# ADP-RIBOSE -1"-PHOSPHATASE ACTIVITIES OF THE HUMAN CORONAVIRUS 229E AND SARS CORONAVIRUS X DOMAINS

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## 1. INTRODUCTION

Coronavirus RNA synthesis is mediated by the viral replicase, a huge multienzyme complex comp. of several cellular proteins and up to 16 viral nonstructural proteins (nsp1–16). For the majority of these proteins, the available functional and structural information is extremely limited.[1] Coronavirus nsp3, the largest viral subunit of the coronavirus replicase, has been predicted to contain several conserved domains, including an N-terminal domain enriched in Glu and Asp residues ("acidic domain"), one or two papain-like proteases ($PL1^{pro}$ and $PL2^{pro}$), the X domain, and a C-terminal conserved domain ("Y domain") containing putative transmembrane and metal ion-binding domains.[2] The X domain has been predicted to be a phosphatase that converts ADP-ribose-1"-monophosphate (Appr-1"-p) to ADP-ribose (Appr).[3] Appr-1"-p is a downstream metabolite of cellular tRNA splicing. It is generated from ADP-ribose-1", 2"-cyclic-phosphate (Appr>p) by a cyclic phosphodiesterase (CPDase) activity.[4,5] Coronavirus X domain homologs are conserved in very few plus-strand RNA viruses, excluding the closely related arteri- and roniviruses.[3,6] There is a large number of poorly characterized cellular homologs that constitute the so-called macrodomain protein family. Recently, the crystal structure of one of these homologs, the *Archeoglobus fulgidus* AF1521 protein, has been determined[7] and the *Saccharomyces cerevisiae* Poa1p has been demonstrated to have Appr-1"-pase activity.[8] To gain insight into the biochemical properties of coronavirus X domains, we expressed and characterized recombinant forms of the human coronavirus 229E (HCoV-229E) and severe acute respiratory coronavirus (SARS-CoV) X domains.

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## 2. MATERIALS AND METHODS

*Cloning and expression of coronavirus X domains*: X domain coding sequences (HCoV-229E nucleotides 4085 to 4600 and SARS-CoV nucleotides 3262 to 3783, respectively) were amplified by reverse transcription-PCR using poly(A) RNA isolated from HCoV-229E-infected MRC-5 cells and SARS-CoV-infected Vero cells, respectively. Each of the PCR reverse primers contained a translation stop codon followed by an EcoRI restriction site. The PCR products were treated with T4 DNA polymerase, polynucleotide kinase, and EcoRI, and ligated with XmnI/EcoRI-digested pMal-c2 plasmid DNA (800-64S; New England Biolabs). The resulting plasmids encoded fusion proteins consisting of the *E. coli* maltose-binding protein (MBP) and the respective coronavirus X domain. Using PCR-based methods, a mutant derivative of the HCoV-229E X domain was generated. This protein contained substitutions (by Ala) of the pp1a/pp1ab residues, Asn1302 and Asn1305. *E. coli* TB1 cells transformed with the appropriate plasmid were grown to an $OD_{595}$ of 0.5, and protein expression was induced with 1 mM IPTG for 3 hours at 18°C. The MBP-X fusion proteins were purified by amylose-affinity chromatography and cleaved with factor Xa. The protein purification and storage buffer contained 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol.

*Appr-1"-pase activity assay*: To produce Appr-1"-p, chemically synthesized Appr>p was treated with *Arabidopsis thaliana* cyclic nucleotide phosphodiesterase as described previously.[4] The purified coronavirus X domains (0.5 μM) were incubated with Appr-1"-p (2 mM) in buffer containing 35 mM Tris-HCl (pH 7.5), 0.005% Triton X-100, 0.5 mM EDTA, 100 mM NaCl, and 0.5 mM dithiothreitol at 30°C for 3 hours. In control reactions, 0.5 U/μl alkaline phosphatase from calf intestine (Roche) was used. Reactions products were separated by cellulose thin-layer chromatography [saturated $(NH_4)_2SO_4$/3M sodium acetate/isopropyl alcohol (80:6:2)] and visualized under UV light.

