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A Chemical Reporter for Protein AMPylation

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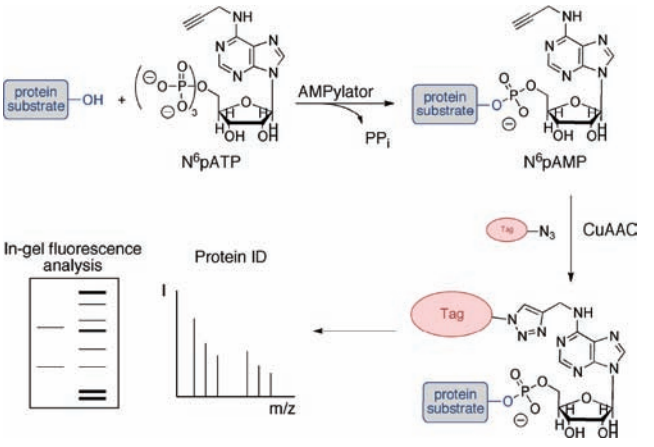
Supporting Information

ABSTRACT: Protein AMPylation is an emerging post-translational modification, which plays key roles in bacterial pathogenesis and cell biology. Enzymes with AMPylation activity, referred to as AMPylators, have been identified in several bacterial pathogens and eukaryotes. To facilitate the study of this unique modification, we developed an alkynyl chemical reporter for detection and identification of protein AMPylation substrates. Covalent functionalization of AMPylation substrates with the alkynyl reporter in lieu of adenylyl 5′-monophosphate (AMP) allows their subsequent bioorthogonal ligation with azide-fluorescent dyes or affinity enrichment tags. We show that this chemical reporter is transferred by a range of AMPylators onto their cognate protein substrates and allows rapid detection and identification of AMPylated substrates.

Protein AMPylation refers to the covalent modification of protein side chain hydroxyl groups with adenylyl 5′-monophosphate (AMP) through a phosphodiester bond. This post-translational modification is installed by the enzymatic transfer of AMP from adenosine-5′-triphosphate (ATP) to the substrate hydroxyl group. This catalytic activity was initially described for *Escherichia coli* glutamine synthetase (GS) adenylyl transferase, which tightly regulates GS activity.[1,2] Additional proteins with AMPylation activity (AMPylators) have been identified that contain either the filamentation induced by cAMP (fic) domain or the adenylyl transferase (ATase) domain, which confer AMPylation activity.[3,4] Many of these characterized AMPylators serve as bacterial virulence factors that are secreted into the mammalian host cell during infection. There, they AMPylate mammalian host proteins to alter their function for the benefit of the pathogen. In particular, secreted bacterial AMPylators, such as VopS (*Vibrio parahemolyticus*), IbpA (*Histophilus somni*), and DrrA (*L. pneumophila*) have been shown to target mammalian small GTPases, like RhoA, Rac1, Cdc42, and Rab1.[5−7] Another bacterial effector protein has been identified that selectively deAMPylates a GTPase, indicating the dynamic nature of this modification.[8,9] The AMPylation of small GTPases, on threonine or tyrosine residues, interferes with their proper function, either by sterically blocking the interaction with downstream signaling components or GTPase activating proteins (GAPs). Interestingly, fic and ATase domains are not limited to bacterial effector proteins, but have been identified in archaea and eukaryotes as well. Most eukaryotic genomes appear to contain a fic domain protein. AMPylation activity has been observed for the human protein HYPE and the *Drosophila* protein dFic.[4,6] The widespread presence of these domains suggests a ubiquitous role for protein AMPylation as a regulated and reversible post-translational modification. While radioactive ATP, targeted mass spectrometry, and specific antibodies can be used to detect AMPylated substrates, more general and efficient analytical tools are still needed for the unbiased identification of new AMPylated substrates and the analysis of their regulation.[10,11] We therefore developed an alkynyl chemical reporter for bioorthogonal detection, enrichment, and identification of AMPylated proteins (Scheme 1).

