A peptide extension dictates IgM assembly

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Professional secretory cells can produce large amounts of high-quality complex molecules, including IgM antibodies. Owing to their multivalency, polymeric IgM antibodies provide an efficient first-line of defense against pathogens. To decipher the mechanisms of IgM assembly, we investigated its biosynthesis in living cells and faithfully reconstituted the underlying processes in vitro. We find that a conserved peptide extension at the C-terminal end of the IgM heavy (IgM-\(\mu\)) chains, termed the tailpiece, is necessary and sufficient to establish the correct geometry. Alanine scanning revealed that hydrophobic amino acids in the first half of the tailpiece contain essential information for generating the correct topology. Assembly is triggered by the formation of a disulfide bond linking two tailpieces. This induces conformational changes in the tailpiece and the adjacent domain, which drive further polymerization. Thus, the biogenesis of large and topologically challenging IgM complexes is dictated by a local conformational switch in a peptide extension.

antibody | IgM structure | protein complex assembly | immunoglobulin fold | disulfide bond linkage

Secretory IgMs are polymeric antibodies that provide a first-line defense in vertebrates against invading microorganisms and other pathogens (1). Like in other Ig classes, two \(\mu\) heavy (H) and two light (L) chains assemble covalently into \(\mu_2L_2\) subunits (often also referred to as “monomers” in the Ig context). The assembly pathways vary in different isotypes. In IgM, \(\mu\)-L assembly precedes dimerization, while in IgG1, H dimerization precedes assembly with L (2, 3). In plasma cells, IgM monomers are further arranged into \((\mu_2L_2)_{\lambda}\) pentamers in the absence of the J chain, or into \((\mu_2L_2)_{\lambda}\) J pentamers in its presence (4, 5). The \(\mu\) chains can form oligomers that are able to incorporate the J chain in the absence of L chains. However, \(\mu\) oligomers are binding immunoglobulin protein (BiP)-associated, and are therefore not secreted (6). Abundant \(\mu_2\) intermediates accumulate in plasma cells, with their further polymerization being limited by efficient endoplasmic reticulum (ER)-associated degradation (7).

The polymeric structure increases the avidity for antigen and complement factors such as C1q, compensating for the lower affinity of most IgM (8, 9). The IgM H (Ig-\(\mu\)) chains contain four constant domains (C\(\mu_1\)–C\(\mu_4\)) with no hinge region. The C\(\mu_4\) domain harbors a C-terminal extension, the so-called \(\mu\) tailpiece (\(\mu tp\)), which is essential for polymerization (10–13). A related peptide is found in IgA, an isotype that also binds J chains can undergo anti-\(\mu\) and anti-\(\lambda\) antibodies (Fig. 1L and Fig. S1B). The four mutants with a markedly reduced hydrophobicity (Y562A, V564A, L566A, and I567A), were secreted exclusively as \(\mu_2L_2\) oligomers from the Golgi back to the ER (20, 25). C575 is also the site for the covalent attachment of J chains to IgM and IgA polymers (26–28).

We have previously solved the structures of all individual IgM constant domains at atomic resolution and presented a model for the IgM oligomer (13). We showed that the C\(\mu_4\) domain and the \(\mu tp\) are necessary and sufficient for the specific polymerization into hexamers of covalently linked dimers (i.e., dodecamers). However, the molecular mechanism of how the \(\mu tp\) promotes oligomerization was still unclear. In this study, we determined the principles underlying polymer formation and, by replacing every amino acid in the \(\mu tp\), we identified the residues essential for polymer assembly in vitro and in cell culture.

Results

The \(\mu tp\) Dictates Oligomerization of the Entire IgM. The regulated formation of IgM hexamers is still a mysterious process. Previous work has shown that the C\(\mu_4\) domain extended by the 18-aa C-terminal \(\mu tp\) is sufficient to drive hexamer formation (13). To define the role of the \(\mu tp\) amino acids in the polymerization of the entire IgM, we replaced every single residue in the \(\mu tp\) by alanine and analyzed the effects of these and other point mutations in cultured cells and in vitro.

For the cell culture analyses, the point mutations were inserted into full-length IgM-\(\mu\) and coexpressed in HEK-293T cells with Ig-\(\lambda\) L chains to form 4-hydroxy-3-nitrophenoxy acetyl-binding IgM assemblies (12). The molecular composition of intracellular and secreted subunits was analyzed by Western blotting with anti-\(\mu\) and anti-\(\lambda\) antibodies (Fig. 1L and Fig. S1B). The four mutants with a markedly reduced hydrophobicity (Y562A, V564A, L566A, and I567A), were secreted exclusively as \(\mu_2L_2\) polymers of over 1,200 kDa consisting of six antibody subunits (or five in the presence of the J-chain protein). These are arranged in a ring-like structure connected by disulfide bonds. Here, we show that in vitro and in cell culture, a short peptide extension of the IgM heavy chain is sufficient to steer the formation of the hexameric complex. The formation of a disulfide bond triggers conformational changes in the peptide extensions, which involve specific hydrophobic residues. Our study reveals the redox-controlled assembly of a large protein complex via structural rearrangements in a peptide as a design principle.

Significance

How protein assemblies with complex topologies are formed is an important question in structural biology. An intriguing example is IgM, a complex of over 1,200 kDa consisting of six antibody subunits (or five in the presence of the J-chain protein). These are arranged in a ring-like structure connected by disulfide bonds. Here, we show that in vitro and in cell culture, a short peptide extension of the IgM heavy chain is sufficient to steer the formation of the hexameric complex. The formation of a disulfide bond triggers conformational changes in the peptide extensions, which involve specific hydrophobic residues. Our study reveals the redox-controlled assembly of a large protein complex via structural rearrangements in a peptide as a design principle.

