Polyprotein Context Regulates the Activity of Poliovirus 2C^{ATPase} Bound to Bilayer Nanodiscs

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Positive-strand RNA viruses generally replicate in large membrane-associated complexes. For poliovirus, these replication complexes are anchored to the membrane via the viral 2B, 2C, and 3A proteins. 2C is an AAA+ family ATPase that plays a key role in host cell membrane rearrangement, is a putative helicase, and is implicated in virion assembly and packaging. However, the membrane-binding characteristics of all of these viral proteins have made it difficult to elucidate their exact roles in virus replication. We show here that small lipid bilayers known as nanodiscs can be used to chaperone the *in vitro* expression of soluble poliovirus 2C, 2BC, and 2BC3AB polyproteins in a membrane-bound form. ATPase assays on these proteins show that the activity of the core 2C domain is stimulated ~60-fold compared to the larger 2BC3AB polyprotein, with most of this stimulation occurring upon removal of 2B. The proteins are active over a wide range of salt concentrations, exhibit slight lipid headgroup dependence, and show significant stimulation by acetate. Our data lead to a model wherein the replication complex can be assembled with a minimally active form of 2C that then becomes fully activated by proteolytic cleavage from the adjacent 2B viroporin domain.

The picornaviruses are a family of human and mammalian pathogens that include hepatitis A virus, the rhinoviruses, foot-and-mouth disease virus (FMDV), coxsackievirus, and poliovirus. Poliovirus remains a health threat in many parts of the world, where it is being kept in check by extensive immunization efforts aimed at its eventual eradication (1). Poliovirus has a 7.5-kb single-stranded, positive-sense RNA genome that encodes a single ~250-kDa polypeptide. Upon translation, this viral polyprotein undergoes a series of proteolytic cleavages to generate a total of 11 different proteins plus several important functional intermediates (Fig. 1A). The four proteins in the P1 region (VP1 to VP4) are the structural proteins that make up the virion capsid, while the remaining seven nonstructural proteins in the P2 and P3 regions (2A–2C and 3A–3D, respectively) are involved in viral replication biochemistry (2). Processing of the polyprotein plays an important role in mediating protein function as several viral precursor proteins have different functional roles and biochemical activities than the fully processed proteins. For example, the cleavage of poliovirus 3CD^{Pro} generates and activates 3D^{Pol}, the RNA-dependent RNA polymerase that carries out replication of the viral RNA (3–5). Similarly, the 2C and precursor 2BC proteins act in concert to trigger a massive rearrangement of intracellular membranes that results in the formation of extensive “membranous web” structures (6–8), on the surfaces of which these proteins form the viral replication centers that are the sites of RNA synthesis and virion assembly.

The viral 2B, 2C, and 3A proteins all contain membrane-binding regions. Both 2BC and 2C are able to induce membrane rearrangement in living cells (9–11); however, coexpression of a combination of these membrane-binding proteins is needed to recapitulate the double-walled membranes observed during virus infection (12). 2B contains a pair of predicted integral membrane helices and is thought to form a viroporin structure (13–15), 2C is anchored to the membrane via a N-terminal amphipathic helix (10, 16–18), and 3A contains an amphipathic sequence within its C-terminal 22 residues (19, 20). There is evidence that these three membrane-anchored proteins interact with one another both *in*

vivo and *in vitro*; substitution of the 2C amphipathic helix from other picornaviruses into a poliovirus background results in compensatory mutations in the membrane anchoring sequences of both 2B and 3A (21, 22). In addition to its roles in membrane rearrangements, 2C has also been implicated in initiation of negative-strand RNA synthesis (23), interacting with the VP3 capsid protein (24), and virion packaging (25).

2C is comprised of 329 amino acids and contains an N-terminal amphipathic helix, two predicted RNA-binding regions, three motifs implicated in NTP binding, and a cysteine-rich putative zinc finger motif (Fig. 1B) (18, 26, 27). Two of the NTP binding motifs are Walker A and B, common to AAA+ ATPases, while the third is motif C/Sensor-1 that shares homology with the superfamily III helicases (28). 2C has been shown to have ATPase activity and may also have low-level GTPase activity (26, 28, 29). Electron microscopy images of 2C proteins from poliovirus and FMDV show hexameric ring structures, and activity assays indicate that oligomerization of FMDV 2C is important for ATP hydrolysis (30, 31). There are no atomic level structures for the picornaviral 2C proteins, and sequence homology searches do not reveal convincing matches for proteins other than those from closely related viruses. Structure prediction using the Phyre engine (32) that utilizes profile-profile sequence matching and structure-based threading to do homology modeling shows a high probability that residues 100 to 260 fold into a AAA+ family ATPase. The AAA+ (ATPases associated with diverse cellular activities) superfamily of enzymes are often considered protein workhorses because they are involved in a multitude of basic cellular functions, including protein degradation, membrane rear-

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FIG 1 Poliovirus polyprotein processing and nanodisc chaperoned expression. (A) Organization of the poliovirus single-stranded positive-sense RNA genome encoding a single open reading frame for the viral polyprotein. Dashed arrows indicate viral proteins known to interact with the 2C^{ATPase} and gray bars identify membrane-binding (MB) regions. (B) Schematic representation of the five viral proteins that were generated for the present study. All constructs contained a C-terminal hexahistidine tag and were made both with and without a N-terminal ubiquitin domain ($\pm$ Ub). Diagrammed below are known 2C functional domains, including the NTP interaction regions that comprise the core AAA + ATPase motifs. (C) Depiction of the nanodisc-based *in vitro* expression protocol showing the initial assembly of discs from pure phospholipids and two copies of the amphipathic helix-based membrane scaffold protein (MSP) that are then added to bacterially based *in vitro* transcription/translation expression reactions (derived with permission from references 38 and 39).

rangement, DNA replication, and disassembly of protein complexes (33, 34). These enzymes tend to oligomerize (33), forming structures that are consistent with the aforementioned electron microscopy data showing 2C ring assemblies.

