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Emerging Role of ISG15 in Antiviral Immunity

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Abstract

Cells express a plethora of interferon-stimulated genes (ISGs) in response to viral infection. Among these is ISG15, a ubiquitin-like protein (UBL), that can be covalently attached to both host and viral proteins. Here we review recent advances towards understanding the role and mechanism of ISG15 modification in antiviral defense.

Introduction

Secretion of type I interferons (IFNs) from virus-infected cells is a hallmark of antiviral immunity. Cells that receive these signals increase expression of interferon-stimulated genes (ISGs), preparing the cells for impending infection. ISG15, a 15-kDa ubiquitin-like protein (UBL), has recently emerged as an important tool in the struggle against many viral pathogens (reviewed by Jeon et al. 2010). The ISG15 structure consists of two ubiquitin-like moieties linked by a short hinge. Like ubiquitin and other UBLs, ISG15 is attached to target proteins through a C-terminal Gly-Gly motif. Conjugation of ISG15, commonly referred to as ISGylation, is a three-step enzymatic cascade (Figure 1A).

The ISG15 E1 enzyme is UBE1L, which specifically activates ISG15 but not ubiquitin, and the E2 enzyme is UBCH8. The predominant E3 enzyme appears to be the HECT domain protein HERC5, because RNA interference against HERC5 abolishes most IFN-induced ISGylation. In addition, co-expression of UBE1L, UBCH8, HERC5, and ISG15 is sufficient to produce a level of ISGylation similar to that of IFN stimulation. However, biochemical evidence that HERC5 directly transfers ISG15 to substrates is still lacking. Like other UBLs, addition of ISG15 is reversible; indeed, UBP43 was identified as a deISGylation enzyme. Notably, expression of UBE1L, UBCH8, HERC5, and UBP43 is also induced by IFN.

The function of ISG15 since its discovery in the 1980's remained enigmatic until very recently. Over the past few years significant advances have led to a clearer understanding of the physiological function of ISG15 and several potential antiviral mechanisms.

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Genetic Evidence linking ISG15 and Antiviral Immunity

The robust induction of ISG15 in response to IFN treatment or viral infection implies a role for ISG15 in antiviral defense, yet initial analyses of mice lacking ISG15 or UBE1L revealed no apparent defect in defense against vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) (Kim et al., 2006; Osiak et al., 2005). Nevertheless, a growing body of work strongly suggests a role for ISG15 in defense against many viral pathogens. ISG15 overexpression in cell culture has broad antiviral effects, such as suppressing the replication of HIV and the budding of Ebola VP40 virus-like particles. Also consistent with a role for ISG15 in antiviral defense, several viruses express proteins that antagonize the ISGylation machinery (reviewed by Jeon et al. 2010). Here we focus primarily on recent results from mouse models of viral infection, and the interaction between the influenza B non-structural protein 1 (NS1B) and the ISGylation machinery.

Functional insight from mouse models of viral infection

Strong evidence that ISG15 protects mammals from viral infection came from studies using a recombinant chimeric Sindbis virus system (Lenschow et al., 2005). Exogenous expression of ISG15 in mice lacking the IFN-α and -β receptors conferred protection against systemic infection and lethality. Importantly, mutation of the two C-terminal glycine residues of ISG15 to alanines (GG>AA) abrogates this protective effect, suggesting that ISG15 conjugation is important for protection against Sindbis virus. In addition, mice lacking ISG15 succumb more readily than wild type mice to infection with several viruses, including Sindbis virus, influenza A and B virus, herpes simplex virus type 1 (HSV-1) and murine gammaherpesvirus 68 (γHV68). The impaired defense against Sindbis virus is rescued in ISG15 knockout mice by expressing wild type ISG15, but not the GG>AA mutant (Lenschow et al., 2007). Consistent with a critical role of ISG15 conjugation in antiviral defense, mice lacking UBE1L are susceptible to infection with Sindbis virus, and mutation of ISG15 Arg151, a residue critical for interaction with UBE1L, abrogates the protective effect of ISG15 (Giannakopoulos et al., 2009). UBE1L deficient mice are also susceptible to infection with influenza B (Lai et al., 2009). Taken together, these results implicate ISG15 conjugation as a key component of mammalian antiviral immunity. Interestingly, bone marrow transplantation experiments show that ISG15 exerts its antiviral function exclusively in cells of non-hematopoetic origin (Lai et al., 2009).

