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Emerging Roles of Protein Deamidation in Innate Immune Signaling

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Protein deamidation has been considered a nonenzymatic process associated with protein functional decay or "aging." Recent studies implicate protein deamidation in regulating signal transduction in fundamental biological processes, such as innate immune responses. Work investigating gammaherpesviruses and bacterial pathogens indicates that microbial pathogens deploy deamidases or enzyme-deficient homologues (pseudoenzymes) to induce deamidation of key signaling components and evade host immune responses. Here, we review studies on protein deamidation in innate immune signaling and present several imminent questions concerning the roles of protein deamidation in infection and immunity.

Innate immunity is the first line of defense against invading pathogens. Central to host immune responses is the detection of pathogen-associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs) (1). Retinoic acid-induced gene I (RIG-I) is a cytosolic receptor that senses double-stranded RNA (dsRNA) originating from pathogens such as viruses (2–5). Binding to dsRNA disrupts an intramolecular interaction that keeps RIG-I in an autoinhibitory state (6, 7), triggering an overall conformational change that releases the N-terminal CARD domain (8, 9). The N-terminal CARD of RIG-I undergoes homotypic oligomerization and heterooligomerization with that of the mitochondrion antiviral signaling (MAVS) adaptor molecule (10). Oligomerized MAVS forms prion-like filaments that are capable of activating two kinase complexes, IκB kinase alpha beta gamma (IKKαβγ) and IKKε–TBK-1, which, in turn, activate NF-κB and interferon (IFN) regulatory factors (IRFs) (11–13). Along with other transcription factors, NF-κB and IRFs upregulate the expression of intrinsic antiviral molecules (e.g., Mx and viperin) and the secretion of various cytokines (e.g., interferon) that further induce the expression of a network of a few hundred antiviral genes (14). Given the potent activity of RIG-I in inducing inflammatory responses, it is not surprising that RIG-I activation is regulated by multiple mechanisms in response to viral infection. For example, noncovalent binding and covalent conjugation of the Lys63-linked polyubiquitin chain to the CARD domain are reported to activate RIG-I (15–17), whereas phosphorylation by protein kinase C and casein kinase represses and dephosphorylation promotes RIG-I-mediated signaling (18–20). These are key cellular events that have been evolved to tightly regulate RIG-I-mediated immune activation in response to viral infection.

Viruses often evolve intricate mechanisms to deflect host immune responses. While RNA viruses deploy various proteins to blunt RIG-I-mediated innate defenses by hampering key signaling components such as RIG-I and MAVS, DNA viruses can manipulate the signaling cascade to benefit their infection (21–23) (Fig. 1). Studies of RNA viruses have identified distinct viral factors that target RIG-I and MAVS. Influenza virus NS1 derails RIG-I ubiquitination by nullifying the essential TRIM25 E3 ligase (24). Notably, hepatitis C virus uses its NS3/4A protease to cleave MAVS and release it from the mitochondrial membrane (25–27), thereby halting RIG-I-dependent antiviral immune responses. A similar strategy is employed by hepatitis G virus, hepatitis A virus, enterovirus 71, and coxsackievirus to derail IFN production (28–31). DNA viruses utilize strategies that are more intricate than those utilized by RNA viruses to evade these innate immune signaling cascades. The manipulation of innate and adaptive immune responses by herpesviruses has been previously well reviewed (21). One interesting example is murine gammaherpesvirus 68 (γHV68), which requires MAVS for efficient lytic replication. γHV68 is a model herpesvirus for human Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). With a combination of genetic and biochemical analyses, Dong et al. showed that the downstream IKKβ kinase is usurped to phosphorylate viral replication *trans*-activator (RTA) and thereby promotes viral lytic gene expression (32). Additionally, IKKβ is also coopted to phosphorylate p65 (also known as RelA), which primes p65 for proteasome-mediated degradation in conjunction with the RTA E3 ligase, thereby terminating NF-κB activation (33, 34). Thus, MAVS-dependent signaling is critical for efficient productive replication of γHV68.