The propensity of 2C to associate with membranes has made it difficult to study and much remains unknown about both its structure and its function during viral replication. Thus far, poliovirus 2C has been generated using glutathione S-transferase (GST) (28) or maltose-binding protein (30) fusions to improve expression and solubility, but these proteins remained difficult to work with. FMDV 2C has been purified in a soluble form by deleting the N-terminal membrane-binding region to generate a soluble and biochemically active core ATPase fragment (31). Attempts by our laboratory to express poliovirus 2C using similar approaches in both *Escherichia coli* and baculovirus systems resulted in protein that was largely insoluble and had little or no ATPase activity. In addition, our GST-2C fusion construct could not be efficiently cleaved using PreScission protease, a derivative of the picornaviral rhinovirus 3C protease, suggesting the protein may not have been natively folded.

To circumvent these difficulties with expression and purification, we explored a new approach for isolating 2C by expressing it *in vitro* on small lipid bilayers known as nanodiscs (Fig. 1C). Developed by the Sligar laboratory, nanodiscs are soluble nanolipoprotein particles comprised of a lipid bilayer held together by an amphipathic helix membrane scaffold protein (MSP) that forms a belt around the lipid acyl chains (35, 36). Nanodiscs provide a native bilayer surface to which membrane proteins can adhere, thus reducing the likelihood of aggregation, while being small

enough to remain fully soluble and tumble rapidly enough to enable a variety of spectroscopy experiments (37–39). Most importantly, this system has allowed us to biochemically characterize 2C activity when bound to true membrane bilayers instead of detergent micelles. In addition, this allowed us to explore 2C activity in the context of its membrane-associated polyprotein neighbors to examine how processing may affect 2C function.

In the present study, we demonstrate the *in vitro* expression and subsequent purification of poliovirus 2C, 2BC, 2C3A, 2BC3AB, and 3AB proteins on nanodiscs composed of dimyristoyl phospholipid bilayers with choline, glycine, and serine headgroups. Using an NADH-coupled ATPase assay, we show that the 2C^{ATPase} is active over a broad range of pH values and its activity is stimulated by the presence of acetate while being slightly inhibited by monovalent salts. Notably, we observe a ~60-fold activation of 2C ATPase activity by the fully processed 2C compared to the 2BC3AB polyprotein precursor, suggesting that proteolytic processing of the viral polyprotein plays a major role in activating 2C.

MATERIALS AND METHODS

Cloning. Constructs were cloned from pUC57-2BC3AB, a bacterial codon optimized synthetic gene made by Genscript, Inc., encoding poliovirus 2BC3AB, followed by a C-terminal GSSS-His₆ tag sequence. The construct includes a SacII restriction site immediately preceding the start codon and a NotI site following the His₆ sequence. Initially, the 2C-containing constructs were cloned into a pET26 expression vector developed by the Cameron laboratory for expression of poliovirus 3D^{pol} (40). With this vector the protein is initially translated as a fusion with an N-terminal ubiquitin domain (Ub), but this domain is cleaved off by the ubiquitin-specific carboxyl-terminal protease Ubp1 that is coexpressed in the *E. coli*

pCG1 strain, resulting in a processed polymerase with a N-terminal glycine residue that mimics the natural cleavage of the poliovirus polyprotein at Gln-Gly sites. The 3D^{pol} gene was removed from this vector and replaced with the gene for 2BC3AB-GSSS-His₆ via the SacII and NotI sites, resulting in pET26-Ub-2BC3AB-GSSS-His₆. 5' and 3' deletions within the open reading frame were then used to generate expression vectors for Ub2BC, Ub2C, Ub2C3A, and Ub3AB. A second set of these expression vectors where the N-terminal ubiquitin fusion was replaced by a single methionine start codon were also generated, and the integrity of all constructs was verified by sequencing.

For the *in vitro* translation experiments, we found that the pET26-based constructs resulted in very low protein yield compared to the elongation factor Ts produced from the pEFT control plasmid provided in the EasyXpress kit (Qiagen, Inc.). Further analysis showed this to be the result of inefficient RNA transcription, which can have a major effect on *E. coli*-based *in vitro* translation systems due to high endogenous RNase activity. To increase RNA levels in the reactions, we cloned all of our constructs into the pEFT control vector by simply replacing the elongation factor Ts open reading frame. The subsequent mutations to the predicted AAA+ ATPase NTP-binding motifs in 2C were made by the QuikChange mutagenesis protocol to produce K135A (Walker A), D177A (Walker B), and T222A and N223A (motif C/Sensor-1) in both the pEFT-Ub2C and pEFT-Ub2BC vectors.

Nanodisc assembly. Nanodiscs were expressed and assembled with the MSP1E3D1 protein (41) and dimyristoyl phosphatidylcholine (DMPC) lipids—unless noted otherwise—using 1:150 molar ratio of MSP1E3D1:DMPC according to the protocols provided by the Sligar laboratory (sligarlab.life.uiuc.edu). Briefly, MSP1E3D1 was expressed from the vector distributed by Addgene as plasmid number 20066 (www.addgene.org/20066) and purified by nickel affinity chromatography. The N-terminal His₆ tag on the purified MSPs was removed using TEV protease that also contained a His₆ tag. After cleavage, the TEV/MSP mixture was run over a second nickel affinity column, from which the only the MSPs lacking the His₆ tag were collected in the unbound fraction. MSPs and DMPCs were then cosolubilized using cholate detergent, cholate was removed using hydrophobic gel permeation BioBeads (Bio-Rad, Inc.) to initiate nanodisc assembly, and the resulting discs were purified over a Superdex S-200 gel filtration column where elution also confirmed that the nanodiscs were of the expected size with a Stokes diameter of ~12 nm (42). Nanodiscs containing dimyristoyl phosphatidylserine (DMPS) or dimyristoyl phosphatidylglycine (DMPG) lipids were assembled using the same MSP protein and assembly protocols, with the difference that lipid solubilization and assembly were carried out just above the appropriate lipid transition temperatures of 23°C for DMPC and DMPG and 35°C for DMPS. We attempted to assemble nanodiscs using dimyristoyl phosphatidylethanolamine (DMPE), but they did not remain soluble when brought below the 50°C transition temperature. All lipids were purchased from Avanti Polar Lipids.

