

Synthesis of and Specific Antibody Generation for Glycopeptides with Arginine N-GlcNAcylation**

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Abstract: As a unique and unappreciated protein posttranslational modification, arginine N-glycosylation was recently discovered to play an important role in the process that bacteria counteract host defenses. To provide chemical tools for further proteomic and biochemical studies on arginine N-glycosylation, we report the first general strategy for a rapid and cost-effective synthesis of glycopeptides carrying single or multiple arginine N-GlcNAcylation groups. These glycopeptides were successfully utilized to generate the first antibodies that can specifically recognize arginine N-GlcNAcylation peptides or proteins in a sequence-independent manner.

As one of the most complex and extensively studied posttranslational modifications (PTMs),^[1] protein glycosylation is involved in a wide range of biological processes including cell migration, cell adhesion, and signal transduction.^[2] Up to now, most studies on protein glycosylation have focused on the O-glycosylation of Ser, Thr, and Tyr residues or N-glycosylation of Asn residue,^[3] whereas the N-glycosylation of Arg residue in proteins has only been reported in very rare cases.^[4] In this context, a surprising recent discovery by one of our groups^[5a] and Hartland's group^[5b] was that an entero pathogenic *Escherichia coli* (EPEC) type III secretion system effector protein, NleB, exhibited an unusual arginine GlcNAc (N-acetylglucosamine) transferase activity toward multiple proteins with death

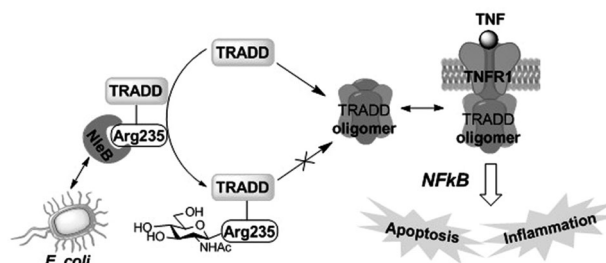


Figure 1. *E. coli* protein NleB caused arginine N-GlcNAcylation of multiple proteins with death domain, e.g., Arg235 of TRADD. Crystallographic study of TRADD carrying Arg235 N-GlcNAcylation revealed that the linkage between the glycan and guanidyl group has β -configuration (unpublished result). TNF = tumor necrosis factor; TNFR1 = tumor necrosis factor receptor 1; NF κ B = nuclear factor κ B.

domain (Figure 1). For example, NleB could specifically modify a conserved arginine (Arg235) in the TNFR1-associated death domain protein TRADD, thereby blocking the homotypic/heterotypic death domain interactions and the assembly of the oligomeric TNFR1 complex. This event blocked host cell death by disrupting the TNF signaling in EPEC-infected cells, including NF- κ B signaling, apoptosis, and necroptosis. The finding revealed a previously unappreciated PTM, which may be involved in many other important signaling processes. However, it is currently not possible to monitor the amount of global arginine N-GlcNAcylation within a native proteome, although the available biochemical, pharmacological, and proteomic tools for studying O-GlcNAcylation of Ser, Thr, and Tyr have been well developed.^[6]

To address the above challenge we propose to develop specific antibodies against Arg N-GlcNAcylation for further study of the occurrences and mechanisms of such unique PTM events. This proposition poses a hitherto unsolved problem of how to efficiently synthesize Arg N-GlcNAcylation peptides to help induce antibodies. Another critical question is whether the Arg-GlcNAc antibodies can be generated to specifically recognize Arg-GlcNAc without interference of any peptide sequence or related O-GlcNAcylation. Herein we report the first general strategy for the synthesis and specific antibody generation of glycopeptides with arginine N-GlcNAcylation (Figure 2). Our synthesis features a silver-promoted solid-phase glycosylation process, which enables rapid and cost-effective production of glycopeptides with single or multiple Arg-GlcNAc sites from readily available starting materials. These synthetic glycopeptides were then successfully used to generate the first antibodies that can

