Covalent Homodimers of Murine Secretory Component Induced by Epitope Substitution Unravel the Capacity of the Polymeric Ig Receptor to Dimerize Noncovalently in the Absence of IgA Ligand*

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Recombinant secretory immunoglobulin A containing a bacterial epitope in domain I of the secretory component (SC) moiety can serve as a mucosal delivery vehicle triggering both mucosal and systemic responses (Corthésy, B., Kaufmann, M., Phalipon, A., Peitsch, M., Neutra, M. R., and Kraehenbuhl, J.-P. (1996) J. Biol. Chem. 271, 33670–33677). To load recombinant secretory IgA with multiple B and T epitopes and extend its biological functions, we selected, based on molecular modeling, five surface-exposed sites in domains II and III of murine SC. Loops predicted to be exposed at the surface of SC domains were replaced with the DYKDDDDK octapeptide (FLAG). Another two mutants were obtained with the FLAG inserted in between domains II and III or at the carboxyl terminus of SC. As shown by mass spectrometry, internal substitution of the FLAG into four of the mutants induced the formation of disulfide-linked homodimers. Three of the dimers and two of the monomers from SC mutants could be affinity-purified using an antibody to the FLAG, mapping them as candidates for insertion. FLAG-induced dimerization also occurred with the polymeric immunoglobulin receptor (pIgR) and might reflect the so-far nondemonstrated capacity of the receptor to oligomerize. By co-expressing in COS-7 cells and epithelial Caco-2 cells two pIgR constructs tagged at the carboxyl terminus with hexahistidine or FLAG, we provide the strongest evidence reported to date that the pIgR dimerizes noncovalently in the plasma membrane in the absence of polymeric IgA ligand. The implication of this finding is discussed in terms of IgA transport and specific antibody response at mucosal surfaces. Clinical relevance IgAs can be produced by hybridomas and have been shown in animal models to be protective against challenge with mucosal pathogens (1–7). Association in vitro of recombinant secretory component (SC) and dimeric IgA (IgA2)

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Recent, we designed a novel potential delivery shuttle for combined active/passive immunization by assembling in vitro IgA2 with recombinant rabbit SC bearing a pathogen-derived epitope in the E-F loop of domain I (10). Mice immunized orally with this complex developed an antibody response directed at both the heterologous SC and the epitope, which was surface-exposed. However, the antigenized SC was very poorly secreted and mostly retained in the early secretion pathway. This study revealed a novel function for domain I in SC/pIgR secretion, besides its role in the initial, high affinity, binding to IgA1 (11–17).

This unexpected drawback prompted us to identify other potential sites of insertion for linear epitopes that will not affect secretion or association with IgA1. We concentrated our mapping analysis on domains II and III of SC because they do not play any documented role in IgA binding and are even missing in certain rabbit alleles coding for a truncated, yet active, pIgR (17, 18). Domain I of rabbit SC exhibits two anti-parallel β-pleated sheets shaped by nine rigid successive β-strands connected by protruding flexible loops (17). Molecular models of mSC domains II and III were constructed and shown to fold with a similar pattern.

A selection of loops was replaced by the FLAG octapeptide for two major reasons. 1) The peptide is highly hydrophilic and thus more prone to remain exposed on the molecule surface; 2) recognition of the peptide by the commercial monoclonal antibody M2 confirms its surface exposure. To facilitate the interpretation of the data, target sites were modified individually. Surprisingly, epitope substitution in most locations inside SC domains II and III induced the formation of SC homodimers through disulfide bridges. In the SC dimer, structural constraints or buried residues modulate the M2 reactivity against each mutant differentially, yielding clues about its topology of association. This implies that several insertion sites in the new dimeric SC we have generated and analyzed either expose a...
was deleted according to the strategy described in Fig. 1B, using primers mSCBglMet(+), and mSCKpn1(−) (Table I), yielding plasmid pCB6mpIgR(-5′). To allow expression of wild type mSC and mSC-FLAG:Cterm, the 3′ Kpn1-EcoRI fragment of the constructs in Fig. 1A was introduced into pCB6mpIgR(-5′) cut with the same two enzymes. (a) Receiver into recombinant PCR (23) to introduce into all the BglII sites in one copy of the FLAG coding sequence at sites defined according to the molecular models (Table II and Fig. 2). “Inside” primers were designed to contain the FLAG coding sequence as the overlapping region, e.g. primers FLD1cdr3(−) and FLD1cdr3(+) (Table I). “Outside” primers were designed to permit the amplification of products exhibiting restriction sites adapted to the substitution of wild type mSC sequences in pCB6. The pairs of outside primers and restriction enzymes used were: mSCBglMet(+), and mSCNhe1(−), with BglII and NheI (domain I mutant); mSCKpn1(+) and mSCNhe1(−), with Kpn1 and NheI (domain II mutant); mSCNhe1(+) and mCB6(−), with NheI and EcoRI (domain III mutant). The FLAG coding sequence was inserted between domains II and III (between residues 215 and 216) by using two successive PCRs. The PCR product amplified with primers mpIgRFLin(−) and mpIgRFLout(−) was recovered from an agarose gel and reamplified with primers mSCNhe1(+) and D3D2FLout(−) to elongate the domain II carboxyl terminus by the FLAG sequence and a NheI site. Insertion of the Kpn I-NheI-digested product resulted in the generation of plasmid pCB6mpIgR(-5′). The regions that have been amplified by PCR are schematized in Fig. 3. For tagging the receptor with a FLAG epitope following Asp947 (deleting the last five residues), mpIgR coding sequences were first amplified by PCR using mSCNhe1(+) and mpIgRFLout(−) (Table I). The gel-purified PCR fragment was reamplified using primers mSCNhe1(+) and mpIgRFLin(−) (Table I). The same strategy was followed to append five histidine residues after His59 of mpIgR (deleting the last six residues), using primers mpIgRFLin(+), mpIgRFLout(−), and mpIgRFLin(−), and mpIgRFLout(−) in the two sequential PCR amplifications, yielding plasmid pCB6mpIgR:FLAG(5′-5′). The same strategy was followed to append five histidine residues after His59 of mpIgR (deleting the last six residues), using primers mpIgRFLin(+), mpIgRFLout(−), and mpIgRFLin(−), and mpIgRFLout(−) in the two sequential PCR amplifications, yielding plasmid pCB6mpIgR:FLAG(5′-5′). The same strategy was followed to append five histidine residues after His59 of mpIgR (deleting the last six residues), using primers mpIgRFLin(+), mpIgRFLout(−), and mpIgRFLin(−), and mpIgRFLout(−) in the two sequential PCR amplifications, yielding plasmid pCB6mpIgR:FLAG(5′-5′). The same strategy was followed to append five histidine residues after His59 of mpIgR (deleting the last six residues), using primers mpIgRFLin(+), mpIgRFLout(−), and mpIgRFLin(−), and mpIgRFLout(−) in the two sequential PCR amplifications, yielding plasmid pCB6mpIgR:FLAG(5′-5′).
acids, 0.1% transferrin (Life Technologies), and 10 mM of the pIgR (see “Results”). The cell lysate was transferred to a fresh
of PBS. The addition of leupeptin was required to preserve the integrity
and lysed by scraping in the presence of 2 ml of 10 mM Tris-HCl (pH
sion of mpIgR derivatives, cells were then seeded into a 175-cm² T-flask
(Qiagen) according to the manufacturer's instructions. To allow expres-
the lower line.