***In vitro* expression and purification.** 2C^{ATPase} and the other 2BC3AB-based proteins were produced using the Invitrogen Expressway *in vitro* bacterial expression system. Protein expression reactions (0.1 to 2 ml) were incubated in an Eppendorf Thermomixer at 30°C and 1,200 rpm for 3 h using 1 µg of DNA per 100-µl final reaction volume (RV), and nanodiscs were added to a final concentration of 15 µM. Half of the required small molecule “feed mix” was added 30 min after starting the reaction, and the other half was added after 2 h. The completed reactions were then centrifuged for 10 min at room temperature. The supernatant was batch purified with one-quarter RV of settled Ni-NTA resin (Qiagen) pre-equilibrated in low imidazole buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 10 mM Imidazole, 0.02% NaN₃, 10 to 20% glycerol) by incubation at 4°C for at least 30 min. After mixing, the supernatant was removed and the resin washed with three RVs of low imidazole buffer. Protein was batch eluted in multiple sequential quarter-RV fractions of high-imidazole (500 mM) buffer. TCEP reducing agent (Pierce) was added to each elution to a final concentration of 10 mM, samples were analyzed by

SDS-PAGE, and elution fractions containing significant amounts of protein were combined, divided into aliquots, and stored at -80°C for future use. Note that since the His₆ tag had previously been cleaved from the MSPs, only nanodiscs bound to the 2C constructs are copurified in the elution fractions, and this represents a small portion of the 15 µM nanodiscs that were added to the translation reaction.

Several constructs were later subjected to more rigorous rounds of purification in order to reduce the amount of contaminant proteins that coeluted with 2C during the batch purification process. In this purification process, 1 to 2 ml (final RV) of the Expressway reaction were purified by liquid chromatography using an AKTA purifier system first over a 1-ml HisTrap HP nickel resin column (GE Life Sciences) in the same low- and high-imidazole buffers used for batch purification. Peak fractions were pooled, diluted 5-fold, and further purified over a 1-ml HiTrap Q HP ion-exchange column (GE Life Sciences) with a gradient of low- to high-salt Q buffer (25 mM Tris [pH 8.5], 50 mM/1 M NaCl or KCl, 20% glycerol). 2C protein was typically eluted in ~300 mM salt and then stored as stated above.

2C yield and concentration determination. Determination of the specific activity of each ATPase construct requires knowing the precise concentration of ATPase in the purified samples. Since the purified samples contained the 2C construct, the nanodisc MSP, and some contaminant proteins from the batch-based purification scheme, we could not accurately determine the 2C concentration by simple 280-nm absorbance or other overall protein content assays. We therefore determined the concentrations of both the viral proteins and MSP by separating them on 12 to 15% SDS-PAGE gels alongside known concentrations of the ProSieve Unstained Protein Marker II standards (Lonza Group, Ltd.). The gel was stained with IRDye blue protein stain (LiCor Bioscience) and imaged on an Odyssey infrared imaging system at 700 nm. The intensities of the protein standard bands were quantified and plotted against total amount of protein in each band to yield a linear relationship between staining intensity and protein content (Fig. 2). The protein concentration of the *in vitro* expressed His-tagged viral proteins and the nanodisc MSPs that copurified with them were then obtained by interpolating their band intensities on this standard curve.

ATPase Activity assay. The rate of ATP hydrolysis was determined using an ATP/NADH coupled assay (see Fig. 5A) combined with an ATP regeneration system that allows us to assay steady-state 2C^{ATPase} activity over a wide range of ATP concentrations (43). In this assay, the conversion of ATP to ADP + P_i by an ATPase is coupled to ATP regeneration via the pyruvate kinase (PK) catalyzed conversion of phosphoenolpyruvate (PEP) + ADP to pyruvate + ATP and the lactate dehydrogenase (LDH) catalyzed conversion of pyruvate + NADH to lactate + NAD⁺. The rate of ATP hydrolysis is then monitored by the loss of NADH absorbance at 340 nm (Fig. 5B) with a molar extinction coefficient of 6.22 mM⁻¹ cm⁻¹. Coupled reactions consisted of 5 mM PEP, 0.024 U of PK/µl, 0.036 U of LDH/µl, and 0.4 mM NADH, resulting in an initial absorbance of ~2.0 at 340 nm that is linearly reduced to ~0.25 upon complete oxidation of the NADH as a result of ATPase turnover.

The ATP hydrolysis assays were carried out at 37°C in a Varian Bio50 spectrophotometer with a thermostated multicell holder using a 0.5 to 40 nM 2C^{ATPase} domain in a base buffer containing 75 mM KCl, 50 mM magnesium acetate, 20 mM HEPES (pH 7.0), 400 µM NADH, and 5 mM dithiothreitol (DTT). Steady-state data were collected for 30 to 60 min with points measured approximately every 30 to 45 s, depending on how many assays were being conducted in the 18-cell multicell holder at the time. Experiments to characterize the monovalent salt, pH, and magnesium dependence of 2C^{ATPase} activity were done by various individual components of this buffer as needed. To verify that the buffer conditions tested did not adversely affect the coupled reporter system assay, 0.2 mM ADP was directly added to control reactions, and the subsequent loss of NADH absorbance was instantaneous and always significantly faster than the rate of ADP production by 2C.

FIG 2 Protein concentration determination. (A) Protein concentrations were determined by running various dilutions of the ProSieve Unstained Protein Marker II standard alongside dilutions of 2C protein constructs on reducing acrylamide SDS gels. The gels were stained with LiCor IRDye blue protein stain, imaged by scanning on a LiCor Odyssey infrared fluorescence imaging system, and multiple bands from the standards, the 2C constructs, and nanodisc MSP were outlined for quantitation as shown. (B) Example standard curve generated from the correlation of the protein standards intensities (open circles) with the amount of protein loaded and then fit to a linear curve whose parameters are listed in the inset box. The concentrations of the various 2C construct dilutions were then interpolated on this line, as per the three proteins shown here (solid symbols), and averaged to obtain the concentration in the original sample. Independent gels with standards were analyzed for every purified protein, allowing us to calculate specific activities in the ATPase assays based on the amount of 2C protein added.

