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Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation

Yoshikatsu Kaneko,* Falk Nimmerjahn,* Jeffrey V. Ravetch†

Immunoglobulin G (IgG) mediates pro- and anti-inflammatory activities through the engagement of its Fc fragment (Fc) with distinct Fc γ receptors (Fc γ R). One class of Fc-Fc γ R interactions generates pro-inflammatory effects of immune complexes and cytotoxic antibodies. In contrast, therapeutic intravenous gamma globulin and its Fc fragments are anti-inflammatory. We show here that these distinct properties of the IgG Fc result from differential sialylation of the Fc core polysaccharide. IgG acquires anti-inflammatory properties upon Fc sialylation, which is reduced upon the induction of an antigen-specific immune response. This differential sialylation may provide a switch from innate anti-inflammatory activity in the steady state to generating adaptive pro-inflammatory effects upon antigenic challenge.

Immunoglobulin G (IgG) is the major serum immunoglobulin and is principally responsible for the recognition, neutralization, and elimination of pathogens and toxic antigens. It is a glycoprotein, composed of two identical heavy chains and two light chains, which in turn are composed of variable and constant domains.

The variable domains form the antigen recognition site, whereas the constant domains of the heavy chain form the effector arm of the molecule as an Fc domain. A single N-linked glycan is found at Asn²⁹⁷ in the Fc domain, and this covalently linked complex carbohydrate is composed of a core biantennary heptapolysaccharide

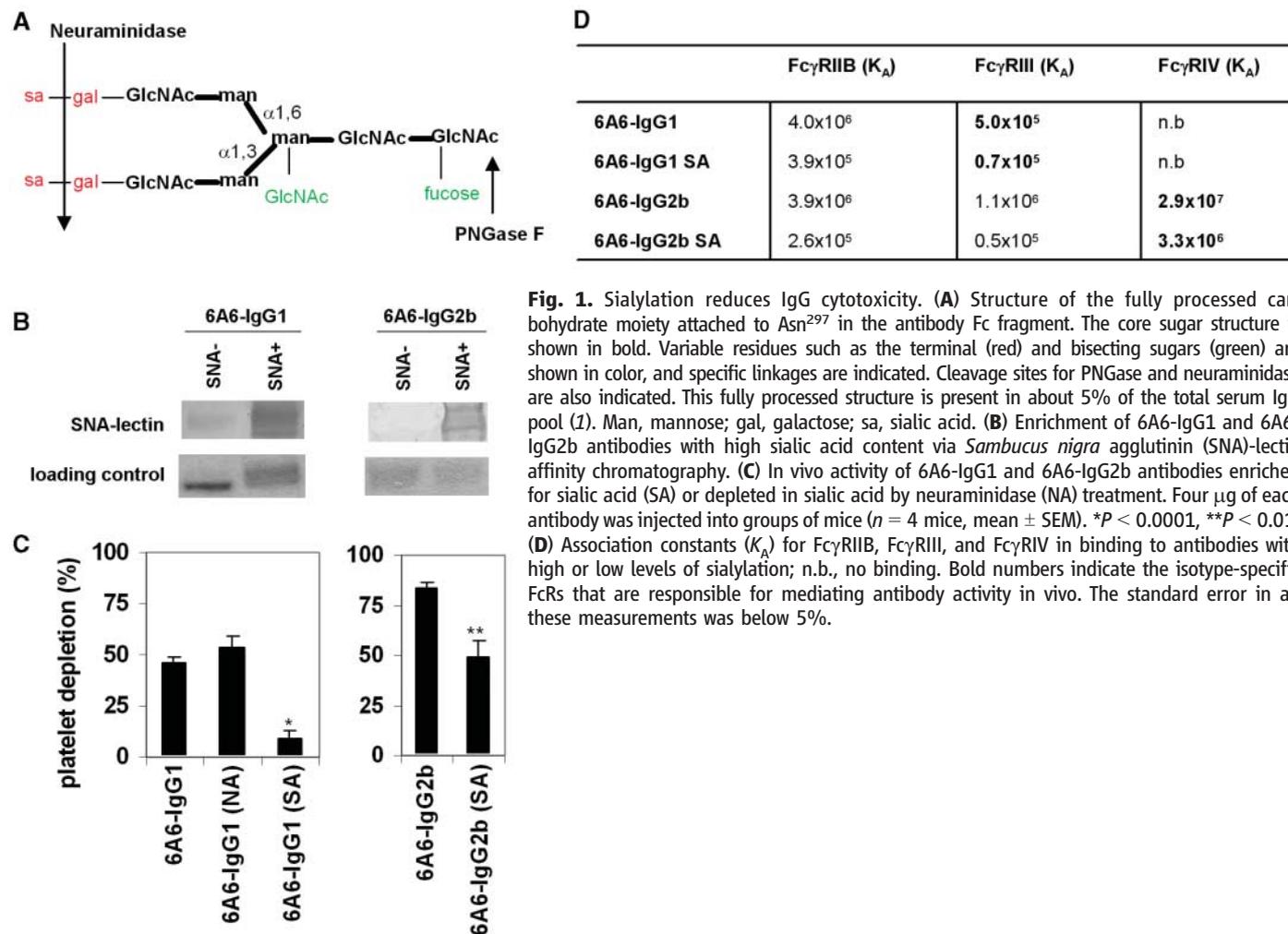
containing *N*-acetylglucosamine (GlcNAc) and mannose. Further modification of the core carbohydrate structure is observed in serum antibodies, with fucose, bisecting GlcNAc, galactose, and terminal sialic acid moieties being variably present (Fig. 1A). The fully processed form of the carbohydrate moiety exists in about 5% of the total serum IgG pool (1), and over 30 different covalently attached glycans have been detected at this single glycosylation site (1). Glycosylation of IgG is essential for binding to all Fc γ receptors (Fc γ R) (2) through maintenance of an open conformation of the two heavy chains (3). This absolute requirement of IgG glycosylation for Fc γ R binding accounts for the inability of deglycosylated IgG antibodies to mediate in vivo triggered inflammatory responses (4).

