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Alternative pathways of disulfide bond formation yield secretion-competent, stable and functional immunoglobulins

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Abstract

Disulfide bonds within and between proteins are responsible for stabilizing folding and covalent assembly. They are thought to form by an obligatory pathway that leads to a single native structure compatible with secretion. We have previously demonstrated that the intradomain disulfide in the C_H1 domain of the Ig γ 2b heavy chains was dispensable for secretion (Elkabetz et al., 2005). Here we show that the heavy chain-light chain interchain disulfide is also dispensable. γ 2b with mutated Cys128, which normally disulfide bonds with the light chain, still assembled with λ I light chain into a secretion-competent, tetrameric IgG2b. This assembly comprised of a covalent homo-dimer of mutant heavy chains (C128S₂) accompanied non-covalently by a covalent homo-dimer of light chains (λ ₂). The λ ₂ homo-dimer formed only upon association with C128S₂, through disulfide bonding of the two "orphan" heavy chain-interacting Cys214 in λ I. The unique Ig tetramer was secreted efficiently as a functional antibody whose antigen binding capacity resembled that of normal IgG2b. Therefore, disulfide bonding of Ig manifests considerable plasticity and can generate more than one functional structure that is considered native by the cellular quality control system.

Keywords

IgG; assembly; disulfide bonds; secretion

1. Introduction

Disulfide bonds within proteins are responsible for stabilizing folded structure and disulfide bonds between polypeptides are responsible for covalent assembly. Immunoglobulins (Igs) serve as an excellent model to study disulfide bond formation because, based on a large database of Ig sequences and structures, it is evident that these oligomeric proteins contain highly conserved disulfide bonds within their heavy chains (HC) and light chains (LC) and between HC and LC. A single disulfide bond within each variable or constant domain of HC

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or LC stabilizes the typical β sandwich fold of these domains. In addition, two types of interchain disulfide bonds stabilize the tetrameric structure of functional Igs; one that links two HC to each other and another that bridges each HC to LC (Edelman and Gall 1969). Assembly of LCs and HCs is initiated by non-covalent interactions between their respective V_L and V_H variable regions (Percy et al. 1975; Secher et al. 1977; Hamel et al. 1984; Chothia et al. 1985; Hamel et al. 1987). This is followed by hydrophobic interaction between C_L and C_{H1} , the constant domains of LC and HC, respectively (Janeway 1994), which is eventually stabilized by a covalent interchain disulfide between these two constant domains (Hamel et al. 1987; Hendershot et al. 1987; Leitzgen et al. 1997). The C_{H1} - C_L interchain disulfide forms between Cys214 of λ C_L (or Cys215 of κ C_L) and the most N-terminal among three cysteines in C_{H1} . In mouse IgG2b, the interchain disulfide with LC is formed by Cys128, which is located in the loop connecting the first and second β -strands of C_{H1} , as suggested by both conserved cysteine positions (Amzel and Poljak 1979) and the roles assigned to them in the C_{H1} of the mouse γ 2b HC (hereafter referred to as γ) (Rose et al. 1993). The interaction of this loop with C_L appears to play a role in the assembly of HC with LC. Known exceptions are IgG1 and human IgA2(n), in which the LC-interacting cysteine is the most C-terminal, hinge domain-proximal cysteine in C_{H1} or the hinge-proximal cysteine in the C_{H2} domain (Chintalacharuvu et al. 2002), respectively.

The usual assembly pathway of IgG2b in Ig producing cells is $HC-LC \rightarrow HC_2LC \rightarrow HC_2LC_2$ (Scharff et al. 1970). An alternative IgG2b assembly pathway, which predominates in COS-7 cells, is $HC \rightarrow HC_2 \rightarrow HC_2LC \rightarrow HC_2LC_2$ (Elkabetz et al. 2005). This pathway is also followed during assembly of other Ig isotypes (Scharff et al. 1970). These alternative pathways show that formation of the interchain disulfide bond is not an autonomous feature of the subunits, but is influenced by the cellular environment, a conclusion that was also reached recently by (Chintalacharuvu et al. 2007). Regardless of the assembly pattern, most naturally occurring Igs are secreted either as HC_2LC_2 covalent tetramers or as HC_2LC accompanied by one non-covalent LC, but not as HC_2 accompanied non-covalently by two LCs. This phenomenon may reflect rapid covalent assembly of HC_2 into HC_2LC and negative cooperativity in the covalent assembly of the second LC with the HC_2LC intermediate (Kazin and Beychok 1978). It is also plausible that a disulfide bond between HC_2 and at least one of the two accompanying LCs is required to pass the scrutiny of the quality control mechanisms operating in the endoplasmic reticulum (ER). However, while the covalent assembly via disulfides is the norm, the existence of exceptions like human IgA2m(1), which is secreted largely without a covalent bond between HC and LC (Chintalacharuvu et al. 2002), shows that alternative structures are possible.

