

COOH Terminus of Membrane IgM Is Essential for an Antigen-specific Induction of Some but Not All Early Activation Events in Mature B Cells

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Summary

Transfectants of mature B cell lines that bind phosphorylcholine were made in order to understand the role of the COOH terminus of the μ chain of membrane IgM (mIgM) in generation of antigen-specific signals. A chimeric receptor (I-A α tail) was constructed by replacing 40 amino acids from the μ COOH terminus with that of major histocompatibility complex class II I-A α chain. The effect of wild-type and chimeric tails were studied on representative immediate-early antigen-specific signals. The I-A α tail hybrid, but not the wild-type receptor, was defective in antigen-driven Ca²⁺ mobilization, although it could effectively endocytose ligand-receptor complexes. Signal(s) transduced through the wild-type receptor led to transient induction of selected immediate-early gene messages (*Egr-1*, *c-fos*, *Jun*) above basal levels. However, the signal(s) generated after crosslinking of the I-A α tail receptor either showed no effect (*c-fos*) or actually repressed basal level expression of *Egr-1* and *Jun*. Thus, we have established that receptor-mediated endocytosis can be distinguished from other early events associated with B cell activation, based on their differential dependence upon the structural fidelity of the COOH-terminal sequence of mIgM.

IgM molecules on the B cell surface serve as antigen-specific receptors (1). Crosslinking of these receptor molecules by antigen or antiidiotypic antibody generates a complex series of biochemical reactions (2, 3). One of the immediate reactions includes activation of phospholipase C-catalyzed hydrolysis of phosphatidyl inositol into inositol 3-phosphate and diacylglycerol, followed by a rise in intracellular Ca²⁺ levels (4). One of the next documented consequences is induction of various immediate-early gene messages such as *Egr-1*, *c-fos*, *Jun*, *c-myc* (5–7). Recently, several laboratories have identified phosphorylation of proteins on tyrosine residues after crosslinking of mIgM (8–10), indirectly implicating the activation of tyrosine kinase(s). While these and various other biochemical changes are taking place, the mIgM receptor-ligand complex is endocytosed, ligand is processed, and some of the ligand peptides are presented to T cells in a MHC class II-restricted fashion (11). Eventually, B cells undergo either growth arrest (12) or proliferation and differentiation, often requiring help from T cells (13).

The extracellular NH₂ terminus of membrane IgM (mIgM)¹

endows specificity for antigen, whereas the membrane-associated COOH terminus in concert with associated membrane proteins (reviewed in reference 14) is responsible for initiating various biochemical changes inside the cell. The μ heavy chain COOH terminus consists of 12 extracellular, 26 transmembranal, and three intracellular residues, all unique to the membrane form (Fig. 1). We have been interested in understanding the structure-function relationship of mIgM and in dissecting out various complex biochemical changes that take place upon antigen stimulation of mature B cells. The experimental approach we have taken is to replace the 40-amino acid COOH-terminal segment of the μ heavy chain (15) with that of the MHC class II I-A α chain (16). In a previous study, we showed that this I-A α chimeric receptor, with T15 idiotype, when transfected into an immature B cell line, CH33, did not lead to growth arrest upon crosslinking with anti-T15 antibody (17). To study the effect of this replacement on generation of immediate-early signals in mature B cells, we have now transfected these chimeric and wild-type mIgM molecules of identical PC specificity into tumor models of mature B cells (M12.4, CH12.Lx). We show here that the COOH terminus of mIgM is required for an induction of second messenger, Ca²⁺, and some of the immediate-

¹ Abbreviations used in this paper: HRP, horseradish peroxidase; mIgM, membrane IgM; PC, phosphorylcholine.

early gene messages. In contrast, another mIgM-mediated function, endocytosis of antigen, is not dependent upon the precise structure of the mIgM COOH terminus.

