Mapping the Interaction Between Murine IgA and Murine Secretory Component Carrying Epitope Substitutions Reveals a Role of Domains II and III in Covalent Binding to IgA

(Received for publication, February 4, 1999, and in revised form, August 16, 1999)

Pascal Crottet‡‡ and Blaise Corthésy‡‡‡

From the ‡ Institut Suisse de Recherches Expérimentales sur le Cancer, CH-1066 Épalinges and † Division d’immunologie et d’allergie, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

We have identified sites for epitope insertion in the murine secretory component (SC) by replacing individual surface-exposed loops in domains I, II, and III with the FLAG sequence (Crottet, P., Peitsch, M. C., Servis, C., and Corthésy, B. (1999) J. Biol. Chem. 274, 31443–31455). We had previously shown that epitope-carrying SC re-associated with dimeric IgA (IgAd) can serve as a mucosal delivery vehicle. When analyzing the capacity of SC mutants to associate with IgAd, we found that all domain II and III mutants bound specifically with immobilized IgAd, and their affinity for IgAd was comparable to that of the wild type protein (IC50 = 1 nM). We conclude that domains II and III in SC are permissive to local mutation and represent convenient sites to antigenize the SC molecule. No mutant bound to monomeric IgA. SC mutants exposing the FLAG at their surface maintained this property once bound to IgAd, thereby defining regions not required for high affinity binding to IgAd. Association of IgAd with SC mutants carrying a buried FLAG did not expose de novo the epitope, consistent with limited, local changes in the SC structure upon binding. Only wild type and two mutant SCs bound covalently to IgAd, thus implicating domains II and III in the correct positioning of the reactive cysteine in SC. This establishes that the integrity of murine SC domains II and III is not essential to preserve specific IgAd binding but is necessary for covalency to take place. Finally, SC mutants existing in the monomeric and dimeric forms exhibited the same IgAd binding capacity as monomeric wild type SC known to bind with a 1:1 stoichiometry.

The first line of defense against pathogens consists of mucosal secretions, with secretory IgA being one of the main effectors. The latter is transported by the polymeric immunoglobulin receptor (pIgR)1 from submucosal sites into the lumen. The receptor can bind dimeric IgA (IgAd), polymeric IgA (IgA p), and IgM (which are produced by subepithelial plasma cells) but not monomeric IgA (IgA m) or IgG. The complex is internalized at the basolateral surface of the epithelium and transcytosed to the apical plasma membrane, where the extracellular portion of the receptor called secretory component (SC) is released by proteolytic cleavage and remains bound to IgA within a complex termed secretory IgA (sIgA) (2, 3).

All three loops, referred to as B-C, D-E, and F-G in domain I are particularly implicated in binding to IgA (4, 5); in addition, some residues of loop E-F in the opposite face appear to be important for IgA binding. In contrast, deletion of domains II and III or individual deletion of domain IV and V of rabbit pIgR did not prevent binding to IgA in a qualitative cell-ligand binding assay (5). A natural variant of rabbit SC lacking domains II and III can bind to IgA, although only in a noncovalent fashion (6, 7). In human sIgA, a disulfide bridge within domain V is also involved in covalent binding to the Co2 domain of IgA through a disulfide exchange mechanism (8–10). Species variations in the level of covalency between pIgR and IgA have been reported (11, 12). Thus, it is tempting to speculate that although not critical for the initial recognition of IgA, domains II to IV appear to position domain V such that disulfide exchange with IgA can take place.

The mechanism for the selective recognition of IgAd over IgAm remains an open question. We have generated murine SC mutants with predicted exposed loops of domains I, II, and III replaced with the FLAG epitope (13) to evaluate their IgA binding properties. We show that although the affinity of these mutants for IgA and the selectivity for IgAd over IgAm are both preserved, covalent binding is lost in all mutants but one. Given the pinpointing strategy designed here, this suggests that domains II and III are essential to the proper positioning of domain V. However, because binding to the anti-FLAG mAb is preserved in both free and IgAd-bound reactive SC mutants, we postulate that minor structural changes occur upon IgAd-mSC interactions. Dimeric SC-FLAG mutants were shown to be as good IgAd binders as their monomeric counterparts. In the same assay, we found by direct comparison of mSC and pIgR mutants that mSC binds to IgAd with much reduced stringency. We conclude that mSC-FLAG mutants are useful 1) to study the topology of SC-IgAd complexes, 2) as short epitope carrier once combined with IgAd, 3) to tackle the binding specificity and stoichiometry of SC/pIgR IgAd complexes.