HIJACKING RIG-I TO EVADE CYTOKINE PRODUCTION VIA DEAMIDATION

In order to characterize the virus-host interactions that instigate MAVS-dependent signaling, a screen for open reading frames from γHV68 that activate MAVS-dependent signaling revealed that ORF75C is a potent activator of RIG-I and MAVS signaling (35). Genetic and biochemical studies demonstrated functional and direct physical interactions between ORF75c and RIG-I. ORF75 genes are highly conserved in all gammaherpesviruses. While the KSHV genome encodes ORF75 and EBV encodes BNRF1, ORF75 genes are duplicated in herpesvirus saimiri (ORF3 and ORF75) and triplicated in murine γHV68 (i.e., ORF75a, -b, and -c). Accumulating studies describe antagonism of members of the promyelocytic leukemia (PML) family as a common immune evasion function of gammaherpesvirus ORF75 proteins (36–38). These studies have been extensively reviewed elsewhere (70). Here, we discuss the deamidating activity of ORF75 proteins of gammaherpesviruses.

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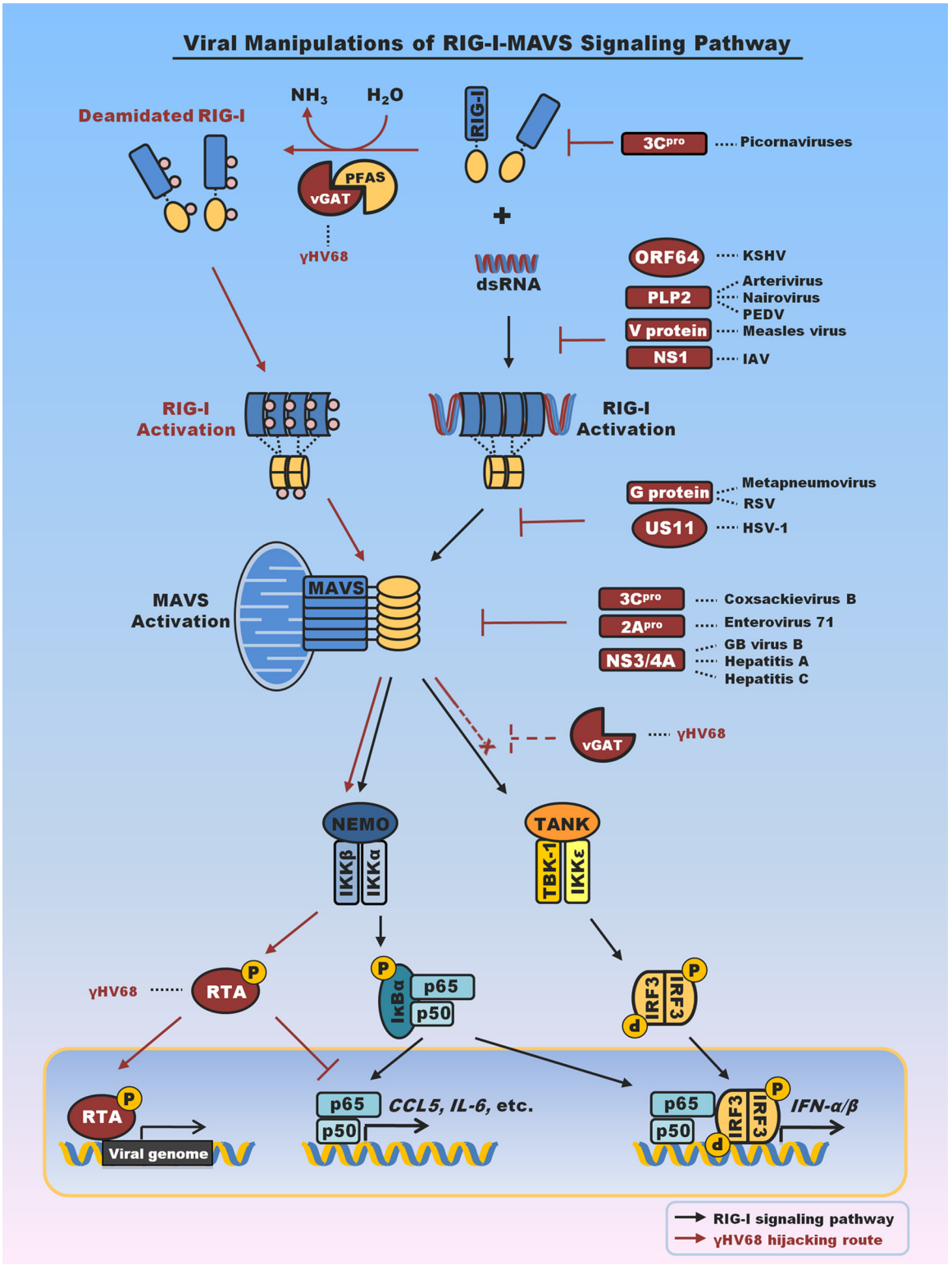

