Mechanistic reconstruction of glycoprotein secretion through monitoring of intracellular N-glycan processing

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N-linked glycosylation plays a fundamental role in determining the thermodynamic stability of proteins and is involved in multiple key biological processes. The mechanistic understanding of the intracellular machinery responsible for the stepwise biosynthesis of N-glycans is still incomplete due to limited understanding of in vivo kinetics of N-glycan processing along the secretory pathway. We present a glycoproteomics approach to monitor the processing of site-specific N-glycans in CHO cells. On the basis of a model-based analysis of structure-specific turnover rates, we provide a kinetic description of intracellular N-glycan processing along the entire secretory pathway. This approach refines and further extends the current knowledge on N-glycans biosynthesis and provides a basis to quantify alterations in the glycoprotein processing machinery.

INTRODUCTION

Protein secretion in eukaryotic cells is mediated by a complex set of compartmentalized reactions. The process initiates in the endoplasmic reticulum (ER) and proceeds toward the Golgi apparatus, the plasma membrane, or the lysosome by vesicular transport. Posttranslational modifications (PTMs) are a hallmark of secretory proteins, and the processing machinery is specifically localized in the different compartments. N-linked protein glycosylation, present in all domains of life (1), is a complex biosynthetic pathway that involves organelle-specific reactions (2). The process begins in the ER lumen, where a defined glycan (Glc3Man9GlcNAc2) is covalently linked to the asparagine residue in the N-X-T/S consensus sequence of newly synthesized poly-peptides by the oligosaccharyltransferase (OST) enzyme (3). Glycosyl hydrolases that are part of the glycan-directed folding and quality control machinery of the ER trim three glucoses and up to four mannose residues from the initial Glc3Man9GlcNAc2 glycan (4). The properly folded glycoproteins are transferred to the Golgi (5), where multiple enzymes trim and elongate the N-glycan to yield the secreted glycoprotein. It is a characteristic property of the N-glycosylation process to start with the transfer of a defined oligosaccharide (Glc3Man9GlcNAc2), which is then processed to generate a species- , cell-, and site-specific heterogeneous ensemble of N-linked glycans. The composition of the final glycan output is determined by the activities and localization of processing enzymes as well as the structure of the glycoprotein itself (6, 7). N-glycan heterogeneity poses a great analytical challenge and affects the development and production of therapeutic glycoproteins (8). Mass spectrometry (MS)–based approaches have emerged as valid technologies to characterize N-glycan profiles (9).

Current knowledge about the N-glycosylation reaction machinery relies (i) on in vitro experiments that determine the activity and substrate specificity of various glycosyl transferase and hydrolases (10–14) and (ii) on microscopy and biochemistry data that provide the localization of the different enzymes and substrates within the ER and the Golgi (15–20). In addition, metabolic studies in combination with mathematical models have contributed to a better understanding of the N-glycosylation process (21, 22). Nevertheless, these models have so far been restricted to mere correlations between inputs and outputs (22–25) and have been developed without the use of empirical information regarding the dynamics of intracellular N-glycan processing.

We present a novel experimental approach for the characterization of the secretory pathway in mammalian cells by monitoring and quantifying intracellular, site-specific N-glycan processing. Our methodology combines parallel reaction monitoring (PRM), a quantitative targeted MS approach to quantify glycopeptides, with dynamic SILAC (stable isotope labeling of amino acid in cell culture) (26), a protein-labeling technique to follow the kinetics of protein-bound glycans. The experimental data were used to formulate a kinetic model for intracellular glycoprotein processing, the retrieval of activity-based localization profiles, as well as the relative kinetic parameters of glycosyl transferases and hydrolases in the N-glycosylation reaction network. Our results confirm and refine concepts on glycoprotein processing in the ER and the Golgi, suggesting an additional pathway for the export of folding intermediates from the ER directly to the lysosome.

RESULTS

SILAC coupled with nano-UPLC-PRM allows for reliable quantification of glycopeptides from intracellular and secreted recombinant IgG

Immunoglobulin Gs (IgGs) containing a single N-glycosylation site at each heavy chain (HC) were purified from CHO cell extracts or culture supernatant via protein A capture. Because this purification method requires the initial folding of the CH2 and CH3 domains of the HC (27), the detection of some glycopeptides (i.e., Glc3Man9GlcNAc2) is possibly biased since it can be attributed not only to the action of the OST but also to the folding of the aforementioned domains. For this reason, the species Glc3Man9GlcNAc2 (generated by ER glucosidase I and II) were excluded both from the data acquisition and from the model (see Material and Methods for more details).

Glycoproteomics analysis was performed after tryptic digestion and reverse–phase chromatography separation. For all detectable IgG tryptic glycopeptides, we performed data-dependent acquisition (DDA) with higher-energy C-trap dissociation (HCD), as described previously (28). DDA data were used to generate the target list for PRM analysis (table S1).
Solutions containing different concentrations of (glyco)peptides from secreted IgG samples were tested (10 μM to 200 μM, equivalent to 100 pmole to 1 fmoles of glycopeptides per analysis). Whereas the most abundant glycopeptides were still detectable at 200 μM, less abundant ones, such as the sialylated glycopeptides, had a signal-to-noise ratio that prevented quantification at concentrations below 100 nM total IgG (fig. S1). Our limit of detection (200 pM IgG) was comparable to values obtained in previous studies (29). Nevertheless, for a complete glycopeptide quantification, our limit of quantification was at 100 nM IgG. To obtain the best fragmentation into Y ions (intact peptides with fragmentation at the glycans level), we tested different normalized collision energies (NCEs). NCE = 16%, as opposed to NCE = 22%, gave the best fragmentation pattern: it led to decreased abundance of the dominant Y1 ion (peptide with one N-acetylgalactosamine residue) and the oxonium ions (N-acetylgalactosamine ions and di-saccharide ions) and increased the abundance of characteristic Y ions and unfragmented precursor ion (fig. S2) (30).