Species specificity in the ISGylation system

Two reports this year have introduced the intriguing prospect of species specificity in the ISG15 system, including key differences between mice and humans (Sridharan et al. 2010; Versteeg et al. 2010). The influenza NS1B protein can antagonize host cell ISGylation, one of the earliest indications that the ISGylation system might be antiviral (Yuan and Krug, 2001). Indeed, NS1B can bind directly to ISG15 (Chang et al., 2008). However, as mentioned above, ISG15 and UBE1L deficient mice are more susceptible to influenza B than their wild type counterparts. This finding suggests that in wild type mice NS1B fails to protect the virus from ISGylation. A potential explanation for this finding has recently been uncovered; NS1B cannot bind to mouse ISG15. The binding of NS1B to human ISG15 involves residues within the N-terminus and the short hinge region of ISG15. The five residues in this hinge region are highly conserved among primates, but divergent in other mammalian species including mouse and dog. Indeed, NS1B can only bind to ISG15 from humans and non-human primates. Remarkably, substitution of residues from the human hinge region with the corresponding mouse residues abolishes this binding (Sridharan et al., 2010). Consistent with the species selectivity of the NS1B-ISG15 interaction, NS1B cannot antagonize mouse ISGylation (Versteeg et al. 2010). Substitution of the N-terminus of

mouse ISG15 with the human N-terminus restores the NS1B-ISG15 interaction. This report also reveals that HERC6 is the apparent E3 protein in mice, whereas mouse HERC5 does not support ISGylation. These findings warrant careful attention in studies utilizing mice or mouse cells to study the role, and mechanism(s) of action, of ISG15. It will be of interest to determine the extent to which the species specificity of ISG15 and ISGylation machinery contributes to the different responses among mammals to viral infection.

Biochemical mechanisms of antiviral defense by ISG15

Proteomics studies have identified more than 150 proteins as putative ISGylation targets, a few of which have been validated under conditions of endogenous expression (Zhao et al., 2005). Notably, several of the ISGylation substrates identified are themselves IFN-induced proteins, such as MxA (myxovirus resistance A) and RIG-I (which senses viral RNA). However, even for proteins whose ISGylation can be confirmed, it has been difficult to determine if this modification exerts a functional consequence, in part because only a very small fraction of any cellular protein is modified by ISG15. In principle, ISGylation could lead to a gain of function, loss of function, or dominant negative effect. A gain of function or dominant negative effect could allow a small fraction of ISGylated proteins to exert a strong effect. On the other hand, a loss of function of a small fraction of proteins is unlikely to have a functional consequence, unless ISGylation occurs preferentially on an ‘active’ pool of proteins. In some cases studied so far, ISGylation appears to impair the function of target proteins. For example, ISGylation of filamin B impairs its ability to support IFN-induced Jun N-terminal kinase (JNK) activity and apoptosis (Jeon et al., 2009).

There are at least two examples in which ISGylation results in a gain of function of a cellular target protein. 4EHP binds to the cap structure of mRNA and inhibits translation by competing with the translation initiation factor eIF4E. ISGylated 4EHP binds to the mRNA cap with greater affinity than the unmodified protein. It has been postulated that ISGylation of 4EHP leads to selective inhibition of viral RNA translation, which may partly account for the inhibition of viral protein synthesis by IFN (Okumura et al., 2007).

A recent study has uncovered a role for ISGylation by HERC5 in the regulation of IRF3, a transcription factor that controls the production of IFN (Shi et al. 2010). HERC5 interacts with IRF3 and promotes its ISGylation. This ISGylation stabilizes IRF3 by inhibiting its interaction with PIN1, a protein that promotes IRF3 ubiquitination and degradation. Consistent with a gain of function mechanism, HERC5 promotes expression of IRF3-dependent genes during viral infection, and attenuates replication of several viruses, including VSV.

In addition to cellular ISGylation targets, recent reports implicate viral proteins as targets of ISG15 modification. These studies provide fresh insights into the antiviral mechanisms of ISG15.

Specific targeting of influenza A NS1 protein

To determine whether targeting of any viral proteins is involved in ISG15-mediated impairment of influenza A replication, Krug and colleagues co-expressed influenza A proteins with the ISGylation machinery and find that the NS1 protein of the H3N2 influenza A/Udorn/72 (Ud) virus is an ISG15 substrate (Zhao et al. 2010). ISGylation of NS1A could also be observed following infection of IFNβ-treated cells with Ud virus. Moreover, NS1A binds specifically to HERC5 but not the closely related HERC4 and HERC6. Similarly, Wang and colleagues find that NS1A interacts with HERC5, and that HERC5 promotes its ISGylation (Tang et al. 2010). NS1A is a virulence factor that can inhibit host cell pre-mRNA processing and the IFN-induced 2’ to 5’ oligo(A) synthetase/RNase L pathway.