In addition to the vital contribution of intradomain and interchain disulfide bonds to the stability and functional properties of antibodies, they play a key role in quality control mechanisms that operate during Ig secretion. Formation of disulfide bonds in Ig structure occurs in the lumen of the ER, and as with other secreted proteins, disulfide bonds are considered to be formed by an obligatory pathway that involves a large array of ER thiol oxidoreductases. This obligatory pathway leads to a single unique native structure, which is stable and functional and is the only structure assumed to be compatible with secretion. This quality control phenomenon is attributed to the C_{H1} domain, which is unable to complete its folding and dissociate from the ER chaperone BiP (Vanhove et al. 2001). Only in the presence of LC does C_{H1} fold and dissociates from BiP, completing the formation of an immunoglobulin that is then secretion-competent (Haas and Wabl 1983; Bole et al. 1986; Hendershot 1990; Leitzgen et al. 1997; Lee et al. 1999; Kaloff and Haas 1995). We have previously shown that delayed C_{H1} folding can be explained by disulfide bonds formation. Cysteine residues that form both interchain and intradomain disulfides in the mouse IgG2b were mutated: Cys128, Cys140 and Cys195 in the C_{H1} domain of the γ HC and Cys214 in the C_L domain of the λ LC (designated λ). Studying assembly and secretion of these

mutant chains, we showed that neither the LC-HC interchain nor the C_H1 intradomain disulfides formed independently and instead, Cys128-Cys214 and Cys140-Cys195 oxidized coordinately, so that the completion of HC folding was coupled to its assembly with LC (Elkabetz et al. 2005). Moreover, in this study we have demonstrated that the intradomain disulfide in the C_H1 domain of the γ HC was dispensable for secretion (Elkabetz et al. 2005).

In the current study we investigated whether the interchain disulfide bond between Cys128 of γ HC and Cys214 of λ LC was essential to attain a functional three-dimensional IgG2b that is compatible with secretion. By substituting Cys128 in γ and/or Cys214 in λ , the two cysteines that form the HC-LC disulfide in IgG2b, we demonstrated that any disulfide bond between γ and λ was dispensable for IgG2b secretion. Moreover, in the absence of the LC-interacting Cys128, alternative pathways of disulfide bond formation yielded secretion-competent, stable and functional IgG2b. The predominant structure was a non-covalent hetero-dimer formed between two covalent homo-dimers, LC₂ and HC₂. The data presented here and in (Elkabetz et al. 2005) provide solid evidence for the remarkable plasticity of Ig molecules, which can follow alternative patterns of disulfide bonds formation in both folding and assembly, yet generating secretion-competent antigen-binding antibodies.

2. Materials and methods

2.1. DNA construction

All mutant γ constructs were based on pJDEV_{NP}C γ _{2b}, encoding full-length murine γ HC genomic DNA carrying the B1.8 V_H (Reth et al. 1978) and a known constant region (Yamawaki-Kataoka et al. 1980) (accession No. j00461). The C_H1 exon, flanked by intronic sequences, was excised from pJDEV_{NP}C γ _{2b} with *EcoRI* and *PvuII*. A *NoI*-containing linker was ligated to the vector at the *EcoRI* and *PvuII* sites, generating the C_H1-lacking plasmid pJDEV_{NP}C γ _{2b} Δ C_H1. Exons C_H1, C_H2 or C_H3 were PCR-amplified, using primers (Table 1) and pJDEV_{NP}C γ _{2b} as a template, and were sub-cloned into pBlueScript (pBS; Stratagene) at the *EcoRI* and *XbaI* sites, to generate pBS-C_H1, pBS-C_H2 and pBS-C_H3, respectively. Site-directed mutageneses of C_H1 were performed using pBS-C_H1 as a template and either forward mutagenic primer or the complementary (reverse) mutagenic primer (Table 1). The mutated C_H1 was cut by *EcoRI* and *XbaI* and inserted into pBS. All DNA constructs were sequenced. Wild-type and mutated C_H1, as well as C_H2 and C_H3, were excised from the respective pBS constructs and re-introduced into pJDEV_{NP}C γ _{2b} Δ C_H1 with *EcoRI* and *NoI*. The pJDE λ I plasmid encodes genomic full-length murine λ I LC (Dul and Argon 1990). The λ I A60VF62S mutant (replacements of Ala60 by Val and of Phe62 by Ser) was previously described and is designated here as F62S (Dul and Argon 1990). The cDNAs encoding wild-type, C214A and C214S λ I were generated by amplification and mutagenesis performed by PCR (see primers in Table 1), using as a template pTM1- λ I cDNA (Elkabetz et al. 2003), and cloned into pCDNA3 (InvitroGen) at *EcoRI* and *NoI* sites.

2.2. Plasmids and transfection

COS-7 cells grown in DMEM supplemented with 10% fetal calf serum, penicillin and streptomycin at 37°C in humidified 5% CO₂ atmosphere were transfected at ~50% confluence by the calcium-phosphate method (Chen and Okayama 1988). Empty vectors (mock) or various γ constructs (5 μ g) were introduced with or without equimolar amounts of wild-type λ I or the F62S, C214A or C214S λ I mutants. Cells were analyzed 42–48h post-transfection.

