Structural, Mechanistic, and Antigenic Characterization of the Human Astrovirus Capsid

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ABSTRACT

Human astroviruses (HAstVs) are non-enveloped, positive-sense, single-stranded RNA viruses and are a leading cause of viral gastroenteritis. HAstV particles display T=3 icosahedral symmetry formed by 180 copies of the capsid protein (CP), which undergoes proteolytic maturation to generate infectious HAstV particles. Little is known about the molecular features that govern HAstV particle assembly, maturation, infectivity, and immunogenicity. Here we report the crystal structures of the two main structural domains of the HAstV CP: the core domain at 2.60-Å resolution and the spike domain at 0.95-Å resolution. Fitting of these structures into the previously determined 25-Å resolution electron cryomicroscopy density maps of HAstV allowed us to characterize the molecular features on the surfaces of immature and mature T=3 HAstV particles. The highly electropositive inner surface of HAstV support a model in which interaction of the HAstV CP core with viral RNA is a driving force in T=3 HAstV particle formation. Additionally, mapping conserved residues onto the HAstV CP core and spike domains in the context of the immature and mature HAstV particles reveals dramatic changes to the exposure of conserved residues during virus maturation. Indeed, we show that antibodies raised against mature HAstV have reactivity to both the HAstV CP core and spike domains, revealing for the first time that the CP core domain is antigenic. Together these data provide new molecular insights into HAstV that have practical applications for the development of vaccines and antiviral therapies.
Astroviruses are a leading cause of viral diarrhea in young children, immunocompromised individuals, and the elderly. Despite the prevalence of astroviruses, little is known at the molecular level how the astrovirus particle assembles and is converted into an infectious, mature virus. In this paper, we describe the high-resolution structures of the two main astrovirus capsid proteins. Fitting these structures into previously determined low-resolution maps of astrovirus has allowed us to characterize the molecular surfaces of the immature and mature astrovirus. Our studies provide the first evidence that astroviruses undergo viral RNA-dependent assembly. We also provide new insight into the molecular mechanisms that lead to astrovirus maturation and infectivity. Finally, we show that both capsid proteins contribute to the adaptive immune response against astrovirus. Together, these studies will help to guide the development of vaccines and antiviral drugs targeting astrovirus.
INTRODUCTION

Human astroviruses (HAstV) are a leading cause of viral gastroenteritis in children, elderly, and immunocompromised patients (1-9), with approximately 3.9 million cases of HAstV gastroenteritis per year in the United States alone (10). There are eight canonical human serotypes, HAstV-1 through HAstV-8, where HAstV-1 is the most common serotype worldwide (1, 11, 12). Divergent strains of HAstV have been associated with encephalitis (13-15). The Astroviridae family also includes many non-human astroviruses (AstVs) that cause infections in mammals and birds, causing a range of symptoms including gastroenteritis, fatal hepatitis, and neurological disease (16, 17).

AstVs are non-enveloped, positive-sense, single-stranded RNA viruses with three ORFs. ORF1a and ORF1b encode two nonstructural polyproteins (18, 19), and ORF2 encodes the capsid protein (CP) (20-22). The Ast CP is composed of several domains, including a highly basic N-terminal domain, a core domain, a spike domain, and a C-terminal acidic domain (Fig. 1A). Newly synthesized HAstV CP is 87-90kDa (VP90) and assembles into immature HAstV particles inside infected cells (23, 24). HAstV CP undergoes a multi-step maturation process via proteolytic cleavage events that are required for virus release and infectivity. First, intracellular caspases remove the C-terminal acidic domain of CP to generate VP70 (25, 26) (Fig. 1A). After immature HAstV virus release from cells, the CP is further processed by host extracellular proteases to produce mature HAstV. In cell culture, trypsin has been used to produce mature HAstV, whose infectivity is $10^5$-fold higher than that of immature HAstV not treated with trypsin (25, 27, 28). Mature HAstV is composed of three predominant proteins, VP34, VP27, and VP25 (25, 28-30) (Fig. 1A). The mechanism by which
proteolysis increases HAstV infectivity is unknown. Mature HAstV has recently been shown to gain entry into host cells via clathrin-mediated endocytosis (31).