## 3. RESULTS AND DISCUSSION

### 3.1. The HCoV-229E nsp3-Associated X Domain Is a Phosphatase That Converts Appr-1"-p to Appr

The phylogenetic relationship of coronavirus X domains with cellular proteins of the macrodomain family[3] and the recent biochemical characterization of two of the cellular homologs[8,9] suggested that coronavirus X domains might be enzymes that dephosphorylate Appr-1"-p to Appr. To confirm this hypothesis, the HCoV-229E X domain was expressed as an MBP-fusion protein in *E. coli* and its activity was examined in an *in vitro* Appr-1"-pase assay. Based on sequence aligments with other coronavirus X domains[2] and many more cellular homologs and guided by the crystal structure of the *Archeoglobus fulgidus* AF1521 protein,[7] the functional HCoV-229E X domain was predicted to encompass the pp1a/1ab residues Glu 1265 to Val 1436. Based on our prediction that Asn 1302 and Asn 1305 residues are part of the X domain's active site, we expressed a mutant protein in which these two Asn residues were replaced with Ala and used it as a negative control in subsequent experiments. Following IPTG-induced protein expression in *E. coli*, the fusion proteins were purified by amylose-affinity

chromatography and cleaved with factor Xa to separate the X domains from MBP. SDS-PAGE analysis of cell lysates obtained from IPTG-induced or noninduced bacteria as well as protein samples from the amylose-affinity purification and factor Xa cleavage (Fig. 1A and 1B) showed that sufficient amounts of soluble HCoV-229E X domain could be obtained for biochemical studies (Fig. 1A and 1B). To test the Appr-1”-pase activity, the factor Xa-cleaved proteins were incubated with Appr-1”-p and the products were analyzed by thin-layer chromatography. As Fig. 1C illustrates, Appr-1”-p was processed by the HCoV-229E X domain (but not the negative control protein containing two putative active-site substitutions) to a product that comigrated with Appr. As expected, the same product was seen when the positive control, alkaline phosphatase, was used (Fig. 1C). Taken together, the data show that (i) the HCoV-229E nsp3-associated X domain has Appr-1”-pase activity and (ii) Asn residues 1302 and 1305 are essential for enzymatic activity.

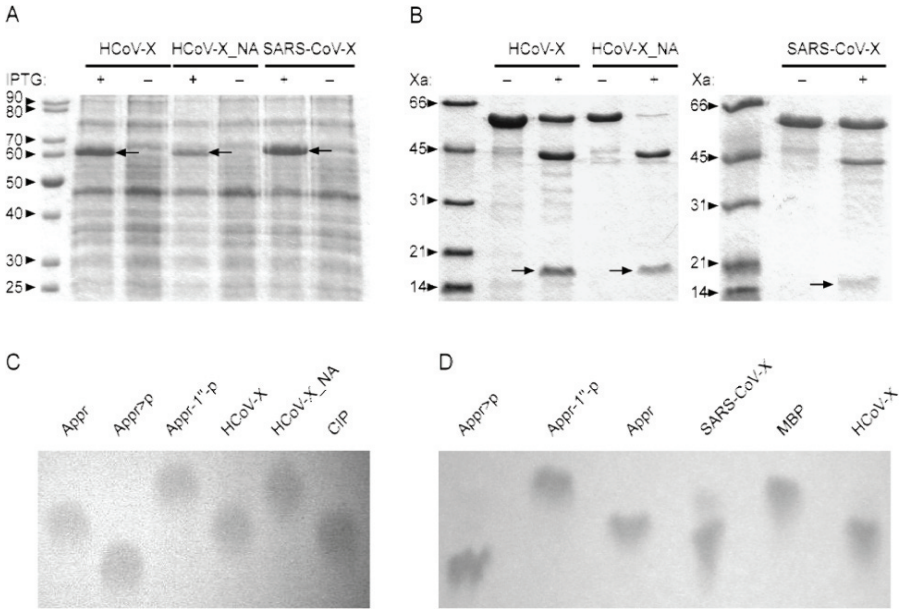


**Figure 1.** Expression, purification, and ADP-ribose-1”-phosphatase activity of the HCoV-229E and SARS-CoV X domains. A, The HCoV-229E X domain (HCoV-X), a mutant form of the HCoV-229E X domain containing two presumed active-site Asn substitutions (HCoV-X_NA, see text for details), and the SARS-CoV X domain (SARS-CoV-X) were expressed in *E. coli* as MBP fusion proteins. Total lysates obtained from IPTG-induced and noninduced *E. coli* TB1 cells transformed with the appropriate expression plasmids were analyzed in a 12.5% SDS-polyacrylamide gel which was stained with Coomassie brilliant blue. Arrows indicate the overexpressed MBP-X fusion proteins. B, Factor Xa cleavage of amylose affinity-purified MBP-X domain fusion proteins. Arrows indicate the proteolytically released X domains. C, Cellulose thin-layer chromatography analysis of the Appr-1”-pase activities of HCoV-X, HCoV- X_NA, and calf intestine phosphatase (CIP). D, Analysis of the Appr-1”-pase activities of SARS-CoV-X, maltose-binding protein (MBP), and HCoV-X. Markers: Appr>p, Appr-1”-p, and Appr.