Importantly, both groups find evidence that ISGylation of NS1A impairs influenza replication, although different conclusions were reached regarding the mechanism(s) of this impairment.

Through a combination of affinity purification, mass spectrometry, and mutagenesis, Krug and colleagues find that NS1A Lys 41 appears to be the major ISG15 acceptor site. As this lysine lies within the region of NS1A responsible for binding to double-stranded RNA and the nuclear import factor importin-α, the authors assayed the ability of ISGylation to affect either of these interactions. Whereas ISGylated NS1A binds as well as non-ISGylated NS1A to polyI:C, it fails to interact with importin-α, suggesting that ISGylation of NS1A causes a specific loss of function. Importantly, K41R mutation significantly enhances the ability of the virus to replicate in the presence of IFNβ, suggesting that specific targeting of NS1A protein by ISG15 impairs influenza A replication through a loss of function mechanism (Figure 1B).

By contrast, mutagenesis results from Wang and colleagues indicate that ISGylation of multiple lysines on NS1A contributes to the impairment of viral replication. Moreover, ISGylation of NS1A appears to cause a severe impairment in the binding to U6 snRNA and dsRNA. In addition, ISGylation also impairs self-interaction of NS1A.

The reasons for the discrepancies regarding NS1A's ISGylation site(s) and the ability of ISGylated NS1A to bind to RNA are unclear. It is noteworthy that the influenza viruses used by the two groups differ in origin, so their interactions with the host cell may be different. In any case, these reports identify the first viral ISG15 target, and suggest that ISGylation of this target impairs viral replication through a loss of function mechanism. It is at present not clear how ISGylation of a small percentage of NS1A leads to such a dramatic impairment in viral replication.

Broad targeting of newly synthesized viral proteins

A recent article in *Molecular Cell* suggests an intriguing model for understanding the antiviral activity of ISG15 (Durfee et al. 2010). Only a minority of constitutively expressed proteins from the aforementioned proteomics study could be confirmed as ISGylation substrates at their endogenous levels, even when ISG15 and the ISGylation enzymes are overexpressed. By contrast, most of these proteins are confirmed as ISGylation substrates when they are exogenously expressed along with the ISGylation machinery. In fact, most (but not all) exogenously expressed proteins, including bacterial proteins and the TAP affinity tag, are also ISGylated using this method. These results raise doubts regarding the physiological significance of putative ISGylation substrates.

Yet Huibregtse and colleagues embraced what could easily have been dismissed as a technical artifact. Their subsequent results suggest that a key variable determining whether or not a protein gets ISGylated is its new synthesis in the presence of ISG15 and ISGylation machinery. Proteins that are newly synthesized, for instance those that are expressed from a transfected plasmid, in the presence of the ISGylation machinery are readily ISGylated. Moreover, multiple fragments of a protein that are expressed as deletion mutants appear equally susceptible to ISGylation, suggesting a lack of rigid specificity determinants within the protein structure as might have been presumed. A potential explanation of these results is that newly-synthesized proteins are targets for ISGylation; indeed, fractionation of cytosolic extracts reveals that HERC5 is associated with ribosomes. Thus the authors propose that HERC5 broadly, and at least somewhat non-specifically, targets newly-synthesized proteins for ISGylation (Figure 1C).

This idea implies that some viral proteins will be ISGylated during replication. As some viral structural proteins, such as those that make up the capsid, must precisely assemble into higher order structures, it is possible that ISGylation of a small fraction of these proteins could have a dominant negative effect. Indeed, using human papillomavirus (HPV) pseudovirus system, in which the HPV L1 and L2 capsid proteins are able to package a plasmid expressing green fluorescent protein and deliver it to new cells, the authors show that ISGylation of approximately 10% of L1 protein is associated with a 70% decrease in infectivity. The mechanistic basis of the infectivity impairment by ISG15 remains to be determined; perhaps entry of the virus into new cells or release of the nucleic acids into the infected cells is impaired. In any case, the results suggest that ISG15 can indeed cause a dominant negative impairment of viral protein function, an appealing idea that might explain how ISGylation of a small fraction of a given protein can have potent antiviral effects. In addition, as postulated by the authors of this report, these findings suggest that ISGylation of some, perhaps most, host proteins could be a by-product of the cell's effort to maximize ISGylation of viral proteins.

