Differential Structure-Function Requirements of the Transmembranal Domain of the B Cell Antigen Receptor

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Summary

By generating phosphorylcholine (PC)-specific, wild-type (μ), and chimeric (μ-I-Aα) antigen receptor transfectants of mature B cells, we have shown that the COOH terminus of the μ heavy chain is essential for three major functions: immediate signal transduction (measured as changes in intracellular Ca^{2+}), antigen presentation, and induction of immunoglobulin M secretion. A more detailed analysis of structural requirements of the COOH-terminal domains contributing to these functions was achieved by systematically replacing the spacer, cytoplasmic, and transmembranal domains of the μ-I-Aα chimeric chain with those of μ. Using this rescue approach, we show that the carboxyl two-thirds of the transmembranal domain (proximal to the cytoplasmic domain) is required for induction of intracellular Ca^{2+}, whereas the complete transmembranal domain is required for the function of antigen presentation but is dispensable for induction of antibody secretion.

Membrane IgM (mIgM)[1] molecules serve as antigen-specific receptors on mature B cells (1). The crosslinking of these receptors with antigen induces a complex series of biochemical reactions (2, 3). Some of the documented immediate biochemical changes include hydrolysis of phosphatidyl inositol into inositol 1,4,5 triphosphate and diacylglycerol (DAG), followed by a rise in intracellular Ca^{2+} levels (4). DAG and Ca^{2+} lead to activation of protein kinase C (PKC), which might be involved in induction of various immediate early genes (5–7). In addition, recent studies indicate activation of tyrosine kinase(s) immediately after crosslinking of mIgM (8–10). While these and various other biochemical changes are taking place, the mIgM receptor is endocytosed, ligand is processed, and some of the ligand peptides are presented to T cells in a MHC class II–restricted fashion (11). The resulting cognate B-T cell interaction leads to lymphokine secretion (12), which in turn helps mature B cells to differentiate into plasma cells (13).

The extracellular NH_2 terminus of mIgM endows it with antigen specificity, whereas the membrane-associated COOH terminus, in concert with associated proteins, appears to be involved in initiating the cascade of reactions mentioned above (14). We have shown that the COOH terminus is required for antigen-specific induction of intracellular Ca^{2+} levels, induction of immediate early gene messages, and growth arrest in CH33 cells (15), but not for endocytosis of ligand-receptor complexes (16). This was achieved by replacing 40 amino acids of the COOH terminus of mIgM with the equivalent segment from the COOH terminus of MHC class II I-A$^b\alpha$ (17). The COOH terminus can be divided into three hypothetical domains: spacer, transmembranal (TM), and cytoplasmic (Fig. 1). Here, by domain shuffle mutagenesis (DSM), we have tried to define the structural requirements of the TM domain (summarized in Fig. 1 *c*) for different functions, such as induction of intracellular Ca^{2+} levels, antigen presentation, and induction of secretion.

Materials and Methods

Reagents. All reagents except hypoxanthine, xanthine, thymidine, adenine, and acetomethyl-ester of indo 1 (Indo 1-AM) were purchased from Gibco Laboratories (Grand Island, NY). The Indo 1-AM was purchased from Molecular Probes (Eugene, OR), and the remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Purified anti-T15-idiotype (Id)$^+$ antibody, AB1-2 (18), and fluorescein-conjugated goat anti-mouse IgG1 were obtained from Southern Biotechnology Associates (Birmingham, AL).

[1] *Abbreviations used in this paper:* DSM, domain shuffle mutagenesis; HEL, hen egg lysozyme; Id, idiotype; mIgM, membrane immunoglobulin M; PKC, protein kinase C; TM, transmembranal.