FIG 1 Summary of viral factors that interfere with or hijack RIG-I-mediated innate immune signaling. Emphasis is placed on viral proteins that interfere with RIG-I or MAVS to evade antiviral cytokine production. Notably, viral proteins that target TBK-1 and IRF3 to block interferon production are not included here. vGAT proteins of KSHV and γHV68 recruit PFAS to deamidate and activate RIG-I. Activation of RIG-I and its downstream signaling events, specifically, those associated with IKKβ, result in p65 degradation and suppress inflammatory cytokine production (33–35). vGAT appears to blunt IRF activation by an unknown mechanism (indicated by dashed inhibition sign). PEDV, porcine epidemic diarrhea virus; IAV, influenza A virus; RSV, respiratory syncytial virus; HSV-1, herpes simplex virus 1; Hepatitis A, hepatitis A virus; Hepatitis C, hepatitis C virus; GB virus, hepatitis G virus; pro, protease; NEMO, NF-κB essential modulator; TANK, TRAF family member-associated NF-κB activator. RTA, replication and transcription activator.

The gammaherpesvirus ORF75 shares homology with phosphoribosyl-formylglycinamidine synthetase (PFAS; also known as FGARAT of phosphoribosyl-formylglycinamidine [FGAM]), a cellular glutamine amidotransferase (GAT), and is thus also referred to as vGAT. PFAS catalyzes reaction 4 of the 10 steps of purine *de novo* synthesis. However, the vGAT proteins of γHV68 cannot complement cells deficient in PFAS (40). The carboxyl-terminal GAT domain of vGAT is sufficient to interact with RIG-I but fails to activate RIG-I. Coupled with the fact that vGAT proteins share homology with cellular GATs, this observation suggests that vGAT-induced RIG-I activation may require the enzymatic activity of GAT. Indeed, treatment of cells expressing vGAT with a GAT inhibitor specifically diminished signaling downstream of RIG-I, but not that downstream of IKKβ, indicating that GAT activity is specifically required for events upstream of IKKβ (e.g., RIG-I). Two-dimensional gel electrophoresis showed that vGAT reduced the RIG-I charge, and mass spectrometry analysis identified three site-specific deamidations within RIG-I: Q10 in the CARD domain and N245 and N445 within the helicase 1 domain. Simultaneous deamidation of all three residues led to constitutive activation of RIG-I in the absence of RNA (35), revealing a new means to activate the RIG-I receptor. Consistent with previous findings on MAVS and IKKβ (32, 33), activated RIG-I is usurped to evade antiviral cytokine production via inducing the degradation of p65, the key subunit of the transcriptionally active NF-κB dimer. Whether deamidated and activated RIG-I is required for efficient replication of γHV68 remains an open question.

vGAT proteins lack the catalytic triad essential for amidotransfer/deamidation catalysis (35), suggesting that vGAT may recruit a cellular enzyme(s) to induce the deamidation and concomitant activation of RIG-I. Enzymes involved in nucleotide biosynthesis have a propensity to form homo-oligomers in order to regulate enzymatic activity (39, 41), so it is possible that vGAT proteins recruit cellular PFAS to deamidate RIG-I. Indeed, vGATs of KSHV, EBV, and γHV68 interact with PFAS, but only vGATs of KSHV and γHV68 induce RIG-I deamidation and activation. Depletion or pharmacological inhibition of PFAS recapitulates the phenotype of fibroblasts deficient in RIG-I and MAVS, i.e., increased cytokine production in response to γHV68 infection (33–35). Furthermore, purified vGAT and PFAS deamidated RIG-I *in vitro*. Neither vGAT nor PFAS alone was sufficient to induce RIG-I deamidation, suggesting that vGAT activates PFAS by an intrinsic mechanism. For the first time, these studies demonstrated that a PRR is activated by a deamidase consisting of a metabolic enzyme and a viral pseudoenzyme rather than by its conventional ligand. This work also describes a new function of the cellular metabolic PFAS enzyme in deamidating asparagines and glutamines of RIG-I to regulate innate immune signaling (35), suggesting that protein deamidation could play pivotal roles in regulating innate immune signaling.