* This work was supported by Biotechnology Priority Program Grant 5002-38009 and Swiss National Science Foundation Grant 3100-050912.97. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated as a “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ Current address: Biozentrum, University of Basel, CH-4056 Basel, Switzerland.

§ To whom correspondence should be addressed: Division d’immunologie et d’allergie, CHUV, BH18–701, Rue du Bugnon, CH-1011 Lausanne, Switzerland.

¶ The abbreviations used are: pIgR, polymeric immunoglobulin receptor; mPlgR, murine pIgR; IgAd, dimeric IgA; IgAm, monomeric IgA; IgA, polymeric IgA; sIgA, secretory IgA; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; SC, secretory component; hSC, human SC; mSC, murine SC; IC50, 50% inhibitory concentration; mAb, monoclonal antibody; PBS, phosphate-buffered saline; pIgR is expressed by epithelial cells in a variety of mucosal tissues and in rodents is further implicated in transport into bile through the liver (1). The receptor can bind dimeric IgA (IgAd), polymeric IgA (IgA p), and IgM (which are produced by subepithelial plasma cells) but not monomeric IgA (IgAm) or IgG. The complex is internalized at the basolateral surface of the epithelium and transcytosed to the apical plasma membrane, where the extracellular portion of the receptor called secretory component (SC) is released by proteolytic cleavage and remains bound to IgA within a complex termed secretory IgA (sIgA) (2, 3).

PAGE, polyacrylamide gel electrophoresis.
EXPERIMENTAL PROCEDURES

The structure and production of the various recombinant proteins and most analytical procedures as well as the source of most antibodies and reagents are described in the accompanying article (13).

Recovery of Recombinant mSC and mpIgR—COS cell culture supernatants containing mSC proteins were harvested as described (13). In some experiments, cells were incubated for 36 h with fresh medium containing 2.5 mM L-carnosine (Life Technologies, Inc.), and the culture supernatants were supplemented with 30 mM iodoacetamide. Murine SC proteins were recovered from the supernatants by concanavalin A-agarose chromatography (Vector Laboratories; Ref. 13) and stored at 4 °C in PBS with 0.02% (w/v) sodium azide. Such preparations were used for all the experiments. Cell lysates containing mpIgR proteins were obtained as described (13).

Antibodies—Purified IgGα and IgGμ from the murine hybridoma ZAC3 (14) are kind gifts of Dr. E. Lüllau (Ecole Polytechnique Fédérale, Lausanne, Switzerland). Murine IgG1 MOPC-31c, biotinylated goat IgG to murine α chain, mAb to human SC, and horseradish peroxidase (HRP)-conjugated reagents were obtained from Sigma. Rabbit antiserum to mSC and murine mAb to the FLAG were as described (13). Anti-FLAG mAb M2 was used for all the experiments. Cell lysates containing mpIgR proteins were obtained as described (13).

Biotinylation of Proteins—To specifically label sialic acid residues, 250 μg of affinity-purified mSC-FLAG:Cterm (36) in 0.5 ml of 0.1 M sodium acetate (pH 5.5) were incubated with 2 mM sodium periodate on ice for 30 min (15). Oxidation was quenched with 15 mM glycerol, which was then removed by several washes with 0.1 M sodium acetate (pH 5.5) using a Centricon-50 filtration unit (Amicon). Biotin-LC-hydrazone (Pierce) in dimethyl sulfoxide was added to a 5 mM final concentration and incubated at room temperature for 2 h. The buffer was exchanged with containing 0.05% (w/v) sodium azide using a Centricon-50 filtration unit. Proteins were stored at 4 °C and used within 2 weeks following biotinylation. Purified IgGμ from hybridoma ZAC3 (250 μg) was biotinylated using the same procedure with the exception that 10 mM sodium periodate was used for oxidation of the sugar moieties.

Two mg each of purified rabbit IgG against unfolded mSC, respectively against the native form of mSC, were biotinylated using sulfosuccinimidyl NHS-LC-biotin (Pierce) as described by the manufacturer and recovered and stored as indicated above for the mSC and Igα proteins.