Anti-IgM $F(ab')_2$ fragments were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Plasmid Construction. All plasmid constructs contain *Escherichia coli* ampicillin and guanine phosphoribosyl transferase genes and murine genomic sequences for productively rearranged VHS107-$C\mu$ and V_κ 22-C_κ Ig chains (19). The chimeric plasmid with the I-$A^b\alpha$ tail (Fig. 1 *b*) was made by replacing a 1.7-kb fragment spanning m1 and m2 exons of the μ heavy chain with a 2.8-kb fragment spanning the membrane exon and 3′ untranslated region of the MHC class II I-$A^b\alpha$ chain (17). Similarly, all other mutant plasmids were made by replacing the 1.7-kb μ membrane exons containing fragment with mutant fragments (see below).

Domain Shuffle Mutagenesis. The 700-bp fragment containing only the membrane exon of the class II I-Aα chain was cloned into M13 bacteriophage. Single-stranded DNA was generated and used to construct domain shuffle mutant μ-S-C (Fig. 1), by standard site-directed mutagenesis (20). This 700-bp mutant fragment was then cloned in front of the 2.1-kb 3′ untranslated region of the MHC class II I-Aα chain. This 2.8-kb fragment carrying mutation was then used to replace 1.7-kb μ membrane exons containing fragment. Mutants TM1 and TM2 (Fig. 1) were made by the PCR sowing technique (21). Initially, the mutant μ-S (μ-spacer, I-Aα TM, and cytoplasmic) mutant was made by standard site-directed mutagenesis. This mutant fragment and the 1.7-kb μ membrane exons fragment were used as templates for the PCR sowing technique. All the mutants were sequenced by the dideoxy sequencing method to confirm the changes made.

Parental Cells and T15-Id$^+$-transfected Cell Lines. BCL1 (μ^+, δ^+, I-A^d) (22) and CH12.LX (μ^+, δ^+, I-A^k, Ly-1^+) (23) murine B cell lymphomas were used as parental cell lines in all experiments. T15-Id$^+$ transfectants were generated by electroporating 10–15 μg of linearized plasmid DNA into the parental cells (24). Transfected cells were selected by their resistance to mycophenolic acid and then were cloned by limiting dilution.

Immunofluorescent Staining. Surface expression of the T15-Id on transfected cells was determined by staining with AB1-2 followed by fluorescein-conjugated goat anti–mouse IgG1 antibody (18). As controls, nontransfected parental cells were stained for T15-Id and transfectants were stained with fluorescein-conjugated goat anti–mouse IgG1 alone. Staining profiles were analyzed by FACScan® (Becton Dickinson & Co., Mountain View, CA).

Calcium Analysis. Changes in Ca^{2+} levels were measured by cell sorter (Ortho Diagnostics Systems, Westwood, MA) as described (25). Briefly, cells grown to log-phase were loaded with the Indo-1-AM (10 μM/6–7 $\times$ 10^6 cells) for 25 min and were then allowed to equilibrate in 10-fold excess medium for 25 min in the dark at 37°C. The cell sorter analysis was done at the rate of ~350 cells/s. The base line was established for 1 min before activating cells with the antigen PC-KLH (5 μg/2 $\times$ 10^6 cells/2 ml). Within 10–20 s after addition of antigen, recording of changes in levels of Ca^{2+} was resumed. Anti-IgM $F(ab')_2$ fragments were used as a positive control antibody to check the ability of the cells to induce a rise in intracellular Ca^{2+}. Data were analyzed using the "Cicero Ca^{2+} analysis" program.

Antigen Presentation. Antigen presentation was carried out as previously described (26). Briefly, 5 $\times$ 10^5 cells of the T cell hybridoma, CPC-1-8, derived from BALB/c mice (27), were cocultured with 10^6 cells of BCL1 parental or transfected cells in a total volume of 200 μl in the presence of various concentrations of PC–hen egg lysozyme (PC-HEL) or PBS at 37°C for 20–24 h. The ability of the culture supernatants to support the growth of an IL-2-dependent cell line, CTLL-2, was then determined. 100 μl of culture supernatant was incubated with 5 $\times$ 10^3 CTLL-2 cells in total volume of 200 μl RPMI 1640 supplemented with 5% FCS. After 20–24 h, cells were pulsed with [^{3}H]dTR (1 μCi/well) for 12–16 h and harvested on glass filters (Whatman Inc., Clifton, NJ). The [^{3}H]dTR uptake was measured in a scintillation counter (Beckman Instruments, Inc., Palo Alto, CA).

