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Immunology

Altered I-A protein-mediated transmembrane signaling in B cells that express truncated I-A^k protein

(major histocompatibility class II molecule/B-cell activation/protein kinase C/site-directed mutagenesis)

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ABSTRACT **Recent evidence suggests that the major histocompatibility complex class II molecules of B lymphocytes function as signal-transducing receptors during the generation of T lymphocyte-dependent humoral immune responses. By analogy with other receptors, we postulate that perturbation of the class II molecules is coupled to the generation of intracellular second messengers through interactions involving the transmembrane and/or cytoplasmic domains of the class II molecules. We report a series of experiments that assess which amino acids of the class II molecule I-A^k are required for coupling it to the signal-transduction pathway. We prepared a series of B-lymphocyte transfectants that express I-A^k molecules with COOH-terminal truncations of either the A_α^k or A_β^k chain or both. The ability of each transfected class II molecule to transduce a signal after being bound by monoclonal antibody was found by monitoring the translocation of protein kinase C from the cytosol to the "nuclear compartment" of the transfected B lymphocyte. Results indicate that the A_β^k chain plays the dominant role in signal transduction and that the 6 cytoplasmic amino acids of A_β^k chain most proximal to the inner plasma membrane are of greatest importance in coupling I-A^k molecules to the molecules of the signaling cascade.**

Class II molecules (Ia in the mouse) encoded in the major histocompatibility complex genes are cell-surface glycoprotein heterodimers composed of a 35- to 36-kDa α chain and a 27- to 29-kDa β chain that are pivotal in generating thymus-dependent immune responses (1). The best-documented function of Ia molecules is their role in peptide–antigen binding and presentation to T lymphocytes (2). An additional, and more recently described, role for Ia molecules is to act as transmembrane signal transducers (3). Thus, while formation of the trimolecular complex of antigen, Ia, and T-cell receptor initiates T-cell activation (4), Ia engagement through this complex, and perhaps also through the interaction of CD4 with Ia molecules, appears to initiate B-lymphocyte signaling. Evidence supporting the role of Ia molecules in signaling includes the following: (*i*) stimulation of interleukin 4 (IL-4)- and anti-IgM-primed normal B lymphocytes with immobilized anti-Ia antibodies leads to proliferation (5); (*ii*) treatment of CH12.LX lymphoma cells or normal B lymphocytes with anti-Ia monoclonal antibodies (mAbs) leads to immunoglobulin secretion and enhanced humoral immune responses, respectively (6, 7); (*iii*) crosslinking of membrane Ia leads to elevation of intracellular cAMP and translocation of protein kinase C (PKC) to the nuclear compartment (3, 8).

The involvement of PKC in several receptor-mediated signaling systems has been documented (9, 10) as has its perinuclear binding, presumably to nuclear proteins (3, 8). Perhaps importantly, PKC has structural motifs—i.e., zinc fingers—which resemble those associated with DNA-binding proteins (11). Further, after stimulation of B lymphocytes with anti-IgM mAb, a subunit of PKC (PKM) has been postulated to be translocated to the nucleus where it phosphorylates laminin B in a manner similar to the phosphorylation of laminin B by PKM *in vitro* (12). These observations together suggest that PKC translocation is a functionally relevant step in the Ia-mediated signal pathway. Therefore, we have used PKC translocation as an indicator of signal transmission in an attempt to identify the structural elements of Ia required for signaling.

The transmembrane and cytoplasmic domains of both the α and β chains of Ia show extensive portions of sequence that are conserved among alleles and species (13). This high degree of conservation suggests that a conserved structural or enzymatic function—e.g., interacting with secondary signal-transducing molecules—is provided by the transmembrane or cytoplasmic domains of Ia. To determine which structural element(s) of Ia molecules is (are) required for signal transduction, we generated B-lymphocyte transfectants that express I-A^k molecules with truncated α or β chains or with a combination of truncated α and β chains; then we examined the ability of these molecules to transduce a signal as indicated by PKC translocation to the nucleus after binding of surface I-A^k with anti-I-A^k mAb.

