Biochimica et Biophysica Acta 1808 (2011) 554–560

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Comparative NMR studies demonstrate profound differences between two viroporins: p7 of HCV and Vpu of HIV-1

Gabriel A. Cook, Hua Zhang, Sang Ho Park, Yan Wang, Stanley J. Opella *

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA

ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 3 August 2010
Accepted 5 August 2010
Available online 18 August 2010

Keywords:
Hepatitis C virus
p7
Membrane protein
Vpu
Human immunodeficiency virus
Nuclear magnetic resonance

ABSTRACT

The p7 protein from hepatitis C virus and the Vpu protein from HIV-1 are members of the viroporin family of small viral membrane proteins. It is essential to determine their structures in order to obtain an understanding of their molecular mechanisms and to develop new classes of anti-viral drugs. Because they are membrane proteins, it is challenging to study them in their native phospholipid bilayer environments by most experimental methods. Here we describe applications of NMR spectroscopy to both p7 and Vpu. Isotopically labeled p7 and Vpu samples were prepared by heterologous expression in bacteria, initial isolation as fusion proteins, and final purification by chromatography. The purified proteins were studied in the model membrane environments of micelles by solution NMR spectroscopy and in aligned phospholipid bilayers by solid-state NMR spectroscopy. The resulting structural findings enable comparisons to be made between the two proteins, demonstrating that they have quite different architectures. Most notably, Vpu has one trans-membrane helix and p7 has two trans-membrane helices; in addition, there are significant differences in the structures and dynamics of their internal loop and terminal regions.

Contents

1. Introduction

1.1. Viroporins

Viroporins are relatively small viral membrane proteins whose functions are crucial to the lifecycle of viruses [1,2]. Proteins classified as viroporins include Vpu from the Human immunodeficiency virus 1

* Corresponding author.
E-mail address: sopella@ucsd.edu (S.J. Opella).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.08.005

(HIV-1), p7 from Hepatitis C virus (HCV), M2 from the influenza virus A, poliovirus 2B, and alphavirus 6K. Although these proteins are not involved in replication, they appear to have multiple biological activities, and are essential for viral infectivity by promoting virus release. Structural investigations of viroporins are at an early stage, with M2 from influenza being the most advanced [3–5]. However our NMR studies of Vpu [6–14] and of p7 [15–17] have revealed many of their principal structural features in membrane environments, and the properties of these two proteins are compared here. In general, all of the viroporins have several common features, including at least one hydrophobic trans-membrane helix and conserved basic and/or aromatic residues. There is evidence that at least some of these proteins oligomerize to form ion channels [18–21], several of which are inhibited by the addition of established channel blocking compounds [21–27]. Also, mutations in these proteins can lead to non-viable viruses [28–31]. These proteins are highly conserved across genotypes, and these experimental results suggest that they are potential targets for anti-viral drugs.

1.2. Viroporin proteins p7 of HCV and Vpu of HIV-1

Hepatitis C virus, a member of the Flaviviridae family, infects over 170 million people worldwide. Infections are often chronic, resulting in cirrhosis, end-stage liver disease and hepatocellular carcinoma. HCV's viral RNA, which is 2.6 kb, encodes a single polyprotein that is proteolytically cleaved into ten proteins that are responsible for the functions of the virus life cycle [32,33]. One of these proteins, p7, a small hydrophobic protein with 63 residues, has been shown to play a role in the release of mature virus particles from infected cells [28]. Topology predictions suggest that p7 has two hydrophobic trans-membrane helices connected by a short inter-helical loop that contains the two highly conserved basic residues, K33 and R35 [34–36]. p7 has been shown to form hexamers in phospholipid bilayers, and several groups have demonstrated that the resulting ion channels can be blocked by compounds such as amantadine [21], hexamethylene amiloride [23] and long acyl-chain iminosugar derivatives [24].