Plaque-forming Colony Assays. Log-phase transfectants, washed thrice with RPMI, were seeded into a 96-well microtiter plate (1.5 $\times$ 10^3 cells/well) and incubated with anti-I-E^k antibody (14-4-4S; 50 ng/ml) and medium alone, or with anti-I-E^k antibody and one of the following: anti-CH12-Id$^+$ antibody (5E3; 300 ng/ml), anti-T15 antibody (AB1-2; 300 ng/ml), or with PC-KLH (1 μg/ml) for 72 h at 37°C in an atmosphere of 5% CO_2. After 72 h, cells were washed with RPMI, and a fraction of cells was then analyzed for their ability to form plaques as previously described (28).

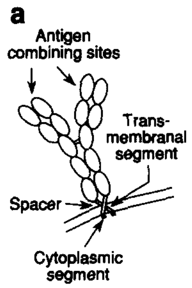
Results

Establishment of Antigen-specific, Wild-Type and Mutant Cell Lines. Antigen-specific transfectants were generated by electroporating wild-type and mutant plasmid DNAs into parental cell lines. The μ-I-Aα plasmid was made by replacing 40 amino acids comprising three domains: spacer, TM, and cytoplasmic, with the analogous amino acids from the MHC class II I-Aα (Fig. 1 *b*). The I-Aα COOH terminus was chosen because the genomic clone was available, it used the same RNA-splicing site as μm, and there is no significant homology to μm in any of the COOH-terminal domains. The other DSMs (Fig. 1 *b*) were generated either by site-directed mutagenesis or by a PCR sowing technique. Wild-type and mutant constructs were transfected into BCL_1 (μ^+,δ^+,I-A^d) (22) and CH12.LX (μ^+, δ^+, I-A^k) (23) B lymphoma cell lines. BCL_1 was used for studying an immediate signal transduction event (induction of Ca^{2+} levels) and for antigen presentation. Parental and transfected BCL_1 cells express an MHC haplotype for which T cell clones specific for PC-HEL are available (27), and they have been shown to present antigen (26). CH12.LX cells were used to study an induction of IgM secretion, a measure of B cell differentiation. These cells can be activated by antigen (or antiidiotypic antibody) plus anti-MHC class II I-E^k antibody, which acts as a surrogate for T cell help (23). This phenomenon of induction of secretion of antibody is not restricted to CH12.LX cells since normal B cells can also be triggered to secrete using the similar induction protocol. (29, 30).

The surface expression of transfected receptors was determined by immunofluorescence staining with an antiidiotypic antibody, AB1–2 (18) (Fig. 2). Besides TM1 and TM2 receptors, all other mutant receptors on BCL1 cells were expressed at levels generally equivalent to wild-type (Fig. 2 *a–c*, *g*, and *h*). Mutant receptors on CH12.LX cells were expressed at lower levels (Fig. 2, *d–f*) than those on BCL1 cells but were higher than wild-type, dismissing the possibility that differential functional activities could be ascribed to differential cell surface expression.