MATERIALS AND METHODS

DNA Constructs. Genomic DNA sequences for the A_α^k and A_β^k chains of I-A^k were isolated from an EMBL3 library. The pSV2-neo (14) DNA was a gift from Peter Southern (Scripps Clinic and Research Foundation). To perform *in vitro* site-directed mutagenesis genomic DNA fragments were subcloned into plasmid vectors; the A_β^k clone has been described (15), and the 9.0-kilobase A_α^k DNA fragment was subcloned into pUC13 that was modified to lack *Eco*RI and *Sst* I restriction sites.

Oligonucleotide Site-Directed *in Vitro* Mutagenesis. The method of Taylor *et al.* (16) was used to introduce nucleotide changes that resulted in premature stop codons indicated by the vertical bars in the primary amino acid sequence of A_α^k and A_β^k chains (Fig. 1). For mutagenesis small DNA fragments encoding the transmembrane/cytoplasmic domains of the A_α^k and A_β^k chains were subcloned into pTZ19U and M13mp19, respectively. After mutagenesis random clones were selected and sequenced by the dideoxy chain-termination method to ensure that only the correct nucleotide

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Abbreviations: mAb, monoclonal antibody; PKC, protein kinase C; wt, wild type; IL-2 and -4, interleukin 2 and 4, respectively; HEL, hen egg lysozyme.

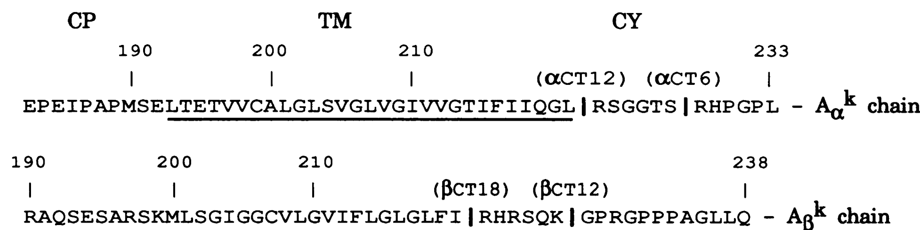

FIG. 1. Amino acid sequence (in single-letter code) for the α and β chains of I-A^k from the connecting peptide sequence to the COOH-terminal amino acids. The Eisenberg algorithm (17) was used to show the connecting peptide (CP), transmembrane (TM), and cytoplasmic (CY) amino acids. Amino acids predicted to be in the TM regions are underlined. Above the CY sequences for each chain are the designations of the truncations introduced into the individual chains—e.g., αCT6 is a truncation of 6 amino acids counting from the COOH-terminal amino acid of the α chain.

changes had been made. After reconstruction of mutated genes, a restriction enzyme-fragment analysis was performed on wild-type (wt) I-A^k and mutant I-A^k DNA to ensure the genes were assembled in correct orientation.

Transfection of DNA into M12.C3 Cells. Electroporation was used to introduce DNA into the Ia-negative B-cell lymphoma M12.C3 (18). A Baekon 2000 electroporator was used to transfect 5 × 10^6 M12.C3 cells with 20 μg each of linearized A_α^k (digested with *Hin*dIII) and A_β^k (digested with *Cla* I) genomic DNA constructs as well as 20 μg of pSV2-neo (digested with *Eco*RI). Transfected cells were selected with a final concentration of G418 at 300 μg/ml.

Flow Cytometric Analysis of Transfectants. Individual transfectants resistant to G418 were analyzed with either A_α^k (39J or 3F12)-specific mAb (19, 20) or A_β^k (11-5.2 or 10-3.6)-specific mAb (21) that was directly conjugated with fluorescein isothiocyanate. Cells (5 × 10^5) were stained with saturating amounts of mAb (20 μg/ml) at 4°C for 30–60 min and then washed twice in phosphate-buffered saline/2% fetal calf serum and analyzed by using a Coulter Epics C flow cytometer with a three-decade logarithmic scale with 256 channels. A difference of 25 channels in mean channel fluorescence corresponds to a 2-fold difference in I-A^k protein expression.