RESULTS

Lipid dependence of nanodisc-based *in vitro* expression. Nanodiscs were critical for viral protein synthesis, and we could not detect poliovirus proteins from expression reactions done in the absence of nanodiscs (Fig. 3). We expressed both Ub2BC and Ub2C in the presence of nanodiscs made with DMPC, DMPS, or DMPG and found there were marked differences in the expression levels of the different proteins in the presence of the different lipids. Ub2BC expressed the best with DMPC and DMPS nanodiscs, and expression levels were reduced ~2-fold in the presence of DMPG nanodiscs (Fig. 3A). Alternatively, the best Ub2C expression was seen with DMPG discs and yield was reduced ca. 2- to

FIG 3 Phospholipid dependence of Ub2C and Ub2BC expression and activity. (A and B) Batch nickel purification elution fractions E1 and E2 from 100 μ l *in vitro* expression of Ub2BC (A) and Ub2C (B) in the presence of nanodiscs assembled with DMPG, DMPS, or DMPC lipids or in the absence of nanodiscs entirely. Lane L contains ProSieve Unstained Protein Marker II. (C) Relative expression levels and ATPase activities obtained with the different nanodiscs for both Ub2C and Ub2BC proteins. ATPase assays were carried out at 37°C in the presence of 1 mM ATP.

3-fold when in the presence of DMPS or DMPC (Fig. 3B). Activity assays showed that the headgroup type had up to ~3-fold effects on the ATPase k_{cat} values, and the lipid giving maximal activity varied depending on the polyprotein being assayed; Ub2C shows the highest activity with DMPC, Ub2BC prefers DMPG, and both proteins exhibit half of their respective maximal activities with DMPS nanodiscs (Fig. 3C). Ultimately, we decided to use DMPC nanodiscs for further experiments since Ub2C demonstrated the highest ATPase activity on these discs and Ub2BC activity was only reduced by ~25% on DMPC discs compared to its maximal activity observed on DMPG discs.

Expression of 2C constructs. In the presence of DMPC nanodiscs, the average final batch purification yield for the various protein constructs was ~11 μ g/ml of translation reaction, with the different constructs varying from 1 to 40 μ g/ml in their yields. The lowest yield was observed for 2C alone, and this protein had a strong tendency to aggregate into a pelleting fraction, but the yield

FIG 4 Purification of *in vitro*-translated 2C proteins. (A and B) Batch nickel resin elution fractions (E1-E4) from DMPC nanodisc chaperoned *in vitro* expression of wild-type 2C constructs (A) and N-terminal ubiquitin fusion constructs (B). The bands for the desired 2C protein are marked by single-sided arrows, and the nanodisc MSP protein is denoted by double-sided arrows. Constructs were expressed in 100- μ l reactions, purified in batch with Ni^{2+} affinity resin, and 5- μ l samples of each 25- μ l elution fraction were analyzed on 12% acrylamide-SDS gels. The ladder molecular weight shown beside the 2BC3AB is the same for all gels except for Ub2C3A. (C and D) FPLC purification of Ub2C via HisTrap nickel affinity (not shown) and HiTrap Q anion exchange columns. The primary 2C elution position is shown by the arrow on the HiTrap Q chromatogram (C), and SDS-PAGE analysis of fractions 17 to 23 revealed two major proteins eluting immediately after 2C (D). Mass spectrometry was used to identify these proteins as bacterial DnaK, a protein-folding chaperone, and glucosamine fructose-6-phosphate aminotransferase (GF6PA).

was improved by including polypeptide neighbor proteins, i.e., 2B and 3A (Fig. 4A). N-terminal Ub fusion proteins generally resulted in higher expression levels and better yield of soluble protein (Fig. 4B). Indeed, the soluble protein yield was typically lowest for 2C at ~ 1 μ g/ml of *in vitro* translation reaction, but this was increased ~ 5 -fold for Ub2C, and even further by the addition of viral polypeptide neighbors, resulting in up to ~ 40 μ g/ml of reaction mixture for Ub2BC3AB. Thus, we primarily used the Ub fusion constructs for our ATPase activity assays. We also made alanine point mutations to the AAA⁺ ATPase motifs predicted to exist within the 2C domain: Walker A (K135A), Walker B (D177A), and motif C (T222A, N223A). These mutants demonstrated a 2- to 30-fold increase in protein yield compared to the wild type, with the most significant increase seen for the Walker A mutant. Furthermore, Ub3AB expressed at higher levels than any of the 2C-containing constructs, resulting in up to 60 μ g of protein/ml of *in vitro* expression reaction.

To reduce the probability of having multiple viral proteins associated with a single disc, the *in vitro* translation reactions were carried out in the presence of a large excess nanodiscs (15 μ M), only 2 to 5% of which were recovered by copurification with the expressed hexahistidine-tagged 2C constructs. Quantitation of the purified protein bands from multiple expressions showed overall mole ratios of one to two 2C constructs per pair of MSPs

needed for each nanodisc. The protein-nanodisc complexes were soluble, and little sample loss was observed upon centrifugation at 4°C, except for the 2C protein that was prone to precipitation. This was exacerbated in the fast-performance liquid chromatography (FPLC)-purified samples that had a clear tendency to aggregate upon storage, but was improved somewhat by the addition of the N-terminal ubiquitin fusion. Note that we were able to stoichiometrically cleave the Ub domain from Ub2C using exogenously added Ubp2 protease (generously provided by Robert Cohen), consistent with the fusion protein being properly folded (data not shown). This is in contrast to our earlier attempts to express 2C as a GST fusion in insect cells, where the resulting protein was not very soluble and could not be cleaved from GST, suggesting these proteins may have been misfolded or aggregated.