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“monomers.” In addition to the more abundant monomers, M568A and C575A transfectants secreted some covalent complexes of higher molecular weight (MW). Other mutants with increased hydrophobicity (N563A, S565A, and D570A), the former two of which destroy the glycosylation site characterized by the amino acid sequence NVS, secreted mainly hexamers and higher MW species.

The μ-L2 complexes secreted by Y562A, V564A, L566A, I567A, and M568A could have been part of bigger noncovalent oligomers, which were dissociated during SDS/PAGE. To test this possibility, the corresponding supernatants (SNs) were fractionated by sucrose gradient density centrifugation. No signal was detected in fractions 6–8, where covalent wild-type (wt) IgM polymers migrate (Fig. S1A). Thus, we can exclude the secretion of noncovalent polymers for these mutants, as well as for C575A (16). Further evidence for their inability to form oligomers was obtained by SDS/PAGE analyses of the intracellular fractions. Few, if any, intermediates of higher MW than (μ-L2)2 dimers could be observed for the hydrophobic mutants (Fig. L4).

With the exception of Y562A, V564A, L566A, I567A, M568A, and C575A, the other mutants were secreted mainly as pentamers and hexamers in different proportions. In cell lysates, IgM formed a smear in the upper part of the gels. These high-MW species were present also in M568A and C575A (Fig. L4 and Fig. S1B). These findings highlight the stringency of the IgM quality control mechanisms. These ensure that, on the one hand, only oligomers of correct size are secreted and, on the other hand, that all intermediates, including μ-L2 monomers, are prevented from secretion (12, 25). Therefore, the mutants secreted as μ-L2 monomers must be somehow able to escape the thiol-dependent control machinery.

Owing to their differential accessibility to the modifying enzymes in the Golgi complex, the processing of the N563 glycans depends on the oligomeric state of IgM transiting through the secretory pathway (11, 29). Thus, C575A Ig-μ chains show slower electrophoretic mobility under reducing conditions due to extensive processing of the N563 glycans by Golgi enzymes (29). When analyzed by reducing gels, the L566 mutant secreted exclusively as μ-L2 displayed slow mobility (Fig. 1B). The M568A mutant, which was secreted, in part, as pentamers and hexamers, migrated instead as a doublet under reducing conditions. Interestingly, when the same samples were run under nonreducing conditions, L566A and M568A showed a faster electrophoretic mobility than C575A (Fig. 1B), suggesting that mutants in which a hydrophobic residue was replaced adopted a more compact conformation. One possibility is that formation of C575-dependent disulfides within the same subunit reduces their hydrodynamic volume. The presence of an additional intrasubunit bond may also explain the paucity of secreted μ-L “hemimers” in the SNs of L566A and M568A transfectants.

Alanine replacement of either N563 or S565, two mutations that prevent attachment of the highly conserved N-glycan, caused secretion of hexameric and higher MW complexes. The N563 glycans serve important roles in determining the extent and velocity of polymerization (20). To further investigate the influences of glycosylation on polymer size, the mutant N563Q was generated, as glutamine closely resembles the wt asparagine, but N-glycosylation is no longer possible (Fig. 1A). N563Q formed fewer high-MW species than N563A or S565A. The stronger aggregation tendency of mutants with an A than Q at position N563 may reflect their higher overall hydrophobicity. To mimic the environment normally encountered in plasma cells, we coexpressed J chains with WT, L566A, M568A, or C575A μ chains. No polymers or intermediates containing J chains were secreted by the mutants (Fig. S2). Thus, cysteine 575 is not reactive with J chains in the mutants with increased hydrophobicity.

**Cu4tp Forms Cu4tp2 Hexamers via a Trimeric Intermediate in Vitro.** To achieve more detailed insight into IgM assembly, we performed in vitro experiments with the minimum oligomerization module, the Cu4 domain with the attached μtp extension (i.e., Cu4tp) (13). In vitro, the Cu4tp domains associated into hexamers of covalent dimers ([Cu4tp]2) without any additional factors. To identify potential intermediates, we followed the association of the Cu4tp domains by analyzing the process at defined time points by size exclusion (SE)-HPLC (Fig. 2A).

The chromatograms indicated the presence of four main species: the Cu4tp monomer, Cu4tp dimers (Cu4tp2), an intermediate species corresponding to trimers of dimers (i.e., Cu4tp hexamers ([Cu4tp]3)), and hexamers of dimers (i.e., Cu4tp dodecamers ([Cu4tp]2)). These species were present over the period of the analysis, but their relative abundance changed. Whereas monomers progressively decreased over the time course of the experiment, dodecamers increased. Cu4tp- and Cu4tp2- reached a plateau early on (~37% and ~2%, respectively), and remained almost constant, suggesting that those species are intermediates in the polymerization process. Our data show that
certain Cμ4tp threshold concentration is needed to allow hexamer formation. Nonreducing SDS/PAGE analyses revealed the presence of only two species: monomers, whose intensity gradually decreased during the experiment, and covalent dimers, whose intensity increased (Fig. 2B). Together, these results clearly indicate that the end product [i.e., (Cμ4tp)₃], the (Cμ4tp)₃ inter-

mediate] are noncovalent assemblies of the first intermediate to appear, the covalent dimer (i.e., Cμ4tp₂).

