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Zinc Binding in Pestivirus N^{pro} Is Required for Interferon Regulatory Factor 3 Interaction and Degradation

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Pestiviruses, such as bovine viral diarrhea virus and classical swine fever virus (CSFV), use the viral protein N^{pro} to subvert host cell antiviral responses. N^{pro} is the first protein encoded by the single large open reading frame of the pestivirus positive-sense RNA genome and has an autoproteolytic activity, cleaving itself off from the polyprotein. N^{pro} also targets interferon regulatory factor 3 (IRF3), a transcription factor for alpha/beta interferon genes, and promotes its proteasomal degradation, a process that is independent of the proteolytic activity of N^{pro}. We determined that N^{pro} contains a novel metal-binding TRASH motif consisting of Cys-X_{21}-Cys-X_3-Cys (where X is any amino acid) at its C-terminus. We also found that N^{pro} coordinates a single zinc atom as determined by graphite furnace–atomic absorption spectrophotometry and inductively coupled plasma–mass spectrometry. Mutational and biochemical analyses show that the cysteine residues in the TRASH motif are required for zinc binding and protein stability. Individual substitutions of the cysteines in the TRASH motif of CSFV N^{pro} abolished the interaction of N^{pro} with IRF3 and resulted in the loss of virus-mediated IRF3 degradation in CSFV-infected cells. Thus, the zinc-binding ability of N^{pro} in pestiviruses appears to be essential for the virus-mediated degradation of IRF3.

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Introduction

Viral infection triggers a complex signal transduction cascade that activates the cellular antiviral defense mechanisms. For example, double-stranded RNA (associated with viral replication or transcription) triggers the alpha/beta interferon (IFN-α/β) pathway, leading to antiviral responses such as destruction of viral RNA, inhibition of cellular transcription and translation, and ultimately apoptosis.[1,2] IFN-α/β induction depends on activation of the interferon regulatory factor (IRF) family of transcription factors.[3] IRF3 is ubiquitously expressed in the cytoplasm and is activated in

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Abbreviations used: ANS, 1-anilino-8-naptalenesulfonate; BDV, border disease virus; BVDV, bovine viral diarrhea virus; CAT, chloramphenicol acetyltransferase; CSFV, classical swine fever virus; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EMEM, Earle's minimal essential medium; GF-AAS, graphite furnace–atomic absorption spectrophotometry; ICP-MS, inductively coupled plasma–mass spectrometry; IFN, interferon; IRF3, interferon regulatory factor 3; MALDI-MS, matrix-assisted laser desorption and ionization mass spectrometry; UTR, untranslated region.

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response to viral infection. When activated, IRF3 is phosphorylated at multiple sites, dimerizes, and translocates into the nucleus where it associates with a complex of transcription factors to form the enhanceosome that binds to the IFN-α/β promoters. This triggers expression and secretion of IFN molecules that bind type I IFN receptors, which results in activation of antiviral responses.[4] To counter this antiviral response, many viruses—including pestivirus, hepatitis C virus, bovine herpes virus, rotavirus, Bunyamwera virus, Ebola virus, influenza virus, human cytomegalovirus, and human rhinovirus (for selected reviews, see Refs. 1, 2, 5, and 6)—have evolved strategies to interfere with the IRF3 pathway, thus inhibiting the induction of IFN-α/β. The mechanisms of IRF3 antagonism vary, including proteasomal targeting of IRF3, inhibition of IRF3 phosphorylation, nuclear translocation of IRF3, and transcription complex assembly.[7] Pestiviruses use their protein N^{pro} to interact with IRF3 resulting in its proteasomal degradation.[8–11]