Materials and Methods

Parental Cells and T15-Id⁺-transfected Cell Lines. M12.4 (I-A^{k+}, surface Ig⁻) (18), CH12.Lx (I-A^{k+}, μ⁺, Ly-1⁺) (19) murine B cell lymphomas were used as parental cell lines in all experiments. The T15-Id⁺ transfectants were generated by electroporating 10–15 μg of linearized plasmid DNAs into parental cells (20). All plasmid constructs contain *Escherichia coli* ampicillin and guanine phosphoribosyl transferase genes and murine genomic sequences for productively rearranged V_HS107-C_μ and V_κ22-C_κ Ig chains (21). The chimeric plasmid I-A_α tail, as shown in Fig. 1, was made by replacing the 1.7-kb fragment spanning the μ membrane exons with a 2.8-kb fragment spanning the membrane exon of the MHC class II I-A_α chain (22).

Reagents. All reagents except hypoxanthine, xanthine, thymidine, adenine, and acetomethyl-ester of Indo 1 (Indo 1-AM) were purchased from Gibco Laboratories (Santa Clara, CA). The Indo 1-AM was purchased from Molecular Probes (Eugene, OR), and the remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The antigen, phosphorylcholine (PC) conjugated to KLH, was kindly provided by Dr. M.C. Yang (University of Texas Southwestern Medical Center, Dallas, TX). Purified anti-T15-Id⁺ antibody, AB1-2 (23), fluorescein-conjugated goat anti-mouse IgG1, and anti-μ antibody were obtained from Southern Biotechnology Associates (Birmingham, AL). Another anti-T15-Id antibody F6 was obtained from Dr. R. E. Ward (Roswell Park Memorial Institute, Buffalo, NY) (24).

Immunofluorescent Staining. The T15-Id⁺ surface positivity of transfected cell lines was established by staining transfectants either with AB1-2 or F6 as a primary antibody and fluorescein-conjugated goat anti-mouse IgG1 as a secondary antibody (23). As controls, transfectants were stained with secondary antibody alone, and untransfected parental cell lines were stained with anti-

T15-Id⁺. Staining profiles were analyzed by FACS[®] (Becton Dickinson & Co., Mountain View, CA).

Calcium Analysis. The method used to measure changes in Ca²⁺ levels by FACS[®] (Ortho 50HH FACS[®] and associated 2150 computer; Ortho diagnostic systems, Westwood, MA) was as described (25). Analysis was done at the rate of ~300 cells/s. The baseline was established for 1 min before activating cells with either antigen (PC-KLH), anti-μ antibody, or anti-T15-Id⁺ antibody. Within 10–20 s after addition of ligand, recording of changes in levels of Ca²⁺ was resumed. The data were analyzed using “Normcon” and “cyto2D” programs.

Northern Blot Analysis. Cells grown to log phase were harvested, and 2 × 10⁶ cells/0.9 ml of medium were distributed into the wells of a 24-well plate. They were rested for 3–4 h and were then induced by addition of 1 μg of PC-KLH in 0.1 ml of medium. Cells were harvested at indicated times except for a 0-h sample, which was harvested along with the 0.5-h sample. This was done in order to confirm that induction was due to the addition of antigen only and not due to the accompanying manipulations. Total RNA was isolated by the hot phenol method as described by Maniatis et al. (26). Total RNA samples (10 μg/lane) were subjected to electrophoresis in a 1.2% formaldehyde-agarose gel and were transferred and covalently linked to Gene-Screen membrane (DuPont Co., Wilmington, DE). The filters were hybridized with ³²P-labeled probes (*Egr-1*, *c-fos*, *Jun*) under the conditions recommended by the manufacturer. The washes were done at 42°C for 1 h in 0.1× SSC and 0.5% SDS, and then blots were exposed to Kodak XAR-5 x-ray film with an intensifying screen at -70°C for 2–3 d. The *Egr-1* probe was provided by Dr. J. Monroe (University of Pennsylvania, Philadelphia, PA) whereas *c-fos* and *Jun* probes were obtained from Dr. B. Ozane (BICR, Glasgow, Scotland).

Monitoring Ligand Endocytosis. Horseradish peroxidase (HRP)-labeled PC-KLH and an anti-T15-Id⁺ antibody were used to monitor endocytosis. HRP labeling was performed as described by Huru and Chantler (27). Endocytosis of HRP-labeled ligands by mIgM was determined and quantitated according to a variation of a method (28) previously reported by Antoine and Avrameas (29).