Initially, all of the 2C constructs were only batch purified using nickel affinity resin; however, we consistently noticed a ~ 70 -kDa contaminant protein coeluting with the purified protein. This band was excised from an SDS-PAGE gel, analyzed by liquid chromatography-tandem mass spectrometry at the Proteomics and Metabolomics Facility at Colorado State University, and identified as the bacterial chaperone protein DnaK (Hsp70), a known protein folding chaperone and ATPase (44). Concerned that DnaK could be contributing to the observed ATPase activity in our assays, we subjected several constructs to a more rigorous

TABLE 1 Michaelis-Menten parameters of batch-purified proteins

	Mean $\pm$ SEM		
Construct	K_m (mM ATP)	k_{cat} (μ mol of ATP/ μ mol of protein/ min)	Fold activation ^a
Wild-type constructs			
Ub2BC3AB	0.55 $\pm$ 0.06	1,290 $\pm$ 30	1.00
Ub2C3A	1.8 $\pm$ 0.4	1,850 $\pm$ 90	1.4
2BC	0.78 $\pm$ 0.06	3,120 $\pm$ 60	2.4
Ub2BC	0.47 $\pm$ 0.08	3,200 $\pm$ 120	2.5
Ub2C	0.63 $\pm$ 0.07	10,200 $\pm$ 300	7.9
2C	0.9 $\pm$ 0.1	7,300 $\pm$ 200	5.7
Ub2BC AAA+ mutants			
Ub2BC-K135A	1.1 $\pm$ 0.1	1,550 $\pm$ 50	0.48
Ub2BC-D177A	0.7 $\pm$ 0.1	2,520 $\pm$ 80	0.79
Ub2BC-T222A	0.8 $\pm$ 0.2	2,300 $\pm$ 100	0.72
Ub2BC-N223A	0.90 $\pm$ 0.08	2,290 $\pm$ 50	0.72
Ub2C AAA+ mutants			
Ub2C-K135A	0.7 $\pm$ 0.1	290 $\pm$ 10	0.03
Ub2C-D177A	0.88 $\pm$ 0.07	8,200 $\pm$ 200	0.80
Ub2C-T222A	1.0 $\pm$ 0.2	6,700 $\pm$ 300	0.66
Ub2C-N223A	0.9 $\pm$ 0.1	4,400 $\pm$ 200	0.43

^a That is, the fold activation from Ub2BC3AB (wild-type constructs), Ub2BC (Ub2BC AAA+ mutants), and Ub2C (Ub2C AAA+ mutants), respectively, as indicated in column 1.

FPLC-based purification scheme that included both a nickel affinity column and an ion exchange column (Fig. 4C and D). Ub2C, Ub2BC, Ub2BC3AB, Ub3AB, and Ub2C-K135A were all purified in this manner and used for the final Michaelis-Menten kinetic analyses (see Table 2). Batch-purified proteins were used to determine ATPase assay conditions and for initial kinetic comparisons (Table 1).

ATPase assay. Enzymatic activity was determined using a coupled assay in which the rate of ATP hydrolysis is linked to a stoichiometric conversion of NADH to NAD⁺ (Fig. 5A). Figure 5B shows the time dependence of NADH depletion measured by absorbance at 340 nm in an assay containing Ub2BC with ATP concentrations ranging from 0 to 10 mM. The lowest ATP concentration tested in this experiment, 0.25 mM, results in a NADH oxidation rate that is about twice that of background, and the rates then increase with increasing ATP concentration. At 4 mM ATP and above the total amount of ATP hydrolyzed in 30 to 60 min often exceeded the 0.4 mM NADH added to the assay, and the absorbance data therefore reached a minimal signal plateau at $\sim$ 0.25 absorbance units. The raw data curves do not have the same Y-intercept because there is contaminating ADP in the ATP solution that is immediately converted back to ATP at the expense of NADH upon assay assembly. The concentration of this contaminating ADP increases along with that of the ATP, resulting in

FIG 5 ATPase activity of 2C proteins bound to nanodisc bilayers. (A) Reaction scheme of ATPase assay in which ATP hydrolysis is coupled to the oxidation of NADH via pyruvate kinase (PK) and lactate dehydrogenase (LDH). Loss of absorbance at 340 nm reflects the loss of NADH due to ATP hydrolysis. (B) Raw absorbance data for a set of Ub2BC assays with various ATP concentrations that were performed simultaneously using a Cary 50 Bio spectrophotometer with a thermostated (37°C) multicell holder. (C) Monovalent salt concentration dependence of Ub2C ATPase activity in the presence of NaCl or KCl. Reactions were carried out with 3 mM ATP and 1 nM batch-purified Ub2C protein and normalized to the activity in the absence of added salt. (D) pH dependence of Ub2C ATPase activity examined with 2 mM ATP and 5 nM batch-purified Ub2C, normalized to activity at pH 7. (E) Temperature dependence of Ub2C and Ub2BC ATPase activity carried out with 2 mM ATP and 5 nM batch-purified protein. (F) Magnesium and acetate dependence of Ub2C ATPase activity. The magnesium acetate data are a combination of three sets of assays that were carried out with 1 mM ATP and 2 to 4 nM batch-purified Ub2C. MgCl₂ and potassium acetate assays started with 75- μ l reactions containing 2 mM ATP and 3 mM MgCl₂ into which either MgCl₂ or potassium acetate were titrated with the total added volume added not exceeding 5 μ l.

lower starting absorbance values at higher ATP concentrations, as shown in Fig. 5B.

Salt, pH, and temperature dependence. To determine the optimal conditions for 2C ATPase activity assays, we examined the rate of ATP hydrolysis by batch-purified Ub2C under different monovalent salt (KCl and NaCl), magnesium, acetate, pH, and temperature conditions (Fig. 5C to F). To verify that the temperature and pH conditions being tested were not affecting the enzymatic activity of the PK and LDH components of the coupled assay, ADP was added to a final concentration of 0.2 mM, corresponding to half of the starting NADH concentration. In every case, the loss of NADH absorbance at 340 nm in these control reactions was immediate, indicating that turnover within the reporter system was not rate limiting.

