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An unfolded C_H1 domain controls the assembly and secretion of IgG antibodies

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Summary

A prerequisite for antibody secretion and function is the assembly into a defined quaternary structure, composed of two heavy and two light chains for IgG. Unassembled heavy chains are actively retained in the endoplasmic reticulum (ER) until they associate with light chains. Our mechanistic analysis of this critical quality control step revealed that, unlike all other antibody domains studied, the C_H1 domain of the murine IgG1 heavy chain is an intrinsically disordered protein in isolation. It adopts the typical immunoglobulin fold only upon interaction with its cognate partner, the C_L domain. Structure formation proceeds *via* a trapped intermediate, can be accelerated by the ER-specific peptidyl-prolyl isomerase cyclophilin B, and is modulated by the molecular chaperone BiP. BiP recognizes incompletely folded states of the C_H1 domain and competes for binding to the C_L domain. *In vivo* experiments demonstrate that requirements identified for folding the C_H1 domain *in vitro*, including association with a folded C_L domain and isomerization of a conserved proline residue, are essential for antibody assembly and secretion in the cell.

Introduction

In eukaryotic cells, proteins destined for secretion mature within the endoplasmic reticulum and are subject to rigorous quality control prior to their transport to the Golgi (Helenius et al., 1992). This usually involves surveillance of the folding status, the correct posttranslational modifications and proper oligomerization (Helenius et al., 1992; Ellgaard and Helenius, 2003; Christis et al., 2008). A prominent example of this is immunoglobulin G (IgG), the most abundant antibody in the blood. It is a heterotetrameric glycoprotein assembled from two light and two heavy chains that are comprised of two and four compact Ig-domains, respectively, which are structurally almost identical (Huber et al., 1976). Each domain shows a β-barrel topology, a two-layer sandwich structure composed of seven to nine antiparallel β-strands (Huber et al., 1976; Amzel and Poljak, 1979). The fold is stabilized by an internal disulfide bridge (Goto and Hamaguchi, 1979) that is located in the

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hydrophobic core and lies perpendicular to the β-sheets (Huber et al., 1976). Most of these structural characteristics are shared by the ubiquitous members of the immunoglobulin (Ig) superfamily (Bork et al., 1994), which perform a broad variety of extracellular recognition functions (Williams and Barclay, 1988; Rougon and Hobert, 2003; Aricescu and Jones, 2007). The evolutionary success of the Ig superfamily has fueled a vast scope of investigations on its biophysical properties. In particular, the folding pathways of diverse members of the Ig superfamily have been studied in detail (Goto and Hamaguchi, 1982; Freund et al., 1996; Thies et al., 1999; Cota et al., 2001; Paci et al., 2003; Feige et al., 2004) and have provided insights into determinants of robust folding (Hamill et al., 2000; Feige et al., 2008) as well as potentially harmful misfolding of this class of proteins (Kameda et al., 2005; Jahn et al., 2006; Qin et al., 2007).

To become secreted from the cell and fulfill their biological functions, the individual domains of an antibody not only have to fold into their native tertiary structure, but furthermore must assemble into a defined quaternary structure (Porter, 1973; Huber et al., 1976). While isolated antibody light chains can be secreted from the endoplasmic reticulum (Coffino et al., 1970; Melchers, 1971), unpaired heavy chains are actively and efficiently retained in the ER (Bole et al., 1986; Hendershot et al., 1987). Antibody heavy chain and light chain synthesis occur asynchronously during B cell development (Burrows et al., 1979), and only completely assembled molecules can both bind to antigen and carry out effector functions. Therefore, tight quality control of their assembly prior to secretion is vital. It is known that the first constant domain of the heavy chain, the C_H1 domain, plays an important role in this retention process (Hendershot et al., 1987; Kaloff and Haas, 1995). If deleted or replaced with another antibody domain, isolated heavy chains can be secreted, as occurs in the case of the rare heavy chain diseases (Wolfenstein-Todel et al., 1974; Adetugbo, 1978; Hendershot et al., 1987), or naturally in camelid antibodies, which do not contain light chains (Hamers-Casterman et al., 1993). In the context of the whole IgG molecule (Fig. 1A), the C_H1 domain is associated with the constant domain of the light chain (C_L) and shows the typical immunoglobulin fold (Huber et al., 1976). *In vivo*, C_H1 is the only antibody domain that is stably bound to the molecular chaperone BiP and remains in a reduced form before assembly with light chain (Vanhove et al., 2001). The basis for the unusual behavior of the C_H1 domain has remained enigmatic.

Here we set out to study the role of the C_H1/C_L association for correct antibody assembly and secretion. To our surprise we found that C_H1 is an unfolded protein in isolation, which gains structure only upon interaction with its cognate partner C_L. Based on this finding, we analyzed the association-coupled C_H1 folding pathway and its modulation by the chaperone BiP in detail and provide a comprehensive picture for the control of antibody secretion in the cell.

Results

The IgG1 C_H1 domain is intrinsically disordered

Antibodies are modular structures composed of a series of structurally highly homologous domains (Fig. 1A). These domains can usually be produced and studied separately as they represent independent structural units (Goto and Hamaguchi, 1982;Lilie et al., 1995). Surprisingly, analysis of the murine IgG1 C_H1 domain revealed that, in marked contrast to all antibody domains studied thus far (Goto and Hamaguchi, 1982;Thies et al., 1999;Feige et al., 2004;Rothlisberger et al., 2005), the isolated C_H1 domain is an unfolded protein, irrespective of whether its internal disulfide bridge is formed or not (Fig. 1B, supplemental Fig. 1 and supplemental Fig. 2). To further characterize the unfolded state of the C_H1 domain under physiological conditions, iodide fluorescence quenching experiments were carried out. The experiments indicate no significant differences in the burial of tryptophan

residues between C_H1 in PBS and in 3 M GdmCl. Additionally, NMR experiments were recorded on a highly deuterated C_H1 sample (data not shown). Deuteration enables detection of long range NOEs even if they belong to only a subset of conformers. In these experiments, no long range NOEs could be determined and consequently no preferential conformation of C_H1 seems to persist. Taken together, these data argue against the presence of a significant amount of stable structure in the isolated C_H1 domain. However, the pattern changed completely when the C_L domain, the cognate association partner of C_H1 in the antibody, was added. Only then did we observe folding of the C_H1 domain to a well defined β-sheet structure (Fig. 1B). Thus, the C_L domain is necessary and sufficient to induce structure formation in C_H1. This folding process was observed only if the internal disulfide bridge in the C_H1 domain was present (supplemental Fig. 2). Based on these findings, a key role of this association-coupled folding reaction for correct antibody assembly can be anticipated, which we aimed to elucidate in more detail.

The mechanism of induced folding of the C_H1 domain

To understand how binding to C_L and folding of C_H1 are coupled the thermodynamic and kinetic parameters for this reaction were established. The dissociation constant of the two domains was determined to be 6.2 ±0.4 μM (Fig. 1C) by the change in intrinsic fluorescence emission upon C_L-induced C_H1 folding (Fig. 1C, inset). A moderate affinity is expected since C_H1 has to fold upon binding to C_L. One should bear in mind that the observed dissociation constant is orders of magnitude lower than the antibody concentration in the ER of plasma cells (Cenci and Sitia, 2007), and thus association will readily occur *in vivo*. The analysis of the kinetics of secondary, tertiary and quaternary structure formation using far-UV CD spectroscopy (Fig. 1D), near-UV CD spectroscopy and analytical HPLC (supplemental Fig. 1) showed that all three processes occur with virtually identical time constants of $\tau = 60 \pm 10$ min at 25°C. Hence, all these processes are likely rate-limited by the same slow reaction. This slow folding reaction could be accelerated by the ER-specific peptidylprolyl isomerase cyclophilin B (Fig. 1D). Thus, the slow folding phase can be attributed to the isomerization of peptidyl-prolyl bonds within the C_H1 domain, which possesses an unusually high number of three *cis* prolines in the native state (Augustine et al., 2001). Prior to the slow folding to the native structure, the C_H1 domain forms an intermediate with the C_L domain in a concentration-dependent reaction (Fig. 1E). As this complex could be detected by fluorescence anisotropy measurements but not by the other techniques outlined above, it is likely a dynamic species with an only marginally folded C_H1 domain. In the complete antibody, a disulfide bridge covalently links the C_H1 domain with the C_L domain (Fig. 1A). If the bridge forming Cys residues were included in the C_H1 as well as the C_L domain, no change in the folding state of the isolated domains and the C_L-induced folding of the C_H1 domain was observed (supplemental Fig. 3) but formation of covalent dimers could be readily followed by SDS-PAGE. As covalent dimers were formed with the same rate as the slow C_H1 folding reaction and the reaction could be accelerated by cyclophilin B (Fig. 1F), it is clearly limited by proline isomerization and hence complete folding of the C_H1 domain.