As opposed to canonical tandem MS methods, where the precursor isolation window (Q1) is maintained as low as possible (31), we adjusted the analytical procedure and increased the Q1 isolation window to 6 Th, so that both heavy and light glycopeptide precursors were simultaneously isolated and fragmented (Fig. 1A). To evaluate the consistency of MS2-level quantification among all the different glycoforms, we analyzed different mixing ratios of heavy and light IgGs and compared peak area or peak height-based quantification. The ratio of all glycoforms was consistent with the original IgG mixtures, we analyzed different mixing ratios of heavy and light IgGs (32). To evaluate the agreement with enzyme localizations resolving into a more robust analysis for low-abundant glycoforms and unfragmented precursor ion (fig. S2) (30).

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We compared the N-glycan distribution of secreted and intracellular IgG gained with MS1 quantification (28) with the MS2 quantification method described above (Fig. 1C). For both MS1- and MS2-based quantification, the N-glycan profile of secreted IgGs consisted of $80%$ complex N-glycan, whereas the intracellular IgGs presented $80%$ of high-mannose N-glycan structures. The consistency between MS1- and MS2-based quantification and the agreement with precedent studies on secreted or intracellular IgG glycan profiling using different MS approaches (23, 32) proved the suitability of our PRM methodology for N-glycan profiling (representative MS2 spectra for each glycan structure are shown in fig. S3).

To monitor intracellular IgG glycan processing, we coupled the PRM-based analytical setup to a dynamic SILAC regime. We purified IgGs from cell extracts that were lysed at different time points after switching to heavy SILAC medium. The fractional labeling of the different tryptic glycopeptides is reported in Fig. 2A. Labeled IgG glycopeptides bearing intermediates synthesized early in the N-glycosylation/secretory trajectory were detected shortly after the pulse (i.e., high-mannose structures detected already after 10 min). In contrast, more complex Golgi-derived fucosylated, galactosylated, or sialylated N-glycopeptides appeared only after 20 to 60 min (Fig. 2A), indicating delayed accumulation/synthesis of the corresponding intracellular glycoform (17–19).

**Development of a mathematical model enabled the derivation of quantitative kinetic information and refinements of the canonical N-glycosylation network**

Our fractional labeling data provided information about the turnover rates of the intracellular pools of defined IgG-bound glycans but cannot directly reveal the kinetic information and enzymatic activity windows along the secretory pathway. Therefore, we developed a mathematical model (detailed in the Supplementary Materials). The best-fitting turnover reactions (Fig. 2A), the intracellular steady-state N-glycan distribution (fig. S4A), and the final secreted N-glycan profiles (fig. S5A) were produced with the ER and Golgi networks presented in Fig. 2 (B and C). A simple N-glycosylation model assuming a bare sequential order of glycosylation reactions did not fit the data successfully. To correctly reproduce the experimental data, it was necessary to include spatially separated pools of intracellular IgGs that carry the same high-mannose (Man$_9$-5) glycans. The different pools are related because a high mannose–bearing IgG can be found in both the ER and the cis-Golgi, and within the ER, high-mannose isoforms can account for different folding states of the protein. In the ER, high-mannose structures are generated by the collaborative action of ER-localized alpha-mannosidases (ER-mannosidase I and/or EDEMs) implicated in the buildup of the degradation signal present on the not properly folded glycoproteins (33–35). In contrast, a distinct Golgi-localized pool of Man$_8$$_{-5}$ structures is generated by the Golgi-mannosidase I that trims Man$_9$ to Man$_5$ with slightly different specificities (36).

Our data implied a faster turnover of the Golgi-generated structures as compared with the oligomannose structures in the ER (Fig. 2A). Mechanistically, the fast turnover of Golgi species can be explained by short residence time of glycoproteins in the Golgi cisternae. Conversely, slow ER turnover can be attributed to a slow export of the Golgi or the cytosol. This was also confirmed by the model, which, on the one side, predicted slower kinetic parameters for the formation and degradation of the Man$_7$$_{-5}$ structures (table S2), and on the other side, predicted a bigger IgG pool for folding intermediates in the ER as compared with the pool of folded IgG in the Golgi (fig. S5B). Unexpectedly, we observed a Man$_9$GlcNAc$_2$ glycans with an early onset and fast turnover. This structure has not been characterized in detail, and there are controversial opinions regarding its generation either by Golgi alpha-mannosidases (37, 38) or by lysosomal mannosidases (39). Neither ER-mannosidases nor Golgi-mannosidase I can trim further than Man$_9$ due to their $\alpha$-1,2 specificity (10). It has been reported that protein aggregates, generated in the ER, are cleared from the ER and transported in single-membrane vesicles directly to the lysosome (40, 41). Thus, we hypothesized the Man$_9$GlcNAc$_2$ structure is generated by the action of a lysosomal mannosidase on IgG HC that are cleared from the ER in a pathway that differs from the well-characterized ER-associated degradation (ERAD) pathway (represented by the collaborative action of ER-mannosidase 1 and EDEMs). The integration of this pathway into the model allowed for accurate fitting of the Man$_9$GlcNAc$_2$ kinetics (Fig. 2A). Moreover, the addition of MGI32, a potent inhibitor of the proteasome 26S complex, showed a marked accumulation of ERAD-relevant Man$_7$-Man$_5$ species without affecting the Man$_9$GlcNAc$_2$ turnover (fig. S6A), thereby excluding it as an ERAD intermediate.