2.3. Steady-state analyses

Secretion was monitored by adding 1.5ml fresh DMEM and media were collected after 3–5h. Collected media were centrifuged (800×g, 8 min, 4°C) and cells were washed twice with ice-cold PBS and lysed (300μl/2×10⁶ cells) in ice-cold NET buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM EDTA, 0.5% NP40) supplemented with 2mM phenylmethanesulfonyl fluoride (Sigma) and 100 U/ml aprotinin (Roche). For samples analyzed by non-reducing SDS-PAGE, the PBS and NET buffers included freshly added N-ethylmaleimide (20mM; Sigma). Following 30min incubation at 4°C, cell lysates were centrifuged (20,000×g, 20min, 4°C) and supernatants were collected.

2.4. Immunoprecipitation, immunoabsorption, electrophoresis and immuno-blotting

Quantitative isolation of γ2b and its associated proteins from cell lysates or media was by immunoabsorption with protein A-Sepharose (Repligen). λI and its associated proteins were isolated by immunoprecipitation with rabbit anti-λI (Southern Biotech) followed by protein A-Sepharose. Samples were rotated for 4h at 4°C, washed three times with borate wash buffer (Kaloff and Haas 1995) and once with PBS. Proteins were resolved by SDS-PAGE under reducing or non-reducing conditions. For immuno-blotting, resolved proteins were electroblotted onto nitrocellulose, membranes were blocked with 10% low-fat milk in PBS and probed with horseradish peroxidase (HRP)-conjugated anti-mouse γ2b (SouthernBiotech) or HRP-conjugated anti-mouse λI (SouthernBiotech), and the HRP was visualized by enhanced chemiluminescence (ECL).

2.5. Size fractionation

Equal amounts of transfected cells were incubated 43 h post transfection in fresh serum-free medium that was collected after 5 h and size-fractionated on HR-200 Sephacryl column (Amersham Pharmacia Biotech), using FPLC (BioRad). The column was calibrated with aldolase (232kDa), alcohol dehydrogenase (150kDa) and bovine serum albumin (66kDa). Fractions (40 fractions of 3ml) were collected, aliquots were analyzed by dot-blots on nitrocellulose membrane blocked with 10% low-fat milk in PBS and probed with the HRP-conjugated anti-γ2b and anti-λI antibodies. Positive fractions were immunoabsorbed to protein A-Sepharose, analyzed by SDS-PAGE and immunoblotted with the HRP-conjugated anti-γ2b and anti-λI antibodies.

2.6. Binding of the antigen NP

For isolation via the hapten-binding site, NP bound to BSA (a generous gift from N. Hollender, Tel Aviv University) was conjugated to activated Sepharose. Samples were rotated for 4h at 4°C, washed three times with borate wash buffer (Kaloff and Haas 1995) and then once in PBS. For ELISA measurements, 96-well ImmunoTM-plates (Nunc) were coated over-night at 0°C with NP-BSA (10μg/ml; 50μl/well), blocked for 1h at 37°C with 1% BSA/0.02% NaN₃ in PBS and washed 3 times with Buffer B (0.5M NaCl/0.1% Tween20 in PBS). Media samples of 30, 60 or 90μl collected from cells expressing λ with WT or C128S were added to wells, incubated for 2h, washed 3 times with Buffer B, incubated for 1hr with HRP-conjugated anti-γ2b antibody (SouthernBiotech), washed again 3 times with buffer B and incubated for 15min with freshly made 0.7mg/ml o-phenylenediamine in 33mM citric acid/66mM Na₂HPO₄ (pH 5.0)/0.5μl 30% H₂O₂. Reaction was terminated by addition of 50μl/well of 1M H₂SO₄ and absorption was measured in an ELISA reader at 490 nm.

3. Results

3.1. An alternative Ig-like structure that is functional and secreted

In the course of studying the folding of the C_H1 domain of the γ_2 b HC and its effects on Ig assembly, we have previously noticed that upon mutating Cys128, the residue in C_H1 that forms the disulfide bond between the C_H1 and C_L domains, two alternative Ig assemblies were formed ((Elkabetz et al. 2005) and Fig. 1A). One assembly had the electrophoretic migration of the usual $\gamma_2\lambda_2$ covalent tetrameric IgG and its intermediate $\gamma_2\lambda$. Since the normal interchain disulfide could not form in the absence of Cys128, we attributed this assembly to non-conventional disulfide bonds (discussed in (Elkabetz et al. 2005)). In addition, a second assembly was formed by the C128S HC: a prominent secreted species was a γ_2 homo-dimer in non-covalent association with a unique ~47 kDa λ -containing species (Fig. 1A). This 47 kDa species represented a disulfide-bonded λ_2 homo-dimer, as shown by its estimated molecular weight, sensitivity to reduction by dithiothreitol and reactivity with anti- λ antibodies (Fig. 1B). It was formed only in cells expressing C128S and was efficiently secreted as a stable complex with the C128S₂ homo-dimers, based on its precipitation by protein A and on its distribution between the medium and the cell lysate (Fig. 1A).

