Unusual architecture of the p7 channel from hepatitis C virus

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The hepatitis C virus (HCV) has developed a small membrane protein, p7, which remarkably can self-assemble into a large channel complex that selectively conducts cations1–2. We wanted to examine the structural solution that the viroporin adopts in order to achieve selective cation conduction, because p7 has no homology with any of the known prokaryotic or eukaryotic channel proteins. The activity of p7 can be inhibited by amantadine and rimantadine3,4, which are potent blockers of the influenza M2 channel6 and licensed drugs against influenza infections6. The adamantane derivatives have been used in HCV clinical trials5, but large variation in drug efficacy among the various HCV genotypes has been difficult to explain without detailed molecular structures. Here we determine the structures of this HCV viroporin as well as its drug-binding site using the latest nuclear magnetic resonance (NMR) technologies. The structure exhibits an unusual mode of hexameric assembly, where the individual p7 monomers, i, not only interact with their immediate neighbours, but also reach farther to associate with the i+2 and i+3 monomers, forming a sophisticated, funnel-like architecture. The structure also points to a mechanism of cation selection: an asparagine/histidine ring that constricts the narrow end of the funnel serves as a broad cation selectivity filter, whereas an arginine/lysine ring that defines the wide end of the funnel may selectively allow cation diffusion into the channel. Our functional investigation using whole-cell channel recording shows that these residues are critical for channel activity. NMR measurements of the channel–drug complex revealed six equivalent hydrophobic pockets between the peripheral and pore-forming helices to which amantadine or rimantadine binds, and compound binding specifically to this position may allosterically inhibit cation conduction by preventing the channel from opening. Our data provide a molecular explanation for p7-mediated cation conductance and its inhibition by adamantane derivatives.

Many viruses have developed integral membrane proteins to transport ions and other molecules across the membrane barrier to aid various steps of viral entry and maturation5–10. These membrane structures, known as viroporins, usually adopt minimalist architectures that are significantly different from those of bacterial or eukaryotic ion channels. Therefore, understanding the structural basis of how viroporins function broadens our knowledge of channels and transporters while generating new opportunities for therapeutic intervention.

The viroporin formed by the HCV p7 protein has been sought after as a potential anti-HCV drug target11–13. p7 is a 63-residue membrane protein that oligomerizes to form ion channels with cation selectivity, for Ca2+ over K+ and Na+ (refs 2, 3, 12, 13), and a more recent study has also reported p7-mediated H+ intracellular conductance14. The p7 channel is required for viral replication15; it has been shown to efficiently assemble and release of infectious virions16,17, although the precise mechanism of these functions remains unclear. The channel activity can be inhibited by adamantane and long alkyl chain iminosugar derivatives and hexamethylene amiloride in vitro, with varying reported efficacies12,13,15. In addition to ion conduction, p7 has been shown to specifically interact with the non-structural HCV protein NS2, indicating that its channel activity could be regulated14,15.

There is not yet a detailed structure of the p7 channel, although a number of NMR studies showed that the p7 monomer has three helical segments: two in the amino-terminal half of the sequence and one near the carboxy terminus12,13. A single-particle electron microscopy (EM) study obtained a 16 Å resolution electron density map of the p7 oligomer using the random conical tilting approach12. The map shows that the p7 channel is a 42-kDa hexamer and adopts a flower-like shape that does not resemble any of the known ion channel structures in the database.

It is not known how the small p7 polypeptide assembles into what appears to be a complex channel structure, and whether the viroporin has adopted novel structural elements for cation selectivity and channel gating. Amantadine or rimantadine blocks the influenza M2 channel by binding to the small pore formed by four transmembrane helices21–23, but the pore of the p7 hexamer is expected to be much bigger and it is thus unclear how these small molecules would fit. We sought to address these questions by determining detailed structures of the p7 hexamer and its drug-binding site.

We systematically tested p7 amino acid sequences from various HCV genotypes and found that the sequence from genotype 5a (EUH1480 strain) generated samples that were sufficiently soluble for structure determination (Supplementary Fig. 1). This p7 construct, designated here as p7(5a), could be efficiently reconstituted in dodecylphosphocholine (DPC) micelles at near physiological pH and generated high-quality NMR spectra (Supplementary Fig. 2). Negative-stain EM of the DPC-reconstituted p7(5a) in NMR buffer showed hexameric, flower-shaped particles that are similar to those in the electron micrographs of the p7 (JFH-1 strain, genotype 2a) hexamer in dihexanoyl-phosphatidyl-choline (DHPC) micelles used earlier for single-particle reconstruction18 (Supplementary Fig. 3). Moreover, iso-thermal titration calorimetry and NMR chemical shift perturbation analyses of p7(5a)–rimantadine interaction showed that the drug binds specifically to the reconstituted protein with a binding constant (Kd) from 50 to 100 μM at 3 mM detergent concentration (Supplementary Figs 4 and 5). The above results together indicate that the p7(5a) polypeptides reconstituted in DPC micelles form structurally relevant hexamers.

