Membrane Immunoglobulins Are Stabilized by Interchain Disulfide Bonds Occurring within the Extracellular Membrane-Proximal Domain

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ABSTRACT: Membrane-bound immunoglobulins have, in addition to the transmembrane and cytoplasmic portions, an extracellular membrane-proximal domain (EMPD), absent in the secretory forms. EMPDs of immunoglobulin isotypes α, γ, and ε contain cysteines whose role has so far not been elucidated. Using a genetic strategy, we investigated the ability of these cysteines to form disulfide bridges. Shortened versions of human membrane immunoglobulins, depleted of cysteines known to form intermolecular disulfide bonds, were constructed and expressed on the surface of a B-cell line. The resulting membrane proteins contain a single chain fragment of variable regions (scFv) linked to the dimerizing domain from the immunoglobulin heavy chains (CH3 for α and γ or CH4 for ε isotypes), followed by the corresponding EMPD and the transmembrane and cytoplasmic domains. The two functional membrane versions of the ε chain, containing the short and long EMPD, were analyzed. Our results show that the single cysteine within α1L and γ1 EMPD and the short version of ε EMPD form an interchain disulfide bond. Conversely, the cysteine resident in the ε transmembrane domain remains unreacted. ε-long EMPD contains four cysteines; two are involved in interchain bonds while the remaining two are likely forming an intrachain bridge. Expression of a full-length membrane ε heavy chain mutant, in which Cys^{121} and Cys^{209} within domain CH2 (involved in interchain bridges) were mutated to alanines, confirmed that, within the complete IgE, EMPD cysteines form interchain disulfide bonds. In conclusion, we unveil evidence for additional covalent stabilization of membrane-bound immunoglobulins.

Immunoglobulins (Igs)[1] are expressed as secretory or membrane-bound proteins, depending on the stage of differentiation of B-cells. On the surface of B-lymphocytes, membrane-bound Igs constitute the antigen-specific component of B-cell receptors (BCR) (*1*). Conversely, when produced by differentiated plasma cells, Igs are actively secreted, becoming relevant components of the serum. The constant (C) region of immunoglobulin heavy (H) chain is composed of different domains, of which CH1, in all isotypes, associates with the C region of light (L) chains. CH2 and CH3 domains for IgG and IgA and CH2, CH3, and CH4 for IgM and IgE constitute the so-called Fc fragments, common to both membrane and secretory forms. Membrane Igs, however, contain three additional C-terminal domains, namely, the extracellular membrane-proximal domain (EMPD), the transmembrane domain (TMD), and the cytoplasmic domain (CytoD).

In most Ig isotypes (with the exception of α) these three domains are encoded by two additional exons called M1 and M2. Exon M1 encodes the EMPD and TMD while M2 codes for the cytoplasmic C-terminal domain. In the case of the human εH-chain gene, alternative splicing in the 3′ part of the ε locus involving exons M1 and M2 generates a number of different ε-mRNAs coding for two functional secretory (named ϵ_{S1} and ϵ_{S2}) (*2*, *3*) and two functional membrane isoforms (named ε-short, ϵ_{Sh}, and ε-long, ϵ_L) (*4*−*7*). Membrane ϵ_{Sh} and ϵ_L result from two alternative splice acceptor site usage in exon M1 and differ only in the EMPD length. While ϵ_{Sh} EMPD is composed of 14 residues, ϵ_L EMPD has 52 additional amino acids at the N-terminus (66 in total). A similar mechanism appears to take place in the α1 and α2 H-chain transcripts, generating a long (α_L) and a short (α_{Sh}) EMPD (*8*). Among all Ig isotypes, EMPDs have different lengths (13 for μ, 18 for γ1, γ2, γ3, and γ4, 27 for δ, 28 for $\alpha1_{Sh}$ and $\alpha2_{Sh}$, 34 for $\alpha1_L$ and $\alpha2_L$, 14 for ϵ_{Sh}, and 66 for ϵ_L), share abundance of acidic residues, and present a low degree of homology (Figure 1). The extra 52 amino acids of ϵ_L EMPD have no homology to any other EMPD isotype, thus excluding a possible gene duplication origin of the EMPD stretch. TMDs are highly homologous within Igs, while the cytoplasmic domains show little similarities with only three amino acids for μ and δ up to 12 for α, 26 for ε, and 28 for γ.

An interesting structural feature of γ, α_L, and ε EMPDs is the presence of cysteine residues, albeit in different numbers. While $\alpha1_L$, γ, and ϵ_{Sh} EMPDs have only one

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[1] Abbreviations: Ig, immunoglobulin; BCR, B-cell receptor; CH, heavy chain constant domain; CytoD, cytoplasmic domain; ECL, enhanced chemiluminescence; EMPD, extracellular membrane-proximal domain; FACS, fluorescence-activated cell sorter; Fc, fragment of constant regions; FITC, fluorescein isothiocyanate; H-chain, heavy chain; NEM, *N*-ethylmaleimide; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinyl difluoride; RT, reverse transcription; scFv, single-chain fragment of variable regions; SIP, small immune protein; TMD, transmembrane domain.