Formation of C575 Disulfide Bonds Is a Prerequisite for Oligomerization in Vitro. To investigate whether and how redox conditions affected oligomerization, we monitored the kinetics of assembly under conditions in which formation of C575 bonds was not possible (Fig. 3A–C). To this end, we analyzed the association properties of Cμ4tp monomers in which C575 was irreversibly blocked by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) by SE-HPLC (Fig. 3A). No dimers became detectable even at a high concentration (15 mg/mL), indicating that the oligomerization process was not initiated when the formation of covalent Cμ4tp species was prevented.

Second, we added an excess of AMS at different time points during oligomerization, thus blocking the remaining free C575 (Fig. 3B). Clearly, upon irreversible modification of C575, no further conversion of Cμ4tp monomers into (Cμ4tp)₂₆ was observed over time. This implies that both dimerization and further oligomerization were blocked. The amount of Cμ4tp monomers and (Cμ4tp)₂₆ present before the addition of AMS remained unchanged. Altogether, these data are strong evidence for the formation of covalent dimers as the first and essential step for further noncovalent oligomerization into (Cμ4tp)₃.

Third, we tested whether noncovalent interactions could drive oligomerization in the absence of C575, comparing two mutants in which the cysteine was replaced with serine (isosteric and polar) or with alanine [isovolumetric and hydrophobic (30)]. At all protein concentrations investigated, for both the C575A and C575S mutants, the main species (96–99%) was the monomer. At high concentration (15 mg/mL), indicating that the formation of covalent Cμ4tp species was prevented.

Finally, to assess how stable the noncovalent interactions are within (Cμ4tp)₂₆, after disruption of the C575 S-S bond, we treated the dodecamers with a reducing agent (1 mM DTT) and analyzed the oligomeric state by SE-HPLC at different time points. As shown in Fig. 3D, after addition of DTT (Cμ4tp)₂₆ decreased progressively, while monomers increased to become the prevalent species. Thus, by disrupting the C575 S-S bonds, noncovalent interactions are not able to persist.

Hydrophobic μtp Residues Are Essential for Oligomerization of Covalent Cμ4tp in Vitro. Unlike most other Ig C1 domains, Cμ4 is prevalently monomeric in solution (13, 31). Since Cμ4tp fragments assemble into (Cμ4tp)₂₆ complexes (13) (Fig. 3), the 18-aa-long μtp is the element that confers the ability to oligomerize, likely allowing formation of alternate covalent and noncovalent linkages with adjacent Cμ4tp. So far, we assessed the effects of the μtp extension on IgM polymerization in cultured cells. Furthermore, we established in vitro that the penultimate cysteine is important for assembly. To verify our cell culture results and to gain a deeper understanding of the structural bases leading to oligomerization, each μtp amino acid in the Cμ4tp context was replaced by alanine, and the effects of these mutations on oligomerization were then analyzed in vitro.

All alanine mutants, except C575A, were able to form covalent dimers. Based on their ability to oligomerize, the mutants can be grouped in three categories: (i) mutants with no ability to oligomerize at all, (ii) mutants capable of oligomerization, and
(iii) mutants with a tendency to aggregate (Fig. 4A, Fig. S4A, and Table 1). Interestingly, mutants that formed covalent dimers but completely lost the ability to assemble (Y562A, Y564A, L566A, and I567A) were those where a hydrophobic residue was replaced by alanine (Fig. 4B). This perfectly matches our cell culture studies and supports the fact that hydrophobic residues are essential for oligomerization. To assess whether loss of oligomerization could be overcome in an environment that enhances hydrophobic interactions, mutants were analyzed in the presence of 1 M NaCl (Fig. S4B). Under these conditions, only I567A showed a detectable oligomerization tendency at higher protein concentrations. Thus, the effects of mutating single hydrophobic residues could not be compensated by favoring hydrophobic interactions.

A detailed analysis of the mutants revealed interesting features. P559A, T560A, and L561A exhibited a severely compromised ability to form polymers in vitro (Fig. 4A and Fig. S4A). The hydrophobic mutant M568A formed mainly decamers (Cµ4tp)₆ of 168 kDa (Table 2). It should be noted that the covalent dimerization of Ig-μ observed in HEK-297T cells depends primarily on intrasubunit disulfide bonds between C337 in the Cµ2 domain.

