Copper-catalyzed azide-alkyne cycloaddition (click chemistry)-based Detection of Global Pathogen-host AMPylation on Self-assembled Human Protein Microarrays* [§]

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AMPylation (adenylylation) is a recently discovered mechanism employed by infectious bacteria to regulate host cell signaling. However, despite significant effort, only a few host targets have been identified, limiting our understanding of how these pathogens exploit this mechanism to control host cells. Accordingly, we developed a novel nonradioactive AMPylation screening platform using high-density cell-free protein microarrays displaying human proteins produced by human translational machinery. We screened 10,000 unique human proteins with *Vibrio parahaemolyticus* VopS and *Histophilus somni* IbpAFic2, and identified many new AMPylation substrates. Two of these, Rac2, and Rac3, were confirmed in vivo as bona fide substrates during infection with *Vibrio parahaemolyticus*. We also mapped the site of AMPylation of a non-GTPase substrate, LyGDI, to threonine 51, in a region regulated by Src kinase, and demonstrated that AMPylation prevented its phosphorylation by Src. Our results greatly expanded the repertoire of potential host substrates for bacterial AMPylators, determined their recognition motif, and revealed the first pathogen-host interaction AMPylation network. This approach can be extended to identify novel substrates of AMPylators with different domains or in different species and readily adapted for other post-translational modifications. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M114.041103, 3164–3176, 2014.

Protein AMPylation (adenylylation) was recently discovered in bacteria-host interactions where virulence factors catalyze AMPylation using either a conserved Fic domain (e.g., VopS, *Vibrio parahaemolyticus* (V. para) and IbpA, *Histophilus somni* or an adenyl transferase domain (e.g., DrrA, *Legionella pneumophila*). These bacterial AMPylation enzymes, or AMPylators, are secreted into the host cells by bacterial secretion systems and transfer AMP from ATP to Tyr or Thr residues of their respective substrates (1–3). In the case of VopS and IbpA, several Rho family GTPases (Rac1, RhoA, and Cdc42) are known substrates and AMPylation disrupts the binding of the GTPase to its downstream effectors, for example, PAK1 (2–6). Considering the conservation of AMPylation domains in both prokaryotic and eukaryotic organisms, we expect that AMPylation plays an important role in a wide range of cellular processes (2, 4, 5, 7–9). Nevertheless, our understanding of this post-translational modification (PTM) is still limited to only a handful of known eukaryotic AMPylation substrates, exclusively belonging to the Rho and Rab GTPase families (10–14). Determining the repertoire of substrates modified by AMPylators will help illuminate both the functional consequences of AMPylation and the mechanistic strategies of pathogens that employ them (6).

Significant effort has been devoted to identifying AMPylation substrates. Li et al. systematically investigated the fragmentation patterns of chemically synthesized peptides with Thr, Ser, and Tyr AMPylation using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). They detected AMPylation sites with high confidence and selectively scanned AMPylated peptides in protein mixtures (10). Hao et al. produced a polyclonal antibody that specifically recognized proteins with AMPylation at threonine residues (11). Grammel et al. synthesized an ATP analog, N6pATP (N6-propargyl adenosine-5'-triphosphate), which allows the labeling of AMPylated proteins with azide-functionalized fluorescein or...
a cleavable biotin enrichment tag (ortho-hydroxy-azido-ethoxy-azobiotin) based on copper-catalyzed azide-alkyne cycloaddition (CuAAC). The identification of new substrates for VopS in HeLa cell lysates was explored by a combination of AMP-specific pull-down and LC-MS (12). Using the same approach, Lewallen et al. tried to identify the substrates of VopS in MCF7 cell extracts by employing a commercial N^6-(6-amino)hexyl-ATP-5-carboxyl-fluorescein (F1-ATP) and anti-fluorescein antibody. With these efforts combined, four potential new VopS substrates have been identified (SCCA2, NAGK, NME1, and PFKP), though not yet confirmed. These approaches might miss substrates because of temporal and spatial expression or low abundance in cell lysate, poor recognition by the capture molecules or loss during pull-down procedures (12, 14).

Protein microarrays offer a promising approach to identify candidate substrates because they display thousands of unique proteins in a high-throughput and reproducible format (15–17). However, producing arrays with consistent levels of well-folded proteins is challenging because of limitations of protein production, purification, and storage, particularly for mammalian proteins (18).

