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Protein AMPylation

A new chemical handle for protein AMPylation at the host-pathogen interface

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Post-translational modification (PTM) of proteins plays a role in regulation and control of all major cellular processes.^[1] This phenomenon involves (generally enzymatic) covalent attachment of a specific moiety, e.g. a phosphate, carbohydrate, or lipid, to a particular amino acid side-chain of a target protein. This in turn alters its properties, for example enzymatic activity, sub-cellular localization, proteolytic stability, or ability to interact with other proteins. A common characteristic feature of PTMs is their dynamic nature, and the existence of enzymatic activities that reverse PTMs can allow regulation of cellular signaling. A number of recent reports have demonstrated how chemical approaches can be particularly powerful in unraveling the process of PTM, with protein lipidation providing a paradigm example.^[2, 3]

AMPylation, the covalent attachment of adenosine monophosphate (AMP) to hydroxyl side-chains of proteins via a phosphodiester bond, is yet another class of PTMs. It was initially observed in the 1970s in the context of metabolic regulation of *Escherichia coli* glutamine synthetase by glutamine synthetase adenylyl transferase,^[4] but its potential beyond metabolic regulation went largely unexplored for the next four decades. It is only very recently that the field of protein AMPylation has been revitalized by the discovery that pathogenic bacteria utilize specific effector proteins (AMPylators) to catalyze the transfer of AMP from adenosine triphosphate (ATP) to host guanosine triphosphate hydrolases (GTPases) and disrupt their interactions with cognate binding partners.^[5] Host GTPases are a major target of pathogenic bacteria as they are involved in various defense mechanisms, including apoptosis, pyroptosis, phagocytosis, and phagosome trafficking.^[6-8] Key bacterial AMPylators characterized to date include VopS, a cytotoxin of *Vibrio parahaemolyticus*, that AMPylates Rho family GTPases at a conserved threonine residue;^[9] IbpA secreted by *Histophilus somni*, that AMPylates the same target protein but modifies a tyrosine residue instead of a threonine;^[10] and finally DrrA (also known as SidM), a

Legionella pneumophila effector that AMPylates Rab1 GTPases also at a tyrosine.^[11] Both VopS and IbpA mediate AMPylation using a so-called Fido catalytic motif (canonical sequence: HPFx[D/E]GN[G/K]R)^[12] and target the host cell cytoskeleton, whilst DrrA utilizes an adenylyl transferase domain (canonical sequence: Gx₁₁DxD)^[11] and targets host cell protein trafficking. In 2011, the first de-AMPyating enzyme, or 'protein AMPylase', was discovered in *L. pneumophila*, confirming the importance and the dynamic nature of post-translational AMPylation.^[13,14] Apart from its role in pathogenicity, there is growing evidence that AMPylation may operate as a general intracellular signaling mechanism in normal cell function. Bacterial Fido domain proteins are involved in cell division, whilst HYPE, a human Fido motif protein, may regulate interactions of small GTPases with their binding partners.^[10] Furthermore, metabolic labeling experiments using ³²P-α-ATP show numerous radioactive protein bands in human cell lysates, suggesting the presence of additional AMPylating enzymes.^[9]

AMPylation is rapidly emerging as a fundamental mechanism serving to regulate protein-protein interactions and cell signaling in normal cells and during the invasion of mammalian cells by bacteria. However, without robust tools to identify and manipulate both AMP transferases and their AMPylated protein substrates our understanding of this complex signaling network will remain superficial. So far the majority of reported and predicted AMPylators in bacteria and eukaryotic cells have been found via bioinformatic methods utilizing sequence/structure similarities to Fido and adenylyl transferase domains.^[15] In turn, AMPylated substrate proteins have been identified by the combination of radioactive isotope labeling and targeted mass spectrometric analysis both on the MS and MS/MS level.^[9-11, 16] Two additional developments that complement the above-mentioned approaches include the elucidation of mass spectrometric fragmentation characteristics of AMPylation based on the analysis of synthetic peptides modified at serine, threonine, and tyrosine residues,^[17] and the generation of a polyclonal antibody specific towards threonine-AMPylation.^[18]

In a very recent report, a potentially more general, chemistry-based methodology for labeling AMP transferase substrates has been proposed by Hang, Orth and co-workers.^[19] Specifically, the authors took advantage of the chemical proteomic approach that has previously been applied for mapping the substrates of other PTMs, such as

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glycosylation, prenylation, and acylation, among others.^[20] The process relies on the enzymatic transfer of a chemical handle, a so-called bioorthogonal reporter (usually an azide or an alkyne), from a synthetic substrate analogue onto the target protein. This biotransformation is followed by a non-enzymatic but highly chemoselective reaction, in this case a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), with an appropriate ligation partner in order to introduce secondary labels (affinity tag or fluorophore) that allows target enrichment and/or detection. It is important to emphasize that this overall process of chemo-enzymatic labeling introduces a covalent linkage between chemical tag and the modified proteins, allowing far more stringent washing following pull-down than permitted by immunoprecipitation. In their work Orth and Hang^[19] have utilized an ATP analogue (AMP donor) that has been equipped by means of organic synthesis with the alkynyl tag at the N⁶ position of adenine ring (N⁶pATP), a site selected based on the structural data available for a selection of active Fido domains, like IbpA,^[21] VopS,^[22] and BepA, a *Bartonella henselae* effector.^[23] The presence of the alkynyl moiety in the ATP analogue enables subsequent CuAAC of tagged AMPylated substrates with azide-bearing capture reagents (Figure 1).