Two mutants, N563A and D570A, displayed a strong tendency to aggregate, as also observed in cultured cells. In N563A, the attachment site for N-glycosylation is mutated. The effects of glycosylation at position 563 on polymerization were suggested above. D570A lacks the only negative charge present on the μt, which might act as a gatekeeper that prevents aggregation (32–34). Interestingly, with the exception of C575A, all mutants showing defective oligomerization carry a mutation in the N-terminal half of the μt, spanning residues 559–570. To gain further information on their biophysical properties, we interrogated programs designed to predict the β-aggregation propensity of polypeptide sequences (35, 36). Analysis of the wt μt and its Ala mutants identified a region located in the N-terminal half of the sequence, encompassing residues 559–569 (TANGO) and 559–570 (AGGRESCAN), which influences the β-aggregation tendency of the μt (Fig. 4C). Both programs predicted a strikingly higher aggregation propensity for the N563A mutant and a significantly lower aggregation propensity for L561A, Y562A, Y564A, L566A, and I567A (Fig. S4C). Remarkably, with the exception of C575A, the behavior of all IgM and Cµ4tp mutants was in line with
To assess the effects of region prone to mutants in PBS. c(s), concentration of species as a function of sedimentation; Sed. coefficient, sedimentation coefficient. (Pasalic et al. PNAS Fig. 4. Oligomerization ability of C\textsubscript{4}tp alanine mutants in vitro. (A) Schematic representation of their oligomerization ability in vitro. Mutants were refolded, and monomers were isolated as previously described. To accelerate oligomerization, monomers were concentrated to 15 mg/mL and then run over a preparative SE column. Fractions containing the oligomer were pooled, and the concentration was adjusted to 0.5 mg/mL for analytical ultracentrifugation (aUC) and to 1 mg/mL for SEC-MALS analysis. When no oligomers formed, fractions containing monomer and dimers were pooled and analyzed at the same concentrations. For mutants able to oligomerize the height of the dodecamer, the peak was normalized with respect to that of the wt dodecamer peak. Red indicates C\textsubscript{4}tp, orange indicates C\textsubscript{4}tp\textsubscript{2}, yellow indicates (C\textsubscript{4}tp\textsubscript{2})\textsubscript{6}, and green indicates (C\textsubscript{4}tp\textsubscript{2})\textsubscript{6}. (B) SEC-MALS (a) and aUC (b) profiles of nonoligomerizing mutants in PBS. c(s), concentration of species as a function of sedimentation; Sed. coefficient, sedimentation coefficient. (C) Schematic representation of the region prone to β-aggregation predicted by TANGO and by AGGRESCAN of the wt C\textsubscript{4}tp peptide and 18 alanine C\textsubscript{4}tp mutants.

TANGO and AGGRESCAN predictions. Thus, residues in the C\textsubscript{4}tp dictate the extent of IgM oligomerization.

Replacement of Hydrophobic Residues Alters the C\textsubscript{4}tp Secondary Structure. To assess the effects of C\textsubscript{4}tp mutations on the isolated C\textsubscript{4}tp, we synthesized 18-aa-long peptides encompassing residues 559–576 and carrying the respective point mutations. The C\textsubscript{4}tp mutants were tested for oligomerization, either in the absence or presence of a redox system (GSSG/GSH) and analyzed by SE-HPLC (Fig. 5A). All mutants except C575A and C575S formed covalent dimers upon oxidation. However, not even the wt C\textsubscript{4}tp was able to assemble further into oligomers, suggesting that interactions between C\textsubscript{4}tp and C\textsubscript{4}tp of the same chain or adjacent chains are important for polymerization. When wt monomers or dimers were mixed with C\textsubscript{4}tp and incubated, no oligmeric species were detected, indicating that in cis, C\textsubscript{4}tp-C\textsubscript{4}tp continuity is required for oligomerization.

The secondary structure of the peptides was assessed by far-UV CD spectroscopy. The wt peptide showed a CD spectrum consistent with a β-strand motif (Fig. 5B, a). Upon oxidation, the CD spectrum changed, yielding a more pronounced minimum of ellipticity at 215 nm. In agreement with TANGO and AGGRESCAN, the PSIPRED prediction for secondary structure (http://bioinf.cs.ucl.ac.uk/psipred/) suggests the presence of a β-strand motif on the N-terminal half of the C\textsubscript{4}tp from 560 to 569 (Fig. 5B, b). The mutants in which hydrophobic residues were substituted exhibited different characteristics (Fig. 5C). These hydrophobic residues are located in the stretch from 560 to 569, which contributes to the formation of a structure required for oligomerization in the context of the C\textsubscript{4}tp domain. An altered conformation might thus explain the fate of aggregating and nonoligomerizing mutants.

C575 Reactivity and Y576 Hydrophobicity Control IgM Polymerization. Next, we assessed the impact of the C-terminal doublet, CY. When Y576 was replaced with alanine, the oligomerization ability of both IgM in HEK-297T cells and C\textsubscript{4}tp in vitro was significantly affected (Figs. 1A, 4A, and 6). To determine whether higher hydrophobicity at this position would instead favor oligomerization, Y576 was replaced with residues of increasing hydrophobicity: glycine, tryptophan, or phenylalanine. In both C\textsubscript{4}tp and IgM, an increased hydrophobicity of the C-terminal residue promoted oligomerization, mainly (μ2−Lc)\textsubscript{15} pentamers being formed in cultured cells (Fig. 6A, a and B, b).

To test whether and how the presence of a charged residue at this position affects polymerization, we replaced Y576 with either lysine or glutamate (Fig. 6A, b and B, c). The presence of a positive charge (Y576K) supported the formation of hexameric...
Table 1. Nonoligomerizing, oligomerizing, and aggregating C4tp mutants with the molecular mass and $S$ value of the biggest species formed