Transfectants chosen for analyses are referred to by clone name followed with the I-A^k truncation phenotype: M12.C3 (Ia negative), M12.C3-3-D8 (α^d/β^k), M12.C3-F6 (wt/wt), M12.C3-4-D5 (αCT6/wt), M12.C3-10-B3 (αCT12/wt), M12.C3-5-A2 (wt/βCT12), M12.C3-3-B6 (αCT12/βCT12), and M12.C3-10-D2 (αCT12/βCT18). The nomenclature αCT12 or βCT12 refers to COOH-terminal truncation of 12 amino acids in the A_α^k and A_β^k chains, respectively.

Antigen Presentation and Interleukin 2 (IL-2) Assay. The antigen-presentation assay and IL-2 assay have been described (22). Briefly, transfectants were cultured with T-cell hybrids that were specific for tryptic fragments of hen egg lysozyme (HEL) in the presence of a HEL tryptic digest at 300 μg/ml. After overnight incubation at 37°C, supernatants were harvested and diluted 2-fold serially for culture with HT-2 cells that are dependent on IL-2 for growth. The reciprocal of the last dilution that supports 90% growth of HT-2 cells is the IL-2 titer.

PKC Assay. Operationally, we define Ia receptor-mediated cell signaling as the translocation of PKC from the cytosolic compartment to the nuclear compartment. The phorbol-binding assay is a convenient method to quantitate the translocation of PKC that can also be seen by staining intact nuclei with mAb specific for PKC or by measuring PKC phosphorylation activity (3, 8, 23).

Typically, cells are incubated with anti-A_α^k mAb at 37°C for various times, then rapidly cooled and washed, followed by lysis with 0.5% Nonidet P-40. Nuclei are isolated by centrifugation through a 60% sucrose cushion and sonicated; then the protein concentration of the sonicate is determined. The sonicates (≈30 μg of protein) are added to 200 μl of the PKC assay reaction mixture (see ref. 8 for details). After incubation at 37°C for 1.5–2.0 hr, cell protein is captured by filtration and washing in 96-well Millititer HA (Millipore). Bound radioactivity ([^{3}H]phorbol) is quantified by liquid scintillation counting. The SEM of three individual samples per time point is shown.

RESULTS

Characterization of M12.C3 Transfected with $A_\alpha^kA_\beta^k$ DNA. The anti-A_α^k and anti-A_β^k staining profiles of the transfection recipient M12.C3 and that of two transfectants that express either high levels (M12.C3-F6, wt/wt) or intermediate levels (M12.C3-9-D4, wt/wt) of I-A^k are shown (Fig. 2 *a* and *b*). As control for the PKC assay, we intentionally produced a transfectant expressing mixed I-A molecules by transfecting M12.C3 (expresses an endogenous A_α^d gene) with A_β^k and pSV2-neo DNA only. The resultant transfectant (M12.C3-3-D8) expresses I-A molecules that are comprised of A_α^d and A_β^k chains (Fig. 2*b*). This mixed molecule serves as a specificity control for the translocation of PKC mediated by A_α^k-specific mAb (see below). Transfectants used in the PKC analyses were selected based on their expression of I-A^k molecules at levels similar to that of the two transfectants expressing full-length I-A^k molecules (Fig. 2 *c* and *d*).

Antigen-Presentation Capacity of I-A^k Transfectants. All transfectants were analyzed for their ability to present antigen to HEL-specific T-cell hybridomas (Table 1). Each transfectant shown in Fig. 2 could present antigen to the designated T cells as measured by IL-2 production by the hybridomas, although the T-cell hybridoma kLy-7.11 responded slightly less well to transfectants expressing αCT12/wt and αCT12/βCT12 phenotypes than it did to wt/wt transfectants. In contrast to the transfectants in Fig. 2, the transfectant M12.C3-10-D2 (αCT12/βCT18) does not present