The secretion of this unusual Ig-like assembly suggested that it passed the quality control scrutiny of the cell. Even more importantly – this assembly was functional, because when Sepharose conjugated to NP, the specific antigen of the IgG antibody studied here, was used as a capture reagent, the entire population recovered from the media by protein A-Sepharose could also bind NP, regardless of whether these molecules were composed of λ with WT HC or with C128S HC (Fig. 1A). As expected, when either WT or C128S HCs were expressed alone, neither was secreted and intracellular HCs could only be captured by protein A-Sepharose, but not NP-Sepharose (Reth et al, 1978 and data not shown). As shown in Fig. 3C, below, when λ LC was expressed alone, it was secreted, but was neither captured by protein A-Sepharose nor by NP-Sepharose and was precipitated only by an anti- λ antibody. The similar capacity of the wild-type and the unusually assembled C128S-containing antibodies to bind the nominal antigen NP was also demonstrated by ELISA (Fig. 1C), where similar levels of NP-bound secreted proteins were captured. This result underscores the biological activity of the C128S alternative Ig assemblies.

3.2. Covalent λ_2 homo-dimers assembled non-covalently with C128S₂ homo-dimers to form a tetrameric complex

Size-exclusion chromatography of the secreted assembly species demonstrated that both WT (Fig. 2, A and B) and C128S (Fig. 2, C and D) were secreted to a similar extent when complexed with wild-type λ . More importantly, WT and C128S formed comparable complexes of ~150kDa (Fig. 2, E and F, circles and squares, respectively) that were immunoadsorbed to protein A and displayed overlapping chromatographic patterns of γ (Fig. 2, B, D and F) and λ (Fig. 2, A, C and E). Hence, these 150kDa complexes contained γ associated with λ forming tetrameric structures with IgG stoichiometry of 2HC+2LC. Nonetheless, the tetrameric structures of WT and C128S were very different (Fig. 2, G and H). When distribution was observed with anti- γ (Fig. 2H), approximately 60%, of WT γ was in covalent $\gamma_2\lambda_2$ tetramers and almost 40% were in the second common assembly species of $\gamma_2\lambda$. The same distribution was observed with anti- λ (Fig. 2G), while 40% of λ distributed equally between $\gamma_2\lambda$ and λ . γ_2 comprised less than 2% of the secreted material, as expected from the intracellular retention of unassembled HC, and no λ_2 was detected, even though LC dimers are normally secreted (Knittler and Haas 1992; Dul et al. 1996). We conclude that, as shown before (Scharff et al. 1970), the final steps in WT γ assembly involve the step-wise disulfide bonding with the λ chains. In contrast, a major assembly

form of the C128S HC was a non-covalent heterodimer of two disulfide-linked homo-dimers, γ_2 and λ_2 . Approximately 50% of the species detected by anti- γ were γ_2 (Fig. 2, D and H, black bars, *) and ~50% of the species detected by anti- λ were λ_2 (Fig. 2, C and G, black bars, **), with very little $\gamma_2\lambda$. Approximately 40% of C128S assembled into covalent $\gamma_2\lambda_2$ even though they lacked the usual HC-LC disulfide bond (Fig. 2, G and H, white bars). These non-conventional HC-LC disulfide bonded assemblies were analyzed in (Elkabetz et al. 2005)). We conclude that secretion-competent Ig is composed of two γ HCs and two λ LCs, regardless of the number or configuration of disulfides between LCs and HCs.

3.3 The molecular requirements for the formation of the covalent λ_2 homo-dimer

The unusual feature of the alternative assembly pathway is the formation of a covalent λ_2 homo-dimer. We therefore investigate the molecular requirements for the formation of the covalent λ_2 homo-dimer. Cys214 of λ is known to be the residue that forms the interchain disulfide bond with γ HC. We asked if Cys214 was also responsible for the alternative disulfide bonding of two LCs into λ_2 . When Cys214 was mutated to serine or alanine, neither C214S nor C214A LC formed λ_2 homo-dimers when co-expressed with the C128S HC (Fig. 3A). Moreover, during normal Ig assembly, an initial non-covalent V_L - V_H interaction is a prerequisite for the formation of the HC-LC interchain disulfide bond (Percy et al. 1975; Secher et al. 1977; Hamel et al. 1984; Chothia et al. 1985; Hamel et al. 1987). To determine whether a similar V_L - V_H interaction was required to initiate the formation of λ_2 in the presence of C128S, we used the F62S mutant of λ . In this LC mutant, oxidation of the V_L domain was inefficient (Fig. 3B–C), hindering the V_L - V_H interaction, without affecting the conformation of the C_L domain (Dul and Argon 1990). When F62S λ LC was co-expressed with WT HC, it was capable of assembly into $\gamma_2\lambda$ and $\gamma_2\lambda_2$ species and mostly $\gamma_2\lambda_2$ was secreted, albeit less efficiently than the corresponding WT LC (Fig. 3B). However, when paired with C128S HC, the F62S LC did not form the λ_2 homo-dimers (Fig. 3B). These results indicate that the formation of λ_2 homo-dimers requires the HC-interacting Cys214, the presence of an HC that lacks its LC-interacting cysteine and the V_L - V_H interaction that initiates normal Ig assembly.

In this regard, contrary to the reported capability of λ to homo-dimerize in the absence of any HC (Knittler and Haas 1992; Dul et al. 1996), HC-independent, spontaneous homo-dimerization of LC was negligible in our experimental system, even upon deliberate over-expression of λ (Fig. 3C; see also (Dul et al. 1996)). As expected, such spontaneous λ_2 homo-dimers were precipitated by the anti- λ antibody but not by protein A (Fig. 3C).