PROTEIN DEAMIDATION

Protein deamidation was initially reported more than half a century ago (42). Early work focused on the nonenzymatic deamidation of asparaginyl and, to a lesser extent, glutaminyl residues of proteins *in vivo* and *in vitro*. Analyzing a large set of proteins, Robinson and Robinson showed that the rate of asparaginyl deamidation was determined by the primary sequence, secondary and tertiary structures of the protein, and cellular environment (such as pH) (43). The ubiquitous distribution of asparaginyl/glutaminyl residues and frequent deamidation thereof in proteins, coupled with the finding that surrounding sequences determine the rate of protein deamidation, prompted the postulation that nonenzymatic protein deamidation serves as an internal clock to time the biological events of a particular protein (44). Thus, nonenzymatic deamidation may be a built-in clock to time protein functional decay or "aging."

(i) Enzymes (deamidases) that catalyze protein deamidation. Emerging studies indicate that microbes deploy protein deamidation to manipulate key signaling components to promote their infection. Microbial enzymes constitute the founding members of the protein deamidase family and offer an opportunity to answer fundamental questions concerning protein deamidation. Work on the PFAS-vGAT protein-deamidating complex has described a new regulatory function of the metabolic PFAS enzyme, and likely other glutamine amidotransferases, in innate immune signal transduction. This work raises a number of imminent questions that are fundamental to enzymology and innate immune signaling. First, how does the amidotransferase activity of PFAS in purine synthesis correlate with its protein-deamidating activity in virus-infected cells? PFAS is known to catalyze amidotransfer from free glutamine to synthesize phosphoribosyl-formylglycinamidine (FGAM) in purine synthesis (45), whereas the PFAS-vGAT complex is capable of deamidating asparaginyl and glutaminyl residues of RIG-I (35). Cellular GAT enzymes catalyze the biosynthesis of nucleotides, amino acids, glycoprotein, and NAD (46). The fact that PFAS can participate in both purine synthesis and RIG-I activation suggests that nucleotide metabolism is linked to innate immune signaling. Indeed, inhibitors targeting dihydroorotate dehydrogenase, a key enzyme of the *de novo* pyrimidine synthesis pathway, reduced viral replication (47–49). Later, it was shown that this antiviral activity relies on an elevated cellular immune response (50). The molecular link between reduced pyramidine biosynthesis and increased antiviral gene expression remains unknown. Presumably, the nucleotide pool within cells infected by a virus *in vivo* is small, so activating nucleotide biosynthesis is imperative for efficient viral transcription and genome replication. An elegant example is how cellular SAMHD1 nucleotide hydrolase restricts human immunodeficiency virus (HIV) via depleting the nucleotide pool available for HIV replication (51). If vGAT activates PFAS for purine biosynthesis and RIG-I-dependent evasion of antiviral cytokine production, a proper division between the two enzymatic activities (i.e., protein deamidation and purine synthesis) is critical for viral lytic replication. In support of this notion, murine γHV68 robustly upregulates the protein expression of PFAS, partly by increasing its mRNA level (35). Future work concerning PFAS regulation in virus-infected cells will likely further elucidate the possible cross talk between innate immune signaling and nucleotide biosynthesis.

Second, how does RIG-I access the active site of the PFAS deamidase domain? Cellular glutamine amidotransferases consist of a GAT domain and a metabolite synthetase domain connected by an ammonia channel. Structural analysis of the *Salmonella enterica* serovar Typhimurium homolog of PFAS revealed a relatively buried catalytic triad for deamidation facing the internal ammonia channel (39). If human PFAS is structurally similar to *Salmonella* PFAS, binding to vGAT perhaps triggers a conformational change that exposes the catalytic triad to accommodate as-

