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Expression, purification, and in vitro activity of an arterivirus main proteinase

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Abstract

To allow the biochemical and structural characterization of the chymotrypsin-like "main proteinase" (non-structural protein 4; nsp4) of the arterivirus prototype Equine Arteritis Virus (EAV), we developed protocols for the large-scale production of recombinant nsp4 in *Escherichia coli*. The nsp4 proteinase was expressed either fused to maltose binding protein or carrying a C-terminal hexahistidine tag. Following purification, the nsp4 moiety of MBP-nsp4 was successfully used for structural studies [Barrette-Ng, I.H., Ng, K.K.S., Mark, B.L., van Aken, D., Cherney, M.M., Garen, C, Kolodenko, Y., Gorbalenya, A.E., Snijder, E.J., James, M.N.G, 2002. Structure of arterivirus nsp4—the smallest chymotrypsin-like proteinase with an alpha/beta C-terminal extension and alternate conformations of the oxyanion hole. J. Biol. Chem. 277, 39960–39966]. Furthermore, both forms of the EAV proteinase were shown to be proteolytically active in two different *trans*-cleavage assays. Recombinant nsp4 cleaved the cognate nsp6/7- and nsp7/8 site in in vitro synthesized substrates. In a synthetic peptide-based activity assay, the potential of the recombinant proteinase to cleave peptides mimicking the P9–P7′ residues of six nsp4 cleavage sites was investigated. The peptide representing the EAV nsp7/8 junction was used to optimize the reaction conditions (pH 7.5, 25 mM NaCl, 30% glycerol at 30 °C), which resulted in a maximum turnover of 15% of this substrate in 4 h, using a substrate to enzyme molar ratio of 24:1. The assays described in this study can be used for a more extensive biochemical characterization of the EAV main proteinase, including studies aiming to identify inhibitors of proteolytic activity.
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1. Introduction

Many viruses with a single-stranded, positive-sense RNA genome regulate their genome expression by synthesizing large precursor polypeptides (or polyproteins) that are subsequently subjected to limited proteolysis to produce functional viral proteins (for reviews, see Dougherty and Semler, 1993; Gorbalenya and Snijder, 1996; Krausslich and Wimmer, 1988; Spall et al., 1997). RNA virus polyproteins that include replicative proteins are often processed autocatalytically, although in some virus groups cellular proteinases are also involved. In this manner, the expression of the non-structural proteins (or "replicase" complex) can be regulated in time and space, e.g. to produce alternative cleavage products or stable processing intermediates with unique functions (de Groot et al., 1990; Jore et al., 1988; Lemm et al., 1994; Ypma-Wong et al., 1988).

Viruses in the order *Nidovirales*, which unifies the enveloped, positive-stranded *Arteriviridae*, *Coronaviridae*, and *Roniviridae* (Snijder et al., 2005; Spaan et al., 2005), have a similar polycistronic genome organization, share a conserved array of homologous replicase domains, and use common transcriptional and (post)-translational strategies to regulate their genome expression. Among these strategies, one of the most crucial is the proteolytic maturation of the replicase polyproteins pp1a and pp1ab that are translated from the incoming genome (den Boon et al., 1991; Ziebuhr et al., 2000).

Equine arteritis virus (EAV) is the prototype of the arterivirus family and has a genome of approximately 12.7 kb, of which

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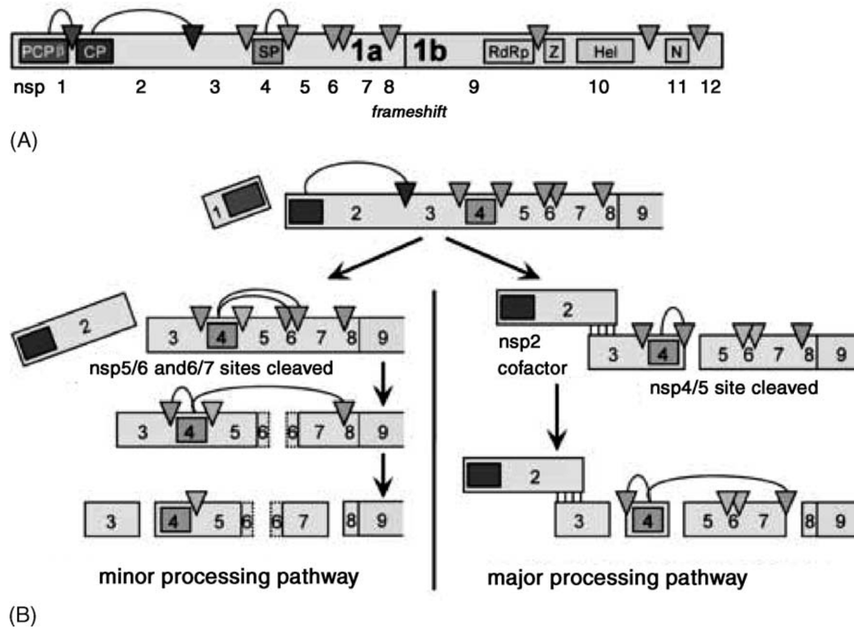