Briefly, the procedure was carried out between 2 and 4°C unless otherwise specified. HRP-labeled ligands were bound to cells for 30 min on ice, and endocytosis was initiated at 37°C in an atmosphere of 5% CO₂ for various periods of time. Endocytosis was stopped by placing cells on ice, and excess free ligand was removed by washing. The samples, in which the total amount of cell-associated ligand was to be measured, were treated with 1% NP-40 in PBS solution for 10 min at room temperature. The other samples were kept on ice during this time. The amount of ligand was determined by measuring HRP activity at pH 6.0 in the dark for 15 min at room temperature and in the presence of 5 mM O-phenylenediamine, HCl, and 0.015% H₂O₂ (28). The HRP reaction was stopped by addition of one drop of 6 N HCl. The reaction mixture was then centrifuged to remove cell debris. The optical absorbance of the supernatant was measured at 492 nm, and all values in an experiment were normalized to a value of 100%, representing the total amount of ligand initially bound to the cells (30). The distribution of HRP-labeled ligand was determined by measuring the amount of HRP on the external surface of the cells, and the total amount of cell-associated HRP detected after lysis of the cells. The amount of ligand internalized by the cell was then determined by subtracting the amount of ligand detected on the external surface of the cell from the total amount of ligand detected after cell lysis. The difference between the amount of ligand detected after cell lysis and the amount of ligand bound initially was considered as amount of antibody shed.

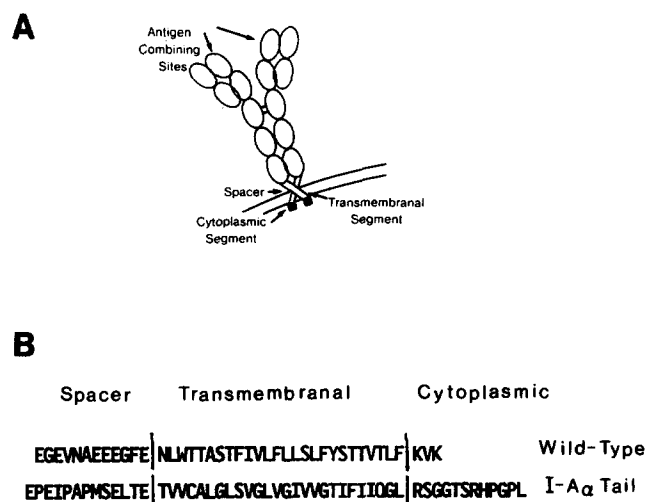


Figure 1. Schematic representation of a mIgM molecule (wild type). (A) Illustrated are the antigen-combining sites and the membrane-anchored COOH terminus, which is divided into three hypothetical domains: spacer, transmembranal, and cytoplasmic. (B) Comparison of amino acid sequences of the COOH termini of wild-type and I-A_α tail mutant.

Cytochemical Staining for Light Microscopy. The method used to visualize the morphological distribution of the HRP-labeled ligands was that of Graham and Karnovsky (31). The procedure for spectrophotometrically monitoring ligand endocytosis was followed up to and including the point where the cells were placed on ice after the 37°C incubations. For microscopy, the cells were attached to an alcian blue-coated cover slip (32), fixed, and then stained with substrate and H₂O₂ for 45 min in the dark. Analysis was performed with a microscope at $\times 1,000$ (Swift Instruments Inc., San Jose, CA).

Results

Establishment of Antigen-specific, Wild-type, and Mutant Cell Lines. The COOH terminus of the μ chain can be divided into three domains: spacer, transmembranal, and cytoplasmic (Fig. 1 A). The entire 40 amino acids comprising these domains were replaced with the analogous amino acids from the MHC class II I-A α (Fig. 1 B). The I-A α COOH terminus was chosen because the genomic clone was available, it used the same RNA-splicing site as μ m, and there is no significant homology to μ m in any of the COOH-terminal domains (Fig. 1 B).