Taken together, the C_L-induced folding of the C_H1 domain can be dissected into three reactions: first, oxidation of the internal C_H1 disulfide bridge has to take place. Then, a transient heterodimeric intermediate is formed, and subsequently peptidyl-prolyl isomerization is required to allow folding to the native state and covalent assembly with C_L (Fig. 1G).

An atomic level description of the C_H1 folding pathway

To resolve the specific recognition and the folding pathway of the intrinsically disordered C_H1 domain at the level of atomic resolution, NMR experiments were performed.

The ^{15}N-^{1}H HSQC spectrum of the isolated C_H1 domain is characteristic of an unfolded protein (Fig. 2A, red spectrum) confirming the results described above. In contrast, after induced folding by C_L, the C_H1 domain shows well-dispersed spectra (Fig. 2A, blue spectrum). The backbone assignment of the C_H1 domain in the complex was achieved by a combination of triple resonance experiments and NH residual dipolar couplings (RDCs). All obtained NMR data, the carbon chemical shifts, the NH RDCs and the MEXICO (Gemmecker et al., 1993) water exchange rates (data not shown) agree with an all-β structure for the C_H1 domain in the presence of C_L, like that observed in the crystal structure of IgG antibodies (Augustine et al., 2001). Because complete folding of the C_H1 domain is limited by proline isomerization, and hence associated with a high activation energy, the final folding step is significantly decelerated at low temperatures. This allowed us to characterize the trapped intermediate and resolve the association-coupled folding process using real time ^{15}N-^{1}H HSQC experiments. For each assigned residue, changes of the amplitudes over time could be described by a single exponential function (Fig. 2A, inset). Notably, some residues already exhibit significant intensities in the first spectrum recorded after 20 min (Fig. 2B, red bars). These residues are likely to already adopt a native-like backbone conformation prior to the slow peptidyl-prolyl isomerization reaction. Altogether ten residues, which are part of the β-sheets that form the mature structure, were found to be in a native-like environment in the intermediate. Mapping these residues on the crystal structure of the C_H1 domain of a murine IgG1 Fab fragment revealed how the association-coupled folding reaction of this antibody domain might proceed. Residues Thr22, His49, Ser65 and Thr67 in the C_H1 domain, which form part of the C_L interface, seem to be already correctly positioned in the intermediate (Fig. 2C, left panel). Importantly, His49 and Ser65 are involved in hydrogen bonds with the C_L domain in the native state. The interaction with C_L apparently initiates the formation of a hydrophobic cluster in the C_H1 domain including Val21, Val68, Trp73 and Val78 (Fig. 2C, right panel). Additionally, interaction of Val48 and Val66 might also be involved in this hydrophobic cluster, although this could not be directly addressed due to peak overlap for Val66. Thus, a few key interactions between C_L and C_H1 establish an interface between the two domains in the intermediate, which allows the formation of a hydrophobic folding nucleus in the C_H1 domain, and subsequent prolyl isomerization paves the path to the native state.

To identify the residues responsible for the slow folding reaction of the C_H1 domain, each of the three proline residues (Pro32, Pro34, and Pro74) that adopt a *cis* conformation in the native state (Fig. 2C, right panel) was individually mutated to alanine. ^{15}N-^{1}H HSQC spectra were recorded for each of the mutants in the absence and in the presence of C_L. All three mutants showed almost indistinguishable spectra compared to the wild type C_H1 domain in the absence of C_L (Fig. 2D). Importantly, two of the mutants, Pro34Ala and Pro74Ala, displayed well dispersed HSQC spectra in the presence of C_L that are very similar to that of wild type C_H1 in the presence of C_L (Fig. 2D), arguing that isomerization of these two prolines is not essential for C_H1 domain folding. However, when the Pro32Ala mutant was similarly examined, identical spectra were obtained in the presence and in the absence of C_L (Fig. 2D). Thus, isomerization of Pro32 from *trans* to *cis* is a prerequisite for C_L-induced folding of the C_H1 domain.

The role of the molecular chaperone BiP in C_H1/C_L assembly

Although association-coupled folding of the C_H1 domain is an intrinsic feature of this protein, the folding, assembly and subsequent secretion of IgG molecules in the cell involve additional factors (Meunier et al., 2002). To retain the unassembled heavy chain in the ER, a protein recognizing the unfolded C_H1 domain is needed. The molecular chaperone BiP, a member of the Hsp70 chaperone family that is present at high concentrations in the ER, plays a crucial role in this process *in vivo* (Haas and Wabl, 1983; Lee et al., 1999). After

synthesis and prior to assembly with the light chain, the C_H1 domain of the heavy chain remains in a reduced state (Vanhove et al., 2001). In line with this finding, BiP formed stable complexes with the reduced C_H1 domain *in vitro* with a dissociation constant of 4.2 ±0.4 μM (Fig. 3A). Oxidation of C_H1 only slightly reduced the affinity for BiP to K_d = 12.6 ±0.7 μM (Fig. 3A), which is consistent with this domain remaining unfolded. This is further supported by our finding that reduced C_H1 could form its intradomain disulfide bridge while in the BiP bound state (data not shown). Oxidized and reduced C_H1 both bind to BiP in a two-state, concentration-dependent reaction guarantying a fast association in the ER (Fig. 3B). To assess whether C_L can form stable triple complexes with BiP and C_H1, FRET experiments were carried out with ATTO594-labeled BiP and ATTO532-labeled C_L to which unlabeled C_H1 was added (supplemental Fig. 4). No FRET signal was detected even though BiP readily associates with C_H1 under these conditions (supplemental Fig. 4) and C_H1 interacts with C_L (Fig. 1). Thus, the presence of stable BiP:C_H1:C_L complexes is very unlikely even though the light chain can trigger the dissociation of BiP:C_H1 complexes *in vivo* (Lee et al., 1999).

The antibody domain folding status controls binding to BiP and secretion from the ER *in vivo*

It has long been appreciated that the C_H1 domain is central to correct assembly and transport of IgG molecules and other immunoglobulin isotypes (Wolfenstein-Todel et al., 1974; Adetugbo, 1978; Hendershot et al., 1987; Shaffer and Schlissel, 1997). Deletion of this domain allows secretion or surface expression of free heavy chains and various Ig assembly intermediates (Hendershot et al., 1987), which shows that quality control is focused on this domain. Our data put the unexpected unfolded nature of the C_H1 domain at the center of the secretion control mechanism of IgG antibodies. To test this notion in a cellular context, we first expressed the MAK33 κ light chain, which contained the C_L domain that was used in the *in vitro* experiments, in COS-1 cells and performed metabolic labeling and immunoprecipitation assays. As expected, this wild type light chain (LC_{wt}) was detected not only in the cells but also in the medium, indicating that LC_{wt} was secreted efficiently (Fig. 4A, lanes 1 and 3). When we replaced the C_L domain of the light chain with the C_H1 domain, this light chain (LC_{CH1}) now behaved like a heavy chain in terms of retention in the ER and interaction with BiP as demonstrated by an increase in the amount of the altered light chain that co-precipitated with BiP and its absence in the medium (Fig. 4A, lanes 4–6). This shows that the structural characteristics of the C_H1 domain and its role in antibody retention are intrinsic, context-independent features. To more directly address the structural prerequisites for antibody retention, we exchanged the small helical elements of the C_L domain, which have been reported to play a crucial role in the folding of this domain (Feige et al., 2008), against the corresponding elements of the C_H1 domain. This exchange transformed the C_L domain into an unfolded protein *in vitro* (data not shown). When a light chain containing this altered C_L domain (LC_{CLmut}) was expressed in COS-1 cells, it strongly interacted with BiP *in vivo* and was no longer secreted from the cell (Fig. 4A, lanes 7–9), which argues that the folding status of an antibody domain is key for its retention.