We next asked whether the formation of covalent λ_2 homo-dimers specifically depended on the presence of a C_H1 domain. As shown in Fig. 4A, disulfide bonded λ_2 dimers did not form when C_H1 was deleted or replaced by an additional C_H2 or C_H3 domain. They only formed in the presence of C128S harboring a mutated C_H1 domain. We therefore conclude that the formation of the alternative interchain disulfide between Cys214 of two λ LC, to generate the λ_2 covalent homo-dimers, required as a template a C_H1 -containing γ that lacks Cys128.

3.4. Hierarchy in disulfide bonds formation

The tendency of two λ HCs to engage in the alternative disulfide bond formation was estimated from the calculated $\lambda_2:\lambda$ ratio in samples from cells expressing λ alone, or in a combination with either WT or C128S. Total λ and λ_2 , regardless of association with HC, was recovered by direct immunoprecipitation with the anti- λ antibody, whereas λ and λ_2 associated non-covalently with HC were co-precipitated by protein A-Sepharose. As shown in Fig. 4B, when directly immunoprecipitated from cells expressing λ and WT, only ~10%

of λ formed λ_2 (lane 3; $\lambda_2:\lambda = 0.11$), resembling cells expressing λ alone (Fig. 3C), and no λ_2 was co-precipitated protein A-Sepharose. On the other hand, in cells expressing λ and C128S, ~50% of λ formed λ_2 and more importantly, similar amounts were co-precipitated as HC-associated λ_2 (Fig. 4B, lane 5; $\lambda_2:\lambda = 0.49$) or directly immunoprecipitated by the anti- λ antibody (Fig. 4B, lane 7; $\lambda_2:\lambda = 0.53$). These data showed that the alternative disulfide bond formation of λ LC with another λ to generate the λ_2 homo-dimers took place only when λ LCs associated non-covalently with the C128S₂ HC dimers and that this non-covalent interaction was stable enough to endure protein A co-precipitation.

The ability of Cys214 to form an interchain disulfide bond with either HC or LC implied a hierarchy in disulfide bonds formation. Evidence for such hierarchy was obtained by expressing λ with a combination of WT and C128S HCs. When given such a “choice”, LC homo-dimerization diminished in the presence of WT HC and assembly into conventional $\gamma_2\lambda$ and $\gamma_2\lambda_2$ was enhanced (Fig. 4C). The decreased λ_2 did not reflect limiting amounts of λ , but rather a limiting amount of C128S, which was further competed by the WT HC (Fig. 4C). This result indicates that the preferred interaction is Cys214 of LC with Cys128 of HC. Only when Cys128 is unavailable, Cys214 interacts with a neighboring Cys214 to form LC homo-dimers.

4. Discussion

Assembly of HC and LC has long been known to be essential for the B-cell receptor activity and is important in two respects: it creates a functional antigen-binding pocket and it enables the assembled B-cell receptor, or its secreted version, to exit the ER and reach the surface of B-cells or be secreted from plasma cells, respectively. Here we provide evidence that IgG2b with conventional HC₂LC₂ stoichiometry can actually assemble in a non-conventional manner and yet exhibit properties of a functional antibody. Moreover, this unique IgG2b structure, which comprises the usual disulfide-bonded HC₂ homo-dimer associated non-covalently with the unusual disulfide-bonded LC₂ homo-dimer, can pass the scrutiny of the quality control system and be efficiently secreted. Our study argues for a significant plasticity in the quality control mechanisms that govern antibody production and secretion, exhibiting hierarchic pair wise disulfide bonding. Although the λ studied here is inherently inefficient in spontaneous homo-dimerization, (Dul et al. 1996), λ_2 seems as the major assembly species in the presence of HC that must contain C_H1 that lacks the LC-interacting cysteine. LC dimerization does not occur if C_H1 is either deleted or replaced and when Cys128 is present, the HC-LC interchain disulfide bond between Cys128 and Cys214 is the preferred, normal mode of assembly. Only when Cys128 is absent, an alternative mode of disulfide bond formation generating LC₂ homo-dimers is sufficient to stabilize a novel structure.

It is currently accepted that HC-LC assembly begins by non-covalent interactions between the hydrophobic surfaces of V_H and V_L domains (Percy et al. 1975; Secher et al. 1977; Chothia et al. 1985; Hamel et al. 1984; Hamel et al. 1987), which is subsequently stabilized by the formation of an interchain disulfide bond between C_H1 and C_L domains. The non-covalent interaction between the HC and LC variable domains also seems to be an early step in the formation of the alternative tetrameric Ig described here. Interchain disulfide bond cannot be formed between Cys214 of two LC when they harbor the F62S mutation in V_L. This mutation does not preclude the normal interchain disulfide bonding of LC with Cys128-containing HC and has only a local effect on V_L conformation (Dul and Argon 1990). When the λ_2 homo-dimers do not form, the LC harboring the F62S mutation does not assemble with the C128S homo-dimers. Thus, like the normal pathway, the alternative Ig assembly pathway requires a series of interactions between HC and LC domains that are

affected by the folding of these domains, which, in turn, affects the completion of the folding of the individual chains.