Electron cryomicroscopy (cryo-EM) studies of immature and mature HAstV particles reveal a ~35nm T=3 icosahedral structural core projecting knob-like spikes (32). The immature HAstV particle shows remarkable resemblance to the T=3 hepatitis E virus (HEV) particle (32, 33). VP34 composes the structural core that forms the protein shell around the virus and VP25 and VP27 form the spikes that protrude from the structural core. Comparison of immature and mature HAstV reveals a dramatic difference with respect to the surface spikes: immature HAstV particles contain 90 dimeric spikes whereas mature HAstV particles contain only 30 spikes at the icosahedral 2-fold axes (32). Our lab and others have previously determined the crystal structures of the AstV CP spike domains from HAstV-8 and turkey AstV-2 (34, 35), however the structure of the CP core domain that composes VP34 has remained elusive.

To further explore the structural features of the AstV CP, we have determined the crystal structures of the HAstV-1 CP core and spike domains to 2.60-Å and 0.95-Å resolution, respectively. Fitting of these structures into the cryo-EM density maps of immature and mature HAstV enabled characterization of the conserved molecular features on the surface of the virus. Our studies reveal new insights into the mechanisms of AstV particle assembly and maturation.

MATERIALS AND METHODS

HAstV-1 CP core production. cDNA corresponding to HAstV-1 capsid protein residues 80 to 429 (Accession# AAC34717.1) was cloned into pET52b in frame with a C-terminal
thrombin cleavage site and 10-histidine purification tag. The plasmid was transformed into *Escherichia Coli* strain BL21 (DE3) pLysS, and HAstV-1 CP core expression was induced with 1mM IPTG at 18°C for 16 h. Selenomethionine-substituted HAstV-1 CP core was expressed in *E. coli* strain B834 (DE3) pLysS in M9 minimal media supplemented with selenomethionine. *E. coli* cells were lysed by ultrasonication in 20mM Tris, pH 7.5, 1M NaCl, 5% (v/v) glycerol, 1mM dithiothreitol (DTT), and 20mM imidazole. HAstV-1 CP core was purified from soluble lysates by HisTrap metal-affinity chromatography. HAstV-1 CP core was further purified and characterized by size-exclusion chromatography on a Superdex 75 column in 10mM Tris, pH 7.5, 1M NaCl, 5% (v/v) glycerol, and 1mM DTT. Pure HAstV-1 CP core was concentrated to ~11 mg/ml.

**HAstV-1 CP core structure determination.** HAstV-1 CP core crystals were grown by the hanging-drop vapor diffusion method at 22°C in a well solution containing 1M LiCl, 0.1M sodium acetate, and 16.2% (v/v) polyethylene glycol (PEG) 6000. Crystals were transferred to a cryo solution of well solution and 25% (v/v) ethylene glycol before being frozen in liquid nitrogen. Diffraction data were collected at cryogenic temperature at the Advanced Light Source Beamline 5.0.1. Data were processed with HKL-2000 (36) (Table 1).

The HAstV-1 CP core structure was solved by single-wavelength anomalous dispersion (SAD) (Table 1). Selenomethionine crystal data were collected at the Advanced Light Source Beamline 5.0.2 under cryogenic temperatures. Data were processed with HKL-2000 (36). PHENIX (37) was used to determine the location of 10
selenium sites and to autobuild an initial structural model. Although 14 sites were expected (7 per molecule in asymmetric unit), four sites were not observed because they were located in flexible loops or position one. Using the initial model and native data, the HAstV-1 CP core structure was refined and manually rebuilt using Phenix (37) and Coot (38), respectively.

**HAstV-1 CP spike production.** cDNA corresponding to HAstV-1 capsid protein residues 429 to 645 (Accession# AAC34717.1) was cloned into pET52b in frame with a C-terminal thrombin cleavage site and 10-histidine purification tag. The plasmid was transformed into *Escherichia Coli* strain BL21 (DE3), and HAstV-1 CP spike expression was induced with 1mM IPTG at 18°C for 16 h. *E. coli* cells were lysed by ultrasonication in 20mM Tris, pH 8.0, 300M NaCl, 1mM dithiothreitol (DTT), and 20mM imidazole. HAstV-1 CP spike was purified from soluble lysates by HisTrap metal-affinity chromatography. HAstV-1 CP spike was further purified by size-exclusion chromatography on a Superdex 75 column in 10mM Tris, pH 8.0, 150mM NaCl, and 1mM DTT. Pure HAstV-1 CP spike was concentrated to ~18mg/ml.