Fig. 1. Proteolytic processing of the EAV replicase. (A) Processing map of the 3175-amino acid EAV replicase polyprotein pp1ab. The three EAV proteinases (PCPβ, CP and SP), their cleavage sites and the EAV nsp nomenclature are depicted. PCPβ, nsp1 papain-like Cys proteinase; CP, nsp2 Cys proteinase; SP, nsp4 Ser proteinase; RdRp, RNA-dependent RNA polymerase; Z, zinc finger; Hel, helicase; N, nidovirus-specific endoribonuclease (NendoU). (B) Overview of the two alternative processing pathways that apply to EAV pp1a (Wassenaar et al., 1997). The association of cleaved nsp2 with nsp3–8 (and probably also with nsp3–12) was shown to be a cofactor in the cleavage of the nsp4/5 site by the nsp4 proteinase (major pathway). Alternatively, in the absence of nsp2, the nsp5/6 and nsp6/7 sites are processed and the nsp4/5 junction remains uncleaved (minor pathway). The status of the small nsp6 subunit (fully cleaved or partially associated with nsp5 and/or nsp7) remains to be elucidated. Adapted from Barrette-Ng et al., 2002.

the 5′-proximal three quarters contain the replicase gene. The 3′-proximal region of the genome includes seven open reading frames encoding the viral structural proteins, which are expressed from a nested set of subgenomic mRNAs (de Vries et al., 1990; Snijder and Meulenberg, 2001). Replicase gene expression ultimately yields (at least) 12 non-structural proteins (Fig. 1A; Snijder et al., 1994; van Dinten et al., 1996; Wassenaar et al., 1997), which are produced from two primary genome translation products, the large polyproteins pp1a (1728 amino acids) and pp1ab (3175 amino acids), with the latter being expressed following a ribosomal frameshifting event (den Boon et al., 1991). The replicase subunits are released from the polyproteins by three virus-encoded proteinases (Fig. 1), of which the one located in nsp4 is responsible for the processing of the polypeptides that remain after nsp1 and nsp2 have been autocatalytically released from pp1a and pp1ab (Snijder et al., 1992, 1994, 1995, 1996; van Dinten et al., 1999; Wassenaar et al., 1997). Since nsp4 controls the production of the viral RNA-dependent RNA polymerase and RNA helicase, it has been termed the EAV "main proteinase" (Gorbalenya et al., 1991; Ziebuhr et al., 2000).

The arterivirus nsp4 main proteinase belongs to the 3C-like serine proteinases, a distinct group of viral chymotrypsin-like proteolytic enzymes (Barrette-Ng et al., 2002; Snijder et al., 1996; Ziebuhr et al., 2000). The arterivirus proteinase combines the catalytic triad His/Asp/Ser of canonical chymotrypsin-like proteinases with the substrate specificity of the 3C-like cysteine proteinases, a subgroup of chymotrypsin-like enzymes named after the picornavirus 3C proteinases. In the cleaved, 204-residue EAV nsp4 (which equals Gly-1065 to Glu-1268 of the EAV replicase polyproteins) the catalytic triad is formed by His-39, Asp-65, and Ser-120. Cleavage sites recognized by the nsp4 proteinase carry a Glu at the P 1 position (Gln in one case) and a small amino acid (Gly, Ser, Ala) at the P 1′ position (using the cleavage site nomenclature of Schechter and Berger, 1967). Furthermore, nsp4 possesses a unique C-terminal domain (CTD) extension of unknown function, which is not found in most other chymotrypsin-like proteinases and might be involved in modulation of nsp4 activity (Barrette-Ng et al., 2002).

Arterivirus replication depends on the regulation of replicase gene expression in time and space, in which nsp4 plays a key role. Notably, an elegant (presumably) regulatory mechanism connected to nsp4-driven proteolysis was documented in the form of the differential processing of the nsp4–8 part of the EAV replicase polyproteins (Fig. 1B; Wassenaar et al., 1997). Together, the two pathways yield a variety of products, whose role in the viral life cycle remains to be studied in detail.

The biochemical characterization of the arterivirus main proteinase requires the large-scale expression and purification of an active form of the enzyme. In this paper, we describe the production of active, recombinant EAV nsp4 from *Escherichia coli* (*E. coli*), allowing the design of an in vitro cleavage assay, which was used to characterize the properties of nsp4 and its interaction with substrates. The expression system also formed the basis for structural studies, which led to the elucidation of the three-dimensional structure of EAV nsp4 by X-ray crystallography (Barrette-Ng et al., 2002). Together, these studies are a major step towards dissecting the structure–function relation-

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