To add support to the key role played specifically by the interactions between C_H1 and C_L domains, we first performed additional *in vivo* experiments using two different full length heavy chains (the MAK33 γ1 heavy chain and a humanized mouse IgG γ1 heavy chain (Liu et al., 1987)) in combination with both the wild type (LC_{wt}) and the mutated MAK33 light chain (LC_{CLmut}). The wild type MAK33 light chain assembled with both heavy chains and allowed their secretion from cells, whereas the mutant light chain was unable to assemble or induce secretion of the heavy chains (Fig. 4B and supplemental Fig. 5). This is in keeping with our *in vitro* data showing that the mutant C_L domain was unfolded and therefore unable to induce the folding of the C_H1 domain. To determine if this was also the case *in vivo*, we

used a truncated version of the chimeric heavy chain consisting of only the V_H and C_H1 domains, which allows us to monitor oxidation of the C_H1 domain based on an increase in its mobility (Lee et al., 1999). Indeed we found that the wild type MAK33 light chain induced oxidation and secretion comparable to a different λ light chain that was used in previous studies (Lee et al., 1999) (Fig. 4C, compare lanes 4 and 6 and lanes 5 and 7). However the mutant light chain was unable to induce either oxidation or secretion of this truncated heavy chain (Fig. 4C, lanes 8 and 9). These data clearly show that the correct folding of the C_L domain is absolutely required to induce the folding and oxidation of the C_H1 domain as well as their covalent assembly *in vivo*, which is in line with our *in vitro* data. Furthermore, these data demonstrate that the template-assisted folding of C_H1 is a general and interchangeable control element functioning in murine and human antibodies.

Isomerization of a conserved proline residue in the C_H1 domain is essential for assembly and secretion of IgG molecules *in vivo*

To directly assess the role of proline isomerization in the association-coupled folding reaction of the C_H1 domain *in vivo*, each single *cis* proline residue in the C_H1 domain was exchanged against alanine in the context of the full length heavy chain and metabolic labeling experiments were conducted. For the MAK33 wild type heavy chain and two of the heavy chains mutated in the C_H1 domain, Pro34Ala and Pro74Ala, all Ig assembly intermediates could be detected (Fig. 5A, lanes 1,2,4,5,10 and 11), and importantly, completely assembled Ig molecules were secreted (Fig. 5A, lanes 3,6 and 12), demonstrating that isomerization of neither of these prolines was critical for C_H1 domain folding *in vivo*. In fact, mutation of proline 74 to alanine actually increased the assembly and secretion of the heavy chain. In contrast, when proline 32 in the C_H1 domain was mutated to alanine no significant amount of heavy chain and light chain assembly were detected nor was any heavy chain secreted (Fig. 5A, lanes 7–9). This is not due to poor expression of either the light chain or mutant heavy chain in this experiment (Fig. 5B). These results are in excellent agreement with our *in vitro* data (Fig. 2D) and reveal a key role of the *trans* to *cis* isomerization of Pro32 in the C_H1 domain for the assembly, interchain disulfide bridge formation and secretion of IgG molecules. It should be noted that this critical proline residue is highly conserved in the various murine Ig isotypes as well as in the immunoglobulins of different species.

Discussion

In this work we show that the C_H1 domain of the murine IgG1 antibody is an intrinsically disordered protein. As C_H1 does not possess an unusual number or distribution of charged or hydrophobic residues, it can still form a well defined globular structure once folded and does not show the typical sequence signature usually associated with intrinsically disordered proteins (Ward et al., 2004; Yang et al., 2005; Fink, 2005; Dunker et al., 2008). The large number of proline residues, also in a part of the C_H1 domain that was recently identified as forming small helical elements important for antibody domain folding (Feige et al., 2008), might be one determinant contributing to its unfolded nature. Our data thus suggest that the C_H1 domain is a representative of a novel class of intrinsically disordered proteins.

We demonstrate that the C_L domain, which is the cognate partner of the C_H1 domain in the complete IgG molecule, is required to fold C_H1 to the structure observed in IgG antibodies. The detailed analysis of the underlying pathway suggests that the reaction is initiated by the recognition of a few key interface residues between C_L and C_H1, which then promotes the formation of a hydrophobic core in C_H1. Both reactions render each other energetically more favorable and thereby allow the entropically demanding structuring of an unfolded polypeptide chain. *In vitro*, this folding reaction requires the presence of the internal disulfide bridge in C_H1. Therefore one might assume that a roughly preformed topology or

residual structure, which could not be detected in this study, might play a role in the folding process. Folding of the C_H1 domain is rate-limited by proline isomerization as observed for most isolated antibody domains (Goto and Hamaguchi, 1982; Thies et al., 1999; Feige et al., 2004). The presence of several *cis* proline residues in the native state and the overall very large number of proline residues, however, sets the C_H1 domain apart from most other antibody domains and may reflect the special role played by this domain. The key step in C_H1 folding, the isomerization of a single highly conserved proline residue in the loop between strand B and C, is likely to be essential for the folding and secretion of most Ig classes and potentially of other Ig superfamily members.

We show that BiP, a major ER chaperone, strongly binds to C_H1 *in vitro*, in agreement with previous *in vivo* studies (Hendershot et al., 1987). Oxidation of the internal C_H1 disulfide bridge was possible in the BiP-bound state and BiP still binds to the oxidized C_H1 domain *in vitro*, although this form has not been detected *in vivo*. After release from BiP, C_H1 can complete folding upon association with C_L and, if successful, form an interchain disulfide bridge with C_L. Since heavy chains of most isotypes that are devoid of the C_H1 domain can be secreted (Coffino et al., 1970; Hendershot et al., 1987), the essential steps controlling IgG assembly described here are likely to be general for all antibodies. Our data allow us to propose a possible order of events for this quality control mechanism *in vivo*. First, the C_H1 domain binds to BiP as it enters the ER cotranslationally in the reduced state. Then, likely triggered by association with light chain (Lee et al., 1999), the oxidation of the internal disulfide bridge between Cys25 and Cys80 takes place, which brings at least two of the residues (Val21 and Val78) that are involved in the formation of the hydrophobic folding nucleus in close proximity to each other. Only after release from BiP can the oxidized C_H1 domain complete its folding in association with C_L. This scenario is in agreement with the fact that some residues in the C_H1 domain found to be involved in the initial interaction with C_L were among those identified as putative BiP binding sequences in this domain in a previous study (Knarr et al., 1995). Even though most antibody domains possess BiP binding sequences (Knarr et al., 1995), they interact with BiP in the cell only transiently or not at all due to the competing, rapid folding reaction (Hellman et al., 1999). In contrast, the continued unfolded status of the C_H1 domain in the absence of light chain association allows it to permanently expose binding sites for BiP predisposing it for a stable interaction in the ER. In the complete antibody, the C_L and the C_H1 domain are covalently cross-linked *via* a disulfide bridge. We found that once this intermolecular disulfide bridge is formed, BiP no longer associates with the C_H1 domain *in vitro* (data not shown), because formation of this interchain disulfide bridge is rate-limited by proline isomerization and hence depends on the complete folding of C_H1. Thus, folding-dependent, covalent assembly provides yet another checkpoint for monitoring the proper maturation of Ig molecules in the ER and allows the assembled heavy chain to escape thiol-mediated retention mechanisms in cells (Sitia et al., 1990). It is conceivable that in the ER, association, oxidation and folding of C_H1 are tightly coupled by the immunoglobulin assembly machinery, as no oxidized C_H1 was found to be bound to BiP *in vivo* and ATP-induced release of BiP from unassembled heavy chains results in the formation of disulfide linked heavy chain aggregates (Vanhove et al., 2001).

