Cysteines in \( \text{CH}1 \) Underlie Retention of Unassembled Ig Heavy Chains

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Conformation, structure, and oligomeric state of immunoglobulins not only control quality and functional properties of antibodies but are also critical for immunoglobulins secretion. Unassembled immunoglobulin heavy chains are retained intracellularly by delayed folding of the \( \text{CH}1 \) domain and irreversible interaction of BiP with this domain. Here we show that the three \( \text{CH}1 \) cysteines play a central role in immunoglobulin folding, assembly, and secretion. Remarkably, ablating all three \( \text{CH}1 \) cysteines negates retention and enables BiP cycling and non-canonical folding and assembly. This phenomenon is explained by independent formation of intradomain and interchain disulfides, although both bonds are dispensable for secretion. Substituting Cys-195 prevents formation not only of the intradomain disulfide, but also of the interchain disulfide bond with light chain, BiP displacement, and secretion. Mutating the light chain-interacting Cys-128 hinders disulfide bonding of intradomain cysteines, allowing their opportunistic bonding with light chain, without hampering secretion. We propose that the role of \( \text{CH}1 \) cysteines in immunoglobulin assembly and secretion is not simply to engage in disulfide bridges, but to direct proper folding and interact with the retention machinery.

Immunoglobulins (Igs) are key molecules of the immune response, and, being oligomeric secretory proteins, Igs are excellent models to relate fidelity of folding and chain assembly to intracellular trafficking. Not only do conformation, structure, and oligomeric state control quality and functional properties of antibodies, but they are also critical for Ig secretion. Two heavy (HC) and two light (LC) chains form disulfide-bonded HC\(_2\)LC\(_2\) molecules. Each Ig chain consists of several similar compact domains, each comprising two twisted \( \beta \) sheets stabilized by a single intradomain disulfide (1). IgG assembly is stabilized by an interchain disulfide between C\(_L\), the LC constant domain, and C\(_H\), the first HC constant domain (2). The C\(_H\)1 domain confers retention in the endoplasmic reticulum (ER) on unassembled HCs, and despite its high degree of similarity to other constant domains, only C\(_H\)1 is retarded in folding and is unable to cycle from the ER chaperone BiP (3). Only upon LC expression is BiP displaced from C\(_H\)1, which completes its folding, allowing assembly of HC\(_2\)LC\(_2\) and secretion (4–7).

Although BiP binds transiently a wide variety of proteins (8), its associations with Ig HC and LC are among the best characterized. In vivo, BiP binds transiently to nascent LC via the unfolded V\(_L\) domain and is released when this domain folds (9–12). Non-secreted LC mutants display more persistent association with BiP (13–19). BiP also binds HC, where in addition to its interaction that seems irreversible with C\(_H\)1, it binds transiently other domains. In vitro studies defined specific heptameric sequences throughout LC and HC, which serve as BiP binding sites (18, 20). The affinity for binding sites in C\(_H\)1 is not obviously different from that for other heptamers, so presumably, BiP binding sites in the incompletely folded C\(_H\)1 remain exposed, enabling continued interaction with BiP and ER retention (3, 7, 21). It has been suggested that C\(_H\)1 is unique in its propensity to expose BiP binding sites following C\(_H\)2 and C\(_H\)3 homodimerization, because C\(_H\)1 is the only constant domain unable to homodimerize (7, 20).

Recognizing the unique folding status of C\(_H\)1, we undertook to identify structural elements within this domain, which control folding, chaperone interactions, and retention of unassembled HC. Several lines of evidence focused our attention on cysteines in C\(_H\)1. Contribution of exposed thiols to ER retention is well documented (22–27). Thus, in the absence of LC, C\(_H\)1-conferred retention could have resulted from continued exposure of the LC-interacting cysteine. However, even without such a cysteine, a truncated \( \gamma \)1 HC containing only V\(_L\) and C\(_H\)1 was still not secreted (21). Although this construct could no longer form the C\(_L\)-C\(_H\)1 disulfide, its C\(_H\)1 oxidative folding, reflected by intradomain disulfide bond formation and consequent secretion, still required LC (21). Hence, possible contribution of the LC-interacting cysteine to C\(_H\)1-conferred retention should be in the context of the two additional C\(_H\)1 cysteines, which form the intradomain disulfide.

