Cryo-EM structure of the SARS-CoV-2 3a ion channel in lipid nanodiscs

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Abstract
SARS-CoV-2 encodes three putative ion channels: E, 3a, and 8a. In related SARS-CoV-1, 3a is implicated in viral release, inflammasome activation, and cell death and its deletion reduces viral titer and morbidity in animal models, suggesting 3a-targeted therapeutics could treat SARS and COVID-19. However, the structural basis for the function of 3a is unknown. Here, we show that SARS-CoV-2 forms large conductance cation channels and present cryo-EM structures of dimeric and tetrameric SARS-CoV-2 3a in lipid nanodiscs. 3a adopts a novel fold and is captured in a closed or inactivated state. A narrow bifurcated exterior pore precludes conduction and leads to a large polar cavity open to the cytosol. 3a function is conserved in a common variant among circulating SARS-CoV-2 that alters the channel pore. We identify 3a-like proteins in \textit{Alpha-} and \textit{Beta-coronaviruses} that infect bats and humans, suggesting therapeutics targeting 3a could treat a range of coronaviral diseases.

Introduction
Coronavirus disease 2019 (COVID-19), caused by the SARS-CoV-2 virus, is an ongoing global pandemic. Neutralizing the virus is the focus of a multi-pronged approach, including behavioral, medical, and basic research efforts around the world. Vaccine and therapeutic development are predominantly focused on the essential virus-encoded Spike, main protease, and RNA-dependent RNA polymerase proteins. These targets have well characterized functions and, particularly for Spike, evidence for neutralizing antibodies in convalescent patient serum. The availability of high-resolution structures of these targets, some in complex with drug candidates or neutralizing antibodies, has yielded mechanistic insight into their function and provided a platform for structure-guided drug design\textsuperscript{1-6}. However, expanding of the range of SARS-CoV-2 drug targets may accelerate therapeutic discovery and increase diversity of available drugs to mitigate against the potential evolution of drug-resistant viral strains\textsuperscript{7}.

The SARS-CoV-2 genome encodes three putative ion channels (viroporins)\textsuperscript{8}, E, 3a, and 8a\textsuperscript{9}. Vi-
Roporins are generally believed to modify host membrane permeability to promote viral assembly and release, among other functions\(^8,10\). Many successful drugs targeting ion channels have been developed\(^11\) and targeting viroporins with small molecule inhibitors, in particular the influenza M2 proton channel, has demonstrated therapeutic success\(^12-14\).

In this study, we focus on the SARS-CoV-2 3a channel\(^15\). The ORF3 genomic region contains coding sequence for multiple open reading frames and exhibits high diversity among coronaviruses compared to neighboring regions\(^16\). Notably, 3a is highly conserved within the Betacoronavirus sub-genus Sarbecovirus which includes SARS-CoV-1 and related bat coronaviruses that are thought to be the zoonotic source of human-infecting SARS coronaviruses (Fig. S1)\(^17\). SARS-CoV-1 3a has been reported to form an emodin-sensitive K\(^+\)-permeable cation channel\(^15,18\) and has been implicated in inflammasome activation\(^19\) and both apoptotic\(^20\) and necrotic cell death\(^21\). In mouse models of SARS-CoV-1 infection, genomic deletion of ORF3a reduced viral titer and morbidity\(^9\). SARS-CoV-1 3a has therefore been considered a potential target for therapeutics to treat SARS.

3a has three predicted transmembrane helices followed by a cytosolic domain with multiple β-strands per protomer chain\(^15\). Each of its domains: N-terminal, Transmembrane, and C-terminal have been proposed to play roles in SARS biology and pathogenesis\(^15,19\). 3a has been shown to form dimers, tetramers, and potentially higher order oligomers of 31 kDa subunits\(^15,21\). No structural information exists for 3a proteins nor are there structures of close homologs that could be used to generate structural models, impeding both computational discovery and design of inhibitors as well as a mechanistic understanding of 3a function. To better understand the basis for 3a function, we have determined structures of dimeric and tetrameric SARS-CoV-2 3a in lipid nanodiscs by cryo-electron microscopy (cryo-EM) and characterized 3a channel activity in a reconstituted system. This could provide a framework for the design of drugs which target 3a and have the potential to serve as COVID-19 therapeutics.

**Results**

Full length SARS-CoV-2 3a was heterologously expressed in *Spodoptera frugiperda* (Sf9) cells with a cleavable C-terminal GFP tag. Whole cell currents recorded from 3a-expressing cells were difficult to distinguish from control cells, likely because the majority of 3a protein is present in intracellular membranes. To better assess 3a channel function, we purified 3a in detergent, reconstituted it into phosphatidylcholine lipids, and recorded currents across excised patches pulled from proteoliposome blisters.

3a-containing patches generated currents with modest outward rectification in symmetric [K\(^+\)] (rectification index = 1.28±0.02, mean ±s.e.m. (n=5)), consistent with preferential sidedness of rectifying channels in the membrane after reconstitution (Fig. 1A,B). We evaluated selectivity of 3a for different cations by replacing the K\(^+\)-containing bath solution with solutions containing Na\(^+\), NMDG\(^+\), or Ca\(^2+\). Solution exchange resulted in reversal potential shifts from 0.3±0.3 mV in K\(^+\) to -6.7±0.5 mV in Ca\(^2+\), -13.5±1.8 mV in Na\(^+\), and -31.0±1.1 mV in NMDG\(^+\) (Fig. 1A,C). These shifts correspond to the following permeability ratios (P\(_X\)/P\(_K^+\)):

\[
\begin{align*}
\text{Ca}^{2+} \quad & (2.04±0.06) > \text{K}^+ \quad (1.0) > \text{Na}^+ \quad (0.59±0.04) > \\
\text{NMDG}^+ \quad & (0.29±0.01)
\end{align*}
\]

(Fig. 1D). We conclude that SARS-CoV-2 3a is a cation channel with modest selectivity for Ca\(^2+\) and K\(^+\) over Na\(^+\).