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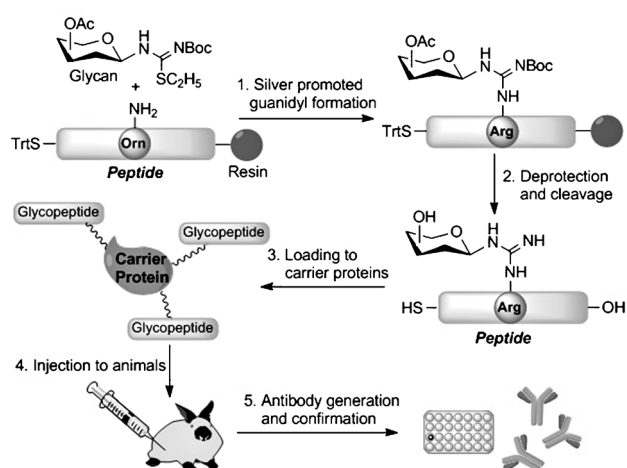
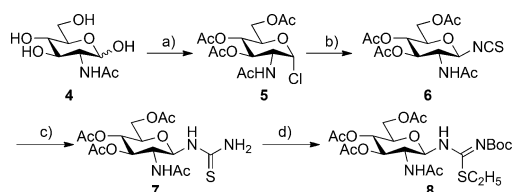


Figure 2. Synthesis of glycopeptides with arginine *N*-GlcNAcyl groups and antibody-generation strategy. Trt = trityl; Boc = *tert*-butoxycarbonyl.

specifically recognize arginine *N*-GlcNAcylated peptides or proteins in a sequence-independent manner.

Our study began with the attempt to synthesize a suitable protected Arg(*N*-GlcNAc) building block for solid-phase peptide synthesis (SPPS). However, after many tests we concluded that such synthesis would be lengthy and inefficient due to the need to change the protecting groups on the guanidine group repeatedly. Accordingly we turned to an alternative strategy for glycopeptide synthesis that involves direct glycosylation of amino acid side chains on the solid phase,^[7] which has been successfully used to prepare glycopeptides with Asn *N*-glycosylated groups.^[8] To implement this strategy we were interested in the silver-promoted guanylation reaction between an *S*-alkyl-isothiourea and an amine, which emerges as an effective approach for the construction of guanidine moieties in natural product synthesis.^[9] Our task was to examine whether this guanylation reaction is compatible with the glycosylation of preassembled peptides.

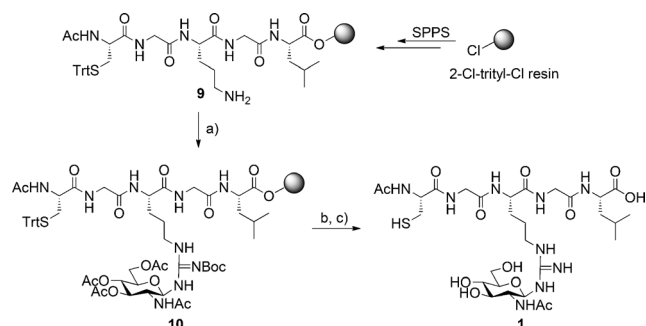
The synthesis of the key building block **8**, that is, an *N*-glycosyl-*S*-alkyl-isothiourea, is shown in Scheme 1. From 2-acetamino-2-deoxy-D-glucose **4**, we obtained glycosyl chloride **5** in 68% yield by using well-established procedures.^[10] Then, glycosyl isothiocyanate **6** was prepared in 61% yield by treatment of **5** with potassium thiocyanate (KSCN) and tetrabutylammonium iodide (TBAI) in anhydrous acetonitrile.^[11] Next, **6** was treated with ammonia in tetrahydrofuran to yield *N*-glycosyl-thiourea **7** in an almost quantitative



Scheme 1. Synthesis of building block **8**. Reagents and conditions: a) acetyl chloride, rt, 2 days, 68%; b) KSCN, TBAI, CH₃CN, reflux, 3 h, 61%; c) NH₃, THF, 1 h, 99%; d) EtI, MeOH, reflux, 3 h; then Boc₂O, Et₃N, CH₂Cl₂, 84%.

yield.^[12] Finally, treatment of **7** with ethyl iodide and Boc anhydride afforded **8** in a two-step, one-pot procedure with a very good yield of 84%.^[13] The overall yield of **8** from **4** was 34%.