In this work we studied possible interrelations between all three C\(_H\)1 cysteines, and, as shown below, substituting each of them with serine, singly or in combinations, yielded surprising structure-function relationships with respect to folding, assembly, and secretion. Remarkably, a mutant chain lacking all three C\(_H\)1 cysteines was secretion-competent. We provide a mechanistic explanation for this phenomenon by showing that, although in C\(_H\)1 neither the intradomain nor the interchain native disulfide was essential for secretion, these bonds affect each other’s formation. In addition to providing a molecular basis for the coordinated folding of C\(_H\)1 and its assembly with LC (7), we discuss a model in which C\(_H\)1 cysteines employ thiol-mediated retention, and their interplay maintains the stable BiP binding of unassembled Ig HC.
Primers were designed based on the published genomic sequence of murine γ2b (accession No. J00461). Designed restriction sites are italicized and underlined. Primers to amplify CH1, CH2, and CH3 were designed to include the relevant exon flanked by 50–100 bp of intronic sequences to ensure functional splicing. The reverse primer for CH1 was designed such that the stop codon was skipped over and the C1 donor splice site was added. All primers were synthesized by Invitrogen.

**MATERIALS AND METHODS**

**Plasmid Construction**—All mutant γ constructs were based on pJDEVNγ2b, containing genomic DNA encoding full-length murine γ HC with a known constant region (Ref. 28; accession number J00461). The CH1 exon, flanked by intronic sequences, was excised with EcoRI and PvuII. A NotI-containing linker was ligated to the vector at the EcoRI and PvuII sites, generating the CH1-less pJDEVNγ2b.C1γ1. Exons C1γ1, C2γ1, or C3γ1 were PCR-amplified, using primers (Table I) and pJDEVNγ2b, as a template, and subcloned into pBluescript (pBS, Stratagene) at EcoRI and XbaI sites, to generate pBS-C1γ1, pBS-C2γ1, and pBS-C3γ1, respectively. Site-directed mutagenesis analyses of CH1 were performed using pBS-C1γ1 as a template and either forward mutagenic primer (Table I) or the complementary (reverse) mutagenic primer. The mutated CH1 was cut by EcoRI and XbaI and inserted into pBS. Double and triple mutations in CH1 were introduced successively. All DNA constructs were sequenced. Wild-type and mutated CH1, as well as C2γ1 and C3γ1, were excised from the respective pBS constructs and re-introduced into pJDEVNγ2b.C1γ1 with EcoRI and NotI. Amplification and mutagenesis of Al was performed by PCR (see primers in Table I) on pTM1-Al cDNA (29) and cloned into pCDNA3 (Invitrogen) at the EcoRI and NotI sites.

**Cell Culture and Transfection**—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin at 37 °C in humidified 5% CO2 atmosphere. Modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin at 37 °C in humidified 5% CO2 atmosphere. Cells (50% confluent) were transfected using the calcium phosphate method (30), with 5 μg of the various γ constructs, with or without equimolar amounts of the pJDEVI vector encoding genomic full-length murine Al LC (14) or CDNA of wild-type or mutant γ. Where indicated, 8 μg of constructs encoding HA-tagged wild-type or T19G BiP (31) was co-transfected. Cells were analyzed by densitometry. Subsequently, blots were probed with the following antibodies: horseradish peroxidase (HRP)-conjugated anti-mouse γ (SouthernBiotech), HRP-conjugated anti-mouse Al (SouthernBiotech), biotin-conjugated anti-BiP (SouthernBiotech) followed by HRP-conjugated anti-mouse IgG (Jackson). The HRP was visualized by enhanced chemiluminescence (ECL).