**HAstV-1 CP spike structure determination.** HAstV-1 CP spike crystals were grown by the hanging-drop vapor diffusion method at 22°C in a well solution containing 1M ammonium sulfate, 6% PEG400, and 0.1M MES pH5.6. Crystals were transferred to a cryo solution of well solution and 35% (v/v) glycerol before being frozen in liquid nitrogen. Diffraction data were collected at cryogenic temperature at the Advanced Photon Source Beamline 23-ID-B. Data were processed with HKL-2000 (36) (Table 1).
The HAstV-1 CP spike structure was solved by molecular replacement using the HAstV-146 CP spike structure (PDB ID 3QSQ) (34) and the program Phaser (39). The HAstV-1 CP spike structure was refined and manually rebuilt using Phenix (37) and Coot (38), respectively.

Modeling HAstV-1 CP core and spike domains into the immature and mature HAstV cryo-EM maps. The HAstV-1 CP core was structurally aligned to the three quasi-equivalent HEV CP molecules from the T=3 model determined previously by cryo-EM (33). This initial T=3 HAstV-1 CP core model was then fit into the 25-Å resolution cryo-EM density map of HAstV-1 (32) using the program Chimera (40). Assessment of the T=3 HAstV-1 CP core model revealed a number of intermolecular clashes between core domains. Thus, sequential fitting between the three quasi-equivalent HAstV-1 CP core molecules, in combination with symmetric fitting, was performed with Chimera to reduce the number of clashes. Sequential rounds of fitting of the three quasi-equivalent HAstV-1 CP core molecules into the cryo-EM map were then performed. This final T=3 HAstV-1 CP core model was used for construction of complete models of both immature and mature T=3 HAstV-1 models. To construct the immature T=3 HAstV-1 model, 30 HAstV-1 CP spike dimers at the icosahedral two-fold symmetry axes and 60 HAstV-1 CP spike dimers at the icosahedral five-fold vertices were fitted into the immature HAstV-8 cryo-EM density map (32). To construct the mature T=3 HAstV-1 model, 30 HAstV-1 CP spike dimers at the icosahedral two-fold symmetry axes were fitted into the HAstV-1 cryo-EM map (32).
Mapping CP sequence conservation onto immature and mature T=3 HAstV-1 models. Accession numbers used for sequence alignments of the AstV CP are listed as follows: HAstV-1-8 (O12792, Q82446, Q9WFZ0, Q3ZN05, Q4TWH7, Q67815, Q96818, Q9IFX1), select mammalian astroviruses (BAN62843, yP_006905854, ADH93577.1, YP_003090288.1, YP_006792628.1, YP_006905860.1, YP_006905857, ACX85472.1, ACX85474.1, ACX85476.1, AGO50636, Q80KJ6, BAA90309, AED89600.1, ADO67579.1, NP_059946.1, AII82243.1, CAR82567, ACR54274, AEM05826, ACF75865, AEV92822, YP_005271209.1, ADJ38391.1, ADX97522.1, ACR54280.1), and select avian astroviruses (YP_002728003, CBY02488, CBY02492, ADG45753, AEB15599, AEE88305, ABX46584, ABX46566, AAV37187). Sequence alignments were performed by Clustal Omega and conservation level was mapped onto the immature and mature T=3 HAstV-1 models using Chimera (40).

TAstV-2 CP core and spike production. cDNA corresponding to TAstV-2 CP residues 77-423 (core) or 424-631 (spike) (Accession NP_987088) were cloned, expressed, and purified using the same methods as those for HAstV-1 CP core and spike, respectively. The only difference in methods was the use of E. Coli strain Rosetta 2 (DE3) pLysS for expression.

Enzyme-linked immunosorbent assay (ELISA). Purified HAstV-1 or TAstV-2 CP core and spike (18.7 pmol/well) were incubated overnight at room temperature in their respective buffers in 96-well ELISA microtiter plates. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBST). Wells were blocked by adding 150
μL Thermo Scientific StartingBlock blocking buffer followed by three PBST washes. The blocking and washing step was repeated a total of three times. Anti-HAstV-1 polyclonal rabbit serum (41) was initially diluted 1:20 with blocking buffer, and then subsequently diluted 1:4 in series with blocking buffer. Wells were incubated 150 μL of diluted polyclonal serum and incubated for one hour at room temperature. Plates were washed three times with PBST and then incubated with a HRP-conjugated, secondary goat anti-rabbit IgG antibody, diluted 1:5,000 in blocking buffer. Plates were washed three times with PBST and then developed by adding peroxidase substrate o-phenylenediamine dihydrochloride (OPD) for 10 min at room temperature. The reactions were stopped with 3M sulfuric acid, and the absorbance was measured at 490 nm.

Protein structures accession numbers. The HAstV-1 CP structural models and structure factors have been deposited into the online Protein Data Bank (PDB; www.pdb.org) as PDB entry 5EWN (CP core domain) and 5EWO (CP spike domain).