Structure determination of the p7(5a) hexamer by NMR used an approach taken earlier for oligomeric membrane proteins24–26, which involves: (1) determination of local structures of the monomers; and (2) assembly of the oligomer with intermonomer distance restraints and orientation constraints. The NMR-derived restraints define an ensemble of structures with backbone root mean squared deviation (r.m.s.d.) of 0.74 Å (Fig. 1a). Each monomer consists of an N-terminal helix (H1) from residues 5–16, a middle helical segment (H2), with a

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polar residues with salient structural features (Fig. 2b). One is Asn 9, which forms a ring of carboxamide that constricts the conical region of the channel (Fig. 2c). Residue 9 is asparagine in all strains except in genotype 2 viruses, where it is substituted with histidine. Both asparagines and histidines have affinity for monovalent and divalent cations. We propose that the Asn 9 ring serves as a broad selectivity filter that dehydrates cations, allowing them to pass the hydrophobic ring formed by Ile 6. The Ile 6 ring defines the narrowest point of the channel and probably serves as a hydrophobic gate. Another feature is the Arg 35 ring that defines the wider, C-terminal end of the channel and probably serves as a hydrophobic filter that dehydrates cations, allowing them to pass the hydrophobic ring formed by Ile 6. The Ile 6 ring defines the narrowest point of the channel and probably serves as a hydrophobic gate. Another feature is the Arg 35 ring that defines the wider, C-terminal end of the channel (Fig. 2b). Placement of a positively charged ring on the other end of the pore was incomprehensible to us initially because it can repel cations. But the recent structure of an Orai Ca$^{2+}$ channel also revealed a stretch of basic residues in the ion-conducting pore$^{22}$. We propose that one role of Arg 35 is to bind and obstruct anions at the pore entrance while allowing cations to diffuse into the pore. In this model, cation conduction is unidirectional from the C- to N-terminal end of the channel.

To test the above hypotheses, we established an assay that uses the two-electrode voltage-clamp technique to record p7-mediated current in Xenopus oocytes (see Methods). Owing to the poor stability of oocytes that overexpress p7(5a), p7 (JFH-1 strain, genotype 2a) was used instead for these experiments. As expected because of the proposed role of residue 9 in selectively dehydrating cations, replacing His 9 of p7(2a) with alanine caused a ~70% reduction in channel conductance at +80 mV (Fig. 2d). The proposed role of Arg 35 indicates that placing negatively charged residues at the channel entrance would bind cations and hinder their diffusion into the pore, and indeed the Arg35Asp mutation also reduced conductance by ~70% (Fig. 2d).

We next investigated the mechanism of amantadine binding to the p7 channel using proteins that are $^{15}$N-labelled and deuterated so that nuclear Overhauser enhancement (NOE) between the protein backbone
amid protons and drug protons could be measured unambiguously. At 10 mM amantadine (not corrected for drug partitioning to detergent micelles), the $^{15}$N-edited nuclear Overhauser enhancement spectroscopy (NOESY) spectrum showed NOE crosstalks between the adamantane protons and the amide protons of Val 26, Leu 55, Leu 56 and Arg 57 (Fig. 3a). We then identified contacts between the drug and protein side chains using protein that is $^{1H}/^{13}C$-labelled at the methyl positions of alamines, valines and leucines but is otherwise deuterated. In this case, the $^{13}C$-edited NOESY showed several methyl-drug NOEs (Fig. 3b).

These NOEs were used to dock amantadine into the structure determined in the absence of drug. In doing so, we emphasize that the relevance of the p7–amantadine complex is confined to only the drug-binding region because we do not know how and to what degree does drug binding alter the global conformation of the channel. The relatively poor stability of the protein–drug complex at the current stage of our study precludes full-scale structure determination. Nonetheless the available NMR data show that the drug adamantane binds to six equivalent hydrophobic pockets between the pore-forming and peripheral helices (Fig. 3c). The pocket consists of Leu 52, Val 53 and Leu 56 from H3, and Phe 20, Val 25 and Val 26 from H2. The amantadine amino group on average points to the channel lumen. The same NOESY spectrum as above recorded using a sample with 5 mM rimantadine indicates that rimantadine binds to the same pocket with the methyl and amino groups pointing to the lumen (Supplementary Fig. 6).