Human Immunodeficiency Virus (HIV) is a lentivirus in the retrovirus family. HIV can lead to the acquired immune deficiency syndrome (AIDS) by destroying crucial immune functions. In 2008, an estimated 33.4 million people were infected with HIV worldwide. HIV-1 and HIV-2 are the two principal types of HIV; HIV-1 is more virulent, has higher infectivity, and contains the gene for Vpu. Vpu is an 81-residue membrane protein that is responsible for two biological activities that contribute to the pathogenicity of HIV-1 infections in humans; it accelerates the degradation of CD4 receptors and enhances the release of newly formed virus particles from infected cells [37–40]. These activities are associated with separate domains of the protein. The C-terminal cytoplasmic domain of the protein modulates CD4 degradation via the phosphorylation of two conserved serine residues (S52 and S56). The N-terminal transmembrane domain is responsible for enhancing the release of virus particles. The mechanism by which Vpu enhances virus release is under active investigation in a number of laboratories [18,41–44]. The results of molecular dynamics simulations and channel activity studies have shown that the hydrophobic transmembrane domain of Vpu can form pentamers or tetramers [45]. Notably, a single mutation of alanine at position 18 of Vpu by a histidine (A18H) has been shown to make the virus infection sensitive to rimantadine, a M2 channel blocker [14].

1.3. Applications of NMR spectroscopy to p7 and Vpu

Information about the structure and dynamics of these proteins has the potential to provide insights into their mechanisms of action and contribute to the rational design of drugs. NMR spectroscopy can be used to characterize the structure of these proteins at atomic resolution in micelle and bilayer environments [14,46–48], something that is not possible using other experimental methods of protein structure determination. Another advantage of NMR spectroscopy is that experiments are available that can be used to monitor molecular motions on multiple timescales to provide a detailed description of the dynamics of the proteins. Recent developments in instrumentation, specifically, the design and construction of solid-state NMR probes that reduce the sample heating due to high frequency radiofrequency irradiations and provide higher sensitivity facilitate the study of samples with high dielectric constants like viroporins in their native environments of fully hydrated phospholipid bilayers [49].

2. Expression, isotopic labeling, and purification of p7 and Vpu

2.1. Fusion protein expression

The over expression of polypeptides with sequences corresponding to those of p7 and Vpu in bacteria is challenging because in high concentrations they can disrupt the integrity of cell membranes, resulting in the early death of rapidly growing bacteria. The use of fusion proteins overcomes this obstacle, since the over expressed fusion proteins are generally not toxic to bacteria because they are sequestered in inclusion bodies, which keeps them away from the vulnerable cell membranes. Fusion partner proteins such as Trp-leader (TrpΔLE) and ketosteroid isomerase (KSI) are commonly used, and have been successfully employed in the bacterial expression of p7 and Vpu.

The methods used for the expression and purification for p7 and Vpu have evolved from those previously described by Park et al. [10] and by Cook et al. [15]. Coding sequences corresponding to p7 from HCV genotype J4 and to Vpu from HIV-1 genotype NL43 were designed with codon optimization to facilitate heterologous expression in *Escherichia coli.* Amplified sequences were inserted into plasmid vectors containing N-terminal fusion proteins. The p7 sequence was fused to that of TrpΔLE and that of Vpu to KSI. Both constructs include histidine tags to enable facile purification by nickel affinity chromatography. The plasmids were transformed into BL21 (DE3) cells. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) when the growth media reached an optical density of 0.5–0.6. Cells were allowed to grow an additional 4–6 h and then harvested by centrifugation. The expression of the fusion proteins is visualized in the SDS-PAGE shown in Fig. 1A and D, where the total cell proteins before and after 4 hours of induction by the addition of IPTG are compared.

2.2. Isotopic labeling

NMR experiments require milligram quantities of highly purified isotopically labeled proteins. The expression of membrane proteins as fusion proteins in *E. coli* can be carried out in a variety of media for this purpose. M9 minimal media with ^{15}N–ammonium sulfate, ^{13}C-glucose and/or deuterium oxide can be used to produce uniformly ^{15}N, ^{13}C and ^{2}H-labeled proteins as required. M9 media can also be prepared with specific isotopically labeled amino acids along with other unlabeled amino acids to produce selectively labeled (by residue type) proteins.

2.3. Purification

Following expression in *E. coli*, the purification of the polypeptides is accomplished in several discrete steps. Following the disruption of the cells, the fusion proteins are separated from soluble proteins and membrane fractions using a detergent-containing resuspension buffer. A denaturing buffer containing guanidine hydrochloride is then used to solubilize the fusion proteins. Metal affinity column with nickel is used to separate the fusion protein from contaminants in the inclusion bodies. To separate the polypeptide sequence of interest from its fusion partner, site-specific cleavage is accomplished either as