4 °C until use.

TABLE I

| Loop replaced in mSC | Sequence | Position | Net change in charge |
|----------------------|----------|----------|---------------------|
| Domain I, loop P–G (CDR3-like) | GLGTSENRG | 93–100 | –4 |
| Domain II, loop B–C (CDR1-like) | PPFSENVY | 135–142 | –4 |
| Domain II, loop D–E (CDR2-like) | FMKGTDLT | 176–183 | –3 |
| Domain III, loop C’-C’ | RMMKET | 254–259 | –4 |
| Domain III, loop D–E (CDR2-like) | TFPDDNQR | 281–288 | –3 |
| FLAG epitope | DYYKDDDDK | | |

* The global charge is compared with that of wild type mSC. Note that an excess of three negative charges is introduced when the FLAG is placed
in the lower line.

Residues are numbered according to Piskurich et al. (20). Boldface residues are conserved after replacement with the FLAG sequence given in the lower line.

**Purification of mSC Proteins Engineered with the FLAG Epitope**

All chromatographic steps were performed at 4 °C. Glycosylated recombinant mSC-FLAG proteins were enriched from COS-7 cell-conditioned media on 2-mL ConA agarose (Vector Laboratories) columns equilibrated in binding buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂). After extensive washing, elution was performed following an overnight incubation at 4 °C with binding buffer containing 0.5 mM α-methyl-mannopyranoside (Sigma), and the eluates were concentrated using Centricon-50 cartridges in PBS containing 0.02% (w/v) NaN₃.

A portion of each preparation was then applied to individual 1-mL M2 affinity gel columns equilibrated in TBS buffer (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl (pH 7.5)). After extensive washing with TBS, mSC-FLAG proteins were eluted with 7 column volumes of TBS containing 50 μg/mL FLAG peptide (DYKDDDDK, synthesized at the Institut de Biochimie, Université de Lausanne, Switzerland). The peptide was washed out by repeated passages over a Centricon-50 cartridge (Amicon), whereas the buffer was exchanged for PBS containing 0.02% (w/v) NaN₃. Purified proteins were stored at 4 °C until further use. The columns could be used several times after sequential treatment with 0.1 M glycine (pH 3.0) and 1 M Tris-HCl (pH 8.0) without loss of binding capacity. Gel Filtration Chromatography

Gel filtration chromatography was performed at 4 °C. To study the monomeric or polymeric nature of mSC-FLAG polypeptides, 0.2-ml samples were injected into a 30 × 1-cm Superose 12 HR 10/30 column coupled to a fast protein liquid chromatography system (Amersham Pharmacia Biotech) and run in PBS at a flow rate of 0.5 ml/min. Fractions of 250 μl were siliconized 1.5-ml tube, and cell debris and nuclei were pelleted at 3,000 × g by centrifugation. Aliquots of the clarified lysate were stored at –20 °C before use.
recovered, and 15-µl aliquots were analyzed by immunodetection using the antisera described above.

**Capture ELISA for mSC**

The wells of Nunc MaxiSorp ELISA plates were coated overnight at 4 °C with 50 µl of affinity-purified IgG to native mSC (54) (2 µg/ml in 50 mM sodium carbonate/bicarbonate (pH 9.6)). Wells were blocked for 30 min at room temperature with 0.2 ml of TBS buffer containing 5% (w/v) nonfat dry milk and 0.05% (w/v) Tween-20 (Bio-Rad). Samples containing mSC proteins were serially diluted into 50 µl of PBS and incubated for 1 h at room temperature. 50 µg/well of purified mSC-FLAG:Cterm (0 to 200 ng/ml) was used as a standard. After washing with TBS containing 0.05% Tween-20, bound mSC was detected using biotinylated IgG (40 µg/ml) to native mSC. HRP-coupled ExtrAvidin (Sigma; 1:1,000) was developed with 50 µl of 1,2-phenylenediamine as a chromogen. The reaction was stopped with 50 µl of 2 M H2SO4, and plates were read at 492 nm using 620 nm as reference wavelength.

**Immunoblotting of mSC-FLAG Proteins**

Crude lysates, mSC-FLAG preparations, affinity-purified proteins, or immunoprecipitated materials were separated in 6 or 8% denaturing polyacrylamide gels with or without 0.1 mM dithiothreitol. SDS-PAGE and immunoblotting were performed as described in Rindisbacher et al. (8) using polyvinylidine difluoride membranes (BDH Laboratory supplies). Primary antibodies used were the rabbit IgG to denatured or native mSC (5 µg/ml, purified on protein A-Sepharose) or the mAb M2 to FLAG (0.75 µg/ml). Bound antibodies were detected using appropriate HRP-conjugated secondary reagents (all used at 1:3,000 dilution) and the ECL reagents from Amersham Pharmacia Biotech.

**Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI MS)**

Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was obtained from Aldrich, HPLC grade trifluoroacetic acid was purchased from Pierce; HPLC grade water came from Romil Ltd (Cambridge, U.K.), and acetonitrile was provided by Biosolve Ltd (Barneveld, Netherlands). All the other chemicals were of the highest purity and were used without further purification.

The buffer of protein samples was exchanged against 50 mM ammonium bicarbonate using a Centricon-50 cartridge, lyophilized, and dissolved in 0.1% aqueous trifluoroacetic acid at a concentration of 12.5 pmol/µl. 1 µl of the protein solution was mixed with 9 µl of sinapinic acid (40 mg/ml) in 0.1% trifluoroacetic acid, H2O:acetonitrile (2:1 v/v). 1 µl of the mixture was applied on the gold-plated target and air-dried before transfer into the source of the mass spectrometer. MALDI mass spectra were obtained on a Perseptive Biosystems Voyager RP™ mass spectrometer using a 337-nm nitrogen laser, a 25-kV accelerating potential, and a delayed extraction time of 300 ns. Samples were optimally controlled using a video camera. External calibration of the MALDI spectra in the linear mode was carried out using bovine serum albumin.

**Immunoprecipitations**

mSC—Identical amounts of each mSC protein were incubated for 4 h at 4 °C under gentle rocking with 0.5 ml of TBS buffer containing 60 µl of M2 affinity gel. After centrifugation, beads were washed with TBS and eluted overnight at 4 °C with 45 µl of 0.1% trifluoroacetic acid, H2O:acetonitrile (2:1 v/v). 1 µl of the mixture was applied on the gold-plated target and air-dried before transfer into the source of the mass spectrometer. MALDI mass spectra were obtained on a Perseptive Biosystems Voyager RP™ mass spectrometer using a 337-nm nitrogen laser, a 25-kV accelerating potential, and a delayed extraction time of 300 ns. Samples were optimally controlled using a video camera. External calibration of the MALDI spectra in the linear mode was carried out using bovine serum albumin.

**FIG. 2. Ribbon representation of domains of wild type and engineered mSC.** Panel A represents a ribbon model of domain II and III of wild type mSC. Panels B–F depict ribbon models of mSC mutants carrying the FLAG epitope in surface-exposed areas of domain II and domain III. B, mSC-FLAG:D2:BC; C, mSC-FLAG:D2:DE; D, mSC-FLAG:D2-D3; E, mSC-FLAG:D3:DE; F, mSC-FLAG:D3:CC. Amino acid sequences comprising the FLAG octapeptide are colored in red. Orange rods connecting the blue peptide backbone represent disulfide bridges.

**Ligand-independent Dimerization of Murine pIgR**

Crude lysates, mSC-FLAG preparations, affinity-purified proteins, or immunoprecipitated materials were separated in 6 or 8% denaturing polyacrylamide gels with or without 0.1 mM dithiothreitol. SDS-PAGE and immunoblotting were performed as described in Rindisbacher et al. (8) using polyvinylidine difluoride membranes (BDH Laboratory supplies). Primary antibodies used were the rabbit IgG to denatured or native mSC (5 µg/ml, purified on protein A-Sepharose) or the mAb M2 to FLAG (0.75 µg/ml). Bound antibodies were detected using appropriate HRP-conjugated secondary reagents (all used at 1:3,000 dilution) and the ECL reagents from Amersham Pharmacia Biotech.

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The buffer of protein samples was exchanged against 50 mM ammonium bicarbonate using a Centricon-50 cartridge, lyophilized, and dissolved in 0.1% aqueous trifluoroacetic acid at a concentration of 12.5 pmol/µl. 1 µl of the protein solution was mixed with 9 µl of sinapinic acid (40 mg/ml) in 0.1% trifluoroacetic acid, H2O:acetonitrile (2:1 v/v). 1 µl of the mixture was applied on the gold-plated target and air-dried before transfer into the source of the mass spectrometer. MALDI mass spectra were obtained on a Perseptive Biosystems Voyager RP™ mass spectrometer using a 337-nm nitrogen laser, a 25-kV accelerating potential, and a delayed extraction time of 300 ns. Samples were optimally controlled using a video camera. External calibration of the MALDI spectra in the linear mode was carried out using bovine serum albumin.

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mSC—Identical amounts of each mSC protein were incubated for 4 h at 4 °C under gentle rocking with 0.5 ml of TBS buffer containing 60 µl of M2 affinity gel. After centrifugation, beads were washed with TBS and eluted overnight at 4 °C with 45 µl of TBS containing 100 µM FLAG peptide. 15-µl aliquots of eluate were removed and boiled with 1 volume of gel-loading buffer (100 mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate, 0.2% (w/v) bromphenol blue, 20% (v/v) glycerol) for immunoblotting with rabbit IgG to denatured mSC.

mpIgR—Cell lysates were diluted 6-fold with cold TBS (final volume 480 µl) and combined in siliconized 2.2-ml tubes with 60 µl of anti-FLAG slurry (M2 affinity gel) equilibrated in TBS, 0.02% sodium azide. After an overnight incubation at 4 °C, immunoprecipitates were washed 4 times with TENT buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 150 mM NaCl, 1% Triton X-100), and the beads were specifically eluted with 50 µg/ml FLAG peptide DYKDDDDK. The
eluate was mixed with SDS-PAGE loading buffer with or without dithiothreitol, boiled for 3 min, and loaded onto an 8% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes, and after blocking in TBS-0.05% Tween 20, the blot was sequentially incubated with 0.1 μg/ml Penta-His™ mAb and 1:3,000 goat anti-mouse IgG (Fc-specific), HRP-conjugated. Enhanced chemiluminescence was used to visualize the precipitated receptors. Alternatively, immunoprecipitation was performed with 1 μg/ml Penta-His™ mAb. After an overnight incubation at 4 °C, the pIgR-antibody complexes were precipitated with 60 μl of precleared protein G-Sepharose bead slurry (Amersham Pharmacia Biotech). The beads were washed as above and specifically eluted with 50 μg/ml peptide DHDFHHHHHHK. The associated pIgR-FLAG protein was detected using anti-FLAG mAb M2 at 2 μg/ml, with all other steps and reagents kept identical. Immunodetection with anti-mSC was performed as described above.