The binding site is overall consistent with a mutational study showing that mutations in residues 50–55 significantly reduce drug sensitivity of the channel. It is also consistent with a Leu20Phe mutation in genotype 1b virus originally identified in clinical trials that confers amantadine resistance. In the p7(5a) structure, residue 20 is an integral part of the drug pocket and is in direct contact with the drug adamantane. Therefore, replacing Leu 20 in p7(1b) with phenylalanine is expected to reduce hydrophobic interaction with the drug. Elucidation of previous functional data in the context of the structure suggests that the binding site shown in Fig. 3c is relevant to drug inhibition and that interactions between the drug adamantane and protein hydrophobic residues are critical for inhibition. Variations in the hydrophobicity of the binding pocket among the p7 variants (Supplementary Fig. 7) thus explain the large differences in drug efficacies observed between different HCV genotypes.

We have learnt from KcsA and other channels that a gated ion channel generally adopts two essential features: pore elements that provide ion selectivity, and a gating mechanism that can transiently

Figure 2 | The pore properties of the p7(5a) channel. a, The pore surface calculated using the program HOLE, showing the shape and constrictions of the pore. b, Sectional view of the channel showing the pore-lining residues with residues in red being strongly conserved. The numbers next to the helical segments represent the monomers to which the helices belong. Colour scheme as in Fig. 1. c, A close-up view of the rings formed by Asn 9 and Ile 6 that constrict the N-terminal end of the channel. d, The current–voltage relationships of wild-type (WT) p7(2a) and the His9Ala and Arg35Asp mutants. Each data point is the mean ± s.e.m. (standard error of mean) calculated over measurements from six different oocytes (n = 6).

Figure 3 | NMR characterization of the amantadine binding site. a, Representative strips from the three-dimensional $^{15}$N-edited NOESY- TROSY (transverse relaxation optimized spectroscopy) spectrum (300 ms NOE mixing time) recorded using a sample containing $^{15}$N-, $^{2}$H-labelled p7(5a) and 10 mM amantadine, showing amantadine NOEs to the backbone amide protons of Val 26, Leu 55, Leu 56 and Arg 57. b, Representative strips from the three- dimensional diagonal-suppressed $^{13}$C-edited NOESY-HSQC spectrum recorded using a sample that is $^{1H}$-, $^{13}$C-labelled at the methyl positions of alamines, valines and leucines but is otherwise deuterated, showing drug NOEs to the side-chain methyl protons of Val 26, Leu 52 and Val 53. The spectra in a and b were recorded at $^{1}H$ frequency of 900 MHz. c, Amantadine docked into the p7(5a) hexamer using restraints from NOEs in a and b (left) and a close-up view of amantadine in the binding pocket (right). Colour scheme as in Fig. 1.
open the channel to allow ion permeation. By virtue of being a funnel, the p7 structure indicates that the tip of the funnel represented by the Ile 6 and Asn 9 rings is the key region for channel gating (Fig. 4). The role of the Asn 9 ring is to provide ion selectivity by recruiting and dehydrating cations near the funnel exit, whereas the Ile 6 ring is a hydrophobic constriction that would prevent water from freely passing through. Channel activation may involve reorientation of the H1 helices that widens the funnel tip, analogous to the dynamic C-terminal helix of KcsA30, and such structural rearrangement can be afforded by the flexible hinge between H1 and H2, the intervening loop between H2 and H3, and the C-terminal tail that ‘latches’ onto H1. We thus propose that binding of adamantane derivatives inhibit channel activity by restricting the structural rearrangement. Our NMR titration data (Supplementary Fig. 5c) are consistent with this proposal, which shows that in the absence of rimantadine, the Ile 6 methyl resonance is split into an intense and weak peak, possibly corresponding to the open and closed state, respectively, and that increasing the drug concentration shifts the equilibrium that make the weak peak stronger. Although rigorous testing of the model is needed, the preliminary observation suggests the existence of multiple states of the p7 channel.

METHODS SUMMARY

The p7 sequence from HCV genotype 5a was mutated at five unconserved positions to improve protein stability and to facilitate NMR analyses (see details in Supplementary Fig. 1). The protein was expressed as a fusion to His9–trpLE that formed inclusion bodies, and purified and reconstituted in DPC as previously described12,15. A typical NMR sample contains 0.8 mM protein (monomer concentration), 200 mM DPC and 25 mM MES (pH 6.5).

For structure determination, the secondary structures of the p7(5a) monomers in the hexamer were first determined using standard NOE experiments. We then used a mixed sample in which 50% of the monomers are (15N/2H)-labelled and 50% 13C-labelled to measure exclusively NOEs between the 1H-attached protons of one subunit and the 13C-attached protons of the neighbouring subunits. This experiment provided key intermonomer NOEs between adjacent H1 and H2 helices for assembling the central cavity of the hexamer (Supplementary Fig. 8a).