The mathematical model was used to calculate the trajectory of IgGs through the entire N-glycosylation network (Fig. 2, B and C). The model predicted the distribution of the activity profiles of the main Golgi enzymes (Fig. 3A) and the distribution of the respective N-glycan substrate abundance (Fig. 3B) along the Golgi compartment. On the one side, the agreement with enzyme localizations resolved by microscopy (15–18) was appreciated, and on the other side, our data provided experimental support for inferred mathematical model outputs (22, 24, 25).
Fig. 1. Validation of the dynamic SILAC-PRM methodology. (A) Typical MS2 spectrum of an IgG glycopeptide obtained during SILAC-PRM acquisition using the broad isolation window (6 Th) and normalized collision energy (NCE) of 16% to achieve glycan-level fragmentation of light and heavy glycopeptides simultaneously (ratio, 1:5). Schematic representation of the glycopeptides of interest is shown above the corresponding peak. Gray long squares indicate the peptide backbone (with N, asparagine, indicating the N-glycosylation site; and R, arginine), blue squares indicate N-acetylglucosamine residues, green circles indicate mannose residues, and red triangles indicate fucose residues. Masses and charge states of the peak signals are reported. Mass differences between peaks that correspond to sugar moieties are indicated with the corresponding geometric representation of the sugar. (B) SILAC-PRM data acquisition of 100% heavy and 100% light IgG glycopeptides mixed in a 5:1 ratio prior to MS injection. Quantification of defined IgG glycoforms (individual bars) was conducted by averaging the peak area (black bars) or peak height (gray bars) of defined glycotransitions (fragment ions at the glycan level) (n = 3). Details about the glycoforms and the glycotransitions used for the quantification are listed in table S1. (C) N-glycan profiling analysis of purified intracellular and secreted IgGs. After PRM data acquisition, quantification was performed either on the MS1 level (light gray), by averaging the intensity of the extracted ion chromatograms, or on the MS2 level, by averaging the intensity of defined glycotransitions (dark gray) (n = 3). The relative abundance of each N-glycoform (x axis) compared with the sum of all the glycoforms is reported (y axis) for secreted (top graph) and intracellular (bottom graph) IgGs.
Fig. 2. Intracellular N-glycan processing. (A) Intracellular IgG molecules were harvested at the time indicated after the switch of the cells from light to heavy SILAC medium (x axis) and analyzed by SILAC-PRM. The fractional labeling (y axis) of intracellular pools of IgG peptides bearing different N-glycan intermediates (shown as symbols) is given over time (n = 3; except for complex sialylated structures, n = 2). The modeled turnover kinetics are shown as curves. (B) IgG fluxes through the ER processing pathway calculated by the model. The size of the arrows is proportional to the flux through each reaction indicated (numerical values predicted by the model are indicated in the figure as percentage). Upper rows reflect folded IgGs transported to the Golgi, middle rows reflect folding intermediates in the folding/ERAD pathway, and the lower rows refer to the lysosome degradation of aggregates (left) and cytoplasmic degradation by proteasome (right). Blue proteins refer to folded, and purple proteins indicate partially folded IgGs. Different N-glycan structures are shown as symbols. (C) IgG flux through the Golgi N-glycan processing pathway. The size of the arrows is proportional to the flux through each reaction indicated. The colors of the arrows indicate the different enzymes catalyzing the reaction (for the color code, see Fig. 3A). Circles highlight the major glycoforms found on secreted IgGs. Gray glycoproteins refer to IgG glycostructures that were included in the data measurements but did not provide reliable signals due to low abundance (below limit of quantification), preventing a flux calculation (no arrows).
The refined N-glycosylation network correctly predicts the effect of N-glycan processing inhibitors

To evaluate the robustness of our model, we perturbed the system by the addition of well-defined processing inhibitors and monitored the effect on the intracellular N-glycan processing of IgGs. The experimental data were used to develop a mathematical model for N-glycoprotein processing in CHO cells. The model was based on the current knowledge of the N-glycosylation pathway in mammalian cells (42), and upon a refinement of the canonical network, we were able to deduce a robust mathematical description of the process. In the ER, N-glycan processing is primarily involved in the folding and quality control process that ensures the exit of correctly folded protein from the ER. In contrast, Golgi processing is characterized by spatial separation of enzymes and a continuous flow of the substrate through the organelle.

The correct reproduction of the Man$_4$GlcNAc$_2$ kinetic was only possible with the incorporation of an additional degradation pathway that directly acts early in the ER (as Man$_4$GlcNAc$_2$ has an early turnover rate). On the basis of previous ER-phagy studies (43) and recent descriptions of a vesicular transport from the ER to the lysosome (41), we propose that mannose trimming to Man$_4$GlcNAc$_2$ (and possibly further, to not protein A capture purifiable glycopeptides) reflects the quality control process involved in protein aggregate clearance from the ER, referred to as ERAD (ER-to-lysosome–associated degradation) (41). Our glycoproteomics approach differentiates this pathway from ERAD, which can be accurately followed by N-glycan processing down to Man$_5$ (cf. Fig. 2A and fig. S7A). From our data, ERAD acts much more rapidly than ERAD, indicating a much longer half-life for ERAD substrates as compared with their ERAD counterparts. This observation is in line with previously described models, where a stochastic association of unfolded protein and chaperones with the signal-generating EDEMs determines the half-life of ERAD substrates (44, 45). In contrast, glycoprotein aggregates interacting with calnexin (through Glc$_1$Man$_4$GlcNAc$_2$ N-glycans) are selected for export to the lysosome (41). On the basis of the kinetic data and the model predictions, we propose that aggregation of polypeptides occurs after translocation into the ER lumen, where nascent polypeptides fail to associate with folding chaperones and therefore aggregate. We hypothesize that the N-glycan profile on these aggregates is a mixture containing at least some Glc$_1$Man$_4$GlcNAc$_2$, as

**DISCUSSION**

We monitored the intracellular N-glycan processing of IgGs. Notably, neither the SWA nor KIF addition affected the turnover kinetics of the Man$_4$GlcNAc$_2$ glycoprotein, confirming a processing pathway independent of ER- or Golgi-localized mannosidases (fig. S7A). To test this hypothesis, we treated cells with bafilomycin (BAF), a substance known to affect pH homeostasis in the lysosome and to reduce lysosomal hydrolases activity. BAF treatment had a single effect on the turnover of the Man$_4$GlcNAc$_2$, strongly reducing its turnover (fig. S7B), whereas the turnover of Man$_5$GlcNAc$_2$ (fig. S7C), an ER-generated structure, or FA2, a Golgi-generated N-glycan (fig. S7D), did not show deviations from the control turnover data (Fig. 2A), except for a shift on the time axis (a delay), probably due to an overall effect of reduced biosynthesis and secretion speed under BAF treatment. Under SWA and KIF treatment, the production of Golgi-generated glycan is prevented due to the absence of the correct substrate. The turnover of Man$_4$GlcNAc$_2$, an ER-generated structure, did not show significant deviation from the control conditions under SWA treatment, but a delay was observed under KIF treatment, as expected (fig. S7C). These data supported the hypothesis of a direct export from the ER to the lysosome.