The idea that individual glycoforms of IgG may contribute to modulating inflammatory responses has been suggested by the altered

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affinities for individual Fc γ R that have been reported for IgG antibodies containing or lacking fucose (5) and their consequential effects on cytotoxicity (6). In contrast, aglycosylated IgG retains FcRn binding and in vivo half-life (5). IgG glycosylation differs in patients with rheumatoid arthritis and several forms of autoimmune vasculitis with decreased Fc galactosylation and sialylation when compared to normal individuals (7–10). Variations in IgG glycoforms have also been reported to be associated with aging (11) and upon immunization (12). Thus, although it has been proposed that individual IgG glycoforms may play a role in modulating antibody effector function in vivo (13), an underlying mechanism that could account for the disparate observations is, as yet, lacking.

To determine whether specific glycoforms of IgG are involved in modulating the effector functions of antibodies, we explored the role of specific Asn²⁹⁷-linked carbohydrates in mediating the cytotoxicity of IgG monoclonal

antibodies of defined specificity. The antibodies to platelets, derived from the 6A6 hybridoma, expressed as an IgG1, IgG2a, or IgG2b switch variant in 293 cells as previously described (6, 14, 15), were analyzed by mass spectrometry to determine their specific carbohydrate composition (figs. S1 and S2). These antibodies contained minimal sialic acid residues, and because it has been suggested that antibodies with decreased levels of terminal sugar residues might be more pathogenic (7–10), we investigated the influence of sialic acid on antibody activity in vivo. Enrichment of the sialic acid-containing species by *Sambucus nigra* lectin affinity chromatography yielded antibodies enriched 60- to 80-fold in sialic acid content (Fig. 1B and fig. S2). Comparison of the ability of 6A6-IgG1 and 6A6-IgG2b antibodies to mediate in vivo platelet clearance revealed an inverse correlation with sialylation (Fig. 1C). Sialylation of 6A6-IgG antibodies resulted in a 40 to 80% reduction in biological activity, which could be