**Limited Proteolysis**—Limited proteolysis of intracellular HCs was carried out on Protein A precipitates under optimized time, temperature, and enzyme concentrations. Papain (Sigma) was added at 5 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml elastase (ICN). Metabolic Labeling and Steady-state Analyses—Limited proteolysis of intracellular HCs was carried out on Protein A precipitates under optimized time, temperature, and enzyme concentrations. Papain (Sigma) was added at 5 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml elastase (ICN). Metabolic Labeling and Steady-state Analyses—Limited proteolysis of intracellular HCs was carried out on Protein A precipitates under optimized time, temperature, and enzyme concentrations. Papain (Sigma) was added at 5 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml elastase (ICN). Metabolic Labeling and Steady-state Analyses—Limited proteolysis of intracellular HCs was carried out on Protein A precipitates under optimized time, temperature, and enzyme concentrations. Papain (Sigma) was added at 5 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml elastase (ICN). Metabolic Labeling and Steady-state Analyses—Limited proteolysis of intracellular HCs was carried out on Protein A precipitates under optimized time, temperature, and enzyme concentrations. Papain (Sigma) was added at 5 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml elastase (ICN). Metabolic Labeling and Steady-state Analyses—Limited proteolysis of intracellular HCs was carried out on Protein A precipitates under optimized time, temperature, and enzyme concentrations. Papain (Sigma) was added at 5 μg/ml of trypsin (Difco) or at 37 °C with 0.1 μg/ml elastase (ICN).

**Results**

The coordination between C1γ1 oxidative folding and its covalent assembly with CH1 (7) and the well-documented contribution of exposed thiolis to ER retention raised the possibility that the 3 C1γ1 cysteines are key determinants in these processes. This hypothesis was tested by Cys-to-Ser substitutions of relevant cysteines, singly, or in combinations. We disrupted formation of either intradomain or interchain disulfides and investigated in vivo the interrelations between these bonds. Based on conserved cysteine positions in multiple Ig constant domains (1, 33), we anticipated that in C1γ1 of mouse γ2b HC (designated γ), Cys-140 and Cys-195 formed the intradomain disulfide, whereas Cys-128 formed the interchain bond with LC (see Fig. 6, below). Each mutant was expressed with or without Al LC (designated λ) in non-lymphoid COS-7 cells, where its fate could be assayed in the absence of endogenous HC and LC.

We first validated COS-7 cells as a system to study the intracellular fate of γ, based on retention of wild-type HC (WT; Fig. 1A, lanes 1 and 13; Fig. 1B; for intracellular expression of the non-secreted WT, see Fig. 3A, lane 1) and efficient secretion of a C1γ1-deleted HC (ΔC1γ1; Fig. 1A, lanes 1 and 14; Fig. 1B) or upon replacement of C1γ1 by either C2γ1 or C3γ1 domains (Cγ1/2γ 2 or Cγ1/3γ2, respectively; Fig. 1A, lanes 3, 15, 4, and 16; Fig. 1B). These experiments confirm and extend previous reports, that, despite their similar fold, neither C1γ2 nor C1γ3 can confer retention even when grafted in place of C1γ1. Importantly, all secreted HC consisted mostly of γ2b, indicating that C1γ1 absence did not hamper HC homodimerization.

 secretion of WT was restored in the presence of λ LC (Fig. 1, B and C). Because λ was not directly precipitated by Protein A (Ref. 12 and Fig. 1D), its co-precipitation reflected covalent assembly into the secreted species γλ, γ3λ, and γ4λ (Fig. 1C, lanes 1, 13, and 25). In addition, λ escorted the γ2b intermediate hetero-covalently, as shown by co-precipitation of monomeric
FIG. 1. Secretion of the various HC mutants in the absence or presence of LC. COS-7 cells were transfected with an empty vector (mock) or with genomic constructs encoding the indicated HC mutants in the absence (-λ; A) or presence (+λ; C) of λ-encoding genomic construct. Cells were metabolically labeled for 4h with [35S]methionine, and γ was precipitated by Protein A-Sepharose (IP: Protein A) from the medium. Precipitated proteins were resolved by SDS-PAGE under non-reducing conditions (PAGE: NR) and electroblotted, and blots were autoradiographed ([35S]; lanes 1–12). Blots were then probed with anti-γ2b antibody (IB: anti-γ; lanes 13–24) and re-probed with anti-λ antibody (IB: anti-λ; lanes 25–36). The data are representative of seven independent experiments. B, for comparative secretion, the seven experiments were quantified by
λ in addition to a 125kDa species (Fig. 1C, lanes 1 and 25). This conclusion was verified by size-exclusion chromatography, where all secreted WT species were ~150-kDa tetramers, regardless of their covalent assembly status (Supplemental Fig. S1). HCs lacking CH1 (either deleted or replaced) did not interact with co-expressed λ (Fig. 1C, lanes 26–28) nor was their secretion improved by λ (Fig. 1, A–C), corroborating their LC-independent secretion. The association of BiP with intra-cellular WT, but not when CH1 was either deleted or replaced (Fig. 1E), confirmed that the CH1-conferred retention was due to BiP binding. BiP was displaced from WT by co-expression of λ, and to a lesser extent by ATP (Fig. 1E, lanes 1–3; Fig. 1F). The only partial dissociation of HC and BiP by ATP (Fig. 1F) likely reflects their rebinding as nucleotide is degraded (see Ref. 18).