Wild-type and I-A α mutant molecules with specificity for PC were transfected into the B cell lines, M12.4 and CH12.Lx. The expression of transfected receptors was analyzed by surface immunofluorescence staining with an antiidiotypic antibody, AB-1, whose recognition requires both transfected heavy and light chains and thus does not recognize endogenous mIgM or molecules formed by the mixing of transfected and endogenous heavy and light chains. The surface staining of the parental cell line, as shown in Fig. 2, was nega-

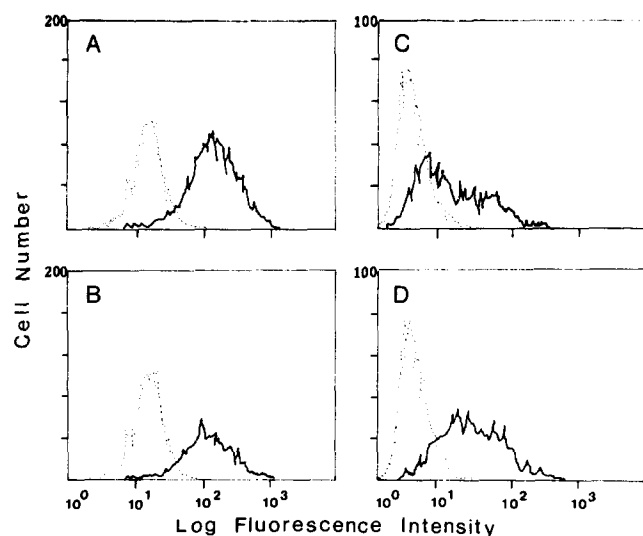


Figure 2. Wild-type and I-A α receptors are expressed equivalently on the cell surface of transfected cell lines. Shown are the surface expression profiles of T15 idiotype-bearing receptors on M12.4 and CH12.Lx cells transfected with wild-type (A and C) and I-A α tail mutant receptors, respectively (B and D). The untransfected M12.4 and CH12.Lx parental cells used as control are designated by the dotted line. Cells were stained with anti-T15 idiotype-specific antibody followed by FITC-labeled goat anti-mouse IgG1.

Table 1. Endocytosis of Anti-T15-Id HRP

Distribution of anti-T15-Id HRP	M12.4 wild type	M12.4 I-A α tail
External	59 +	45
Internal	37	37
Shed	04	19

HRP-labeled anti-T15-Id antibody was bound to cells on ice and then incubated for 1 h at 37°C to allow endocytosis. The distribution of HRP-labeled antibody was determined by measuring the amount of HRP activity on the external surface of the cells and the total amount of cell-associated HRP detectable after lysis of the cells. The amount of antibody internalized by the cells was determined by subtracting the amount of ligand detected on the external surface of the cells from the total amount of cell-associated ligand. The difference between the amount of HRP-labeled antibody initially bound and the amount detected after the cell lysis was accounted as "Shed" antibody. The amount of HRP-labeled antibody bound to control parental cells was below the level of detection.

tive, whereas both wild-type and I-A α tail receptor molecules were generally expressed equivalently. The fact that the expression of the I-A α tail receptor in CH12.Lx cells was higher than the wild-type receptor further confirmed that differential functional activities (see below) could not be ascribed to differential cell surface expression.

The I-A α Receptor Endocytoses Ligand-Receptor Complex Efficiently. mIgM-mediated endocytosis is the first important step in the sequential process of antigen presentation. The replacement of the COOH terminus of mIgM did not have any effect on mIgM-mediated endocytosis. Wild-type and I-A α tail transfectants of M12.4 cells endocytosed HRP-labeled antiidiotypic antibody or PC-KLH equally well, as analyzed by a spectrophotometric assay, in which ligand was quantified (Table 1). The same results were obtained comparing wild-type and I-A α tail transfectants in CH12.Lx cells (data not shown). Cytochemical staining monitored by light microscopy showed that neither HRP-conjugated PC-KLH nor the anti-T15-Id⁺ antibody bound the parental cell line M12.4, and that no internalization of either ligand was detectable (Fig. 3, A and B). These results sustain the notion that the internalization observed with both the wild-type and I-A α tail transfectants was due to receptor-mediated endocytosis and not by pinocytosis. Binding of HRP-labeled ligand to the surface of wild-type and mutant transfectant cells was observed, and the HRP-labeled ligand was endocytosed by both cell lines after 60 min of incubation at 37°C (Fig. 3, C, D, and E). We conclude that endocytosis of the receptor-ligand complex of the B cell is not dependent upon the COOH-terminal sequence of mIgM.