**Fig. 3.** Empirical enzyme activities and substrates distribution along the Golgi. (A) The apparent activities of the different glycosyl hydrolases and transferases as calculated by the model along the normalized Golgi, from cis to trans, are shown. Inset: Enlarged y axis to reveal galactosyl- and sialyl-transferase activities. ManI, Golgi alpha-mannosidase I (light green); GnTI, N-acetylglucosamine transferase I (light blue); ManII, alpha-mannosidase II (dark green); FucT, α-1,6-fucosyl-transferase (red); GnTII, N-acetylglucosamine transferase II (dark blue); GalT, beta1,4-galactosyl-transferase (yellow); SiaT, alpha2,3 and 2,6-Sialyl transferase (purple). (B) Intracellular distribution of the different N-glycoforms of IgG calculated by the model along the normalized Golgi is shown. Abbreviations and the corresponding structures are exemplified in table S1.
inhibition of downstream glycan processing (with KIF and SWA) does not influence the Man4GlcNAc2 kinetics (assumed to be indicative of ERLAD).

Our SILAC-PRM methodology allowed us to follow the N-glycan maturation along the entire secretory pathway with a spatial-temporal resolution. For each of the routes taken by a newly synthesized IgG, we can now assign an N-glycan “marker”: The secretion trajectory is characterized by hybrid or complex N-glycan structures (46), ERAD by Man7-Man5 species, and, as suggested by our data, ERLAD is characterized by paucimannose structures, like Man4GlcNAc2 (reflecting degradation intermediates).

The mathematical model retrieved in vivo kinetic information for many enzymes along the N-glycosylation network. On the basis of the product formation, we obtained a functional map of enzyme activity distribution along the Golgi and noted a full agreement of the spatial localization of the enzymes determined by microscopy experiments (17–19). The measured Golgi enzyme activity in our experimental setup reflected a combination of multiple factors (catalytic activity of the enzyme, enzyme concentration exposure time, and glycan substrate accessibility) rendering our model cell, protein, and even glycosylation site specific. An example of this specificity was evident in the SWA inhibition experiment (Fig. 4, C and D), where the activity-based model output shifted the localization and activity of GalT and SiaT. We speculate that this shift did not represent an altered localization of the enzymes but rather increased accessibility of the glycan substrate to the enzymes. Crispin and colleagues (47) showed that the protein-glycan interaction in the Fc region of IgG is reduced for hybrid as compared with complex N-glycans, allowing better galactosylation of hybrid glycan structures.

Our methodology and the new insights presented here allow for the calculation of the effects of altered processing enzyme level or changed enzyme localization on site-specific N-glycan composition.
This is relevant to assess the quality of recombinant glycoprotein production in cell culture. On the other side, it offers a more reliable basis for the implementation and optimization of mathematical models used in the product design of glycoproteins as biopharmaceuticals.

**MATERIALS AND METHODS**

**Preparation and purification of heavy- and light-labeled human IgG**

CHO-S cells stably expressing IgG (provided by Biopharma Merck AG, Switzerland) were cultivated in suspension in expansion medium (customized medium by Biopharma Merck AG) at 320 rpm, 37°C, 5% CO₂ in a shaking incubator (Adolf Kuhner AG, Birsfelden, Switzerland). For the SILAC labeling, cells were centrifuged for 3 min at 300 relative centrifugal force (rcf) and resuspended at 5 × 10⁵ cells/ml either in light or in heavy SILAC medium (containing light l-arginine and l-lysine or 13C-arginine and 13C,15N-lysine, respectively). Cells were subcultured in SILAC medium every 2 days for 6 days to reach complete heavy isotope labeling. Cells (5 × 10⁷) from light or heavy culture were collected after centrifugation and lysed with 10 ml of lysis buffer [3% Triton X-100, 110 mM KAc, 20 mM Heps, 2 mM MgCl₂ (pH 7.2), and 1X complete EDTA-free protease inhibitor cocktail; Roche]. Protease inhibitor cocktail tablets (complete EDTA-free protease inhibitor cocktail tablets; Roche) were added to the cleared supernatants. The IgGs were purified via protein A capture by adding 200 μl of protein A-Sepharose 4 Fast Flow beads (GE Healthcare Life Science) to the cell lysates and culture supernatant solutions. Batch binding was performed under continuous rotation for 3 hours. Beads were then washed by centrifugation with 10 ml of 0.02 M sodium phosphate buffer. IgGs were eluted by shaking the beads for 10 min in 800 μl of 0.1 M citric acid in a 1.5-ml Eppendorf tube. Eluates were placed onto 30K cutoff Micro filter (Millipore) and washed three times with water.

**SILAC labeling**

CHO-S cells growing in expansion medium were transferred to light SILAC medium as described above and subcultured every 2 days for 6 days. For the last subculturing prior to the experiment, the cells were seeded to a cell density of 1 × 10⁶ cells/ml. On the day of the heavy chase experiment, cells (at concentration 5 × 10⁷ cells/ml) were resuspended in heavy SILAC medium (hereby referred to as time 0, t₀). Then, cells were incubated at 320 rpm, 37°C, 5% CO₂ for 4 hours. At different time points, 8 ml of culture (corresponding to roughly 5 × 10⁶ cells) was sampled and subjected to centrifugation for 3 min at 300 rcf. Cell pellets and supernatants were flash frozen with liquid nitrogen and kept at −80°C prior to protein purification. For protein A capture, the cells were lysed with 10 ml of lysis buffer, and protease inhibitor cocktail tablets were also added to the cleared supernatants. The IgGs were purified as described above.