RESULTS

Structure of the HAstV-1 CP core domain.

Assuming that the HAstV-1 CP core would form an independently folded structural domain, we attempted to express it in Escherichia coli. An expression construct composed of HAstV-1 CP residues 80-429 produced soluble protein and remained stable at high concentrations in buffers containing at least 1M sodium chloride and 5% glycerol. HAstV-1 CP core exhibited an apparent molecular weight of ~20 kDa as examined by gel filtration chromatography, consistent with it being a compact monomeric protein in
solution. Higher molecular weight peaks corresponding to possible capsomeres or virus-like particles were not observed, however this may be due to the high salt and glycerol buffer required to prevent protein precipitation.

Purified HAstV-1 CP core was crystallized in the space group P2_12_12_1 and a native diffraction dataset was collected to 2.6-Å resolution (Table 1). Attempts to determine the structure by molecular replacement using the structure of the Hepatitis E virus (HEV) CP S and P1 domains, which have only 18% sequence identity, or homology models of HAstV-1 CP core domain, were unsuccessful, suggesting structural differences. We therefore generated a selenomethionine-substituted sample and used single-wavelength anomalous dispersion methods to determine the 2.6-Å resolution structure of the HAstV-1 CP core (Fig. 1B and Table 1). Two molecules in the asymmetric unit differ by an RMSD of 1.0 Å, as assessed by the DaliLite server (42), with notable differences observed in the β4-β5, β10-β11, and α4-β15 loops that we predict are involved in intermolecular contacts in HAstV particle formation (see below) (Fig. 2A). Our final HAstV-1 CP core model contains amino acid residues 80-411, with a disordered loop at residues 390-396 being the only loop not visualized in at least one of the two molecules in the asymmetric unit (Fig. 1B). The DALI server (43) identified the HEV CP as the closest structural homolog with a RMSD of 3.3 Å and a Z score of 27.1 (with >2.0 being significant) (33, 44, 45).

Structure of the HAstV-1 CP spike domain.

HAstV-1 CP spike, composed of residues 429-645, was expressed in E. coli and purified. HAstV-1 CP spike exhibited an apparent molecular weight of ~50 kDa as
examined by gel filtration chromatography, consistent with dimer formation. Purified HAstV-1 CP spike was crystallized in the space group P4_121_2 and a native diffraction dataset was collected to 0.95-Å resolution (Table 1). The previously determined structure of the HAstV-8 CP spike was used as a molecular replacement model (34). Structural alignment of the HAstV-1 and HAstV-8 CP spike dimer structures by the DaliLite server (42) revealed nearly identical structures, with a RMSD of 0.8 Å (Fig. 2B). One noteworthy difference was an unexpected disulfide bond formed at the interface between the two protomers of the HAstV-1 CP spike dimer, despite the presence of reducing agents throughout protein purification (Fig. 1D). Sequence alignments of HAstV-1-8 capsid proteins reveal that this disulfide bond is unique to serotype HAstV-1.

Models of immature and mature T=3 HAstV-1 particles.

To investigate the molecular features on the surfaces of immature and mature HAstV particles, we first created an initial T=3 HAstV-1 CP core model by aligning to the three quasi-equivalent CP molecules from the T=3 HEV cryo-EM model (33) and fitting this model into the 25-Å resolution cryo-EM density map of HAstV-1 (32). We then performed iterative rounds of fitting of the three quasi-equivalent CP molecules into the cryo-EM density map using the program Chimera (40). Using this T=3 HAstV-1 CP core model, we then added and fit HAstV-1 CP spike dimers into the protrusions of immature HAstV-8 and mature HAstV-1 cryo-EM density maps (32). Our final immature and mature T=3 HAstV-1 models show striking consistency with cryo-EM density maps (Fig. 3). Correlation coefficients measuring the agreement between a simulated map of
our models at 25-Å resolution with the cryo-EM maps (also at 25-Å resolution) were 0.898 for immature HAstV and 0.902 for mature HAstV.

Comparison of HAstV-1 and HEV CPs.

The HAstV-1 CP core structure reveals two linear domains similar to the S and P1 domains of the HEV capsid (Figs. 1B and 4A). Structural alignment of the S domains (inner core) from HAstV-1 CP and HEV CP using the DaliLite server (42) revealed a Z-score of 20.0 and a RMSD of 2.4 Å (Fig. 4A). The S domains adopt a typical jelly-roll β-barrel fold that is commonly found in CPs from small RNA viruses. Interestingly, another unexpected disulfide bond was observed in the HAstV-1 CP core linking strands β1 and β12, despite the presence of reducing agents throughout purification, and this disulfide is conserved throughout serotypes HAstV-1-8 (Fig. 1B).