Surface Biotinylation

1 × 10⁷ transfected COS-7 cells were washed in cold PBS, resuspended in 2 ml of cold PBS containing 0.5 mg/ml sulfo-NHS-LC-Biotin (Pierce), and incubated for 30 min at 4 °C. The cells were washed twice with PBS, resuspended in 1 ml of Dulbecco’s modified Eagle’s medium (Pierce), and incubated on ice for 10 min to quench any residual biotinylation reagent. Cells were lysed using 1 ml of biotinylation lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% bovine serum albumin, 1% Triton X-100, 1 mM leupeptin, 50 μl of Complete™ (reconstituted from 1 pellet in 50 ml PBS). Cleared lysates were incubated at 4 °C for 2 h with streptavidin-agarose (Sigma) to harvest biotinylated pIgR. The beads were washed three times with biotinylation buffer and boiled for 3 min in 2× SDS sample buffer, and the eluate was analyzed by immunoblotting using rabbit anti-mSC as described above.

Receptor Dimerization

Cross-linking by boiling in SDS was performed as described in Hirt et al. (26). 1 × 10⁶ transfected COS-7 cells were washed in PBS, scraped, pelleted, lysed with 100 μl of hot 3% SDS, and then boiled for 5 min. The lysate was diluted with dialysis buffer (10 mM Tris-HCL (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) then immunoprecipitated with anti-FLAG beads (M2 affinity gel) as above and analyzed by 8% SDS-PAGE. Immunodetection of blotted proteins was carried out with rabbit anti-mSC.

Other Analytical Procedures

Proteins were quantitated with the bicinchonninic acid assay (27) using bovine serum albumin as a standard (Pierce). Silver staining of SDS-PAGE gels was performed according to Morrissey (28).

RESULTS

Prediction of Surface-exposed Loops in mSC Using Molecular Modeling—Based on the approach described in Coyne et al. (17) and Cortés et al. (10), we constructed molecular models of domain II and III of wild type mSC (Fig. 2A). Both domains exhibit the characteristic structure of Ig superfamily members, consisting of nine strands (termed A, B, C, C′, C″, D, E, F, G) connected by loops of variable size. Within each domain, a disulfide bond links strand B with strand F, and a second disulfide bridge constraints the C-C′ loop. The folding pattern in the first four domains of pIgR has been classified in the V set (variable domain) of Ig-like domains (29).

Guided by this model, we selected four internal loops for replacement with the FLAG octapeptide DYKDDDDK (Ref. 30; see Table II and Fig. 3). The FLAG sequence was aligned to optimally match the charge and hydrophathy of amino acids in the loops. The tyrosine residue in the FLAG sequence served as an “anchoring point,” always replacing a hydrophobic residue of SC (Fig. 2). Loops for substitution were also selected on the basis of (i) their length (6–8 amino acids), (ii) the degree of variability of the SC sequence between species including human, rat, and rabbit, and (iii) the absence of the N-linked glycosylation signal Asn-Xaa-Thr/Ser in the case of domain II. As a negative control, we engineered the F-G loop of domain I (CDR3-like), whose replacement with another sequence abolished binding of IgA to rabbit pIgR without strongly altering the overall conformation (17). Models of domains II and III modified with the FLAG epitope (Fig. 2, B–F) were constructed to take into account the local changes due to amino acid substitution.

The absence of a “linker” between domains II and III probably reduces their mobility in a back-to-back conformation. This prompted us to insert (rather than substitute) the FLAG sequence as a linker between domain II and III. Accordingly, the model of the resulting mutant shows an extension of the G strand of domain II projecting into the A strand of domain III (Fig. 2D). Finally, our positive control consisted of inserting the FLAG sequence at the carboxyl terminus of mSC (20).

Efficient Secretion of mSC-FLAG Mutants Produced In
Expression of the various recombinant mSC proteins. Preparations of mSC mutants secreted by COS-7 cells were concentrated by chromatography on ConA-agarose columns. Equivalent amounts of mSC were subjected to electrophoresis onto 8% polyacrylamide gel under denaturing and reducing conditions (Panels A and B) or denaturing and nonreducing conditions (Panel C). Proteins were blotted onto polyvinylidene difluoride membranes and detected with rabbit IgG against mSC (panels A and C) and mouse mAb M2 to the FLAG (panel B). Antigen-antibody binding was detected with appropriate secondary antibodies coupled to HRP using enhanced chemiluminescence. Molecular size markers expressed in kDa are indicated on the left of each panel.

COS-7 Cells—COS-7 cells were transiently transfected with the pCB6 expression vector comprising the wild type or mutated mSC constructs depicted in Fig. 3. The amount of recombinant mSC polypeptides secreted into the cell culture medium was measured by capture ELISA as described under “Experimental Procedures.” All the recombinant proteins were efficiently secreted by COS-7 cells (9 to 16 µg/ml) to a level similar to wild type SC (12 µg/ml). Substitutions in domains II and III were thus a suitable option, overcoming the limited production resulting from replacement of the E-F loop of domain I (10). Recombinant mSC-FLAG proteins in cell culture supernatants were enriched by ConA-agarose chromatography, before fractionation by SDS-PAGE under reducing conditions. Immunodetection with IgG to mSC revealed a single band for the wild type and mutant mSC (Fig. 4A); consistently, mAb M2 recognized the FLAG epitope in the mutants (Fig. 4B). Significant differences in the apparent M, of the various mutants were observed (Table III). In particular, the two mutants bearing a substitution of the D-E loop migrated much faster than the other proteins. Such notable differences could not simply be explained from the local sequence and charge substitutions imposed by the mere presence of FLAG. Full deglycosylation with peptide:N-glycosidase F, which cleaves N-linked glycans, still resulted in mSC species with highly variable M, values in SDS-PAGE. Additional analysis of mSC mutants with wheat germ agglutinin (31, 32) and endo-β-N-acetylglucosaminidase H indicates that the presence of the FLAG motif affects both the terminal glycosylation and/or the surface exposure of sialic acid residues in the protein mutants (summarized in Table III).

