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Immunology

Cytoplasmic domain affects membrane expression and function of an Ia molecule

(major histocompatibility complex/class II gene/antigen presentation)

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ABSTRACT **The association of foreign antigen with Ia molecules on the surface of antigen-presenting cells is necessary for the interaction with the clonally distributed antigen receptor on T cells and is therefore critical in the initiation and regulation of immune responses. Ia polypeptides (α and β) are composed of two extracellular domains, a transmembrane domain and a cytoplasmic domain. Although exon-shuffling experiments have demonstrated that antigen associates with the NH_2-terminal $\alpha 1$ and $\beta 1$ domains, the roles that the other domains play in Ia function are still poorly understood. The B-hybridoma cell line 2B1 was selected in a series of positive and negative immunoselection steps for a mutation in the E^k_β polypeptide. It was found to fortuitously contain a mutation in the A^k_α polypeptide as well. Sequence analysis of the A^k_α gene showed that a single base transition (C→T) resulted in a stop codon at amino acid residue 222. This caused the loss of 12 amino acids from the cytoplasmic domain of the mature polypeptide. This mutation results in a decreased level of A^k_α polypeptide expression on the cell surface (50% of wild-type levels), an increased half-life of A^k_α polypeptide in the cell, and a specific limited defect in antigen presentation.**

The class II (Ia) molecules encoded by the major histocompatibility complex are transmembrane glycoproteins expressed predominantly on the surface of B lymphocytes and macrophages (antigen-presenting cells, APCs) (1). In the mouse, two isotypic forms of Ia exist: I-A and I-E, each a heterodimer composed of noncovalently associated α (A_α or E_α) and β (A_β or E_β) chains. Each chain is composed of two external domains, a hydrophobic transmembrane region, and a short intracytoplasmic tail. A remarkable property of Ia molecules is the extensive intraspecies polymorphism present in the membrane-distal (NH_2-terminal) external domain. This allelic variation is the basis for Ia-restricted antigen recognition by T cells (2, 3).

The mutant cell line 2B1 was derived from Ia^+ ($H\text{-}2^{d/k}$) antigen-presenting B–B hybridoma TA3 after negative immunoselection (4). Although selected with anti-E^k_β monoclonal antibodies (mAbs), the 2B1 mutant was coincidentally found to have reduced binding, about 50% of wild-type, to every A^k_α-specific mAb examined (4). In spite of this reduction in surface expression of the A^k_α polypeptide, the A^k_β polypeptide continued to be expressed at wild-type levels on the cell surface, presumably as part of a hybrid $A^{d,k}_\alpha$·A^k_β heterodimer as well as a homozygous A^k_α·A^k_β heterodimer (4). Two-dimensional nonequilibrium pH gradient–$NaDodSO_4$/PAGE showed that the A^k_α polypeptide from the 2B1 mutant migrated more acidically than the wild-type polypeptide (4), and one-dimensional $NaDodSO_4$/PAGE showed that it had a decreased apparent molecular weight (unpublished data).

In this report, we demonstrate that a mutation deleting the cytoplasmic tail of the A^k_α polypeptide accounts for the more acidic migration and decreased apparent molecular weight and results in a decreased level of I-A^k expression in the membrane. This decrease in expression is associated with a specific, limited loss of APC function by this B-hybridoma cell line. Possible explanations for the reduction in surface expression of the A^k_α polypeptide are addressed.

MATERIALS AND METHODS

Cell Lines and T-Cell Assays. To produce the 2B1 cell line, the antigen-presenting Ia^+ B–B hybridoma TA3 was mutagenized by treatment with ethyl methanesulfonic acid (5, 6). The mutagenized cells were then subjected to several rounds of negative immunoselection by complement-mediated lysis using an anti-E^k_β mAb and then to positive immunoselection by electronic cell sorting using a second anti-E^k_β mAb (4). The wild-type TA3 was derived from a fusion between the M12.4.1 ($H\text{-}2^d$) lymphoma and spleen cells from an $H\text{-}2^{d/k}$ mouse and therefore has only one copy of the $H\text{-}2^k$ genes (7, 8). The panel of T-cell hybridomas used in these studies was produced and examined for antigen specificity and Ia restriction as described (8–10). T cells were tested for activation in a standard interleukin 2 release assay involving secondary culture of supernatant with HT-2 cells (8). Dose–response experiments with APCs were performed to ensure maximal stimulation and interleukin 2 release. The antigens hen egg-white lysozyme (Sigma), ovalbumin (Sigma), and keyhole limpet hemocyanin (Calbiochem–Behring) were used in culture as described (8). The AODH3.4, SKK2.3, SKK45.10, and SKK9.11 cell lines were provided by P. Marrack (National Jewish Hospital, Denver), the Mdk5.1 and Mdk15.3 cell lines by G.-K. Sim (National Jewish Hospital, Denver), and the 2127.24 and K31.H2 cell lines by T. Briner (Massachusetts Institute of Technology, Cambridge, MA).