Ablation of All Three CH1 Cysteines Negates CH1-conferred Retention—Substituting each of the 3 CH1 cysteines with serine, singly or in combinations, yielded surprising structure-function relationships with respect to folding, assembly, and secretion. When expressed without LC, these mutants could be divided into two categories. The majority resembled WT and were retained intracellularly, whereas a few mutants were secreted, resembling HCs that lacked CH1 altogether. The most remarkable example of a mutant that was secreted efficiently in the absence of LC was the triple mutant C128S/C140S/C195S (Fig. 1A, lanes 11 and 23; Fig. 1B). In this mutant all 3 CH1 cysteines, which normally stabilize the conserved Ig fold, were ablated. Moreover, C128S/C140S/C195S did not interact with λ LC, even non-covalently (Fig. 1C, lane 35), and neither its interaction with BiP (Fig. 1F) nor its secretion (lanes 11 and 23 in Fig. 1, A and C; Fig. 1B) were affected by LC.

Several lines of evidence confirmed the bona fide secretion of C128S/C140S/C195S. First, similar maturation of N-glycans, reflected by retarded mobility and resistance to endo H, was observed for WT secreted in the presence of LC (Fig. 2A, lanes 3, 4, 7, and 8) or for C128S/C140S/C195S secreted without LC (Fig. 2A, lanes 11, 12, 15, and 16). Interestingly, secreted C128S/C140S/C195S was resolved into γ and a unique higher assembly species designated γ<sub>+</sub> (see below), both resistant to endo H and sensitive to peptide: N-glycosidase F (Fig. 2A, lanes 11, 12, 15, and 16; arrows). Second, C128S/C140S/C195S acquired galactose residues, as detected by Ricinus communis agglutinin<sup>2</sup>, indicating that this mutant traversed the trans-Golgi. Third, secretion of both γ and γ<sub>+</sub> was completely blocked by brefeldin A (Fig. 2B). Although γ<sub>+</sub> was preferentially detected in the medium, its accumulation in brefeldin A-treated cells (Fig. 2B, lane 3) indicated that it assembled within cells and was immediately secreted.

Mutation of Cys-195 Hampers Interchain Disulfide Formation, Causes Misfolding, and Prevents Secretion—The secretion of C128S/C140S/C195S may appear as a hierarchical contribu-