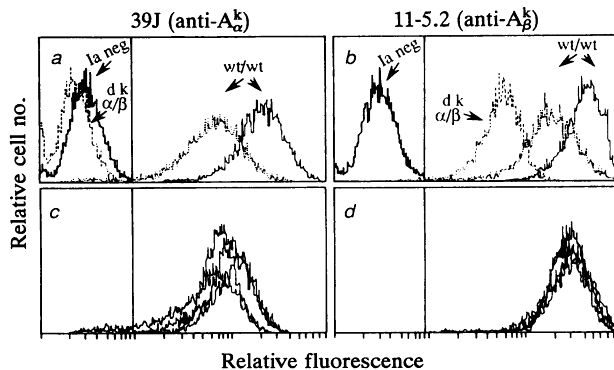

FIG. 2. Histograms illustrating relative fluorescence of representative M12.C3 transfectants stained with mAb specific for the A_α^k chain (39J; *a* and *c*) or the A_β^k chain (11-5.2; *b* and *d*). (*a* and *b*) Control cells M12.C3 (—), M12.C3-F6 (—), M12.C3-9-D4 (· · ·), and M12.C3-3-D8 (- - -). (*c* and *d*) Experimental transfectants M12.C3-4-D5, M12.C3-10-B3, M12.C3-5-A2, and M12.C3-3-B6.

Table 1. Antigen presentation to HEL peptide-specific hybrids by M12.C3 transfectants

Antigen presenting cell	Surface Ia phenotype	T-cell hybridoma response, units of IL-2 per ml		
		C11.A3	kLy-7.11	kLy-11.10
M12.C3	Ia^-	<10	<10	<10
M12.C3-3-D8	α^d/β^k	40	<10	<10
M12.C3-F6	wt/wt	640	1280	160
M12.C3-9-D4	wt/wt	1280	640	640
M12.C3-4-D5	αCT6/wt	640	320	160
M12.C3-10-B3	αCT12/wt	640	80	80
M12.C3-5-A2	wt/βCT12	1280	640	320
M12.C3-3-B6	αCT12/βCT12	1280	40	160
M12.C3-10-D2	αCT12/βCT18	10	<10	<10

Units of IL-2 are represented as the reciprocal of the highest dilution of supernatant from the antigen presentation well that would support HT-2 cells, an IL-2-dependent T cell (23). Only IL-2 titers that differ from wt by 4-fold are significantly different. Three different T-cell hybridomas were used as responders. C11.A3 cells can recognize HEL(34–45) presented by $I\text{-}A^k$ as well as the mixed molecule ($A^d_\alpha A^k_\beta$) and appear to possess a very high-affinity receptor. C11.A3 was a gift from Laurie Glimcher (Department of Cancer Biology, Harvard School of Public Health). kLy-7.11 and kLy-11.10 cells recognize the HEL(46–61) peptide only in the context of $I\text{-}A^k$. No T-cell hybridoma produces detectable IL-2 without HEL peptides, regardless of the transfectant used as antigen-presenting cell.

antigen to T-cell hybridomas with the possible exception of C11.A3 (which express the highest affinity T-cell receptor of the group). This lack of presentation is probably due to the low level of $I\text{-}A^k$ expressed by M12.C3-10-D2 (see Fig. 4). The $I\text{-}A^k$ expression by M12.C3-10-D2 approximates that of resting B cells, which also present antigen poorly (24). M12.C3-10-D2 does express $I\text{-}A^k$ protein as evidenced by flow cytometry (Fig. 4 *c* and *d*) and by our ability to precipitate radiolabeled $I\text{-}A^k$ (data not shown).

The combined flow cytometry and antigen-presentation data provide strong evidence that not only do the transfectants (except for αCT12/βCT18 phenotype) express surface levels of truncated $I\text{-}A^k$ molecules comparable to wild-type transfectants, but also that no significant structural abnormalities exist in the extracellular portions of these molecules.