Formation of Covalent Homodimers upon Introduction of the FLAG Epitope Within Domain I, II, or III of mSC—When analyzed by nonreducing SDS-PAGE, mSC-FLAG:D1:FG, mSC-FLAG:D2:BC, mSC-FLAG:D2-D3, and mSC-FLAG:D3:CC' migrated as high molecular weight complexes along with the monomeric form (Fig. 4C). These high M, forms are reversibly cross-linked by disulfide bonds because they were absent when samples were boiled in 0.1 M dithiothreitol before SDS-PAGE (Fig. 4A). The mere presence of the foreign sequence in mSC-FLAG:Cterm was not responsible for its lack of cross-linking since wild type mSC did not form oligomers either. Overexpression in COS-7 does not explain the phenomenon either, because not all the mutants are secreted as oligomers. The appearance of intermolecular disulfide bridges seems to correlate with the FLAG sequence introduced in the vicinity of the C-C' loop. The disulfide bond encompassing loop C-C' is the most surface-exposed in SC domains (Fig. 2) and, therefore, could be more prone to local changes.

To determine whether monomeric forms of mutants could exist as noncovalent dimers, we chromatographed mSC-FLAG: D2:BC on a sizing column run under native conditions. Column fractions analyzed by immunoblotting showed that only the covalent dimer elutes early (Fig. 5A). mSC-FLAG:D2:DE, mSC-FLAG:D3:DE, mSC-FLAG:Cterm, and wild type mSC were eluted at the position of the monomer exclusively. Therefore, covalency appears necessary to stabilize mSC as a dimer.

To provide conclusive evidence that the dimeric forms were not artifacts of electrophoresis, we subjected the purified mSC-FLAG:D2:BC protein to MALDI MS. Fig. 5B shows the MALDI spectrum obtained in the linear mode using sinapinic acid as a matrix. The spectrum displays a molecular ion peak centered at 77 kDa for the M + ion and 38 kDa for the M 2+. Note that a weak amount of monomer was present in this preparation as shown in the inset of Fig. 5B. In addition to the M + ion, the 2M + was observed with a molecular weight of 153 kDa, corresponding to dimeric mSC-FLAG:D2:BC. The multiple signals reflect the presence of different glycoforms. This heterogeneity is attributed to native structure diversity and not to spectroscopic fragmentation (33). With a calculated M, of 66,194, post-translational modifications contribute about 14% of the mass in both monomeric and dimeric mSC-FLAG:D2:BC.

Surface Accessibility of the FLAG Epitope Introduced Inside SC Domains—To test the surface exposure of the FLAG epitope in the various mutants, we first performed immunoblotting under nonreducing conditions using the anti-FLAG mAb M2. Fig. 6A shows that mAb M2 detected only a subset of the species observed in Fig. 4C using a polyclonal antibody to mSC. Strong signals were observed for mSC-FLAG:Cterm, dimeric mSC-FLAG:D1:FG, and both forms of mSC-FLAG:D2-D3. Dimeric mSC-FLAG:D2:BC and monomeric mSC-FLAG:D1:FG yielded signals of intermediate intensity.

To directly assess the reactivity of the epitope in monomers and dimers in solution, we performed immunoprecipitation by using agarose-bound M2. Data in Fig. 6B indicate that all immunoreactive mutants in Fig. 6A were also precipitable, yet to various extents. In addition, detection of mSC-FLAG:D2:BC and mSC-FLAG:D3:CC' species was improved in this assay. This indicates that dimerization of mSC-FLAG:D1:FG, mSC-FLAG:D2:BC, and mSC-FLAG:D2-D3 does not mask FLAG-containing sites and maps those as candidates for B and T epitope insertion. The lack of binding to agarose-bound M2 of the two mutants with the D-E loop replaced with FLAG implies that the peptide epitope was probably in a constrained conformation no longer adapted for recognition by the mAb.

The same four mSC-FLAG mutants (D1:FG, D2:BC, D2-D3,
could be reproducibly purified to homogeneity using the mAb M2 coupled to agarose beads and mild elution with the FLAG octapeptide. The purity and integrity of these preparations was verified by SDS-PAGE and subsequent silver staining (Fig. 6C, left panel). The protein preparations were homog-

### Table III

| Protein              | Secretion<sup>a,b</sup> | Molecular mass:<sup>c</sup> | M2 signal, Nonreduced | Types of N-linked carbohydrates<sup>d,e</sup> | Binding to wheat germ agglutinin |
|----------------------|--------------------------|-----------------------------|-----------------------|---------------------------------------------|---------------------------------|
|                      | µg/ml<sup>g</sup>        |                             |                       |                                             |                                 |
| mSC-FLAG:D1:FG       | 9                        | 95                          | 100                   | +<sup>d</sup>                               | H/HM<sup>*, complex</sup>       |
| mSC-FLAG:D2:BC       | 16                       | 85                          | 95<sup>f</sup>        | ++                                         | Complex                        |
| mSC-FLAG:D2:DE       | 10                       | 75                          | 85                    | +/−                                       | Complex                         |
| mSC-FLAG:D2–D3       | 11                       | 85                          | >200<sup>g</sup>      | ++                                         | –                               |
| mSC-FLAG:D3:CC<sup>c</sup> | 10                       | 85                          | 95                    | −/−                                       | Complex                         |
| mSC-FLAG:D3:DE       | 11                       | 75                          | 85                    | −/−                                       | –                               |
| mSC-FLAG:Cterm       | 12                       | 90; 80                      | 100; 90               | ++                                         | H/HM, complex                   |
| Wild type mSC        | 12                       | 80                          | 90                    | −                                         | Not determined                  |

<sup>a</sup> Determined by capture ELISA of culture supernatant.