Induction of Rise in Ca^{2+} Can Be Rescued by the Carboxy Two-Thirds of the TM Domain. An immediate rise in intracellular Ca^{2+} was observed when the wild-type IgM receptors on the surface of BCL_1 transfectants were cross-linked with the antigen PC-KLH (Fig. 3 *b*). The μ-I-Aα tail

a



b

	Spacer	Transmembranal (TM3 | TM2 | TM1)	Cytoplasmic
μ	EGEVNAEEEGFE	NLWTTASTFIVLFLLSLFYSTTVTLF	KVK
μ-I-Aα	epeipapmselte	tvvcalglsvglvgivvgtifiiqgl	rsggtsrhpgpl
μ-S-C	EGEVNAEEEGFE	tvvcalglsvglvgivvgtifiiqgl	KVK
μ-TM1	EGEVNAEEEGFE	tvvcalglsvglvgivvFYSTTVTLF	KVK
μ-TM2	EGEVNAEEEGFE	tvvcalglFIVLFLLSLFYSTTVTLF	KVK

c

Constructs	Δ Ca^{2+}	Antigen presentation	Δ Secretion
μ	+	+	+
μ-I-Aα	−	−	−
μ-S-C	−	−	+
μ-TM1	−	−	N.D.
μ-TM2	+	−	N.D.

Figure 1. Summary of structures, mutations, and results of B cell antigen receptor domain shuffling. (*a*) Schematic presentation of mIgM molecule denoting the antigen combining sites and the membrane-anchored COOH terminus of the μ heavy chain, which is divided into three hypothetical domains: spacer, TM, and cytoplasmic. (*b*) Comparison of amino acid sequences of the COOH terminus of μ and I-Aα^k chains and DSMs. The TM domain is divided into three subdomains: TM1, TM2, and TM3. (*c*) Tabular presentation of μ and DSMs and their abilities (+) or inabilities (−) to perform immediate (rise in intracellular Ca^{2+}), late (antigen presentation), and differentiative (secretion) functions when induced by antigen.

receptor lacked this capacity completely (Fig. 3 *c*), thus establishing the requirement of the COOH terminus. We could not rescue this function either by reinserting both μ cytoplasmic and spacer domains (Fig. 3 *d*) or by reinserting μ cytoplasmic and spacer domains as well as the TM subdomain, TM1 (Fig. 3 *e*). However, the replacement of both cytoplasmic and spacer domains and two TM subdomains, TM1 and TM2 (Fig. 3 *f*), restores the Ca^{2+} response.

The Antigen Presentation Function Requires the Entire μ TM Domain. When the same BCL_1 transfectants were analyzed for their ability to present antigen (PC-HEL) to appropriately MHC class II–restricted T cells (31), only wild-type (Fig. 4 *b*), but none of the mutants (Fig. 4, *c–f*), were able to activate T cells to produce IL-2. The wild-type receptor could present specific antigen (PC-HEL) 10–15 times more efficiently than nonspecific antigen (HEL). Our assay condi-