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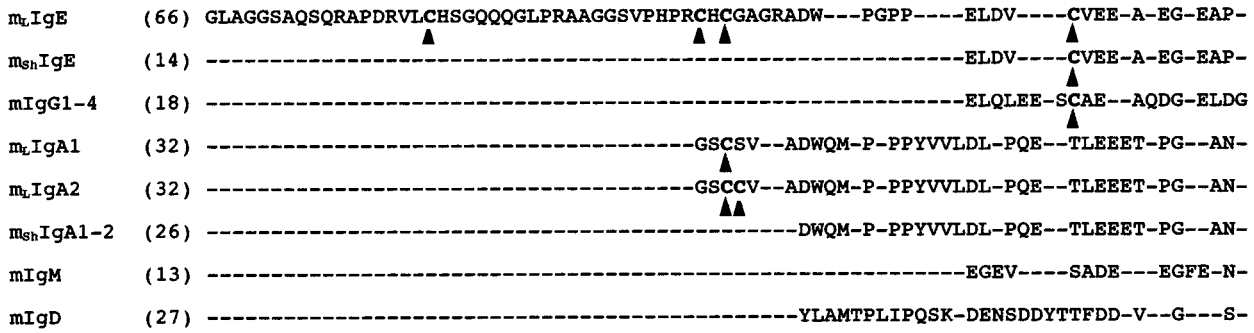

FIGURE 1: Sequence alignment of EMPDs of human membrane Ig isotypes. The number of amino acids is shown in parentheses. Arrowheads indicate cysteine residues; dashes represent missing amino acids at the corresponding positions. EMPD multiple sequence alignment was produced with ClustalW (*26*), visually inspected and manually corrected where necessary.

cysteine, $\alpha 2_L$ has two and ϵ_L EMPD has a total of four ($\alpha 1_{Sh}$ and $\alpha 2_{Sh}$ have no EMPD cysteines). In γ and ϵ_{Sh} EMPDs the unique cysteine is located in a conserved position, whereas $\alpha 1_L$-Cys and one of the two adjacent $\alpha 2_L$-Cys are more related either to Cys^{147} or to Cys^{149} of ϵ_L (Figure 1). Although the membrane long isoform of IgA2 has been described (*8*), we were unable to obtain the corresponding cDNA fragment by RT/PCR.

To analyze the disulfide bond formation in the EMPDs of the different isotypes, we followed a genetic strategy, using the membrane versions of our previously described small immune proteins (SIP). These molecules are recombinant miniantibodies where a scFv is fused to the dimerizing domain of the H-chain Fc (*9*); in the membrane version they also contain the isotype-specific EMPD, TMD, and CytoD, determining cell surface localization (mSIP, Figure 2a).

By the use of mutants of this class of molecules, we showed that the unique cysteine in α, γ, and ϵ_{Sh} EMPD and two (out of four) cysteines of ϵ_L EMPD form stable interchain disulfide bridges between the two H chains of membrane-bound immunoglobulin. On the contrary, the cysteine present in ϵ TMD is not involved in disulfide bridge formation. As a consequence, this feature should lead to a covalent stabilization of membrane Igs, in addition to the well-known hinge interchain bridges.

EXPERIMENTAL PROCEDURES

Plasmids Construction. The construction of the vectors for the expression of complete membrane ϵ heavy chains (whose structure, for the long EMPD version, is schematized in Figure 2b-I), either with the long or the short EMPD (pCIG-CϵCH4-M1′-M2 and pCIG-CϵCH4-M1-M2, respectively), has already been described (*6*, *7*). From both of these plasmids a *Bsu*36I/*Xba*I fragment, containing the ϵ CH4, EMP, TM, and cytoplasmic domains, was excised and inserted in the eukaryotic expression vector pcDNA-SIP (*9*), containing a scFv derived from a human lymphoma, arranged with the VL gene 5′ to the VH gene, and the secretion form S_2 of the human ϵ CH4 domain (J. Sepulveda and O. R. Burrone, unpublished results; *3*). The resulting constructs (pcDNA-ϵ_L-mSIP, Figure 2b-III, and pcDNA-ϵ_{Sh}-mSIP, Figure 2b-VII, respectively) contain the recombinant genes under the control of the cytomegalovirus promoter for expression in eukaryotic cells.

The cDNAs of the human $\gamma 1$ and $\alpha 1_L$ CH3 with their EMP, TM, and cytoplasmic domains were amplified by RT/PCR from mRNA extracted from normal human lymphocytes with primers 5G1 (TACTCCGGAGGCTCTGGCGGGCAGCCCCGAGAACCACA) and 3G1 (CTGAATTCTTAGGCCCCCTCTCCGATCATGT) for the $\gamma 1$ and 5A1 (TACTCCGGAGGCTCTGGCGGAAACACATTCCGGCCCGA) and 3A1 (CTGAATTCTTAGTACTGGGGGCCCTCCCT) for the $\alpha 1$. The amplified fragments were cloned with *Bsp*EI/*Eco*RI into the pcDNA-ϵ_{Sh}-mSIP vector, thus substituting the ϵ-specific portion of the molecule with the corresponding γ and α. The resulting constructs are shown in Figure 2b-XI ($\gamma 1$-mSIP) and Figure 2b-XII ($\alpha 1$-mSIP).

The plasmid pcDNA-ϵ-mSIP/ΔEMPD (Figure 2b-IX) was obtained by removing a *Xma*I/*Kas*I fragment from pcDNA-ϵ_L-mSIP and inserting a linker with the sequence CCCGGGGGATCCGCGGCGCC, containing a *Bam*HI site for further constructions.

The plasmid pcDNA-ϵ_{Sh}-mSIP/C113A (Figure 2b-VIII) was constructed by PCR amplification of the ϵ_{Sh} EMPD with primers MS-CA (GAGCTGGACGTGGCCGTGGAGGA) and MS-END (TCGGTACCTCAGAATGGGAGTGA) and cloning of the resulting fragment in pcDNA-ϵ_L-mSIP digested *Sma*I/*Sac*I.