**Cell treatment with glycosylation enzyme inhibitors**

Cells were pretreated in SILAC light medium with SWA at 20 μM final concentration (48) for 2 hours. At time point t₀, the cells were switched to the SILAC heavy medium containing 13C-arginine and 13C,15N-lysine, and 20 μM swainsonine was added to maintain the SWA concentration. Cells (5 × 10⁷) were collected at different points (0, 10, 20, 30, 60, 90, 120, 180, and 240 min). IgGs were purified via protein A capture and subjected to SILAC-PRM analysis. The same procedure was followed for KIF treatment using 10 μM final concentration (48). For the BAF and MG132 treatments, 100 nM and 80 μM final concentrations were used, respectively (49, 50).

**Sample preparation for MS analysis**

IgGs were further processed according to the FASP (filter-aided sample preparation) procedure modified by Wisniewski et al. (51). Shortly, 25 to 100 μg of IgGs was reduced with 100 mM dithiothreitol in 50 mM ammonium bicarbonate (ABC) buffer (pH 8.5) for 30 min at 37°C and alkylated with 130 mM iodoacetamide in 50 mM ABC buffer for 25 min at 37°C. Sequencing-grade trypsin (Promega) was used to digest proteins at the ratio of 1:80 overnight at 37°C. Peptides were collected by centrifugation, and the filters were washed once with water and once with 10% acetonitrile in ddH₂O. All flow-through fractions were pooled and dried via SpeedVac centrifugation (Thermo Fisher Scientific). All samples were desalted by C18 ZipTip (Millipore) prior to MS analysis.

**Nano-UPLC-DDA and nano-UPLC-PRM for glycopeptide identification and quantification**

The discovery of all glycoforms was first performed on the LTQ-Orbitrap Velos (Thermo Fisher Scientific) as previously described (28). All quantitative experiments were performed with the nanoACQUITY UPLC (ultra performance liquid chromatography) system (Waters), coupled online to a calibrated Q Exactive HF mass spectrometer (Thermo Fisher Scientific) with a PicoView nanospray source 300 model (New Objective). The tryptic samples were dissolved in 2% acetonitrile/0.1% formic acid, loaded onto a nanoACQUITY UPLC 2G C18 trap column (180 μm by 20 mm, 100-Å, 5-μm particle size), and separated on a nanoACQUITY UPLC BEH130 C18 column (75 μm by 250 mm, 130-Å, 1.7-μm particle size) at a constant flow rate of 300 nl/min, with a column temperature of 50°C and a linear gradient of 1 to 35% acetonitrile/0.1% formic acid in 42 min, followed by a sharp increase to 98% acetonitrile in 2 min and then held isocratically for another 10 min. For the DDA analysis, one scan cycle comprised a full scan MS survey spectrum, followed by up to 10 sequential HCD scans based on the intensity. Full-scan MS spectra [800 to 2000 mass/charge ratio (m/z); for inhibitory experiments, 500 to 2000 m/z] were acquired in the FT-Orbitrap at a resolution of 60,000 at 400 m/z, while HCD MS/MS spectra were recorded in the FT-Orbitrap at a resolution of 30,000 at 400 m/z. HCD MS/MS spectra were performed with a target value of 5 × 10⁶ by the collision energy setup at an NCE of 22%.

For PRM analysis, one scan cycle comprised a full-scan MS survey spectrum, followed by 10 sequential PRM scans based on the inclusion list. Full-scan MS spectra (800 to 2000 m/z) were acquired in the FT-Orbitrap at a resolution of 60,000 at 400 m/z, while PRM MS/MS spectra were recorded in the FT-Orbitrap at a resolution of 30,000 at 400 m/z. PRM MS/MS spectra were performed with a target value of 5 × 10⁶ by the collision energy setup at an NCE of either 22 or 16%. Automatic gain control target values were 5 × 10⁶ for full FT-MS. For SILAC-PRM experiments, the isolation lists can be found in table S1. Briefly, the setup of each precursor ion was the average of observed light-labeled glycopeptides from Velos measurement and their theoretical corresponding heavy-labeled m/z. The quadrupole isolation window for each precursor was 6 m/z.

**Data analysis and SILAC-nano-HPLC-PRM methodology evaluation**

To set up and evaluate the SILAC-PRM methodology for glycopeptide quantification, heavy-labeled IgG glycopeptides were mixed with...
light-labeled ones at the ratios of 5:1, 4:2, 2:4, and 1:5 before MS analysis. Data were first analyzed with the XCalibur 3.0 (Thermo Fisher Scientific) software manually. For quantification, one or three fragment (transition) ions of each precursor were used for manual extraction and inspection based on the abundance, interference-free signal, and representative potential to each structure. Mass tolerance (10 ppm) was used for peak extraction in each PRM spectrum. For some structures, only two transitions were used for quantification. Both peak area and height of each transition were evaluated as quantification methods.

**Semiautomated glycopeptide analysis using skyline**
MS2-level quantification was performed with the software Skyline (52), similarly to Pan and colleagues (32), by manually inserting the unique glycotransitions into the software (fig. S2B). All fragment ions were defined on the basis of their chemical formula and added into Skyline manually. In addition, the presence of glycan oxonium ions was also used to confirm all types of glycopeptides with the same principle as described for XCalibur analysis. The peak height of each transition was used for quantification.

**Fractional labeling quantification and analysis**
For control experiments and SWA-treated cells, the fractional labeling for a given glycopeptide at each time point was defined as the ratio between the measured intensity of the heavy-labeled species H and the sum of the heavy- and light-labeled species (H + L). We noticed that this type of normalization produced biased values for the heavy-to-light ratio in KIF-treated cells since a large intracellular pool of Man8–s glycans was still present 6 hours after the addition of the ER-mannosidase I inhibitor. This resulted in a significant underestimation of the fraction labeling for most of the high-mannose species, which was not due to reduced enzyme kinetics (for example, Golgi-mannosidase I is not affected by KIF) but due to the residual amount of light species in the system. To account for this, the values of heavy- and light-labeled species at intermediate time points were normalized with respect to the steady-state value of heavy- and light-labeled fractions.