densitometry, monitoring γ secreted in the absence (white bars) or presence (black bars) of LC. The calculated secretion is relative to 100% set for secretion of ΔCH1 and is presented as averages ± S.D. Note the comparable levels of intracellular γ (Figs. 1E, 2A, 3A, and 5B). D, COS-7 cells were transfected with an empty vector (−λ) or with an excess (30 μg of DNA) of genomic constructs encoding λ (λ). Cells were incubated for 4 h in fresh medium, and proteins were precipitated from identical samples of medium (m) and lysed cells (c) by either goat anti-λ antibody followed by Sepharose-conjugated mouse anti-goat antibody (anti-λ) or by Protein A-Sepharose (PA). Precipitated proteins were resolved by SDS-PAGE under non-reducing conditions (PAGE: NR) and electrophobotted, and blots were probed with an anti-λ antibody (IB: anti-λ). E, COS-7 cells transfected with an empty vector (mock) or with constructs expressing WT, ΔCH1, (C128), or (C130), with (+) or without (−) λ-conedating construct, were labeled for 4 h with <sup>35</sup>S]methionine, γ was precipitated by Protein A-Sepharose (IP: Protein A) from lysed cells, and BiP was co-precipitated. Precipitated proteins were resolved by SDS-PAGE under reducing (PAGE: R) and electrophobotted, and blots were autoradiographed (22). Where specified, ATP was added to lysates. F, for all γ mutants, bands corresponding to intra-cellular γ and co-precipitated BiP detected by autoradiography (see Fig. 1E for WT) were quantified by densitometry and BiP/γ ratio was calculated relative to the value 1 set for cells expressing the respective HC alone (see Fig. 1E, lane 1 for WT). BiP/γ ratio from four independent experiments is presented as averages ± S.D. for lysates incubated with ATP prior to precipitation (white bars) or for lysates from cells expressing γ+λ (black bars). γ, HC monomers; γ<sub>λ</sub>, homodimers; γ<sub>λλ</sub>, assembly species; λ, LC monomers precipitated by anti-λ or co-precipitated by protein A as non-covalently bound LC; λ<sub>γ</sub>, LC homodimers; BiP, co-precipitated BiP.

*Y. Elkabetz, Y. Argon, and S. Bar-Nun, our unpublished observation.*
terchain disulfide by substituting Cys-128. Without LC, C128S was secreted only marginally (5% of ΔC141 secretion; Fig. 1B). However, C128S was not nearly as misfolded as C195S, because it resembled WT (Fig. 3A). In the presence of LC, C128S dissociated from BiP (Fig. 1F) and was secreted efficiently (Figs. 1B, 1C (lanes 5, 17, and 29), and 3A). As expected, the major secreted covalent form of C128S was γ2, rather than γλ or γλλ (Figs. 1C (lanes 17 and 3A) and 3A). Because WT γ2 was hardly secreted (Fig. 3A, lane 4), it suggested that when a LC-interacting cysteine was absent, the secreted γ2 species of C128S was associated non-covalently with two λ LCs, as corroborated by the abundant free LC co-precipitated by Protein A (Fig. 3A, lane 16). Interestingly, a λ-containing species of ~47kDa, which was not formed when λ was expressed alone (Fig. 1D), was also co-precipitated from cells and media of C128S-expressing cells (Fig. 3A, lanes 15 and 16). This species is most likely disulfide-bonded λλ homodimers that are formed only when Cys-128 is absent, as discussed elsewhere.3 A minor, yet significant, fraction of C128S was surprisingly assembled into covalent γλλλ and γλλλ, as revealed by probing with anti-γ (Fig. 3A, lanes 7 and 8) and anti-λ (Fig. 3A, lanes 15 and 16) antibodies. Because LC was not disulfide-bonded to HCs that lacked C141 (see Fig. 1C), this result indicated that, when Cys-128 was absent, C141 cysteines that usually formed the intradomain disulfide could form opportunistic interchain disulfides with λ, precluding intradomain disulfide between Cys-140 and Cys-195. Indeed, consistent with altered oxidative folding (34), all species containing covalently linked C128S and λ (γ2λλ, γλλλ, and γλλλ) exhibited altered electrophoretic migration in non-reducing gels (Fig. 3A, lanes 4, 8, 12, and 16). Formation of opportunistic interchain disulfides is probably allowed by inefficient engagement of Cys-140 with Cys-195 when Cys-128 is absent, as shown for a small fraction of intracellular HC monomers. These assembly species, which have not yet covalently bound to either α or γ, could be resolved into oxidized and unoxidized forms (Fig. 3A, lanes 1–8, upper and lower panels). Whereas WT monomers were fully oxidized (lane 3), C128S monomers appeared to be mostly reduced (lane 7), yet only the oxidized monomers were secreted (lane 8). That the distorted and retarded oxidative folding was due to the mutant C128S HC and not to its opportunistic interchain disulfides is confirmed by the mutation of Cys-141, and the residue that interacts with HC (Fig. 3B). This mutation abolished not only the normal disulfide with WT, but also the opportunistic interchain disulfides with C128S. Nonetheless, WT HC monomers remained oxidized and C128S monomers remained reduced (Fig. 3B, lanes 1–12, upper and lower panels), indicating that only Cys-214 could engage a HC cysteine, either the native Cys-128 or an opportunistic partner (see Fig. 6).