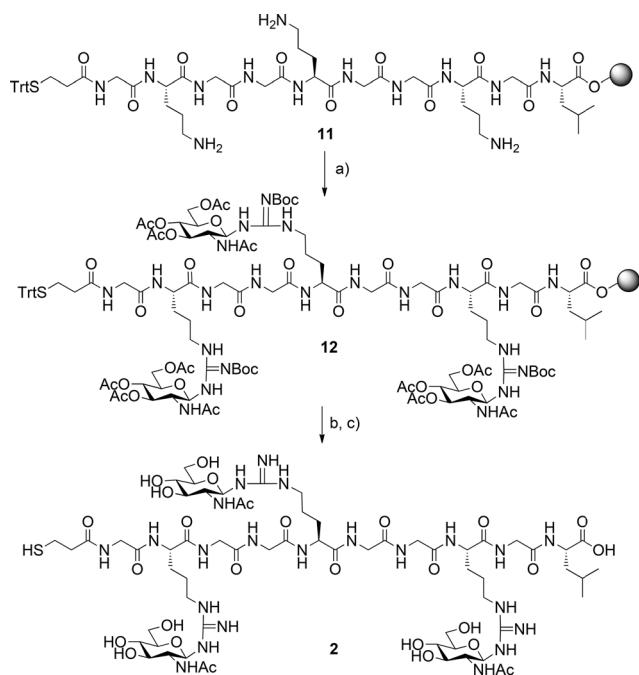
As shown in Scheme 2, an Arg *N*-GlcNAcylated pentapeptide was designed for Arg-GlcNAc antibody generation. The free mercapto group of the Cys residue at the N terminal was used for coupling to the carrier protein. The two Gly residues were inserted into the sequence to avoid interactions between antibodies and amino acid side chains around Arg-*N*-GlcNAc. The Leu residue at the C terminal was selected to tune the polarity of the glycopeptide for HPLC separation.



Scheme 2. Solid-phase synthesis of monoglycosylated peptide **1**. Reagents and conditions: a) TEA, DMF, AgNO₃, **8** (3 equiv), room temperature; b) 5% NH₂NH₂ in DMF; c) 5% TIPS in TFA. TEA = triethylamine.

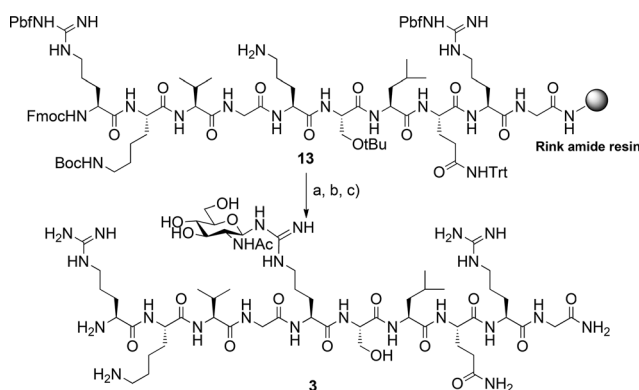
With **8** in hand, we then performed the synthesis of glycopeptide **1** using the on-resin glycosylation strategy (Scheme 2). First, the linear peptide was prepared by using the standard Fmoc (9-fluorenylmethyloxycarbonyl) SPPS procedures with 2-chlorotrityl resin as the solid support. Fmoc-Orn(Alloc)-OH was used as the precursor for the *N*-GlcNAcylated Arg residue. After the peptide assembly was completed, the Alloc group was removed using tetrakis(triphenylphosphine)palladium(0) to yield compound **9** on resin.^[14] The free amino side chain of **9** was then treated with AgNO₃ and **8** to afford glycopeptide **10** on resin. Next, the acetyl groups on GlcNAc were removed with 5% NH₂NH₂ in dimethylformamide (DMF).^[15] The resin was treated with 5% triisopropylsilane (TIPS) in trifluoroacetic acid (TFA) to release glycopeptide **1**, which was purified by preparative reverse-phase HPLC. The overall isolated yield of **1** was 39% as calculated from the resin loading, manifesting the good efficiency of the on-resin glycosylation process. All the key intermediates were monitored by analytical HPLC and successfully characterized by ESI-MS (Figure S1). The final product was fully characterized by 1D- and 2D-NMR spectroscopy as well as high-resolution quadrupole time-of-flight mass spectrometry (HR-Q-TOF-MS). The β -configuration of the glycosidic linkage was confirmed by NOESY (Figure S2).