Induction of Intracellular Ca²⁺ Levels Requires the COOH Terminus of mIgM. Crosslinking of mIgM leads to a rapid increase in levels of intracellular Ca²⁺. This induction is considered to be a major step in receptor-mediated signaling pathways (33). Neither M12.4 (Fig. 4 A) nor CH12.Lx (data not shown) parental cell lines mobilize Ca²⁺ upon induc-

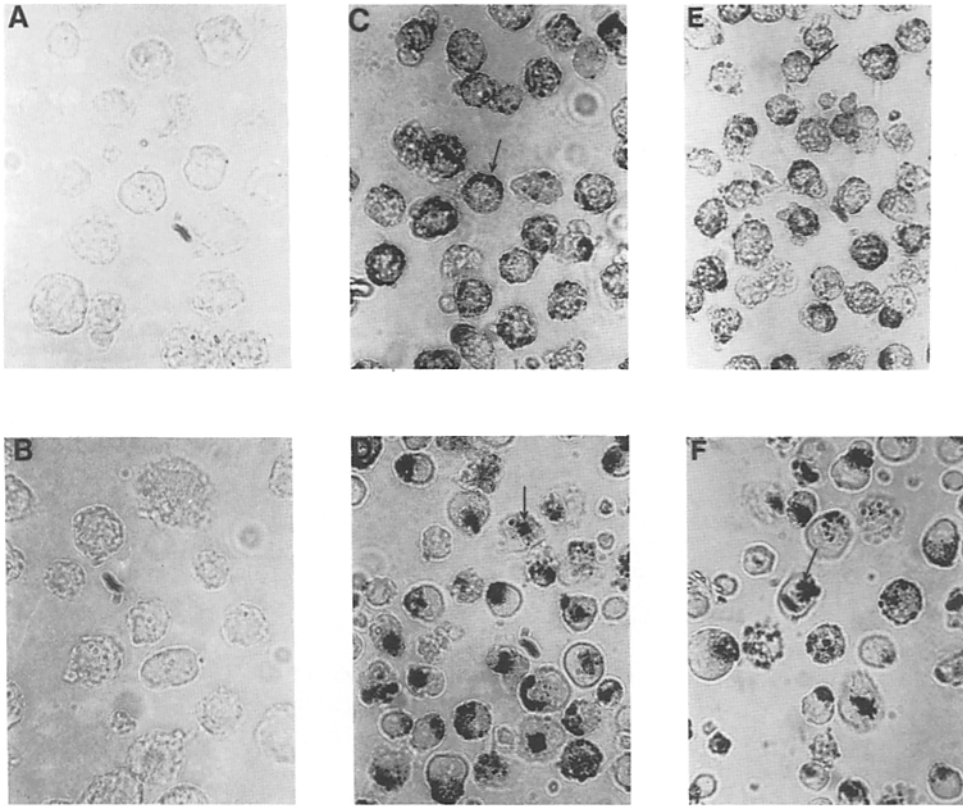


Figure 3. Both wild-type and mutant mIgM mediate endocytosis efficiently. Microscopic analysis of the endocytosis of anti-T15 antibody by M12.4 parental (A and B), M12.4 wild-type (C and D), and M12.4 I-A α tail mutant (E and F) cells. The cells were incubated with HRP-labeled anti-T15 antibody for 0 min (A, C, and E) or 60 min (B, D, and F) at 37°C. The surface-bound (C and E) and internalized (D and F) HRP anti-T15 molecules are indicated with arrows.

tion with either PC-KLH or antiidiotypic antibody, and, in the case of M12.4, with anti- μ antibody. An immediate rise in Ca^{2+} was observed upon the crosslinking of wild-type receptor (Fig. 4 B), whereas the mutant receptor was completely defective (Fig. 4 C). Since the chimeric receptor is

competent in endocytosis of antigen, its defect in Ca^{2+} induction indicates mechanistic segregation of these two early activation-associated events.