To circumvent these limitations, cell-free protein arrays, which do not require protein purification, have been developed over the past decade (19–22). These methods provide rapid and economical approaches of fabricating protein arrays in terms of cost, shelf life, and storage (23, 24). In cell-free protein arrays, a nucleotide template is printed on the slide and used to produce proteins in vitro with cell-free expression systems from several organisms such as E. coli, wheat germ, and rabbit reticulocyte lysate, etc. (24, 25). These proteins can be engineered to contain fusion tags that enable their capture to the array surface with an appropriate agent. Of these cell-free protein array methods, the Nucleic Acid Programmable Protein Array (NAPPA) is the most advanced, having achieved both high-density and high content containing ~2300–8000 proteins per slide (20, 26, 27). In NAPPA, a plasmid-based cDNA configured to include an epitope tag is printed on a microscope slide along with the corresponding tag-specific binding reagent, such as an anti-tag antibody, and stored. At the time of experimentation, the cDNA is transcribed/translated into recombinant protein and captured/displayed in situ by the binding reagent. Using a rabbit reticulocyte lysate-based cell-free expression system, NAPPA has been applied toward the identification of novel protein-protein interactions and disease-related antibody biomarkers (20, 26, 28, 29). However, cell-free protein arrays have yet to be employed in the study of PTMs.

In this work, we established a novel, nonradioactive unibased AMPylation screening platform by developing a novel click chemistry-based detection assay for use on high-density cell-free protein microarrays displaying human proteins. Labelling AMP-modified substrates covalently with a fluorophore coupled with the use of human ribosomal machinery and chaperones to produce proteins achieved much higher sensitivity and signal to noise (S/N) ratio compared with previous studies. We screened 10,000 human proteins with two bacterial pathogen AMPylators, VopS and IbpAFic2, identifying more than twenty new substrates each. Two novel Rho GTPases (Rac2 and Rac3) were validated in vivo as substrates of the virulence factor VopS in HEK293T cells during V. para infection. Using mass spectrometry, we verified that a non-GTAPose protein, ARHGDIB/LyGDI, was AMPylated by VopS on its threonine 51, which is located in a highly regulated part of this protein. This modification inhibited phosphorylation of LyGDI by Src kinase in vitro. Finally, the identification of these new targets allowed us to build the first bacteria-host interaction AMPylation network and may reveal signaling interactions that could potentially be important for bacterial pathogenesis in the future functional studies.

**EXPERIMENTAL PROCEDURES**

*Preparation of Bacterial Fic AMPylators and Click Reagents—VopS and Rac1 proteins were generated as previously described (2). IbpAFic2 proteins were expressed as a His-SUMO fusion in the e-SUMO vector in BL21 (DE3) E. coli and captured with His-Select Nickel Affinity gel (Sigma, St. Louis, MO), washed with 25 column volumes of lysis buffer and eluted with lysis buffer plus 250 mM Imidazole. They were further purified on monoc Q column and Superdex 75 PG columns. Proteins were concentrated, adjusted to 10% glycerol, flash frozen, and stored at −80 °C. The click reagents, including N^6-pATP and az-rho, were generated as previously described (12).*

*Preparation of Plasmid DNA and Fabrication of NAPPA Arrays—All plasmid DNA on the microarray spots was removed by incubating the array with 100 μl 0.5 M NaOH for 1 h at room temperature, followed by incubation with 160 μl solution (HeLa lysates, Accessory proteins, Reaction mix, and Nuclease-free Water) for 1.5 h at 30 °C and 0.5 h at 15 °C.*

After washing with PBST (PBS, 0.2% Tween) three times, the plasmid DNA on the microarray spots was removed by incubating the array with 200 μl DNase (Sigma, St. Louis, MO) for 20 min at room temperature with a coverslip. Then the resulting protein array was blocked with 1% BSA solution for 1 h at room temperature.

To load the GTAPases, the array was treated with 2.5 ml 50 μM GTP/S solution (50 mM Tris-HCl and 7.5 mM EDTA, pH 7.5) for 15 min at 30 °C, followed with the addition of 12.5 μl 1 M MgCl₂ for

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1 The abbreviations used are: AMP, Adenosine monophosphate; az-rho, azide-rhodamine; CB, coomassie staining; CuAAC, copper-catalyzed azide-alkyne cycloaddition; Fic, filamentation induced by cAMP; GO, Gene ontology; GST, Glutathione S-transferase; HRP, horse radish peroxidase; IVTT, in vitro transcription and translation; NAPPA, Nucleic Acid Programmable Protein Array; N^6-pATP, N^6-propargyl adenosine-5’-triphosphate; PANTHER, Protein Analysis Through Evolutionary Relationships; SUMO, Small ubiquitin-related modifier; TBTAs, Tris(1-benzyl-1H-1,2,3-triazol-4-yl)methylamine; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; UniProt, The Universal Protein Resource; VopS, Vibrio outer protein S.
additional 5 min to stop the reaction and washing two times with washing solution (50 μM HEPEs and 5 mM MgCl₂, pH 7.4)(2).

NAPPA AMPylation Assay—The AMPylation reaction was performed by incubation of the prepared NAPPA protein array with 40 μg/ml AMPylators and 250 μM N₆P-ATP in 160 μL AMPylation solution (20 mM HEPEs pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.1 mg/ml BSA, and 1 mM DTT) for 1 h at 30 °C. Following by washing three times with PBST, the detection was performed with the 160 μL click reagents containing 250 μM azido-rodhamine (az-rho), 1 μM TCEP, 0.1 mM TBTA, and 1 mM CuSO₄, as previously described (12) for 1 h at room temperature. After washing overnight, the microarray was scanned with a Tecan’s PowerScanner (Männedorf, Switzerland). The fluorescent signal was quantitated using Array-Pro Analyzer, version 6.3 (Media Cybernetics, Bethesda, MD).