partially restored upon removal of these sialic acid moieties with neuraminidase (fig. S3). Surface plasmon resonance binding analysis revealed a 5- to 10-fold reduction in binding affinity for the sialylated forms of these antibodies to their respective activating or inhibitory Fc γ Rs as compared to their asialylated counterparts (Fig. 1D), whereas no differences in binding affinity for the antigen were observed (fig. S4). Thus, sialylation of the Asn²⁹⁷-linked glycan structure of IgG resulted in reduced binding affinities to the subclass-restricted Fc γ Rs and thereby reduced their in vivo cytotoxicity.

We next examined the role of N-linked glycans on the anti-inflammatory activity of immunoglobulins with established anti-inflammatory activity. Intravenous gamma globulin (IVIG) is a purified IgG fraction obtained from the pooled serum of 5000 to 10,000 donors that is widely used to treat inflammatory diseases through administration at high doses (16). The anti-inflammatory activity of IVIG therapy is a

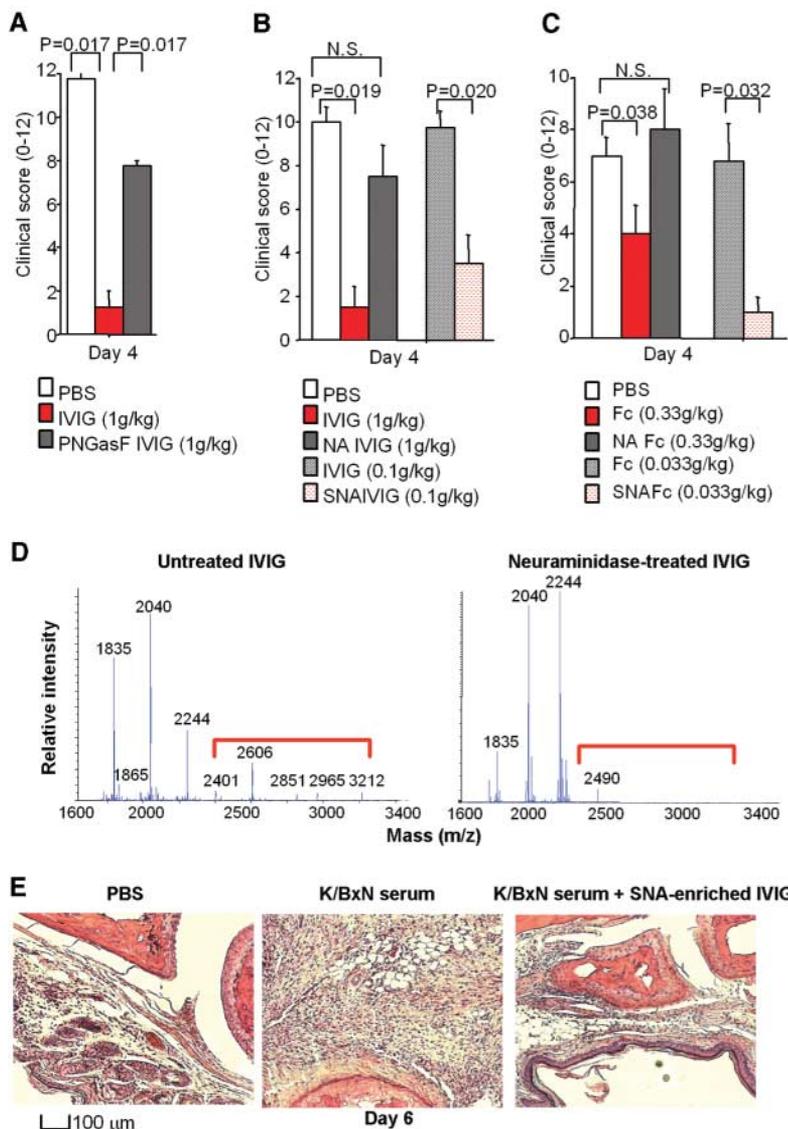


Fig. 2. The anti-inflammatory activity of IVIG requires sialic acid. **(A)** Clinical scores of K/BxN-serum-induced arthritis in mice treated with phosphate-buffered saline (PBS), IVIG, and PNGaseF-treated IVIG (PNGaseF IVIG). **(B)** In addition to the treatment described in **(A)**, mice were treated with neuraminidase-treated IVIG (NA IVIG) or SNA-enriched IVIG (SNA-IVIG). **(C)** Clinical scores of mice treated with the Fc fragment of IVIG, neuraminidase-treated Fc (NA Fc), or SNA-enriched Fc (SNA Fc) ($n = 4$, mean \pm SEM). **(D)** Carbohydrate profiles of IVIG preparations. MALDI-TOF mass spectrometry profiles of N-glycans derived from untreated or neuraminidase-treated IVIG are shown. Peaks that contain sialic acid residues are indicated (red brackets), and the carbohydrate composition of the peaks is presented in fig. S1. m/z , mass/charge ratio. **(E)** Representative