Species Specificity of the NS1 Protein of Influenza B Virus

NS1 BINDS ONLY HUMAN AND NON-HUMAN PRIMATE UBIQUITIN-LIKE ISG15 PROTEINS*

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Influenza B viruses, which cause a highly contagious respiratory disease every year, are restricted to humans, but the basis for this restriction had not been determined. Here we provide one explanation for this restriction: the species specificity exhibited by the NS1 protein of influenza B virus (NS1B protein). This viral protein combats a major host antiviral response by binding the interferon-α/β-induced, ubiquitin-like ISG15 protein and inhibiting its conjugation to an array of proteins. We demonstrate that the NS1B protein exhibits species-specific binding; it binds human and non-human primate ISG15 but not mouse or canine ISG15. In both transfection assays and virus-infected cells, the NS1B protein binds and relocates only human and non-human primate ISG15 from the cytoplasm to nuclear speckles. Human and non-human primate ISG15 proteins consist of two ubiquitin-like domains separated by a short hinge linker of five amino acids. Remarkably, this short hinge plays a large role in the species-specific binding by the NS1B protein. The hinge of human and non-human primate ISG15, which has a sequence that differs from that of other mammalian ISG15 proteins, including mouse and canine ISG15, is absolutely required for binding the NS1B protein. Consequently, the ISG15 proteins of humans and non-human primates are the only mammalian ISG15 proteins that would bind NS1B.

Influenza A and B viruses cause a highly contagious respiratory disease in humans. Influenza A viruses infect a wide variety of species, whereas influenza B viruses infect only humans (1). Many, but not all, of the proteins encoded by these two groups of species, whereas influenza B viruses infect only humans (1). The on-line version of this article (available at http://www.jbc.org) contains supplementary Fig. S1.

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2 The abbreviations used are: IFN, interferon; GST, glutathione S-transferase; GFP, green fluorescent protein.

* This work was supported, in whole or in part, by National Institutes of Health Grant AI11772 (to R. M. K.).

** The on-line version of this article (available at http://www.jbc.org) contains supplementary Fig. S1.

Received for publication, December 16, 2009, and in revised form, January 19, 2010
Published, JBC Papers in Press, January 21, 2010, DOI 10.1074/jbc.C109.095703

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MARCH 12, 2010

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 11, pp. 7852–7856, March 12, 2010
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7852 JOURNAL OF BIOLOGICAL CHEMISTRY
Species-specific Binding of ISG15 by NS1B Protein

**RESULTS**

**Influenza B Virus NS1 Protein Binds to Specific Mammalian ISG15 Proteins**—The protein sequence of ISG15 is poorly conserved across different mammalian species. For example, canine and mouse ISG15 proteins are only 69 and 65% identical to human ISG15, respectively. In contrast, monkey ISG15 exhibits 92% identity to human ISG15. We hypothesized that only those ISG15 proteins that exhibit high homology with human ISG15 would bind to the NS1B protein. To test this hypothesis, human HEK 293T cells were co-transfected with plasmids encoding NS1B and either GST alone or GST fused to the N terminus of human, mouse, canine, or monkey ISG15, and the cell extracts were subjected to GST selection (Fig. 1). Immunoblotting of the GST-selected extracts showed that NS1B binds not only human ISG15 but also monkey ISG15 (top panel, lanes 2 and 5). In contrast, binding of NS1B to either mouse or canine ISG15 was not detected (top panel, lanes 3 and 4). Immunoblots with anti-GST antibody showed that comparable amounts of the different GST-ISG15 proteins were selected (middle panel). Immunoblotting of transfected cell extracts with anti-NS1B antibody confirmed that the expression of the NS1B was comparable in all the samples (bottom panel). These results demonstrate that NS1B binds ISG15 in a highly species-specific manner. The same species-specific binding was observed when this transfection assay was carried out in mouse NIH3T3 cells (lanes 6–10) and in canine Madin-Darby canine kidney cells (lanes 11–15), demonstrating that the binding of NS1B to ISG15 is independent of species-specific host factors.