Bead-based AMPylation Assay—The proteins with GST or Flag tag was expressed using the expression solution and captured to the anti-GST or anti-Flag antibody coupled Dynabeads (Invitrogen, Carlsbad, CA) (supplemental Materials and Methods ). 10 μL GST-proteins conjugated beads were used for each reaction. The supernatant of GST-protein conjugated magnetic beads was removed using a magnetic stand and GFPase conjugated beads were loaded with GFP-MS solution as in NAPPA. After washing with GFP washing buffer, 15 μL AMPylation solution (20 mM HEPEs pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.1 mg/ml BSA, and 1 mM DTT) containing 40 μg/ml AMPylators and 250 μM N₆P-ATP was added and the incubation was performed at 30 °C for 1 h. After washing again, 20 μL click reagents were added (250 μM az-rho, 1 μM TCEP, 0.1 mM TBTA, and 1 mM CuSO₄), and the incubation was carried out for 1 h at room temperature. The reaction was stopped by addition of 20 μL 1× SDS loading buffer containing 10% 2-mercaptoethanol. Samples were boiled 5 min and analyzed in a 4–15% Tris-Criterion™ Precast Gel (Bio-Rad, Hercules, CA).

Protein gel images were obtained using an Amersham Bioscience Typhoon 9400 variable mode imager (excitation 532 nm, 580 nm filter, 30 nm band-pass). All image adjustments were performed using Adobe® Photoshop® CS4 software (Adobe Systems, San Jose, CA).

The protein expression was examined in the same gel followed with Western blot using 1:3000 dilution of mouse anti-GST antibody (Cell Signaling Technology, Danvers, MA) and HRP conjugated sheep anti-mouse antibody (Jackson ImmunoResearch, Labs, West Grove, PA) separately. The Chemiluminescence detection was performed with SuperSignal West Fento Luminol/Enhancer Solution (Pierce, Rockford, IL).

Data Analysis—Before statistical analysis, all the fluorescent microarray images were examined for the spot shape, dust, and nonspecific binding to remove false positive signals. We then normalized the raw signal intensity to decrease the variation caused by different slides’ background. The normalization was performed by subtracting the background signal attributable to nonspecific binding of AMPylators or fluorescent molecules, which was estimated by the first quartile of the ground signal attributable to nonspecific binding of AMPylators or background. The normalization was performed by subtracting the background signal intensity to decrease the variation caused by different slides’ background. The normalized value was calculated by dividing the signal above the median were considered positive signals.

Probing of Proteins for AMPylation using anti-AMP Antibodies—The recombinant proteins for RhoGDI1, LyGDI, and LyGDI T51A were produced using bacterial coexpression experiments (supplemental Materials and Methods). To probe the proteins for AMPylation, 20 μl of precapture lysate or 100 ng of each eluted protein was loaded onto miniProtean SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes. Membranes were incubated for 1 h in blocking buffer (TBS-T containing 5% milk), washed three times in TBS-T, and then incubated with primary antibodies in blocking buffer for 2 h. Membranes containing eluted proteins were probed with 1:1000 dilution of anti-His (Life Technology, Carlsbad, CA), anti-T-AMP (11), or anti-Y-AMP, whereas precapture lysates were blotted with anti-VopS (peptide antibody generated at UTSW antibody core, 1:1000). Membranes were again washed three-times, probed for 1 h with either anti-rabbit or anti-mouse HRP (1:20,000), washed three-times, incubated in homemade luminol substrate and exposed to film. Rabbit anti-Y-AMP antibody was made in house using a chymotrypsin-digested peptide, TTNAFPGYE(AMP)PTV, from IbpA AMPylated Rac1 via Invitrogen.

Intact Mass Measurement—Intact mass measurements of His-LyGDI proteins expressed with empty vector or VopS in solution were obtained by the UT Southwestern Proteomics Core using an Agilent 6540 UHD Accurate-Mass QTOF machine. A mass shift of 328.05 was observed for His-LyGDI coexpressed with VopS (supplementary Fig. S6).

Tandem LC-MS/MS—An SDS-PAGE excised band containing His-LyGDI protein coexpressed with VopS was digested with trypsin and run on the Q-Exactive mass-spectrometer for LC-MS/MS analysis at the UT Southwestern Medical Center Proteomics Core. Resulting data file was searched using in house Central Proteomics Facilities Pipeline and phosphoadenosine (AMPylation) definitions present in the Unimod database were used as variable modifications in the searches. GDIR2 human sequence was identified with 740 spectra/41 unique peptides, yielding 97.5% sequence coverage. AMPylation of Threonine T51 was confidently identified. 34 identifications of the "TLGDGVPVDPK peptide or mis-cleaved variants were found with AMPylation present on the threonine 51. Identification were made from charge states 2+, 3+, 4+, and characteristic AMPylation reporter ions from Li et al. are visible in spectra (10) (supplementary Fig. S7 and supplementary Table S7).