Taken together, our data provide a detailed mechanism by which BiP and an intrinsically disordered antibody domain control the secretion of murine IgG1 antibodies (Fig. 6). The comprehensive model incorporates previous *in vitro* (Lilie et al., 1995;Mayer et al., 2000) and *in vivo* findings (Bole et al., 1986;Hendershot et al., 1987;Lilie et al., 1995;Lee et al., 1999;Mayer et al., 2000;Vanhove et al., 2001) and suggests regulatory hubs where additional components may come into play *in vivo* (Vanhove et al., 2001;Elkabetz et al., 2005) to orchestrate folding, oxidation, assembly and secretion.

A rigorous assembly control mechanism is particularly important for antibody molecules, which undergo a developmentally asynchronous expression of heavy and light chain genes. In pre-B cells, heavy chain genes are rearranged first and the resultant proteins are largely retained in the cell (Burrows et al., 1979), except for a limited number that assemble with the surrogate light chain (Pillai and Baltimore, 1987). The developmentally more mature B cell expresses light chains, which assemble with heavy chains and allow their transport to the cell surface. Finally, the terminally differentiated plasma cell produces enormous quantities of Ig molecules (Cenci and Sitia, 2007), which must be appropriately assembled to bind specifically to antigens and fulfill their effector functions. Accordingly, the nature of the reactions that govern C_H1 folding and assembly with C_L allow efficient and accurate assembly of antibodies prior to secretion and might hint towards the co-evolution of substrates and folding factors in the ER as well as a general mechanism of quality control for oligomeric proteins.

Experimental Procedures

Protein production

All antibody domains were produced similar to published protocols (Feige et al., 2004; Feige et al., 2007). Details can be found in the Supplemental Materials section. Numbering of the C_H1 domain begins with 1 in this work.

Hamster BiP (Wei and Hendershot, 1995) was mutated by site-directed mutagenesis to the murine sequence. Expression was carried out in HB101 cells for 3 h at 37°C. Following cell disruption, Ni-NTA affinity purification was performed in 50 mM Hepes/KOH, pH 7.5, 400 mM NaCl, 50 mM Imidazole. BiP was eluted with an Imidazole gradient from 0.05 M to 1 M. BiP containing fractions were applied to a Superdex 200pg (26/60) gel filtration column (GE Healthcare, München, Germany) equilibrated in HKM buffer (50 mM Hepes/KOH, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$) and finally to a Superdex 200 10/300GL HPLC column (GE Healthcare, München, Germany) equilibrated in the same buffer. The cyclophilin B (CypB) gene was amplified without the signal sequence from the murine cDNA (imaGenes, Berlin, Germany) and inserted into the pET28a vector. Expression was carried out for 3 h at 37°C in BL21-DE3 cells. The cell pellet was dissolved in 50 mM Hepes/KOH, pH 7.0, 10 mM EDTA and the cleared lysate was applied to a SP-Sepharose column equilibrated in the same buffer. The protein was eluted with a 0–1 M NaCl gradient. Subsequently, CypB containing fractions were applied to a Superdex 75pg (26/60) gel filtration column equilibrated in HKM buffer. All vectors were sequenced and protein masses were verified by mass spectrometry.

Optical spectroscopy

A Jasco J-720 spectropolarimeter was used for all CD measurements (Jasco, Gross-Umstadt, Germany). Far-UV CD spectra were recorded in a 0.2 mm quartz cuvette, far-UV kinetics in a 1 mm quartz cuvette. Spectra of the isolated domains were recorded at 45 μM protein concentration, for the spectrum of the complex 15 μM C_H1 in the presence of 45 μM C_L was used. Far-UV CD kinetics were recorded at 10 μM protein concentration of each domain and followed at 205 nm. Spectra of the C_H1 domain in the complex were calculated by substraction of the spectrum of the isolated C_L domain from the spectrum of the complex, measured after a 4 h equilibration step at 25°C. All spectra were averaged 16 times and buffer corrected. Fluorescence measurements were carried out in a Spex FluoroMaxIII spectrofluorimeter (Jobin Yvon, München, Germany) in a stirred 1 cm quartz cuvette. Kinetics and titrations were measured by the change in the intrinsic tryptophan fluorescence, excited at 280 nm and detected at 350 nm. For titrations, varying concentrations of C_L were added to 2 μM C_H1 and immediately as well as after a 4 h equilibration step at 25°C, the

fluorescence of the same samples was determined. The difference between initial and final fluorescence emission was normalized and analyzed according to a one-site binding model. Iodide quenching experiments were performed as published (Feige et al., 2007).

For anisotropy measurements, 1 μM lucifer yellow labeled C_L Ala113Cys and varying concentrations of C_H1 were used. Lucifer yellow fluorescence was excited at 430 nm and detected at 525 nm. The change in quantum yield of the chromophore was less than 5% upon association of the labeled C_L domain with C_H1. Individual traces were fitted by single exponential functions. The obtained rate constants were fitted to a linear equation to derive the k_{on} and the k_{off} value.

NMR spectroscopy

Spectra of the C_H1 domain in complex with the C_L domain were recorded at 25°C on Bruker DMX600 and DMX750 spectrometers (Bruker, Rheinstetten, Germany), whereas spectra for the assignment of the unfolded C_H1 domain were measured at 12.5°C on a Bruker AVANCE900 spectrometer (Bruker, Rheinstetten, Germany). Backbone sequential assignment of the isolated C_H1 domain was obtained by standard triple resonance experiments implemented with selective proton flip-back techniques for fast pulsing (Diercks et al., 2005). To gain information about any preferential conformations present in the disordered C_H1 domain, an NNH-NOESY spectrum and a ^{15}N-HSQC-NOESY spectrum were recorded on a highly deuterated sample with a mixing time of 600 ms in order to detect long range H^N-H^N NOEs (Mok et al., 1999).

For all measurements of the folded C_H1 domain in association with C_L, a twofold excess of unlabeled C_L was added to ^{15}N or ^{15}N, ^{13}C labeled C_H1. Prior to steady state measurements, samples were incubated for at least 6 h at room temperature to ensure complete folding of the C_H1 domain. Backbone sequential assignment of the assembled C_H1 domain was achieved with standard triple resonance experiments with selective proton flip-back techniques for fast pulsing. The assignment of the carbon chemical shifts was limited to the C' and C^α chemical shifts due to the relaxation properties of the whole protein complex. To verify the backbone resonance assignment NH residual dipolar couplings (RDCs) were determined. The sample was prepared as described above and aligned with nonionic liquid crystalline media (Ruckert and Otting, 2000). NH RDC values were extracted from IPAP-HSQC spectra using Bruker pulse sequences. The sequential information based on the C' and C^α chemical shifts as well as the NH RDC values and the crystal structure of the folded C_H1 domain (pdb code: 1ORS) served as input for the software MARS (Jung and Zweckstetter, 2004b; Jung and Zweckstetter, 2004a).

In order to characterize the folding pathway of the intrinsically disordered C_H1 domain, ^{15}N-HSQC spectra were recorded at 12.5°C every 14 min immediately after adding unlabeled C_L to ^{15}N labeled C_H1 using selective proton flip-back techniques for fast pulsing. Identical processing of all spectra measured during the folding process was performed with the software TOPSPIN 1.3 (Bruker Biospin). Peak intensities were analyzed using the software SPARKY (www.cgl.ucsf.edu/home/sparky) and normalized to the corresponding intensities in the final spectrum after 36 h. The backbone resonance assignment was transferred from 25°C to 12.5°C recording a HSQC temperature series.