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Oligomer size</th>
<th>MM, kDa</th>
<th>$S$ value</th>
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<tbody>
<tr>
<td>WT</td>
<td>12x</td>
<td>201</td>
<td>8.5</td>
</tr>
<tr>
<td>C575A</td>
<td>1x</td>
<td>14</td>
<td>1.7</td>
</tr>
<tr>
<td>Y562A</td>
<td>2x</td>
<td>28</td>
<td>2.5</td>
</tr>
<tr>
<td>V564A</td>
<td>2x</td>
<td>29</td>
<td>2.5</td>
</tr>
<tr>
<td>L566A</td>
<td>2x</td>
<td>28</td>
<td>2.5</td>
</tr>
<tr>
<td>IS67A</td>
<td>2x</td>
<td>28</td>
<td>2.6</td>
</tr>
<tr>
<td>P559A</td>
<td>12x</td>
<td>211</td>
<td>8.5</td>
</tr>
<tr>
<td>T560A</td>
<td>12x</td>
<td>192</td>
<td>8.4</td>
</tr>
<tr>
<td>L561A</td>
<td>12x</td>
<td>195</td>
<td>8.4</td>
</tr>
<tr>
<td>S564A</td>
<td>12x</td>
<td>179</td>
<td>7.7</td>
</tr>
<tr>
<td>M568A</td>
<td>10x</td>
<td>168</td>
<td>7.5</td>
</tr>
<tr>
<td>S569A</td>
<td>12x</td>
<td>191</td>
<td>8.1</td>
</tr>
<tr>
<td>T571A</td>
<td>12x</td>
<td>198</td>
<td>8.4</td>
</tr>
<tr>
<td>G572A</td>
<td>12x</td>
<td>197</td>
<td>8.2</td>
</tr>
<tr>
<td>G573A</td>
<td>12x</td>
<td>193</td>
<td>8.3</td>
</tr>
<tr>
<td>T574A</td>
<td>12x</td>
<td>200</td>
<td>8.5</td>
</tr>
<tr>
<td>Y576A</td>
<td>12x</td>
<td>191</td>
<td>8.6</td>
</tr>
<tr>
<td>N563A</td>
<td>Aggregates</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D570A</td>
<td>Aggregates</td>
<td>—</td>
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MM and $S$ value were determined by SEC-MALS and aUC, respectively. aUC, analytical ultracentrifugation; MM, molecular mass.

C4tp and $\mu_2$-L2. The Y576D mutant carrying a negative charge close to C575 had opposite effects. In the C4tp context, no oligomerization occurred and the efficiency to form covalent dimers was reduced, most likely due to the repulsion of the negative charges in the adjacent positions 575 and 576. These results could also reflect effects on the $p_K$, of C575, and hence its reactivity. In the IgM context, a negative charge in the last position favored secretion of $\mu_2$-L2 subunits, as did also upstream C575 (14).

Finally, to investigate the relevance of the position of the cysteine within the $\mu$tp, and therefore the position of disulfide bonds within the polymers, we generated mutants in which the cysteine was replaced at the very C-terminal position or at increased distances from the C terminus. When the CY sequence was inverted (C575YY576C), both the C4tp and the IgM mutants formed polymers. In particular, the YC mutant formed more C4tp2 hexamers than the wt in vitro, while in the IgM context, hexamers were favored over pentamers (Fig. 6d, b and B, d). In contrast, the three C4tp double-point mutants, where C575 was replaced by serine and a cysteine was inserted at position 559, 565, or 569, displayed a stronger propensity to aggregate. Thus, shortening the distance between the $\mu$tp cysteine and the C4 domain is deleterious to oligomerization.

Taken together, these experiments reveal further constraints for the composition of the $\mu$tp and the positioning of specific residues.

Discussion

Our data reveal that the C-terminal $\mu$tp contains crucial information for IgM assembly, which matches its evolutionary conservation. In a previous study, we had demonstrated that extending the C4 domain with a $\mu$tp is sufficient to drive the formation of hexamers of dimers in vitro without any additional factors (13). In this work, we analyzed the underlying mechanism by determining the role of individual residues within the $\mu$tp. An important conclusion is that disulfide linkage between the penultimate cysteines (C575) of two C4tp monomers is a prerequisite for assembly. Only after this covalent connection occurs can noncovalent interdimer associations start and continue until hexamers of covalent (C4tp2)2 are formed (Fig. 7).

Strikingly, the results obtained for the assembly of tailpiece mutants were almost identical in vitro and in cell culture. The effects observed for C4tp in vitro faithfully phosphorylated those for IgM in living cells, demonstrating directly that all of the information required for IgM assembly is contained in this module. However, the slow kinetics of oligomerization in vitro support the notion that additional intrinsic factors like other domains or N-glycosylation and/or cellular factors such as ERp44 and ERGIG53 (25) further assist IgM biogenesis in cultured cells.

While impeding the formation of the C575-disulfide bond in vitro prevents oligomerization, none of the mutations that abolish further noncovalent assembly prevent the covalent linkage. Importantly, this indicates that covalent association of two C4tp monomers via the tailpiece must precede the establishment of noncovalent interactions leading to dimerization and oligomerization. Since (C4tp2)6 formation is topologically quite challenging and the amount of dimers stays constant in vitro, this suggests that a certain C4tp2 threshold concentration is needed to allow formation of the dodecamer. The mutations severely affecting oligomer formation are replacements of L561A, V564A, L566A, M568A, and Y575A, with Y562A and IS67A completely hindering assembly. A likely explanation is that these hydrophobic residues are involved in different interactions in the absence of the C575 disulfide bond. Thus, formation of the disulfide bridge seems to induce rearrangements within the C4tp, which impose steric constraints that, in turn, allow hexamer formation. However, oligomerization is possible only when the $\mu$tp is anchored to the C4 domain: Isolated $\mu$tp exclusively formed covalent dimers, and no further association was observed when C4 and $\mu$tp were incubated in trans. Our data do not allow us to discriminate whether the noncovalent interactions leading to oligomerization occur between two $\mu$tps or between a $\mu$tp and C4 domain (Fig. 7). Interestingly, the peptides generated upon mutation of the highly hydrophobic residues seem to be less structured than the wt $\mu$tp. In cultured cells, the mutants show increased electrophoretic mobility under nonreducing conditions, pointing to a more compact conformation, possibly due to intrasubunit C575 disulfide bonding. This bond might be formed by wt $\mu$ chains as well, but recognized as nonnative and isomerized into intersubunit linkages by ERp44 or other oxidoareductases of the early secretory pathway.