In K\(^+\) - and Na\(^+\)-containing solutions, channels remained open for long durations with infrequent closures. In contrast, in Ca\(^2+\)-containing solutions, channels exhibited “flickery” behavior with frequent transitions to closed or subconductance states (Fig. 1F). Contrary to previous studies of SARS-CoV-1 3a in cells, SARS-CoV-2 3a activity was not inhibited by Ba\(^2+\)\(^15\) or by the small molecule emodin\(^18\) (Fig. S2F,G). Alkaline pH modestly increased channel activity (by 31 ± 2% at -80 mV in symmetric K\(^+\), Fig. S2H,I,J). Gating was not observed in response to acidic pH (Fig. S2J). After a variable amount of time (on the order of minutes), we observed an increase in “flickery” closures followed by loss of channel activity in each patch, perhaps as a result of channel inactivation (Fig. 1G). The basis for this loss of channel activity remains to
Figure 1 - Function of purified and reconstituted SARS-CoV-2 3a

(A) Current-voltage relationship from a 3a-proteoliposome patch. Pipette solution was 150 mM K\(^+\) and external solution was K\(^+\) (black), Na\(^+\) (pink), Ca\(^{2+}\) (orange), or NMDG\(^-\) (blue) (mean ± s.e.m., n=4-6 patches). (B) Rectification index (I\(_{100\text{mV}}\) / I\(_{-100\text{mV}}\)) in symmetrical K\(^+\). (C) Reversal potential from (A). (D) Permeability ratios (P\(_X\) / P\(_{K^+}\)) calculated from reversal potential shifts in (C). (E) apparent single channel current at -80 mV with K\(^+\) pipette and Na\(^+\) bath solution. (F) Gap-free current recording held at -80 mV during bath solution exchanges indicated in the bar above the current trace. (G) Gap-free current recording held at -80 mV with K\(^+\) pipette and Na\(^+\) bath solution (right) and corresponding current histogram (left). Differences assessed with a one-way ANOVA with Dunnett correction for multiple comparisons, **p<0.01, *** p<0.001, ****p<0.0001.

be determined. Current reduction occurred in discrete steps of 30.0 ± 0.8 pA from which we estimate the single channel conductance of 3a to be 375 pS at -80 mV under bi-ionic conditions with K\(^+\) in the pipette and Na\(^+\) in the bath (Fig. 1E,G).

Purification of 3a in detergent resulted in two species separable by gel filtration (Fig. S3). A majority of 3a runs at a position consistent with a dimer of 62 kDa (Fig. S3A) and 5-10% runs as a 124 kDa tetramer (Fig. S3D). A similar degree of tetramer formation was observed at low concentrations of 3a by florescence size-exclusion chromatography, indicative of a biochemically stable species rather than concentration-dependent nonspecific aggregation (Fig. S3E). These data are consistent with previous reports of dimeric and tetrameric SARS-CoV-2 3a observed by western blot\(^{15,21}\).

We separately reconstituted dimeric and tetrameric SARS-CoV-2 3a into nanodiscs made from the scaffold protein MSP1E3D1 and a mixture of POPC, DOPE, and POPS lipids and determined their structures by cryo-EM (Fig. 2A-H). We also determined the structure of dimeric 3a in the presence of 100 μM emodin, but observed no significant structural changes from dimeric apo 3a or any indication of bound emodin. This is consistent with the lack of emodin inhibition observed in proteoliposome recordings (Fig. S2F,J). The final dimeric reconstruction (with C2 symmetry applied) had an overall resolution of 2.9 Å and permitted de novo modeling of 195 of the 275 amino acids per protomer chain (Fig. 2B). The N-terminus (amino acids 1-
Figure 2 - Structure of 3a in lipid nanodiscs (A) Cryo-EM map of the 3a dimer in MSP1E3D1 nanodiscs at 2.9 Å nominal resolution viewed from the membrane plane. One subunit is colored gray and the second subunit is colored with transmembrane region orange and cytosolic domain (CD) pink. Density from the nanodisc is drawn transparent. (B,C) Model of dimeric 3a viewed (B) from the membrane (as in (A)) and (C) from the extracellular or lumenal side. (D) Zoomed in view of the interaction between subunits in the CD with residues forming the hydrophobic core indicated. (E) Schematic of a 3a monomer. Secondary structure elements are indicated and unmodeled termini and a 5 amino acid β3-β4 loop are shown with dashed lines. (F) Two-dimensional class average of tetrameric 3a in MSP1E3D1 lipid nanodiscs (above) and cryo-EM map at 6.5 Å nominal resolution (lower). (G) Two copies of the dimeric 3a structure rigid body docked into the tetrameric 3a cryo-EM map. (H) Zoomed in view of the interface between two dimers with residues positioned to make contacts indicated.

3a adopts a fold that is, to our knowledge, novel among available protein structures. Querying the protein structure database for structural homologs with DALI returned only weak hits for fragments of 3a domains. Viewed from the membrane plane, 3a is approximately 70 Å tall with a 40 Å high transmembrane region and a cytosolic domain (CD) extending 30 Å out of the membrane (Fig. 2B). The transmembrane region connects to the CD per protomer. The N-termini are oriented on the extracellular or luminal side and C-termini on the cytosolic side of the bilayer. 3a is thus a Class IIIA viroporin according to the classification system of Nieva et al. Viewed from the extracellular side, the transmembrane helices (TMs) trace the circumference of an ellipse with TM1-3 from one protomer followed by TM1-3 of the second protomer in a clockwise order (Fig. 2C). TM1s and TM2s pack against each other across the elliptical minor axis with TM3s positioned at the major axis vertices. TM1-TM2 and TM2-TM3 are joined by short intracellular and extracellular linkers, respectively.
Figure 3 - The 3a channel pore
(A) View of a 3a dimer from the membrane plane with the bifurcated pore connecting the exterior solution to the channel cavity colored in pink and orange. (B) Radii of the pores shown in (A) as a function of distance along the conduction axis. (C-E) Zoomed in views from the external side looking down the pore of six constrictions labeled in (B). (I) View of 3a dimer (upper) from the membrane as in (A) and (lower) from the cytosol with the three pairs of tunnels connecting the channel cavity to the interior solution shown in blue. (J) View from the membrane of the upper tunnels. (K) Radius of the upper tunnel as a function of distance from the conduction axis. (L) View from the cytosol into the upper tunnel with residues contributing to the tunnel surface indicated. (M-O) Same as (J-L) for the intersubunit tunnels. (P-R) Same as (J-L) for the lower tunnels.
through a turn-helix-turn motif following TM3. Each protomer chain forms a pair of opposing β-sheets packed against one another in an eight stranded β-sandwich (Fig. 2B,D). The outer sheet is formed by strands β1, β2, β6 and the N-terminal half of β7. The inner sheet is formed by strands β3, β4, β5, β8, and C-terminal half of β7. The inner sheets from each protomer interact through a large (940 Å² of buried surface area per chain) and highly complementary interface with residues V168, V225, F230, and I232 forming a continuous buried hydrophobic core (Fig. 2D). The interaction between β-sandwiches from each protomer thus forms a strong and stable link between monomers in the dimer.