Isolation and Sequencing of the A^k_α Gene from a cDNA Library. A cDNA library was constructed from the 2B1 mutant as described (11). After first-strand/second-strand synthesis from poly(A^+)-enriched mRNA, *Eco*RI linkers were added and the cDNA was ligated into the bacteriophage λgt10 (Stratagene, La Jolla, CA). An unamplified library (4.8×10^5 phage) was screened by Southern blot analysis with an A^k_α cDNA probe (12). A clone containing a full-length A^k_α insert was identified from the initial pool of clones by restriction fragment length polymorphism analysis. The A^k_α

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Abbreviations: APC, antigen-presenting cell; FPR, fluorescence photobleaching recovery; mAb, monoclonal antibody.

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insert was then subcloned into the appropriate M13 vectors (Amersham) for sequencing by the dideoxy chain-termination method (13).

Computer Resources. Computer resources used to carry out our studies were provided by the National Institutes of Health-sponsored BIONET National Computer Resource for Molecular Biology.

Pulse–Chase Analysis. Cells (12×10^7) were biosynthetically labeled by incubation with 1.2 mCi (44.4 MBq) of [^{35}S]methionine, essentially as described (14). After 4 hr of incubation, cells were washed twice with RPMI-1640 containing 10% (vol/vol) fetal bovine serum (complete medium) and then incubated at 37°C in complete medium. At each time point examined, 2×10^7 cells were harvested by centrifugation, and the cell pellets were lysed for 30 min on ice in 0.75 ml of phosphate-buffered saline containing 0.5% Trasylol and 1% Triton X-100. After centrifugation, lysates were precleared of IgG by incubation with *Staphylococcus aureus* Cowan strain 1 cells coupled to Sepharose and then were incubated overnight at 4°C with anti-A^k_α mAb 39J (15) coupled to Sepharose. Radioactively labeled I-A^k molecules were eluted from exhaustively washed Sepharose conjugate and analyzed by NaDodSO$_4$/PAGE. Gels were fixed and dried, and autoradiographs were made. Gels from 2B1 lysates were exposed for 48 hr; those from TA3 lysates were exposed for 24 hr. Exposed films were scanned with an Ephortec Joyce–Loebl densitometer to determine the relative area (amount of radioactivity) of A^k_α polypeptide in each lane.

Fluorescence Photobleaching Recovery. The lateral mobility of I-A^k in the membrane was measured by fluorescence photobleaching recovery (FPR; ref 16). Our experimental apparatus and analytical methods have been described in detail (5, 17). In brief, $0.5–1 \times 10^6$ cells were incubated in the dark for 30 min at 4°C with 3–5 μl of anti-I-A^k mAb 39J (15) that had been conjugated to fluorescein isothiocyanate according to protocol provided by A. H. Stolpen (personal communication). Labeled cells were washed and FPR measurements and data analysis were performed as described (5, 17). Fluorescence of the medium was 1–10% of membrane fluorescence, and this background was subtracted from the measured fluorescence. Punctate cell-associated fluorescence was not observed. Experiments were repeated on three separate days with no significant differences in results.

RESULTS

Sequence Analysis of the A^k_α DNA. Preliminary biochemical analysis of the 2B1 mutant had identified a mutation in the A^k_α polypeptide (4). Therefore, A^k_α-encoding DNA was closed from a cDNA library derived from the 2B1 cell line. Sequence analysis showed that the 2B1 A^k_α gene contained a single C→T nucleotide transition in the coding strand that resulted in a premature stop codon at position 222 (Fig. 1). This mutation caused the deletion of the 12 amino acids comprising the cytoplasmic domain of the A^k_α polypeptide, including the three basic residues Arg-222, Arg-228, and His-229. This loss of the cytoplasmic tail explains both the lower apparent molecular weight and the increased acidity of the mutant A^k_α polypeptide.