To construct the plasmids pcDNA-ϵ_L-mSIP/C147A;C165A (Figure 2b-IV) and pcDNA-ϵ_L-mSIP/C149A;C165A (Figure 2b-V), the ϵ_L EMPD was first PCR-amplified with primer pairs EMPD-UP (TCACAGACCGTCCAGCGAGCGGTGTCT)/Cys2A (GGCTCCACAGTGAGCGCGGGGGT), EMPD-UP/Cys3A (CCGGCTCCAGCGTGGCAGCG), and EMPD-UP/Cys4A (ACGGCGCCTCGCCCTCGGCCTCCTCCACAGCCACGTCCA), then the fragment from amplification with EMPD-UP/Cys4A was cut with *Apa*I, and the longer portion (corresponding to the 3′ end of the EMPD cDNA) was annealed to either of the other two fragments and reamplified with primers EMPD-UP/Cys4A. The two resulting fragments were then cloned with *Xma*I/*Kas*I in the vector pcDNA-ϵ-mSIP/ΔEMPD.

In the so obtained plasmid pcDNA-ϵ_L-mSIP/C147A;C165A an 806 bp *Ppu*MI fragment from pcDNA-ϵ_L-mSIP was substituted, thus producing the construct pcDNA-ϵ_L-mSIP/C165A (Figure 2b-VI).

The plasmid pcDNA-ϵ-mSIP/γEMPD (Figure 2b-X) was obtained by annealing the two primers γEMPD-1 (GATCCGAGCTGCAACTGGAGGAGAGCTGTGCGGAGGCGCAGGACGGGGAGCTGGACG) and γEMPD-2 (GATCCGTCCAGCTCCCCGTCCTGCGCCTCCGCACAGCTCTCCTCCAGTTGCAGCTCG), to reconstitute a synthetic γ EMPD cDNA, and cloning the resulting fragment in the *Bam*HI site of the vector pcDNA-ϵ-mSIP/ΔEMPD.