**Mathematical model of intracellular N-glycan processing**
**Theory and assumptions**
The IgG used in the study contains a single N-glycosylation site located at the Asn297 consensus sequence of each HC, leading to a glycoprotein with two separate glycan moieties. Because of sample preparation for MS analysis, the information about the combination of glycans present on a single IgG is lost. Therefore, the mathematical model only deals with single glycosylated HCs rather than describing the behavior of single glycan on dimerized monoclonal antibodies (mAbs). The model further assumes the intracellular machinery to operate at constant concentrations of enzymes and substrates, with the latter only varying with respect to its isotopic composition (i.e., heavy- or light-labeled peptides). The network included N-glycan processing reactions, catalyzed by hydrolases and glycosyl transferases, protein folding reactions of IgG molecules with a given glycan structure in the ER, transport between organelles, and degradation reactions. In contrast to the ER, we assumed a nonhomogenous distribution of both substrates and enzymes in the Golgi, mathematically represented by a spatial distribution reflecting a cisternal maturation model (53). From an engineering perspective, the ER behaves similarly to a well-mixed continuous reactor, which is continuously fed unglycosylated peptides and produces correctly folded, glycan-bearing proteins with the maximum possible efficiency (i.e., it is characterized by a high enzyme-to-substrate ratio). On the other hand, the Golgi acts as a low-conversion plug flow reactor with limited residence time, where the output is largely dominated by N-glycan accessibility and yields site-, protein-, and cell-specific processing.

**Modeling of the N-glycan pathway in the ER**
After the addition of the isotopic tracers in the medium, the incorporation of heavy-labeled amino acids (H-AA) on peptides occurs in the cytoplasm during RNA translation. Although the extracellular AA composition switch is very rapid, assuming an instantaneous switch from 100% light- to 100% heavy-labeled peptide production leads to inaccurate predictions for the kinetics of the ER species in the model (data not shown). This is probably due to the presence of an intracellular pool of light-labeled amino acids (L-AAs) with a delayed consumption kinetic. To account for this, we assumed that the production of H-labeled HCs ($q^H_p$) follows a first-order kinetic behavior (Eq. 1)

$$q^H_p = \frac{q_{p}^{\text{max}}}{\tau} e^{-t/\tau}$$

where $q_{p}^{\text{max}}$ indicates the maximum cell productivity at steady state and $\tau$ is the time constant representing the delay in the turnover of intracellular H-AA. This time constant was arbitrarily set to 10 min as measured by $^{13}$C flux analysis on CHO cells (54).

The N-glycosylation pathway starts in the ER with the attachment of the Glc$_3$Man$_9$GlcNAc$_2$ glycan on the HC N-S-T motif catalyzed by the OST enzyme. For the sake of simplicity, this step was assumed to occur cotranslationally with the peptide synthesis so that the production of the H-Glc$_3$Man$_9$GlcNAc$_2$ glycan is equal to $q^H_p$.

The Glc$_3$Man$_9$GlcNAc$_2$-bearing HC is a transient species where the peptide is in a linear chain conformation deriving from the translocation into the ER. Since the purification method used (protein A affinity capture) requires the initial folding of the CH$_2$ and CH$_3$ domains of the HC (27), the detection of Glc$_3$Man$_9$GlcNAc$_2$ glycopeptides is possibly biased, since it can be attributed not only to the action of the OST but also to the folding of the aforementioned domains. For this reason, the species Glc$_3$Man$_7$GlcNAc$_2$ (generated by ER glucosidase I and II) were excluded from the model, and the kinetic constants relative to ER glucosidase I and II were set to be 100 times faster than the cell-specific productivity $q_{p}^{\text{max}}$, due to the impossibility of correctly identifying their values.

After glucose trimming, we assume the HCs to reach a partially folded, protein A–binding state represented by the Man$_9$GlcNAc$_2$ structure. This structure can either enter the quality control machinery of the ER (and lose mannose residues due to the action of EDEMs) or fold correctly and become available for translocation to the Golgi. It is important to mention that the folding step included in the model and in the network presented in Fig. 2B lumps together a series of transitions, which include HC folding, light chain (LC) folding, HC-LC dimerization, and disulfide bond formation, which are indistinguishable from an N-glycosylation point of view. For the sake of simplicity, the reaction rate representing the single-step transition from partially to completely folded is assumed to follow a first-order kinetic (Eq. 2) (55) and to share the same kinetic constant $k_{\text{folding}}$ for all the partially folded glycoprotein isoforms in the ER (denoted by the term $S^{\text{NF}}$)

$$r_{\text{folding}} = k_{\text{folding}} S^{\text{NF}}$$
The transport to the Golgi apparatus was assumed to be selective for all correctly folded species \( S^r \) (this is mathematically equivalent to assuming a perfect recycling of nonfolded species) and to follow first-order kinetics (with constant \( k_{\text{Transport}}^{\text{Golgi}} \)), similarly to folding (Eq. 3). To prevent parameter unidentifiability, the transport from the ER to the Golgi was assumed to be much faster than the folding step, and \( k_{\text{Transport}}^{\text{Golgi}} \) was constrained to be \( 10^4 \) times \( k_{\text{folding}} \).

Noncorrectly folded species in the ER can enter two different degradation pathways: the ERAD pathway, leading to cytosolic degradation, and a secondary pathway, leading to lysosomal transport and degradation (see Results and Discussion).