Two-dimensional class averages of tetrameric 3a show a side by side arrangement of two dimers with well-separated TMs and close juxtaposition of CDs (Fig. 2F). Our tetrameric 3a reconstructions had lower final resolutions (6.5 Å) than dimeric 3a (Fig. 2F). However, the tetrameric map was sufficiently featured in the ICDs to enable rigid-body docking of two copies of the 3a dimer model (Fig. 2G). The best fit models show that TM3-CTD linkers and β1-β2 linkers from neighboring dimers form a continuous interface (300 Å² buried surface area per dimer). While the exact positions of side chains cannot be determined at this resolution, residues W131, R134, K136, H150, T151, N152, C153, and D155 are poised to form a network of hydrophobic, polar, and electrostatic interactions which could mediate tetramerization (Fig. 2H).

Tetramerization of SARS-CoV-1 3a observed by western blot was abolished by reducing agents and a C133A mutation resulted in the loss of tetramerization, membrane localization, and whole-cell currents. However, expression of the C133A mutant was also dramatically reduced, so it may be that these results are a consequence of destabilizing 3a. In SARS-CoV-2 3a, C133 is located in a notable cysteine-rich pocket adjacent to the tetramerization interface (Fig. S11A). At the base of TM3, C133 projects back towards the top faces of β1 and β2 in close proximity to solvent exposed C148 and buried C157. Due to geometric considerations, we modeled C133 and C157 as reduced sulfhydryls, but note that they are nearly within disulfide-bonding distance (Cα distance 6.9 Å) (Fig. S11A). While it is unlikely a single conserved disulfide mediates tetramerization in SARS-CoV-1 3a and SARS-CoV-2 3a, it may be that disruption of this cysteine-rich pocket with cysteine modifying agents or mutations disfavor oligomerization in both channels.

Analysis of possible conduction pathways through 3a reveals a narrow, bifurcated pore which stretches through the outer half of the TM region and is connected to a large and polar cavity open to the cytosol (Fig. 3). The pore has a series of six constrictions and narrows to 1 Å in radius, too small for the conduction of permeant cations. We therefore conclude this 3a structure represents either a closed or inactivated channel conformation.

From the extracellular side, the first four constrictions are hydrophobic (Fig. 3A-F). Opposing F43 residues at the top of TM1 create two paths into the channel which taper to 1.5 Å in radius. The paths then merge and go through a series of tight (1 Å radius) constrictions lined by L46 and I47, V50 and V58, and finally L53 and L85. The final two constrictions are polar (Fig. 3G,H). The close juxtaposition of Q57s from opposing TM1s splits the pore into two paths of 1 Å radius lined by C81s from TM2s. The final and widest constriction (2 Å radius) before the pore opens to the cavity is lined by S60, H78 and K61 from TM1 and H78 from TM2. Opening the channel would require conformational changes in TM1 and/or TM2 to both expand the hydrophobic constrictions and rearrange Q57 at the inner hydrophilic constriction. The polar cavity within the inner half of the TM region is continuous with the cytosol and surrounding bilayer through three pairs of openings: the upper, intersubunit, and lower tunnels (Fig. 3I-R). The upper tunnels are formed between TM2 and TM3 within each protomer and narrow to 2 Å in radius (Fig. 3J,K,L). Judging by the relative position of the nanodisc in the EM maps and hydrophobic character of the upper tunnel exterior, in a cell membrane they likely open to the surrounding lipid bilayer. The intersubunit tunnels run between TM1 and TM3 from different protomers, just above the CD, and narrow to 2.5 Å in radius (Fig. 3M,N,O). With a modest expansion, the intersubunit tunnels...
could permit even large cations like NMDG to access the channel cavity. The intersubunit tunnels open to the membrane-cytosol interface. Tubular shaped-densities are present in both the upper and intersubunit which are consistent with lipid acyl chains, but are not sufficiently featured to confidently model as such (Fig. S11B). The lower tunnels run underneath the TM1-TM2 linker and above the CD and narrow to 2 Å in radius (Fig. 3P,Q,R). The lower tunnels open well into the cytosol and are open paths for ion movement between the cell interior and channel cavity.

The N-terminal 41 residues of each chain constitute the majority of the extracellularly or lumenally exposed 3a protein and thus could be involved in retention of 3a to internal membranes in cultured cells. To test this, we generated an N-terminal deletion construct lacking the first 41 amino acids (3aΔN) and compared its localization to wild-type 3a in HEK cells. Indeed, 3aΔN-EGFP shows reduced localization to internal membranes and bright foci and increased plasma membrane expression (Fig. 4A and Fig. S12). While we were unable to model the N-terminal 39 residues of 3a, an unassigned density feature in the cryo-EM maps that stretches between subunits just above the extracellular entrance to the pore that could correspond to a portion of these unmodeled N-terminal residues (Fig. 4B). We speculated that if the N-terminal region was stably positioned above the mouth of the pore it could influence channel properties. 3aΔN was therefore purified, reconstituted into proteoliposomes and compared to wild type 3a in patch recordings. Aside from a modest decrease in the relative permeability of Ca^{2+} to K^{+}, no significant differences were observed in 3aΔN properties compared to wild-type 3a (Fig. 4C,D,E). These results are consistent with the N-terminal region of 3a being a determinant of its subcellular localization without influencing channel properties.