**Influenza B Virus Infection Causes Intracellular Relocalization of Human and Monkey ISG15 Proteins**—During transfection and at early times after infection, the NS1B protein localizes to the nucleus and accumulates in intranuclear compartments known as splicing or SC35 speckles (23). We determined whether NS1B specifically relocalizes only human and monkey ISG15 to speckles during transfection and virus infection. When HeLa cells were co-transfected with plasmids encoding GFP-NS1B and either 3xFLAG-tagged human ISG15 or 3xFLAG-tagged mouse ISG15, GFP-NS1B accumulated in nuclear speckles (Fig. 2A, GFP fluorescence). Only human ISG15, but not mouse ISG15, was relocalized from the cytoplasm to nuclear speckles (FLAG fluorescence), as verified in the Merge panels. The same species specificity was seen in influenza B virus-infected cells. HeLa cells were transfected with a plasmid encoding a 3xFLAG ISG15 protein 24 h prior to infection with influenza B virus. Immunofluorescence of infected cells with anti-FLAG antibody showed that human and monkey ISG15, but not mouse or canine ISG15, were relocalized to nuclear speckles in infected cells (Fig. 2B, middle and right columns). To verify that these cells were infected, immunofluorescence was carried out using anti-influenza B virus antibody, which detects all viral proteins, including the NS1B protein that is localized in nuclear speckles (right column). These results show that the NS1B protein in infected cells specifically binds and alters the localization of only human and monkey ISG15.

**The Critical Role of the Hinge of Human ISG15 in Binding the NS1B Protein**—We took advantage of the species specificity of ISG15 binding by the NS1B protein to identify regions of the human ISG15 molecule that are required for NS1B binding. We confirmed that the N-terminal domain (plus the hinge) of...
human ISG15 is sufficient to bind the NS1B protein (11) and showed that this was also the case for monkey ISG15 (supplemental Fig. S1). Remarkably, canine ISG15 acquired strong NS1B binding simply by replacing its hinge with the human hinge (Can/Hum-H) (Fig. 3A, lanes 1–3), demonstrating the critical role of the human hinge in NS1B binding. The results with mouse ISG15 were different. Substitution of the human hinge in mouse ISG15 (Mou/Hum-H) led to little or no increase in ISG15 binding (lanes 4–6). We can conclude that region(s) in the N-terminal domain of ISG15 are also recognized by NS1B and that the N-terminal domain of mouse ISG15 lacks the appropriate binding sequence(s), in contrast to human, non-human primate, and canine ISG15 (see under “Discussion”).

Substituting the mouse ISG15 hinge for the human hinge in human ISG15 resulted in a complete loss of NS1B binding (Fig. 3B, lane 2), confirming the critical role of the human hinge in NS1B binding. To determine the role of individual amino acids in the human hinge, we made the indicated human-to-mouse amino acid substitutions (D76Q, K77N, and D79S). Each of these substitutions resulted in essentially a total loss of NS1B binding (lanes 3–5). Consequently, only those mammalian ISG15 molecules that contain the human ISG15 hinge sequence would be able to bind the NS1B protein. The hinge sequence of the ISG15 proteins of non-human primates is identical to that of humans, except that Lys-77 is replaced by Arg in the African green monkey hinge (Fig. 3C). This Lys-to-Arg replacement does not affect NS1B binding because African green monkey ISG15 binds NS1B (Fig. 1). In contrast, the hinge sequences of the other mammalian ISG15 proteins show substantial deviation from the human/non-human primate ISG15 hinge sequence. Consequently, these other mammalian ISG15 proteins would not be expected to bind NS1B, as already documented for the canine and mouse ISG15 proteins.

