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An Endoglycosidase with Alternative Glycan Specificity Allows Broadened Glycoprotein Remodelling

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Supporting Information

ABSTRACT: Protein endoglycosidases are useful for biocatalytic alteration of glycans on protein surfaces, but the currently limited selectivity of endoglycosidases has prevented effective manipulation of certain *N*-linked glycans widely found in nature. Here we reveal that a bacterial endoglycosidase from *Streptococcus pyogenes*, EndoS, is complementary to other known endoglycosidases (EndoA, EndoH) used for current protein remodeling. It allows processing of complex-type *N*-linked glycans +/− core fucosylation but does not process oligomannose- or hybrid-type glycans. This biocatalytic activity now addresses previously refractory antibody glycoforms.

Glycan display is critical for development and physiology of many living systems.[1] Protein glycosylation is a diverse modification comprising 50% of cellular proteome and 90% of secreted proteome.[2] Of proteins currently in clinical trials, 70% are glycoproteins,[3] typically with "core fucosylation" (Figure 1). Natural glycoproteins exist as mixtures of glycoforms—same peptide backbone but different glycosylation pattern and site—making isolation of well-defined glycoproteins complicated.[4] Since certain glycoforms are more active,[5] access to specific uniform types can confer therapeutic advantages. In the case of antibodies (Abs), altering IgG-Fc glycosylation modulates binding to cellular receptors, thus tuning cytoxicity[6] and determining anti-inflammatory activity.[7]

Recombinant expression systems and chemoenzymatic and chemical methods can supply some homogeneous synthetic glycoproteins.[8] One powerful approach is enzymatic remodeling: initial heterogeneous glycoform mixtures are treated with an endoglycosidase ("Endo") to trim off the variable portions of the oligosaccharides attached to the first *N*-acetylglucosamine (GlcNAc) residue at *N*-linked site(s) (Figure 1b). Subsequent enzyme-mediated glycosylation of the exposed GlcNAc residue can then produce a more homogeneous sample.[8d,9] However, Endo enzymes currently used to attach glycans to proteins in this way show restricted sugar selectivity that prevents such combined removal and attachment of the complex-type glycans normally found on mammalian proteins or glycans that contain "core fucose".[10−12]

To identify new activities, we surveyed known and putative Endo (E.C. 3.2.1.96) orfs (putative *ndo* genes). While those in the CAZy glycoside[13] hydrolase family GH85 are most commonly used in current synthesis, orfs exist in other families, implying possibly different substrate specificities. The *ndoS* gene from *Streptococcus pyogenes* (GH18) codes for production of an apparently immunomodulatory evasion factor that prolongs bacterial survival and acts upon four subclasses of IgG.[19,20] Codon-optimized *ndoS* gene was generated (Table S1) and subligated into pGEX-4T-1 vector, which allowed efficient (∼10 mg/L) heterologous expression of EndoS in *N*-terminally-GST-tagged form using *Escherichia coli* BL21(DE3).

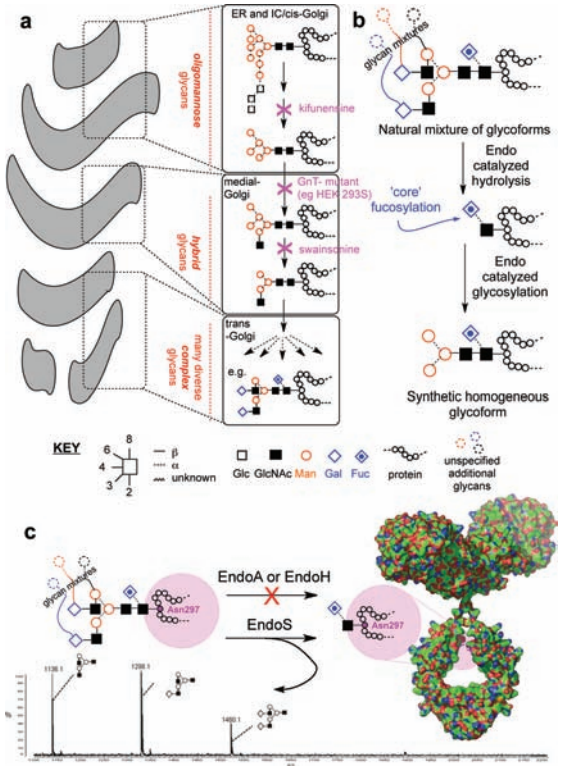

Figure 1. (a) *N*-linked glycoprotein biosynthesis and potential corresponding methods (magenta) to access altered glycans.[14] (b) Glycan remodeling using Endo enzymes (here idealized complex-type; key from ref 15). (c) Trimming of human IgG glycans by EndoS, not EndoA/H. MALDI-TOF MS ($[M + Na]^+$) confirms unique complex-type processing (see also Figure S8).