Taken together, this detailed analysis of single, double, and triple cysteine substitutions shows that the effect on folding and secretion of mutating one cysteine is not equivalent to mutating the other, even when the effect on disulfide bond formation is the same. Hence, when either interchain or intradomain disulfide bond in C141 is disrupted, formation of the other disulfide bond is affected, and thus formation of these two disulfide bonds in C141 is interdependent. The C128S/C140S/C195S Heavy Chain Exhibits Unusual Folding and Assembly—The triple mutant C128S/C140S/C195S was secreted independently of LC and in addition ex-
hindered a unique assembly pattern. First, the secreted molecules were mostly typical 100-kDa γ2 homodimers, indicating that this Cys-less C_{H1} did not interfere with HC homodimerization. Yet, a significant fraction migrated as an unusual 200-kDa species (Fig. 1, lanes 11 and 23), which contained HC exclusively, as even in cells co-expressing C214S, they were reactive only with anti-γ but not with anti-λ antibodies (Fig. 1C, lanes 11–24). Excising both assembly species from non-reducing gels and individually subjecting them to reducing SDS-PAGE resolved the 100-kDa species into the expected 50-kDa γ2 monomers, whereas the 200-kDa species gave rise to a 100-kDa species (Fig. 4A). The latter probably represented atypical dimers (γ2*) held together via SDS-resistant interactions. Two such γ2* molecules appeared to undergo conventional homodimerization into 200-kDa γ2* tetramers (see Fig. 6).

The secretion of the Cys-less C_{H1} mutant suggested that its C_{H1} domain folded into a distinct structure that passed the scrutiny of the cellular quality control. To test this possibility, intracellular HCs of the secretion-competent C128S/C140S/C195S and secretion-incompetent WT were each subjected to limited proteolysis with elastase or trypsin. Partial elastase digestion yielded a 85-kDa intermediate only from C128S/C140S/C195S (Fig. 4B, lane 5, arrow). An additional 70-kDa elastase product was generated from both WT and C128S/C140S/C195S, but was more pronounced in C128S/C140S/C195S (lane 5) than in WT (lane 2). Limited proteolysis with trypsin also revealed differences between intracellular C128S/C140S/C195S and WT. Although two major fragments, ranging between 80 and 90 kDa, were generated from the mutant (Fig. 4C, lanes 8–12, arrows), only a single
major fragment was generated from WT (lanes 2–6). To address the conformation of the secreted C128S/C140S/C195S, it was compared with the secreted (CH3)2, because both HCs could be collected from the medium independently of LC expression. Papain digestion yielded a 50-kDa anti-/H9253-reactive product in both (CH3)2 and C128S/C140S/C195S, but only C128S/C140S/C195S gave rise to a predominant 70-kDa product (Fig. 4D, lanes 8–12, arrow). The altered proteolytic sensitivity demonstrated that the fold of intracellular C128S/C140S/C195S was distinct from that of intracellular WT, and the fold of secreted C128S/C140S/C195S was distinct from that of secreted (CH3)2. An alternative interpretation to the altered proteolytic sensitivity is that C128S/C140S/C195S could interact with a unique set of auxiliary proteins. Either way, the data points to an alternate conformation of the Cys-less CH1 mutant that is correlated with unusual assembly into /H92534* and is evidently compatible with secretion of HCs that are not assembled with LC.