hematoxylin/eosin staining of the ankle joints of control mice or mice with K/N-induced arthritis treated with or without SNA-enriched IVIG (0.1 g/kg). The extensive neutrophil infiltration observed in K/N-treated mice is absent from IVIG-SNA (0.1 g/kg)-treated mice. **(F)** Lectin blotting of the control Fc fragment of IVIG, neuraminidase-treated Fc (NA Fc), and Fc with high sialic acid content via SNA affinity chromatography (SNA Fc).

property of the Fc fragment (17) and is also protective in murine models of immunothrombocytopenia, rheumatoid arthritis, and nephrotoxic serum nephritis (18–20). To define the potential role of the glycan structure on the Fc in the anti-inflammatory activity of IVIG, these carbohydrates were removed with peptide N-glycosidase (PNGase) F, and the resulting ability to inhibit inflammatory responses was assessed in the K/N serum model of rheumatoid arthritis. Deglycosylated IVIG was unable to mediate anti-inflammatory activity in vivo (Fig. 2A). To further characterize this glycan requirement, IVIG was treated with neuraminidase to remove the terminal saccharides, and the composition of

the complete sugar removal was confirmed by mass spectrometry (Fig. 2D and fig. S1). These IgG preparations were then tested for their ability to protect mice from joint inflammation in the K/N serum arthritis model (21). Desialylation with neuraminidase abrogated the protective effect of the IVIG preparation in this model, at levels similar to those seen after removal of the entire glycan structure (Fig. 2B). This loss of activity was not the result of reduced serum half-life of the desialylated IgG preparations or the result of changes in the monomeric composition or structural integrity of the IgG (fig. S5). The unchanged FcRn binding affinity (5) and serum half-life of the

deglycosylated or desialylated IVIG variants strongly argue against a role for FcRn in the mechanism of IVIG action (fig. S5) (14).