Signal Transduction by the $I\text{-}A^k$ Transfectants. Each transfectant cell line was examined for its ability to carry out $I\text{-}A^k$-initiated signaling as assessed by the kinetics of PKC translocation from cytosolic to nuclear compartment after the cell was treated with A^k_α-specific mAb. Fig. 3 *Left a* shows the kinetics of translocation for transfectants that are either Ia negative (M12.C3), express wt $I\text{-}A^k$ (M12.C3-F6, wt/wt), or express $I\text{-}A^k$ with a truncated α chain missing either 6 (M12.C3-4-D5, αCT6/wt) or 12 (M12.C3-10-B3 and M12.C3-5-C4, αCT12/wt) amino acids from the cytoplasmic domain. As shown, M12.C3 (Ia negative) does not translocate PKC from the cytosol under conditions that result in normal translocation kinetics of PKC in the transfectants (wt/wt) that express full-length $I\text{-}A^k$. Transfectants of the αCT6/wt phenotype translocate PKC as do transfectants that express wt $I\text{-}A^k$. Transfectants lacking all amino acids of the cytoplasmic domain of the α chain (αCT12/wt) reach maximal PKC translocation at 2 min after mAb addition, with a slightly reduced maximal response. Changes in the last 4 min of the αCT12/wt transfectant response are not significant because transfectants expressing wt $I\text{-}A^k$ can respond similarly (data not shown).

The first significant effect on PKC translocation is seen in the two transfectants of the wt/βCT12 truncation phenotype, M12.C3-5-A2 and M12.C3-7-B3. The peak PKC response is shifted by 4 min and reduced by 30% compared with the response of M12.C3-9-D4 (wt/wt) (Fig. 3 *Left b*). The negative control in this experiment expresses the mixed haplotype I-A molecule A^d_α A^k_β. Low levels of A^d_α A^k_β molecules could be expressed in the other transfectants; however, these