Structural alignment of the P1 domains (outer core) from HAstV-1 CP and HEV CP revealed lower structural homology compared to the S domain, with a Z-score of 11.0 and a RMSD of 2.9 Å (Fig. 4A). While both P1 domains form a six-stranded β-barrel flanked by several α helices, a 10-Å distance was observed between the HAstV-1 CP helix η2 and the equivalent helix α6 in HEV CP (Fig. 4A). As a result, HAstV has a more compact P1 domain compared to that in HEV.

To understand and compare the potential role of viral RNA in interacting with CP and promoting virus assembly, we investigated the electrostatic potential on the inside surface of the T=3 HAstV-1 and HEV CP core models (Fig. 4). We observed a striking difference between the HAstV-1 and HEV surfaces, with HAstV-1 having a dramatically more electropositive surface. Notably, the disulfide bond observed in the HAstV-1 CP
core (Fig. 1B) lies on the inner surface and may stabilize nearby basic residues Arg84, Lys136, and Lys138 (Fig. 4B). These data suggest that the HAstV CP core may be directly involved in viral RNA binding.

**Location of trypsin sites on the HAstV-1 CP.**

To better understand the composition of the CP in mature HAstV, we mapped the location of trypsin cleavage sites onto the HAstV CP core P1 domain (Fig. 1B). Most of the 11 trypsin cleavage sites are exposed on surface-accessible loops, suggesting that many sites may become cleaved during maturation. Consistent with this idea, SDS-PAGE studies of purified mature HAstV-1 show an absence of sizeable bands corresponding to the P1 domain (28, 32). However, the remarkable fit of the full HAstV-1 CP core structure into the cryo-EM map of mature HAstV-1 also suggests that trypsin cleavage does not remove this region of the HAstV-1 CP entirely.

**Conserved features on the surfaces of immature and mature AstV T=3 virions.**

To gain insight into the conserved features on the surface of AstV that may play important functional roles in the AstV life cycle, we carried out multiple sequence alignments and mapped conservation onto the immature and mature T=3 HAstV-1 models (Fig. 5). We first mapped out sequence conservation between canonical human serotypes HAstV-1-8 (Fig. 5A). There is a surprising lack of accessibility to conserved features on the surface of immature HAstV compared to mature HAstV. In addition, we observe almost complete sequence conservation on the inside surface of the virion,
further supporting our electrostatics analyses that the inside surface of HAstV may play a key role in virus assembly.

We then mapped out sequence conservation between both canonical and non-canonical HAstV strains (Fig. 5B). The non-canonical HAstVs include the HAstV-MLB and HAstV-VA/HMO clades (14). The remarkable drop in surface conservation compared to that of canonical HAstVs highlights the evolutionary divergence between these HAstV strains. Indeed, these non-canonical HAstVs are more closely related to other mammalian AstVs than the canonical HAstVs, and this is apparent by the minimal changes in surface conservation when adding numerous other mammalian AstV CP sequences to the comparison (Fig. 5C). Finally, we mapped out sequence conservation between all AstV strains, which includes avian AstV strains (Fig. 5D). Although we observe high sequence divergence on the outside surface, there is a remarkable level of conservation remaining on the inside surface, again supporting the concept that the inside surface of AstVs may play a key role in binding viral RNA and promoting virus assembly, and that this assembly mechanism may be conserved across all AstVs.

Examination of polyclonal antibody binding and cross-reactivity.

Almost nothing is known about the location of epitopes on the surface of AstVs. Only two reports describe the isolation of anti-HAstV monoclonal antibodies, and all were found to immunoprecipitate VP25 or VP27, which we now know compose the HAstV spike (29, 41). To investigate the antigenicity of both the HAstV-1 CP core and spike, we performed enzyme-linked immunosorbent assays (ELISAs) and assessed binding to anti-HAstV-1 polyclonal antibodies, which exhibit high neutralizing activity.
against HAstV-1 (41) (Fig. 6). Consistent with previous studies with monoclonal antibodies, we find that the HAstV CP spike is antigenic. Additionally, we show that the HAstV CP core is also antigenic, although to a lesser extent. These data indicate that the mature HAstV-1 virion contains epitopes in both the CP core and spike domains that elicit an antibody response.