Our results provide a novel insight into the hierarchy of disulfide bonds formation between C_L and C_{H1} . Prior to engagement with LC, all three C_{H1} cysteines are unoxidized (Kaloff and Haas 1995). Upon initial encounter of LC with C_{H1} , the preferred disulfide bonding of Cys214 of λ LC is with Cys128 of C_{H1} . Only when Cys128 is absent does Cys214 interact with other cysteines. The next preferred cysteine is Cys214 on the neighboring LC. This allows the formation and secretion of an unusual, yet stable and functional form of antibody. For this disulfide to form, the carboxy-termini of the two LCs have to be sufficiently proximal, which may occur if the assembling Ig chains are in a T-like configuration, rather than the usual Y-configuration (Harris et al. 1998), at the price of restricted flexibility of the Ig tetramer. A second, less preferred option for the orphan Cys214 is to bond with other cysteines in the γ HC, probably with Cys140 or Cys195 that normally make up the intradomain disulfide of C_{H1} (Elkabetz et al. 2005). The apparent hierarchy in the options of cysteines to form disulfide bonds is likely controlled by conformational and spatial constraints, favoring folding and assembly that juxtapose Cys214 to Cys128 and the C_{H1} intradomain cysteines to one another. It appears that the retarded folding built into the C_{H1} domain (Kaloff and Haas 1995) allows a folding intermediate to position Cys128 so that it can preferentially form a disulfide bond with Cys214. Evidently, when an intradomain disulfide bond is oxidized rapidly, as happens when C_{H2} or C_{H3} grafted in place of C_{H1} , the disulfides are not reshuffled when presented with the unoxidized Cys214. Only a mutated C_{H1} domain lacking Cys128 serves as a proper template for two Cys214 to form the alternative interchain disulfide that generates the LC_2 homo-dimer.

An important feature of the unique tetrameric IgG structure described here is its ability to be secreted. This shows that interchain disulfide bonding of γ with either of the two λ is dispensable to pass the scrutiny of the cellular quality control system. We have previously shown the secretion of wild-type γ as disulfide bonded HC_2 assembled non-covalently with two C214S(A) LCs (Elkabetz et al. 2005). This species is similar to the HC_2 accompanied by two non-covalently associated LCs, which had previously been shown to be the predominantly secreted structure of naturally occurring human IgA2 isoforms (Chintalacharuvu et al. 2002). Our observations extend the findings of Chintalacharuvu et al. (2002) that homo-dimers of κ LC were associated with an $\alpha(n)$ HC upon mutating its Cys241, one of the two hinge-proximal cysteines in $C_{\alpha 2}$ that contributed to the disulfide bonding of $\alpha 2$ and κ (Chintalacharuvu et al. 2002). Our study shows that mutating the residue in a γ HC that actually forms the disulfide bond with LC has a similar effect and that λ LC can form homo-dimers similar to κ LC. Taken together, the two studies show that alternative pathways of disulfide bonding that generate functional antibodies appear to be a general property of Igs, reflecting their plasticity. Clearly, assembly of both IgG and IgA via alternative pathways of disulfide bonding leads to distinct Ig molecules, which are stable and functional and exhibit similar antigen-binding capacity, but are structured by completely different patterns of disulfide bonds. Presumably, the non-covalent interaction with the mutant HC positions each LC so that two Cys214 residues are close enough to form the alternative disulfide. Finally, the formation of alternative Ig-like structures that are still functional and bind their nominal antigens is non-trivial. In the case of IgG2b studied here, the rotation of HC and LC involved in their templated assembly into a dimer of homo-dimers could have easily inhibited the formation of the antigen-binding pockets, which in the case of the NP response is known to require both V_H and V_L (Reth et al. 1978).

This study was conducted in a surrogate non-lymphoid cell line, because an efficient transient transfection system that allows phenotypic observation of multiple combinatorial outcomes was needed. Although it is conceivable that different covalent assemblies will

form in different cellular environments, there is compelling evidence that our finding represent a phenomenon general to all eukaryotic cells. The use of COS cells as hosts for antibody assembly and secretion has been amply validated over the years, confirming that Ig molecules ectopically expressed in COS cells behave similarly to their native counterparts in B-cells. This applies to wild type Ig assembly pathways, phenotypes of Ig mutants, and the mechanism by which Ig molecules are handled by ER quality control. First, both of the Ig assembly pathways that are found in B-cells (Laskov and Scharff 1970) are also found in COS-7 cells, with the predominant IgG pathway being $HC \rightarrow HC_2 \rightarrow HC_2LC \rightarrow HC_2LC_2$ (Elkabetz et al. 2005). Second, the phenotype of all structural Ig mutants that affect secretion in B lymphoid cells is recapitulated in COS cells: in both cell systems free HCs are retained in the absence of LC (Milstein et al. 1978; Vanhove et al. 2001). HCs of any isotype lacking C_{H1} are rapidly secreted regardless of the presence of LC (Elkabetz et al. 2005), and secretion-incompetent LC mutants inhibit secretion of assembled IgG and IgM (Wu et al. 1983; Dul and Argon 1990). Third, the association of both LC and HC with chaperones like BiP is similar in lymphoid cells and in COS cells, as is the displacement of BiP from the HC by the LC (Vanhove et al. 2001; Elkabetz et al. 2005). Finally, as this study shows, even the existence of an alternative disulfide assembly is similar, as Chintalacharuvu et al. described an alternatively configured IgA in myeloma cells (Chintalacharuvu et al. 2002). A follow-up study showed that while myeloma cells secreted predominantly HC_2LC_2 species of IgA2m(2), Chinese hamster ovary (CHO) cells secreted in addition the tetrameric $HC_2LC + LC$ and minute amounts of HC_2 (Chintalacharuvu et al. 2007). However, this phenomenon appeared to be restricted to the IgA2m(2) allotype, because IgA1 and IgA2m(1) produced in CHO cells resembled serum IgA in covalent structure (Chintalacharuvu et al. 2007). Thus, the quality control of antibody secretion seems to rely on universal components rather than on B cell-specific factors and it is likely that the alternative IgG tetramer described here represents a property inherent to immunoglobulin folding.