Effect of the A^k_α Mutation on Antigen Presentation. Deletion of the cytoplasmic domain of the A^k_α polypeptide did not alter the ability of the 2B1 cell line to present exogenous antigen to I-A^k-restricted T-cell hybridomas (Table 1). The 2B1 cells also stimulated two alloreactive T-cell hybridomas. In contrast, the 2B1 cells failed to stimulate two out of eight I-A^k-restricted autoreactive T-cell clones that were stimulated by the parent line TA3 (ref. 4; Table 1).

Turnover Rate for I-A^k in the Mutant 2B1 and Wild-Type TA3 Cells. Biosynthetically labeled I-A^k was precipitated from the 2B1 mutant and TA3 wild-type parent to determine whether deletion of the cytoplasmic domain affects the turnover rate of the A^k_α polypeptide. The A^k_α and A^k_β polypeptides are resolved by one-dimensional NaDodSO$_4$/PAGE into two components: a band at higher molecular weight that represents fully glycosylated protein and a band at lower molecular weight that represents the core glycosylated protein (Fig. 2*a*). The polypeptides shift from the core glycosylated (high-mannose) form to the fully glycosylated form as they are transported from the endoplasmic reticulum through the Golgi complex. The increased time required for processing from the core to the fully glycosylated forms of A^k_α and A^k_β indicates that the 2B1 mutant has a reduced rate of intracellular transport of I-A^k relative to the TA3 parent. The longer exposure required for an approximately equal film exposure (48 hr for A^k_α from the 2B1 mutant vs. 24 hr for A^k_α from the TA3 parent) indicates that fewer I-A^k molecules can be precipitated from the cell lysate of the 2B1 mutant relative to