In Vitro Kinase Assays—20 μL radioactive kinase assays were prepared using a 4x Src kinase buffer (100 mM Tris pH 7.2, 125 mM MgCl₂, 25 mM MnCl₂, 8 mM EGTA, 250 μM NaVO₄, and 2 mM DTT), 100 mM ATP, 5 μCi [γ-³²P] ATP (Perkin Elmer 6000Ci/mmol), 4 μg recombinant LyGDI protein, and 3 units Src p60c-src (Millipore cat #14–117, Billerica, MA). Prior to assay setup, His-LyGDI proteins were cleaved with thrombin as the His tag was found to be inhibitory to phosphorylation compared with GST tagged wild type LyGDI. Proteins were incubated at 30 °C for 45 min and reactions were stopped by the addition of Laemmli buffer and boiled 2 min. Samples were loaded onto miniProtean SDS-PAGE gels (Bio-Rad, Hercules, CA) transferred to PVDF membranes, and exposed to film (Phenix, Candler, NC) for between 2–24 h until an exposure in the linear range
was obtained. Densitometry was calculated using ImageJ, and phos-
phorylation bands were normalized to the coomassie stained PVDF
bands. A student’s 1 tailed $t$ test with unequal variance ($p = 0.034$)
was used to compare relative phosphorylation of LyGDI WT and
AMPylated proteins from three independent experiments.

RESULTS

Development of an AMPylation Assay on NAPPA Arrays—
The principle of our click chemistry-based AMPylation assay
on NAPPA is shown in Fig. 1A. Briefly, the protein expression
plasmids were printed on slides and stored anhydrously at
room temperature. At the time of the AMPylation experiment,
we produced proteins by mammalian lysate-based cell-free
expression system. The slides were then treated with DNase
to eliminate potential cross-reaction of detection agents with
the DNA. After incubation with N6pATP and AMPylators for 1h
at 30 °C to allow the transfer of AMP to the substrates, the
AMPylated proteins on the NAPPA slides were detected with
az-rho following CuAAC labeling (12).

We initially compared the ability to detect AMP modification
by VopS between this click chemistry and a mix of anti-T-
AMP and anti-Y-AMP antibodies using a NAPPA array dis-
playing ~2000 human proteins produced from human HeLa
lysat based cell-free expression system (Fig. 1B). In contrast
to the antibody-based assay, where we observed high back-
ground noise presumably because of nonspecific binding, we
readily identified three known VopS substrates (Rac1, RhoA,
and Cdc42) and two new RhoGTPase substrates (Rac2 and
Rac3) using N6pATP. By comparing the normalized signal
intensity, which was calculated by using the signal intensity of
each feature divided by the median background-adjusted
value for all the proteins on the array, we found the click
chemistry had much higher sensitivity, specificity, and a wider
dynamic range than the anti-AMPylation antibodies in the
detection of proteins with AMPylation on NAPPA (Fig. 1B).

Using VopS and its inactive H348A mutant as a control, we
systematically optimized the experimental conditions of the
AMPylation assay on NAPPA. First, we tested the influence of
DNase and RNase treatment on the AMPylation assay. Treat-
ments of either DNase alone or combined with RNase re-
moved nearly all DNA when estimated by PicoGreen staining
(Fig. 2A and supplemental Fig. S1A). The pilot assay showed
that AMPylation of VopS targets (Rac2, Rac3, and Cdc42)
could be detected at high S/N ratios only after removal of DNA
molecules from the slides. However, no additional benefit was
observed after the addition of RNase (supplemental Fig. S1B,
S1C). Second, we tested the effect of various time lengths for