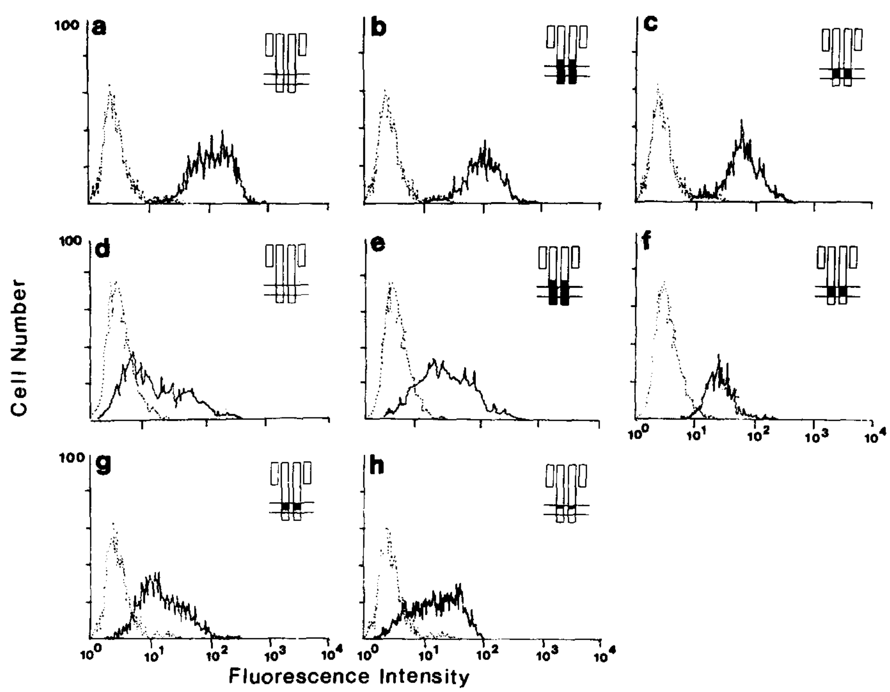


Figure 2. Wild-type and mutant receptors expression on the surface of transfected cell lines. In the schematic inserts, filled regions denote I-Aα^k sequences. Representative data of BCL_1 (*a–c*, *g*, and *h*), and CH12.LX (*d–f*) transfectants. The data shown for a single clone of each receptor are representative of multiple clones analyzed.

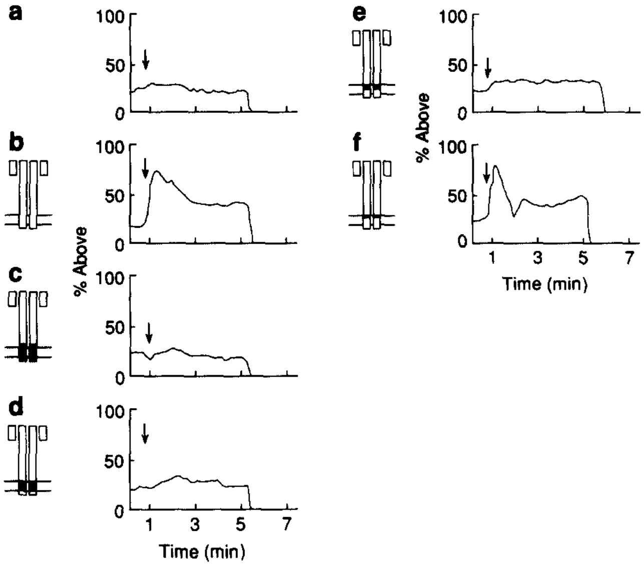


Figure 3. The TM1 and TM2 subdomains are essential for induction of Ca^{2+}. Insets designate the transfected receptor under analysis: (*a*) no receptor; (*b*) μ; (*c*) μ-I-Aα; (*d*) μ-S-C; (*e*) μ-TM1; and (*f*) μ-TM2. At least two clones for each receptor were analyzed and the data shown are representative. Changes in intracellular Ca^{2+} levels were measured by FACS® (Ortho 50HH cell sorter and Cicero computer; Cicero Cytomation, Fort Collins, CO) as described (29). The data were analyzed using the Cicero Ca^{2+} analysis program.