Analytical HPLC experiments

For all experiments, a Shimadzu HPLC system (Shimadzu, München, Germany) was used. Complex formation between BiP and C_H1 was analyzed on a Superdex 200 10/300GL column in HKM buffer at a flow rate of 0.5 ml/min. For the determination of the dissociation constant as well as binding kinetics, peak intensities at the retention time of

28.4 min corrected for baseline drifts were plotted against the C_H1 concentration respectively the incubation time and normalized. The rate constants k_{obs} of the binding reaction were determined from single exponential fits and evaluated with a linear equation to derive k_{on} and k_{off}. Detection of all proteins was performed by the intrinsic fluorescence excited at 280 nm and monitored at 350 nm. Incubation steps were performed in HKM buffer with 1 mM ADP.

Cell culture experiments

The murine IgG1 MAK33 light chain (LC_{WT}) and heavy chain (HC_{WT}) cDNAs were obtained with an intact signal sequence for expression in mammalian ER. Two LC mutants, one in which the C_H1 domain was substituted for the C_L domain (LC_{CH1}) and the other where structural features of the C_H1 domain were substituted for the corresponding regions of the C_L domain (LC_{CLmut}) were produced and all constructs were inserted into the pSVL vector (GE Healthcare, München, Germany). An HA-epitope tag was engineered at the C-terminus of the wild type light chain and the two mutants for immunoprecipitation purposes. Heavy chain proline exchange mutants were generated by site-directed mutagenesis. The cDNA for a chimeric humanized heavy chain was used as published (Liu et al., 1987) and a truncated version of this heavy chain containing only the V_H and C_H1 domains was produced previously (Lee et al., 1999) as was a mouse lambda light chain cDNA (Hellman et al., 1999). The recombinant plasmids, along with a pMT vector encoding hamster BiP (Lee et al., 1999) were introduced into COS-1 cells (Gluzman, 1981) that were cultured as described (Lee et al., 1999) using FuGENE 6 transfection reagent (Roche, Indianapolis, USA) following the manufacturer's protocol. Metabolic labeling, cell lysis, immunoprecipitation, and visualization of the proteins were performed as described previously (Lee et al., 1999). Anti-rodent BiP antiserum (Hendershot et al., 1995), a monoclonal anti-HA (12CA5) antibody (kindly provided by Dr. Al Reynolds, Vanderbilt University, USA), and a goat anti-mouse Ig κ and goat anti-mouse γ antibodies (Southern Biotech, Birmingham, AL, USA) and Protein A Sepharose beads were used for immunoprecipitations. For metabolic labeling experiments, cells were cultured in DMEM lacking methionine and cysteine and labeled with ^{35}S Translabel (MP Biomedicals, Irvine, CA, USA) for the indicated times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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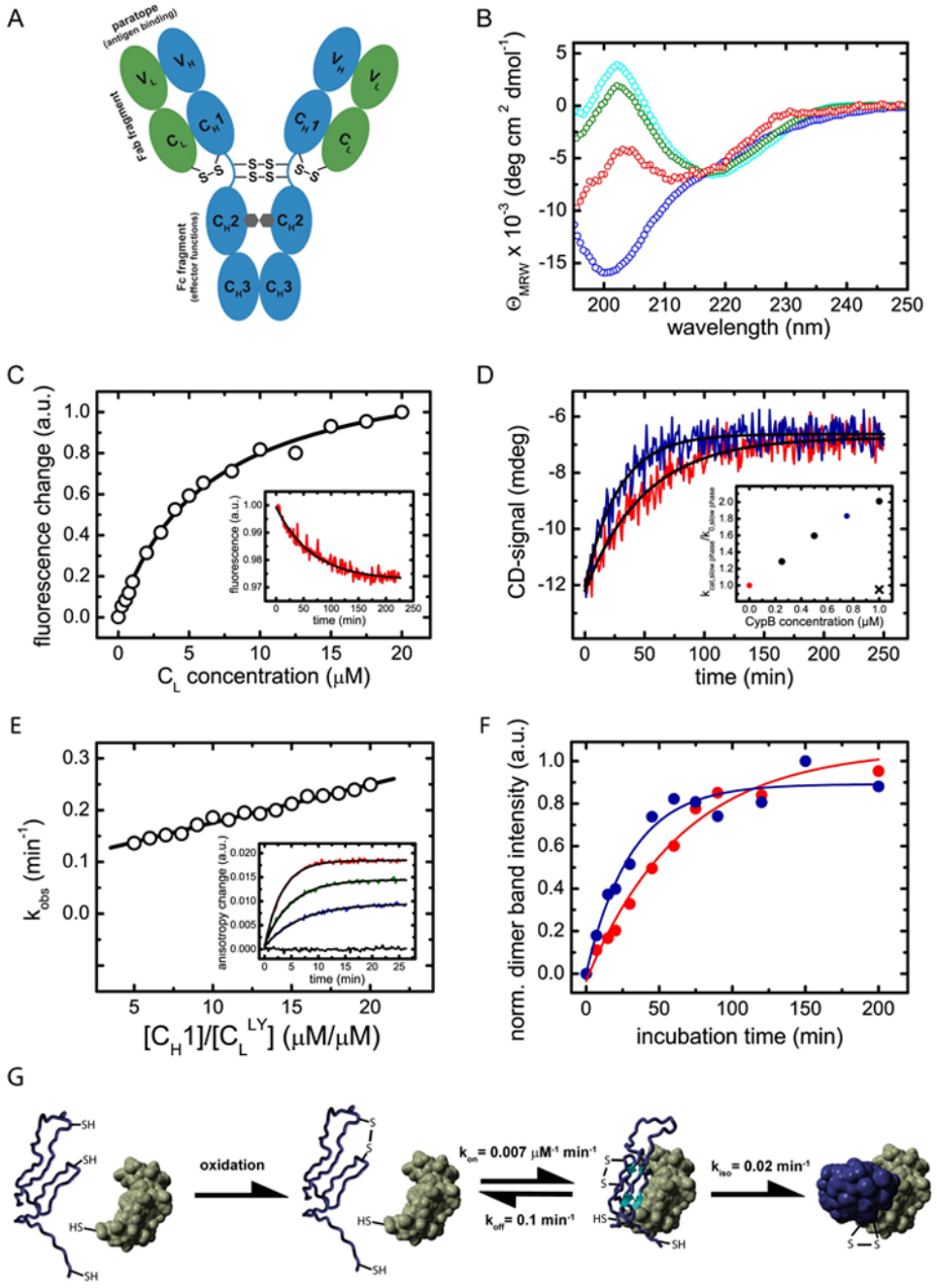

Figure 1. Structural characteristics of the C_H1 domain and its assembly mechanism with the C_L domain

(A) shows a schematic representation of the IgG1 molecule. The IgG molecule is composed of two heavy chains (blue) and two light chains (green). The heavy chain consists of the V_H, C_H1, C_H2 and C_H3 domain and the light chain of the V_L and the C_L domain (listing from N- to C-terminus of each chain). The C_H2 domains of the heavy chains are glycosylated with complex biantennary oligosaccharides (depicted in grey). Each domain possesses an internal disulfide bridge (omitted for clarity) and additional disulfide bonds link the two heavy chains in the flexible hinge region. A single disulfide bond covalently connects C_H1 with the C_L domain. The two identical antigen binding sites (paratopes) are made up by the two