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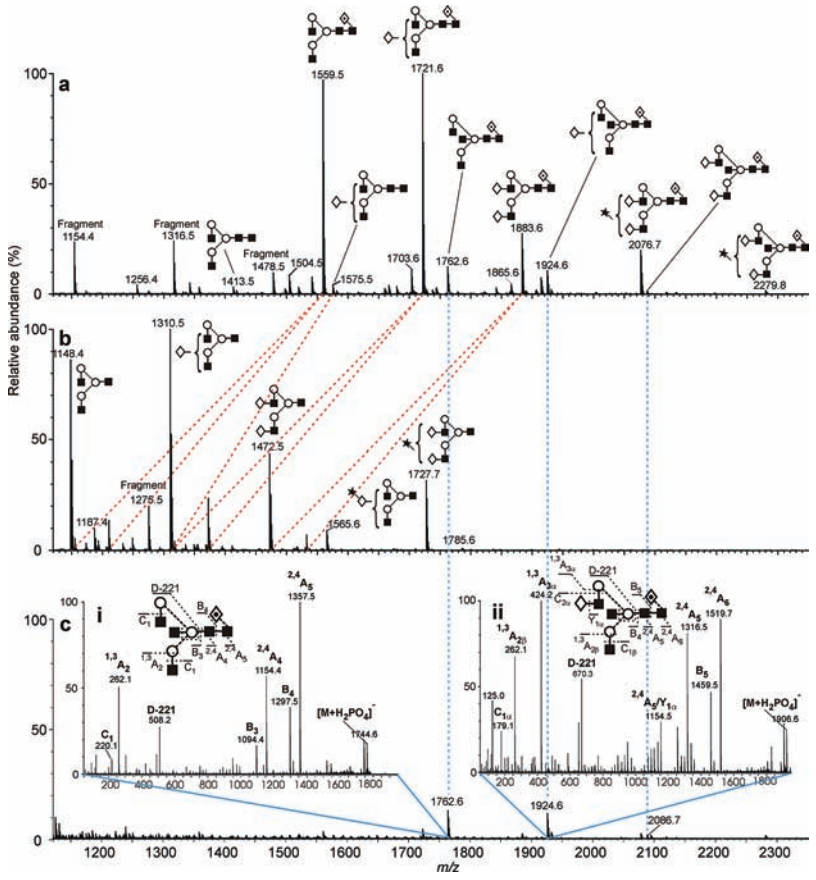

Figure 2. EndoS digestion of IgG glycans. ESI(−)-MS of human serum IgG *N*-linked glycans released by (a) PNGase F, (b) EndoS, and (c) PNGase F after EndoS. Inset in (c): ESI(−)-MS/MS of (i) *m*/*z* 1762.6 and (ii) *m*/*z* 1924.6. Fragment ions are labeled according to the scheme proposed by Domon and Costello;[16] structural identification is as described by Harvey.[17,18]