Fig. 1. Development of AMPylation screening platform on NAPPA arrays. A, The principle of AMPylation assay on NAPPA arrays. B, Comparison of AMPylation assay on NAPPA arrays using click chemistry and anti-AMPylation antibody. The experiment was performed by using VopS enzyme and NAPPA GST2 arrays comprising 1823 human proteins. 1:50 dilution of anti-T-AMP antibody and anti-Y-AMP antibody were mixed and used as the primary antibody, and 1:100 dilution of Alex647 chick anti-rabbit IgG antibody was used as the secondary
detection antibody. X-axis and Y-axis represent the normalized signal intensity produced by taking the signal intensity for each feature divided by the median background-adjusted value for all the proteins on the array as described in the methods. C–G, Optimization of the assay parameters for AMPylation assay on NAPPA arrays, including the cell-free expression system, blocking buffer, N6pATP, and az-rho
G, respectively. The signal to noise ratio (S/N) ratio was calculated using MSP-1, which expresses a 25 kDa protein with the HA tag and is not captured to the anti-GST coated array surface, as a negative control. CuAAC, copper-catalyzed azide-alkyne cycloaddition.
incubation and washing steps, and found that an overnight washing step after the az-rho treatment improved the signal and S/N ratio of the AMPylation assay, presumably by reducing nonspecific binding of fluorescent dyes (supplemental Fig. S2). Third, we achieved a significant improvement in candidate substrate display by using a new cell-free expression system based on human HeLa cell lysate, which has a theoretical advantage of using human ribosomes and chaperones to produce human proteins in their native forms, compared with the initially used rabbit reticulocyte lysate system (25, 30). Indeed, we found that both protein yields and the S/N ratio of the AMPylation assay were markedly improved with the human HeLa system (Fig. 1C, 1D). Finally, further optimizations were performed using different blocking buffers (PBS, Superblock, 1%BSA, and 5%Milk), different concentrations of N6pATP (0, 31, 63, 125, 250, and 500 μM) and different concentrations of az-rho (0, 25, 50, 100, 200, and 400 μM). These combined experiments yielded optimized conditions using 1% BSA, N6pATP at 63 μM, and az-rho at 100 μM (Fig. 1E–1G).

In addition to the signals from candidate substrates, we reproducibly observed signal from a human AMPylator, HYPE, detected even in the absence of the AMPylators, presumably because of auto-AMPylation (Fig. 1F–1G and supplemental Fig. S3). The wild type HYPE showed extremely low auto-AMPylation activity in previous studies with autoradiography, presumably because an inhibitory α-helix containing a conserved (S/T)XXXE(G/N) motif before the Fic domain prevents the binding of ATP to Fic (3, 9). Mutation of this inhibitory motif (E3G) increased HYPE auto-AMPylation and its AMPylation of other substrates significantly (9). This result demonstrates our method has the potential to identify novel mammalian AMPylators with auto-AMPylation regardless of Fic and adenylyl transferase motifs (6, 7, 9, 31).

Global Identification of Substrates for Bacterial Fic AMPylators with Human 10K NAPPA Arrays—To execute a high-throughput screen for AMPylation substrates, we fabricated high-density NAPPA arrays containing 10,000 human proteins on five arrays, four with C-terminal GST-tagged proteins and one with N-terminal Flag-tagged proteins (Fig. 2A). DNA quantity before expression and after DNase treatment was measured to ensure the quality of newly fabricated NAPPA arrays (Fig. 2A, 2B). In addition, we probed several arrays in each printing batch with either a monoclonal anti-GST antibody or an anti-Flag antibody to assess the amount of protein displayed (Fig. 2A, right). Using the mean plus two standard deviations of the signal for negative control features (supplemental Fig. S3).
(MSP-1 protein, which lacks the GST tag) as a cutoff, about 95% of the human proteins were successfully displayed (supplemental Fig. S4), consistent with previous studies (26, 32). The correlation of GST signals (i.e., protein levels) between arrays was $r = 0.93$, indicating a high reproducibility in the fabrication of NAPPA protein arrays (Fig. 2C).

We then performed high-throughput screens using the Fic AMPylators (VopS or IbpAFic2) with the N$^6$pATP substrate (Fig. 2D). Two known substrates (Rac1 and RhoA) for VopS and IbpAFic2 gave strong fluorescent signals, which were not observed on NAPPA with buffer alone. We also observed several features with fluorescent signals that corresponded to novel candidate substrates (Fig. 2D).

Before statistical analysis, all fluorescent microarray images were visually examined to ensure their quality to exclude false positive signals. Normalized signals showing a 1.2-fold increase over the buffer-only control were considered positive. Only the features showing a positive ratio in all three experiments on different days were selected as final candidates. With these criteria, a total of 20 and 21 substrates for VopS and IbpAFic2 were discovered from the screening, respectively (supplemental Tables S1, S2). The distribution of these candidate substrates’ molecular weights agreed with previous findings using gel-based AMPylation assays (Fig. 2E, 2F) (2, 3, 12, 14). The annotations showed that many of the VopS and IbpAFic2 substrates were RhoGTPases, 8 and 7, respectively. In addition, we found dozens of novel potential non-GTPase substrate proteins.

Validation of Candidate Substrates of AMPylators with a Bead-based Assay—To further assess the AMPylation substrates, we developed an independent AMPylation assay based on a magnetic bead platform (Fig. 3A). Briefly, a GST-tagged target protein was expressed in solution with the HeLa cell-free expression system and then captured by a polyclonal anti-GST antibody coupled to magnetic beads. Next, N$^6$pATP and either active or inactive mutant AMPylators were added and incubated for an hour at 30 °C. After addition of az-rho reagents, fluorescently labeled proteins were visualized and protein levels were confirmed by Western blotting on the same gel using anti-GST antibody. Of the 20 and 21 candidates for VopS and IbpAFic2 identified by the NAPPA screening, 11 and 8, respectively, were validated with the bead-based AMPylation assays (Fig. 3B, 3C). Interestingly, the confirmed in vitro substrates included several non-GTPase proteins, ARHGDB/LyGDI, MAP1LC3A, LENG1, and MAGEA3.