variable domains V_H and V_L. The overall IgG molecule can be divided into two Fab fragments (composed of V_H, C_H1, V_L and C_L) and one Fc fragment (composed of two C_H2 and two C_H3 domains). (B) The isolated C_L domain (cyan) displays a typical all-β far-UV CD spectrum whereas the isolated C_H1 domain (blue) shows a random coil spectrum. To assess if C_L induces structural changes in C_H1, the spectrum of co-incubated C_L and C_H1 was recorded (green). From this spectrum and the far-UV CD spectrum of the isolated C_L domain, the spectrum of the C_H1 domain in the presence of C_L was calculated (red) which shows the characteristics of β-sheet secondary structure. (C) The affinity between C_H1 and C_L was determined by the change in the intrinsic fluorescence upon C_L induced folding of the C_H1 domain, recorded before and after a 4 h equilibration step. A one-site binding model was used to fit the data. The inset shows a representative single exponential trace observed after the addition of 1 μM C_L to 2 μM C_H1. A single exponential reaction with a very similar rate was observed by far-UV CD spectroscopy (D, red trace). The folding reaction could be accelerated by the PPIase CypB (red trace: 10 μM C_L and 10 μM C_H1 alone, blue trace: in the presence of 0.75 μM CypB). The inset shows the dependence of the slow reaction on CypB concentration. The observed rate in the absence of CypB is denoted as k_0, in the presence of CypB as k_{cat}. If 1μM CypB was inhibited by 2 μM cyclosporine A, no acceleration was observed (black cross). (E) Association of the C_H1 domain with a lucifer yellow labeled C_L domain was followed by the change in the lucifer yellow anisotropy signal. The observed rate constants were fitted with a linear function to yield the k_{on} value and the k_{off} value of 0.007 ±0.0002 μM^{-1} min^{-1} respectively 0.1 ±0.01 min^{-1}. The inset shows individual single exponential traces after the addition of 0 μM (black), 5 μM (blue), 10 μM (green) and 20 μM (red) C_H1 to 1 μM labeled C_L. (F) To assess the formation of the C_L/C_H1 interchain disulfide bridge, non-reducing SDS-PAGE experiments were carried out and the dimer band intensity was quantified. 25 μM of each domain were used. In the absence of CypB (red), a time constant of τ = 63 ±7 min was observed for the formation of covalent C_L/C_H1 dimers. In the presence of 5 μM CypB, a time constant of τ = 31 ±5 min was obtained. In (G), the overall C_H1/C_L assembly mechanism is shown. Only after formation of the internal disulfide bridge in the C_H1 domain (blue), the fast formation of a dimeric intermediate with the C_L domain (green) is observed. Subsequently, prolyl isomerization limits complete folding and formation of the interchain disulfide bridge. All measurements were carried out at 25°C in PBS.

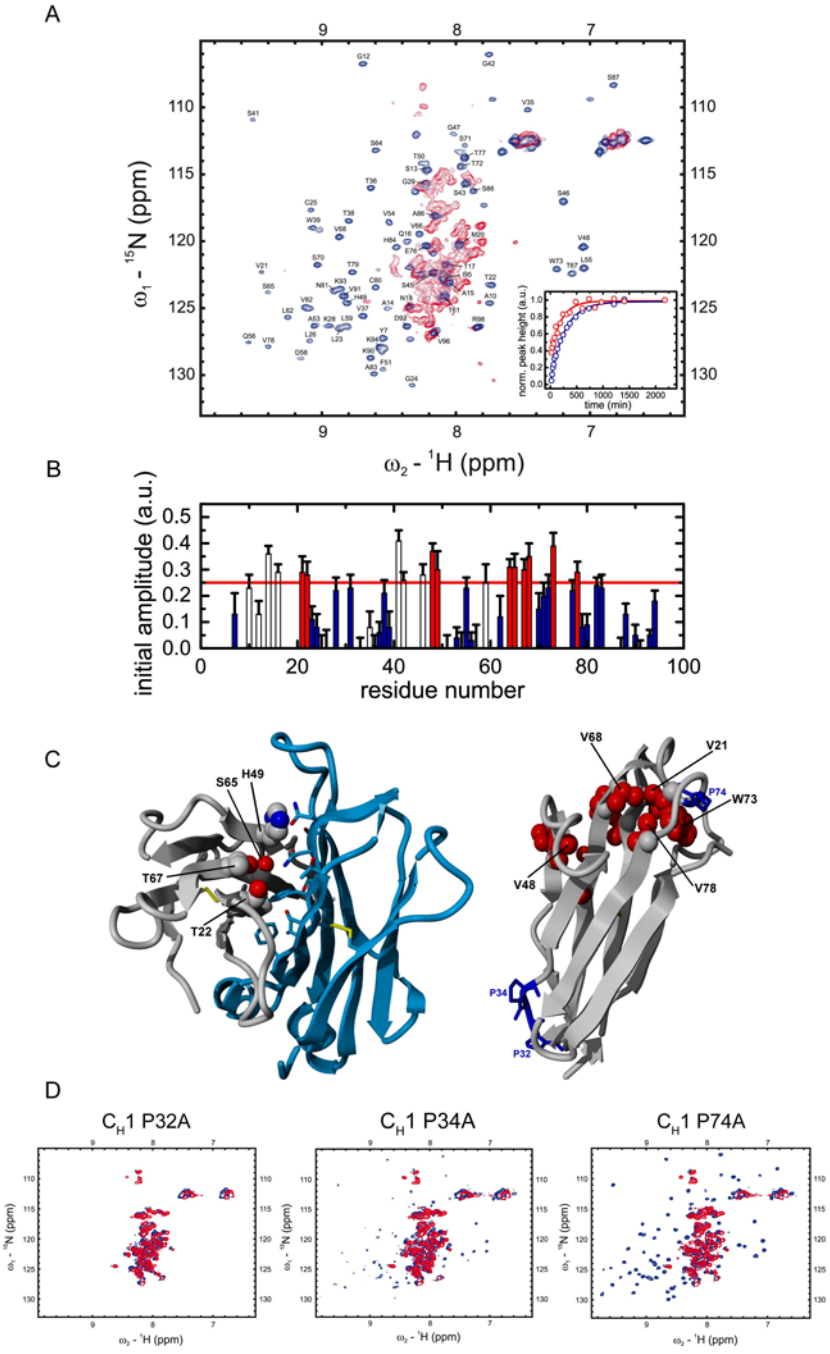

Figure 2. NMR spectroscopic characterization of C_L induced C_H1 folding

(A) ^{15}N-^{1}H HSQC spectra of the isolated C_H1 domain (red) and the assigned C_H1 domain in complex with the C_L domain (blue) are shown. In order to characterize the folding pathway of the intrinsically disordered C_H1 domain, time dependent HSQC intensities upon addition of unlabeled C_L to ^{15}N labeled C_H1 were measured for each assigned residue at the native chemical shift position and fitted by a single exponential function. Two representative traces for Val68 (red) and Lys90 (blue) are shown in the inset. (B) Initial amplitudes for each assigned C_H1 residue were derived from the fitted exponential functions. Residues with an initial HSQC amplitude below a threshold of 25% native intensity are colored in blue and residues above the threshold in red (open bars: residues in loop regions / filled bars: residues

in structured regions). Errors indicate standard deviations from single exponential fits. In (C) C_H1 residues with intensities above the threshold in the intermediate are mapped on the crystal structure of the C_H1/C_L dimer (pdb code 1Q9K). The dimerization interface between C_H1 (grey) and C_L (blue) is shown on the left with only residues of C_H1 indicated that are involved in this interaction and above the HSQC amplitude threshold. On the right, internal C_H1 residues above the 25% threshold are shown in red, the three *cis* proline residues in C_H1 are depicted and labeled in blue. (D) The HSQC spectra of the C_H1 Pro32Ala, Pro34Ala and Pro74Ala mutants show, that only the Pro32Ala mutant is not able to fold in the presence of C_L anymore (blue spectrum). For the other two mutants, Pro34Ala and Pro74Ala, well dispersed spectra and hence folding are observed in the presence of C_L (blue spectra). In the absence of C_L (red spectra), all three mutants show typical HSQC spectra of unfolded proteins. All measurements were carried out in PBS at 25°C except for the folding kinetics which where recorded at 12.5°C.