Over forty thousand SARS-CoV-2 genomes have been sequenced to date and analyses of mutations...
across time and geography have identified a large number of coding variants, some of which may have experienced selective pressure during viral evolution. To date, mutations that result in amino acid changes at 17 residues in 3a have been observed in different SARS-CoV-2 genomes. Thirteen of these residues are represented with colored spheres overlaid on the 3a structure in Figure 5A (the remaining four positions: K16N/L, P36L, P240L/S/H, and P258L are unresolved in our structure). The most prevalent is a Q57H variant found in 25% of sequenced viruses, yet not observed in the earliest sequences or in related bat coronaviruses. Strikingly, as described above, Q57 forms the major hydrophilic constriction in the 3a pore (Fig. 3G and Fig. 5A). We asked whether this mutation had functional consequences by purifying 3a-Q57H, reconstituting it into proteoliposomes, and comparing channel activity to wild-type (Fig. 5B,C,D). No significant difference was observed in the expression, stability, conductance, selectivity, or gating behavior of 3a-Q57H (Fig. 5B,C,D and Fig. S13A,B,C). We conclude that the presence of a histidine at this position does not influence channel properties. The remaining variants are much less common, being observed in <0.1% of sequenced viruses to date. Nine of these are unlikely to impact 3a structure or func-
tion as they are located in loops, lipid facing positions on TMs, and/or are conservative in nature (L41F/I/P, L53F, L96F/H, P104L/S/H, A110V/S, I123V, M125K/L/T, I232F/K/T and N234I/K). The remaining three are at the tetramerization interface (W131C/R/L, R134L/C/H, D155Y/H) (Fig. 2H, 5A). Whether these influence oligomerization or channel function remains to be determined.

3a is very well conserved in the Betacoronavirus subgenus Sarbecovirus that includes SARS-CoV-1 and SARS-CoV-2 (Fig. S1). Structurally related proteins have not been identified in other coronaviruses (including the other five species known to infect humans: MERS-CoV, HCoV-NL63, HCoV-229E, HCoV-HKU1, and HCoV-OC43) by sequence homology. We asked whether we could identify more distant homologs using structure prediction algorithms and the SARS-CoV-2 3a structure. Coronaviridae are classified into 4 genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. 3a homologs were not detected in any Gammacoronavirus or Deltacoronavirus species or in the Betacoronavirus subgenus Embecovirus (which includes HCoV-HKU1 and HCoV-OC43). Distant homology to the CD was identified in the membrane protein ORF5 found in Betacoronavirus subgenus Merbecovirus species including MERS-CoV. In contrast, high confidence structural homologs were predicted in all remaining Betacoronavirus subgenera in proteins annotated ORF3 or NS3 and in all Alphacoronavirus subgenera in proteins annotated ORF3, NS3, ORF4, or NSP3B (including in HCoV-229E and HCoV-NL63 (Fig. S14)). Several of these homologs have been previously demonstrated to have ion channel activity.24–26 Strikingly, all coronaviruses with 3a structural homologs are derived from the bat gene pool, while all those without 3a structural homologs derive from rodent, avian, or pig gene pools. This suggests coevolution of 3a with coronaviruses that have bats as their principal reservoir and may reflect a unique aspect of bat coronavirus biology. The true extent of 3a structural and functional conservation awaits further experimental confirmation. Still, this analysis suggests 3a or 3a-like proteins are more broadly present in coronaviruses than previously recognized and antiviral drugs targeting these proteins could potentially treat diseases associated with multiple known human coronavirus. Further experiments are that resolve the role of 3a in coronavirus biology and pathology could aid in the development of therapeutics targeting 3a channels.
Methods

Cloning and protein expression
The coding sequence for the 3a protein from SARS-Cov-2 was codon optimized for Spodoptera frugiperda (SF9 cells) and synthesized (IDT, Newark, NJ). The sequence was then cloned into a custom vector based on the pACEBAC1 backbone (MultiBac; Geneva Biotech, Geneva, Switzerland) with an added C-terminal PreScission protease (PPX) cleavage site, linker sequence, superfolder GFP (sfGFP) and 7xHis tag, generating a construct for expression of 3a-SNS-LEVLFQGPRGSGGSGG-sfGFP-GSS-7xHis. Mutants and truncation were also introduced into this construct using PCR. MultiBac cells were used to generate a Bacmid according to manufacturer’s instructions. SF9 cells were cultured in ESF 921 medium (Expression Systems, Davis, CA) and P1 virus was generated from cells transfected with Escort IV reagent (MillaporeSigma, Burlington, MA) according to manufacturer’s instructions. P2 virus was then generated by infecting cells at 2 million cells/mL with P1 virus at a MOI 0.1, with infection monitored by fluorescence and harvested at 72 hours. P3 virus was generated in a similar manner to expand the viral stock. The P3 viral stock was then used to infect SF9 cells at 4 million cells/mL at a MOI 2–5. At 72 hours, infected cells containing expressed 3a-sfGFP protein were harvested by centrifugation at 2500 x g for 10 minutes and frozen at -80°C.

Protein purification
For preparation of the 3a dimer and mutant constructs, infected SF9 cells from 1 L of culture (15-20 mL of cell pellet) were thawed in 100 mL of Lysis Buffer containing 50 mM Tris, 150 mM KCl, 1 mM EDTA, 1% n-Dodecyl-β-D-Maltopyranoside (DDM, Anatrace, Maumee, OH), pH 8). A 10% solution of DDM was dissolved and clarified by bath sonication in 200 mM Tris pH 8 prior to addition to buffer to the indicated final concentration. Membrane pellets were then homogenized in Extraction Buffer and this mixture (150 mL final volume) was gently stirred at 4°C for 1 hour. The extraction mixture was centrifuged at 33,000 x g for 45 minutes and the supernatant, containing solubilized membrane protein, was bound to 4 mL of Sepharose resin coupled to anti-GFP nanobody for 1 hour at 4°C. The resin was then collected in a column and washed with 10 mL of Buffer 1 (20 mM HEPES, 150 mM KCl, 1 mM EDTA, 0.025% DDM, pH 7.4), 40 mL of Buffer 2 (20 mM HEPES, 500 mM KCl, 1 mM EDTA, 0.025% DDM, pH 7.4), and 10 mL of Buffer 1. The resin was then resuspended in 6 mL of Buffer 1 with 0.5 mg of PPX protease and rocked gently in the capped column for 2 hours. Cleaved 3a protein was then eluted with an additional 8 mL of Wash Buffer, spin concentrated to 500 µl with Amicon Ultra spin concentrator 10 kDa cutoff (Millipore), and then loaded onto a Superdex 200 increase column (GE Healthcare, Chicago, IL) on an NGC system (Bio-Rad, Hercules, CA) equilibrated in Buffer 1. Peak fractions containing 3a channel were then collected and spin concentrated prior to incorporation into proteoliposomes or nanodiscs. For the tetramer, the preparation was carried out in a similar manner, except with overnight protease cleavage and collection of a peak of larger hydrodynamic radius (see Fig. S2).