The dependence of IVIG on sialic acid for its anti-inflammatory activity is distinct from the well-known property of sialic acid of masking structural determinants and rendering them inaccessible to ligand interactions (22). Rather, in this situation, sialic acid is responsible for the acquisition of new IgG activity in suppressing inflammation via the induction of inhibitory FcγRIIB expression (19). Because sialic acid appeared to be required for the anti-inflammatory activity of IVIG, we reasoned that the basis for the high doses [1 g per kilogram of body weight (g/kg)] needed for this anti-inflammatory activity might be the limiting concentration of sialylated IgG species in the total IVIG preparation (1, 7). We fractionated IVIG on an *Sambucus nigra* agglutinin (SNA)-lectin affinity column to obtain IgG molecules enriched for sialic acid–modified glycan structures and confirmed this idea using mass spectrometric analysis. In the K/N arthritis model, these sialic acid–enriched fractions showed a 10-fold enhancement in protection, so that equivalent protection was obtained at 0.1 g/kg of SNA-enriched IVIG as compared to 1 g/kg of unfractionated IVIG (Fig. 2, B and E). The protective activity was limited to the sialylated Fc fragment (Fig. 2, C and F). Serum half-life and IgG subclass distribution were equivalent in both fractions (fig. S6) and the anti-inflammatory effect was specific to IgG; sialylated N-linked glycoproteins such as fetuin or transferrin, with similar biantennary complex carbohydrate structures, had no statistically significant anti-inflammatory activity at equivalent or higher molar concentrations of IgG (fig. S7). Finally, the mechanism of protection of the sialylated IVIG preparation was similar to that of unfractionated IVIG in that it was dependent on FcγRIIB expression and resulted in the increased expression of this inhibitory receptor on effector macrophages (fig. S8). The high dose requirement for the anti-inflammatory activity of IVIG can be attributed to the minor contributions of sialylated IgG present in the total preparation. Thus, enhancing the sialylation of IgG may be an effective means of increasing the anti-inflammatory activity of IVIG. It remains to be determined whether both the 1,3 and 1,6 arms contribute equally to the anti-inflammatory property and whether preference for either the 2,3, 2,6 or 2,8 sialic acid–galactose linkages is involved in this activity.

Because passive immunization with IgG antibodies indicated that IgG can switch from a pro-inflammatory to an anti-inflammatory species by changing the extent of sialylation of the N-linked glycan on the Fc domain, we next determined whether IgG sialylation is regulated during an active immune response by using the nephrotoxic serum nephritis model (20). In this model, mice are presensitized with sheep IgG and then challenged with a sheep anti-mouse