Immediate-early Gene Induction Requires Signal(s) Transduced by the COOH Terminus of mIgM. The transient induction

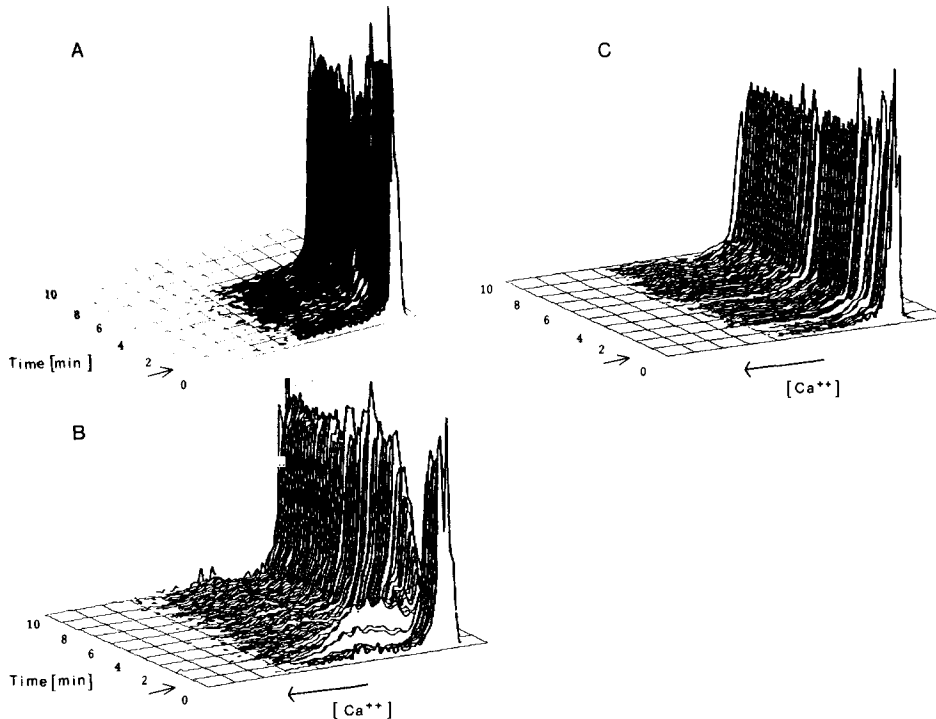


Figure 4. Crosslinking of wild-type but not I-A α tail mutant induces Ca^{2+} mobilization. Comparison of changes in intracellular Ca^{2+} levels in M12.4 parental (A) cells ($2 \times 10^6/ml$) in response to anti-IgM antibody ($10 \mu g$), M12.4 wild-type (B), and M12.4 I-A α tail mutant (C) cells ($2 \times 10^6/ml$) in response to PC-KLH ($1 \mu g$). Similar results were obtained when wild-type and I-A α tail mutant cells were induced with anti-IgM antibody ($10 \mu g$) or parental cells were induced with PC-KLH ($1 \mu g$) (data not presented). Ca^{2+} levels were monitored for 1 min before induction with ligand (arrow).