Proteoliposome formation
For proteoliposome patching experiments, we incorporated protein into lipid and generated proteoliposome blisters for patch recordings using dehydration and rehydration as described previously with the following modifications27. 3a dimer was first purified into Buffer 1. Protein was then exchanged into lipid with the addition of Biobeads SM2 and an overnight incubation at a protein:lipid ratio of 1:10 (corresponding to 0.5 mg purified 3a dimer and 5 mg of cleared Soybean L-α-phosphatidylcholine.
(Soy PC, MilliporeSigma, Burlington, MA) in DR Buffer (5 mM HEPES, 200 mM KCl, pH 7.2).

Electrophysiology

All electrophysiology recordings were made from 3a-reconstituted L-α-Phosphatidylcholine (Sigma P5638) proteoliposomes. Patches formed in an inside-out configuration and were quickly (within 5-10 seconds) transferred to a solution exchange chamber. Recordings were made at room temperature using Clampex 10.7 data acquisition software with an Axopatch 200B Patch Clamp amplifier and Digidata 1550B digitizer (Molecular Devices) at a bandwidth of 1 kHz and digitized at 500 kHz. A pressure clamp (ALA Scientific) was used to form seals. Potassium pipette and bath solution was 5 mM HEPES pH 7.2, 150 mM KCl, 5 mM EGTA, 1 mM MgCl2. Sodium bath solution was 5 mM HEPES pH 7.2, 150 mM KCl, 1 mM MgCl2, 1 mM CaCl2. NaCl in the bath solution was substituted for 150 mM NMDG-Cl or 75 mM CaCl2 for permeability experiments. Borosilicate glass pipettes were pulled and polished to a resistance of 2-5 MΩ when filled with pipette solution. For cation permeability experiments, liquid junction potentials were calculated and data were corrected offline. For current-voltage plots, the following voltage protocol was applied: $V_{\text{hold}} = 0$ mV mV; $V_{\text{test}} = -100$ to $+100$ mV, $\Delta V_{20}$ mV, $t_{\text{test}} = 1$ second. Currents from each patch correspond to mean values during the step to the indicated voltage.

Permeability ratios were calculated according to Goldman-Hodgkin-Katz relationship. For monovalent cations, permeability ratios were calculated as $P_{X^+} / P_{K^+} = \exp(\Delta V_{\text{rev},F}/RT)$. For divalent cations, permeability ratios were calculated as: $P_{X^{2+}} / P_{K^+} = \alpha_{K^+} [K^+] \exp(\Delta V_{\text{rev},F}/RT)(1 + \exp(\Delta V_{\text{rev},F}/RT))/4\alpha_{X^{2+}} [X^{2+}]$ Where $V_{\text{rev}}$ is the reversal potential, F is Faraday’s constant, R is the universal gas constant, and T is absolute temperature (where $RT/F = 25.2$ mV at room temperature), and $\alpha$ is the ion activity coefficient (assumed to be 0.75 for K$^+$ and 0.25 for Ca$^{2+}$).

Nanodisc formation

Freshly purified 3a dimer in Buffer 1 was reconstituted into MSP1E3D1 nanodiscs with a mixture of lipids (DOPE:POPS:POPC at a 2:1:1 molar ratio, Avanti, Alabaster, Alabama) at a final molar ratio of 1:4:400 (Monomer Ratio: 3a, MSP1E3D1, lipid mixture). First, 20 mM solubilized lipid in Column Buffer (20 mM HEPES, 150 mM KCl, 1 mM EDTA pH 7.4) was mixed with additional DDM detergent and 3a protein. This solution was mixed at 4°C for 30 minutes before addition of purified MSP1E3D1. This addition brought the final concentrations to approximately 15 µM 3a, 60 µM MSP1E3D1, 6 mM lipid mix, and 10 mM DDM in Column Buffer. The solution with MSP1E3D1 was mixed at 4°C for 10 minutes before addition of 200 mg of Biobeads SM2 (Bio-Rad). Biobeads (washed into methanol, water, and then Column Buffer) were weighed after liquid was removed by P1000 tip (damp weight). This mix was incubated at 4°C for 30 minutes before addition of another 200 mg of Biobeads (for a total 400 mg of Biobeads per 0.5 mL reaction). This final mixture was then gently tumbled at 4°C overnight (12 hours). Supernatant was cleared of beads by letting large beads settle and carefully removing liquid with a pipette. Sample was spun for 10 minutes at 21,000 x g before loading onto a Superdex 200 increase column in 20 mM HEPES, 150 mM KCl, pH 7.4. Peak fractions corresponding to 3a protein in MSP1E3D1 were collected, 10 kDa cutoff spin concentrated and used for grid preparation. MSP1E3D1 was prepared as described without cleavage of the His-tag. Tetrameric 3a in nanodiscs was prepared similarly, except with a ratio of 1:2:200 (Monomer Ratio: 3a, MSP1E3D1, lipid mixture).

Grid preparation

Dimeric 3a in MSP1E3D1 was prepared at final concentration of 1.1 mg/mL. For the sample with emodin (MilliporeSigma, Burlington, MA, Catalog E7881), a stock solution of 50 mM emodin in DMSO added to protein sample for final concentrations of 1.1 mg/mL 3a and 100 µM emodin and 1% DMSO. Concentrated sample was cleared by a 10 minute 21,000 x g spin at 4°C prior to grid making. For freezing grids, a 3 µl drop of protein was applied to freshly glow discharged Holly Carbon, 300 mesh R 1.2/1.3 gold grids (Quantifoil, Großlöbichau, Germany). A FEI Vitrobot Mark IV (ThermoFisher Scientific) was used with 4°C, 100% humidity, 1 blot force, a wait time of 5 seconds, and a 3 second blot time, before plunge
freezing in liquid ethane. Grids were then clipped and used for data collection. Tetrameric 3a in MSP1E3D1 was frozen at 0.7 mg/mL with the same grid preparation.