<sup>b</sup> Applies to monomer and dimer.

<sup>c</sup> As determined from sensitivity to endo-β-N-acetylglucosaminidase H.

<sup>d</sup> 2, absent; +/−, weak; +, intermediate; ++, strong.

<sup>e</sup> Hybrid/high mannose glycans.

<sup>f</sup> MALDI MS analysis yielded a molecular mass of 77 and 153 kDa, respectively (see Fig. 5B).

<sup>g</sup> Immunoprecipitated by the M2 antibody (see Fig. 6B).

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**Fig. 5.** Dimerization of mSC-FLAG mutants is mediated by disulfide bridges. Panel A, mSC-FLAG:D2:BC concentrated on ConA-agarose was chromatographed on Superose 12 as described under “Experimental Procedures,” and fractions were analyzed as in Fig. 4C using rabbit IgG to mSC as the primary antiserum. M, monomer; D, dimer. Molecular size markers are expressed in kDa. Panel B, mSC-FLAG: D2:BC produced in COS-7 cells and purified by affinity chromatography on mAb M2-agarose beads was analyzed by MALDI MS as detailed under “Experimental Procedures.” M<sub>mono</sub>, monoprotonated mSC mutant monomer; M<sub>dp</sub>, diprotonated mSC mutant monomer; 2M<sub>mono</sub>, monoprotonated mSC mutant dimer. Inset, a silver-stained gel of the preparation applied shows the presence of some monomer (M) alongside with dimer (D). Because of the different molecular weights of the proteins in the sample, the relative signal intensities in the MALDI spectrum do not relate directly to their abundance.

**Fig. 6.** FLAG reactivity in mSC-FLAG mutants. Panel A, preparations of mSC mutants concentrated by ConA chromatography and containing equivalent amounts of mSC were analyzed by immunoblotting of a denaturing, nonreducing 6% polyacrylamide gel using mAb M2. Panel B, culture supernatants containing mSC proteins were incubated with agarose-bound mAb M2, and the bound fraction was analyzed by immunoblotting using rabbit IgG to mSC. Panel C, mAb M2 affinity-purified mSC-FLAG mutants were electrophoresed on a denaturing 6% polyacrylamide gel under reducing or nonreducing conditions, and proteins were visualized by silver staining. M, monomer; D, dimer. Molecular size markers are indicated in kDa.
In an attempt to relieve inappropriate disulfide cross-linking of mutants leading to intracellular accumulation, cells were cultured for 36 h in the continued presence of 2.5 mM 2-mercaptoethanol. Such a treatment allowed the release of free immunoglobulin light chain (36) and of unpolymerized IgM (37) or IgA (38), which are normally sequestered in the ER. No dramatic effect on the degree of secretion and on the molecular aspect of recombinant mSC:FLAG could be seen (Fig. 7C) as compared with the material recovered from COS-7 cells kept under normal conditions (Fig. 4C). Exposure of cells to 2-mercaptoethanol produced a decreased reactivity of the FLAG in mSC-FLAG:D2-D3 molecular species, whereas dimeric mSC-FLAG:D3:CC' and, particularly, mSC-FLAG:D1:FG became more immunoreactive (Fig. 7D).

Covalent Dimerization of the Complete Receptor with a FLAG Replacing Loop B-C of the Second Domain—So far dimers were obtained using recombinant mSC mutants, a nonnatural form of the pIgR that normally exists as a transmembrane protein. To exclude the possibility that SC dimers represented an artificial form appearing as a consequence of the absence of the cytoplasmic and transmembrane domain, we produced recombinant mpIgR with the FLAG replacing the B-C loop of domain II (mpIgR-FLAG:D2:BC) in COS-7 cells. The structure of pIgR was analyzed by immunoblotting of detergent-solubilized cellular proteins under nonreducing conditions (Fig. 8A). Wild type and mutant monomers both migrated with a Mr of 115. As expected, the mpIgR mutant showed an additional band with a double apparent Mr. Thus, dimerization induced by the presence of the FLAG takes place whether the protein is being secreted or integrated in the membrane, and the local change in charge and sequence alone is responsible for the formation of novel disulfide bond(s). Furthermore, only the dimeric form of mpIgR-FLAG:D2:BC was immunoprecipitated by the anti-FLAG antibody.

Immunoprecipitation Studies Identify pIgR Homodimers—Our data provide evidence that pIgR forms homodimers within the plasma membrane; however, it remains to be demonstrated that this happens with wild type pIgR. So far, for this interaction to be detected, forced cross-linking or discrete modifications in the primary structure of the pIgR have had to be used (Refs. 26 and 39; this study). Furthermore, the possibility that pIgR associates with another protein of the same molecular weight could not be excluded. To demonstrate that pIgR indeed forms homooligomers, we performed co-immunoprecipitation studies on COS-7 cells cotransfected with pCB6 constructs encoding mpIgR:6xHis and mpIgR-FLAG carrying a different epitope tag at the carboxyl terminus (Fig. 3). We first determined that COS-7 cells expressed the tagged receptors similarly to the wild type untagged pIgR (Fig. 8B), yet a significant portion of pIgR (115 kDa) in the cell extract was rapidly converted into SC (85 kDa). Three cross-reactive bands were also detected, forced cross-linking or discrete modifications in the primary structure of the FLAG receptor. The structure of pIgR was analyzed by immunoblotting of detergent-solubilized cellular proteins under nonreducing conditions (Fig. 8A). Wild type and mutant monomers both migrated with a Mr of 115. As expected, the mpIgR mutant showed an additional band with a double apparent Mr. Thus, dimerization induced by the presence of the FLAG takes place whether the protein is being secreted or integrated in the membrane, and the local change in charge and sequence alone is responsible for the formation of novel disulfide bond(s). Furthermore, only the dimeric form of mpIgR-FLAG:D2:BC was immunoprecipitated by the anti-FLAG antibody.