Alkynyl chemical reporters allow Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) of labeled substrates with azide-functionalized detection and enrichment reagents. This technology has facilitated the analysis of various post-translational modifications and nucleic acid biogenesis.[12] Recent structural studies of AMPylators and previous studies of fluorescent ATP analogues suggested that a modification of the N^6 position of the adenine ring could be tolerated.[13−17] Thus, we synthesized the ATP analogue N^6-propargyl adenosine-5′-triphosphate (N^6pATP) as a potential

Scheme 1. Detection and Identification of AMPylated Substrates with N^6-Propargyl Adenosine-5′-triphosphate (N^6pATP)[a]



[a] N^6pATP, N^6-propargyl adenosine-5′-triphosphate; N^6pAMP, N^6-propargyl adenosine-5′-monophosphate; PP_i, pyrophosphate; CuAAC, Cu(I)-catalyzed azide−alkyne cycloaddition; Tag, rhodamine fluorescence dye or cleavable biotin enrichment tag.[18,19]

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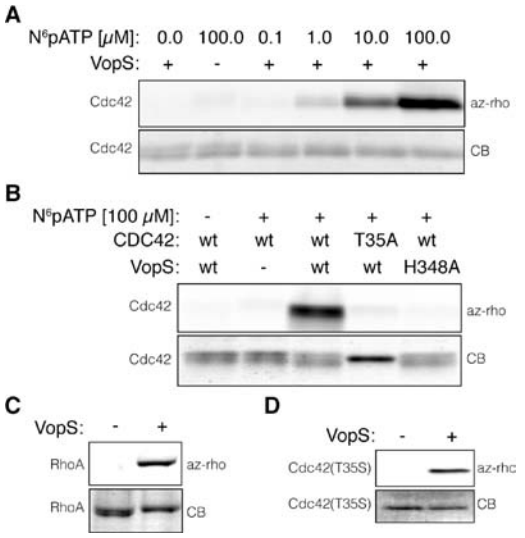

Figure 1. In vitro analysis of VopS activity, using N^6-propargyl adenosine-5′-triphosphate (N^6pATP) as a chemical reporter, by click-chemistry and in-gel fluorescence scanning. All AMPylation reactions were carried out in 15 μL total volume for 1 h at 30 °C. (A) Cdc42 (0.5 μg) was incubated with VopS (1 ng) and increasing concentrations of N^6pATP. (B) Cdc42 (0.5 μg) or Cdc42(T35A) (0.5 μg) was incubated with VopS (1 ng) or VopS(H348A) (1 ng) and N^6pATP. (C) RhoA (0.5 μg) was incubated with VopS (10 ng). (D) Cdc42(T35S) (0.5 μg) was incubated with VopS (10 ng). az-rho, azido-rhodamine fluorescence; CB, Coomassie blue.

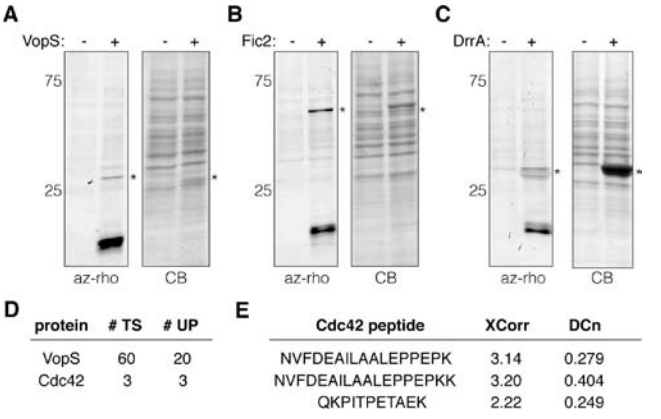

Figure 2. Detection of endogenous AMPylation substrates in cell lysates and proteomic substrate identification. AMPylation reactions (A−C) were carried out in 15 μL for 1 h at 30 °C. (A) VopS (100 ng) was added to HeLa cell lysate (10 μg) in the presence of N^6pATP (100 μM). (B) Fic2 (300 ng) was added to HeLa cell lysate (10 μg) in the presence of N^6pATP (100 μM). (C) DrrA (4 μg) was added to HeLa cell lysate (10 μg) in the presence of N^6pATP (100 μM). (D) Proteomic analysis of VopS substrates in HeLa cell lysate revealed two protein IDs above background, VopS and Cdc42. (E) Identified Cdc42 peptides. # TS, number of total spectra; # UP, number of unique peptides; XCorr, SEQUEST Xcorr score; DCn, SEQUEST ΔCn score; az-rho, azido-rhodamine fluorescence; CB, Coomassie blue; asterisk indicates auto-AMPylation and recombinant AMPylator.

chemical reporter for AMPylation (Supporting Information Scheme 1).