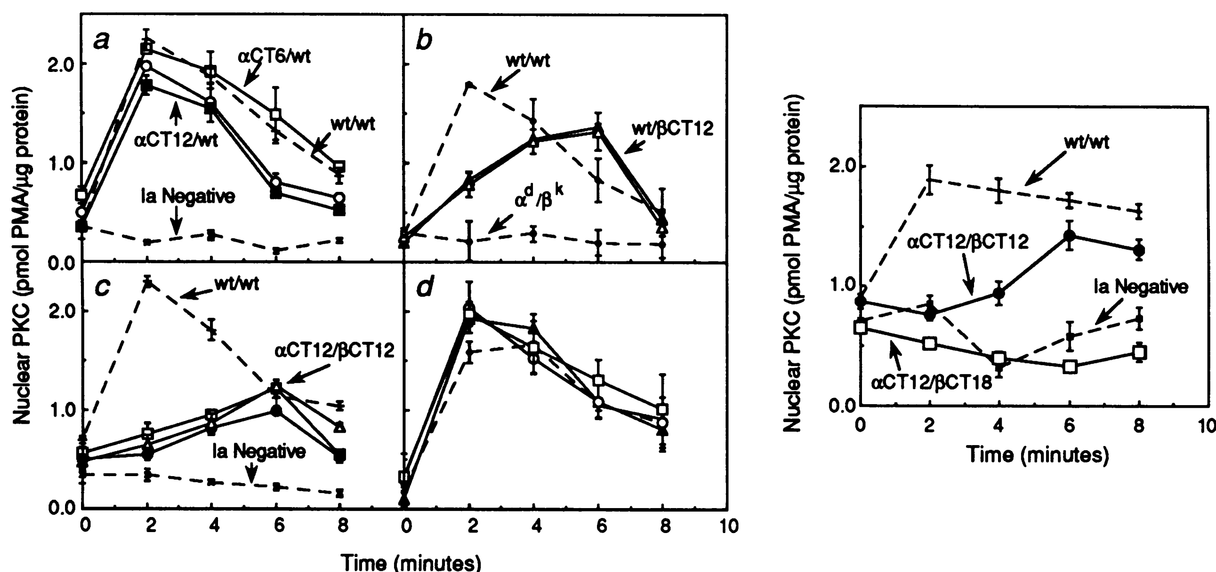

FIG. 3. (*Left*) Nuclear PKC content of transfected M12.C3 cells after stimulation with the anti-A^k_α mAb 39J. (*a*) The α-chain truncation-expressing transfectants M12.C3-4-D5 (αCT6/wt; □), M12.C3-10-B3 (αCT12/wt; ○), and M12.C3-5-C4 (αCT12/wt, ■) are shown. Also shown are the M12.C3-F6 (wt/wt; - - + - -) and M12.C3 (Ia negative; - - × - -) controls. (*b*) Transfectants expressing the wt/βCT12 phenotype (M12.C3-5-A2; △; M12.C3-7-B3; ▲) are shown. Controls include M12.C3-9-D4 (wt/wt; - - ● - -) and M12.C3-3-D8 ($A^d_\alpha A^k_\beta$; - - ○ - -). (*c*) The double truncation (αCT12/βCT12)-expressing transfectants M12.C3-6-D4 (△), M12.C3-3-B6 (□), and M12.C3-5-B2 (●) are shown. Controls include M12.C3-F6 (wt/wt; - - + - -) and M12.C3 (Ia negative; - - × - -). (*d*) Nuclear PKC content of transfected M12.C3 cells after treatment with 1 mM dibutyryl cAMP. Shown are M12.C3-9-D4 (wt/wt; - - + - -), M12.C3-5-A2 (wt/βCT12; ▲), M12.C3-10-B3 (αCT12/wt; ○), and M12.C3-3-B6 (αCT12/βCT12; □). (*Right*) Nuclear PKC content of transfected M12.C3 cells after stimulation with anti-A^k_β mAb. The truncation-expressing transfectants M12.C3-5-B2 (αCT12/βCT12; ●) and M12.C3-10-D2 (αCT12/βCT18, □) are shown. Also shown are the M12.C3-F6 (wt/wt; - - + - -) and M12.C3 (untransfected, - - × - -) controls. Similar results are obtained whether an A^k_α- or A^k_β-specific mAb is used.

molecules are incapable of being signaling elements as shown by the negative results when M12.C3-3-D8 (the mixed molecule expressor) is incubated with A^k_α-specific mAb. All data of Fig. 3 *Left* were obtained with an anti-A^k_α chain mAb to ensure that only signals initiated by I-A molecules of the $A^k_\alpha A^k_\beta$ phenotype would be measured.

The αCT12/βCT12 transfectants were made to assess what effect the introduction of two truncated chains would have on the I-A^k-mediated PKC signaling phenotype. Three independent clones, M12.C3-6-D4, M12.C3-3-B6, and M12.C3-5-B2, of the αCT12/βCT12 truncation phenotype again show that the β chain of I-A^k is dominant in controlling the PKC translocation phenotype (Fig. 3 *Left c*). This result is supported by the fact that transfectants of wt/βCT12 phenotype also reach maximal response at 6 min after mAb addition. The A^k_α chain also evidently plays some role in the PKC signaling phenotype because the slope of the ascending portion of the curve for PKC translocation of αCT12/βCT12 transfectants differs from that of wt/βCT12 transfectant (compare *b* and *c*).

While the differences in the PKC signaling phenotypes could be attributed to some epiphenomenon unrelated to transfected DNA, this explanation is unlikely for three reasons. (*i*) Each truncation genotype has only one PKC translocation phenotype (Fig. 3 *Left a–c*). (*ii*) All transfectants treated with 1 mM dibutyryl cAMP, an Ia-independent inducer of PKC translocation, show equivalent translocation of PKC to the nucleus (Fig. 3 *Left d*). (*iii*) The reported PKC translocation kinetics can be obtained with other A^k_α- and A^k_β-specific mAbs (data not shown).

Removal of the Plasma-Membrane-Proximal 6 Amino Acids of the A^k_β Chain. Clearly, altering the cytoplasmic domains of I-A^k, especially that of the β chain, affects the ability of I-A^k molecules to mediate PKC translocation. However, no transfectant discussed thus far is negative for PKC translocation. We therefore made a group of transfectants that express the αCT12/βCT18 truncation genotype and thus encode I-A^k molecules that lack amino acids in both cytoplasmic domains. I-A^k expression of these transfectants is lower than from other transfectants generated (Fig. 4 *c* and *d*); I-A^k expression is higher than the level of I-A^k on resting AKR B cells (data not shown).