Universal AMPylation of the Rho GTPase Family by Bacterial Fic AMPylators—As the NAPPA set covered about half of all predicted human proteins, we performed bioinformatics analyses to predict additional potential substrates of AMPylators based on the sequences of the hits we identified. As previously shown, Rho GTPases share a homologous sequence within the switch I region (3) where conserved tyrosine and threonine residues are AMPylated by IbpAFic2 and VopS, respectively (2, 3). Sequence alignment of all eight GTPases identified as targets for IbpAFic2 and VopS in the NAPPA screening revealed a highly conserved motif of YxPTVF (Fig. 3D). A motif scan using ScanProsite (http://prosite.expasy.org/scanprosite/) identified seven more proteins with the motif, including six GTPases and a non-GTPase, ERGIC2. Among these potential substrates, expression clones for RhOD, RhOG, and RhOJ were available and their AMPylation by VopS and IbpAFic2 was confirmed (Fig. 3E). In addition, we showed AMPylation of Rnd3 by IbpAFic2, which also shares the motif and was not detected in the initial NAPPA screening. Taken together, these data show that bacterial AMPylators could potentially regulate a wide range of Rho GTPases by targeting the highly conserved switch I regulatory region.

To determine the range of Rho GTPase targets for VopS in cells during an infection, we developed an assay in which candidate substrates are transiently expressed in HEK293T cells, followed by infection with V. para and immunoprecipitation of the target substrate (Fig. 4A). Using a strain that expresses and secretes VopS as the only known T3SS effector (CabS), we found that VopS AMPylated the known substrate Rac1 and two new GTPases, Rac2, and Rac3 (Fig. 4B). This indicates that VopS can AMPylate most Rho GTPase family members during infection and serve as a general inhibitor of all Rho GTPase activity. We also tested AMPylation of three in vitro confirmed non-GTPase substrates (ARHGDB/LyGDI, LC3, and LENG1) in vivo, but did not observe the modification under these conditions. AMPylation of these substrates may be transient or prevented by other PTMs (such as phosphorylation) and we may have failed to evaluate the cells at the relevant time or condition to detect these changes (33). In addition, this measurement is technically challenging because the robust activity of VopS and IbpA for Rho GTPases can mask subtle cellular consequences (3, 9, 12, 13). As a result, we sought to confirm a non-GTPase substrate and elucidate the effect of AMPylation on its potential function in vitro.

Characterization of In Vitro AMPylation of LyGDI AMPylation by VopS—LyGDI was chosen for further study because it represents a new class of substrate that nevertheless plays important roles in GTPase regulation for innate immunity, including T-cell activation, phagocytosis of bacteria by macrophages, and reactive oxygen species production for microbial killing (34–36). LyGDI is primarily expressed in hematopoietic cells and some cancers, and belongs to the RhOGI family of which there are three members: ARHGDA/RhoGDI, LyGDI, and ARHGDA/RhoGDI3. Unlike the GTPase substrates, LyGDI does not have a YxPTVF motif, suggesting that if LyGDI were AMPylated, modification must occur at a novel recognition sequence.

To test these non-GTPase substrates for VopS modification, we co-expressed either LyGDI or RhOGI with VopS, as previously described (2). Using antibodies that recognize AMPylated threonine (T-AMP) or tyrosine (Y-AMP) we ob-
Fig. 3. Validation of identified human substrates from NAPPA screens. A, Schematic illustration of bead-based AMPylation assays. B, C, Validation of identified human substrates for VopS and IbpAFic2, respectively. VopS H348A and IbpAFic2 H359A, both inactive enzymes, were used as their corresponding negative controls. CD48 is an example of a substrate candidate that did not validate in the bead-based assay. D, Prediction of new substrates for VopS and IbpAFic2 by using motif analysis. In Rac1, RhoA, and Cdc42, the conserved Y and T indicated were reported as the AMPylation substrate of IbpA and VopS respectively (2, 49). Additional candidate substrates with the motif were searched in the UniProtKB/Swiss-Prot human database (http://prosite.expasy.org/scanprosite/). E, Validation of predicted substrates using bead-based AMPylation assays. Each experiment was repeated three times on independent days. az-rho, azide-rhodamine; WB, Western blot.
served that purified His-LyGDI co-expressed with VopS, but not empty vector, was strongly recognized by the T-AMP antibody (Fig. 5A). Interestingly, despite strong homology between the RhoGDI family members, RhoGDI was not AMPylated by VopS. Comparison of the apparent mass of His-LyGDI co-expressed with GST-VopS revealed the appearance of a protein population with an increase in molecular weight of 328 Daltons over the LyGDI coexpressed with empty vector (supplemental Fig. S6), indicative of a protein modified with AMP.