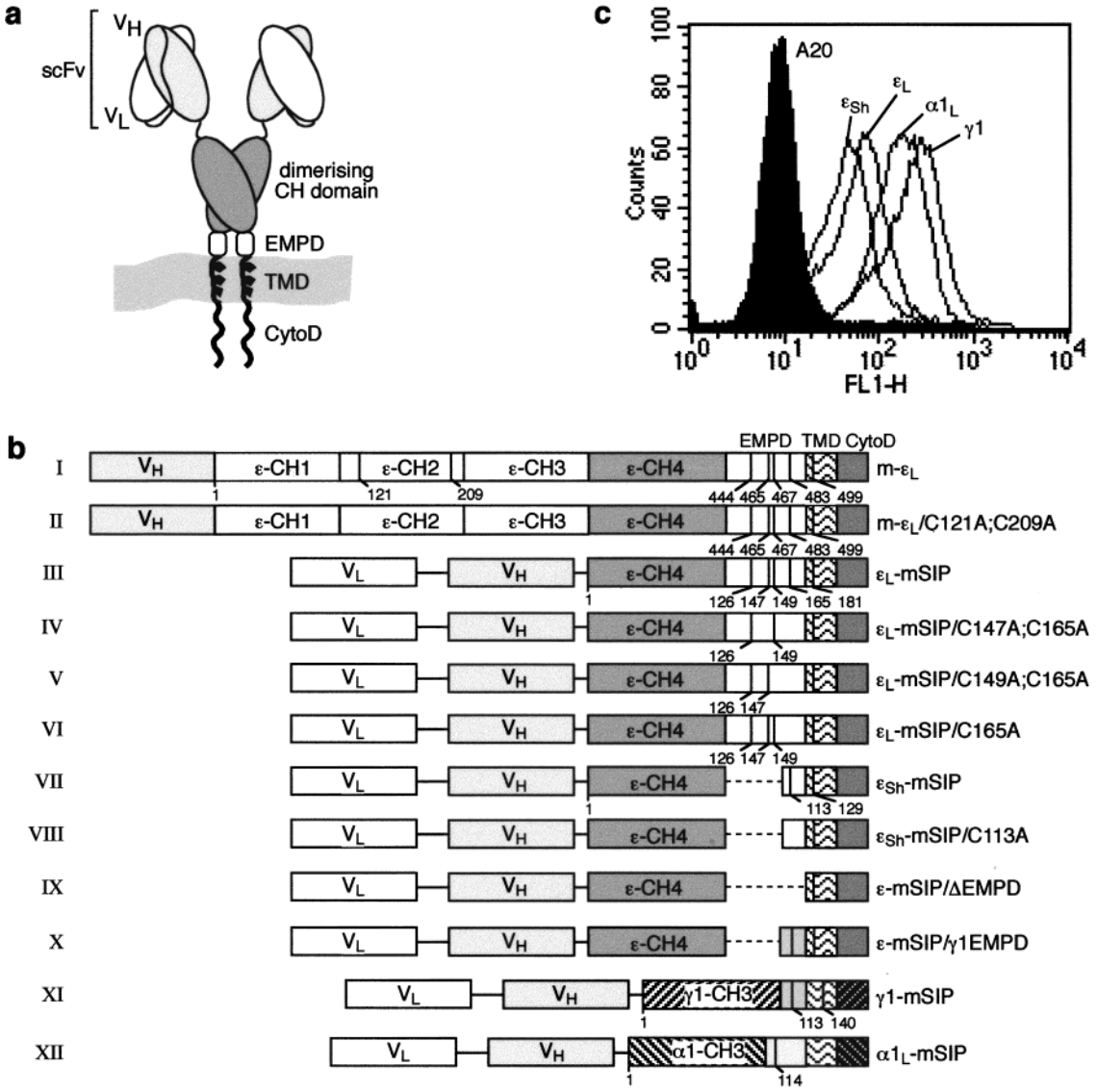

FIGURE 2: Schematic representation of membrane SIPs and FACS analysis. (a) Proposed structure of a cell surface displayed membrane SIP (mSIP). (b) Schematic representation of membrane ϵH-chain and mSIPs constructs. Residue 1 corresponds to the first amino acid on CH1 for the ϵH chain, and on CH3 or CH4 for mSIPs, as indicated. Cysteine residues are shown as vertical bars with the position number. Horizontal dashed line spacings were used to maintain alignments of the ϵ heavy chain regions. (c) Cytofluorometric analysis of A20 cells, wild type and transfected with ϵ_{Sh}-, ϵ_L-, $\alpha 1_L$-, and γ1-mSIP constructs, as indicated.

For the construction of the plasmid containing the full-length ϵ heavy chain mutated in the ϵCH2-Cys (Cys[121] and Cys[209]), pCIG-Cm-ϵ_L/C121A;C209A (Figure 2b-II), the ϵ heavy chain genomic region containing the ϵCH2 domain gene was PCR-amplified with primers pairs C1A-down (TGAAGATCTTACAGTCGTCCGCTGACGGCGGCGGGCACT)/C1A-up (CCACCAGACAGGTGATCGTGGGCGACTTGCGGA) and C2A-down (GACAGCACCAAGAAGGCTGCAGGTACGTTCCCACCTG)/C2A-up (CTGCACTGGTAGGTCTCCCCCTCGATCCAGTCTC), then the two PCR products were annealed and reamplified with primers C1A-down/C2A-up, and the resulting fragment was digested with *Bgl*II/*Hpa*I and used to replace the corresponding *Bgl*II/*Hpa*I fragment in the plasmid pCIG-CϵCH4-M1′-M2.

Cell Transfection and FACS Analysis. For each transfection about 10^6 mouse myeloma A20 (ATCC, TIB-208) cells were resuspended in 0.5 mL of cold PBS (10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.2) and put in a cuvette for electroporation with an electrode gap of 0.4 cm; 10 μg of *Pvu*I-linearized plasmid was added to the cells, and electroporation was performed with a single pulse at 960 μF, 250 V, in a Bio-Rad Gene Pulser equipped with a capacitance extender. After electroporation cells were kept for 5 min on ice, washed, resuspended in 10 mL of culture medium, and seeded in 96-well plates at a density of approximately 10^4 cells/well. After 24 h selective medium containing Geneticin (G-418 sulfate; Life Technologies, Gaithersburg, MD) at a final concentration of 400 μg/mL was added. Selected clones were screened by FACS analysis, following incubation with FITC-conjugated antibodies anti-human IgE, IgG, or IgA (Kirkegaard & Perry Laboratories, Gaithersburg, MD), where appropriated, using a FACSCalibur (Becton Dickinson) and the CellQuest software.

Immunofluorescence Analysis. Transfected A20 cells grown on glass slides (TC Chamber Slides; Nunc, Roskilde, Denmark) were washed twice with PBS and fixed for 20 min in 3% paraformaldehyde in PBS. The fixed cells were washed in PBS, quenched with 0.1 M glycine in PBS, and blocked with 3% bovine serum albumin in PBS. After blocking, cells were incubated for 1 h at room temperature

with the appropriated FITC-conjugated antibodies and then extensively washed in PBS. The slides were mounted in ProLong mounting medium (Molecular Probes, Eugene, OR) and observed with a confocal microscope Axiovert 100 M (Zeiss).

Biotinylation of Membrane Proteins and Immunoprecipitation. Surface membrane proteins were biotinylated with the Amersham ECL protein biotinylation system (Amersham-Pharmacia Biotech), following the directions provided by the manufacturer, with minor modifications. Transfected cells (about 10^7) were washed twice with cold PBS and then resuspended in 0.5 mL of ice-cold biotinylation buffer (40 mM $NaHCO_3/NaCO_3$ buffer, pH 8.6, 100 mM NaCl). Forty microliters of biotinylation reagent (provided with the kit) was next added, and cells were incubated for 30 min at 4 °C on an orbital shaker. After being washed with cold PBS, the cells were lysed in 100 μL of TNN buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% NP-40), containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 0.8 μM aprotinin, 40 μM bestatin, 22 μM leupeptin, and 15 μM pepstatin A, all from Sigma) and 20 mM alkylating agent *N*-ethylmaleimide (NEM, Fluka), which prevents rearrangement of disulfide bonds (*10*). The cell lysates were centrifuged for 10 min at 10000*g* at 4 °C, and the supernatants were preadsorbed onto protein A−agarose (Repligen, Needham, MA), to remove A20 surface IgG, then mixed with 5 μL (5 μg) of the relevant antibody (anti-human IgE for all of the ϵ-mSIP constructs, anti-human IgA and anti-human IgG for the α-mSIP and γ-mSIP, respectively, all from Kirkegaard & Perry Laboratories), and incubated for 2 h at 4 °C. Immune complexes were precipitated with 25 μL of protein A−agarose. Beads were washed with 2 mL of TNN, 2 mL of TNNB (TNN + 1% bovine serum albumin), 2 mL of RIPA buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM $MgCl_2$, 1% NP-40, 1% deoxycholate, 0.1% SDS), and 2 mL of PBS (all washing solutions containing 20 mM NEM). Immune complexes bound to protein A were eluted with 50 μL of the SDS sample buffer (30 mM Tris-HCl, pH 6.8, 1.5% SDS, 10% glycerol, 0.1 mg/mL bromophenol blue, 20 mM NEM), either reducing (with 2.5% β-mercaptoethanol) or nonreducing (without β-mercaptoethanol), and loaded onto 10% SDS−polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a PVDF membrane (Millipore) and detected with peroxidase-labeled streptavidin and the chemiluminescent reagent provided with the ECL kit.