To assess the activity of N^6pATP as a possible chemical reporter for AMPylation, we used a well-established in vitro system, based on the recently identified AMPylator VopS and its cognate mammalian target Cdc42.[20] Cdc42(Q61L), subsequently referred to as Cdc42, was incubated with VopS in the presence of increasing concentrations of N^6pATP under previously reported in vitro AMPylation conditions.[21] N^6-propargyl adenylyl-5′-monophosphate (N^6pAMP) transfer was analyzed by CuAAC with azido-rhodamine fluorescent dye (az-rho) and in-gel fluorescence scanning.[19] Increasing concentrations of N^6pATP yielded a dose-dependent increase in fluorescence labeling of Cdc42 (Figure 1A). The chemical reporter proved to be transferred only in the presence of VopS and appeared to be highly selective for the native substrate Cdc42, as judged by the lack of concomitant BSA labeling (Supporting Information Figure 1). To investigate the selectivity of N^6pATP, we made use of substrate and AMPylator mutants. When VopS was incubated with the T35A mutant of Cdc42, in which the target threonine residue of VopS was mutated to an alanine, no transfer was observed (Figure 1B).[20] In addition, a catalytic inactive mutant of VopS, H348A, failed to transfer the chemical reporter (Figure 1B).[20] The dependence on this previously described catalytic histidine suggests that N^6pAMP is transferred by the same catalytic mechanism as the native AMP group. These data emphasize the distinct selectivity of N^6pATP, which was further demonstrated by competitive inhibition with ATP (Supporting Information Figure 2). N^6pATP appears to be a general cofactor for AMPylation, since it was also utilized by VopS to modify its alternative substrates RhoA (Figure 1C) and Rac1 (Supporting Information Figure 3). Moreover, it served as a chemical reporter for another fic domain AMPylator, IbpA (Fic2, Supporting Information Figure 4). Like VopS, Fic2 modifies Cdc42; however, it transfers AMP onto tyrosine residue Y32.[22] It should be noted that labeling of Cdc42(T35S) by VopS was also observed (Figure 1D), supporting the notion that AMPylation may occur on serine residues. We also tested the ATase domain AMPylator DrrA for its ability to utilize N^6pATP. Indeed, DrrA accepted N^6pATP as cofactor and labeled the reported protein substrate Rab1 (Supporting Information Figure 5).[7] These experiments demonstrate the versatile nature of N^6pATP as a chemical reporter for all known AMPylator families, namely, fic domain and ATase domain AMPylators. N^6pATP labeling appears to be independent of the target protein substrate and could also allow the identification of serine-modified AMPylation substrates.

We next analyzed the ability of VopS, Fic2, and DrrA to modify their substrates with N^6pATP in mammalian cell lysates. It has been reported that addition of purified VopS or Fic2 to HeLa cell lysates in the presence of ^{32}P-α-ATP results in the labeling of one distinct band in the molecular weight range of small GTPases, as assessed by autoradiography.[6,23] We incubated purified VopS, Fic2, and DrrA with Triton X-100 lysed HeLa cell lysates and N^6pATP (100 μM). As previously observed for ^{32}P-α-ATP labeled cell lysates, VopS and Fic2 labeled one distinct protein or protein population at the corresponding molecular weight of small GTPases (Figure 2A,B). Addition of DrrA also resulted in the modification of one distinct protein or protein population at ~21 kDa (Figure 2C), although more recombinant DrrA was needed to observe similar levels of labeling. In the absence of recombinant enzyme, the reporter did not significantly label the cell lysate. All three enzymes displayed auto-AMPylation activity as evidenced by a specific fluorescent signal at the corresponding molecular weight (Figure 2A−C). These results corroborate previous findings,

underscoring the distinctive selectivity of these effector proteins for small host GTPases.[6,7,23]

To explore the utility of N^6pATP for the unbiased identification of AMPylated substrates, we incubated HeLa cell lysate (1 mg) with N^6pATP (100 μM) in the presence or absence of VopS (88 μg). The bulk of the samples was then reacted with a cleavable biotin enrichment tag (*ortho*-hydroxy-azidoethoxy-azo-biotin) and incubated with Streptavidin beads for affinity capture of labeled proteins.[18] Captured proteins were digested on beads and the resulting peptides were collected and subjected to protein identification by tandem mass spectrometry. For data analysis, all protein hits of the negative control were subtracted from the VopS containing sample, which revealed two final protein identifications: VopS and Cdc42, with VopS peptides representing the majority of the identified peptides (Figure 2D,E). The dominant VopS fraction correlates well with the observed fluorescence profiling of the same samples (Supporting Information Figure 6). This data demonstrates that N^6pATP can be used to identify substrates of individual AMPylators in complex protein samples, such as cell lysates, and confirms Cdc42 as an endogenous VopS target.

In summary, we present an efficient chemical reporter for protein AMPylation, which is utilized by all known classes of protein AMPylators and has minimal nonspecific chemical reactivity in cell lysates. N^6pATP enables the unbiased detection and proteomic identification of specific AMPylator substrates in complex protein mixtures and should be a powerful chemical tool for the emerging field of protein AMPylation.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, chemical characterization, supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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