Perspectives

Although ISG15 is the first UBL known to exist, its biological role and mechanism of action are less well understood than most of the other UBLs, such as SUMO or NEDD8. This is in part due to the absence of homologues of ISG15 and its conjugation machinery (e.g, UBE1L) in experimental organisms such as yeast, *Drosophila* or *C. elegans*. Nevertheless, significant progress has been made in the past few years in the identification of the enzymatic machinery that carries out ISGylation and in the elucidation of the role of ISGylation in antiviral defense. The recent findings of the direct antiviral activity of ISG15 through both specific and broad modification of viral proteins represent a major advance in understanding the antiviral mechanisms of ISGylation. Some ISGylated host proteins also appear to mediate its antiviral effects (for example ISGylated 4EHP and IRF3 as mentioned above).

Although upregulating the expression of ISGylation machinery is a primary means of regulating ISGylation, additional regulatory mechanisms clearly exist, for example, NS1B's binding to ISG15 and HERC5's association with ribosomes and specific substrates like NS1A. Biochemical reconstitution of the ISGylation process would potentially facilitate the identification of additional factors that regulate ISGylation.

An emerging theme from the recent mechanistic studies is that ISGylation alters a protein's ability to engage in its typical interactions (such as with other proteins or RNA). The basis for this alteration is as yet unclear. It is likely that the presence of ISG15 could directly interfere with the normal protein-protein or protein-RNA interface. It is also feasible that ISGylation could induce allosteric changes in protein structure, or that ISG15-binding protein(s) may be present in cells and could modulate interactions between ISGylated proteins and their typical partners.

It is noteworthy that mice lacking ISG15 are not as susceptible to viral infection as IFN receptor knockout mice, indicating that ISGylation contributes to, but is not solely responsible for, the antiviral effects of IFN in mice (Lenschow et al., 2007). Recent work demonstrating marked differences in the interaction between influenza B virus and the ISGylation machinery of mice and humans suggests that ISG15 might play a more prominent antiviral role in human. Indeed, blocking ISGylation in human cells severely impairs IFN-induced antiviral activity against Influenza A virus (Hsiang et al., 2009). Future research could also reveal other functions of ISGylation unrelated to its antiviral effect.

Indeed, the levels of ISG15 and its conjugation to cellular proteins are elevated in several tumors and tumor-derived cell lines (Desai et al., 2006).

Understanding the roles and mechanism of action of ISGs, such as ISG15, in antiviral defense may pave the way to more effective antiviral therapies. For example, viral proteins that counter the IFN response by antagonizing ISGylation might make appealing therapeutic targets.

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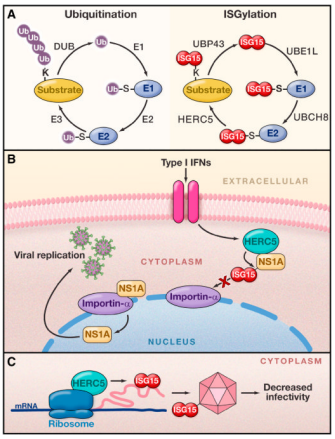

Figure 1. ISGylation and Its Antiviral Mechanisms

A. ISG15, like ubiquitin, is attached to substrates in a three-step enzymatic cascade. In the first step, ISG15 is "activated" by UBE1L in an ATP-dependent process. ISG15 is then transferred to the E2 UBCH8, and subsequently to a target protein through the E3 HERC5. Like ubiquitin, ISG15 is conjugated to a lysine on the target protein through a C-terminal glycine-glycine motif.

B. Type I interferons (IFNs) induce expression of ISG15 and ISGylation machinery including HERC5. During infection with influenza A, nonstructural protein 1 (NS1A) protein is ISGylated on lysine 41. ISGylation inhibits the binding of NS1A to the nuclear import factor importin-α. Mutation of this lysine largely protects influenza A from the antiviral actions of type I IFN.

C. HERC5, likely due to its association with ribosomes, broadly targets newly-synthesized proteins for ISGylation. ISGylation of certain viral proteins, including those that make up the capsid, could have a dominant negative effect by interfering with the precise assembly of higher order structures. Thus ISG15 can cause a significant impairment in viral infectivity despite ISGylation of only a small percentage of the target proteins.