The activity of Ub2C bound to nanodiscs showed a gradual but weak monovalent salt dependence with a fairly flat plateau between 25 and 100 mM KCl and a drop in activity at salt concentrations higher than that (Fig. 5C). The effect was small in the presence of KCl, with activity only reduced by ~10% in the presence of 200 mM salt, whereas NaCl exhibited a greater inhibitory effect with activity decreasing linearly with salt concentration to reach ~60% activity in 200 mM NaCl. We opted to use 75 mM KCl as a standard condition for further ATPase assays since it demonstrated only an ~5% decrease in activity and is fairly close to physiological salt concentrations. We examined the pH dependence of batch-purified Ub2C activity at 11 different values and found a fairly flat activity profile from pH 6 to 7, followed by a decrease in activity as the pH was increased to 9 (Fig. 5D).

The activity of membrane proteins is often affected by the fluidity of the bilayer and can therefore be influenced by lipid-phase transitions, and conversely the presence of proteins can affect phase transition temperatures. The normal phase transition temperature of DMPC is ~23°C, but it is increased by 3 to 4°C in the context of a nanodisc (45). To examine whether a lipid-phase transition near 27°C affected 2C function, we measured ATPase activity at a range of temperatures between 15 and 40°C. This was done using both Ub2C that is anchored to the membrane via an amphipathic surface helix and Ub2BC that is a more integral membrane protein due to the addition of a pair of predicted integral membrane segments. The data show an essentially linear temperature-dependent increase in activity for both proteins with a slight inflection point in the vicinity of 30°C that may correspond to a weak effect from the DMPC-phase transition of a nanodisc bilayer with a bound 2BC or 2C protein (Fig. 5E). The ~3-fold increase in ATPase activity between 15 and 40°C is in contrast to prior studies of GST-2C that have shown a drastic loss of ~25% activity between 37 and ~40°C (28). To ensure that the rates were not reflecting assay reagent instability (e.g., PEP, NADH, and ATP), the temperature dependence experiments were repeated without the addition of Ub2C, and these background rates were subtracted from the observed Ub2C rates to yield the data shown in Fig. 5E (correction was 5 to 10% of observed signal).

Magnesium and acetate dependence. The presence of magnesium acetate had a substantial stimulatory effect on the batch-purified Ub2C ATPase activity, and this effect was further attributed to the addition of acetate. We initially carried out experiments using magnesium acetate in a fairly standard manner of maintaining the Mg^{2+} concentration 1 mM higher than the ATP concentration but discovered that we could not obtain a reliable V_{max} for ATP hydrolysis without going to unusually high

ATP concentrations in the millimolar range. We then independently examined the magnesium and the acetate dependence of ATP turnover by titrating magnesium acetate, magnesium chloride, or potassium acetate in the reaction. The results show a clear stimulation by both magnesium and potassium acetate with activation midpoints of 10 to 15 mM and saturation at ~50 mM concentrations; in contrast, magnesium delivered as $MgCl_2$ strongly inhibited activity (Fig. 5F). We also verified that the magnesium acetate activation was not due to low-level contamination by another divalent metal, such as Zn^{2+} , that could perhaps be binding to the Cys-rich zinc finger-like motif in 2C. To test for this possibility, we added a low concentration of EDTA (1 mM) that would preferentially chelate Zn^{2+} or other divalent ions even in the presence of 50 mM Mg^{2+} (46, 47) and observed that this did not significantly change the ATPase rates (data not shown). High concentrations of magnesium chloride, magnesium acetate, or potassium acetate did not affect the response from the coupled assay reporter system.

Michaelis-Menten kinetics of 2C constructs. To examine the extent to which proteolytic processing modulates the ATPase activity of the core AAA+ domain within 2C, we determined the Michaelis-Menten enzyme kinetic parameters of ubiquitin fusion proteins for 2BC, 2C, 2C3A, 2BC3AB, and 3AB and the nonubiquitin versions of 2BC and 2C (Fig. 6A and B). We also made a series of mutations to the putative AAA+ ATPase and analyzed their effects on ATPase activity in the context of both Ub2C and Ub2BC (Fig. 6C and D). Based on the characterization experiments described above, these assays were carried out using the coupled ATP/NADH assay containing 0.5 to 6 nM 2C protein, 0.1 to 20 mM ATP, 50 mM magnesium acetate, and 75 mM KCl at pH 7.0 and 37°C. The observed ATP hydrolysis rates were converted to specific activities (μmol of ATP/ μmol of protein/min, hereafter referred to as “ min^{-1} ”) by normalizing to the protein concentrations determined by quantitative measurements of band intensities on SDS-PAGE gels (Fig. 2).

Initial ATPase assays were carried out using batch-purified protein; however, these samples contained the contaminant protein ATPase DnaK, a bacterial chaperone. DnaK has a reported K_m of ~1 μM and a k_{cat} ranging from 0.03 to 3 min^{-1} depending on the type of peptide substrate and whether cofactor proteins are present (44). Since our ATPase assays were carried out at 0.1 to 15 mM ATP concentrations that are well above the K_m for DnaK, we chose to semiquantitatively background correct the data by using a floating offset for the Michaelis-Menten curve fits, thus making the assumption that this offset represents a V_{max} rate for any contaminant DnaK in the reaction. This background correction represented only ~3% of the maximal 2C activity for the batch-purified proteins, and the resulting data and kinetic parameters are shown in Fig. 6A, C, and D and Table 1. When the same curve fit was used to determine the Michaelis-Menten parameters for the FPLC-purified proteins the fitted offset values were within error of zero, indicating that these protein preparations did not contain contaminant ATPase activity. The data obtained using the FPLC-purified proteins are shown in Fig. 6B, and the resulting k_{cat} and K_m values for Ub2C, Ub2BC, Ub2BC3AB, Ub3AB, and Ub2C-K135A are reported in Table 2.