The CD spectrum of the wt $\mu$tp peptide presents features of a $\beta$-strand motif that, according to the PSIPRED algorithm, is located in the N-terminal half of the $\mu$tp, encompassing residues 559-569. Also TANGO and AGGRESCAN predicted an increased aggregation propensity for these mutants. Mutations in this region severely compromised oligomerization (i.e., no oligomerization at all or aggregation). Thus, our in vitro experiments reveal that the reduced $\mu$tp is structured and that this structure is rearranged upon oxidation as the starting point for interactions involving the $\mu$tp and the C4 domain that are needed for assembly. Importantly, moreover, the in vitro behavior of the C4tp mutants was identical to that of the corresponding full-length IgM mutants in HEK-297T cells. This proves that the hydrophobic $\mu$tp amino acids are essential for IgM oligomerization and that intracellular cofactors cannot correct this.

Table 2. Synthetic $\mu$tp peptides

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Name</th>
<th>MMcalc, Da</th>
<th>MMobs, Da</th>
<th>Purity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTLYNLVSIMSMTGTCY</td>
<td>wt $\mu$tp</td>
<td>1,935.2</td>
<td>1,934.1</td>
<td>96</td>
</tr>
<tr>
<td>PTLYNLVSSMTGTSY</td>
<td>C575S</td>
<td>1,919.1</td>
<td>1,918.3</td>
<td>93</td>
</tr>
<tr>
<td>PTLYNLVSSMTGTA</td>
<td>C575A</td>
<td>1,903.1</td>
<td>1,901.7</td>
<td>94</td>
</tr>
<tr>
<td>PTLYNLVSSMTGTCY</td>
<td>Y562A</td>
<td>1,843.1</td>
<td>1,842.0</td>
<td>90</td>
</tr>
<tr>
<td>PTLYNLVSSMDSLGGTCY</td>
<td>V564A</td>
<td>1,907.1</td>
<td>1,906.7</td>
<td>89</td>
</tr>
<tr>
<td>PTLYNLVSSMDSLGGTCY</td>
<td>L566A</td>
<td>1,893.1</td>
<td>1,892.1</td>
<td>91</td>
</tr>
<tr>
<td>PTLYNLVSSMDSLGGTCY</td>
<td>IS67A</td>
<td>1,893.1</td>
<td>1,892.2</td>
<td>92</td>
</tr>
</tbody>
</table>

All mutations are highlighted in bold, amino acids double-coupled during synthesis are indicated in italics, and pseudoproline building blocks are underlined. MMcalc, calculated molecular mass; MMobs, observed molecular mass.
It is of special interest that mutants in the hydrophobic core (Y562A, V564A, L566A, I567A, and M568A) are secreted from cells as μ2-L2 subunits. This is surprising because thiol-mediated retrieval normally prevents the secretion of incomplete or incorrectly assembled polymers (16). ERp44 (25, 37) and the C-terminal cysteine C575 (12, 22) are key players in this process. Thus, the essential element required for oligomer formation resides in the region mutated. In these mutants, ERp44 could be unable to recognize C575 due to a collapse of the two μtps that reduces their accessibility and possibly by the formation of intrasubunit C575 disulfide bonds. In cells, the μtps within the same subunit are in close proximity due to the interactions and the covalent bond linking the Cμ4 domains (13, 17, 18). Mechanisms must hence operate that prevent formation of intrasubunit C575 bonds, or isomerize them into native intersubunit interactions.

Our results also shed new light on the effects of glycosylation. The number of subunits in IgM polymers is influenced by the presence of glycan moieties linked to N563. N563A (this work) and S565A (ref. 38 and this work), which lack them, are secreted as hexameric and higher MW species. The presence of glycans in the tightly packed IgM core (13, 39) could limit the number of subunits that can be incorporated into an oligomer or serve as a docking device for ERGIC53, a hexameric lectin shown to promote polymerization in nonlymphoid cells (25). The N563Q mutant tended to form fewer aggregates. Furthermore, of the 18 Cμ4tp mutants analyzed in vitro, all being nonglycosylated, only two formed aggregates (N563A and D570A). This indicates that the increased aggregation propensity of N563A, S565A, and D570A (as predicted by TANGO and AGGRESCAN) is responsible for the formation of high-MW species. N563 glycosylation might be relevant for J-chain incorporation and the formation of pentameric IgM.

Based on our data and the literature, we propose a model for IgM assembly in which the geometry of IgM assembly is determined by the Cμ4 domains together with the tailpiece (Fig. 7). As a committed step, monomeric Cμ4 domains (in isolation or in the context of the H chain) are covalently linked via C575 disulfide bridges. This induces structural changes involving hydrophobic residues in the μtp as essential factors for IgM assembly. These rearrangements trigger events leading to the noncovalent association of the Cμ4tp domains and the oligomerization.
into hexamers. Translated to full-length IgM, this implies that formation of C575 disulfides is required for the formation of intersubunit covalent and noncovalent interactions. In fact, without C575, μ2-L2 subunits are the main secreted species, with few covalent polymers formed via C414. Unexpectedly, these species are absent in the hydrophobic μtp mutants, suggesting an important role for the N-terminal half of the μtp in forming defined polymers. One possibility is that intrasubunit C575 disulfides are formed first as the two μ chains in IgM subunits are linked by C337 disulfides. The intrasubunit C575 disulfides could be isomerized with the help of ERp44 and other cellular factors. The rearranged hydrophobic tailpiece residues would then mediate formation of planar polymers in which isomerization can take place. Thus, this small extension, together with the Cu4 domain, orchestrates the sophisticated assembly of large IgM complexes.