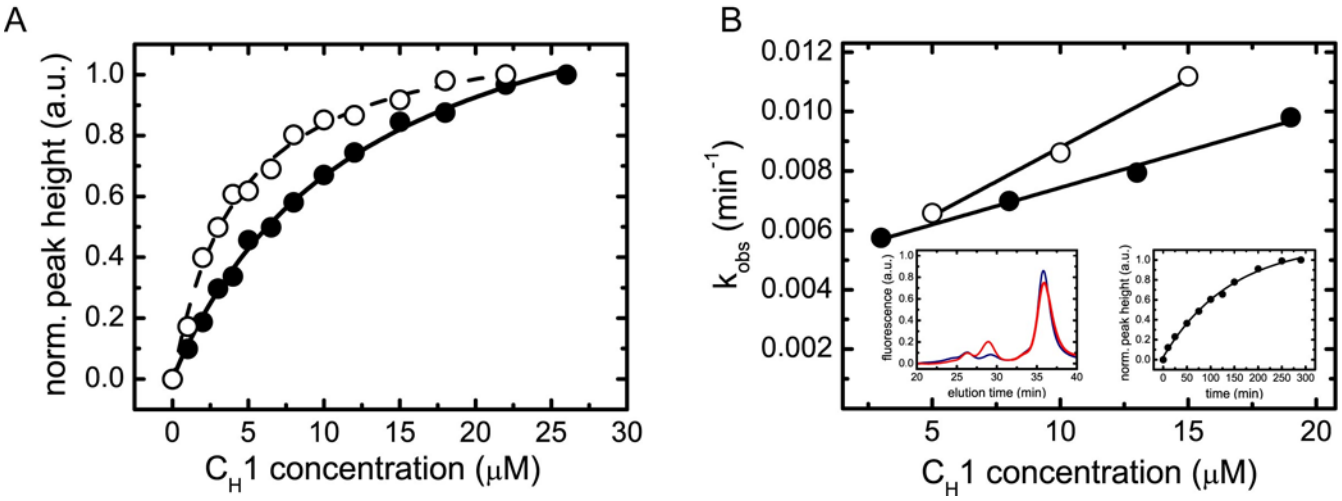

Figure 3. Characterization of the interaction between BiP and C_H1 *in vitro*

(A) The affinity between BiP and oxidized C_H1 (filled circles, straight line) as well as reduced C_H1 (open circles, dashed line) was determined by analytical HPLC experiments. The data were fitted to a one-site binding model to determine the K_d. (B) The association kinetics between 1 μM BiP and varying concentrations of oxidized C_H1 (filled circles) and reduced C_H1 (open circles) were measured to determine the rate constants of the reaction. For oxidized C_H1, k_{on} = 0.00026 ±0.00002 μM^{-1} min^{-1} and k_{off} = 0.0050 ±0.0002 min^{-1} were obtained. For the reduced C_H1 domain, the corresponding values were k_{on} = 0.00041 ±0.00003 μM^{-1} min^{-1} and k_{off} = 0.0047 ±0.0003 min^{-1}. The left inset shows single HPLC runs of 8 μM oxidized C_H1 and 1 μM BiP after 10 min (blue) and 200 min (red) co-incubation. The right inset shows the overall observed single exponential association kinetics between 1 μM BiP and 8 μM oxidized C_H1.

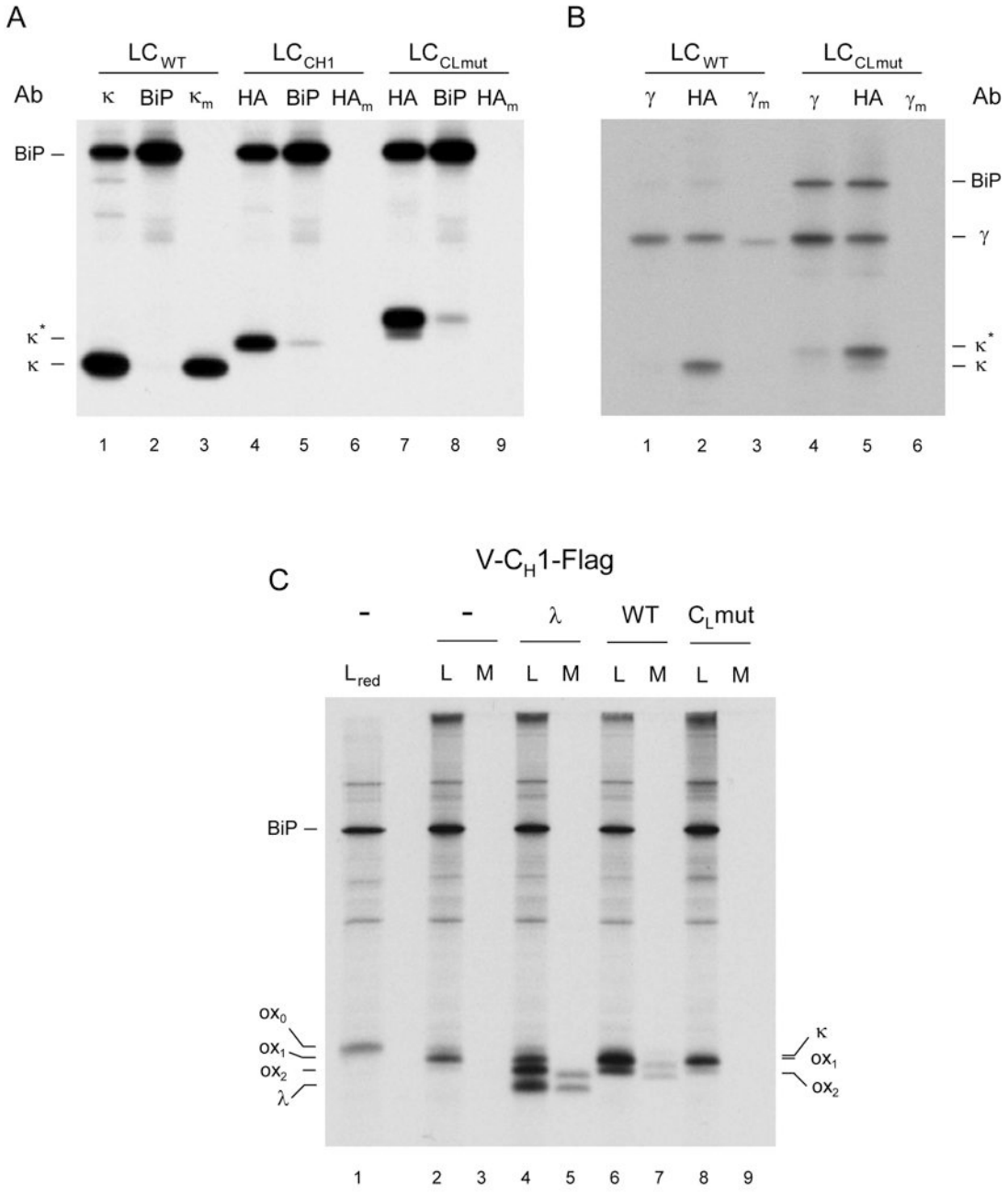

Figure 4. The folding status of an antibody domain controls IgG secretion *in vivo*

(A) COS-1 cells were co-transfected with vectors encoding BiP and either a wild type light chain (LC_{wt}), a light chain containing the C_H1 domain instead of the C_L domain (LC_{CH1}), or a light chain containing an unfolded C_L domain (LC_{CLmut}). Cells were metabolically labeled for 3 h and both cell lysates (no subscript) and culture supernatants (subscript m) were immunoprecipitated with the indicated antisera (Ab). Precipitated proteins were separated by SDS-PAGE under reducing conditions and visualized by autoradiography. (B) COS-1 cells were co-transfected as in (A) except that a vector encoding a chimeric humanized mouse heavy chain was also included. Cells were labeled and analyzed as in (A). (C) COS-1 cells were co-transfected with vectors encoding BiP, a Flag-tagged truncated heavy chain

consisting of only the V_H and C_H1 domains (Lee et al., 1999), and with the indicated light chain constructs (*i.e.*, λ, wild type κ, or C_L mutant κ). Cells were metabolically labeled and both cell lysates (L) and culture supernatants (M) were immunoprecipitated with the anti-Flag antibody. Precipitated proteins were separated by SDS-PAGE under non-reducing condition (except the first lane, which included 2-ME in the sample buffer and is indicated as red) and visualized by autoradiography. Mobilities of completely reduced (ox_0), partially oxidized (ox_1), and fully oxidized (ox_2) forms of truncated heavy chain, as well as those of λ and κ light chains are indicated. The tagged forms of the κ light chain constructs co-migrate with the ox1 form of the truncated heavy chain.