Proteins obtained by both purification methods demonstrate very similar activity patterns among the various 2C constructs and mutants, and these data show that proteolytic processing on both sides of the core 2C domain modulate its ATPase activity. For the

FIG 6 Michaelis-Menten analysis of nanodisc-bound 2C ATPase activity. All data were obtained under standardized conditions of 37°C with 20 mM HEPES (pH 7.0), 75 mM KCl, 50 mM magnesium acetate, and 5 mM DTT. ATP hydrolysis rates were converted to specific activities using gel-based quantitation of protein concentrations and plotted with a uniform y axis scale for ease of comparison. (A) Data from batch-purified proteins with the wild-type polyprotein sequences. (B) Data from FPLC-purified ubiquitin fusion constructs of 2C, 2BC, 2BC3AB, and 2C-K135A, with an expanded-view inset showing details of the data for 2BC, 2BC3AB, and 3AB proteins. (C and D) Data from batch-purified Ub2C (C) and Ub2BC (D) proteins with mutations in AAA+ ATPase Walker A (K135A), Walker B (D177A), and motif C (T222A, N223A) sequences. The K_m and k_{cat} values resulting from the curve fits shown here for batch- and FPLC-purified proteins are listed in [Tables 1](#) and [2](#), respectively.

more reliable FPLC-purified proteins ([Fig. 6B](#), [Table 2](#)), we found that Ub2BC3AB has the lowest turnover rate at $\sim 160 \text{ min}^{-1}$, this increased modestly to 440 min^{-1} upon removal of 3AB to yield Ub2BC and then increased greatly upon removal of the 2B domain to give the highest k_{cat} of $\sim 9,000 \text{ min}^{-1}$ for Ub2C. This 2C rate corresponds to a ~ 20 -fold activation compared to Ub2BC and ~ 60 -fold increase compared to Ub2BC3AB. Only background activity of 5 min^{-1} is observed for Ub3AB, which lacks the 2C domain. The ATPase activity modulation is primarily due to changes in k_{cat} since the K_m values of the purified proteins are fairly consistent at 0.6 to 1.8 mM ATP for all of the constructs.

TABLE 2 Michaelis-Menten parameters of FPLC-purified proteins

Construct	Mean $\pm$ SEM		Fold activation ^a
	K_m (mM ATP)	k_{cat} (μmol of ATP/ μmol of protein/ min)	
Ub2BC3AB	0.83 ± 0.05	158 ± 5	1.0
Ub2BC	1.8 ± 0.4	440 ± 20	2.8
Ub2C	0.63 ± 0.02	$9,000 \pm 900$	57
Ub2C-K135A	0.51 ± 0.04	920 ± 30	6
Ub3AB	1.1 ± 0.3	5.0 ± 0.3	0.03

^a That is, the fold activation from Ub2BC3AB.

Note that the low yield of soluble Ub2C from the FPLC-based protocol made protein quantitation less reliable, and this in turn increases the error of the specific activity measurements for the Ub2C protein to $\sim 10\%$.

Among the mutations of the predicted canonical AAA+ ATPase motifs, the Walker A mutation (K135A) has the most substantial effect on 2C ATPase activity with k_{cat} being reduced by 90 to 95%. Interestingly, the K135A only reduces activity by $\sim 50\%$ in the context of 2BC, suggesting that the active-site geometries of 2C and 2BC may be slightly different. The remaining mutations in the Walker B (D177A) and motif C (T222A and N223A) regions were not as deleterious, and their k_{cat} values are reduced to 40 to 80% of those observed for the wild-type proteins in the batch-purified samples.

DISCUSSION

Among the membrane-anchored poliovirus proteins, both 2C and its precursor 2BC are involved in forming the host membrane structures that serve as the site of viral replication ([9–12](#)); however, the membrane-binding, oligomerization, and aggregation propensities of the picornaviral 2C proteins generally make them very difficult to express and purify for biochemical and structural studies. In the present study, we have shown that nanodisc bilayers can be used to chaperone the expression of soluble poliovirus 2C

by providing membrane surfaces that allow for proper folding and help reduce aggregation by sequestering the proteins on separate bilayers, allowing us to carry out basic biochemical characterization of the enzyme. Processing of the viral polyprotein can be important for regulating protein function, as has been shown for 3D^{pol}, where processing from the 3CD^{pro} protease precursor protein activates the polymerase and even minor changes to its N-terminal residues can have significant deleterious effects on activity (4, 5, 48). To determine whether proteolytic processing plays a similar role in mediating 2C behavior, we also examined the ATPase activity of 2C when expressed as a known functional precursor protein (2BC), with a polyprotein neighbor (2C3A), or in the context of the entire surrounding polyprotein (2BC3AB).

Nanodisc-bound 2C is a highly active ATPase. The *in vitro* expression results clearly show that nanodiscs effectively chaperone viral protein expression, resulting in highly active 2C that could be purified as a 2C-nanodisc complex whose maximal turnover rate is ~200-fold higher than that previously reported, while retaining an ATP K_m of ~0.6 mM that is quite similar to the 0.5 to 0.7 mM values reported for both poliovirus GST-2C and FMDV 2C proteins (28, 31). Previous studies of poliovirus 2C expressed as a GST fusion (28) and FMDV 2C expressed with an N-terminal truncation (31) have reported 50% inhibition of ATPase activity in the presence of 20 and 50 mM NaCl, respectively. In contrast, we found a weaker salt dependence that resulted in only a ~10% reduction of ATPase activity in the presence of 200 mM KCl. Sodium had a stronger inhibitory effect, but even at 200 mM NaCl the nanodisc-anchored proteins retained ~60% activity (Fig. 5C). When testing the magnesium dependence, we found that Mg²⁺ in the form of magnesium acetate stimulated ATPase activity, while Mg²⁺ in the form of MgCl₂ inhibited 2C activity (Fig. 5F), the latter of which is consistent with prior findings of GST-2C fusion construct activity (28). Further analysis showed that the activation was due to acetate stimulation of 2C, where saturable maximal 2C activity was observed at ~50 mM concentrations of either potassium acetate or magnesium acetate (Fig. 5F). The origin of this stimulatory effect is not yet known, but ligand-induced activation is common among AAA+ ATPases where the presence of substrates is often necessary to promote oligomerization and activity (33), suggesting that high concentrations acetate may mimic a natural ligand.