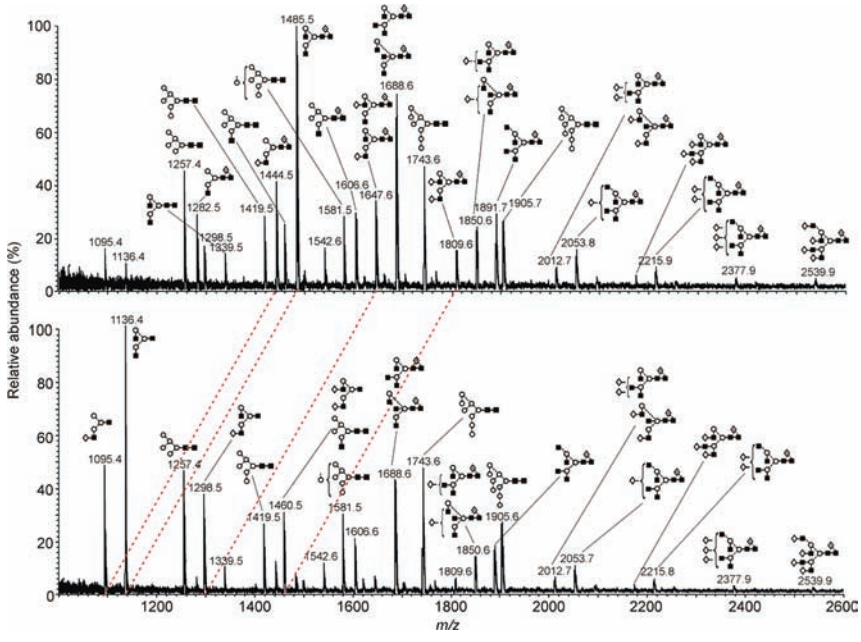

Figure 3. EndoS digestion of free *N*-linked glycans from desialylated recombinant $gp120_{BaL}$. MALDI(+)-MS spectra (a) of total gp120 glycan pool and (b) following digestion with EndoS. The red lines highlight the cleaved species and their products.

A thrombin-cleavable site allowed EndoS to be produced tagged and untagged (Table S2); both were highly active.

To evaluate important targets with relevant glycosylation, we restricted analyses to glycoprotein substrates from mammalian expression. Human IgG (Figures S7 and S8) was incubated with EndoS or widely used EndoA (GH85)[21] and EndoH (GH18)[22] at 37 °C for 1 h in PBS (pH 7.4, 10 mM). Reducing SDS-PAGE and MALDI(+)-MS analyses revealed absence of

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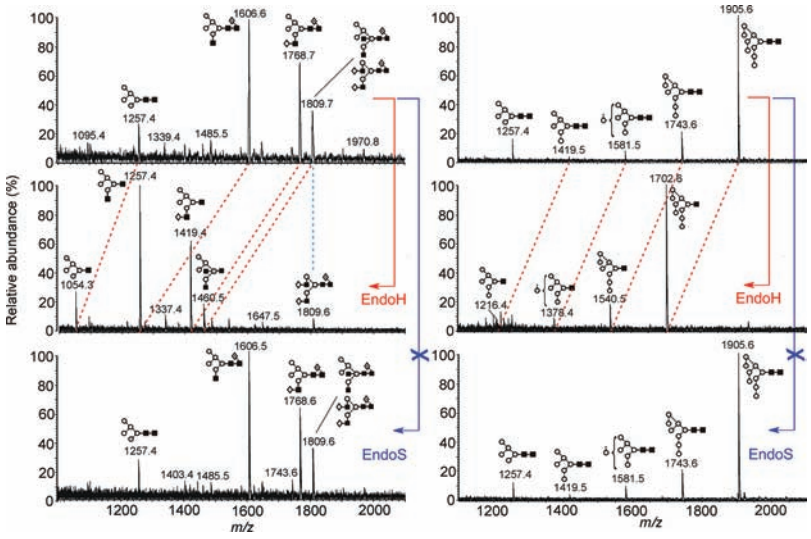

Figure 4. Alternative processing of *N*-glycans (PNGase-F-released) with distributions from modulated glycosylation pathways. Both hybrid (left) and oligomannose $Man_9GlcNAc_2$ (+ $Man_{5-8}GlcNAc_2$) (right) were processed by EndoH, not EndoS.

activity by EndoA and EndoH toward IgG. In contrast, EndoS processed IgG efficiently; glycans were removed even at low concentration (<50 nM, k_{app} > 0.8 s^{-1}). Detailed analyis by ESI(−)-MS (Figure 2) revealed clean release of G0, G1, and G2 glycans (including sialylated variants) by EndoS. Only a trace population (<2%) of bisected biantennary structures was found to be resistant to exhaustive digestion (consistent with tryptic mapping[23]); composition was confirmed by ESI-MS/MS (Figure 2c). The high activity of EndoS against IgG glycans contrasted starkly with lack of cleavage of these sugars by EndoA and EndoH (Figure S8).