Membrane Extract and Western Blotting. About 4×10^7 cells were collected and washed twice in cold PBS. Cells were then resuspended at a concentration of about 10^7/mL in hypotonic buffer (20% PBS containing 0.25 M sucrose, 20 mM NEM, and protease inhibitors as above) and lysed by 3−4 freeze−thawing cycles. The suspension was centrifuged for 10 min at 700*g* to separate nuclei and unlysed cells, and then the supernatant was ultracentrifuged for 1 h at 100000*g*. The membrane pellet was resuspended in 400 μL of TNN buffer (see above) with 20 mM NEM and protease inhibitors. Twenty microliters of this membrane extract was separated in SDS−polyacrylamide gel, either in reducing or in nonreducing conditions, transferred to PVDF membrane, probed with peroxidase-labeled anti-human IgE antibodies (Kirkegaard & Perry Laboratories), and detected with the ECL chemiluminescent reagent (Amersham-Pharmacia Biotech).

RESULTS

To understand the role of EMPD cysteine residues in the assembly of membrane Igs, we followed a genetic strategy. Membrane versions of our previously described small immune proteins (SIPs) were constructed and expressed in the A20 B-cell line. SIPs are dimeric molecules in which a scFv is fused to the dimerizing domain of the H-chain Fc (CH3 for γ and α and CH4 for ϵ). We have previously shown that secretory SIPs are efficiently folded, assembled, and secreted in dimeric form for both γ and ϵ isotypes (J. Sepulveda and O. R. Burrone, unpublished observations; *9*). Secretory ϵ-SIP was derived from both ϵ_{s1} and ϵ_{s2}, the latter being an alternatively spliced secretory isoform (*2*, *3*), containing a distinct CH4 C-terminal tailpiece, with a cysteine in the last position involved in an interchain bridge (*3*). Membrane SIPs (mSIPs) have, in addition to the dimerizing CH3 or CH4 domain, the isotype-specific EMPD, TMD, and CytoD determining cell surface localization (Figure 2a). Schematic representations of the constructs used in this work are shown in Figure 2b. In all of the mSIPs generated, the cysteines participating in H−H interchain disulfide bridges of the full-length H-chain are absent. Therefore, the only possible interchain disulfide bonds should derive from EMPD cysteines or from the single cysteine in γ and ϵ TMD.

Transfection in A20 cells of mSIPs corresponding to ϵ_L, ϵ_{Sh}, γ1, and $\alpha 1_L$ (whose structures are represented in Figure 2b-III, 2b-VII, 2b-XI, and 2b-XII, respectively) yielded efficient constitutive expression of the proteins on the cell surfaces (Figure 2c). These molecules were also efficiently expressed as surface proteins in myeloma Sp2/0 and in CHO cells (*11*).

To investigate whether ϵ_{Sh}-, ϵ_L-, $\alpha 1_L$-, and γ1-mSIPs were expressed on the cell membrane as covalently linked dimers via disulfide bonds, we performed surface biotinylation of living cells followed by immunoprecipitation and SDS−PAGE analysis. As shown in Figure 3a, a single band of the expected size for the monomeric proteins was obtained in reducing conditions for all of the constructs. On the other hand, under nonreducing conditions all of the material recovered had a mobility corresponding to dimers. These results indicated that EMPD and/or TMD cysteines form interchain S−S bridges. However, $\alpha 1_L$ does not contain a TMD cysteine, demonstrating that Cys^{114} of $\alpha 1_L$ EMPD was directly involved in the interchain S−S bond. This suggested that the cysteine residue present in the TMD of other isotypes (i.e., γ and ϵ) may also not participate in covalent bonding. The above assumption was indeed confirmed with two additional ϵ-mSIP constructs: the EMPD deleted version (ϵ-mSIP/ΔEMPD, Figure 2b-IX) and a mutant of ϵ_{Sh} in which the EMPD Cys^{113} was mutated to alanine (ϵ_{Sh}-mSIP/C113A, Figure 2b-VIII). Both ϵ_{Sh}-mSIP/C113A and ϵ-mSIP/ΔEMPD were efficiently expressed on the cell surface and appeared as monomers in nonreducing SDS−PAGE (Figure 3b, lanes 2 and 3), demonstrating that the cysteine in the TMD does not form an interchain S−S bond. In addition, grafting of γ1 EMPD into ϵ-mSIP/ΔEMPD restored the ability of the chimera (ϵ-mSIP/γ1EMPD, Figure 2b-X) to dimerize co-