Binding of mSC-FLAG Mutants to Immobilized IgA—The wells of Nunc MaxiSorp ELISA plates were coated with 50 μl of either purified IgGμ (5 μg/ml) or IgGα (2.5 μg/ml) dissolved in PBS. Control wells were coated with murine IgG (Sigma, 2 μg/ml) or left uncoated. Wells were blocked with 0.2 ml of Tris-buffered saline (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl (pH 7.5)) containing 5% (w/v) nonfat dry milk and 0.05% (w/v) Tween 20 (Bio-Rad). Murine SC prepared by concanavalin A-agarose chromatography (Vector Laboratories; Ref. 13) and culture supernatants were supplemented with 30 mM iodoacetamide.

Murine SC proteins were recovered from the supernatants by concanavalin A-agarose chromatography were incubated with 1 μg/ml of purified IgGμ or IgGα diluted in blocking buffer. The volume was adjusted to 500 μl, 50 μl of streptavidin-agarose slurry (Amersham Pharmacia Biotech), and the tubes were rotated for 4 h at 4 °C. Beads were pelleted by gentle centrifugation and washed four times with TENT buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 150 mM NaCl, 1% Triton X-100) before boiling in 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). Samples were stored at -20 °C until analysis by immunoblotting using rabbit antiserum to mSC and HRP-conjugated mouse anti-rabbit IgG and the chemiluminescence assay (Amersham Pharmacia Biotech).

Overlay Binding Assay—Murine SC-FLAG mutant samples were diluted 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), boiled for 3 min, and fractionated in nonreducing, denaturing 6% polyacrylamide gels. Blotting to polyvinylidiene difluoride Immobilon-P (Millipore) was carried out with transfer buffer lacking SDS. Nonspecific binding sites on the membrane were blocked with Tris-buffered saline containing 0.05% Tween 20 (w/v) and 5% nonfat dry milk (w/v). Membranes carrying mSC-FLAG mutants were incubated overnight at 4 °C with purified IgGμ at a concentration of 5 μg/ml in blocking buffer. Specific binding of IgGμ was detected using biotinylated goat IgG to murine α chain (1:2,000 dilution) followed by HRP-coupled ExtrAvidin (Sigma; 1:3,000 dilution).

Cell lysates containing mpIgR or mpIgR-FLAG:D2:BC mutant were subjected to the same protocol except that biotinylated IgGμ was incubated in the overlay in place of IgGα.

RESULTS

mSC-FLAG Mutants Keep Binding Selectivity for IgAμ over IgGμ—We have generated a series of mSC molecules wherein loops predicted to be surface-displayed had been replaced with the FLAG epitope (13). The IgA binding capacity of the various mSC-FLAG mutans was determined by ELISA. IgGμ or IgGα purified from the mouse hybridoma ZAC3 (14) was coated onto microwells and incubated with a 20-fold molar excess of the various SC-FLAG. Equilibrium was achieved within 1 h of incubation.2 Fig. 1A shows that all mutants and wild type mSC were able to recognize IgGμ, with the exception of mSC-FLAG: D1:FG, used as a negative control. Given that mutation of rabbit IgG domain I loop F-G abolishes binding to IgA (5), the selective binding of other mutants validates the option to select for substitution sites in domains II and III and reflects the sensitivity of the assay. Thus, the loops of mSC that carry the FLAG do not contain essential determinants necessary for specific recognition of IgGμ despite their superficial localization in the molecular models. Furthermore, the lack of binding of the mutants to immobilized IgGα indicates that specific recognition of IgGα is preserved (Fig. 1A).

Accessibility of the FLAG Epitope in IgAμ-bound SC Resembles That of Unbound SC—We next tested using ELISA whether the FLAG epitope was still displayed on the surface when the mutants are bound to IgAμ. Fig. 1B shows that the epitope was detectable in mSC-FLAG:Cterm, mSC-FLAG:D2: D3, or mSC-FLAG:D2:BC associated with IgGμ to an extent similar to the free form (13). Other mutants not reacting with the anti-FLAG mAb M2 as unbound material remained undetectable. No signal was obtained with mSC-FLAG:D1:FG, since the latter does not bind to coated IgGα. This observation together with the absence of anti-FLAG reactivity following coating with IgGαμ demonstrates the specificity of the assay. Results in Fig. 1A and 1B data show that we have designed mutant SC proteins that can both carry epitopes exposed or buried in recombinant sIgA. In terms of assembly, this indicates that the environmental changes induced by the epitope do
not alter the binding of the mutants to IgA, most likely reflecting a remote location with respect to the IgA polypeptides.