Figure 1. Chemo-enzymatic AMPylation of proteins with N⁶pATP.

Following the synthesis of N⁶pATP using a combination of published routes, the authors proceeded to validate the substrate activity and selectivity of the probe. It was demonstrated in the course of *in vitro* studies on purified recombinant proteins that the probe was transferred in a concentration-dependent manner by both Fido family (VopS and IbpA) and adenylyl transferase domain (DrrA) AMPylators onto their cognate mammalian targets, as assessed via CuAAC with azide-rhodamine and in-gel fluorescence. Furthermore, the probe was shown to be specific for its purpose as demonstrated by competitive inhibition with ATP as well as by the lack of probe transfer upon the application of catalytically inactive transferase mutants and transferase substrates in which the target threonine or tyrosine has been replaced by alanine. The performance of N⁶pATP was also verified against a background of mammalian cell lysate. In accordance with previous reports,^[9-11] in-gel fluorescence analysis of HeLa lysates treated with the probe and the above-mentioned recombinant AMP transferases revealed labeling of a distinct band in the molecular weight range of small

GTPases. Finally, in a preliminary experiment the authors applied their probe to mass spectrometry-based identification of an AMPylated substrate within a complex protein mixture. HeLa lysates were incubated with a high concentration of N⁶pATP (100 μ M) in the presence and absence of recombinant VopS (~8% w/w relative to lysate proteins), followed by CuAAC ligation to an azidobiotin reagent that should permit affinity-based enrichment of potential chemo-enzymatically tagged AMPylated substrates, and their subsequent identification by tandem mass spectrometry after on-bead tryptic digest. The proteomic analysis demonstrated the presence of the expected AMPylated target (Cdc42) following enrichment, albeit at relatively low spectral count. On the other hand, VopS itself was readily detected, as a result of its high concentration and apparent auto-AMPylation. This preliminary experiment is interesting, but the results remain somewhat tentative at present; VopS was present at a much higher concentration than it would be the case *in vivo*, a single analysis was performed without reconfirmation by anti-Cdc42 Western blot, and the full list of proteins identified +/- VopS was not disclosed. The probe shows great promise for *de novo* identification of novel substrates, but further experiments are required to confirm these observations.

N⁶pATP is thus a potentially robust tool for labeling, and potentially also identification, of known and novel protein substrates of both classes of known AMP transferases. Importantly, it can label a variety of targets allowing the identification of AMPylation on all three hydroxylated amino acid side-chains: threonine, tyrosine, and serine. The approach complements immunoprecipitation and brute force MS/MS techniques by offering greater flexibility thanks to provision of a versatile handle for further manipulation. As previously seen for lipid modifications, dye visualization or biotin-based enrichment of AMPylated proteins can offer far superior sensitivity and robustness compared to both traditional radiolabeling and PTM-sensitive antibodies, respectively. N⁶pATP itself is perhaps not ideal for *in vivo* applications due to its highly charged triphosphate moiety; however, a second generation of substrate probes optimised for bioavailability may also allow the identification of AMPylated proteins in living systems. This would enable dynamic pulse-chase experiments and direct fluorescence microscopy detection of effector-mediated AMPylation in its native environment, i.e. during infection of mammalian cells. Further probe modification may permit activity-based protein profiling (ABPP),^[24, 25] a complementary technique that could correlate AMPylated proteins with the activities of known AMP transferases, and provide new insights into the activity of as yet undiscovered enzymes in this class.

Intriguingly, very recent work has shown that the Fido domain-containing *Legionella* protein AnkX actually transfers phosphocholine from CDP-choline (to Rab1 and Rab35), rather than AMP from ATP^[26] suggesting that the substrate tolerance of the canonical Fido motif is broader than previously suspected. More generally, a rich variety of novel and chemically diverse PTMs undoubtedly remain to be discovered at the host-pathogen interface that may play key roles in pathogenesis. PTM is an inherently chemical phenomenon, and the work described here furthers the role of chemical proteomics as a central technology in this rapidly evolving field.