The lysosomal degradation pathway leads to the formation of the \( \text{Man}_4 \) glycan. From a kinetic standpoint, the appearance of this species is best explained by assuming its generation deriving solely from \( \text{Glc}_1\text{Man}_9 \). This species undergoes a transition to a terminally misfolded state, possibly linked to aggregation, with kinetic constant \( k_{\text{agg}} \). Because of the lack of information concerning lysosomal transport, this step was assumed to be instantaneous and to follow a first-order mechanism (Eq. 4). After lysosomal translocation, the \( \text{Man}_9\text{Glc}_1 \) glycan was assumed to be trimmed down to a terminal \( \text{Man}_9\text{GlcNAC}_2 \) species (\( k_{\text{Man}}^{\text{Lys}} \), Eq. 5), which is then degraded together with its protein backbone (\( k_{\text{Deg}}^{\text{Lys}} \), Eq. 6). Since the parameter \( k_{\text{Man}}^{\text{Lys}} \) could not be correctly identified by the model, and given that the \( \text{Man}_9\text{GlcNAC}_2 \) appearance is very fast compared with most of the species detected, we assumed aggregation and lysosomal transport to be instantaneous steps in this process and therefore set \( k_{\text{Man}}^{\text{Lys}} \) to be 100 times \( k_{\text{agg}} \).

\[
\begin{align*}
\text{r}_{\text{T}}^{\text{Lys}} &= k_{\text{agg}} \text{Man}_9 \text{Glc}_1 \\
\text{r}_{\text{Man}}^{\text{Lys}} &= k_{\text{Man}}^{\text{Lys}} \text{Man}_9 \text{Glc}_1^{\text{Lys}} \\
\text{r}_{\text{Deg}}^{\text{Lys}} &= k_{\text{Deg}}^{\text{Lys}} \text{Man}_9^{\text{Lys}}
\end{align*}
\] (4-6)

The ERAD degradation pathway is glycan specific, since it is triggered by the exposure of terminal \( \alpha\text{-1,6}-\text{mannose} \) (56). This was implemented in the model by including first transport to the cytosol for \( \text{Man}_{7-8}\text{GlcNAC}_2 \)-bearing nonfolded glycoproteins with first-order kinetics with respect to substrate concentration (\( k_{\text{Cyt}}^{\text{Deg}} \), Eq. 7) and subsequent degradation (\( k_{\text{Cyt}}^{\text{Cyt}} \), Eq. 8)

\[
\begin{align*}
\text{r}_{\text{T}}^{\text{Cyt}} &= k_{\text{T}}^{\text{Cyt}} \text{Man}_{7-8}\text{GlcNAC}_2^{\text{NF}} \\
\text{r}_{\text{Deg}}^{\text{Cyt}} &= k_{\text{Deg}}^{\text{Cyt}} \text{Man}_{7-8}\text{GlcNAC}_2^{\text{Cyt}}
\end{align*}
\] (7-8)

Modeling of the N-glycan pathway in the Golgi apparatus

After translocation from the ER, folded glycoproteins (i.e., correctly folded and dimerized mAbs) travel through the various stacks of the Golgi, where the N-glycans are modified by the action of several compartmentalized enzymes, before being secreted to the extracellular environment. The transport through the Golgi apparatus is assumed to occur at a constant linear velocity (the residence time in the Golgi was fixed at 20 min according to the secretion data), and glycoproteins are secreted only after they reach the end of the entire system. Like all other reaction rates considered, the action of glycosyl transferases is assumed to follow first-order kinetics with respect to the substrate concentration. To correctly represent the spatial compartmentalization of the different enzymes, the kinetic parameters for Golgi-resident enzyme-catalyzed reactions were assumed to vary with respect to the position along the Golgi stacks (represented by the coordinate \( z \)). Although this behavior can, in principle, be modeled using any continuous function, we decided to define the window of activity of all Golgi-resident enzymes using normal Gaussian distributions (Eq. 9)

\[
k_i(z) = E_i^{\text{max}} e^{-\left(\frac{z-z_{\text{max}}}{\omega_i}\right)^2}
\] (9)

where \( k_i(z) \) refers to the value of the kinetic constant for the reaction catalyzed by Golgi enzyme \( i \) at position \( z \), and \( E_i^{\text{max}}, z_{\text{max}}, \) and \( \omega_i \) represent the peak height, the peak position, and the width of the enzymatic window of activity, respectively. The peak height and the peak width of the distribution are highly correlated in defining the total window of the activity (i.e., the area under the curve for a given enzyme). For example, the area under the curve remains the same if the peak height decreases and the peak width increases by a certain amount. For this reason, the parameter \( \omega_i \) was constrained to take the same value for all Golgi-resident enzymes to prevent a priori parameter unidentifiability.

Material balances

The mass balance for the species \( S \) in the ER (either folded or unfolded) assumes homogeneous distribution of both enzymes and substrates (perfect mixing) and perfect selectivity for transport (i.e., only folded proteins move to the Golgi, and only nonfolded proteins are degraded). Mathematically, this translates as follows (Eq. 10)

\[
\frac{\partial S_i}{\partial t} = q_{\text{in}} - \sum_{j=1}^{N_R} v_i r_j - q_{\text{out}}
\] (10)

The terms \( q_{\text{in}} \) and \( q_{\text{out}} \) represent transport in and out of the ER compartment, respectively. Inward transport is zero for all species except for \( \text{Glc}_2\text{Man}_9\text{GlcNAC}_2 \)-bearing peptides, where it equals \( q_p^{\text{H}} \) (Eq. 1), while outward transport depends on the folding and glycosylation processing as previously discussed. The term \( \sum_{j=1}^{N_R} v_i r_j \) accounts for all the reactions described in the ER network. In this term, the indexes \( i \) and \( j \) are used for counting substrates and reactions, respectively, such that \( v_i \) is the stoichiometric coefficient for substrate \( i \) in reaction \( j \) (with sign -1 for reactants and +1 for products) and \( r \) is the corresponding reaction rate.

Contrary to the ER, glycoproteins in the Golgi are not homogeneously distributed but vary in concentration along the entire apparatus. To account for this, the material balances include a term to represent the special distribution of species \( S_i \) as presented in Eq. 11

\[
\frac{\partial S_i}{\partial t} = -\frac{z_{\text{Golgi}}}{\tau_{\text{Golgi}}} \frac{\partial S_i}{\partial z} - \sum_{j=1}^{N_R} v_i r_j
\] (11)
The terms $z_{\text{Golgi}}$ and $\tau_{\text{Golgi}}$ represent the length and residence time in the Golgi, respectively, and their ratio defines the velocity of glycoprotein transport in the Golgi. The Golgi length was normalized to 1, and $\tau_{\text{Golgi}}$ was assumed to be 20 min, independently of the glycan or the position of the mAb. Note that Eq. 11 corresponds to a model of cisternal maturation for the Golgi apparatus (53).