Our work also broadens the scope of the findings of Chintalacharuvu et al. in that we show that the alternative disulfide assembly reflects a templated process on which the entire population of λ_2 is associated with γ_2 . We show that homo-dimerization of λ occurs only when Cys128 in the γ HC is absent, it engages the “orphan” Cys214 in the λ LC and it requires a C_{H1} domain as well as the non-covalent interaction between the V_H and V_L domains. Finally, we demonstrate that the alternative IgG2b structure is indeed tetrameric, exhibiting the conventional stoichiometry of $\gamma_2\lambda_2$ and most importantly, it is secreted as efficiently as the conventional IgG2b.

Our previous (Elkabetz et al. 2005) and current studies of IgG2b show that disulfide bonds within and between proteins are interrelated and illuminate the hierarchy of folding that is involved in Ig chain assembly. We also demonstrate that disulfide bonds can be formed by alternative pathways, all leading to distinct structures, which are nonetheless stable, functional and compatible with secretion. The interaction between γ and λ chains and the generation of the antigen-binding site seems to rely on two very specific, non-covalent interactions, between V_L and V_H and between C_L and C_{H1} . These interactions facilitate the completion of C_{H1} folding and usually culminate in the coordinated formation of intradomain and interchain disulfide bonds. If the HC-LC interchain disulfide bond is precluded by mutating Cys128, LC-LC interchain disulfide is formed, using the HC_2 homo-dimer as a template. This unusual tetrameric IgG, a non-covalent hetero-dimer of two covalent homo-dimers, is nonetheless stable and sufficiently ‘native’ to bind antigen and to pass the scrutiny of the quality control system and be efficiently secreted.

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Abbreviations

ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
γ	mouse γ2b heavy chain
HC	Ig heavy chain
HRP	horseradish peroxidase
λ	mouse λI light chain
LC	Ig light chain

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Fig. 1.

Assembly and efficient secretion of a functional alternative Ig-like assembly. Transfected cells expressing WT or C128S γ , with (+ λ) or without λ , or untransfected cells (mock; C), were incubated for 4 h in fresh media. (A) Media (m) or lysed cells (c) were subjected to immunoabsorption (IA) with protein A-Sepharose (PA). Alternatively, the same samples were adsorbed to NP-Sepharose (NP). Isolated proteins were resolved by non-reducing SDS-PAGE and immuno-blotted (IB) with the indicated HRP-conjugated anti- γ and anti- λ antibodies. Migration of γ , γ_2 , $\gamma\lambda$, $\gamma_2\lambda$, $\gamma_2\lambda_2$, λ , and λ_2 (*) is denoted. (B) Intracellular Ig assembly intermediates were immunoabsorbed (IA) to protein A-Sepharose (PA), resolved by both non-reducing (NR) or reducing (R) SDS-PAGE and immuno-blotted (IB) with

HRP-conjugated anti- λ . The 47kDa homo-dimer band that disappears upon reduction is denoted by *. (C) Equal amounts of cells were incubated for 4 h in fresh serum-free medium and secreted comparable amounts of wild-type and C128S-containing antibodies (see also Figs. 2, 3B). Media were collected and γ bound to NP-coated ELISA plates was quantified in 30 μ l (white), 60 μ l (gray) or 90 μ l (black) aliquots..

Fig. 2.

The secreted Ig-like assembly is a tetramer comprised of HC homo-dimer bound non-covalently to a LC homo-dimer.

Transfected cells expressing wild-type λ together with either WT or C128S γ were incubated for 5 h in fresh serum-free medium and the media were applied to size-exclusion chromatography. Fractions that were positive for γ and λ by dot-blotting were subjected to immunoadsorption (IA) to protein A-Sepharose (PA) of γ and γ-associated λ. Isolated proteins were resolved by non-reducing SDS-PAGE and immuno-blotted (IB) with HRP-conjugated anti-λ (A,C) and re-probed with HRP-conjugated anti-γ (B,D) antibodies. Migration of assembly species is denoted to the left of panels A,C. λ-positive (E, closed symbols) or γ-positive (F, open symbols) bands in fractions of WT-containing (A,B; circles) or C128S-containing (C,D; squares) assembly species were quantified by densitometry. The intensities of all assembly species within each fraction and in all the fractions were summed

and each fraction is presented as its percentages out of the total amount of assembly species in all the fractions. The relative distribution of each λ -positive (G) or γ -positive (H) assembly species is presented as the percentage of that assembly species out of the sum of all assembly species in each fractions, averaged over the 3 fractions in each peak. White, $\gamma_2\lambda_2$: light grey, $\gamma_2\lambda$; black, γ_2 (H, *) or λ_2 (G, **); dark grey, λ .