To further test the scope of the solid-phase glycosylation method, we used **8** to synthesize a glycopeptide **2** bearing three Arg-GlcNAc sites. As shown in Scheme 3, peptide **11** was prepared by standard Fmoc SPPS to contain three



Scheme 3. Solid-phase synthesis of triglycosylated peptide **2**. Reagents and conditions: a) TEA, DMF, AgNO₃, **8** (6 equiv), rt; b) 5% NH₂NH₂ in DMF; c) 5% TIPS in TFA.

ornithine residues. In a single-step treatment with AgNO₃ and **8**, the **11** was successfully converted to glycopeptide **12**. After deprotection of the acetyl groups and cleavage from the resin, the desired glycosylated **2** bearing three Arg–GlcNAc was purified by preparative reverse-phase HPLC and a good overall yield of 16% of **2** was isolated (Figure S3). In addition, we used the above protocol to prepare an Arg–GlcNAc peptide **3** containing Arg, Lys, Ser, and Gln, which were protected with groups including 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf), Boc, *t*Bu, and Trt (Scheme 4). This glycopeptide was successfully obtained in 28% yield, indicating that our method is compatible with various functional side-chain groups. Collectively our experiments establish that the method developed in the present



Scheme 4. Solid-phase synthesis of monoglycosylated peptide **3**. Reagents and conditions: a) TEA, DMF, AgNO₃, **8** (3 equiv), rt; b) 5% NH₂NH₂ in DMF; c) TFA/phenol/water/thioanisole/1,2-ethanedithiol (82.5/5/5/2.5, v/v).

study is a general efficient strategy for fast synthesis of glycopeptides with arginine *N*-glycosylated groups.

With synthetic peptides carrying Arg–GlcNAc residues in hand, we wanted to find conditions for the generation of antibodies that can specifically recognize *N*-GlcNAcylated arginine in glycoproteins. For this purpose, mono-*N*-GlcNAcylated arginine containing peptide **1** was conjugated to the carrier protein keyhole limpet hemocyanin (KLH) through its N-terminal sulfhydryl group and then used to immunize rabbits with a standard protocol.^[16–17] For each immunization 2 mg peptide was used. Production of antibodies recognizing *N*-GlcNAcylated arginine in the inoculated animals was monitored by an enzyme-linked immunosorbent assay (ELISA) analysis of crude serum acquired 7 days after the third immunization. By default, the majorities of the antigen stimulated rabbit antibodies are in the isoform of IgG. In fact, the secondary antibody used in this study was horseradish peroxidase linked antirabbit IgG whole antibody from donkey (NA-934, GE Healthcare Life Science). Therefore the isotype of the Arg–GlcNAc antibody is IgG. To our pleasure, the antibodies from two batches of crude antisera of two immunized rabbits were found to robustly bind with both mono-*N*-GlcNAcylated arginine peptide **1** and tri-GlcNAcylated arginines containing peptide **2** by ELISA analysis. Moreover, these antibodies showed no cross reactivity with the corresponding unmodified peptide (Figure 3). Strong immune reactivity was observed even when the crude antiserum was diluted by more than 100 000 fold, suggesting a very high titer of the Arg–GlcNAc antibodies.

Importantly, the antiserum showed robust reactivity with FADD (Fas-associated death domain) and the death domain of TRADD only after the arginine residue had been GlcNAcylated by the NleB effector (Figure 4).^[5] Because