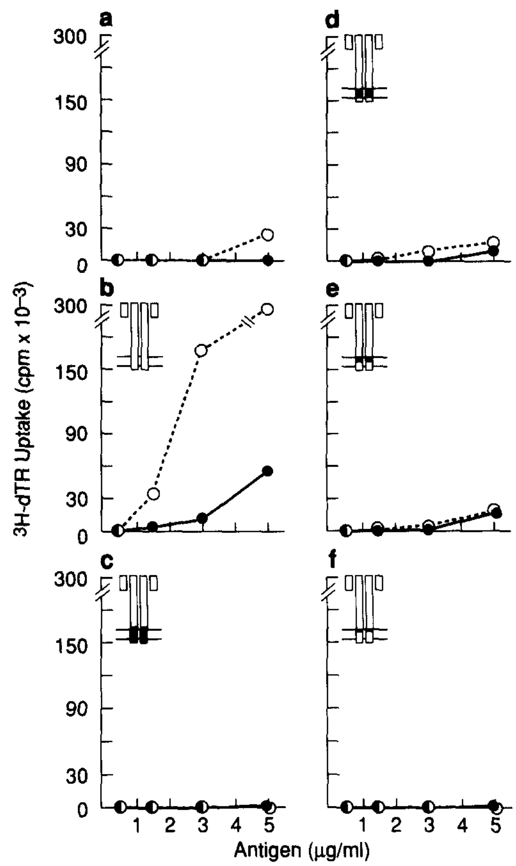


Figure 4. Entire TM domain comprising TM1, TM2, and TM3 subdomains is required for the function of antigen presentation. The ability of the supernatants, from cocultures of BCL1 parental cell (*a*) or BCL1 transfectant (*b*–*f*) and T cell hybridoma CPC-18 in presence of various concentrations of specific antigen (PC-HEL; ○) or nonspecific antigen (HEL; ●), to support the growth of an IL-2-dependent cell line CTLL-2, was considered as a measure of antigen presentation. The insets represent the transfected receptor under analysis: (*a*) no receptor; (*b*) μ; (*c*) μ-I-Aα; (*d*) μ-S-C; (*e*) μ-TM1; (*f*) μ-TM2.

tions yielded specific antigen presentation within the concentration range of 0.1–5 μg of antigen concentration. At higher concentrations, such as 15 μg, all cells could present specific and nonspecific antigens with equal efficiency (data not shown). Thus, the segment of the μ TM domain proximal to the exterior is dispensible for the immediate Ca^{2+} activation event, but the complete TM domain is necessary for the subsequent function of antigen processing.

Induction of Antibody Secretion Does Not Require the μ TM Domain. A 5–10-fold induction in secretion was observed when wild-type transfectants of CH12.LX were induced with either PC-KLH or anti-T15-Id antibody (AB1-2) and anti-I-E^k antibody (Fig. 4). As with other functions, the replacement of all three domains with that of MHC class II I-Aα completely abolished this induction (Fig. 4). However, secretion could be rescued by reinserting the μ spacer and cytoplasmic domains, suggesting that residues and/or conformation unique to the μ TM segment are not required for induction of secretion in these cells.

Discussion

Using the DSM approach, we have segregated differential structural requirements of the TM domain for three major functions of the mIgM antigen receptor. Residues located in all three operationally defined TM subdomains are necessary for antigen presentation, whereas residues in the exterior proximal (TM3) segment can be replaced without affecting Ca^{2+} flux. The complete replacement of the TM domain can support third-function, receptor-mediated antibody secretion. A potential complication in our approach is that the parental cell lines used express their own heavy and light chains. Conceivably these could associate with transfected chains to produce various heterodimeric combinations. Since PC binding requires appropriate heavy and light chains, heterodimers would either be nonfunctional or monovalent. Given the high affinity of T15 chains to reassociate with themselves (32), we do not anticipate a high fraction of heterodimers, and for that matter, their fractions should be effectively the same in wild-type or mutant cells. Furthermore, if heterodimers of the monovalent type existed at measurable levels one might anticipate an artifactually high response in all cells, assuming that a single wild-type TM region is sufficient. That we observe differential responses among mutants is incompatible with this argument.

A point mutational analysis (33) has shown that the cytoplasmic tail as well as a dipeptide (YS) within the TM1 subdomain are essential for Ca^{2+} induction. Our results are