Fig. 3.

The LC homo-dimers are disulfide bonded via Cys214 and their formation requires a V_L - V_H interaction. (A) Cell lysates (c) and media (m) were prepared from transfected cells expressing C128S γ , together with either the WT λ or the C214S or C214A λ mutants. Immunoabsorption (IA) to protein A-Sepharose (PA) was followed by non-reducing SDS-PAGE and immuno-blotting (IB) with the indicated HRP-conjugated anti- γ and anti- λ antibodies. (B) Cell lysates (c) and media (m) from transfected cells expressing WT or C128S γ together with either wild-type λ or the F62S λ mutant were analyzed as in (A). Note that homo-dimerization of λ is seen only when in the combination C128S HC/WT LC. (C) Cell lysates (c) and media (m) from transfected cells expressing either excess (30 μ g

DNA) of WT λ or standard amounts (5 μ g/ml) of the F62S mutant, or from untransfected cells (mock), were subjected to either immunoprecipitation (IP) with anti- λ or immunoadsorption to protein A-Sepharose (PA) or NP-Sepharose (NP), followed by non-reducing SDS-PAGE and immuno-blotting (IB) with HRP-conjugated anti- λ . Note that the intracellular monomers of the secretion-incompetent F62S were a mixture of oxidized (λ_{ox}) and reduced LC (λ_{red}) (see also arrows in panel B, lanes 9–11). Migration of assembly species is denoted.

Fig. 4.

Disulfide bonded LC homo-dimers formed only when templated on C128S. (A) Transfected cells expressing WT, ΔCH1, (CH₂)₂, (CH₃)₂ or C128S γ, together with wild-type λ (+λ), or untransfected cells (mock), were lysed. Immunoabsorption (IA) to protein A-Sepharose (PA) was followed by non-reducing SDS-PAGE, immuno-blotted (IB) with HRP-conjugated anti-γ (upper panel) and re-probed with HRP-conjugated anti-λ (lower panel). (B) Cell lysates (c) and media (m) from transfected cells expressing either WT or C128S γ together with wild-type λ, or untransfected cells (mock), were subjected to immunoabsorption of γ and γ-associated λ to protein A-Sepharose (PA) or immunoprecipitation of λ and λ-associated γ from identical samples by goat anti-λ

antibody, followed by Sepharose-conjugated mouse anti-goat antibody (anti- λ). Isolated proteins were resolved by non-reducing SDS-PAGE and immuno-blotted (IB) with an HRP-conjugated anti- λ . (C) Cell lysates (c) and media (m) from transfected cells expressing wild-type λ together with the indicated combinations of C128S and WT γ (C128S half amount was complemented with either an empty vector (lanes 5,6) or WT half amount (lanes 7,8)), were subjected to immunoadsorption (IA) to protein A-Sepharose (PA), followed by non-reducing SDS-PAGE and immuno-blotting (IB) with an HRP-conjugated anti- λ . Migration of assembly species is denoted.

Table 1

Primers

Mutation	Forward primer	Reverse primer
wild-type C _H 1	GGCCG <u>GAA</u> <u>TTCAA</u> AGCTCACACATTTCTCCTC	CGCAGC <u>TC</u> <u>TAGAT</u> AAATACCTTTTGCCCTCAACTC
C128S	CCACTGGCCCTGGGTCTGGAGATACAACTGGT	ACCAGTTGTATCTCCAGACCCAGGGGCCAGTGG
C _H 2	GGCCG <u>GAA</u> <u>TTCT</u> CCCCAGGAGAAATGGTAAGTG	CGCAGC <u>TC</u> <u>TAGAT</u> TTTATTCTTTATGCCCATCCC
C _H 3	GGCCG <u>GAA</u> <u>TTCT</u> TTCCACTTCAGCCATGACC	CGCAGC <u>TC</u> <u>TAGAC</u> CTCTCACCAAGTTTTTTGTCACCCCGGAGACCGGGGAGATG
Wild-type λ	GGCCGGAA <u>TTCA</u> ATGGCCTGGATTTCACCTTATAC	ATGCGGCCCGCCTAGGAACAGTCGCACGGGAC
C214A λ	GGCCGGAA <u>TTCA</u> ATGGCCTGGATTTCACCTTATAC	ATGCGGCCCGCCTAGGAAGCGTCGCACGGGAC
C214S λ	GGCCGGAA <u>TTCA</u> ATGGCCTGGATTTCACCTTATAC	ATGCGGCCCGCCTAGGAAGAGATCGCACGGGAC

Primers were designed based on the published genomic sequence of murine λ2b (accession No. j00461). Designed restriction sites are italicized and underlined. Primers to amplify C_H1, C_H2 and C_H3 were designed to include the relevant exon flanked by 50–100bp of intronic sequences to ensure functional splicing. The reverse primer for C_H3 was designed such that the stop codon was skipped over and the C_H1 donor splice site was added. All primers were synthesized by Life Technologies.