We also found that phospholipid composition can modulate expression levels and ATPase activities of both 2C and 2BC, although the 2- to 3-fold effects on each are fairly minor (Fig. 3). Expression of the peripherally membrane-anchored 2C was highest with DMPG lipids, while 2BC preferred DMPS or DMPC. Although subtle, the combined ~10-fold effects of phospholipid composition on expression levels and ATPase activity suggest that lipid headgroups can play a role in modulating host membrane interactions with 2C and 2BC. This could perhaps influence the membrane rearrangements that take place during poliovirus infection, and the shift toward DMPS for higher 2BC expression is consistent with prior studies showing that 2B preferably associates with anionic lipids such as phosphatidylserine and phosphatidylinositol that are enriched in the Golgi and ER membranes targeted by the replication proteins (14).

Electron microscopy data of both poliovirus and FMDV 2C proteins showing hexameric ring structures characteristic of this class of enzymes (30, 31) and structural modeling using the Phyre protein fold recognition server (32) indicates with high confi-

dence that 2C has homology to the superfamily III AAA+ ATPases. We mutated canonical residues within the characteristic Walker A, Walker B, and motif C sequences (34) and found that they all reduced activity (Fig. 6, Tables 1 and 2). In particular, K135 in the Walker A motif plays an important role in positioning the ATP γ phosphate at an intersubunit contact, linking oligomerization and ATP hydrolysis (49). Mutating K135 to an alanine not only abolished activity but also significantly increased Ub2C solubility and resulted in ~30-fold increased protein yield from the FPLC purification protocol, all of which are consistent with AAA+ domain oligomerization. Interestingly, the same mutation was not as deleterious in the context of 2BC, where it resulted in only a ~50% activity loss, suggesting that the added 2B domain may not only prevent oligomerization but also cause the ATPase domain to utilize a slightly different active-site geometry that accounts for its lower turnover rate. Furthermore, the Walker B and motif C/Sensor-1 mutations generally affect ATP hydrolysis rates significantly more than ATP binding (34, 49), which is consistent with the relatively small changes we observe in the ATP K_m values for the various mutants.

Based on our findings, we believe that the nanodisc bound 2C protein is indeed forming multimeric structures that are necessary for AAA+ ATPase activity, although we cannot say whether these are discrete hexamers or larger oligomers based on our data. Nanodiscs are quite effective at preventing oligomerization of integral membrane proteins by sequestering the transmembrane segments into individual discs, but it is important to note that 2C is a peripheral membrane protein and that its oligomerization is based on protein-protein interactions outside the lipid bilayer. As a result, the discs may chaperone proper protein folding but do not necessarily prevent oligomerization. Our expression and purification data indicate that the isolated 2C protein is significantly more prone to aggregation or oligomerization than the larger 2BC and 2BC3AB constructs. We postulate that the propensity of 2C to oligomerize will bring multiple nanodiscs together in close proximity, creating a high local concentration that facilitates fusion of the disc membranes into larger bilayer structures and reducing solubility, in effect defeating the advantage of using the nanodiscs. Consistent with this, the K135A mutation that is predicted to disrupt oligomerization results in more soluble material and a significant increase in protein yield. It is also possible that 2C itself may be acting to change the structure of the nanodiscs since it is known to be involved in membrane rearrangement (9–11).

Proteolytic control of 2C ATPase activity. Comparative analysis of enzyme rates by the different protein constructs shows that poliovirus 2C ATPase activity is strongly modulated by the presence of its polyprotein neighbors. Starting with the 2BC3AB protein, ATPase activity is increased ~3-fold by removal of 3AB to generate 2BC and then another ~20-fold upon removal of 2B to generate the fully processed 2C protein. This reflects an overall ~60-fold increase in activity for the fully processed 2C compared to its fully embedded precursor form and indicates that polyprotein neighbors exert allosteric effects on the core 2C domain that can modulate the rate of ATP hydrolysis. It is also notable that both the 2C and Ub2C proteins have higher activities than 2BC, showing that the allosteric effects are specific to having a 2B domain. This is in contrast to the 3D^{pol} proteolytic activation event where any N-terminal modification, even adding a single amino acid residue, will inhibit activity.

Overall, the results from the present study lead to a model in

which precursor forms of 2C, such as 2BC and 2BC3AB, that contain all of the membrane-binding elements of the viral polyprotein, assemble into the viral replication complex in a precursor form. During proteolytic processing, the viral proteases carry out multiple cleavages of the polyprotein, the first of which is 2A^{pro}-mediated cleavage of the P1 region from the polyprotein and this is followed by the 3C^{pro}/3CD^{pro} mediated cleavages of the P2 and P3 regions (50). The exact order in which the P2 and P3 proteins are cleaved is not well established, but the intact P2-P3 protein is rarely observed during infection, and it is thought that the P2-P3 junction (i.e., 2C-3A linkage) is cleaved early during processing. This would make 2BC the predominant precursor form, wherein 2C ATPase activity is kept at a low basal level because the presence of the 2B domain sterically prevents 2C from forming the oligomeric structures required for maximal ATP hydrolysis. This preassembly of 2BC proteins on the membrane surface is then followed by processing of the 2B-2C junction, triggering 2C to assemble into larger oligomeric structures that increase ATPase activity ~20-fold. The true purpose of this ATPase activity during viral replication is not yet known and not addressed by our data, but it may play a role in RNA helicase or translocation activity, capsid assembly, RNA packaging, or perhaps a combination of these functions.

In conclusion, we have shown that the membrane-associated poliovirus proteins can be synthesized in an active and soluble form when anchored to small nanodisc membrane bilayers. The core domain within these proteins is the 2C ATPase, which is shown to exhibit high ATPase activity over a broad range of monovalent salt concentrations, pH values, and temperatures. The activity of this core domain can be modulated by the addition of either N- or C-terminal polyprotein neighbors and the different polyprotein constructs exhibit slight activity preferences for different lipid headgroups. Although issues with oligomerization and solubility continue to present a clear obstacle to structural studies of poliovirus 2C that would require large amounts of purified protein, our nanodisc-based expression data do set the stage for experiments to further understand the interactions of the picornaviral 2C and 2BC proteins with RNA and to study their effects on membrane dynamics by biophysical methods.

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