Removal of the last 6 amino acids of the cytoplasmic domain of the β chain renders these I-A^k molecules (αCT12/βCT18) unable to translocate PKC after stimulation with I-A^k-specific mAb (Fig. 3 *Right*). This result sharply contrasts to the response of the αCT12/βCT12 transfectant that, because of 6 amino acids in the cytoplasmic domain of the β chain, can still translocate PKC, albeit with altered kinetics (Fig. 3 *Right*).

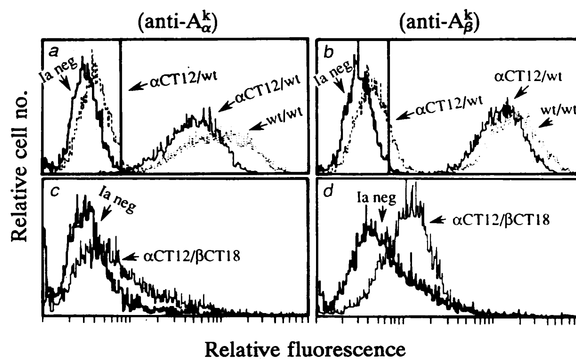

FIG. 4. Fluorescence profiles of transfectants expressing I-A^k gene products stained with either A^k_α (*a* and *c*)- or A^k_β (*b* and *d*)-specific mAb. (*a* and *b*) Transfectants of the αCT12/wt phenotype (M12.C3-10-B3, —; M12.C3-5-C5, - - -) can have different levels of surface expression of I-A^k but still translocate PKC equivalently (see Fig. 3 *Left a*). (*c* and *d*) The M12.C3-10-D2 transfectant of the αCT12/βCT18 phenotype expresses I-A^k 1.5- to 3.3-fold above background staining.

It might be argued that the signal-negative phenotype of the αCT12/βCT18 cells is from the low level of I-A^k molecules expressed. However, resting AKR B cells that express less surface I-A^k than αCT12/βCT18 cells can still fully translocate PKC with kinetics comparable to transfectants that express high levels of wt I-A^k (3, 8). Further support for the results obtained with the αCT12/βCT18 transfectants in the PKC assay comes from the following work: (*i*) we obtained transfectants of the αCT12/wt phenotype that differ markedly in expression of surface I-A^k (Fig. 4 *a* and *b*) but translocate PKC identically (Fig. 3 *Left a*) and (*ii*) a recently isolated subclone of M12.C3-10-D2 (αCT12/βCT18) (sorted for the top 10% expression of I-A^k; 3.3-fold above background) still shows the signaling-negative phenotype, whereas a lower I-A^k-expressing (2.2-fold above background; data not shown) clone of the αCT12/βCT12 phenotype (M12.C3-3-B6) causes PKC to translocate to the nucleus with the magnitude and kinetics of other clones of that phenotype.

DISCUSSION

Major histocompatibility complex class II molecules serve several functions in generating T-cell-dependent immune responses. The best understood of these functions is the role of class II molecules as "restriction elements" for the activation of antigen-specific helper T cells. A second function of class II molecules described recently is that of a signal-transducing receptor for B cells (3, 8). Perturbation of surface class II molecules on B cells with either mAb (6, 7), with cyclosporin A-treated T cells (25), or with T-cell receptor/CD4-containing membranes (26) has been shown to lead to physiologic changes including the induction of proliferation and of immunoglobulin secretion. The pathway by which modulation of surface class II molecules effects these physiologic changes is largely unknown, although two potential components of the signaling cascade have been documented—namely, increased intracellular cAMP and the translocation of PKC to the nuclear compartment (3). While significance of the increase of cAMP remains to be elucidated, involvement of PKC in the activation of genes in other systems (27) suggests that its translocation to the nucleus is a prerequisite for gene activation during B-cell proliferation and/or differentiation.