To investigate the cross-reactivity of anti-HAstV-1 polyclonal antibodies to divergent AstVs, we performed ELISAs with recombinant turkey astrovirus 2 (TAstV-2) CP core and spike (Fig. 6). We observed very little binding to anti-HAstV-1 polyclonal antibodies, suggesting low sequence conservation between epitopes on HAstV-1 and TAstV-2.

**DISCUSSION**

We report here the crystal structures of the HAstV-1 CP core and spike domains at 2.60-Å and 0.95-Å resolution, respectively. By using these structures to model immature and mature T=3 HAstV-1 particles, we provide new molecular insights into HAstV assembly, maturation, immunogenicity, and evolution.

We provide new evidence that HAstVs and HEVs are phylogenetically related and may share commonalities in RNA-driven virus particle assembly. We find that the HAstV-1 CP core is structurally related to the HEV CP core, despite only 18% sequence identity between them. While interaction of the electropositive N-terminus of HEV CP with viral RNA is a known driving force for T=3 HEV particle formation (44), little is known about the role of RNA in HAstV particle assembly. Here, we observe a striking electropositive charge on the inside surface of T=3 HAstV particle, adding to the already
highly positively charged N-terminal 79 residues that precede the HAstV CP core. The inside surface is highly conserved in HAstV-1-8 and even retains partial conservation throughout all AstVs. Previous studies of recombinant HAstV-1 CP, full-length or lacking the first 70 amino acids, revealed virus-like particle formation, suggesting that viral genomic RNA is not necessary for particle formation (23, 24). However, in these studies, the nucleic acid content of these virus-like particles was not examined, and we hypothesize that these particles are stabilized by cellular RNA bound to the electropositive charge on the inside surface. Consistent with this, we have observed that recombinant HAstV-1 CP has a high 260nm absorbance (consistent with the sample containing nucleic acid) and precipitates upon addition of RNase but not DNase (data not shown). Thus, the structural similarities with HEV, the highly electropositive charge, and the high conservation on the inside of the AstV particle support a model in which binding of viral RNA to the AstV CP would drive T=3 AstV particle formation.

Our studies also provide new insights into HAstV maturation and the composition of mature T=3 HAstV particles. Like many viruses, HAstVs undergo proteolytic processing to produce mature, infectious particles. Although the in vivo protease(s) responsible for HAstV proteolysis has yet to be identified, it has been found that trypsin will increase HAstV infectivity by 10^5-fold in cell culture (25, 27, 28). N-terminal sequencing studies have previously identified the trypsin cleavage sites at residues 494 and 324 that produce the VP25 and VP27 proteins of the HAstV-1 CP spike, respectively (25, 29), however the boundaries of VP34 and the composition of the CP core domain in mature HAstV-1 remain elusive. Our data support a model in which the N-terminus of the HAstV CP core is bound to RNA and protected from trypsin cleavage, making
HAstV CP residue 1 the beginning of VP34. Thus, the C-terminus of VP34 is likely made by a trypsin cleavage site occurring after residue 300, resulting in the observed ~34kD band on SDS-PAGE (25, 28, 29). Here, we mapped all 11 trypsin cleavage sites occurring between residues 299 and 394 of the HAstV-1 CP core. Most of the sites lie on surface-exposed loops, suggesting their susceptibility to trypsin digestion, however we do note that the trypsin cleavage sites at 299 and 304 lie on the side of the CP core on helix α4 and may be less susceptible. Thus, we hypothesize that the earliest trypsin cleavage occurs at R313, which is conserved in HAstV-1-8 and would result in a 33.7kD protein band. We also hypothesize that other trypsin cleavages occur, however the remarkable fit of the full HAstV-1 CP core structure into the cryo-EM map of mature HAstV-1 suggests that trypsin cleavage does not remove this region of the HAstV-1 CP entirely. Instead, these data together suggest that the trypsin-matured HAstV1 CP P1 domain exists as proteolytic fragments that remain bound together through hydrophobic core interactions and the hydrogen-bonding network of the β-barrel.

Although the mechanism by which HAstV maturation by proteolysis increases infectivity is still unknown, our data provide insight into several possibilities: (1) Maturation may expose a receptor-binding site at an optimal time in the virus life cycle. Our data show that the surface of immature virus is highly variable between HAstV-1-8, and maturation by trypsin, which removes 60 spikes, exposes a number of conserved sites on both the spike and core domains. A previous report identified three conserved potential receptor-binding sites on the spike domain, P site, S site, and β-turn (34). We mapped out these sites in the context of our immature and mature HAstV models and find that the P-site lies on the most outward side of the virus and is exposed in both immature
and mature HAstV (Fig. 7A). Thus, if the P-site comprises a receptor-binding site, then immature HAstV would likely have the ability to attach to cells. In contrast, both the S site and β-turn lie on the side of the spike and are sterically hindered by other spikes on the immature HAstV. Thus, if the S site or β-turn comprise a receptor-binding site, then immature HAstV would not likely have the ability to attach to cells, and receptor-binding would not occur until after virus maturation. Although the true site of receptor-binding is unknown, having it unexposed until an optimal time could be beneficial for both promoting virus release from cells as well as evading broadly neutralization antibodies.