The pestiviruses are important animal pathogens that include bovine viral diarrhea virus (BVDV) of cattle, classical swine fever virus (CSFV) of pigs, and border disease virus (BDV) of sheep. The genus pestivirus belongs to the family *Flaviviridae*, together with the genus hepacivirus (which includes hepatitis C virus) and the genus flavivirus (which includes dengue, West Nile, and Yellow Fever viruses). The pestiviruses are enveloped viruses containing a single positive-sense RNA genome of ~12 kb.[12,13] The viral RNA comprises a 5′ untranslated region (5′ UTR), a single open reading frame, and a 3′UTR. The 5′UTR acts as an internal ribosomal entry site for cap-independent initiation of translation. The open reading frame encodes a large polyprotein of approximately 3900 amino acids that is processed by viral and cellular proteases into 12 mature proteins whose order is N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. The first protein encoded by pestiviruses is the N-terminal protease, N^{pro}. Importantly, N^{pro} has no counterpart in either hepatitis C virus or flaviviruses. It is an atypical cysteine protease with an autoproteolytic activity that cleaves the bond between Cys168 and Ser169 of the viral polyprotein via cis cleavage, generating the N-terminus of the capsid protein C.[14] After the initial cleavage, N^{pro} is no longer active; no trans activity has been observed.[15] The putative catalytic triad is Glu22-His49-Cys69, as mutations of any of these residues block the autocatalytic cleavage in an *in vitro* translation system.[16] However, sequence alignment with other proteases does not show any significant homology, and a Glu-His-Cys catalytic triad arrangement has never been observed in the active site of other cysteine protease family members.[16] Thus, pestivirus N^{pro} constitutes its own peptidase subfamily, C53, within the cysteine protease family.[17]

N^{pro} is also essential for evading the cellular antiviral defense system. Although N^{pro} is not required for viral replication in cell culture, it protects cells from double-stranded RNA-induced apoptosis.[18–23] N^{pro} from both BVDV and CSFV targets IRF3 for proteasomal degradation, significantly decreasing the amount of available IRF3, and thus disrupting the IFN response.[8–11] N^{pro}-induced IRF3 degradation seems to occur via polyubiquitination, a step required for proteins that are degraded via the proteasome.[8–11] Interaction between N^{pro} and IRF3 has been shown by co-immunoprecipitation and mammalian two-hybrid assay, although it is not known whether N^{pro} interacts with IRF3 directly or indirectly by recruiting cellular proteins.[8,9,24] Recent experiments have identified residues of N^{pro} essential for prevention of IFN-α/β induction. Mutation of the catalytic residue Cys69 to Ala had no effect on IFN induction for both BVDV and CSFV N^{pro}, demonstrating that the protease activity is not involved in IRF3 binding and degradation.[8,10,21,24] In BVDV N^{pro}, single Glu22 to Leu/Val or His49 to Val/Leu substitutions both resulted in a loss of IFN-antagonistic activity, suggesting that the structural changes in these mutants that disable catalysis also disrupt IFN-α/β antagonism.[8,21] Leu8-to-Pro substitution does not affect protease activity but does ablate the effect of the protein on IFN-α/β induction in BVDV N^{pro}.[8,21] Furthermore, N^{pro} deletion mutants that lack either the first 22 amino acids or the last 24 amino acids also lose their ability to inhibit IFN-α/β induction.[8,10,24] Thus, nearly the entire N^{pro} seems to be essential for the suppression of IFN-α/β production in infected cells.[21,24]

We have characterized CSFV and BVDV N^{pro} using a combination of bioinformatics and biochemical and biophysical assays. Analysis of its sequence suggests that N^{pro} consists of two domains and that N^{pro} contains a metal binding TRASH motif at its C-terminus. We have determined the zinc-binding ability of N^{pro}, identified essential residues involved in the metal binding, and tested whether the zinc-binding ability in N^{pro} is directly related to the IRF3 interaction and degradation.

Results

Sequence analysis of N^{pro}

Blast and psi-blast searches[25] with the CSFV N^{pro} sequence found only N^{pro} proteins from pestiviruses, thus indicating that N^{pro} may be a functionally and evolutionarily unique protein. The amino acid sequences of N^{pro} from BVDV, CSFV, BDV, and Bungowannah virus were aligned using the program CLUSTAL W to identify conserved residues that are likely to be important for N^{pro} structure and function.[26] N^{pro} from the Bungowannah virus, the most distinct pestivirus, exhibited only 30% sequence identity to other N^{pro} sequences, while there was 69–73% sequence identity among the N^{pro} sequences from other strains (Fig. 1a).

Depending on the N^{pro} sequences examined, there are a total of either six or eight Cys residues, five of which are conserved (Fig. 1a). The first (Cys69) is the

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