Having confirmed EndoS's atypical ability to process IgG glycans, we tested it against those from other important recombinant glycoproteins. We used HIV-1 glycoprotein, gp120, with a wide range of complex, hybrid, and oligomannose-type glycans when expressed from human embryotic kidney (HEK293T) cells. Analysis of free gp120 *N*-glycans confirmed susceptibility of complex, fucosylated *N*-linked carbohydrates to EndoS (Figure 3). Bisected structures were not cleaved; additional EndoS-resistant glycoforms were identified (e.g., triantennary/oligomannose-type). These alternative specificities of EndoS and EndoH were assayed by expression of recombinant IgG-Fc with oligomannose or hybrid-type glycans (Figure 4), derived through inhibitory modulation of HEK293T glycosylation (Figure 1a, SI). Both were deglycosylated by EndoH but not EndoS, confirming unique and alternative specificity. Thus, protein glycans displayed strikingly different behaviors: EndoH cleaved only oligomannose or hybrid structures, which were untouched by EndoS. Conversely, in the presence of a wide range of *N*-linked glycans, EndoS was uniquely capable of processing complex-type glycans usually found on Abs, presenting a new route to remodeling and synthesis.

Next, we tested EndoS's processing of other glycan types. Core fucosylation modulates effector function[6] and is prevalent in human and therapeutic Abs. IgG-Fc was transiently expressed (see SI) in wild-type human (HEK293T) cells. Processing of glycans in fucosylated, non-fucosylated, and mixed (Figure S9a–c) states by EndoS revealed valuable plasticity for "core" α(1,6)-Fuc. This demonstrated that EndoS has, by virtue of its selectivity, the potential to remodel (Figure 1b) Abs produced in mammalian eukaryotic systems and hence many current therapeutics. To test this promise, we remodeled heterogeneous glycoforms of IgG (Figure 5). Endo-β-*N*-

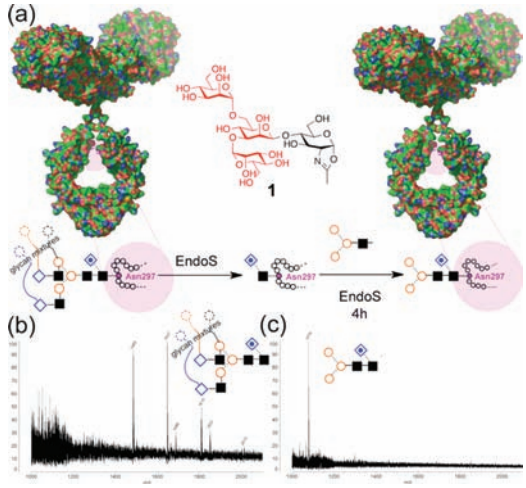

Figure 5. Remodeling of human IgG glycans. (a) EndoS-catalyzed glycosylation with **1** creates unnatural glycoform. (b) Native and (c) remodeled PNGase-cleaved glycan MALDI-MS.

acetylglucosaminidases hydrolyze the glycosidic bond in the *N*,*N*′-diacetylchitobiose core of *N*-linked glycans. Some, e.g., EndoA[24] and EndoM,[25] also possess significant transglycosylation activity and have been used to remodel the *N*-glycoprotein RNase-B.[26] Importantly, the efficiency of glycosylation has been improved[27] using sugar oxazolines as substrates and mutant Endo enzymes. Recently, re-engineering of bacterial glycosylation[9d] or chemical attachment[28] has allowed repositioning of glycosylation site. Notably, previous remodeling of yeast-produced Fc's proved possible due to non-fucosylated, high/oligo-mannose glycoform content.[29] Moreover, other Endo approaches have proven difficult: a system based on innovative adaptation of prokaryotic production gave Fc yields of <5%.[9d]

Tetrasaccharide oxazoline **1** was synthesized (SI) and after trimming was used in EndoS-catalyzed glycosylation of IgG to

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yield essentially a single glycoform (>95%). Transglycosylation remodeling of glycoproteins directly from mammalian eukaryotic sources has not been possible until now; this work usefully complements truncation-only strategies.[30] Importantly, EndoS shows promising tolerance (including to the presence of core fucosylation), confirming our specificity analysis and the broadened synthetic utility.