(2) Maturation may promote virus uncoating. Although the mechanism by which AstV uncoats is unknown, protease maturation may change the ability of AstV to be triggered for uncoating in the endosome and release of viral RNA. One possible mechanism is that the mature virus particle could become less stable, making it primed for uncoating. We observe in cryo-EM maps the presence of electron density between spike dimers of immature HAstV, but not mature HAstV, suggesting that spike-spike interactions may stabilize the immature form (Fig. 3).

(3) Maturation may dampen host immunity. One possible mechanism is that the protease-released AstV spikes serve as decoys to dampen the effect of immune system antibodies. Another possible mechanism is that maturation allows HAstV to inhibit the complement pathway, and mapping of the previously determined C1q binding residues (HAstV-1 CP residues 80-138, or CP1 peptide) reveals that it is only exposed on mature HAstV (46, 47) (Fig. 7B). Although these may play a role in AstV pathogenesis, it is unlikely the sole purpose of protease maturation given its dramatic effect on AstV infectivity.
Finally, our studies reveal for the first time that the HAstV CP core domain plays a role in the adaptive immune response to HAstV. We show that polyclonal antibodies raised against mature HAstV-1 recognize both recombinant HAstV CP core and spike. Furthermore, we show that these antibodies have almost no binding to recombinant CP core and spike from an avian astrovirus (TAstV-2). These findings are consistent with the low level of conservation on the AstV particle surface when mapping out conservation between mammalian and avian AstVs. Together, these antigenic studies have important implications in AstV vaccine development.

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Table 1. Crystallographic Statistics.

| Data collection | Crystal | HAstV-1 CP core | HAstV-1 CP core (SeMet) | HAstV-1 CP spike |
|-----------------|---------|-----------------|------------------------|-----------------|
| Wavelength (Å)  | 0.97741 | 0.97939         | 1.03320                |
| Space group     | P 2 1 2 1 | P 2 1 2 1 | P 4 1 2 1 |
| a, b, c (Å)     | 66.24, 71.10, 158.91 | 66.56, 70.82, 159.06 | 103.23, 103.23, 41.67 |
| α, β, γ (°)     | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å)  | 50.00-2.60 (2.69-2.60) | 79.56-3.20 (3.42-3.20) | 16.31-0.95 (1.0-0.95) |
| Rmerge           | 0.105 (0.558) | 0.163 (0.434) | 0.073 (0.460) |
| I/σI             | 19.66 (3.41) | 17.2 (7.0) | 13.7 (3.1) |
| Completeness (%) | 99.3 (98.4) | 100 (100) | 90.5 (67.4) |
| Redundancy       | 6.9 (6.9) | 13.8 (14.3) | 6.0 (4.5) |
| Selenium site no. | N/A | 10 | N/A |
| Overall figure of merit | N/A | 0.31 | N/A |
| Refinement       |         |         |         |
| Resolution (Å)  | 48.48-2.60 | 16.21-0.95 |
| No. reflections | 23493 | 141071 |
| Rwork/Rfree b    | 0.192/ 0.234 | 0.131/ 0.143 |
| Ramachandran (%) | 95.5 | 96.40 |
| Favored      | 95.5 | 96.40 |
| Allowed      | 4.5 | 2.88 |
| Outliers     | 0.0 | 0.72 |
| Rms deviations |         |         |         |
| Bond lengths (Å) | 0.017 | 0.008 |
| Bond angles (°) | 1.60 | 1.47 |

aData were collected from a single crystal. Values for the highest-resolution shell are shown in parentheses.

bRfree was calculated using 5% of the reflections.
FIGURES LEGENDS

FIG 1 Schematic and crystal structures of HAstV-1 CP core and spike. (A) Schematic of the HAstV-1 CP domain structure and proteolytic processing events. Caspase and trypsin cleavage sites are indicated with white and orange arrows, respectively. (B and D) Crystal structures of HAstV-1 CP core (B) and spike (D). Trypsin cleavage sites are indicated with orange arrows. Disulfide bonds are labeled and colored yellow. N- and C-termini are labeled. (C) Model of mature T=3 HAstV-1 virion. Figures were made with PyMOL.