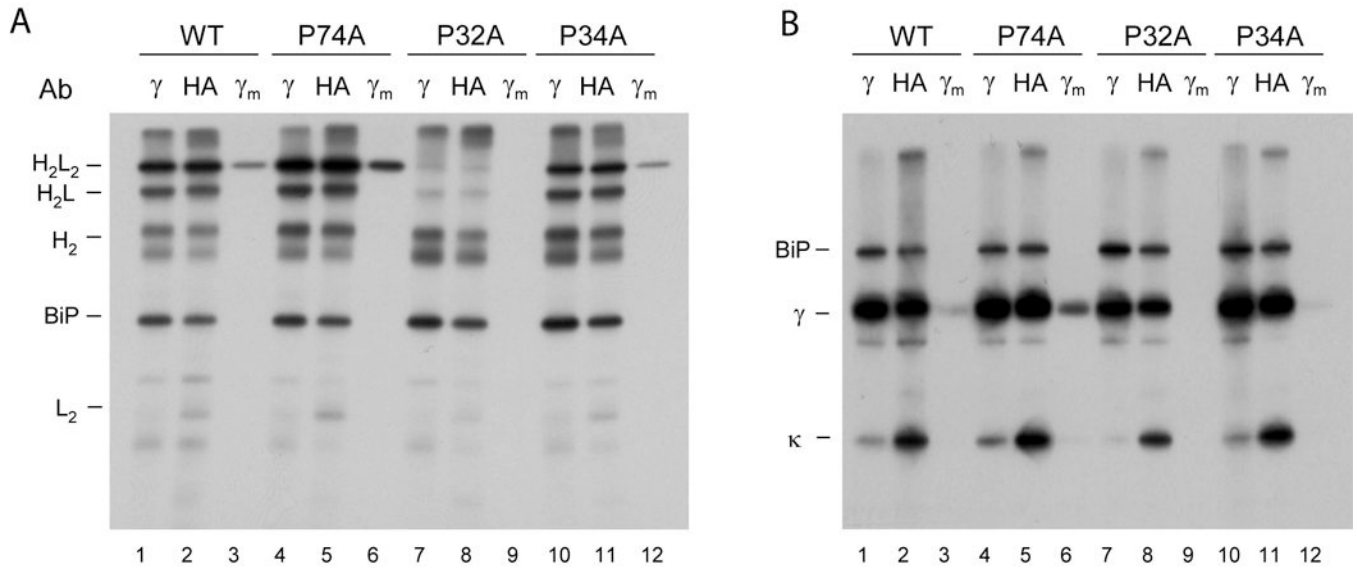

Figure 5. The isomerization of a single proline residue controls the assembly and secretion of heavy chains *in vivo*

COS-1 cells were co-transfected with vectors encoding wild type MAK33 heavy chain (WT) or one of the three Pro to Ala mutants (P74A, P32A, or P34A) together with wild type light chain and BiP. Cells were metabolically labeled and both cell lysates (no subscript) and culture supernatants (subscript m) were immunoprecipitated with the indicated antisera (Ab). Precipitated proteins were separated by SDS-PAGE under non-reducing conditions and visualized by autoradiography. (B) The cell lysates and culture supernatants from (A) were divided in half, immunoprecipitated with the indicated antibodies, analyzed by SDS-PAGE under reducing conditions, and visualized with autoradiography. These data demonstrate that the failure of the P32A mutant to induce assembly and secretion is not because it is expressed poorly, as the signal for this mutant heavy chain is very similar to that of the wild type heavy chain.

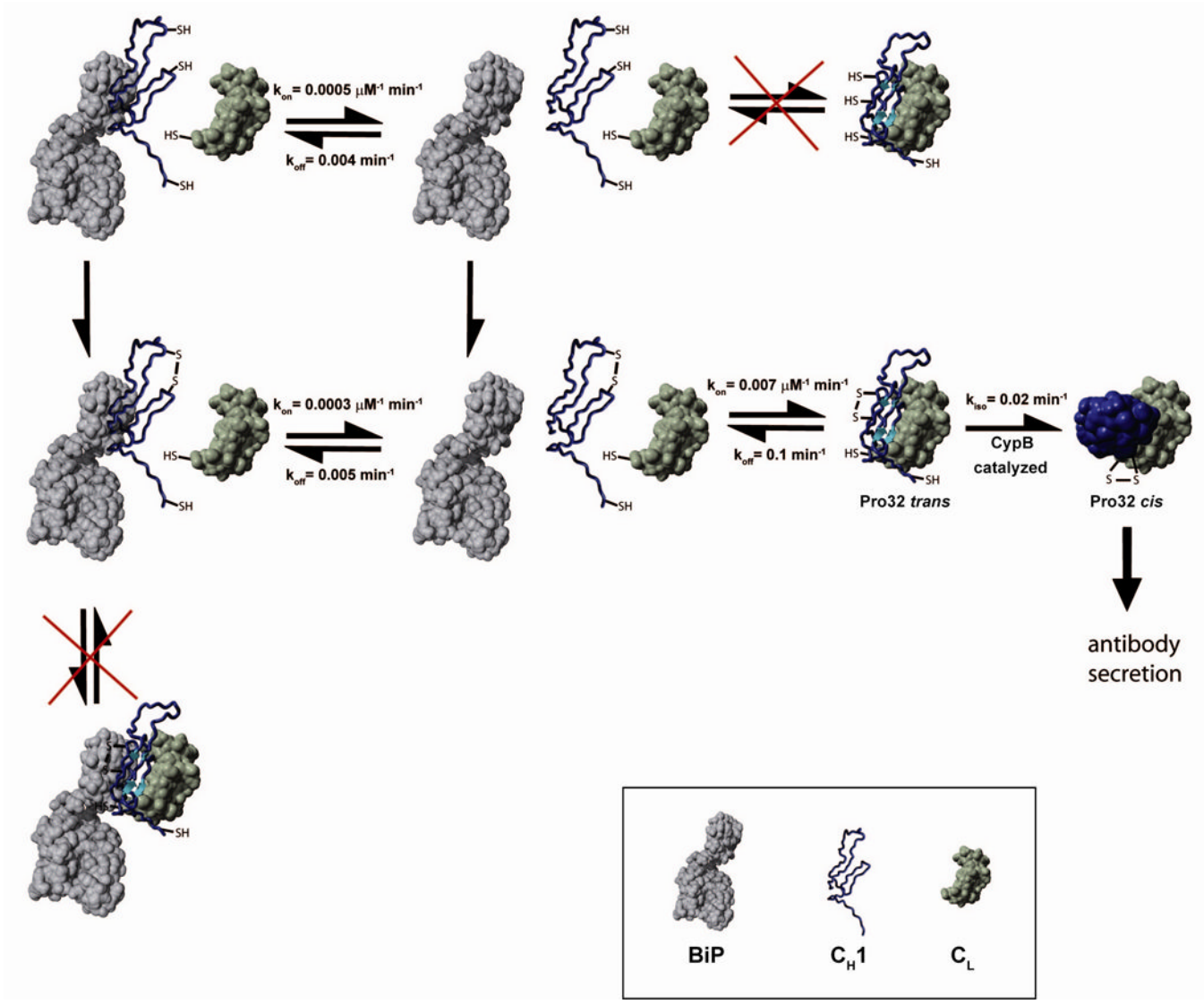

Figure 6. A model for the overall IgG secretion control mechanism

A schematic indicating the possible pathways for the C_H1 domain (blue), its folding and assembly in association with C_L (green) and BiP (grey) is shown. C_H1 has to form its internal disulfide bridge and to be released from BiP before it can associate with C_L. *In vivo*, these processes are tightly coupled and thus cannot be dissected kinetically. Prior to complete folding and irreversible formation of the C_L/C_H1 interchain disulfide bridge, the proline residue 32 has to isomerize from *trans* to *cis*. The isomerization reaction can be accelerated by CyclophilinB. All rate constants were determined at 25°C.