Epitope Replacements In Domains II and III of mSC Preserve High Affinity Binding to IgA—To quantitatively determine the relative affinity of the various SC mutants for IgA, we next performed competition ELISAs (16) using mixtures of a fixed amount of biotinylated mSC-FLAG:Cterm and increasing amounts of each individual mutant. Fig. 2 shows that mutants displaying the FLAG sequence within domains II and III or at the carboxyl terminus were as good inhibitors as wild type recombinant mSC. Murine SC-FLAG:D1:FG was not inhibitory at the highest concentration tested, in agreement with data in Fig. 1. The concentration needed to reach 50% inhibition of binding of biotinylated mSC-FLAG:Cterm (IC50) was about 1 nM for wild type mSC and 1–2 nM for all the mutants except mSC-FLAG:D2-D3, which had a slightly lower affinity (6 nM; Table I). These results are consistent with domains II and III not being required for high affinity recognition of IgA (Fig. 1, A–B; Refs. 5, 17, and 18).

The FLAG-specific mAb M2 Does Not Block Binding of the mSC-FLAG Mutants to IgA—Some mutants did not expose the FLAG, neither in the free form nor when bound to IgA. However, the interaction with IgA was not prevented (Figs. 1 and 2), suggesting that both the FLAG and neighboring residues are distant to the contact area with IgA. We therefore tested whether binding to IgA could be impaired by preincubation of the mutants with a 4-fold molar excess of mAb M2.

Subsequent binding to immobilized IgA was assessed by ELISA. Fig. 3 shows that binding was not inhibited in any case and was even significantly enhanced for mutant mSC-FLAG:D3:DE. The lack of inhibition seen for mSC-FLAG:Cterm, mSC-FLAG:D2-D3, and mSC-FLAG:D2:BC is consistent with the FLAG being at the surface before and after binding to IgA and domains II and III exhibiting a degree of flexibility not affecting IgA recognition. As expected, mutants not recognized by the mAb M2 as free proteins keep binding IgA efficiently. The increased binding of mSC-FLAG:D3:DE in the presence of mAb M2 can be explained by the formation of a ternary complex (IgA SC M2), as detected by sieving chromatography under native (PBS) conditions, with M2 having induced conformational changes favoring association.

The Presence of the FLAG Impairs the Covalent Binding to IgA in Most Mutants—In sIgA isolated from mouse milk, 70–80% of mSC is covalently linked to IgA (19). We thus examined to which extent SC-FLAG mutants can form covalent complexes with IgA. Equimolar amounts of biotinylated IgA and wild type mSC or mSC-FLAG mutants were incubated for 1 h at ambient temperature before precipitation with streptavidin beads. Bound material was resolved by SDS-PAGE under nonreducing conditions, and the presence of free and IgA-associated mSC-FLAG was determined by immunoblotting (Fig. 4). Monomers and dimers of all mutants were co-immunoprecipitated with IgA, confirming that data in Fig. 1 reflect the actual binding of both species. Specificity was confirmed by the absence of signal for mSC-FLAG:D1:FG. No signal arose either when wild type mSC was immunoprecipitated in the absence of biotinylated IgA or in the presence of IgA (19). A higher M, band was detected in the case of mSC-FLAG:D3:DE, mSC-FLAG:Cterm, and wild type mSC, corresponding to covalent IgA SC-FLAG complexes. Immunoprecipitation was quantitative, as reflected by the very similar signals seen for IgA upon detection with HRP-conjugated streptavidin. Thus, even though data in Figs. 1–3 demonstrate preserved IgA

---

3 B. Cortésy, unpublished data.
Domain II-III Mutant SC-IgA Interactions

Table summarizing the data shown in Figs. 1–6. Proteins from culture supernatant that bound to immobilized concanavalin A were used in these experiments. None of the proteins bound significantly to IgAm. mo, mSC monomer; di, mSC dimer; −, no signal; +, intermediate signal; ++/+++, strong signal; ND, not determined.

Table I
IgA binding properties of mSC-FLAG proteins secreted by COS cells

| Protein                  | Normal cell culture conditions | 2-Mercaptoethanol present* |
|--------------------------|-------------------------------|-----------------------------|
|                          | IC [S]                        | Covalency | FLAG detection† | M2 pre- incubation‡ | Overlay with IgAd | FLAG detection† | Overlay with IgAd |
|                          |                               | SC-IgAd |               |               |                 |               |                 |
| mSC-FLAG:D1:FG           | >100                          | No      | −              | −              | −               | −              | −               |
| mSC-FLAG:D2:BC           | 2                             | No      | +              | No effect     | + mo/di         | + di           | + di           |
| mSC-FLAG:D2:DE           | 2                             | No      | −              | No effect     | + mo/di         | −              | ND             |
| mSC-FLAG:D2:D3           | 6                             | No      | ++             | No effect     | + mo/di         | ++             | + di           |
| mSC-FLAG:D3:CC           | 1.5                           | No      | −              | −              | −               | ++             | ND             |
| mSC-FLAG:D3:DE           | 1                             | Yes     | +              | Enhanced      | −               | −              | ND             |
| mSC-FLAG:Cterm           | 1.5                           | Yes     | ++             | No effect     | + mo/di         | + di           | + di           |
| Wild type mSC            | 1                             | Yes     | −              | + mo/di       | n.d.            | ND             | ND             |