H3 was positioned based on orientation restraints from residual dipolar couplings (RDCs) and its intermonomer NOEs to H1 and H2 helices for assembling the central cavity of the hexamer (Supplementary Fig. 8a). Structure calculation statistics are given in Supplementary Table 1. Protein–drug contacts were identified using (15N/2H)-labelled protein so that NOEs between protein backbone amide protons and drug protons could be identified unambiguously. Additionally, NOEs between the drug and protein side-chain methyl groups were measured with a diagonal-suppressed carbon NOESY using ALV labelled protein (see Methods).

Functional p7 mutants were analysed using the standard protocol for two-electrode voltage-clamp of Xenopus oocytes. After expressing the p7(2a) variants in oocytes, we recorded p7 currents across the oocyte plasma membrane at room temperature (~22 °C). For quantitative comparison of currents between the p7 variants, the protein expression levels in oocytes were examined using confocal microscopy.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions B.O. and J.J.C. conceived the study; B.O. prepared samples; M.J.B. performed EM analysis; J.D. and B.O. performed NMR titration; B.O. and J.J.C. collected and analysed NMR data and determined the structure; S.X., X.Z., W.Y. and B.S. designed and performed functional experiments; J.J.C. wrote the paper and all authors contributed to the editing of the manuscript.

Author Information The structure is deposited in the Protein Data Bank under the accession number 2M6X. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.J.C. (chou@cmcd.hms.harvard.edu) or B.S. (bsun@sibs.ac.cn).
Sample preparation. The amino acid sequence of p7 from genotype 5a was slightly modified to allow for efficient reconstitution and protein sample stability. In this sequence, Thr 1 is replaced with Gly, Ala 12 is replaced with Ser, and the three cysteines at positions 2, 27 and 44 are replaced with Ala, Thr and Ser, respectively (Supplementary Fig. 1). The p7(5a) construct was cloned, expressed and purified as previously described35-37. Briefly, the protein was expressed as a fusion to His6-trpLE that formed inclusion bodies. The peptide was released from the fusion protein by CNBr digestion and subsequently separated on a Protein-18C column by reverse-phase chromatography (more details given in Supplementary Methods). The lyophilized peptide was then dissolved in 6 M guanidine and DPC and refolded by dialysis against the NMR buffer. A typical NMR sample contains 0.8 mM protein (monomer concentration), 200 mM DPC and 25 mM MES (pH 6.5).

Assignment of NMR resonances. All NMR experiments were conducted at 30 °C on Bruker spectrometers equipped with cryogenic probes. Sequence-specific assignment of backbone chemical shifts was accomplished using three pairs of triple resonance experiments, recorded using a 15N/1H-13C/1H-labelled sample. The triple resonance experiments were relaxation optimized (TROSY)38, including HNCA, HN(CO),HCACB, HN(CO)CAB, HN(CA)CO and HCNO39. Protein side-chain aliphatic and aromatic resonances were assigned using a combination of NOE restraints including 15N-edited NOESY-TROSY (60 ms NOE mixing time, tNOE) and 13C-edited NOESY-HSQCs (tNOE = 100 ms). Specific stereo assignment of the methyl groups of valines and leucines were obtained from a constant-time 1H-13C-HSQC spectrum recorded using a 15%13C-labelled sample33.

Assignment of local NOEs for determining the secondary structures. The same 15N-edited NOESY-TROSY and 13C-edited NOESY-HSQC above with short tNOE were used to assign local NOEs. Combining the NOE restraints with chemical shifts, we could very precisely define the helical and loop regions of the individual monomers.

Measurement of residual dipolar coupling (RDC) constants. The backbone and side-chain methyl protons was assigned using a sample that was reconstituted with a 1:1 mixture of 15N-, 1H-labelled p7(5a) peptide and 13C-labelled peptide. Recoding a 15N-edited NOESY-TROSY (tNOE = 300 ms) on a 900 MHz spectrometer with this sample allowed exclusive detection of NOE crosspeaks between the 15N-attached protons of one monomer and the 13C-attached protons of other monomers. The intermonomer NOEs between the neighbouring H1 helices and neighbouring H2 helices effectively defined the central alignment in a gel method 35,36. In this experiment the p7(5a) channel in DPC micelles was soaked into a cylindrically shaped polyacrylamide gel (4.5%), initially generating stretched polyacrylamide gels, yielding uniform alignment of proteins within polyacrylamide gels: Diffusional properties and residual alignment by mechanical stress or embedding of oriented purple membranes. J. Biomol. NMR 18, 303–309 (2000).

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