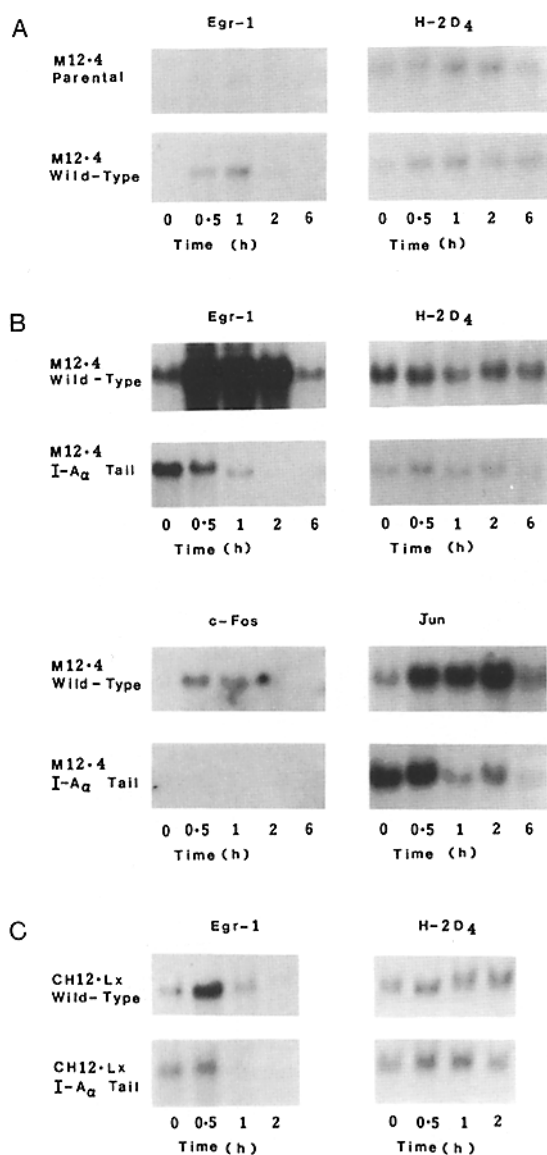


Figure 5. Wild-type but not I-A α tail mutant crosslinking leads to induction of immediate early gene expression. Induction of *Egr-1*, *c-fos*, and *Jun* in response to stimulation with PC-KLH was measured by Northern blot analysis. M12.4 parental cells (A), M12.4 parental cells transfected with wild type (A and B), and with I-A α tail mutant (B) were stimulated with PC-KLH for the indicated times. Each blot was reprobed with a MHC class I probe (H-2D4) to normalize the loads of RNA per lane. To show that the induction of immediate early genes was not restricted to M12.4 cells, a similar analysis was performed on CH12.Lx transfectants (C).

of immediate-early gene messages occurs rapidly after Ca²⁺ mobilization (5–7). Therefore, we analyzed the temporal accumulation of *Egr-1*, *c-fos*, and *Jun* mRNAs upon crosslinking of wild-type and mutant receptors.

First, antigen specificity was confirmed by showing that these messages were not induced in parental cell lines in response to PC-KLH (Fig. 5 A). The steady-state levels of mRNAs of these genes increased within 30 min of crosslinking of wild-type receptor at PC-KLH concentrations as

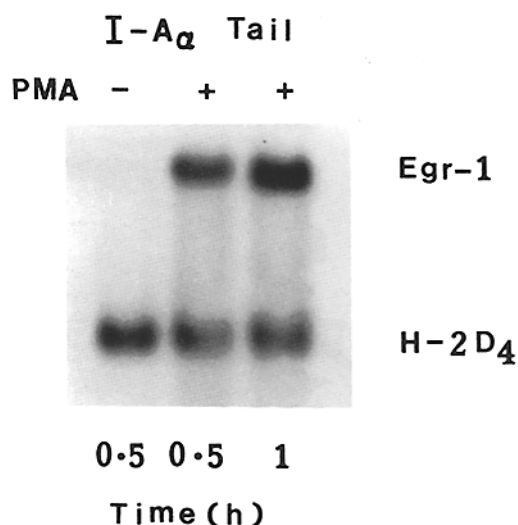


Figure 6. Induction of *Egr-1* message in CH12-I-A α tail mutant cells by PMA. The cells were incubated with PMA (10 ng/2 \times 10⁶/ml) for indicated times. Total RNAs were extracted and analyzed (10 μ g/lane) with *Egr-1* and MHC class I H-2d(H-2D4) probes.

low as 1 μ g per 2 \times 10⁶ wild-type transfected cells (Fig. 5, B and C). The increased rate of accumulation of these messages was comparable, but their degradation kinetics were different. The mRNA of the *c-fos* gene disappeared rapidly by 2 h of induction, whereas *Egr-1* and *Jun* mRNAs had slower rates. After PC-KLH addition to mutant cell lines, these messages did not accumulate above basal expression level. In fact, *Egr-1* and *Jun* mRNAs could not be detected after 2–6 h of induction (Fig. 5, B and C). The mutant cells were fully capable of *Egr-1* message induction after treatment with the protein kinase C activator, PMA (Fig. 6) (34). This rules out the possibility that the defect in transient induction in the mutant cells results from events downstream of the hybrid mIgM receptor.