* Cell cultures were exposed to 2.5 mM 2-mercaptoethanol, and the collected culture media were treated with 30 mM iodoacetamide.
† Relative detectability of proteins bound to immobilized IgA, using the mAb M2.
‡ The binding to immobilized IgAd of proteins preincubated with an excess of mAb M2 was compared with that of control samples.
§ Proteins fractionated by nonreducing SDS-PAGE were blotted and incubated with IgAd in solution.

Differential IgAd Binding to mSC-FLAG Molecular Forms Previously Separated by SDS-PAGE—The relative inaccessibility of the FLAG to immunodetection after SDS-PAGE under nonreducing conditions and transfer (13) suggests that partial renaturation of SC occurs on the blotting membrane. This raises the possibility of examining, using an overlay assay, whether IgAd in solution reassociates preferentially with either mutant SC monomers, dimers, or both. Fig. 5A shows that several mSC mutants could be recognized by IgAd. No signal was detected when IgAm was present in the overlay. IgAm recognized both dimeric and monomeric mSC-FLAG:D2:BC, while mSC-FLAG:D2:DE, dimeric and to a lesser extent monomeric mSC-FLAG:D2:D3, mSC-FLAG:Cterm, and wild type mSC. The pattern of proteins present was verified after stripping of the membrane and immunodetection with IgG to the FLAG and IgG to mSC. The partial discrepancy with binding data obtained using mSC-FLAG proteins in solution (Figs. 1 and 2) together with the efficiency of the assay when using wild type or mSC-FLAG:Cterm suggests that the mere presence of the FLAG precludes renaturation of a selection of mSC mutants. The assay is, however, reliable and convenient to rapidly test supernatants containing recombinant SC or mutant plgR in cell extracts.

IgAd Recognizes SC and pIgR in a Different Manner—We then tested the binding of IgAd to wild type plgR and the plgR-FLAG:D2:BC mutant. Although the SC counterpart of both proteins was recognized by IgAd, only the wild type plgR can serve as a binding site on the membrane (Fig. 5B). After stripping, immunodetection of the same blot with IgG to mSC detected both proteins, including monomeric and dimeric plgR-FLAG:D2:BC. This result is at variance with the recognition by IgAd of both monomeric and dimeric mSC-FLAG: D2:BC (Fig. 5A) and argues in favor of a reduced binding stringency of SC as compared with plgR. Thus, it appears that the mode of recognition of SC and of the complete receptor may differ.

Bioassembly of mSC-FLAG Proteins in the Presence of 2-Mercaptoethanol Modifies Their IgA-binding Properties—The presence of the reducing agent 2-mercaptoethanol during culture did not alter the ability of mSC-FLAG mutants to form dimers.
but changed the immunoreactivity of the epitope in some instances (13). We determined the effect of this treatment on IgA binding properties of the mutants. As seen in Fig. 6A, mSC-FLAG:D2-D3 was the only mutant whose ability to bind immobilized IgA was affected significantly by this treatment. The control mutant mSC-FLAG:D1:FG did not bind to IgA, and no mutants bound above background levels to IgA. Fig. 6B shows a similar experiment using mAb M2 for detection of the FLAG in mSC mutants bound to IgA. Strikingly, the FLAG was well detected in IgA-bound mSC-FLAG:D2-D3 despite the poor binding detected using the antibody to mSC, suggesting an exacerbated surface exposure of the FLAG. A strong signal was also obtained with mSC-FLAG:D3:CC, in sharp contrast to what was seen when the protein was produced under normal conditions (Fig. 1B). In this case, however, the treatment also increased the immunoreactivity of the FLAG in the free protein (13). It is possible, therefore, that the C'-C loop is no longer constrained by a disulfide bond under these conditions. Thus, reduction has selectively altered the structure of some mutants in a manner that is unraveled upon IgA binding.