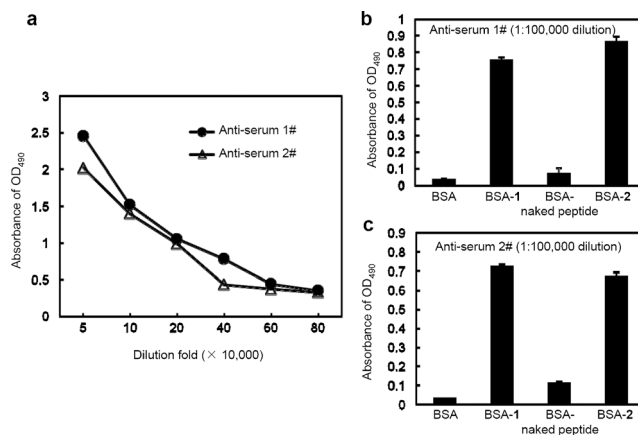


Figure 3. Arg–GlcNAc antiserum immunized by peptide **1** can recognize the arginine GlcNAcylated peptide with no dependence on the peptide sequence. ELISA analysis of two batches of antisera immunized by the arginine GlcNAcylated peptide. Antiserum 1# and antiserum 2# were gradually diluted in (a) or 100 000 fold diluted in (b) and (c) and subjected to indirect ELISA experiments against the peptide **1** conjugated with BSA in (a) or indicated peptides conjugated with BSA in (b) and (c). Carrier protein BSA was included as a negative control. Shown are the absorbance of OD₄₉₀ nm. Representative data from at least three repetitions with similar results are shown.

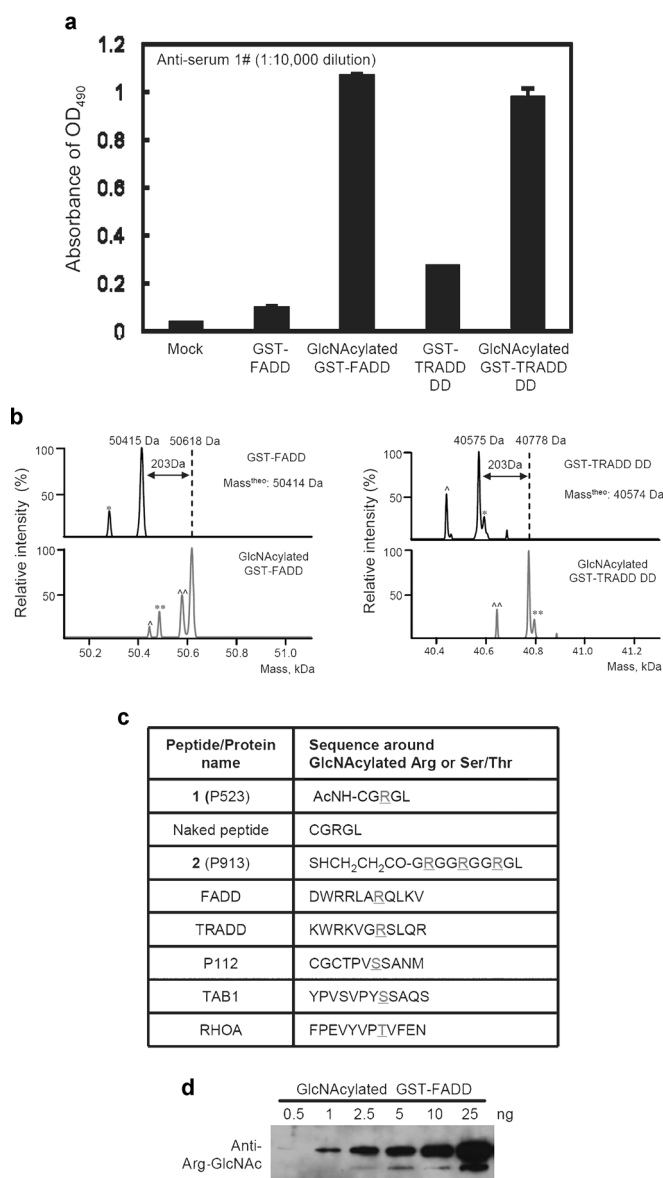


Figure 4. The Arg-GlcNAc antiserum can recognize arginine GlcNAcylated death domain proteins independent of protein sequences. **a)** ELISA analysis of the Arg-GlcNAc antiserum against GlcNAcylated or non-GlcNAcylated death domain proteins. Representative data from at least three repetitions with similar results are shown. **b)** ESI-MS analysis of recombinant proteins used in (a). Peaks marked with ^ and ^^ have a 131 Da mass decrease, corresponding to loss of the N-terminal methionine. Peaks marked with * and ** represent the acetylated form of unmodified and GlcNAcylated GST-FADD or GST-TRADD DD, respectively. **c)** Sequences of GlcNAcylated peptides/proteins used in Figures 3, 4, and 5. The GlcNAcylated arginine or serine/threonine is shown in red. **d)** Immunoblotting of GlcNAcylated GST-FADD used in (a,b) with Arg-GlcNAc antiserum 1# 1:1000 diluted in TBST buffer.

the amino acid sequences surrounding the GlcNAcylated arginine in the synthetic peptides and death domains shared no similarity (Figure 4c), our observation suggests that the antibodies should recognize the GlcNAcylated arginine epitope in a sequence-independent manner. Remarkably, the binding is so strong that even at 1 ng protein loading the

antiserum can still recognize GlcNAcylated FADD (Figure 4d).