**Cryo-EM data acquisition**

Grids were clipped and transferred to a FEI Talos Arctica electron microscope operated at 200 kV. Fifty frame movies were recorded on a Gatan K3 Summit direct electron detector in super-resolution counting mode with pixel size of 0.5685 Å. For the apo 3a dataset, the electron dose was 9.528 e⁻² Å⁻² s⁻¹ and 10.135 e⁻² Å⁻² s⁻¹ and total dose was 50.02 e⁻² Å⁻² and 53.72 e⁻² Å⁻² in the first set and second set of movies respectively. The two different doses are the result of needing to restart the gun during collection. For the 3a with added emodin dataset, the electron dose was 8.991 e⁻² Å⁻² s⁻¹ and total dose was 47.21 e⁻² Å⁻². For the 3a tetramer, the electron dose was 8.841 e⁻² Å⁻² s⁻¹ and total dose was 49.95 e⁻² Å⁻². Nine movies were collected around a central hole position with image shift and defocus was varied from −0.6 to −2.0 µm through SerialEM

**Cryo-EM data processing**

For the apo 3a dimer, motion-correction and dose-weighting were performed on all 6,309 movies using RELION 3.1’s implementation of MotionCor2, and 2x “binned” to 1.137 Å per pixel³⁰⁻³². CTFFIND-4.1 was used to estimate the contrast transfer function (CTF) parameters³³. Micrographs were then manually sorted to eliminate subjectively bad micrographs, such as empty or contaminated holes, resulting in 3,611 good micrographs. Additionally, micrographs with a CTF maximum resolution lower than 4 Å were discarded, resulting in 2,595 remaining micrographs. Template-free auto-picking of particles was performed with RELION3.1’s Laplacian-of-Gaussian (LoG) filter yielding an initial set of particles. This initial set of particles were iteratively classified to generate templates, which were subsequently used to template-based auto-pick 1,750,730 particles.

Template picked particles were iteratively 2D-classified in RELION3.1 and then in cryoSPARC v2³⁴, resulting in 820,543 particles. These particles were subsequently 3D-classified in cryoSPARC v2 with iterative ab-initio and heterogeneous refine-
ment jobs. The resulting maps were visually evaluated with regard to the transmembrane domain density. A set of 86,479 particles were identified, polished in RELION3.1 and refined in cryoSPARC v2 with subsequent homogeneous and non-uniform refinement jobs (maps were low-pass filtered to an initial resolution where TM density was still visible (6-9 Å), and the dynamic mask was tightened with the near (2-5 Å) and far (3-9 Å) parameters), yielding a map with overall resolution of 3.6 Å.

From this set of 86,479 particles, 2D-classification was performed in RELION3.1 to identify a set of particles with subjectively equal view distribution. From the resulting set, 1,000 particles were randomly sampled and their coordinates used for training in the Topaz particle-picking pipeline³⁶. Training, picking, and extraction were performed independently on each subset of the micrographs. 4,134,279 total particles were extracted in RELION3.1 with a box size of 256 pixels and “binned” 4x to 4.548 Å/pixel. These particles were then iteratively 2D-classified in RELION3.1 resulting in 2,674,606 particles which were extracted at 2.274 Å/pixel. 2D-classification was continued in both RELION3.1 and cryoSPARC v2 resulting in 1,429,763 particles. Further classification was performed in cryoSPARC v2 with subsequent ab-initio (4 classes, max resolution 8 Å) and heterogeneous refinement (8 Å initial resolution) jobs. The two best classes were selected and the particles pooled resulting in 743,800 particles which were extracted in RELION3.1 at 1.137 Å/pixel.

Iterative 3D-classification was performed with subsequent ab-initio and heterogeneous refinement jobs as described above. Following each round, 2D classification jobs were used to “rescue” good particles from the worst classes before the next round. After 3 rounds, a final 2D-classification job was used to identify 112,502 particles, which were subsequently pooled with the previous 86,479 RELION3.1 template-picked particles, resulting in 185,871 particles after duplicates were removed. These particles were then refined with subsequent homogeneous and non-uniform refinement jobs resulting in a map with overall resolution of 3.4 Å. This map was post-processed in RELION3.1 using a mask with a soft edge (5 pixel extension, 7
pixel soft-edge), the output of which was used for Bayesian particle polishing in RELION3.1 (training and polishing were each performed independently on each subset of the micrographs). The resulting “shiny” particles were then refined in cryoSPARC v2 with subsequent homogenous refinement (1 extra pass, 7 Å initial resolution) and non-uniform refinement (C2, 1 extra pass, 9 Å initial resolution) to yield a map with 2.9 Å overall resolution.

For 3a dimer with added 100 µM emodin, initial processing was similar to the dimer without added drug (see processing supplement). As with the apo 3a dimer, the critical steps included Topaz particle picking, particle clean-up with cryoSPARC v2 ab-initio and heterogeneous refinement, non-uniform refinement with tightened masking, and RELION3.1 Bayesian particle polishing. However, in contrast to the apo dataset, we observed a set of particles that were included in < 4 Å reconstructions that had discontinuous transmembrane domain density. Removal of these particles with RELION3.1 3D classification without angular sampling led to the best map from the emodin-added dataset. We did not see any evidence of bound emodin, but the 1% DMSO added with drug addition may have contributed to subtype map differences (Fig. S8,S9).

For the 3a tetramer, the initial 7,092 micrographs were first cleaned using manual inspection and removal of images with < 4 Å CtfMaxResolution to obtain a set of 4,324 micrographs. Reference particles for Topaz particle picking were generated by first template picking in RELION3.1, followed by 2D classification in both RELION3.1 and cryoSPARC v2, and subsequent ab-initio in cryoSPARC v2. Particles from various views were then selected from iterative RELION3.1 2D classification to create a set of 6,843 particles. Using these coordinates for training, Topaz particle picking was then performed to generate a set of 1,282,913 initial particles. These particles were then cleaned using 2D classification in RELION3.1 and cryoSPARC v2, followed by rounds of cryoSPARC v2 ab-initio and RELION3.1 3D classification. A major hurdle for tetramer processing was obtaining a reconstruction where most particles were properly oriented in the same direction (i.e. CD domains on the same side of the nanodisc as seen in the 2D classes, see Fig. 2F). Substantial cleanup by 3D-classification was needed to generate a correctly aligned reference map, but this map could then be used as a reference for refinements and classification for larger particle sets. Reconstructions with C1 or C2 symmetry looked similar (see Fig. S10), although no tetramer reconstruction went to high enough resolution to determine symmetry with certainty. Therefore, it is possible that either the tetramer is pseudosymmetric or that different particles have heterogeneous orientations between dimer pairs. For the tetramer, the highest resolution reconstruction came from cryoSPARC v2 non-uniform refinement with a tightened mask, which was subsequently used for dimer-docking and figure preparation.