DISCUSSION

Influenza B virus is predominately, if not totally, restricted to humans (1), but the basis for this restriction had not been determined. Here we provide one explanation for this restriction, namely that the influenza B virus NS1B protein binds only human and non-human primate ISG15. Consequently, the NS1B protein would only be able to protect influenza B virus from the antiviral effects of ISG15 and ISG15 conjugation in humans, and presumably in non-human primates. No protection would be possible in other mammalian species, as already documented by the finding that ISG15 conjugation inhibits influenza B virus replication in mice (14, 18). Because influenza B virus would not be protected from this IFN-induced antiviral system, it would not be able to be maintained in these other mammalian species. However, influenza B virus might also be found in non-human primates, a possibility that has not yet been explored. Future experiments will determine whether other influenza B virus proteins also exhibit human-specific properties.
Critical role of the hinge of human ISG15 in binding the NS1B protein. A, top, schematic representation of ISG15 with the N- and C-terminal domains linked by the hinge (H). HEK 293T cells were co-transfected with plasmids encoding NS1B and the indicated ISG15 proteins fused to GST: human ISG15 (Hum) (lanes 1 and 4), canine ISG15 (Can) (lane 2), canine ISG15 with the human hinge (Can/Hum-H) (lane 3), mouse ISG15 (Mou) (lane 5), and mouse ISG15 with the human hinge (Mou/Hum-H) (lane 6). Cell extracts were analyzed as described in the legend for Fig. 1. WB, immunoblot. B, top, alignment of human and mouse hinge (H) sequences, with the differences between the sequences denoted by asterisks. HEK 293T cells were co-transfected with plasmids encoding NS1B and GST fused to the indicated human ISG15 molecules: wild-type (wt) (lane 1), mouse hinge replacing the human hinge (Mou-H) (lane 2), D76Q mutant (lane 3), K77N mutant (lane 4), and D79S mutant (lane 5). C, alignment of the hinge sequences in the ISG15 molecules of the indicated mammalian species. The human and non-human primate hinge sequences are boxed.

Surprisingly, the small 5-amino acid hinge between the two ubiquitin-like domains of ISG15 is crucial for NS1B binding. Only the human and primate hinges are suitable for binding. Replacement of individual amino acids in the human hinge sequence with the corresponding mouse amino acid was sufficient to eliminate NS1B binding. In addition, substitution of the human hinge sequence for the canine hinge sequence in the canine ISG15 molecule was sufficient to result in optimum binding of the NS1B protein. It has been proposed that the hinge of human ISG15 is flexible and might adopt a different orientation upon binding other proteins (10). Perhaps only the human hinge sequence allows an orientation suitable for the NS1B protein to bind to a region in the N-terminal domain. Alternatively, human hinge amino acids may interact directly with the NS1B protein.

The N-terminal domain of human ISG15 contains a binding site for the NS1B protein, as shown by the results with mouse ISG15. Substitution of the human hinge in mouse ISG15 resulted in little or no NS1B binding, indicating that the N-terminal mouse domain contains sequences that are inhibitory to NS1B binding. Such inhibitory sequences likely include some, or all, of the 8 amino acids in the N-terminal domain of mouse ISG15 that differ from the corresponding amino acids in both human and canine ISG15. To definitively identify the binding site in the N-terminal domain of human ISG15, we and our collaborators are determining the x-ray crystal structure of human ISG15 in complex with an N-terminal fragment of the NS1B protein. This structure should also identify the NS1B amino acids that directly interact with human ISG15 and hence would enable us to generate a recombinant influenza B virus encoding a NS1B protein that does not bind human ISG15. Such a virus would be expected to be susceptible to inhibition by ISG15 conjugation in human cells, thereby enabling us to identify the mechanism by which IFN-induced ISG15 conjugation inhibits influenza B virus replication in human cells.

Acknowledgment—We thank Tien-Ying Hsiang for providing us with the plasmid expressing canine ISG15.

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