**BiP Cycles from the Cys-less CH1 to Allow Secretion of C128S/C140S/C195S** —As established previously and corroborated in this study, C128S/C140S/C195S—As established previously and corroborated in this study, C128S/C140S/C195S could interact with a unique set of auxiliary proteins. Either way, the data points to an alternate conformation of the Cys-less CH1 mutant that is correlated with unusual assembly into /H92534* and is evidently compatible with secretion of HCs that are not assembled with LC.
in the two identified clusters of potential BiP binding sites (Fig. 5A), obtained by multiple alignment of \(\gamma_2b\) C\(_{\text{H}1}\) sequence (28) with likely BiP-binding heptapeptides (32). Therefore, we investigated whether and how BiP-mediated retention operated on the Cys-less C\(_{\text{H}1}\). For WT, the relatively constant levels of co-precipitated BiP during the chase, detected either radioactively or with an anti-BiP antibody (IB: anti-BiP; upper panel). Importantly, BiP levels did not diminish further even if \(\lambda\) was co-expressed in abundance. C, COS-7 cells were transfected with C128S/C140S/C195S construct, along with either wild-type or T19G mutant of HA-tagged BiP. \(\gamma\) was precipitated by Protein A-Sepharose (IP: Protein A) from medium or lysed cells, resolved by SDS-PAGE under reducing conditions (PAGE: R) and electrophblotted, and blots were autoradiographed (\(^{35}\text{S};\) middle and lower panels) and probed with an anti-\(\gamma\)-antibody (IB: anti-\(\gamma\)). The data are representative of five independent experiments. \(\gamma\), HC monomers; \(\gamma^*\), SDS-resistant dimers; \(\lambda\), co-precipitated LC; BiP, co-precipitated BiP.

**DISCUSSION**

The ER retention of unassembled HC is attributed to inherently retarded folding of C\(_{\text{H}1}\) and its irreversible binding to BiP (3). However, what sets C\(_{\text{H}1}\) apart from the structurally similar C\(_{\text{H}2}\) and C\(_{\text{H}3}\) is not fully understood. This work provides three important and surprising findings that help explain the unique nature of C\(_{\text{H}1}\). First, the retarded folding is due to delayed oxidation of the 3 C\(_{\text{H}1}\) cysteines, because formation of both intradomain and interchain disulfide bonds is interdependent. Second, formation of neither disulfide is obligatory for secretion. Third, the retention can be overcome if all 3 C\(_{\text{H}1}\) cysteines are substituted, resulting in efficient LC-independent secretion of a HC harboring a full-length C\(_{\text{H}1}\). These findings demonstrate a link between two well established quality control...
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mechanisms, thiol-mediated and BiP-mediated retention. Furthermore, they highlight the plasticity of the Ig fold.

Cysteine residues can affect Ig folding, assembly, and secretion in a number of ways. Often they participate in disulfide bonds, a characteristic of Ig superfamilies. Usually, disulfides serve to stabilize the fold rather than to catalyze its formation, and can be replaced if the loss of folding stability is at least partially compensated for by other stabilizing mutations (35, 36). In most Ig domains, a disulfide links two β sheets and is buried in the core of the domain, with conserved, hydrophobic side chains packed against it. If this intradomain disulfide fails to form, exposed thiols in this hydrophobic core adversely affect folding, leading to continued exposure of BiP binding peptides and consequent retention (18). This can clearly be the explanation for the compromised folding of all mutants lacking Cys-195, which cannot be rescued by LC expression. However, the non-reciprocal nature of the mutations described here indicates that Cys1 cysteines must also have other effects. Cysteine substitutions other than Cys-195, that disrupt the same intradomain disulfide (C140S; C128S when opportunistically engaged with λ, Fig. 6), can be rescued by LC, at least partially, as judged by BiP displacement and consequent secretion. Moreover, leaving a surface-exposed cysteine, such as Cys-128 reduced, or replacing it with serine, would not normally be expected to have a dramatic destabilizing effect. Nonetheless, C128S displays defects in oxidative folding that are manifested even when LC is present, contrary to expectation. It seems, therefore, that Cys-128 is essential for C1 domains lack Cys-195, as well as being required for linking C1 to LC. A third effect of cysteines may be via thiol-mediated retention (23, 25), where unpaired cysteines can interact with ER proteins (37–39). This mechanism may operate in WT retention, where cysteines and Cys-128 in particular are unpaired, or in some of the single mutants that form opportunistic disulfides and have promiscuously exposed thiols.