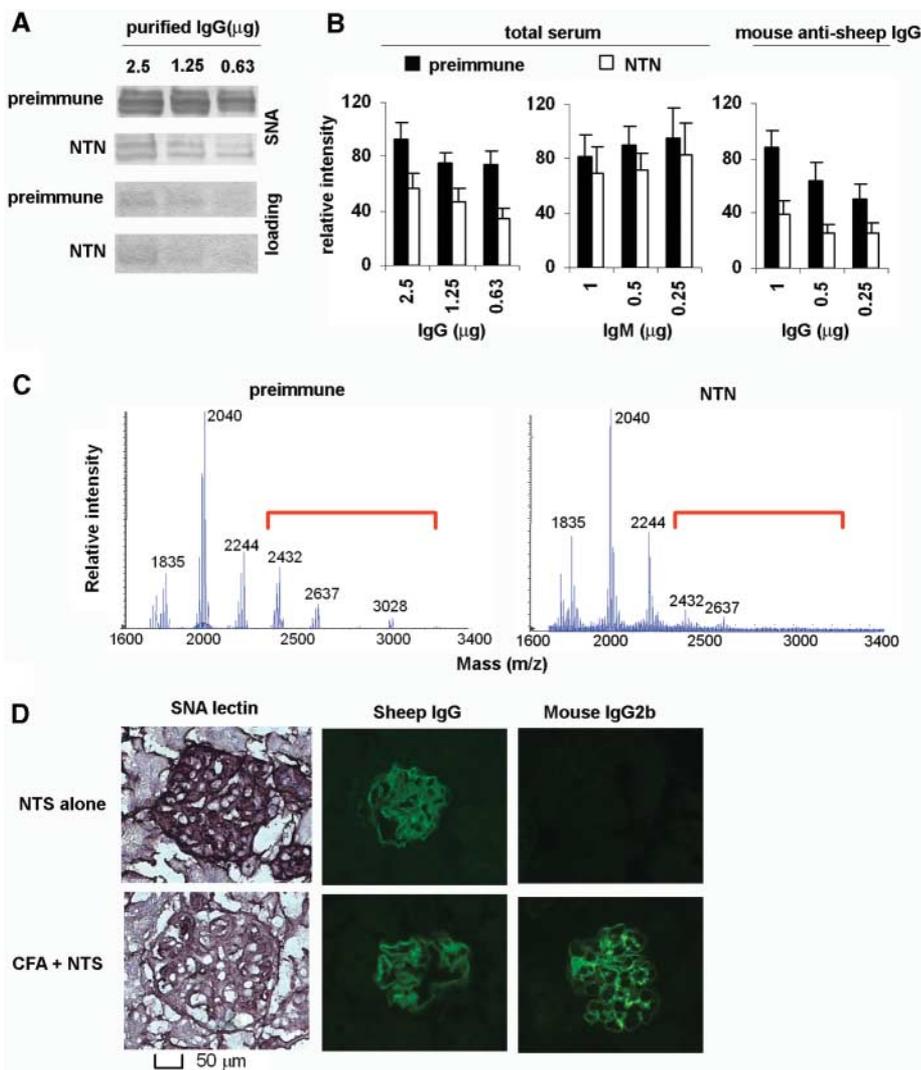


Fig. 3. Active immunization results in reduced IgG sialylation. (A) Serum IgG from untreated (preimmune) or mice with nephrotoxic nephritis (NTN) induced by immunization with sheep IgG and NTS was characterized for sialic acid content by blotting with SNA (14). (B) Quantitation of the level of sialylation of total serum IgG and IgM antibodies and sheep IgG–specific IgG antibodies in untreated and NTN mice (mean ± SEM) as determined by densitometry. No detectable sheep IgG was present in the mouse antibody preparations. (C) MALDI-TOF analysis of sugar residues attached to IgG antibodies from untreated or NTN mice. Sialic acid–containing moieties are indicated (red brackets). The detailed carbohydrate composition of the individual peaks is shown in fig. S1. (D) Detection of sialic acid content in antibodies deposited in the glomeruli of mice injected with NTS with (NTS+CFA) or without (NTS alone) preimmunization with sheep IgG in Freund’s complete adjuvant (CFA).

glomerular basement membrane preparation [nephrotoxic serum (NTS)], thereby inducing a mouse IgG response. Serum IgG and IgM from preimmune and NTS-immunized mice were characterized for sialic acid content; total IgG sialylation was reduced on average by 40% in immunized mice as compared to the unimmunized controls (Fig. 3, A and B). The effect was specific for IgG; sialylation of IgM or transferrin was equivalent before and after immunization (Fig. 3B and fig. S9). This difference in sialylation was more pronounced when the anti-sheep-specific IgG fraction from mouse serum was analyzed, showing a 50 to 60% reduction in sialylation as compared to preimmune IgG (Fig. 3B), and these results were confirmed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis (Fig. 3C). Finally, the mouse IgG2b antibodies to sheep that were deposited in the glomeruli (20) displayed reduced sialic acid content as compared to the preimmunized controls (Fig. 3D).

The regulated sialylation of IgG suggests a mechanism for ensuring that steady-state serum IgG antibodies maintain an anti-inflammatory

state. Upon antigenic challenge by a potential pathogen, the antigen-specific IgG antibodies can switch to a population with reduced sialic acid that is thus capable of mediating antigen clearance and a protective inflammatory response through the engagement of subclass-specific FcγRs on effector cells.

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23. We thank M. Madaio for providing the NTS; C. Bertozzi for helpful discussions and suggestions; A. Datta for glycan analysis; and P. Smith, J. Clowney, A. Kim, and J. Pagan for technical assistance. These studies were supported by grants from NIH (J.V.R.). Y.K. was supported by fellowships from the Naito Foundation, the Kanoe Foundation for Life & Social-Medical Science, and the Uehara Memorial Foundation. F.N. is a fellow of the Cancer Research Institute.