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confirming that the 115-kDa band was indeed pIgR. The same 115-kDa band was revealed when the cell lysate was first precipitated with anti-6xHis mAb, then analyzed with anti-FLAG mAb M2 (Fig. 8C, lanes 6–9). Immunodetection required the presence of the primary antibody (Fig. 8C, lanes 5 and 10), showing the specificity of the interaction between the pIgR forms. Plasma membrane localization of pIgR was assessed by the lack of sensitivity to endo-β-N-acetylglucosaminidase H treatment (Fig. 8D, lanes 1 and 2) and by cell surface biotinylation (Fig. 8D, lane 3). Boiling of the pIgR in 3% SDS resulted in partial dimerization that could be detected under nonreducing conditions (Fig. 8D, lanes 4 and 5). This approach was chosen because chemical cross-linking with either glutaraldehyde or sulfo-SMSP (sulfosuccinimidyl-4-[p-maleimidophenyl]butyrate) was unsuccessful (data not shown).

Although the experiments above all go to the direction of pIgR dimer formation, one can argue that COS-7 cells are not suitable for epitope mapping with a monoclonal antibody (Fig. 8C). Immunoblotting with antibody to mSC under reducing conditions. Conversion of pIgR into SC dimer, covalency might also mask thiols usually acting as interdomain disulfide bridges. Thus, our attempt to seek favorable sites to integrate B and T epitopes in recombinant secretory IgA to be used as a mucosal vaccine (10) led us to the finding that the pIgR exists as a dimer in the absence of IgA ligand. Initially, we chose to identify insertions sites based on modeling of domains II and III known to be dispensable to the pIgR function in rabbit. Five mSC mutants were obtained with the FLAG octapeptide replacing stretches of amino acid predicted to be surface-exposed. Two mutants were also produced containing the FLAG in between the second and third domain and at the carboxyl terminus, respectively. Unexpectedly, all mutants exhibited many particular structural features. First, the interaction between the FLAG and the cognate mAb was modulated depending on the selected site. Second, apparent sizes and glycosylation patterns were heterogeneous. Third, several mutants were partly secreted as covalent dimers. The latter observation prompted us to extend our analysis to the full pIgR, and we could establish that the empty receptor dimerizes in a noncovalent fashion in the plasma membrane of both COS-7 cells and epithelial Caco-2 cells.

FIG. 8. Forced and spontaneous association of pIgR. Panel A, cell lysates of purified wild type mplgR and mplgR-FLAG:D2:BC were detected by immunoblotting with antibody to mSC under nonreducing conditions. Panel B, detection of wild type, 6xHis-tagged, and FLAG-tagged mplgR in COS-7 crude extract analyzed by immunoblotting with antibody to mSC under reducing conditions. Conversion of pIgR into SC indicates that lysates have to be prepared in the presence of 1 mM leupeptin to avoid degradation. Panel C, COS-7 or Caco-2 (bottom part) cells were cotransfected with pCB6mplgR:FLAG (5′) and pCB6mplgR:6xHis (5′), then kept in culture to permit pIgR-tag expression. Immunoprecipitation (IP) of lysed cells was either with anti-FLAG mAb M2 (F) or with Penta-His™ mAb (H), as indicated on the top of the lanes. Western blotting (W. b.) was with F, H, rabbit anti-mSC (SC) or with an irrelevant Ab (−) to demonstrate specificity. Upper panel, COS-7 cells; bottom panel, Caco-2 cells reducing conditions. Panel D, lanes 1 and 2, the lack of endo-β-N-acetylglucosaminidase H (EndoH) sensitivity of mplgR:FLAG immunoprecipitated with anti-FLAG mAb M2 (F) indicates full maturation of the receptor; lane 3, streptavidin (St) precipitation of surface-biotinylated COS-7 cells identifies a single SC-reactive band on blot corresponding to plasma membrane mature pIgR; lanes 4–5, pellets of cells expressing the mplgR:FLAG were processed as indicated under "Experimental Procedures" and analyzed by SDS-PAGE (6% polyacrylamide) in the presence or the absence of dithiothreitol.

The molecular models predict that, in solution, the FLAG is displayed on the surface of individual domains. Mutants D1:FG, D2:BC, D2-D3, and D3:CC′ could indeed be immunoprecipitated with the immobilized anti-FLAG mAb M2, indicating that the inner FLAG epitope was freely accessible, to an extent similar to that of construct Cterm. In immunoblots, the reactivity of mutants D1:FG, D2:BC, and D3:CC′ toward mAb M2 was decreased under nonreducing conditions as compared with samples boiled in the presence of dithiothreitol. This behavior of mAb M2 has been observed before in another protein context (40) and might result from its ability to recognize multiple conformers. Two mutually nonexclusive possibilities, conformation-dependent binding of mAb M2 or masking of the FLAG by interaction with residues of vicinal domains in the same mSc molecule, could explain the lack of reactivity of mutants D2:DE and D3:DE under nonreducing conditions. For those monomeric mutants that are poorly recognized by mAb M2, it can also be postulated that the five domains fold back into a U-shaped conformation, burying the FLAG located in the central portion of the protein. Consistently, mAb M2 reacts strongly with both the dimers and the monomers of mSc-FLAG:D1:FG and msc-FLAG:D2:BC, exhibiting the FLAG in a more exposed environment. Finally, dimeric mSC-FLAG:D3:CC′ is weakly immunoreactive, compatible with the C-C′ loop being very close to the intermolecular disulfide bridge. Also, the monomeric form is poorly reactive, probably because the FLAG replacing the C-C′ loop is strongly constrained by the disulfide bond connecting Cys254 and Cys261. We conclude that mutants D1:FG, D2:BC, D2-D3, D3:CC′, and C-term should preferentially carry B epitopes to be exposed on the surface, whereas mutants D2:DE and D3:DE can be used as carrier of T epitopes solely.