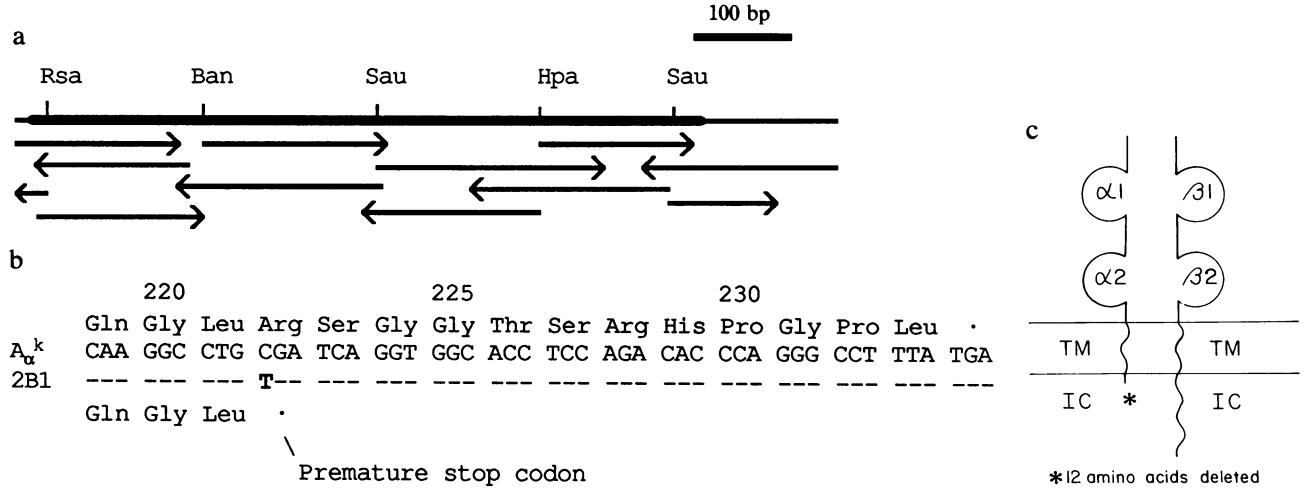

FIG. 1. (*a*) Partial restriction map and sequencing strategy for the A^k_α cDNA. Various constructs were made by using the restriction sites shown, and the A^k_α insert was fully sequenced in both orientations. The coding sequence is depicted by the thick line, whereas partial 5′ leader and 3′ untranslated sequences are depicted by thinner lines. Ban, *Ban* II; Hpa, *Hpa* II; Rsa, *Rsa* I; Sau, *Sau* 3A1; bp, base pairs. (*b*) Nucleotide sequence encoding the cytoplasmic domain of the 2B1 mutant A^k_α compared to the wild-type sequence. The predicted amino acid sequence is also shown. The single base transition at codon 222 was confirmed by sequencing both strands of the cDNA. (*c*) Schematic representation of the tailless A^k_α polypeptide on the membrane as part of an A^k_α·A^k_β heterodimer. α1, α2, β1, and β2 are extracellular domains of the α and β chains; TM, transmembrane; IC, intracytoplasmic.

Table 1. Selective defect in antigen presentation by an A^k_α tailless mutant

T-cell line		APC line	
Specificity*	Name	TA3 (wild-type)	2B1 (mutant)
Autoreactive	E9	+	−
	B8	+	−
	C8	+	+
	E2	+	+
	2127.24	+	+
	E2	+	+
	E8	+	+
	F4	+	+
Alloreactive	Mdk5.1	+	+
	Mdk15.3	+	+
KLH	SKK2.3	+	+
	SKK9.11	+	+
	SKK45.10	+	+
	2C3	+	+
	K31.H2	+	+
	2A1	+	+
	IC3.A5	+	+
Ovalbumin	AODH3.4	+	+
HEL	A6	+	+
	C4	+	+
	B4	+	+
	C11	+	+
	A2	+	+
	C10	+	+
	18.N.30	+	+
	A4	+	+
	3A9	+	+
	19.47	+	+
	2A11	+	+

T cells were tested for activation in a standard interleukin 2-release assay involving secondary culture of supernatant with HT-2 cells (18). +, Responses comparable to those found by using wild-type (TA3) APCs; −, lack of response (background level).
*All the T-cell lines are I-A^k-restricted. KLH, keyhole limpet hemocyanin; HEL, hen egg-white lysozyme.

the wild-type TA3 cell. Fig. 2*b* shows an analysis of the total A^k_α polypeptides in each lane of the one-dimensional gels presented in Fig. 2*a*. The mutant (2B1) $A^k_\alpha \cdot A^k_\beta$ heterodimer has a longer half-life (>18 hr vs. 2 hr) than the wild-type (TA3) heterodimer.

Lateral Diffusion of I-A^k in the Membrane of the 2B1 and TA3 Cells. The lateral mobility of the I-A^k molecule in the membrane of the 2B1 cell was measured to determine whether an altered mobility could contribute to the defect in antigen presentation detected in this variant. The lateral diffusion was assayed by FPR. As shown in Table 2, the lateral diffusion coefficient and fractional mobility of the I-A^k molecule in the 2B1 mutant are indistinguishable from those of the I-A^k molecule in the wild-type TA3 parent.

DISCUSSION

Ia-restricted recognition has been attributed to the polymorphic NH_2-terminal domain of the Ia molecule (2, 3). The inability of the 2B1 mutant to stimulate two autoreactive clones (B8 and E9, Table 1) is a clear demonstration that a mutation in the cytoplasmic domain of the molecule can also affect antigen presentation. Braciale *et al.* (19) reported that the transmembrane sequence of the influenza hemagglutinin molecule is recognized by class I-restricted cytolytic T lymphocytes. This shows that a membrane protein can be processed in such a manner that regions normally embedded in the membrane can be exposed to the extracellular environment in association with major histocompatibility com-