Supporting Online Material

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Materials and Methods
Figs. S1 to S11
References

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N- to C-Terminal SNARE Complex Assembly Promotes Rapid Membrane Fusion

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Assembly of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) syntaxin 1, SNAP-25, and synaptobrevin 2 is thought to be the driving force for the exocytosis of synaptic vesicles. However, whereas exocytosis is triggered at a millisecond time scale, the SNARE-mediated fusion of liposomes requires hours for completion, which challenges the idea of a key role for SNAREs in the final steps of exocytosis. We found that liposome fusion was dramatically accelerated when a stabilized syntaxin/SNAP-25 acceptor complex was used. Thus, SNAREs do have the capacity to execute fusion at a speed required for neuronal secretion, demonstrating that the maintenance of acceptor complexes is a critical step in biological fusion reactions.

The three soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors or SNAREs—syntaxin 1, SNAP-25, and synaptobrevin 2 (also referred to as VAMP2)—are key elements of the molecular machinery mediating the rapid exocytosis of synaptic vesicles in neurons (1, 2). Syntaxin and SNAP-25 are localized within the plasma membrane, and synaptobrevin resides in synaptic vesicles. In vitro, the three proteins assemble into a stable complex with equimolar stoichiometry. It consists of a tight bundle of four α helices aligned in parallel, in which the transmembrane regions of synaptobrevin and syntaxin lie at one end (3). SNARE assembly is thought to be

initiated at the N-terminal end and proceed toward the C-terminal membrane anchors (in a process referred to as zippering). In consequence, the membranes are pulled tightly together, overcoming the energy barrier for fusion (4–6).

Although the zipper hypothesis of SNARE function has received substantial support, the features of SNARE assembly in vitro are difficult to reconcile with the proposed role of SNAREs as catalysts of the final step in exocytotic membrane fusion. SNAREs are able to fuse liposomes, but the fusion rates are very slow, requiring hours for completion (7). Faster rates have been observed upon fusion of liposomes with planar membranes (8–10), but because these reactions do not require SNAP-25, the importance of these results remains questionable. On the other hand, neuronal exocytosis occurs at a submillisecond time scale (11). To resolve this major discrepancy, it has been proposed that, in primed fusion-ready vesicles, the

SNAREs are partially assembled in trans configuration bridging the fusing membranes (12, 13). However, the actual configuration of the SNARE machinery for rapid fusion has remained elusive.

SNARE assembly proceeds in an ordered fashion involving structurally defined intermediates (14, 15). In vitro, syntaxin and SNAP-25 readily form a stable four-helix bundle in 2:1 stoichiometry (16). Because the second syntaxin occupies the position of synaptobrevin (17, 18), the overall slow rate of SNARE assembly may primarily be due to competition between syntaxin and synaptobrevin for binding to a transient 1:1 syntaxin/SNAP-25 heterodimer (15), thus occluding the true reactivity of the final (and fusion-relevant) step in the assembly pathway.

We therefore investigated whether the formation of the 1:1 syntaxin/SNAP-25 heterodimer allowed subsequent binding of synaptobrevin at rates compatible with biological fusion reactions. We monitored the binding kinetics of fluorescently labeled synaptobrevin (19). To increase the concentration of the syntaxin/SNAP-25 heterodimer, we used a large excess of SNAP-25 over the SNARE motif of syntaxin [amino acids 180 to 262 (Syx180–262)]. When synaptobrevin was added, a rapid increase in fluorescence anisotropy was observed ($\sim 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; Fig. 1A). Similarly fast binding was observed when the heterodimer was preformed with the entire cytoplasmic region of syntaxin (Syx1–262; Fig. 1B), demonstrating that syntaxin's autonomous N-terminal domain did not affect synaptobrevin binding (Fig. 1C).

Next, we attempted to stabilize the acceptor site for synaptobrevin in the 1:1 heterodimer. According to the zipper hypothesis, binding of synaptobrevin should initiate at the N-terminal end

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