**Modeling, Refinement, and Analysis**

Apo dimeric 3a cryo-EM maps were sharpened using cryoSPARC and were of sufficient quality for de novo model building in Coot. Real space refinement of the models was carried out using Phenix.real_space_refine. Molprobity was used to evaluate the stereochemistry and geometry of the structure for subsequent rounds of manual adjustment in Coot and refinement in Phenix. Docking of the apo dimeric 3a into the tetrameric 3a cryo-EM map was performed in Phenix using a map in which large empty regions of the nanodisc were erased in Chimera. Similar results were found using maps with only the CDs present. Cavity measurements were made with HOLE implemented in Coot. Comparisons to the structure database was performed with DALI. Structure prediction was performed with Phyre2. Figures were prepared using PyMOL, Chimera, ChimeraX, Fiji, Prism, GNU Image Manipulation Program, and Adobe Photoshop and Illustrator software.

**Fluorescence Size Exclusion Chromatography (FSEC)**

Sf9 cells (4 million) from the third day of infection were pelleted, frozen, and then thawed into extraction buffer (20mM Tris pH 8, 150 mM KCl, all protease inhibitors used for protein purification, 1 mM EDTA, 1% DDM). Extraction was performed at 4°C for 1 hour and lysate was then pelleted at 21,000 x g at 4°C for 1 hour to clear supernatant. Supernatant was then run on a Superose 6 Increase column with fluorescence detection for
GFP into column buffer (20 mM HEPES pH 7.4, 150 mM KCl, 0.025% DDM).

**Transfection and Imaging** The constructs for full length 3a and 3aΔN were cloned into a vector with a CMV-promoter and C-terminal EGFP. Constructs (2 µg) were transfected into HEK293 cells on glass coverslips using Fugene HD (Promega, Madison, WI) per manufacturer’s instructions. Two days after transfection cells were washed with DPBS and then fixed in 4% Formaldehyde in DPBS for 10 minutes. Cells were then washed with DPBS before mounting the coverslip with Prolong Glass Antifade with NucBlue (ThermoFisher Scientific) per manufacturer’s instructions. Fluorescent images were collected using a Zeiss LSM 880 NLO Axio Examiner confocal microscope at either 20X (NA 1.0) or 63X oil immersion objective (NA 1.4). The samples were excited with 488nm argon laser and image analysis was performed using ImageJ.

**Data and reagent availability**

All data and reagents associated with this study are publicly available. For dimeric apo 3a, the final model is in the PDB under 6XDC, the final map is in the EMDB under EMD-22136, and the original micrograph movies and final particle stack is in EMPIAR under EMPIAR-10439. For tetrameric apo 3a, the final map is in the EMDB under EMD-22138 and the original micrograph movies and final particle stack is in EMPIAR under EMPIAR-10441. For dimeric 3a in the presence of emodin, the final map is in the EMDB under EMD-22139 and the original micrograph movies and final particle stack is in EMPIAR under EMPIAR-10440.

**Acknowledgements**

We thank Sonali Mali and Dr. Diana Bautista for providing tissue culture cells, Dr. Hillel Adesnik for providing emodin, and all three for discussions. We thank Paul Tobias for computational resources at the Cal-Cryo EM facility, and Dr. James Hurley and Dr. Eva Nogales for supporting the microscopy work. We thank members of the Brohawn lab for feedback on the manuscript. SGB is a New York Stem Cell Foundation-Robertson Neuroscience Investigator. This work was funded by the New York Stem Cell Foundation, NIGMS grant GM123496, a McKnight Foundation Scholar Award, a Rose Hill Innovator Award, a Sloan Research Fellowship (to SGB), NIGMS grant GM128263 (to DMK), and a Fast Grants Award from Emergent Ventures at the Mercatus Center, George Mason University (to SGB, Hillel Adesnik, and Diana Bautista).

**Author contributions**

BS, DMK, and SGB conceived of the project. BS performed all electrophysiology. DMK performed all molecular biology, biochemistry, proteoliposomes preparation for electrophysiology, and cryo-EM sample preparation. SS performed light microscopy. JPR and DBT collected cryo-EM data. CMH and DMK processed the cryo-EM data. SGB built and refined the atomic models. DK, BS, CMH, and SGB wrote the manuscript with input from all authors.

**References**

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Table S1 - Cryo-EM data collection, processing, refinement, and modeling data
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Table S2 - 3a homologs across Coronaviridae
Figure S1 – Sequence alignment of 3a channels from Betacoronavirus Sarbecovirus

(A) Alignment of fifteen 3a protein sequences colored by conservation in a ramp from white (not conserved) to dark blue (highly conserved). Accession numbers are indicated. Sequences were selected to maximize diversity among annotated Sarbecovirus 3a proteins. Secondary structure for SARS-CoV-2 is drawn above the sequence with unmodeled sequence drawn as dashed lines. (B) Neighbor-joining tree calculated from the alignment in (A).
Figure S2 - Patch recordings from 3a-proteoliposomes (A) Current recordings from a 3a-proteoliposome patch with 150 mM K⁺ in the pipette and 150 mM Na⁺ in bath. Currents were recorded with the following protocol in (A-D, F, H): V_{hold} = 0 mV, V_{test} = -100 to 100 mV, ΔV = 20 mV, t_{test} = 1 sec. (B) Current recordings from a 3a-proteoliposome patch with 150 mM K⁺ in the pipette and 150 mM NMDG⁺ in bath. (C) Current recordings from a 3a-proteoliposome patch with 150 mM K⁺ in the pipette and 75 mM Ca²⁺ in bath. (D) Current recordings from a 3a-proteoliposome patch with 150 mM K⁺ in the pipette and 150 mM Na⁺ or 150 mM Na⁺ and 100 µM Ba²⁺ in the bath. (E) Current-voltage relationship plotted from (D). (F) Current recordings from a 3a-proteoliposome patch with 150 mM K⁺ in the pipette and 150 mM Na⁺ or 150 mM Na⁺ and 100 µM emodin in the bath. (F) Current-voltage relationship plotted from (F). (G) Current sweeps for 150 mM K⁺ in the pipette and in the bath: 150 mM Na⁺ and 150 mM Na⁺ + 100 µM emodin. (H) Current recordings from a 3a-proteoliposome patch with 150 mM K⁺ in the pipette and 150 mM Na⁺ pH 7.2 or 150 mM Na⁺ pH 9.2 in the bath. (I) Current-voltage relationship plotted from (H). (J) Bar chart showing ratio of current change at 100 mV between pH 7.2 and 5.2 or pH of 9.2.
Figure S3 – Purification and reconstitution of 3a
(A) Size exclusion chromatogram of 3a expressed in insect cells and extracted and purified in DDM (left). Pooled fractions corresponding to dimeric 3a are highlighted in blue.
Coomassie-stained SDS-PAGE of pooled dimeric 3a-containing fractions (center) and of 3a following reconstitution into PC lipids (right). (B) Size exclusion chromatogram of dimeric 3a reconstituted into MSP1E3D1 lipid nanodiscs (left). Pooled fractions are highlighted blue. (C,D) Same as (A,B), but for tetrameric 3a. (E) GFP fluorescence chromatogram of 3a expressed in SF9 cells and extracted in DDM detergent. Samples were run on a Superose 6 column.
Figure S4 - Example micrographs and 2D class averages of dimeric apo 3a in MSP1E3D1 lipid nanodiscs (A) Representative micrograph and (B) 2D class averages of dimeric apo 3a in MSP1E3D1 lipid nanodiscs.
Figure S5 - Cryo-EM processing pipeline for dimeric apo 3a in MSP1E3D1 lipid nanodiscs