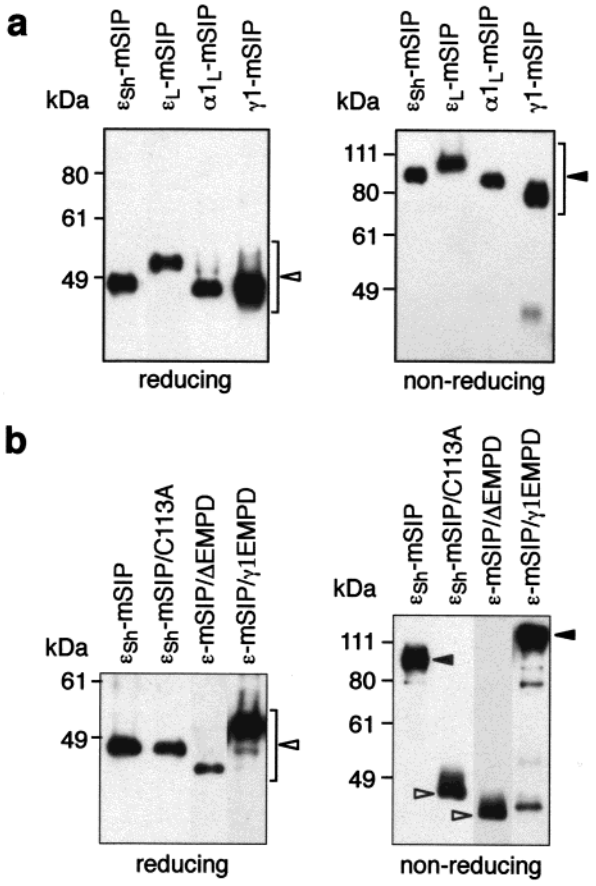

FIGURE 3: SDS−PAGE analysis of immunoprecipitated, surface biotinylated mSIPs. Extracts from surface-biotinylated cells expressing ϵ_{Sh}-, ϵ_L-, $\alpha 1_L$-, and $\gamma 1$-mSIP (a) and ϵ_{Sh}-mSIP, ϵ_{Sh}-mSIP/C113A, ϵ-mSIP/γ1EMPD, and ϵ-mSIP/ΔEMPD (b) were immunoprecipitated with the appropriated antibodies (anti-ϵ, anti-α, or anti-γ chains), separated in SDS−PAGE in reducing and nonreducing conditions, as indicated, and revealed by incubation with peroxidase-labeled streptavidin. Open and filled arrowheads indicate monomeric and dimeric forms, respectively.

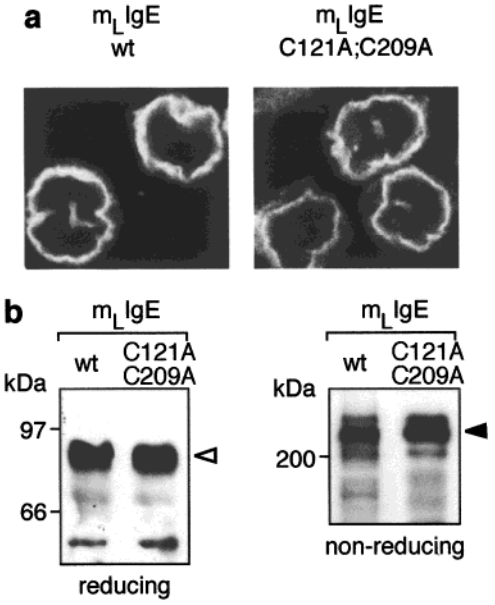

FIGURE 4: Immunofluorescence and Western blotting of cell membrane IgE. (a) Immunofluorescence of optical sections of intact A20 cells expressing wild-type (wt) and mutant (C121A;C209A) m_LIgE, using a FITC-conjugated anti-human IgE, showing cell surface restricted expression of the transfected proteins. (b) Western blotting of membrane extracts from A20 cells expressing wt and mutant m_LIgE, resolved in reducing and nonreducing SDS−PAGE, as indicated, and probed with peroxidase labeled anti-human IgE antibodies. Open and filled arrowheads indicate the band corresponding to ϵH chain and to fully assembled H_2L_2 IgE, respectively.

valently (Figure 3b, lane 4). Thus, our results showed the presence of interchain disulfide bridges formed by cysteines in the EMPDs of membrane-bound Igs, suggesting an important structural role for these moieties.

This conclusion was further confirmed by the expression of a complete membrane IgE mutant, following transfection of a full-length membrane ϵ_L H chain construct in which Cys^{121} and Cys^{209} were mutated to alanines (m-ϵ_L/C121A; C209A, Figure 2b-II). The above cysteines, located respectively N- and C-terminally to ϵCH2, are known to form the interchain bridges between H chains of IgE, basically replacing the hinge region of other Ig isotypes (*12*). This double mutant was expressed on the cell surface, as revealed by surface immunofluorescence staining (Figure 4a). As expected, nonreducing SDS−PAGE analysis demonstrated that m-ϵ_L/C121A;C209A formed covalent dimers (Figure 4b), proving that cysteines in ϵ_L EMPD participate in the disulfide bond stabilization of membrane IgE.

However, ϵ_L EMPD contains three other cysteines in addition to Cys^{165} (equivalent to Cys^{113} in ϵ_{Sh}) in positions 126, 147, and 149. To complete the possible ϵ_L EMPD disulfide pattern, we analyzed three additional ϵ_L-mSIP mutants, two of which were double mutants (ϵ_L-mSIP/C147A;C165A, Figure 2b-IV, and ϵ_L-mSIP/C149A;C165A, Figure 2b-V), with either Cys^{147}/Cys^{165} or Cys^{149}/Cys^{165} simultaneously mutated to alanines, and one was a single mutant (C165A, Figure 2b-VI).

Following cell surface biotinylation and immunoprecipitation, the two double mutants appeared exclusively as monomers in nonreducing SDS−PAGE, whereas the single C165A mutant migrated as a dimer (Figure 5). Taken together, these results indicate that two of the four cysteines are involved in interchain SS bonds (Cys^{165} and either Cys^{147} or Cys^{149}), and the two remaining should likely form an intrachain bond (Cys^{126} linked to either Cys^{147} or Cys^{149}).

Therefore, we have demonstrated that membrane IgA, IgG, and IgE are further stabilized via disulfide bridges occurring within their EMPDs.