FIG 2 Structural alignments. (A) Alignment of the two molecules of HAstV-1 CP core in the asymmetric unit. Notable differences in the loops that form contacts at the five-fold and three-fold symmetry axes in the T=3 HAstV-1 model are indicated with arrows. (B) Alignment of HAstV-1 and HAstV-8 CP spike structures. The disulfide bond observed in HAstV-1 CP spike is shown as sticks.

FIG 3 Immature and mature T=3 HAstV-1 models fit into HAstV cryo-EM maps. (A) Immature T=3 HAstV-1 model fit into HAstV cryo-EM map (left) and a zoomed-in, sliced view (right). (B) Mature T=3 HAstV-1 model fit into HAstV cryo-EM map (left) and a zoomed-in, sliced view (right). Models are shown as cartoon view and colored as in Fig. 1, and cryo-EM maps are shown as gray transparent surfaces. An oval, a triangle, and a pentagon indicate the 2-fold, 3-fold and 5-fold icosahedral axes. For immature HAstV, contour levels were set to encompass the expected volume of 180 copies of VP70.
plus 1 copy of viral RNA genome: $\sim 18.5 \times 10^6 \, \text{Å}^3$. For mature HAstV, contour levels were set to encompass the expected volume of 180 copies of VP34, 60 copies of VP25, and 1 copy of viral RNA genome: $\sim 14.8 \times 10^6 \, \text{Å}^3$. Calculations of volume values are based upon a protein density of 1.35 g/cm$^3$ and a RNA density of 2.0 g/cm$^3$.

**FIG 4** Structural and electrostatic comparisons between CP core domains from HAstV-1 and HEV. (A) Superposition of the HAstV-1 and HEV CP core domains. HAstV-1 CP core is colored as in Fig. 1B, and HEV CP core is colored gray. Significant differences between structures are indicated with arrows. (B and C) Electrostatic potential on the inner surface of the T=3 HAstV-1 CP core model (B) and T=3 HEV CP core model (C).

In the HAstV-1 model, the disulfide bond Cys82-Cys254 is colored yellow to show its proximity to electropositive residues. Note that in the T=3 HAstV-1 CP core model, the first 79 amino acids of the CP are not included. Similarly, in the T=3 HEV CP core model, the first 129 amino acids of the CP are not included. Thus, the electropositive surface comes only from amino acids on the inner core domain (S domain). Figures were made with Chimera (40).

**FIG 5** Conservation on the surface of the immature and mature T=3 HAstV-1 models. (A - D) Surface representation of T=3 immature and mature HAstV-1 models (top, bottom, and sliced in half) with residues colored according to their level of conservation. Residues conserved between canonical HAstV serotypes 1-8 (A), canonical and non-canonical HAstVs (B), mammalian AstVs (C), and mammalian and avian AstVs (D) are
colored by conservation, from cyan (least conserved) to magenta (strictly conserved).

Figures were made with Chimera (40).

**FIG 6** Reactivity of HAstV-1 and TAstV-2 CP core and spike domains to anti-HAstrV-1 polyclonal rabbit serum. (A) Reducing SDS-PAGE analysis of purified proteins used for ELISA. M, Bio-Rad Precision Plus molecular weight markers; 1, HAstV-1 CP spike; 2, TAstV-2 CP spike; 3, HAstV-1 CP core; 4, TAstV-2 CP core. Each lane was loaded with 10μg protein. (B) Comparison of reactivity of purified HAstV-1 CP core (solid line) and TAstV-2 CP core (dashed line) to anti-HAstrV-1 polyclonal rabbit serum by ELISA. (C) Comparison of reactivity of purified HAstV-1 CP spike (solid line) and TAstV-2 CP spike (dashed line) to anti-HAstrV-1 polyclonal rabbit serum by ELISA. Serum dilution 1 was 1/20 in blocking buffer and each subsequent dilution was obtained by a 1:4 in series dilution with blocking buffer.

**FIG 7** Known and predicted functional sites on the surface of the immature and mature T=3 HAstV-1 models. (A) Predicted receptor-binding sites, the P site (red), S site (yellow) and β-turn (blue), mapped onto immature (top) and mature (bottom) T=3 HAstV-1 models. (B) The CP1 peptide (HAstV-1 CP residues 80-138) (cyan) that binds complement C1q, mapped onto immature (top) and mature (bottom) T=3 HAstV-1 models. Figures were made with Chimera (40).