We have shown that covalent homodimers of mutant msc-FLAG proteins are formed early in the secretory pathway. It is likely that mSC exits the ER as multimeric cargo and that extra negative charges provided by a vicinal FLAG induce rearrangement of disulfide bonds. In addition to stabilizing the dimer, covalency might also mask thiol usually acting as intracellular retention elements for unassembled molecules (36). We were unable to detect cross-linking of msc-FLAG mutants.
with ER matrix resident proteins. This argues in favor of a preferential reactivity of interchain thiols in SC mutants over other reactive groups. Biosynthesis of the mutants, performed in the presence of 2-mercaptoethanol added in the cell culture medium, did not abolish covalent dimerization nor improve secretion. The existence of covalent dimers was further established by resolution on sizing columns and mass spectrometry analysis. Interestingly, mutants D2:DE and D3:DE, which do not form covalent homodimers, have in common three conserved (KDD) and one related (R to K) residue found in the mSC D-E loop. Thus, too few sequence changes do not induce major structural differences required for reshuffling of disulfide bonds. In conclusion, the covalent dimers of mSC mutants described in the paper suggest that mSC molecules pair, at least transiently, via the first three domains. Model-based selection of surface elements and their substitution has enabled us to prove this so-far speculative property. In addition, we found that the pIgR counterpart of mutant D2:BC formed covalent dimers as well, indicating that the transmembrane and cytoplasmic domains do not contain motif(s) controlling dimer association.

Forced covalent dimerization of SC/pIgR mutants might reflect a physiological event involving homooligomerization of pIgR in plasma membrane. Only scant evidence supports the notion of pIgR being a dimer. First, polyclonal or mAb directed against the ecto domain of rabbit pIgR only recognized a subset of immature receptor transiting in the ER and/or Golgi (41). We concluded that pIgR oligomerization was compatible with masking of some epitopes, yet conformational changes or association with other components of the secretory machinery represent sound alternatives. Second, rabbit pIgR covalent dimers form upon boiling in 3% SDS (26). By analogy to glycoporphin A, this phenomenon was originally attributed to transmembrane domain residues of pIgR, but the sensitivity to reduction points toward artifactual disulfide bonding (Ref. 39; this study). Similarly, disulfide-bonded dimers of the low density lipoprotein receptor (42) and the 46-kDa mannose 6-phosphate receptor (43) formed post-lysis as revealed by direct, nonreducing SDS-PAGE. In contrast, chemical cross-linking failed to detect the presence of pIgR homodimers in Madin-Darby canine kidney cells (44), COS-7, and Caco-2 cells (this study). Although not detectable by gel filtration at 4.5 μM concentration, dimers of mSC could be shown by mass spectrometry, indicating a tendency for association with increasing concentration (54). In human milk secretion, the concentration of free SC reaches 25 μM (2 mg/ml (45)), therefore suggesting that dimerization could be physiologically relevant. Detection of such dimers is precluded by a dissociation constant falling in the high micromolar to millimolar range, as exemplified also by the ectodomain of CD4 (46).

Recently, Singer and Mostov (39) reported on IgA-mediated dimerization of pIgR resulting in stimulation of their transcytosis and elicitation of cellular signal. In the discussion, they raised the possibility that pIgR preexists as a dimer, but as far as we know, there are no data on the oligomerization of intact pIgR on the membrane. Our data, using immunoprecipitation of membrane pIgR carrying two different epitope tags at the carboxyl terminus, demonstrate that noncovalent dimerization occurs in the absence of IgA ligand in both COS-7 cells and in epithelial Caco-2 cells normally expressing pIgR. The existence of pIgR dimers before IgA binding makes it unlikely that dimerization per se induces the cascade events (stimulation, sensitization) recently reported in the Madin-Darby canine kidney cells model (47). In the absence of IgA, the short domain (amino acids 726–736 in rabbit pIgR) in the cytoplasmic tail regulating IgA-stimulated transcytosis could be masked or assembled in a nonproductive conformation. Under these circumstances, constitutive transcytosis is reduced, enabling the cell to maintain enough of the pIgR available at the basolateral surface. Dimerization occurs equally on the surface of Jurkat cells (39) and COS-7 cells (this study), arguing in favor of an intrinsic, cell-independent property of the pIgR. Epithelial specific trafficking events seen in Madin-Darby canine kidney cells might be attributed to a so-far unknown partner protein (47).

It is possible that pIgR exists in an equilibrium of monomer and dimer, possibly distributed at specific locations within the cell. Studies with the 46-kDa mannose 6-phosphate receptor showed that the receptor is present in several oligomeric states (43), the distribution of which, however, was independent of recycling and binding of ligands (48). We propose that the function of IgA1 may thus be 2-fold: (i) stabilization of pIgR dimers (displacing the equilibrium); (ii) triggering of conformational change(s) leading to activation of the signal transduction cascade and linked transcytosis (49, 50). In terms of mucosal protection, maintenance of a pool of pIgR dimers ready to achieve IgA transport and function could prevent the rate-limiting step occurring after massive production of IgA necessary to fight local infection. In addition, the existence of monomeric and dimeric pIgR on the cell surface could explain the differences observed in terms of affinity and number of binding sites revealed when using dimeric and tetrameric IgA (51). Up-regulation of pIgR expression by inflammatory cytokines might push the equilibrium toward the formation of pIgR dimers and promote transport of the larger IgA polymers carrying more antigen binding sites.

In conclusion, we have successfully designed a strategy to identify useful sites for introducing 8-mer B and T epitopes and possibly larger ones in the sequence of mSC. Surface-exposed, as well as buried sites, both constitute valuable candidates for substitution. Remarkably, except for the domain I mutant, the mSC-FLAG molecules had the expected IgA binding properties (52). In addition, dimerization of certain mutants might represent an asset, both in terms of IgA stabilization (53) and as multiple epitope carriers. Forced covalent dimerization provided indirect clues that SC/pIgR might assemble into oligomeric structures. The biochemical and genetic approach we used gave the strongest evidence reported so far that pIgR exists as noncovalent dimers in the plasma membrane.

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