To examine whether the new antibodies showed any cross reactivity with the canonical *O*-GlcNAcylation, we prepared a synthetic peptide containing a GlcNAcylated serine (**P112**). According to the ELISA assay, our Arg-GlcNAc antibodies, in contrast to the commercial *O*-GlcNAc antibody (CTD110.6),^[18] did not recognize *O*-GlcNAc (Figure 5a).

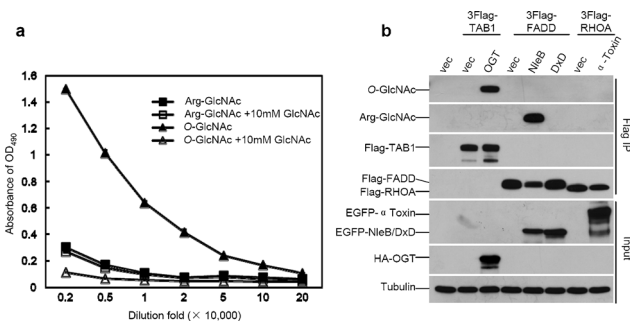


Figure 5. Recognition specificity of Arg-GlcNAc antibodies to Arg-GlcNAc but not *O*-GlcNAc. Representative data from at least three experiments are shown. **a)** ELISA analysis of Arg-GlcNAc antiserum or *O*-GlcNAc antibody (CTD110.6) against synthesized *O*-GlcNAcylated peptide **P112** conjugated with BSA supplemented with or without GlcNAc. **b)** Western blot analysis of effects of three types of GlcNAc transferase on their corresponding substrates detected by Arg-GlcNAc and *O*-GlcNAc antibodies. 293T cells were transfected with indicated combinations of 3 × Flag-tagged substrates (TAB1, FADD, and RHOA) and GlcNAc transferase (OGT, NleB, and α-Toxin) expression constructs. DxD: an enzyme activity dead mutant of NleB.

Furthermore, we used the mammalian *O*-GlcNAc transferase OGT and α-toxin GlcNAc transferase from *Clostridium novyi* to modify Ser395 in TAB1 (TGF-beta activated kinase 1/ MAP3K7 binding protein 1)^[19] and Thr37 in RHOA (ras homolog family member A) protein,^[20] respectively. The modification on TAB1 was readily detected by the *O*-GlcNAc antibody in the immunoblotting assay (Figure 5b). The GlcNAcyl groups on RHOA cannot be recognized by the *O*-GlcNAc antibody, although the modification was verified by mass spectrometry (data not shown). Nonetheless, both *O*-GlcNAcylated proteins were not reactive with the Arg-GlcNAc antibodies (Figure 5b). These data suggest that the Arg-GlcNAc antibodies have the desired specificity for Arg-GlcNAc modification and do not recognize the canonical *O*-GlcNAcyl groups.

Finally, the NleB GlcNAc transferase effector modifies multiple host death-domain proteins including FADD, TRADD, and RIPK1 during EPEC infection, which plays a critical role of suppressing host inflammation and promoting bacterial colonization and virulence. Here we found that the Arg-GlcNAc antibodies can detect infection-induced GlcNAc modification on FADD, TRADD, and RIPK1 only when EPEC strain harboring a catalytically active NleB was used for infection (Figure 6). These observations suggest that the Arg-GlcNAc antibodies are sensitive enough to monitor pathogen-induced modification of host proteins during infection, serving as a potential diagnostic tool.