The mass balances for both ER and Golgi were solved for heavily-labeled species, starting from the initial condition of 100% light-labeled species in the ER. For the Golgi, the initial condition $S_{i,\text{Golgi}}$ was set as nonzero only for the Man$_9$GlcNAc$_2$ glycoproteins (ER transport) and equal to (Eq. 12) at each time point and at $z = 0$

$$S_{i,\text{Golgi}} = \left(k_{\text{Transport}} Z_{\text{ER}}(t_i) - k_{\text{Transport}} Z_{\text{ER}}(t_{i-1})\right) \cdot (t_i - t_{i-1})$$

Model construction and parameter estimation

The system of ordinary differential equations (ODEs) of the ER was solved in MATLAB R2017a (Mathworks Inc., Natick, MA) using the built-in ODE15s solver. The system of partial differential equations of the Golgi was computed in FORTRAN using the solver DLSODES from ODEPACK. The solution was discretized along the Golgi axis $z$ using first-order central finite differences (50 grid points). The entire system was numerically solved from 0 to 500 min after the L-H switch, with a resolution of 5 min. Parameter optimization was performed using the “genetic algorithm” function of MATLAB initialized with a Latin hypercube sampling technique. The objective function was defined as the sum of least square errors between experimental data and the model output for ratios, intracellular fractions, and secreted fractions of every species, except for Glc$_3$Man$_9$GlcNAc$_2$ 

Evaluation of confidence intervals was performed, since the total pool of Man$_9$GlcNAc$_2$ is lower in size than the sum of all other (slower) high-mannose species. Another possibility is that aggregation occurs at the Glc$_1$Man$_9$GlcNAc$_2$ level. This species is known to interact with calnexin and calreticulin, chaperones responsible for disulphide bond formation. Errors in this process might lead to the formation of very unstable intermediates that could possibly be extremely prone to aggregate (fig. S8B). The model confirms that this is a possible behavior, since it can fit the Man$_9$GlcNAc$_2$ data correctly for control, SWA-, and KIF-treated experiments. Alternatively, it is still possible to consider every unfolded glycoprotein in the ER to be eligible for aggregation, but the propensity for this to occur decreases the more units of mannose are trimmed, due to the repeated interactions with chaperones that can stabilize the structure of the proteins (fig. S8C). To test this hypothesis, we assumed the aggregation rate $k_{agg}$ to scale for high-mannose species using the following relation (Eq. 17)

$$k_{agg} = k_{agg, \text{Man}n}\text{Glc} i \cdot x^{-n+i}$$

where the constant $k$ accounts for the enzymatic activity and the competitive inhibition present in the system investigated. This simplification is also valid for more complex kinetic mechanisms, since the cell machinery is supposed to operate at a constant enzyme, total H + L substrate, and inhibitor concentration.

**Kinetic theories for in vivo Man4GlcNAc$_2$ generation**

Considering the light- to heavy-labeled turnover rates for the various ER species, Man$_4$GlcNAc$_2$ is slightly slower than Man$_6$GlcNAc$_2$ but significantly faster than all other high-mannose species. If all the nonfolded glycoproteins in the ER could aggregate with the same kinetic constant (fig. S8A), the model would predict a slower turnover rate of Man$_4$GlcNAc$_2$ compared with the one experimentally detected, since the total pool of Man$_6$GlcNAc$_2$ is lower in size than the sum of all other (slower) high-mannose species. Another possibility is that aggregation occurs at the Glc$_1$Man$_9$GlcNAc$_2$ level. This species is known to interact with calnexin and calreticulin, chaperones responsible for disulphide bond formation. Errors in this process might lead to the formation of very unstable intermediates that could possibly be extremely prone to aggregate (fig. S8B). The model confirms that this is a possible behavior, since it can fit the Man$_9$GlcNAc$_2$ data correctly for control, SWA-, and KIF-treated experiments. Alternately, it is still possible to consider every unfolded glycoprotein in the ER to be eligible for aggregation, but the propensity for this to occur decreases the more units of mannose are trimmed, due to the repeated interactions with chaperones that can stabilize the structure of the proteins (fig. S8C). To test this hypothesis, we assumed the aggregation rate $k_{agg}$ to scale for high-mannose species using the following relation (Eq. 17)

$$k_{agg} = k_{agg, \text{Man}n}\text{Glc} i \cdot x^{-n+i}$$

where $k_{agg, \text{Man}n}\text{Glc} i$ is the aggregation constant for the monoglucosilated glycoproteins, and $n$ and $i$ are scaling factors, and $i$ is the number of mannose units trimmed. For the model to correctly predict the Man$_9$GlcNAc$_2$ kinetics, it was found that the value of $x$ has to be lower than 10$^{-4}$, indicating that aggregation is prevalently happening at the Glc$_1$Man$_9$GlcNAc$_2$ level even if this mechanism is assumed.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/11/eaax8930/DC1

Fig. S1. PRM detection and quantification limit.
Fig. S2. Collision energy optimization and Skyline implementation into the pipeline.
Fig. S3. Representative MS2 spectra of IgG N-glycopeptides from SILAC-PRM analysis.
Fig. S4. Data prediction accuracy by the model: intracellular IgG N-glycan distribution.
Fig. S5. Data prediction accuracy by the model: secreted IgG N-glycan distribution.

Fig. S6. Effects of different perturbations on N-glycan processing.

Fig. S7. ManGlcNAc$_2$ generation.

Fig. S8. Kinetic mechanisms considered for ManGlcNAc$_2$ formation.

Table S1. List of glycans selected for PRM analysis of glycopeptides.

Table S2. ER-related parameters.

View/request a protocol for this paper from Bio-protocol.

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