Discussion

The requirement of the COOH terminus of mIgM for two immediate antigen-specific activational signals, changes in Ca²⁺ levels and immediate-early gene induction, was established in antigen-specific, B cell transfectants of μ wild-type and I-A α chimeric receptors. In contrast, the indifference of endocytosis to the COOH-terminal sequence of mIgM was established. Keeping in mind the limitations of transfected cell lines, these studies were performed in transfectants of M12.4 and CH12.Lx that produced similar results. Moreover, the amount and the kinetics of PC-KLH and anti-idiotypic antibody internalized by the transfectant cell lines were very similar to the rate and amount of antigen endocytosis by a different antigen-specific cell line and to endocytosis of anti-Ig by normal BALB/c splenic B lymphocytes (28).

Endocytosis is the first essential step for mIgM-mediated antigen presentation. Our data suggest that this function of mIgM does not require (and/or potentiate) Ca²⁺ mobiliza-

tion. A role for increased Ca^{2+} levels in ligand-mIgM endocytosis via clathrin-coated pits was suggested previously (35). This was based on the detection of calmodulin in mIgM-containing, clathrin-coated vesicles and the sensitivity of the endocytosis to the calmodulin-directed drug, stelazine. Mutational analysis of calmodulin in yeast (36), and ability of calmodulin to function in the absence of Ca^{2+} in various other eukaryotic systems (37, 38), indicate that calmodulin can perform its essential function without Ca^{2+} binding. Thus, our results with the I-A α -transfected cell lines suggest that either Ca^{2+} binding is not a prerequisite for the function of calmodulin or it can perform its function in the presence of basal or increased levels of Ca^{2+} too low to be detected by FACS[®] analysis. Even though the I-A α receptor did endocytose antigen, we do not know to which endocytic compartment it was delivered. Differences between mutant and wild-type exist at this level, as suggested by our observation that wild-type, but not mutant, cells are able to present antigen to appropriately MHC-restricted T cells (V.S. Parikh et al., manuscript in preparation). Segregation of mIgM functions is not without precedent. Recently, using a site-directed mutagenesis/in vitro transfection approach, Shaw et al. (39) showed, that a conserved tyrosine residue in the mIgM transmembranal segment is essential for antigen presentation but not for Ca^{2+} induction.

Antigen induction of immediate-early gene messages can be detected shortly after Ca^{2+} rises. Several laboratories have studied induction of *c-fos* (6), *Egr-1* (5), and *c-myc* (7) messages both in normal splenic B cells and established cell lines.

The most parallel study to this work was antigen induction of *c-myc* in TNP-specific splenic B cells (7). Although induction of immediate-early messages and rise in Ca^{2+} levels are well documented in vivo and in vitro, whether these events contribute to the same signaling pathway remains unknown (6). Our results, indicating that both events are dependent on structural components of the mIgM COOH terminus, support the idea of linked pathways. The transient induction of immediate-early gene mRNAs is achieved by a fine-tuned balance between transcriptional activation (40) and translational-linked, rapid degradation of transcribed messages (41). The signals generated after crosslinking of the mIgM receptor may be differentially contributing to both levels of regulation, depending on the structure of the mIgM tail. This is based on the unexpected downregulation of *Egr-1* and *Jun* mRNA, which is apparently mediated through the mutant receptor in the absence of detectable Ca^{2+} flux.

Recently, several laboratories have identified at least two glycosylated and disulfide-linked heterodimers associated with mIgM (42-44). Yamanashi et al. (45) coimmunoprecipitated a tyrosine kinase, lyn, with mIgM. Our results here and previous observations from our own (17) and other (39) laboratories implicate the importance of proper association of the mIgM COOH terminus with the above-mentioned and other as yet unidentified accessory polypeptides and kinases in B cell activation. Mutant B cell lines expressing chimeric receptors such as the I-A α should be useful in formally establishing the details of these interactions and their consequences on signal transduction.

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