We propose that C1H1 is unique because its folding requires interdependent and concurrent formation of intradomain and interchain disulfides. This is in contrast to other Ig domains with multiple cysteines, such as C1H, where the intradomain disulfide forms with high fidelity, leaving unpaired the third cysteine responsible for the interchain disulfide. The interdomain disulfide fails to form due to Cys-195 substitution. Cys-128 fails to form interchain disulfide with LC when intradomain disulfide cannot form due to Cys-195 substitution. Reciprocally, in HC whose LC-interacting Cys-128 is absent, intradomain cysteines oxidize inefficiently, even allowing opportunistic engagement of Cys-140 or Cys-195 with LC. Importantly, this inefficient oxidation is inherent to HC and does not occur in LC. Therefore, one possible interpretation is that as long as a fourth cysteine provided by LC is missing, the 3 C1H1 cysteines cannot adopt the proper configuration to form intradomain disulfide and HC cannot dissociate from BiP. However, the finding that LC that lacks this cysteine can still promote folding and secretion of WT HC (as well as of some mutants) demonstrates that the role of C1H1 cysteines in Ig folding, assembly, and secretion is not simply to engage in disulfide bridges, but rather to affect C1H1 folding, including mediating possible interactions with thiol-sensing ER proteins. When LC is provided, a cascade of interdependent folding and assembly events juxtaposes the two pairs of cysteines. Once the positioning of interacting surfaces is accomplished, disulfides form, but only to stabilize the Ig fold. BiP displacement and subsequent secretion are achieved concurrently with proper C1H1 folding and this can be accomplished in the presence of C1H as a folding template.

A mechanistic link between BiP-mediated and thiol-mediated retention of HC is quite probable. The capacity of BiP to
bind HC may well be related to HC binding through unoxidized thiol to thiol oxidoreductases, such as PDI (40, 41), ERP57 (37), or ERP44 (38, 39). Cooperation between BiP and PDI in Ig folding was shown in vitro, suggesting that BiP prevents unfolded Ig chains from collapsing and presents them to PDI (42). A secreted version of PDI (PDI-KDEL) implicated PDI in re-folded Ig chains from collapsing and presents them to PDI (42). The cycling of reversible binding characteristic of WT CH1 (3). The cycling of reversible mode of association with BiP, rather than the irreversible (32). It is likely that association with BiP is not determined by BiP to Cys-less CH1 presumably reflects rapid sequestration of BiP to Cys-less CH1 (3). The cycling of Cys-less CH1 on and off BiP allows this mutant to acquire a secretion-competent fold, which we show to be distinct from that of WT HC. These observations suggest that, although the native structure of CH1, as defined by crystallography, is almost invariant among HC isotypes, there is more than one conformation that the cellular quality control tolerates and considers as secretion-competent.

A correlation between BiP displacement and Ig assembly has been shown for Cys-less CH1 (3). On the other hand, BiP displacement from Cys-less CH1 was correlated with hetero-dimerization with LC (7). Taking into account our results and geometric constrains at the hinge region, it is possible that Cys-less CH1 has acquired the capacity to homodimerize via an atypical SDS-resistant interaction and, together with the usual homodimerization of two C12SS/C140S/C195SS via their hinge regions, a unique tetramer is formed (see Fig. 6). Moreover, the attenuated formation of these tetramers in the presence of the T19G BiP mutant suggests that BiP displacement is required even for this atypical Cys-less CH1 homodimerization. It remains to be established whether reversible BiP binding to Cys-less CH1 reflects rapid folding and secretion that may or may not result in unusual assembly, or whether the latter is the cause of BiP displacement.

Although it is surprising that ablation of conserved disulfides in the case of Cys-less CH1 does not result in a denatured protein that is retained intracellularly, there are precedents for this phenomenon. Na,K-ATPase appears to fold normally and carries a conserved cysteine, which is otherwise easily denatured and susceptible to proteolysis (48, 49). Interestingly, the criteria for correct folding, as manifested by activity, are often less stringent than those recognized by quality control mechanisms that prevent intracellular transport and secretion (48–50). Here we provide evidence that, although the conserved Cys-less CH1 cysteines generate important cues for quality control, in the form of the transiently unstable structure of the normal domain, the quality control mechanism is “doped” when Cys-less CH1 cysteines are substituted. Given the frequent use of Ig domain folds in the human proteome, our model for the relationship between oxidative folding and cellular quality control may be relevant to many receptors and secreted proteins.

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