Although the α1 and β1 domains of the class II molecule are apparently key for its function in antigen presentation, the structural region of the class II molecule responsible for its role in signal transduction was previously unclear. Obvious candidates for such a role would be the transmembrane and/or cytoplasmic domains. We report our initial studies to assess the contribution of these class II molecular regions to transmembrane signaling in B cells. Because preliminary studies showed that removal of half or all of the transmembrane region of either chain prevented expression of the truncated I-A^k molecule at the plasma membrane (W.F.W., unpublished observation), we focused on the cytoplasmic domains of the α and β chains. As shown by our data, the cytoplasmic domain of the β chain exerts the dominant effect in coupling the I-A^k molecule to PKC translocation. However, the data also suggest a role for the α chain in this process, although that role would appear to be one of modulating the interaction between the β chain and the secondary signal-transduction molecule(s) with which it interacts (see Fig. 3 *Left b* and *c* for comparison of the αCT12/βCT12 versus the wt/βCT12 phenotypes). Unfortunately, contribution of the α chain to the negative-signaling phenotype of the βCT18 truncation could not be assessed because we could not obtain plasma-membrane expression of

this truncated β chain paired with the wt α chain (data not shown).

Our results can be contrasted with the recently published results of Nabavi *et al.* (28), who explored a single A_β^k truncation phenotype. When this chain was paired with a full-length A_α^k chain (wt/βCT10), the resultant transfectant showed a PKC translocation phenotype indistinguishable from the wt/βCT12 mutant we described. However, pairing of the βCT10 chain with an αCT12 chain produced a transfectant significantly different from the αCT12/βCT12 transfectant. Although the former transfectant showed less nuclear PKC, its maximal response still occurred at 2 min. In contrast, the αCT12/βCT12 transfectant not only showed a decreased response but also showed altered kinetics with nuclear PKC, which did not reach a maximal level until 6 min. This major alteration in kinetics is caused by the removal of only two amino acids—Gly-227 and Pro-228—from the A_β^k chain (see Fig. 1). This difference underscores the importance of this region of the β chain for signal transduction.

Our data demonstrate that the 6 plasma-membrane-proximal amino acids of the cytoplasmic domain of the β chain are essential for signal transduction by the I-A^k molecule. Within this small region are 3 amino acids, Gln-Lys-Gly (β-chain residues 225–227; see Fig. 1), that are conserved throughout all known class II β-chain sequences except HLA-DP, which has the related sequence Gln-Arg-Gly. This fact would suggest that these amino acids were conserved throughout evolution because they subserve an essential function of the class II molecules—such as interaction with molecules of the signal-transducing pathway. This hypothesis is further supported by studies from one of our laboratories (29) that showed that NH_2-reactive chemical crosslinkers can crosslink the cytoplasmic domain of the I-A^k molecule to molecules that copurify with it—presumably signal-transduction molecules. The only lysine in the cytoplasmic region of either chain is Lys-226 of the β chain, one of the 6 amino acids essential for signal transduction and one of the 3 highly conserved Gln-Lys-Gly amino acids. The crosslinking results imply that this region of the A_β^k chain contacts the molecule(s) of the membrane-proximal end of the Ia-initiated signaling cascade, as the span of the crosslinkers is only $\approx$12 Å.

Data in this report show that the 6 membrane-proximal amino acids of the β-chain cytoplasmic domain are required for signal transduction. In addition, these data strongly support the hypothesis that this region interacts with molecules of the signal-transduction pathway. The data do not, however, address the issues of whether the transmembrane region of the class II molecule also contacts the molecule(s) mediating signal transduction or, alternatively, whether the transmembrane region is the *only* region of the class II molecule to make contact. This last alternative, which we feel is unlikely, would be possible only if the COOH-terminal truncations caused significant conformational changes in the transmembrane region and if the conformational changes were actually responsible for uncoupling the class II molecule from the signal-transduction pathway. Because truncations or deletions in the transmembrane region probably prevent assembly and/or transport of the class II molecule (see above), the role of this region must also be probed through the creation of substitution mutants.

Although this report has focused on the structural features of the class II molecule necessary for its signaling function, the PKC signaling mutants, especially those of the αCT12/βCT18 phenotype, should prove valuable in determining what changes are required for gene regulation to drive β-lymphocyte differentiation after class II molecules interact with the T-cell receptor and/or CD4 molecules.

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