In the context of engineering antibodies with enhanced effector function (40, 41), our work provides a rational basis for the development of peptide appendices able to confer specific oligomerization properties. This would allow turning antibodies into polyvalent or multispecific entities that could be employed as therapeutic agents with improved effector functions.

Materials and Experimental Procedures

Materials. Unless otherwise specified, all experiments in vitro were carried out in PBS [8.09 mM Na2HPO4, 1.76 mM KH2PO4, 137 mM NaCl, and 2.65 mM KCl (pH 7.4)] at 20 °C.

Mutagenesis, Expression, and Purification of the Cμ4tp Domain. The Cμ4tp (E446–Y576) gene was optimized for expression in Escherichia coli by GeneArt and cloned into the pET28a expression vector via the NcoI and HindIII restriction sites. Amino acid numbering is according to UniProt entry P01872 (IGHM_MOUSE) with the variable heavy chain (VH) added. All mutations on the Cμ4tp domain were introduced using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). Expression was performed, and inclusion bodies were prepared, as well as solubilized, as previously described (13, 42). Insoluble components were removed by centrifugation (46,000 × g, 20 min, 4 °C). The SN was filtered and processed over a HiTrap Q FF column (GE Healthcare) equilibrated with 50 mM Tris (pH 7.5), 10 mM EDTA, and 5 M urea. The protein was collected in the flow-through, diluted to a concentration of 1 mg/mL, and refolded by dialysis into 250 mM Tris·HCl (pH 8.0), 100 mM l-cysteine, 10 mM EDTA, 1 mM GSSG, and 0.5 mM GSH overnight at 4 °C. Misfolded protein and remaining impurities were removed by processing the sample on a HiLoad Superdex200 26/60 size exclusion chromatography (SEC) column (GE Healthcare) previously equilibrated in PBS buffer. Fractions containing the monomeric Cμ4tp were pooled to obtain the monomer for kinetic experiments. To obtain the hexamer of dimers, the fractions containing the monomer and the dimer were pooled, concentrated, and processed again over the same SEC column. All constructs were sequenced, and the mass of the purified proteins was confirmed using matrix-assisted, laser desorption ionization, time-of-flight mass spectrometry.

Cell Culture, Transfections, Secretion, and Western Blotting. HEK-293T cells were obtained from the American Type Culture Collection and were cultured in RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM l-glutamine ( Gibco–Invitrogen). Stable transfectants expressing Igλ chains were obtained with pcDNA3.1–λ chain (7) using polyethyleneimine (PEI; Polysciences, Inc.) as previously described (21). Forty-eight hours after transfection, 1 mg/mL G418 was added to the culture medium to select clones with stable insertion of the transgene. As λ is a secreted protein, positive clones were assessed by ELISA of the culture SN. Transient transfection with λ-chain mutants was performed using PEI (21). For analyses of intracellular and secreted IgM mutants, cells were plated in duplicate in six-well plates and transfected. Forty-eight hours after transfection, cells were washed twice with PBS and incubated with minimal essential medium (Opti-MEM) for 4 h. Cell culture SNs were then collected and treated with 10 mM N-ethylmaleimide (NEM) as previously described (21). Forty-eight hours after transfection, 1 mg/mL G418 was added to the culture medium to select clones with stable insertion of the transgene. As λ is a secreted protein, positive clones were assessed by ELISA of the culture SN. Transient transfection with λ chain mutants was performed using PEI (21). For analyses of intracellular and secreted IgM mutants, cells were plated in duplicate in six-well plates and transfected. Forty-eight hours after transfection, cells were washed twice with PBS and incubated with minimal essential medium (Opti-MEM) for 4 h. Cell culture SNs were then collected and treated with 10 mM N-ethylmaleimide (NEM) as previously described (21). Forty-eight hours after transfection, 1 mg/mL G418 was added to the culture medium to select clones with stable insertion of the transgene. As λ is a secreted protein, positive clones were assessed by ELISA of the culture SN. Transient transfection with λ chain mutants was performed using PEI (21). For analyses of intracellular and secreted IgM mutants, cells were plated in duplicate in six-well plates and transfected. Forty-eight hours after transfection, cells were washed twice with PBS and incubated with minimal essential medium (Opti-MEM) for 4 h. Cell culture SNs were then collected and treated with 10 mM N-ethylmaleimide (NEM) as previously described (21). Forty-eight hours after transfection, 1 mg/mL G418 was added to the culture medium to select clones with stable insertion of the transgene. As λ is a secreted protein, positive clones were assessed by ELISA of the culture SN.
Sucrose Density Fractionation. SNs obtained from transiently transfected HEK-293T cells were harvested, and 10 mM NEM was added. After incubation on ice for 20 min, cell SNs were concentrated with TCA, resuspended in 1 mL of homogenization buffer (0.25 M sucrose, PBS, 1% BSA) and fractionated by centrifugation on a continuous sucrose gradient (25-50%) at 273,000 × g in an SW 41 Ti Beckman rotor. Twelve fractions of the gradient were collected. Aliquots from fractions were resolved under nonreducing conditions, transferred to nitrocellulose membrane, and blotted with anti-μ antibody.

Analytical SEC. SEC-HPLC was performed to determine the oligomeric state of the Cμ4tp wt domain, as well all Cμ4tp mutants in kinetic experiments. For all experiments, a Shimadzu HPLC system was used, and the analysis was performed on a Superdex 200 10/300 GL column (GE Healthcare) in PBS buffer at a flow rate of 0.5 mL min⁻¹ at 20 °C. The absorbance at 280 nm was detected. BioRad gel filtration standard (no. 151-1901) was used as a reference. Experimental details of individual experiments are described in the respective figure legends.