### 3.2. Appr-1”-pase Activity of the SARS-CoV X Domain

The pp1a/pp1ab N-terminal region, which also includes nsp3, is the most divergent part of coronavirus replicase polyproteins.[1,3] Consistent with this observation, SARS-CoV features an nsp3 subdomain organization that significantly differs from that of most other coronaviruses. Thus, for example, SARS-CoV employs only one papain-like protease to process the N-proximal pp1a/pp1ab region.[10] Furthermore, next to the X domain, there is a domain called SUD that is not conserved in other coronaviruses. To investigate whether, in this very divergent sequence context, the X domain of SARS-CoV retained its enzymatic activity, we expressed this protein (SARS-CoV pp1a/pp1ab residues Glu 1000 to Lys 1173) as an MBP-fusion protein using the above described protocols. As shown in Fig. 1A and 1B, the SARS-CoV X domain could be expressed and purified in a soluble form. Following proteolytic release from the MBP fusion protein using factor Xa, the SARS-CoV X domain was incubated with Appr-1”-p and shown to dephosphorylate this substrate effectively (Fig. 1D). The reaction product comigrated with the product generated by the activity of the HCoV-229E X domain which, in previous experiments, had been confirmed to be Appr (data not shown). As expected, the negative control, MBP, had no activity on this particular substrate, confirming that the observed activity was mediated by the viral rather than co-purified bacterial protein(s). Taken together, the data suggest that most (if not all) coronavirus X domains mediate highly specific phosphatase activities whose biological significance remains to be investigated.

## 4. REFERENCES

1. Ziebuhr, J., 2005, The coronavirus replicase, *Curr. Top. Microbiol. Immunol.* **287**:57-94.
2. Ziebuhr, J., Thiel, V., and Gorbalenya, A. E., 2001, The autocatalytic release of a putative RNA virus transcription factor from its polyprotein precursor involves two paralogous papain-like proteases that cleave the same peptide bond, *J. Biol. Chem.* **276**:33220-33232.
3. Snijder, E. J., Bredenbeek, P. J., et al., 2003, Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage, *J. Mol. Biol.* **331**:991-1004.
4. Genschik, P., Hall, J., and Filipowicz, W., 1997, Cloning and characterization of the Arabidopsis cyclic phosphodiesterase which hydrolyzes ADP-ribose 1″,2″-cyclic phosphate and nucleoside 2′,3′-cyclic phosphates, *J. Biol. Chem.* **272**:13211-13219.
5. Kumaran, D., Eswaramoorthy, S., Studier, F. W., and Swaminathan, S., 2005, Structure and mechanism of ADP-ribose-1″-monophosphatase (Appr-1″-pase), a ubiquitous cellular processing enzyme, *Protein Sci.* **14**:719-726.
6. Gorbalenya, A. E., Koonin, E. V., and Lai, M. M., 1991, Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses, *FEBS Lett.* **288**:201-205.
7. Allen, M. D., Buckle, A. M., et al., 2003, The crystal structure of AF1521 a protein from Archaeoglobus fulgidus with homology to the non-histone domain of macroH2A, *J. Mol. Biol.* **330**:503-511.
8. Shull, N. P., Spinelli, S. L., and Phizicky, E. M., 2005, A highly specific phosphatase that acts on ADP-ribose 1″-phosphate, a metabolite of tRNA splicing in Saccharomyces cerevisiae, *Nucleic Acids Res.* **33**: 650-660.
9. Martzen, M.R., McCraith, S. M., et al., 1999, A biochemical genomics approach for identifying genes by the activity of their products, *Science* **286**: 1153-1155.
10. Thiel, V., Ivanov, K. A., et al., 2003, Mechanisms and enzymes involved in SARS coronavirus genome expression, *J. Gen. Virol.* **84**:2305-2315.