DISCUSSION

Membrane immunoglobulins (all isotypes) differ from their secretory counterparts for three additional domains, namely, the extracellular membrane-proximal domain (EMPD), the transmembrane domain (TMD), and the cytoplasmic domain (CytoD). The transmembrane domain, in addition to supporting the insertion of immunoglobulins in the cell membrane, may conceal a relevant role for the assembly of the B-cell receptor with the other accessory molecules of the complex, such as CD79a (Igα) and CD79b (Igβ) (*13*−*15*). The cytoplasmic portion of membrane Igs (CytoD) shows high variability in sequence and length, thus excluding possible common features among Ig isotypes. Finally, the structure and function of the extracellular membrane-proximal region (EMPD) of cell-bound Igs are largely unknown. The extracellular location, together with a topology bridging the antibody Fc region to the cell membrane

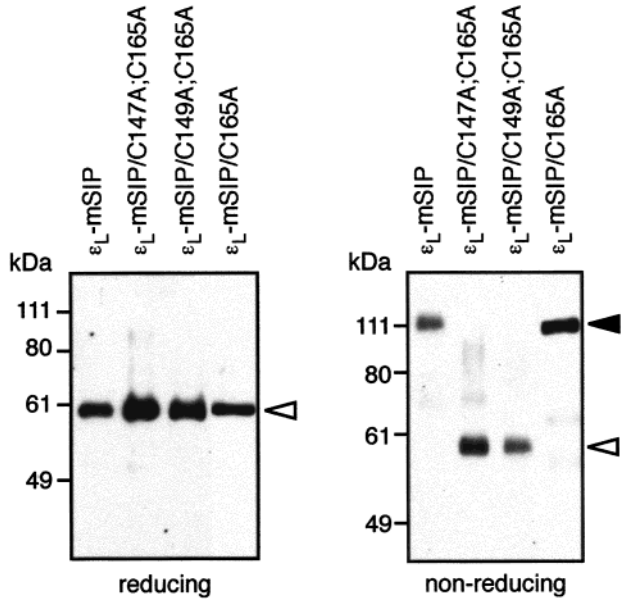

FIGURE 5: Surface biotinylation of ϵ_L-mSIP mutants. SDS−PAGE analysis of immunoprecipitated, surface biotinylated ϵ_L-mSIP, double mutants ϵ_L-mSIP/C147A;C165A and ϵ_L-mSIP/C149A;C165A, and single mutant ϵ_L-mSIP/C165A, analyzed under reducing and nonreducing conditions, as indicated. Open and filled arrowheads indicate monomeric and dimeric forms, respectively.

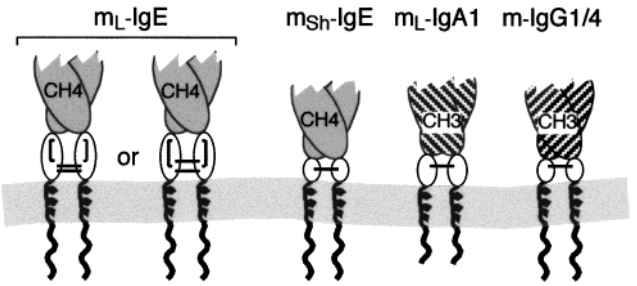

FIGURE 6: Proposed model of disulfide bonds occurring within cysteines of membrane Ig EMPDs. Horizontal and vertical bars indicate intrachain and interchain disulfide bonds, respectively.

environment, likely dictates a crucial role for Igs EMPD in BCR assembly and performance. In this context, EMPD relative sequence similarities may reflect a common structural role, while permitting isotype-specific functions.

In particular, membrane Igs are associated with the accessory glycoproteins Igα and Igβ to form the corresponding BCR (*1*, *16*). We have previously reported that differences in the level of terminal glycosylation of Igα molecules, associated with either the short or the long alternative splice membrane variants of human IgE, depend exclusively on the sequence of the ϵ EMPD (*7*). Similarly, differences in the level of glycosylation of the Igα molecules associated to either IgM or IgD have been described and suggested to depend on the sequences of the corresponding EMPDs (*17*, *18*). In addition, different sets of BCR-associated proteins, specific for the IgE-BCR (named ϵ-BAPs), have been reported, and they were demonstrated to differentially associate with the membrane short and long ϵ-chain through their EMPDs (*7*).

An interesting structural feature of γ, α, and ϵ EMPDs is the presence of cysteine residues, albeit in different numbers. We have investigated the role of cysteine residues present in Ig EMPD in the stabilization of membrane-bound immunoglobulins. Using a genetic strategy, we engineered short antibody-like molecules deprived of any known intermolecular disulfide-forming cysteine. These downsized antibodies represent the membrane version of SIPs (small immune proteins), recombinant molecules in which a scFv is fused to the dimerizing C-terminal heavy chain constant domain of different Ig isotypes (*9*). Isotype-specific SIPs were fused to the corresponding isotype-specific EMP, TM, and Cyto domains, thus obtaining Ig-like membrane molecules.

Here we show that the single cysteine present in $\alpha 1_L$, $\gamma 1$, and ϵ_{Sh} EMPD forms an interchain disulfide bridge. Deletion of the EMPD in the ϵ version resulted in a membrane molecule lacking disulfide bonds despite the presence of the cysteine in the TM domain. Grafting of $\gamma 1$ EMPD into ϵ membrane SIP reconstituted the covalent dimerization in the cell surface exposed protein, indicating that $\gamma 1$ and ϵ_{Sh} EMPD may assume a similar structural role in the context of a membrane Ig.