SEC Coupled to Multiline Light Scattering. A Tosoh TSKgel G3000SW silica SEC column (7.2 × 300 mm, 10-μm bead, 250-Å pore) and a Shimadzu HPLC system were employed for determination of the absolute mass of oligomers of Cμ4tp mutants. The instrument was coupled to a Wyatt Dawn Helios II multiline light scattering (MALS) detector, as well as a Shimadzu refractive index and UV detectors. The column was equilibrated for 24 h to obtain stable baseline signals from the detectors before data collection. The interdetector delay volumes and band broadening, the light-scattering detector normalization, and the instrumental calibration coefficient were calibrated using a standard 2-mg/mL BSA solution (Sigma) run in the same buffer, on the same day, according to standard protocols. Protein samples at concentration of 1 mg/mL (20 μL) were loaded on the column. All experiments were performed at room temperature at a flow rate of 0.3 mL min⁻¹ in PBS buffer. The MW and mass distribution of the sample were then determined using the ASTRA 5 software (Wyatt Technology).

CD Spectroscopy. The secondary structure was determined by CD measurements using a Jasco J-720 spectropolarimeter. Far-UV spectra were recorded from 195 to 260 nm at a protein concentration of 20 μM in 0.5-mm quartz cuvettes. Spectra were accumulated three times and buffer-corrected.

Analytical Ultracentrifugation Sedimentation Velocity Experiments (SV-AUC). Analytical ultracentrifugation was carried out with a ProteomLab XL-I (Beckman) supplied with absorbance optics. All experiments were performed using PBS at 20 °C. Four hundred fifty microliters of the samples was loaded into assembled cells with sapphire windows and 12-mm path length, charcoal-filled, epon double-sector centerpieces and centrifuged at 42,000 × g for 96 h using an eight-hole Beckman Coulter AN50-Ti rotor. Sedimentation was monitored with an UV/VIS spectrophotometer, equipped with a monochromator, at 280 nm. Data analysis was carried out with the program Sedfit (Peter Schuck, NIH, Bethesda, MD), using a non–model-based continuous Svedberg distribution method, with time and radial invariant noise on.

Resuspension and Oxidation of Peptides. All peptides were synthesized manually or on an automated synthesizer (Tribute; Gyros Protein Technologies) by Fmoc-based, solid-phase peptide synthesis using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation in N,N-dimethylformamide (DMF) on Wang resins (44). Double coupling and proline dipeptide building blocks (Iris Biotech) were used to improve synthesis.

Fig. 7. Models for IgM polymerization. (A) Model for Cμ4tp oligomerization in vitro. (a and b) μtp (dark blue tail) conformational changes in Cμ4tp induced by the formation of a C575 disulfide bond (yellow circles). In a, two different possible tailpiece conformations are depicted. Cμ4tp dimerization is coupled to oligomerization (b and c), leading to a hexamer (d). Noncovalent interactions might occur either between two adjacent μtps or between the μtp and the Cμ4 domain in trans. (B) Assembly of IgM in living cells. Cysteines involved in interdomain disulfide bridges are indicated by yellow circles. (a) Fab region of two μ-95 “hemimers” is shown with one of the two μtp arrangements shown in A. Covalent and noncovalent interactions link C337 and surrounding regions in the Cμ2 domains (depicted here in green), limiting the mobility of the C-terminal portions, and hence favoring collisions between pairs of Cμ4 and μtps, to produce a μtp, as depicted in b. Antigen-binding (Fab) fragments attached to the Cμ2 domains were deleted for clarity. (b) Snippet of the IgM Fc dodecamer. Formation of the C575 disulfide results in conformational modifications the Cμ4tp domain, allowing the establishment of noncovalent interactions (red boxes) either between two adjacent μtps or between the Cμ4 and a μtp in trans. The Cμ3 domains in adjacent subunits must become close enough for C414 to form a disulfide bond, a feature favored in hexamers. It is noteworthy that C414 does not form intersubunit disulfides in membrane IgM.
quality as indicated in Table 2. The final peptide resins were washed with methanol, dried under vacuum, and cleaved with a solution of trifluoroacetic acid (TFA). H2O and trifluoroacetamide (92.5:5:2.5) for 3 h at room temperature. Released peptides were precipitated with cold diethyl ether (Et2O), washed twice with Et2O, and dissolved in a 1:1 mixture of water and acetonitrile (ACN) containing 0.1% TFA. After freeze-drying, peptides were dissolved in aqueous 6 M GuHCl buffer with 100 mM NaOAc at pH 4. Before final purification, Tris-(2-carboxyethyl)phosphine was added to cysteine-containing peptides to fully reduce them. Purification was achieved by RP-HPLC on a C4 column running a gradient of 5% ACN (0.08% TFA) in H2O (0.1% TFA) to 65% ACN (0.08% TFA) in H2O (+0.1% TFA). Peptide-containing fractions were identified by electrospray ionization mass spectrometry (ESI-MS) (Waters 3100 Detector) and lyophilized.

Peptide lyophilizates were resuspended in Tris HCl (pH 7.5) and 10 mM EDTA in an appropriate volume to reach the final concentration of 0.4 mg/mL. To stabilize the peptides, 1 mM GSSG and 0.5 mM GSH were added. The samples were then incubated for 2 h at room temperature under stirring.

For material requests or further details on the experimental methods and results, please contact the corresponding authors.

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