were fixed on the membrane by irradiation with UV light (Stratalinker UV crosslinker, Stratagene, Tokyo, Japan) and dried in the air. RNAs on the membrane were hybridized with DIG-labeled probe. DIG-labeled RNAs were detected by using a DIG Northern starter kit (Roche Applied Science) according to the manufacturer's protocol and visualized by the LAS-3000 imaging analyzer, Fujifilm, Tokyo, Japan). The purified template DNA for the probe RNA was prepared by digesting the plasmid carrying 584-base cDNA of HEV ORF2 (5,903–6,486 nucleotides) under the T7 promoter with BamHI. The probe RNA was prepared using the DIG Northern starter kit according to the manufacturer's manual (incorporation of DIG-UTP during RNA transcription).