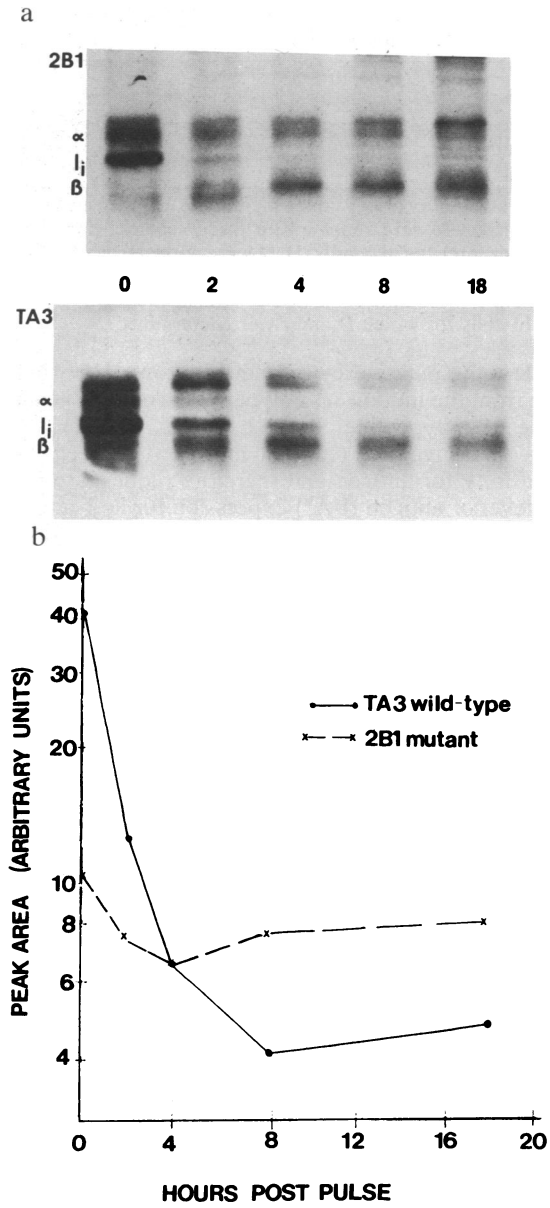

FIG. 2. Turnover rate of I-A^k in the 2B1 mutant and TA3 parent. (*a*) I-A^k immunoprecipitates analyzed by $NaDodSO_4$/PAGE followed by autoradiography. Samples were taken at 0, 2, 4, 8, and 18 hr post-pulse. A^k_α (α), A^k_β (β), and invariant (I_i) chains are marked on each autoradiograph. Their positions are based upon comparison with molecular weight standards run in the same gel. A^k_α has M_r 33,000–34,000; invariant chain, ≈31,000; and A^k_β, ≈29,000. Results are representative of five separate experiments. (*b*) Quantitative determination of the half-life for the A^k_α polypeptides. Exposed autoradiographs from $NaDodSO_4$/polyacrylamide gels were scanned with an Ephortec Joyce–Loebl densitometer to determine the total relative area (amount) for both core and fully glycosylated forms of A^k_α polypeptide in each lane. Time required for a 50% reduction from the initial amount of radioactive A^k_α polypeptide was <2 hr. TA3 wild type and >18 hr for 2B1 mutant.

plex-encoded molecules. This raises the interesting possibility that the deleted cytoplasmic domain of the I-A^k molecule contains the epitope recognized by the autoreactive clones E9 and B8. That is, these two autoreactive clones recognize a processed form of the I-A^k molecule. This interesting possibility does not appear to be the case, however. The cytoplasmic domains of the A^k_α and A^d_α molecules are identical. Therefore, since there is no reason to expect that the processing of I-A^k molecules is different from that of I-A^d

Table 2. Lateral mobility of I-A^k in the cell membranes of the 2B1 mutant and TA3 parent cell lines

Cell	$D \times 10^9$, cm^2/sec	f, %	n
2B1	4.0 ± 2.0	71 ± 9	38
TA3	4.2 ± 2.4	65 ± 9	38

The lateral mobility of I-A^k in the membrane was measured by FPR (17). Experiments were repeated on three separate days with no significant differences in results. Data were pooled from the different experiments. D, diffusion coefficient of the mobile fraction (± 1 SD); f, fraction of labeled molecules free to diffuse laterally (± 1 SD); n, number of cells for which D and f were determined.

molecules, the putative cytoplasmic epitope that would be missing from the mutant A^k_α polypeptide would be generated from the A^d_α polypeptide.