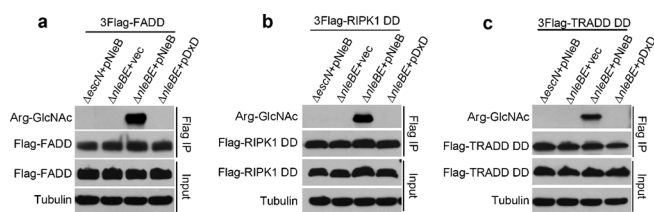


Figure 6. Recognition of Arg–GlcNAc antibodies to pathogen-infection-induced arginine GlcNAcylation. Shown are Western blot analyses of arginine GlcNAcylation catalyzed by type III-delivered NleB on multiple death domains; 24 h after transfection with indicated death domain expressing plasmids (3Flag-FADD in (a), 3Flag-RIPK1 DD in (b), 3Flag-TRADD DD in (c)), 293T cells were infected with indicated EPEC deletion strains complemented with a vector plasmid or a plasmid expressing WT NleB (pNleB) or the D221A/D223A mutant (pDxD). Infected cells were further subjected to anti-Flag immunoprecipitation and immunoblotting.

In conclusion, we report the first synthesis of arginine *N*-GlcNAcylated peptides through a silver-promoted solid-phase glycosylation process. This method was shown amenable to the preparation of glycopeptides with single or multiple Arg–GlcNAc sites in high efficiency. The resulting glycopeptides were used to obtain the first antibodies that can specifically recognize peptides or proteins carrying arginine *N*-GlcNAcylated groups in a sequence-independent manner. Moreover, the new antibodies only recognize Arg–GlcNAc modification without any cross activity toward the canonical *O*-GlcNAcyl groups. Recognition of Arg–GlcNAc antibodies to pathogen-infection-induced arginine GlcNAcylation has been successfully demonstrated. Thus we expect the new antibodies to be useful for the identification of novel arginine *N*-GlcNAcylated proteins and subsequent biochemical and pharmacological studies on pathogen infection as well as other biological processes.

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- [1] K. Moremen, M. Tiemeyer, A. Nairn, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 448–462.
- [2] D. Gill, H. Clausen, F. Bard, *Trends Cell Biol.* **2011**, *21*, 149–158.
- [3] H. Nothaft, C. Szymanski, *Nat. Rev. Microbiol.* **2010**, *8*, 765–778.
- [4] D. G. Singh, J. Lomako, W. M. Lomako, W. J. Whelan, H. E. Meyer, M. Serwe, J. W. Metzger, *FEBS Lett.* **1995**, *376*, 61–64.
- [5] a) S. Li, L. Zhang, Q. Yao, L. Li, N. Dong, J. Rong, W. Gao, X. Ding, L. Sun, X. Chen, F. Shao, *Nature* **2013**, *501*, 242–246; b) J. S. Pearson, C. Giogha, S. Y. Ong, C. L. Kennedy, M. Kelly, K. S. Robinson, T. W. Lung, A. Mansell, P. Riedmaier, C. V. Oates, A. Zaid, S. Mühlen, V. F. Crepin, O. Marches, C. S. Ang, N. A. Williamson, L. A. O'Reilly, A. Bankovacki, U. Nachbur, G.

Infusini, A. I. Webb, J. Silke, A. Strasser, G. Frankel, E. L. Hartland, *Nature* **2013**, *501*, 247–251.