TABLE 1 Emerging discoveries of mammalian protein deamidations

Functional consequence	Deamidated cellular target(s)	Deamidase/other mechanism(s)	Deamidase-encoding species	Reference
		G protein signaling pathways		
Activation of G protein signaling	Rho GTPases ● RhoA ● Rac ● Cdc42	Cytotoxic necrotizing factors (CNFs)	*Escherichia coli* (EPEC) *Yersinia pseudotuberculosis*	60 53
	Heterotrimeric G proteins ($G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$)	*Pasteurella multocida* toxin (PMT)	*Pasteurella multocida*	61
	Rho GTPases ● Rac ● Cdc42	VopC	*Vibrio parahaemolyticus*	62
		Translation/cell cycle progression/apoptosis		
Inhibition of translation	Eukaryotic initiation factor 4A (eIF4A)	*Burkholderia* lethal factor 1 (BLF1)	*Burkholderia pseudomallei*	63
Critical switch (checkpoint) of apoptosis induced by DNA damage	B-cell lymphoma—extra large (Bcl-X_L)	pH change	NA[a]	64
Inhibition of cell cycle progression	Ubiquitin NEDD8	Cycle-inhibiting factors (Cifs)	*Escherichia coli* *Yersinia pseudotuberculosis* *Burkholderia pseudomallei* *Photorhabdus asymbiotica* *Photorhabdus luminescens*	58
		Innate immunity and inflammatory responses		
Inactivation of ubiquitin system and its related pathways, e.g., NF-κB signaling pathway	Ubiquitin	Cycle-inhibiting factors (Cifs)	*Escherichia coli* *Yersinia pseudotuberculosis* *Burkholderia pseudomallei* *Photorhabdus asymbiotica* *Photorhabdus luminescens*	58
Inhibition of UBC13/TRAF6-dependent inflammatory responses	Ubiquitin-conjugating enzyme 13 (UBC13)	OspI	*Shigella flexneri*	59
Activation of RIG-I signaling to evade inflammatory signaling	Retinoic acid-inducible gene 1 (RIG-I)	vGAT (ORF75/ORF75c) + phosphoribosylformylglycinamidine synthase (PFAS)	Murine herpesvirus 68 (ORF75c) Human herpesvirus 8 (ORF75) *Homo sapiens* (PFAS)	35
Recognition by gut-derived T cells to promote intestinal inflammation	Gliadin (wheat), etc.	Tissue transglutaminase (TG2)	*Homo sapiens*	65
		Others		
N-end rule pathway of protein degradation	Model substrate of N-end rule pathway	N-terminal glutamine amidase (NTQA)	*Mus musculus*	66
Alteration of the kinetics of excitatory synaptic transmission	Eukaryotic initiation factor 4E-binding protein 2 (4E-BP2)	pH change	NA	67

[a] NA, not applicable.

paraginyl and glutaminyl residues of RIG-I. The GAT domain is loosely connected to the FGAM synthetase domain (39), which likely provides flexibility and enables substrate accessibility. Such a conformational change may uncouple deamidation from ammonia channeling to the catalytic center of the FGAM synthetase, thereby facilitating the release of free ammonia. Interestingly, mutations blocking the ammonia channel between the GAT domain and the neighboring synthetase domain of imidazole-glycerol-phosphate synthase increased the deamidating activity of the glutaminase domain by more than 3 orders of magnitude (52), suggesting that ammonia release is a mechanism to significantly elevate glutamine hydrolysis. vGAT may deploy a similar mechanism of uncoupling the ammonia channel from the enzymatic domain to deamidate RIG-I, although the detailed mechanism of vGAT activation of PFAS requires further investigation.

Third, it is unclear whether PFAS, and other glutamine amidotransferases, can deamidate proteins in the absence of gammaherpesvirus vGAT proteins. RIG-I deamidation was not observed in cells infected with Sendai virus or γHV68 deficient in vGAT. If PFAS and other GATs can deamidate proteins in mammalian cells, it would be interesting to quantify how viral infection impacts the spectrum of deamidated proteins. This would be best assessed by a proteome-wide deamidation analysis. The herpesvirus proteome offers an excellent platform to systematically ana-

lyze protein deamidation. vGATs of KSHV, EBV, and γHV68 display similar interactions with PFAS, but only EBV vGAT failed to deamidate RIG-I, implying that vGATs and PFAS may have other functions shared by all three gammaherpesviruses such as nucleotide metabolism and evasion of intrinsic antiviral immunity. Although the genomes of herpes simplex viruses (HSV) contain no homolog of vGAT, HSV-1 infection induced a robust reduction in RIG-I's charge, indicative of deamidation. This suggests that herpes simplex viruses may have evolved a different mechanism for inducing RIG-I deamidation. Future studies may reveal a new example of protein deamidation in innate immune signaling.