In addition, by mutating the cysteine in ϵ_{Sh} EMPD, we excluded any reactivity of the cysteine residue present in the transmembrane domain.

In all of the assays performed, disulfide bond interchange after cell lysis was prevented by alkylating the cell extraxts with *N*-ethylmaleimide (NEM; *10*).

Unlike the others, ϵ_L EMPD contains four cysteines. By mutagenesis studies we propose a disulfide pattern in which two cysteines, Cys^{147} (or Cys^{149}) and Cys^{165}, form intermolecular bonds and the remaining Cys^{126} and Cys^{149} (or Cys^{147}) are likely to form an intramolecular bond. Therefore, as opposed to all other isotypes, ϵ_L EMPD presents a more complex conformation, both for its substantially longer sequence and for its pattern of disulfide bonds.

Dimerization via disulfide bridges within an extracellular membrane-proximal stalk appears frequently in Ig-like receptors (e.g., CTLA-4 and gp49A). CTLA-4 presents a striking similarity to membrane Igs, since 3D structure determination of its Ig module (*19*) revealed a dimerization mode very similar to the C-terminal Ig heavy chain domain (e.g., γ CH3). In addition, like mIgA, mIgG, and IgE, CTLA-4 presents an intermolecular disulfide bond in its EMPD (*20*). A recent report on the murine Ig-like receptor gp49A (*21*) revealed the presence of an EMPD cysteine residue undergoing homodimerization. Mutation of this cysteine to alanine (*21*) abrogated the association of gp49A with raft membrane microdomains (*22*, *23*), suggesting a possible parallel with rafts association of BCRs (*24*) mediated by membrane Ig EMPD.

Membrane Ig EMPDs were previously predicted to be in α-helical conformation, although the study was performed with synthetic peptides, without considering the role of cysteine residues (*25*). However, one cannot exclude that the Ig EMPD structure may be influenced by the proximity of the membrane Ig domains and interactions with other BCR components.

In perspective, it will be interesting to see whether, in addition to their structural role, different isotype EMPDs directly regulate BCR functions or influence differential association of membrane Igs with accessory proteins to constitute isotype-specific BCRs.

REFERENCES

1. Reth, M. (1994) *Curr. Opin. Immunol. 6*, 3−8.
2. Zhang, K., Max, E. E., Cheah, H. K., and Saxon, A. (1994) *J. Biol. Chem. 269*, 456−462.
3. Batista, F. D., Efremov, D. G., and Burrone, O. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3399−3404.
4. Peng, C., Davis, F. M., Sun, L. K., Liou, R. S., Kim, Y. W., and Chang, T. W. (1992) *J. Immunol. 148*, 129−136.
5. Zhang, K., Saxon, A., and Max, E. E. (1992) *J. Exp. Med. 176*, 233−243.
6. Batista, F. D., Efremov, D. G., and Burrone, O. R. (1995) *J. Immunol. 154*, 209−218.
7. Batista, F. D., Anand, S., Presani, G., Efremov, D. G., and Burrone, O. R. (1996) *J. Exp. Med. 184*, 2197−2205.
8. Yu, L. M., Peng, C., Starnes, S. M., Liou, R. S., and Chang, T. W. (1990) *J. Immunol. 145*, 3932−3936.
9. Li, E., Pedraza, A., Bestagno, M., Mancardi, S., Sanchez, R., and Burrone, O. (1997) *Protein Eng. 10*, 731−736.
10. Braakman, I., Helenius, J., and Helenius, A. (1992) *Nature 356*, 260−262.
11. Benvenuti, F., and Burrone, O. R. (2001) *Gene Ther.* (in press).
12. Helm, B. A., Ling, Y., Teale, C., Padlan, E. A., and Bruggemann, M. (1991) *Eur. J. Immunol. 21*, 1543−1548.
13. Grupp, S. A., Campbell, K., Mitchell, R. N., Cambier, J. C., and Abbas, A. K. (1993) *J. Biol. Chem. 268*, 25776−25779.
14. Stevens, T. L., Blum, J. H., Foy, S. P., Matsuuchi, L., and DeFranco, A. L. (1994) *J. Immunol. 152*, 4397−4406.
15. Schamel, W. W. A., and Reth, M. (2000) *Immunity 13*, 5−14.
16. Venkitaraman, A. R., Williams, G. T., Dariavach, P., and Neuberger, M. S. (1991) *Nature 352*, 777−781.
17. Campbell, K. S., Hager, E. J., and Cambier, J. C. (1991) *J. Immunol. 147*, 1575−1580.
18. Pogue, S. L., and Goodnow, C. C. (1994) *J. Immunol. 152*, 3925−3934.
19. Ostrov, D. A., Shi, W., Schwartz, J. C., Almo, S. C., and Nathenson, S. G. (2000) *Science 290*, 816−819.
20. Greene, J. L., Leytze, G. M., Emswiler, J., Peach, R., Bajorath, J., Cosand, W., and Linsley, P. S. (1996) *J. Biol. Chem. 271*, 26762−26771.
21. Lee, K. H., Ono, M., Inui, M., Yuasa, T., and Takai, T. (2000) *J. Immunol. 165*, 4970−4977.
22. Simons, K., and Ikonen, E. (1997) *Nature 387*, 569−572.
23. Langlet, C., Bernard, A. M., Drevot, P., and He, H. T. (2000) *Curr. Opin. Immunol. 12*, 250−255.
24. Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999) *J. Exp. Med. 190*, 1549−1560.
25. Major, J., Jr., Davis, F. M., Liou, R. S., and Chang, T. W. (1996) *Mol. Immunol. 33*, 179−187.
26. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.

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