Fig. 6C shows a comparative overlay analysis of proteins that had been synthesized in the presence or absence of reducing agent. Monomeric mSC-FLAG:D2:BC and both forms of mSC-FLAG:D2-D3 have lost their capacity to be recognized in this assay. Therefore, the effect of exposure to 2-mercaptoethanol was more pronounced on the capacity of the mutants to renature (13) than on their ability to bind immobilized IgA, or IgA was determined by ELISA. Protein binding was detected using IgG to native mSC (A) or mAb M2 (B) followed by HRP-coupled secondary reagents. Data are the mean \pm S.D. of triplicate wells. C, overlay of IgA to mSC-FLAG proteins from cultures performed with (+) or without (−) 2-mercaptoethanol (2-ME). Proteins were blotted from a nonreducing gel, and binding was detected with biotinylated antibody to the α chain of IgA and HRP-coupled streptavidin.

**DISCUSSION**

We have studied the IgA binding properties of a panel of mSC mutants with the FLAG octapeptide replacing SC sequences (13). To our knowledge, this is the first report studying the interaction between murine IgA and murine SC. In terms of the structure-function relationship, we demonstrate that the integrity of domains II and III of mSC is not essential for IgA recognition. In contrast, it is necessary to permit formation of covalent binding between mSC and IgA through optimal positioning of domain V in the molecule. Furthermore, although the mSC carrying sequence changes in domain II and III remains an excellent IgA binder, mutated plgR cannot accom-
measured an IC50 of 10 nM for the interaction taking place between either recombinant or milk-derived hSC and the same murine IgAa (16). Thus, it appears that mSC has a higher affinity than hSC for murine IgAa. In the same setting an IC50 in the range of 1 nM, whereas that of rat SC was about 40 nM (4). Scatchard analysis yielded a negative affinities for human IgAa p of bovine and rabbit SC were in the range of 1 nM, whereas that of rat SC was about 40 nM (4). In the same setting an IC50 in the range of 1 nM, whereas that of rat SC was about 40 nM (4).

Wild type mSC and mSC-FLAG mutants were selective for IgAa over IgAm, as was our mutant of loop E-F in rabbit SC (27). Recombinant hSC domain I was shown to bind IgAa, but not IgG (18), suggesting that this domain discriminates among Ig classes, whereas the other domains are responsible for distinguishing the oligomeric state of the Ig. Thus, the loops mutated in domains II and III of mSC do not contain any motif important for the recognition of Ig polymers. In contrast, Bakos et al. (22) showed that tryptic peptides encompassing the first two or first three domains of hSC could bind both IgAa, and IgM but did not recognize IgAa or IgG. A peptide comprising domain II alone could not bind to either molecule. Although such discrepancies could be intrinsic to species differences, we favor the hypothesis that our approach, based on specific sequence modifications, does not drastically perturb the overall conformation and allows more accurate pinpointing of the structure-function relationship in the complex.

As in most species (1), mSC is partially bound in a covalent manner in sIgA isolated from milk (19). Mapping of sIgA isolated from human colostrum indicated that SC was disulfide-bridged via domain V to the Ca2 domain of IgAa (10). Most mSC-FLAG mutants bound IgAa in a noncovalent manner only, indicating that the positioning of the reactive bond was not optimal. The absence of covalence was also seen when rabbit SC lacking domains II-III is bound to IgAa (6, 7) or when the g subclass of rabbit IgA is bound by full-length SC (11). Notably, none of the mSC-FLAG mutant forming dimers could bind covalently to IgAa, suggesting that their overall flexibility was hindered; this represents further evidence that one SC molecule has to be present per sIgA complex. Interestingly, in the two mSC-FLAG mutants that bound IgAa in a covalent manner, the FLAG was located at the end of domain V or was closest to the carboxyl terminus (loop D-E of domain III).

One aim of this study was to engineer mSC-FLAG mutants capable of binding to IgAa and still exposing the epitope on their surface. Our approach relied on the specific replacement of SC sequences with the FLAG epitope that can be traced using a specific mAb. We found that the FLAG was best available in IgAa-bound mutants that already exposed the epitope in their free form (Ref. 13; Fig. 1 and Table I; insertion at the carboxyl terminus of domain V and in between domains II-III represented optimal locations. Excess mAb to the FLAG did not inhibit the binding of mSC proteins to immobilized IgAa and, in the case of mSC-FLAG:D3:DE, even enhanced binding. Studies using mAbs have shown that IgA-induced conformational changes occur in domains II-III of hSC (28). Together, the data reflect the flexibility of the SC in that portion of the molecule and suggest that domain II and domain III do not establish close contacts with the IgA backbone. In contrast, domains II-III might be essential to trigger the “zipper effect” initiated by the interaction of domain I with IgA and progressing to domain V. Given the very different binding properties of mplIgR-FLAG:D2:BC and mSC-FLAG:D2:BC, it is also possible that integral domains II-III are dispensable in SC but mandatory in pIgR. Together, the data demonstrate that mSC “anti-genized” in domains II and III, once combined with IgAa, represent an adequate option to stabilize short antigen sequences for oral administration.