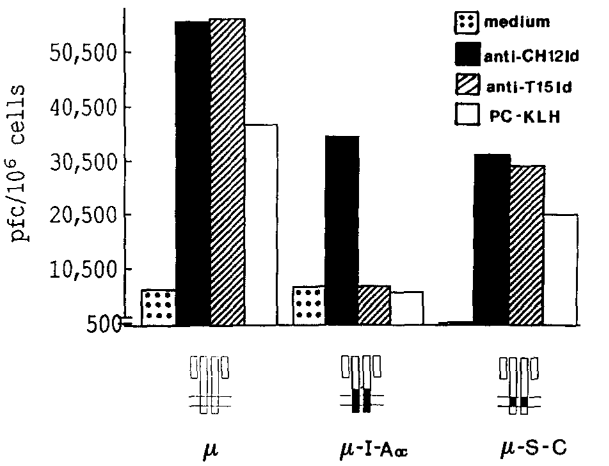


Figure 5. The TM domain is not essential for induction of antibody secretion in CH12.LX B cells. The secretion of IgM was analyzed as plaque-forming colonies (pfc) and is presented as number of pfc/10^6 recovered for wild-type (μ), μ-I-Aα, or μ-S-C transfectant cells. Log-phase transfectants were incubated with anti-I-E^k antibody and medium alone (*dotted bars*), or with anti I-E^k antibody, and one of the following: anti-CH12 Id^+ antibody (*filled bars*), anti-T15 antibody (*stippled bars*), or with PC-KLH (*open bars*). Data shown for one clone of each receptor are representative of two clones analyzed.

consistent with their findings, but indicate further that these TM1 residues alone are insufficient and that TM2 along with spacer and cytoplasmic domains are required for Ca^{2+} induction. There are several polar residues within the μ TM2 subdomain that have no counterparts in I-Aα. These might be associated with B29 (34), mb-1 (35), or other, yet to be described accessory polypeptides. Alternatively, the conformation of the TM1 and TM2 subdomains might be the essential feature.

Antigen presentation first demands endocytosis of ligand-receptor complexes and then routing of these complexes through a defined endocytic pathway. Previously, we have shown that endocytosis of ligand-receptor complexes does not depend upon the μ COOH terminus (16). However, as presented here, the complete TM domain is essential for the ultimate outcome of this process, measured as activation of T cells. Even though ligand-receptor complexes are endocytosed, their delivery to the appropriate endocytic compartment for processing appears to depend on TM residues. A requirement of the tyrosine within the μ TM1 domain and the cytoplasmic tail for antigen presentation has been demonstrated (33). Our experiments implicate a requirement for the TM2 and TM3 segments as well. With respect to the TM3 subdomain, indirect evidence suggests that all or part of the polar segment TAST (Fig. 1 *b*) is associated with an accessory molecule (36). Antigen presentation was not affected by mutation of the carboxyl two residues (TA<u>ST</u> to TA<u>VV</u>) (33) but was completely eliminated when we replaced all eight TM3 residues with those of the I-Aα chain. A proposal that accommodates the above findings is that the NH_2-terminal T of <u>T</u>AST is essential for antigen presentation. This polar residue is an attractive candidate for facilitating interaction between IgM and the putative accessory molecule. Another possibility for loss of function by the TM2 mutant is the alteration in the conformation of the TM3 subdomain.

Given the structural requirements of the μ TM domain for Ca^{2+} mobilization and antigen presentation, its dispensability for induction of antibody secretion was unanticipated. Events regulating secretion have been documented at transcriptional termination, RNA processing, and posttranslational levels (37). The former two mechanisms are known to be susceptible to membrane-generated, signal transduction events (38, 39). The μ-S-C receptor is incapable of immediate signal transduction (Figs. 1 *c* and 3), and we observed no consistent shifts in RNA levels for μm, μs, Cκ, or J chain (data not shown) after antigen induction. Therefore, it is probable that the replacement of antigen receptor spacer and cytoplasmic domains in CH12.LX cells perturbs some downstream event, conceivably by disrupting an interaction with a membrane molecule(s) other than that which interacts with transmembranal residues. Regardless of the mechanism, it is unlikely that this phenomenon is restricted to CH12.LX since normal B cells can be triggered to secrete using a similar induction protocol (29, 30). Mond et al. (40) have shown that Ca^{2+} elevation and PIP_2 hydrolysis can be blocked by the PKC inhibitor, indolactam, without blocking anti-Ig-mediated B cell proliferation. Taken with our results, these data emphasize the importance of defining antigen-induced TM signaling events other than Ca^{2+} flux that are critical for stimulating B cell growth and differentiation.

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