Overview of Cryo-EM data processing pipeline in cryoSPARC and Relion. See Methods for details.
Figure S6 - Cryo-EM validation for dimeric apo 3a in MSP1E3D1 lipid nanodiscs (A) Local resolution estimated in Relion colored as indicated on the final map. (B) Angular distribution of particles used in final refinement with final map for reference. (C) Fourier Shell Correlation (FSC) relationships between (black) the two unfiltered half-maps from refinement and used for calculating overall resolution at 0.143, (red) the final map and model, (gray) half-map one and model, and (blue) half-map and model.
Figure S7 - Example micrographs and 2D class averages of dimeric 3a in MSP1E3D1 lipid nanodiscs with emodin added (A) Representative micrograph and (B) 2D class averages of dimeric 3a in MSP1E3D1 lipid nanodiscs with emodin added.
**3a in MSP1E3D1 + Emodin Dataset**

Micrograph clean-up (Start: 6750 mics)  
Select, CtfMaxResolution sub-4 Å resolution (3676 mics)  
Manual inspection (3405 mics)  

**Topaz picked particles (3,873,767), 4.548 Å/pixel**  
Iterative 2D classification (Relion)  

3,121,137 particles, 2.274 Å/pixel  
Iterative 2D classification (Cryosparc)  

1,895,307 particles  
Ab initio for good and bad templates  
Iterative Heterogeneous Refine  
Until no improvement in resolution  

256,290 particles, re-extract to 1.137 Å/pixel  
**Non-uniform refinement A**  
Starting resolution 10 Å  
Mask near 3 Å, Far 6 Å  
Reference: gaussian filtered lower resolution prior NU  

**Relion Refine3D**  
Reference from cryosparc NU  
Soft mask around protein made in Relion  
Output used for Bayesian Polishing  

**Non-uniform refinement B**  
Settings: As Non-uniform refinement A with polished particles  

**Relion Refine3D**  
Reference from cryosparc NU  
Mask from Cryosparc NU (refine_mask)  

**Relion Class3D**  
3 Classes, Tau 16, No alignments  
Angles from previous Relion Refine3D  
Reference from previous cryosparc NU  
Mask from previews Cryosparc NU (refine_mask)  

51,908 particles (continuous TM density)  
143,375 particles (discontinuous TM density)  
**Non-uniform refinement C and D**  
Settings: Non-uniform refinement A  

Final Map  
Used for post-processing and map and model comparison  

---

**Figure S8 - Cryo-EM processing pipeline for dimeric 3a in MSP1E3D1 lipid nanodiscs with emodin added**  
Overview of Cryo-EM data processing pipeline in cryoSPARC and Relion. See Methods for details.
Figure S9 - Cryo-EM validation for dimeric 3a in MSP1E3D1 lipid nanodiscs with emodin added
(A) Local resolution estimated in Relion colored as indicated on the final map. (B) Angular distribution of particles used in final refinement with final map for reference. (C) Fourier Shell Correlation (FSC) relationships between (black) the two unfiltered half-maps from refinement and used for calculating overall resolution at 0.143, (red) the final map and model, (gray) half-map one and model, and (blue) half-map and model.
Figure S10 - Example micrographs and 2D class averages of tetrameric apo 3a in MSP1E3D1 lipid nanodiscs

(A) Representative micrograph and (B) 2D class averages of tetrameric apo 3a in MSP1E3D1 lipid nanodiscs. (C) Map overview pipeline for final steps of processing (Also see Methods).
**Figure S11 – Structural elements in dimeric 3a** (A) Full model (left) with boxed region zoom-in (right) to show the cysteine-rich region of 3a. (B) Tubular unassigned lipid-like densities in the apo 3a map, colored blue and yellow. Displayed at threshold 0.7, with surface dust hidden (size 6).
**Figure S12 – Localization of 3a-EGFP and 3aΔN-EGFP expressed in HEK cells**

(A) Field of view with multiple cells imaged with 20X objective. Scale bar, 20 µm. (B) Cells imaged with 63X objective with both single plane and brightest-point projections displayed. Scale bar, 10 µm.
Figure S13 – Purification and reconstitution of 3aΔN and 3aQ57H (A) GFP fluorescence chromatogram of 3a, as well as mutants and truncations, expressed in SF9 cells and extracted in DDM detergent. Samples were run on a Superose 6 column. (B) Size exclusion chromatogram of 3a Q57H expressed in insect cells and extracted and purified in DDM (left). Pooled fractions corresponding to dimeric 3a are highlighted in blue. (C) Coomassie-stained SDS-PAGE of pooled dimeric 3a Q57H-containing fractions (center) and of 3a following reconstitution into PC lipids (right). (D,E) Same as (B,C) but for 3aΔN.
Figure S14 - Sequence alignment of 3a-like proteins across *Coronaviridae*(A) Alignment of twenty-eight 3a-like protein sequences colored by conservation in a ramp from white (not conserved) to dark blue (highly conserved). Accession numbers are listed in Table 2. Sequences were selected from representative species from each Coronavirus subgenus. Alphacoronavirus and Betacoronavirus clades are indicated. Within Betacoronavirus the subgenus *Merbecovirus* is also indicated with a bar. (B) Neighbor-joining tree calculated from the alignment in (A).