Binding assays based on blot overlay rely on the capacity of proteins exposed to SDS to refold into a functional state and have been applied frequently in studies involving cytosolic proteins. Examples of membrane-bound ecto-proteins that have been probed by overlay include the low density lipoprotein receptor (29), the asialo-glycoprotein receptor (30), high density lipoprotein-binding proteins (31), and hyaluronan binding proteins (32, 33). Furthermore, the cytosolic domain of pIgR is also bound by calmodulin in overlay experiments (34, 35). We have found that wild type mSC and mplIgR as well as several mSC-FLAG mutants could be recognized by IgAa in solution (see Table I). Since all the mutants but mSC-FLAG:D1:FG were able to recognize IgAa under native conditions, we conclude that only the renaturation of mutants mSC-FLAG:D3:CC and mSC-FLAG:D3:DE were impaired following boiling in SDS, fractionation, and blotting. Notably, both hSC denatured in 6 M guanidinium-HCl or rabbit pIgR boiled in 2% SDS can subsequently regain IgA binding activity (22, 25).

Overlay assays of extracellular domains are sensitive to reduction but favored by blocking the membrane with nonionic detergents (29, 30, 32). In this respect, wild type mSC and mutants thereof, which contain many essential disulfide bridges, behaved accordingly. In contrast to mSC-FLAG:D2:BC, the mplIgR-FLAG:D2:B2 mutant was not recognized by overlay IgAa despite sharing the same extracellular domain. Given that wild type pIgR and mSC-FLAG:D2:BC are recognized by IgAa, it is unlikely that refolding of the ectodomain of mplIgR-FLAG:D2:BC is affected by the presence of transmembrane and cytosolic domains. We rather favor the hypothesis that the mode of IgA binding differs between SC and pIgR, yet preserves the preference for IgAa over IgAm.

Mutants that were secreted with culture medium containing 2-mercaptoethanol were still dimerizing, and the exposure of the FLAG was not dramatically affected (13). These proteins were generally able to bind IgAa under native conditions (the binding of mSC-FLAG:D2:D3 was reduced), but their ability to be recognized in the overlay assay was altered. Thus, the assay revealed subtle folding defects in these mutants that were induced by the reducing agent.

In conclusion, we present evidence that surface loops in domains II and III of mSC do not affect binding to IgAa, in contrast to what was observed for the same loops in domain I (5, 27). Antibody reactivity of the FLAG motif in domains II and III loops in free and IgAa-bound mSC mutants maps multiple insertion sites for epitope substitution. A simple, rapid binding assay we developed based on FLAG-substituted molecules allowed us to dissect the different binding properties of SC and pIgR. The topological study presented here establishes that the integrity of mSC domains II and III is not essential to preserve specific IgAa binding but is necessary for covalency to take place.

Acknowledgments—We thank Dr. Elke Lüllau for the gift of purified IgA preparations and Corinne Tallichet Blanc for skillful technical assistance.

REFERENCES
1. Mestecky, J., and McGhee, J. R. (1987) Adv. Immunol. 40, 515–519
2. Brandztæg, P., Krajée, P., Lamn, M. E., and Kaetzel, C. S. (1994) in Handbook of Mucosal Immunology (P. L. Ogra, J. Mestecky, M. E. Lamn, W. Strober, J. R. McGhee, and J. Bienenstock, eds) pp. 113–126, Academic Press, San Diego, CA
3. Mastov, K. E. (1994) Annu. Rev. Immunol. 12, 63–84
4. Bakos, M.-A., Kuroska, A., Czerwinski, E. W., and Goldblum, R. M. (1993) J. Immunol. 151, 1346–1352
5. Coney, R. S., Siebrecht, M., Peitsch, M. C., and Casanova, J. E. (1994) J. Biol.
Domain II-III Mutant SC-IgA Interactions