It has been suggested (20) that autoreactive T cells have a lower affinity for antigen and therefore require a relatively higher level of antigen (I-A^k) expression for activation than do antigen-specific or alloreactive T cells. Therefore, this limited defect in antigen presentation may be the result of the decreased membrane expression of the I-A^k ($A^k_\alpha \cdot A^k_\beta$) molecule by the 2B1 mutant. We attempted to test this hypothesis by selecting 2B1 cells with wild-type surface levels of A^k_α ($A^k_\alpha \cdot A^k_\beta$). However, in three separate experiments using electronic cell sorting, we were unable to enrich the 2B1 cell line for variants expressing higher levels of A^k_α, although it was possible to enrich the TA3 parent for A^k_α expression after a single sort. This indicates that a wild-type level of A^k_α expression is an inherently unstable phenotype in the 2B1 mutant, and prevents us from directly testing whether the membrane concentration of I-A^k can account for the limited defect in antigen presentation observed.

There are several possible explanations for the reduction in surface expression of the A^k_α polypeptide. These include (*i*) decreased intracytoplasmic transport of the A^k_α-containing heterodimer, (*ii*) decreased stability of the $A^k_\alpha \cdot A^k_\beta$ heterodimer in the membrane, and (*iii*) decreased $A^k_\alpha \cdot A^k_\beta$ interchain pairing. We addressed these points by first measuring the half-life of the A^k_α polypeptide in a pulse–chase experiment. The results (Fig. 2) indicate that the concentration of the A^k_α polypeptide in the cytoplasm of the 2B1 mutant is lower than that in the cytoplasm of the TA3 wild-type parent and that the mutant (2B1) $A^k_\alpha \cdot A^k_\beta$ heterodimer has a longer half-life than the wild-type (TA3) heterodimer. The longer half-life of the mutant I-A^k is due at least in part to the increased time (>18 hr vs. 2 hr for wild-type) required for complete conversion to the higher molecular weight, fully glycosylated forms of the A^k_α and A^k_β polypeptides. Since conversion to the fully glycosylated form occurs in the Golgi complex, this indicates that the rate of intracellular transport for I-A^k is decreased. This finding is in agreement with reports (18, 21–24) that several other transmembrane glycoproteins are transported more slowly from the endoplasmic reticulum to the Golgi complex in the absence of, or with significantly altered, cytoplasmic domains. Our results also demonstrate that an intact A^k_α cytoplasmic domain is not absolutely required for surface expression of an I-A^k molecule, although it may serve to stabilize the molecule and facilitate its transport.

Our work did not specifically address whether deletion of the cytoplasmic tail from the A^k_α polypeptide decreases stability of I-A^k in the membrane or hampers interchain pairing. The latter possibility is supported, however, by the observation that the A^k_β polypeptide associates with both the A^d_α and the A^k_α polypeptides to form the hybrid $A^d_\alpha \cdot A^k_\beta$ heterodimer as well as the homozygous $A^k_\alpha \cdot A^k_\beta$ heterodimer in the 2B1 cell (4). It has been shown (25) that the A^k_β polypeptide preferentially associates with a wild-type A^k_α polypeptide rather than with a mismatched A^d_α polypeptide. The appearance of the hybrid molecule therefore implies that the mutant A^k_α polypeptide has a reduced ability to form the $A^k_\alpha \cdot A^k_\beta$ heterodimer. Residues in the NH_2-terminal domain are critical for heterodimer selection and chain pairing (ref. 26; D.J.M., unpublished data). Our findings may indicate that the cytoplasmic domain also plays a role in chain pairing. In addition, our data suggest that there may be an intracellular pool of the A^k_α polypeptide. This possibility is particularly difficult to confirm since reagents (mAbs) specific for isolated A_α chains do not exist. However, once the A^k_α and A^k_β chains do associate in the cytoplasm, the mutant heterodimer is transported to the cell surface, albeit more slowly than the wild-type molecule, and I-A^k does not accumulate in the cytoplasm (M. Rodriguez and D.J.M., unpublished observation).

The lateral mobility of major histocompatibility complex proteins can affect T-cell recognition of antigen (27). If loss of the cytoplasmic tail from A^k_α affected I-A^k mobility in the membrane, then a limited loss of APC function in the 2B1 mutant might result. We therefore examined the lateral mobility of I-A^k in 2B1 and TA3 cell membranes by using FPR. We found that the mutant I-A^k molecule had a lateral diffusion coefficient and fractional mobility indistinguishable from those of the I-A^k molecule in the wild-type TA3 parent (Table 2). The absence of the A^k_α cytoplasmic domain therefore does not alter the lateral mobility of this molecule.

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