To identify the AMPylated residue(s) on LyGDI, we used LC-MS/MS as previously described (2). Only one residue, threonine 51, was identified in the peptide T51LLGDGPVTDPK to be modified with AMP (supplemental Fig. S7) (10). Analysis of the reporter ions eliminated threonine 60 as the possible site for AMPylation (supplemental Fig. S7). Mutation of threonine 51 to alanine abrogated AMPylation of LyGDI in an identical co-expression experiment, demonstrating that threonine 51 is a specific AMPylation site (Fig. 5A).

The targeting of this specific residue in LyGDI is reminiscent of the way in which VopS precisely targets the threonine in the Switch I region of Rho GTPases to inhibit their interaction with downstream effectors (2).

Sequence analysis of RhoGDIs reveals that the site of AMPylation is present only in LyGDI, not RhoGDI or RhoGDI3 (supplemental Fig. S8A), explaining its specificity. The tertiary structure of RhoGDIs can be divided into two parts: the prenyl binding, immunoglobulin-like C terminus and the dual helix

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**Fig. 4.** VopS AMPylates all Rac proteins during infection. A, Schematic of the V. para infection assay with identified substrates. Cells are transfected with substrate candidate and infected with V. para. Candidate substrates are immunoprecipitated from the lysate and blotted for AMPylation. WT is V. para CAB strain which was generated from POR1 (RIMD 2210633 ΔtdhAS). The CabS strain contains a deletion for the transcriptional regulator VtrA to prevent expression of the second type three secretion system, as well as deletions of the effectors VopQ, VopR, and VP0450 to leave VopS as the only known T3SS effector expressed and secreted. Δ and + are the CabSΔS strain with the deletion of VopS and complemented with a pBAD-VopS expression plasmid, respectively. B, Transfected 3xHA tagged Rac1, Rac2, and Rac3 were immunoprecipitated from infected cells and immunoblotted for AMPylation.
containing N terminus. The latter is the site of multiple PTMs and may be considered the regulatory region of the protein (37, 38) (supplemental Fig. S8B, C). Interestingly, the site of AMPylation of LyGDI by VopS is located in the second N-terminal alpha helix, which is near the location of several PTMs, including phosphorylation by Src kinase (39–42). The diversity of regulation on and around the N-terminal helical region of LyGDI presents multiple avenues by which AMPylation by VopS could disrupt its function.

Inhibition of Src Phosphorylation of LyGDI by VopS AMPylation—To test if AMPylation by VopS might have potential as a competitive PTM, we assessed the ability of AMPylated LyGDI to be phosphorylated by Src. We observed that unmodified LyGDI is readily phosphorylated by Src, but LyGDI previously AMPylated by VopS is phosphorylated at a significantly lower (58%) level than unmodified LyGDI (Fig. 5B, 5C), indicating that AMPylation of LyGDI may be competitive with Src phosphorylation and potentially other sites of modification in the N terminus of LyGDI. A complete loss of phosphorylation was not expected, as the AMPylated-LyGDI sample is a mixed population of modified and unmodified LyGDI, although the relative amounts cannot be deduced from unquantitative mass spectrometry (supplemental Fig. S6). It is possible but not certain the portion resistant to Src phosphorylation also represents the portion of AMPylated LyGDI.

AMPylation of LyGDI during an infection was not observed in preliminary experiments, but further studies will be required to rule it out as a naturally occurring modification. Nevertheless, the ability of this in vitro AMPylation to compete with naturally occurring PTMs presents a tool to understand the regulation of this protein in processes like immunity and cancer.

DISCUSSION

Over 40 years ago, Stadtman and colleagues identified the modification of a protein with AMP as a mechanism to reversibly modulate the activity of glutamine synthetase (43). This modification has been implicated as a fundamental mechanism to regulate protein-protein interactions of modified proteins with downstream signaling machinery. Undoubtedly, the molecular mechanism of AMPylation-mediated cellular changes has not been fully elucidated, because of a limited knowledge of the repertoire of enzymes and substrates (6, 44). Advancement in the identification of novel substrates has been stalled by the dependence on anti-AMPylation antibodies and radioactive detection to find substrates, which have
only yielded a handful of substrates since the discovery of AMPylation (2, 3, 33). To start to address these questions, we established a high-throughput discovery platform by developing a click chemistry-based assay that selectively labels enzyme substrates and works on a cell free protein microarray platform. The alkynyl chemical reporter, N6pATP, allows non-radioactive and robust detection of AMPylated proteins with high sensitivity and specificity (Fig. 1B–1G, Fig. 2, and supplemental Figs. S1–3)(12). Moreover, the covalent bond formed by alkynyl and azido moieties enabled us to examine both the AMPylation status and the expression of target proteins in the same gel using the bead-based AMPylation assay followed by Western blotting (Fig. 3).