1. Solari, R., Kühn, L., and Kraehenbuhl, J.-P. (1985) J. Biol. Chem. 260, 1141–1145
2. Frutiger, S., Hughes, G. J., Fonck, C., and Jaton, J.-C. (1987) J. Biol. Chem. 262, 1712–1715
3. Cunningham-Rundles, C., and Lamm, M. E. (1975) J. Biol. Chem. 250, 1987–1991
4. Lindh, E., and Björk, I. (1976) Eur. J. Biochem. 62, 263–270
5. Fallgreen-Gebauer, E., Gebauer, W., Bastian, A., Kratzin, H. D., Effert, H., Zimmermann, B., Karas, M., and Hilschmann, N. (1993) Biol. Chem. Hoppe-Seyler 374, 1023–1028
6. Knight, K. L., Vetter, M. L., and Malek, T. R. (1975) J. Immunol. 115, 595–598
7. Tamer, C. M., Lamm, M. E., Robinson, J. K., Piskurich, J. F., and Kaetzel, C. S. (1995) J. Immunol. 155, 707–714
8. Crottet, P., Peitsch, M., Servis, C., and Cortheesy, B. J. (1999) J. Biol. Chem. 274, 31445–31455
9. Lallau, E., Heyse, S., Vogel, H., Marison, I., von Stockar, U., Kraehenbuhl, J.-P., and Corthéesy, B. (1996) J. Biol. Chem. 271, 16300–16309
10. Norgard, K. E., Han, H., Powell, L., Kriegler, M., Varki, A., and Varki, N. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1068–1072
11. Crottet, P., and Cortheesy, B. (1998) J. Immunol. 161, 5445–5453
12. Frutiger, S., Hughes, G. J., Hanly, W. C., Kingzette, M., and Jaton, J.-C. (1986) J. Biol. Chem. 261, 16673–16681
13. Bakos, M. A., Widen, S. G., and Goldblum, R. M. (1994) Mol. Immunol. 31, 165–168
14. Parr, E. L., Bozzola, J. J., and Parr, M. B. (1995) J. Immunol. Methods 180, 147–157
15. Rindisbacher, L., Cottet, S., Wittek, R., Kraehenbuhl J.-P., and Cortésy, B. (1995) J. Biol. Chem. 270, 14220–14228
16. Beale, D. (1988) Int. J. Biochem. 20, 873–879
17. Bakos, M.-A., Kurosky, A., and Goldblum, R. M. (1991) J. Immunol. 147, 3419–3426
18. Weicker, J., and Underdown, B. J. (1975) J. Immunol. 114, 1337–1344
19. Kühn, L. C., and Kraehenbuhl, J.-P. (1979) J. Biol. Chem. 254, 11066–11071
20. Kühn, L. C., and Kraehenbuhl, J.-P. (1981) J. Biol. Chem. 256, 12490–12495
21. Song, W., Vaerman, J.-P., and Mostov, K. E. (1995) J. Immunol. 155, 715–721
22. Cortheesy, B., Kaufmann, M., Phalipon, A., Peitsch, M., Neutra, M. R., and Kraehenbuhl, J.-P. (1996) J. Biol. Chem. 271, 35670–35677
23. Bakos, M.-A., Kurosky, A., Woodard, C. S., Denney, R. M., and Goldblum, R. M. (1991) J. Immunol. 146, 162–168
24. Daniel, T. O., Schneider, W. J., Goldstein, J. L., and Brown, M. S. (1983) J. Biol. Chem. 258, 4606–4611
25. Zeng, F. Y., Oka, J. A., and Weigel, P. H. (1996) Glycobiology 6, 247–255
26. Nion, S., Briand, O., Lestavel, S., Terpier, G., Nazih, F., Delbart, C., Fruchart, J.-C., and Clavey, V. (1997) Biochem. J. 328, 415–423
27. Yannariello-Brown, J., Zhou, B., Ritchie, D., Oka, J. A., and Weigel, P. H. (1996) Biochem. Biophys. Res. Commun. 218, 314–319
28. Yannariello-Brown, J., Zhou, B., and Weigel, P. H. (1997) Glycobiology 7, 15–21
29. Chapin, S. J., Enrich, C., Aroeti, B., Havel, R. J., and Mostov, K. E. (1996) J. Biol. Chem. 271, 1336–1342
30. Enrich, C., Jackle, S., and Havel, R. J. (1996) Hepatology 24, 226–232
31. Crottet, P., Cottet, S., and Cortheesy, B. (1999) Biochem. J. 341, 299–306