The complementarity of EndoS to other Endos also raises the possibility of differential processing of mixed glycoform populations; it has been suggested that differently processed antibodies could be used to treat autoimmune diseases.[31] The glycan selectivity and protein efficiency of EndoS will make it attractive in the synthesis of pure relevant glycoforms (e.g., complex-type) of IgG's. The molecular basis and finer details of this activity and use in IgG manipulation are under study. Notably, the direct manipulation of intact IgG shown here complements recent strategies using an elegant indirect approach that exploits inhibition of biosynthesis to control precursor glycoforms (thus far for only Fc).[29b] *S. pyogenes*, the origin of *ndoS*, causes human diseases with high mortality (necrotising fasciitis, sepsis); our studies may also address EndoS's role[19] in pathogenicity.

■ ASSOCIATED CONTENT

Supporting Information

Protocols and further analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ohtsubo, K.; Marth, J. D. *Cell* **2006**, *126*, 855.

(2) (a) Apweiler, R.; Hermjakob, H.; Sharon, N. *Biochim. Biophys. Acta* **1999**, *1473*, 4. (b) Jung, E.; Veuthey, A.-L.; Gasteiger, E.; Bairoch, A. *Proteomics* **2001**, *1*, 262.

(3) Sethuraman, N.; Stadheim, T. A. *Curr. Opin. Biotechnol.* **2006**, *17*, 341.

(4) Rudd, P. M.; Joao, H. C.; Coghill, E.; Fiten, P.; Saunders, M. R.; Opdenakker, G.; Dwek, R. A. *Biochemistry* **1994**, *33*, 17.

(5) Suzuki, T.; Kitajima, K.; Inoue, S.; Inoue, Y. *Glycoconjugate J.* **1995**, *12*, 183.

(6) Jefferis, R. *Nat. Rev. Drug Discov.* **2009**, *8*, 226.

(7) Anthony, R. M.; Nimmerjahn, F.; Ashline, D. J.; Reinhold, V. N.; Paulson, J. C.; Ravetch, J. V. *Science* **2008**, *320*, 373.

(8) (a) Gerngross, T. U. *Nat. Biotechnol.* **2004**, *22*, 1409. (b) Betenbaugh, M. J.; Tomiya, N.; Narang, S.; Hsu, J. T. A.; Lee, Y. C. *Curr. Opin. Struct. Biol.* **2004**, *14*, 601. (c) Kowarik, M.; Numao, S.; Feldman, M. F.; Schulz, B. L.; Callewaert, N.; Kiermaier, E.; Catrein, I.; Aebi, M. *Science* **2006**, *314*, 1148. (d) Bennett, C. S.; Wong, C.-H. *Chem. Soc. Rev.* **2007**, *36*, 1227. (e) Gamblin, D. P.; Scanlan, E. M.; Davis, B. G. *Chem. Rev.* **2009**, *109*, 131.

(9) (a) Witte, K.; Sears, P.; Martin, R.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 2114. (b) Wang, L.-X. *Carbohydr. Res.* **2008**, *343*, 1509. (c) Rich, J. R.; Withers, S. G. *Nat. Chem. Biol.* **2009**, *5*, 206. (d) Schwarz, F.; Huang, W.; Li, C.; Schulz, B. L.; Lizak, C.; Palumbo, A.; Numao, S.; Neri, D.; Aebi, M.; Wang, L.-X. *Nat. Chem. Biol.* **2010**, *6*, 264.

(10) Elegant strategies have used Endos that do not cleave complex or core-Fuc glycans to instead attach them using unnatural substrates under forcing conditions. The processing of core-Fuc has recently been shown on short peptide fragments; see refs 11 and 12.

(11) Huang, W.; Yang, Q.; Umekawa, M.; Yamamoto, K.; Wang, L.-X. *ChemBioChem* **2010**, *11*, 1350.

(12) Huang, W.; Li, J.; Wang, L.-X. *ChemBioChem* **2011**, *12*, 932.