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- [1] E. W. Tate, *J. Chem. Biol.* **2008**, *1*, 17-26.
- [2] H. C. Hang, M. E. Linder, *Chem. Rev.* **2011**, *111*, 6341-6358.
- [3] a) W. P. Heal, B. Jovanovic, S. Bessin, M. H. Wright, A. I. Magee, E. W. Tate, *Chem. Commun.* **2011**, *47*, 4081-4083; b) A. F. Berry, W. P. Heal, A. K. Tarafder, T. Tolmachova, R. A. Baron, M. C. Seabra, E. W. Tate, *ChemBioChem* **2010**, *11*, 771-773; c) B. R. Martin, C. Wang, A. Adibekian, S. E. Tully, B. F. Cravatt, *Nat. Methods* **2011**, *in press*, doi: 10.1038/nmeth.1769.
- [4] M. S. Brown, A. Segal, E. R. Stadtman, *Proc. Natl. Acad. Sci. USA* **1971**, *68*, 2949-2953.
- [5] A. Itzen, W. Blankenfeldt, R. S. Goody, *Trends Biochem. Sci.* **2011**, *36*, 221-228.
- [6] C. Fiorentini, L. Falzano, S. Travaglion, A. Fabbri, *Cell Death Differ.* **2003**, *10*, 147-152.
- [7] T. Bergsbaken, S. L. Fink, B. T. Cookson, *Nat. Rev. Microbiol.* **2009**, *7*, 99-109.
- [8] G. Chimini, P. Chavrier, *Nat. Cell Biol.* **2000**, *2*, E191-196.
- [9] M. L. Yarbrough, Y. Li, L. N. Kinch, N. V. Grishin, H. L. Ball, K. Orth, *Science* **2009**, *323*, 269-272.
- [10] C. A. Worby, S. Mattoo, R. P. Kruger, L. B. Corbeil, A. Koller, J. C. Mendez, B. Zekarias, C. Lazar, J. E. Dixon, *Mol. Cell* **2009**, *34*, 93-103.
- [11] M. P. Muller, H. Peters, J. Blumer, W. Blankenfeldt, R. S. Goody, A. Itzen, *Science* **2010**, *329*, 946-949.
- [12] L. N. Kinch, M. L. Yarbrough, K. Orth, N. V. Grishin, *PLoS One* **2009**, *4*, e5818.
- [13] M. R. Neunuebel, Y. Chen, A. H. Gaspar, P. S. Backlund, Jr., A. Yergey, M. P. Machner, *Science* **2011**, *333*, 453-456.
- [14] Y. Tan, Z. Q. Luo, *Nature* **2011**, *475*, 506-509.
- [15] A. R. Woolery, P. Luong, C. A. Broberg, K. Orth, *Front. Microbiol.* **2010**, *1*, 113.
- [16] B. Zekarias, S. Mattoo, C. Worby, J. Lehmann, R. F. Rosenbusch, L. B. Corbeil, *Infect. Immun.* **2010**, *78*, 1850-1858.
- [17] Y. Li, R. Al-Eryani, M. L. Yarbrough, K. Orth, H. L. Ball, *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 752-761.
- [18] Y. H. Hao, T. Chuang, H. L. Ball, P. Luong, Y. Li, R. D. Flores-Saaib, K. Orth, *J. Biotechnol.* **2011**, *151*, 251-254.
- [19] M. Grammel, P. Luong, K. Orth, H. C. Hang, *J. Am. Chem. Soc.* **2011**, *133*, 17103-17105.
- [20] W. P. Heal, E. W. Tate, *Org. Biomol. Chem.* **2010**, *8*, 731-738.
- [21] J. Xiao, C. A. Worby, S. Mattoo, B. Sankaran, J. E. Dixon, *Nat. Struct. Mol. Biol.* **2010**, *17*, 1004-1010.
- [22] P. Luong, L. N. Kinch, C. A. Brautigam, N. V. Grishin, D. R. Tomchick, K. Orth, *J. Biol. Chem.* **2010**, *285*, 20155-20163.
- [23] D. V. Palanivelu, A. Goepfert, M. Meury, P. Guye, C. Dehio, T. Schirmer, *Protein Sci.* **2011**, *20*, 492-499.
- [24] W. P. Heal, T. H. Dang, E. W. Tate, *Chem. Soc. Rev.* **2010**, *40*, 246-257.
- [25] T. H. Tam Dang, R. P. Fagan, N. F. Fairweather, E. W. Tate, *Bioorg. Med. Chem.* **2011**, *in press*, doi:10.1016/j.bmc.2011.06.042.
- [26] S. Mukherjee, X. Liu, K. Arasaki, J. McDonough, J. E. Galan, C. R. Roy, *Nature* **2011**, *477*, 103-106.

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Entry for the Table of Contents

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HIGHLIGHTS

Tagging protein AMPylation. A new chemical reporter for AMPylation, recently identified as a key post-translational modification during bacterial infection, is a robust tool for detection and identification of AMPylated proteins *in vitro*.

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