Including both the screen and the targeted testing, a total of 27 and 29 potential substrates were identified for VopS and IbpAFic2, respectively, after screening 10,000 human proteins by NAPPA and the following prediction analysis. Among them, 14 and 12 hits showed strong detection using the bead-based AMPylation assay for VopS and IbpAFic2, respectively. The discrepancy of the results between NAPPA and bead-based assays might be caused by the differential sensitivity, resolution and the amount of protein required for signal by these two methods. False positives are also inherent in any screen and careful interpretation of unvalidated hits is required. In addition, although we did not observe any difference for the detection of protein AMPylation between N6pATP analog and native ATP with the substrates we tested, false negatives might still exist in click chemistry based screening in which the alkynyl group on N6pATP may cause steric hindrance and block the transfer of AMP to some potential substrates.

We identified a highly conserved motif (YxPTVF) by sequence alignment of all GTPases targets of IbpAFic2 and VopS (Fig. 3D). Further searching of human protein sequence databases with ScanProsite identified six GTPases and a non-GTPase, ERGIC2. ERGIC2 is an ER-Golgi transmembrane protein with possible functions in transport between ER and Golgi (45). Altogether, these hits expanded the repertoire of host substrates for the bacterial AMPylators to the entire Rho GTPase family and potentially beyond (12, 13).

This motif appears to be specific for GTPase substrates, as it was not present in any of the non-GTPase substrates. The proximal sequence at the documented modification site in LyGDI was KYKKT*LLGDGP. Using the Eukaryotic Linear Motif tool, we could not find a related sequence in the other non-GTPases that had been confirmed in the bead-based assay. Notably, the modified region in LyGDI is alpha helical and the modified threonine residue is facing directly away from the protein, perhaps enabling accessibility to VopS. Nonetheless, there is likely to be additional specificity re-
quired for VopS targeting, as there were less than two dozen observed substrates from among the 10,000 candidates.

A number of other proteins were identified as potential substrates for VopS and IbpA (Fig. 6), covering a diverse range of protein classes in addition to small GTPases, including enzyme modulators, signaling molecules, receptors, and enzymes. These potential substrates localize to diverse subcellular compartments, such as cytoplasm, cell membrane, and the nucleus, and can be lipid-anchored or secreted. VopS has recently been shown to contain a conserved phosphoinositide binding domain (BPD) that localizes it to the cell membrane. However, a mutation of the BPD that renders VopS cytosolic had no appreciable effect on cell rounding during infection (46). Proteins accessible both in the cytoplasm and on the cell membrane are likely to be possible substrates of VopS. The molecular function analysis of the potential substrates reveals a variety of binding activities that might change through AMPylation, for example, GBD domain binding, Wnt receptor binding, BH3 domain binding and thioesterase binding, etc. (supplemental Fig. S9 and supplemental Tables S3, S4). It is important to avoid over-interpretation of the results of a screen without thorough in vivo validation.

LyGDI was confirmed as an in vitro substrate and was found to be specifically AMPylated on threonine 51 by VopS in the highly regulated N terminus of the protein. LyGDI appears to be involved in several processes of the innate immune system and is also implicated as a metastatic factor in several cancers (35, 42, 47) Although we have been unable to confirm LyGDI as an in vivo substrate, its AMPylation may prove to be a competitive PTM (Fig. 5) and could be a powerful tool for understanding its complex roles in immunity and cancer. AvrAC provides a previous precedent for phosphorylation-competitive modifications by Fic proteins, as addition of UMP to the plant kinases Bik1 and RipK by AvrAC masks their phosphorylation sites (33, 48).

We analyzed the biological processes represented by the substrates identified in the screen using BINGO with p value of 0.05, which shows that most of the proteins selected are Rho GTPases (Fig. 7, supplemental Figs. S10, S11 and supplemental Tables S5, S6). The results indicate that the actin cytoskeleton and Rho signaling are the main targets of VopS and IbpA enzymes, which is in accord with previous studies in which the ectopic expression of the bacterial AMPylators induced host cell rounding because of cytoskeletal collapse (2, 3).

Finally, we believe this work contributes major conceptual and methodological advances of cell-free protein arrays, including array fabrication, assay novelty and application in the detection of a novel PTM. We have developed a high-throughput approach for the identification and characterization of proteins modified by AMPylation using a combination of multi-disciplinary technologies that covers proteomics, chemistry, bioinformatics and molecular biology. Furthermore, this study validated the high-density NAPPA based cell-free protein arrays as a powerful method of characterizing PTMs, beginning with the assay development of a fluorescent reporter screen for an enzyme, identification of substrates, analysis of the interaction web of the enzyme, and ending in the validation and preliminary characterization of unexpected and novel substrates. The breadth of information gained about an enigmatic modification like AMPylation demonstrates the great potential of cell-free protein arrays in unbiased, discovery-based research on PTMs.
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