In contrast to viral pseudoenzymes, several bacterial proteins appear to possess intrinsic deamidase activity toward multiple signaling molecules. Cytotoxic necrotizing factors (CNFs) produced by uropathogenic (CNF1) or enteropathogenic (CNF2) *Escherichia coli* deamidate and constitutively activate small G proteins (53–55). Similarly to chemotaxis D (CheD) deamidase, CNFs form a common α/β/β sandwich that contains the catalytic dyad in a shallow cavity at the top of the protein (56, 57). The members of the Cif family of effectors secreted by *Burkholderia pseudomallei* and enteropathogenic *E. coli* and the OspI effector secreted by *Shigella flexneri* can deamidate ubiquitin/Nedd8 and the UBC13 E2 enzyme, respectively (58, 59). Deamidation of these signaling components by bacterial effectors is essential for evading cellular immune responses and the pathogenesis of these microbes. These studies define the structure and function of protein deamidases in pathogen infection.

(ii) Protein targets of enzyme-catalyzed deamidation. Protein deamidation catalyzed by enzymes is generally rapid and tightly regulated. To date, we have understood the functional consequences of deamidation of a small subset of proteins in prokaryotes and mammalian cells (Table 1). Initial studies showed that key cellular signaling molecules are deamidated by pathogenic microbes to facilitate their invasion, which underpins their pathogenesis (68). Small G proteins such as RhoA and Cdc42 require a key glutamine residue for GTP hydrolysis. Deamidation of the conserved glutamine by pathogenic *E. coli* locks these G proteins in a GTP-bound state, resulting in the constitutive activation of G proteins and stress fiber formation (53, 60, 62). Interestingly, deamidation is also crucial for regulating signal transduction in bacterial chemotaxis. In nonenteric bacteria, the chemotaxis C (CheC) phosphatase and methyl-accepting chemotaxis proteins (known as MCPs) are deamidated by the CheD polypeptide and are required for directional chemotaxis (57). Mammalian ubiquitins Nedd8 and UBC13 have recently been shown to be deamidated by effectors secreted by enteric pathogenic *E. coli* and *Shigella*, respectively (58, 59). Deamidation of these cellular signaling molecules inactivates the ubiquitin proteasome system that is critical for signal transduction downstream of tumor necrosis factor alpha (TNF-α), an important component of the host immune defense system. In response to DNA damage, anti-apoptotic Bcl-x_L is targeted for degradation via deamidation (64). Recently, the deamidation of 4E-BP2 was shown to promote its association with mammalian target of rapamycin (mTOR) and modulate neuronal excitatory synaptic transmission (67). It was postulated that the deamidation of Bcl-x_L and 4E-BP2 is a nonenzymatic process and results from an increase in cellular pH. Deamidation of other key proteins is implicated in regulating some fundamental processes (e.g., cell-matrix interaction) and underpins medically important diseases (e.g., Alzheimer's disease) (69).

CONCLUSION AND PERSPECTIVES

Recent studies of enterobacterial effectors and gammaherpesviral pseudoenzymes implicate a new function of protein deamidation in regulating innate immune signal transduction. This is likely just the tip of iceberg concerning the general regulatory roles of protein deamidation in fundamental biological processes. That metabolic glutamine amidotransferases, such as the vGAT-PFAS complex, are capable of deamidating proteins is interesting in that these enzymes may provide a physical and functional link to other biological processes. However, these studies have generated more questions than answers in regard to fundamental principles concerning protein deamidation. For example, how are these cellular GATs regulated and delegated between metabolism and signal transduction in response to infection? What sequence and structural elements of GATs (e.g., PFAS) enable their dual functionality or substrate promiscuity in deamidating free glutamine and glutaminyl/asparaginyl residues? Is there sequence specificity of target deamidated proteins? If yes, is the specificity defined by the primary, secondary, or tertiary structure of the deamidated protein? Last, and importantly, is protein deamidation involved in other fundamental biological processes that are intrinsically linked to nucleotide biosynthesis, such as DNA damage and repair? These microbial studies have unveiled a new function of the simplest posttranslational modification of proteins, deamidation, in immune regulation and will certainly instruct us more in the years to come.

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