(13) www.cazy.org

(14) (a) Reeves, P. J.; Callewaert, N.; Contreras, R.; Khorana, H. G. *Proc. Natl Acad. Sci. U.S.A.* **2002**, *99*, 13419. (b) Chang, V. T.; Crispin, M.; Aricescu, A. R.; Harvey, D. J.; Nettleship, J. E.; Fennelly, J. A.; Yu, C.; Boles, K. S.; Evans, E. J.; Stuart, D. I.; Dwek, R. A.; Jones, E. Y.; Owens, R. J.; Davis, S. J. *Structure* **2007**, *15*, 267.

(15) Harvey, D. J.; Merry, A. H.; Royle, L.; Campbell, M. P.; Dwek, R. A.; Rudd, P. M. *Proteomics* **2009**, 3796.

(16) Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397.

(17) Harvey, D. J. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 647.

(18) Harvey, D. J. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 484.

(19) Collin, M.; Olsen, A. *EMBO J.* **2001**, *20*, 3046.

(20) Allhorn, M.; Briceno, J. G.; Baudino, L.; Lood, C.; Olsson, M. L.; Izui, S.; Collin, M. *Blood* **2010**, *115*, 5080.

(21) Robbins, P. W.; Trimble, R. B.; Wirth, D. F.; Hering, C.; Maley, F.; Maley, G. F.; Das, R.; Gibson, B. W.; Royal, N.; Biemann, K. *J. Biol. Chem.* **1984**, *259*, 7577.

(22) Jefferis, R. *Biotechnol. Prog.* **2005**, *21*, 11.

(23) Goetze, A. M.; Zhang, Z.; Liu, L.; Jacobsen, F. W.; Flynn, G. C. *Mol. Immunol.* **2011**, *49*, 338.

(24) Takegawa, K.; Yamaguchi, S.; Kondo, A.; Iwamoto, H.; Nakoshi, M.; Kato, I.; Iwahara, S. *Biochem. Int.* **1991**, *24*, 849.

(25) Yamamoto, K. J.; Kadowaki, S.; Watanabe, J.; Kumagai, H. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 244.

(26) (a) Fujita, K.; Yamamoto, K. *Biochim. Biophys. Acta* **2006**, *1760*, 1631. (b) Takegawa, K.; Tabuchi, M.; Yamaguchi, S.; Kondo, A.; Kato, I.; Iwahara, S. *J. Biol. Chem.* **1995**, *270*, 3094.

(27) (a) Li, B.; Song, H.; Hauser, S.; Wang, L.-X. *Org. Lett.* **2006**, *8*, 3081. (b) Heidecke, C. D.; Ling, Z.; Bruce, N. C.; Moir, J. W. B.; Parsons, T. B.; Fairbanks, A. J. *ChemBioChem* **2008**, *9*, 2045. (c) Rising, T. W.; Heidecke, C. D.; Moir, J. W.; Ling, Z.; Fairbanks, A. J. *Chem.—Eur. J.* **2008**, *14*, 6444. (d) Ochiai, H.; Huang, W.; Wang, L.-X. *J. Am. Chem. Soc.* **2008**, *130*, 13790. (e) Huang, W.; Li, C.; Li, B.; Umekawa, M.; Yamamoto, K.; Zhang, X.; Wang, L.-X. *J. Am. Chem. Soc.* **2009**, *131*, 2214.

(28) Fernandez-Gonzalez, M.; Boutureira, O.; Bernardes, G. J. L.; Chalker, J. M.; Young, M. A.; Errey, J. C.; Davis, B. G. *Chem. Sci.* **2010**, *1*, 709.

(29) (a) Wang, L. X.; Wei, Y. D.; Li, C. S.; Huang, W.; Li, B.; Strome, S. *Biochemistry* **2008**, *47*, 10294. (b) Zou, G.; Ochiai, H.; Huang, W.; Yang, Q.; Li, C.; Wang, L.-X. *J. Am. Chem. Soc.* **2011**, *133*, 18975.

(30) Mimura, Y.; Church, S.; Ghirlando, R.; Ashton, P. R.; Dong, S.; Goodall, M.; Lund, J.; Jefferis, R. *Mol. Immunol.* **2000**, *37*, 697.

(31) Allhorn, M.; Collin, M. *Ann. N.Y. Acad. Sci.* **2009**, *1173*, 664.