- [6] G. Hart, M. Housley, C. Slawson, *Nature* **2007**, *446*, 1017–1022.
- [7] For some examples, please see: a) A. Schleyer, M. Meldal, R. Manat, H. Paulsen, K. Bock, *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1976–1978; *Angew. Chem.* **1997**, *109*, 2064–2067; b) J. Hudak, H. Yu, C. Bertozzi, *J. Am. Chem. Soc.* **2011**, *133*, 16127–16135; c) P. Wang, S. Dong, J. H. H. Shieh, E. Peguero, R. Hendrickson, M. A. Moore, S. J. Danishefsky, *Science* **2013**, *342*, 1357–1360; d) S. Dedola, M. Izumi, Y. Makimura, A. Seko, A. Kanamori, M. Sakono, Y. Ito, Y. Kajihara, *Angew. Chem. Int. Ed.* **2014**, *53*, 2883–2887; *Angew. Chem.* **2014**, *126*, 2927–2931; e) M. N. Amin, J. S. McLellan, W. Huang, J. Orwenyo, D. R. Burton, W. C. Koff, P. D. Kwong, L. X. Wang, *Nat. Chem. Biol.* **2013**, *9*, 521–526.
- [8] For some examples, please see: a) P. Nagorny, N. Sane, B. Fasching, B. Aussedat, S. Danishefsky, *Angew. Chem. Int. Ed.* **2012**, *51*, 975–979; *Angew. Chem.* **2012**, *124*, 999–1003; b) V. Ullmann, M. Rädisch, I. Boos, J. Freund, C. Pöhner, S. Schwarzing, C. Unverzagt, *Angew. Chem. Int. Ed.* **2012**, *51*, 11566–11570; *Angew. Chem.* **2012**, *124*, 11734–11738; c) T. Conroy, K. A. Jolliffe, R. J. Payne, *Org. Biomol. Chem.* **2009**, *7*, 2255–2258.
- [9] a) D. DeMong, R. Williams, *J. Am. Chem. Soc.* **2003**, *125*, 8561–8565; b) T. Imaoka, O. Iwamoto, K. Noguchi, K. Nagasawa, *Angew. Chem. Int. Ed.* **2009**, *48*, 3799–3801; *Angew. Chem.* **2009**, *121*, 3857–3859; c) D. Ermolat'ev, J. Bariwal, H. Steenackers, S. Keersmaecker, E. Eycken, *Angew. Chem. Int. Ed.* **2010**, *49*, 9465–9468; *Angew. Chem.* **2010**, *122*, 9655–9658.
- [10] D. Macmillan, A. Daines, M. Bayrhuber, S. Flitsch, *Org. Lett.* **2002**, *4*, 1467–1470.
- [11] M. J. Camarasa, P. Fernandez-Resa, M. T. Garcia-Lopez, F. G. Heras, P. P. Mendez-Castrillon, A. S. Felix, *Synthesis* **1984**, 509–510.
- [12] A. Kovalová, M. Ledvina, D. Saman, D. Zyka, M. Kubicková, L. Zidek, V. Sklenár, P. Pompach, D. Kavan, J. Bílý, V. Ondřej, K. Zuzana, L. Martina, L. Ljubina, A. Mária, M. Hynek, R. Daniel, H. Kateřina, K. Vladimír, B. Karel, *J. Med. Chem.* **2010**, *53*, 4050–4065.
- [13] D. Ma, C. Xia, J. Jiang, J. Zhang, W. Tang, *J. Org. Chem.* **2003**, *68*, 442–451.
- [14] Y. C. Huang, Y. M. Li, Y. Chen, M. Pan, Y. T. Li, L. Yu, Q. X. Guo, L. Liu, *Angew. Chem. Int. Ed.* **2013**, *52*, 4858–4862; *Angew. Chem.* **2013**, *125*, 4958–4962.
- [15] C. S. Bennett, S. M. Dean, R. J. Payne, S. Ficht, A. Brik, C. H. Wong, *J. Am. Chem. Soc.* **2008**, *130*, 11945–11952.
- [16] Z. M. Wu, X. Q. Guo, Z. W. Guo, *Chem. Commun.* **2010**, *46*, 5773–5774.
- [17] a) H. Cai, M. S. Chen, Z. Y. Sun, Y. F. Zhao, H. Kunz, Y. M. Li, *Angew. Chem. Int. Ed.* **2013**, *52*, 6106–6110; *Angew. Chem.* **2013**, *125*, 6222–6226; b) U. Westerlind, H. Schröder, A. Hobel, N. Gaidzik, A. Kaiser, C. Niemeyer, E. Schmitt, H. Waldmann, H. Kunz, *Angew. Chem. Int. Ed.* **2009**, *48*, 8263–8267; *Angew. Chem.* **2009**, *121*, 8413–8417.
- [18] F. I. Comer, K. Vosseller, L. Wells, M. A. Accavitti, G. W. Hart, *Anal. Biochem.* **2001**, *293*, 169–177.
- [19] S. Pathak, V. S. Borodkin, O. Albarbarawi, D. G. Campbell, A. Ibrahim, D. M. van Aalten, *EMBO J.* **2012**, *31*, 1394–1404.
- [20] J. Selzer, F. Hofmann, G. Rex, M. Wilm, M. Mann, I. Just, K. Aktories, *J. Biol. Chem.* **1996**, *271*, 25173–25177.