Intracytoplasmic domains of MHC class II molecules are essential for lipid-raft-dependent signaling

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Summary

In addition to their role in antigen presentation, major histocompatibility complex (MHC) class II molecules have been widely described as signaling proteins in diverse antigen-presenting cells (APCs) including B cells and dendritic cells. By contrast, little is known of the signaling function of MHC class II molecules expressed in solid tumors. We describe the functional organization and signaling ability of I-A^k expressed in a sarcoma, and report the recruitment of I-A^k to lipid rafts after MHC class II engagement. Lipid raft integrity was required for I-A^k-mediated reorganization of the actin cytoskeleton and translocation of protein kinase C-α (PKC-α) to the precise site of stimulation via I-A^k. Truncation of the intracytoplasmic domains of I-A^k did not perturb I-A^k recruitment to lipid rafts but abrogated PKC-α translocation and actin rearrangement. PKC-α was detected in lipid microdomains and enrichment of activated PKC-α in lipid rafts was induced by I-A^k signaling. Ordering of the molecular events following engagement of the MHC class II molecules revealed that I-A^k recruitment to lipid rafts precedes signaling. This is consistent with the absence of a requirement for the intracytoplasmic tails for localization to lipid rafts. These data reveal that lipid-rich microdomains play a key role in MHC class II-mediated signaling in a solid tumor.

Key words: Lipid rafts, MHC class II, PKC, Actin, Tumor cell

Introduction

MHC class II molecules are α/β heterodimers constitutively expressed by professional APCs (dendritic cells, B cells and monocytes) and inducible in diverse cell types by interferon γ (IFN-γ). MHC class II proteins can also be expressed either constitutively or inducibly by tumors of non-APC origin (Ruiter et al., 1984; Winchester et al., 1978). MHC class II heterodimers have a well characterized role in the presentation of processed exogenous antigens to CD4$^+$ T cells. In addition to their role in antigen presentation, MHC class II molecules transmit intracellular signals including activation of PKC and protein tyrosine kinases (PTK) (reviewed by Scholl and Geha, 1994). MHC class II-mediated signaling has diverse consequences including proliferation (Cambier and Lehmann, 1989), apoptosis (Newell et al., 1993; Truman et al., 1994), differentiation, synthesis of costimulation molecules (Nabavi et al., 1992), or cytokine release (Mehindate et al., 1995), depending upon the origin of the APC and its state of activation and/or differentiation. A recent in vivo study of a humanized MHC class II monoclonal antibody (mAb) demonstrated that tumoral B cells were susceptible to programmed cell death via MHC class II engagement, whereas resting cells were protected (Nagy et al., 2002).

The PKC family of serine/threonine kinases is classified into three groups of isoenzymes based upon their activation requirements: the classical PKCs (α, βI/II and γ) are activated by diacylglycerol (DAG), phosphatidylserine (PS) and Ca^{2+}; the novel PKCs (δ, ε, η and θ) are activated by DAG and PS; and the atypical PKCs (ζ and λ) only respond to PS. The intracellular tail of the MHC class II β chain is required for activation of the PKC-α and PKC-βII isoforms (Cambier et al., 1987; Harton et al., 1995; Rich et al., 1997; St-Pierre et al., 1989). By contrast, the ability of MHC class II to mediate PTK activation is unaffected by either mutations or truncation of the intracellular part of the β chain in B cells (Harton et al., 1995; Rich et al., 1997).

A role for MHC class II proteins in the antitumoral immune response has been clearly shown in an immunotherapy model wherein vaccination of mice with sarcoma cells transfected with α and β chains of I-A^k molecules (SaI/A^k cells) protects against subsequent challenge with the highly tumorigenic MHC class II$^-$ wild-type sarcoma SaI (Ostrand-Rosenberg et al., 1990). I-A^k expression in this sarcoma enables SaI/A^k cells to present intracellularly expressed antigens directly to CD4$^+$ T cells (Armstrong et al., 1998). By contrast, sarcoma cells transfected with C-terminal-truncated α and β chains (SaI/A^k tr cells) lose their immunogenic properties and remain equally as tumorigenic as the wild-type sarcoma (Ostrand-Rosenberg et al., 1991). Furthermore, single amino acid mutations in the intracellular part of the I-A^k β chain led to distinct antitumoral responses (Laufer et al., 1997).

Recent studies have demonstrated a key role for specific

microdomains of the plasma membrane in signal transduction via MHC class II molecules (Anderson et al., 2000; Huby et al., 1999; Setterblad et al., 2001). These membrane microdomains, highly enriched in cholesterol and glycosphingolipids, are proposed to function as preformed platforms essential for sustaining immunoreceptor signaling and membrane trafficking (reviewed by Harder and Simons, 1997; Simons and Ikonen, 1997). Such detergent-insoluble glycolipid-enriched complexes (DIGs) are also known as glycosphingolipid-enriched membrane microdomains (GEMs), detergent-resistant membranes (DRMs) or lipid rafts. They can be isolated based on their insolubility in non-ionic detergent and their low buoyant density on sucrose gradients. They preferentially recruit and concentrate specific proteins involved in cellular signaling such as doubly acylated Src-family tyrosine kinases, glycosylphosphatidylinositol (GPI)-anchored proteins and proteins with saturated fatty acyl chains, while excluding others such as CD45 (Rodgers and Rose, 1996), thus optimizing activation and subsequent downstream signaling. Aggregation of MHC class II molecules in the THP-1 cell line induced their recruitment to lipid rafts and was required for tyrosine kinase activation (Huby et al., 1999). Ligand binding to HLA-DR in B cells led to HLA-DR and PKC-δ recruitment to DIGs (Setterblad et al., 2001). Anderson et al. implicated lipid raft microdomains in antigen presentation under conditions of low peptide concentration (Anderson et al., 2000). By contrast, Kropshofer et al. proposed that antigen presentation was not dependent on the integrity of MHC class II-containing lipid rafts but rather was dependent upon the MHC class II molecules complexed with tetraspanin molecules (Kropshofer et al., 2002).

We have determined whether I-A^k expressed in a sarcoma transmits signals and whether the intracytoplasmic tail of I-A^k is required. We report that the intracytoplasmic tail of I-A^k is not required for the recruitment of I-A^k to DIGs, whereas PKC-α activation and actin rearrangement, mediated via I-A^k, are abrogated by truncation of I-A^k. Inhibition of PKC activation and of cytoskeletal integrity revealed that recruitment of I-A^k to lipid rafts did not require either PKC-α activation nor actin reorganization, but rather that these events were initiated via I-A^k localized within lipid rafts and were prevented by disruption of lipid rafts.

Materials and Methods

Antibodies and reagents

Polyclonal anti-PKC-α (C-20) and anti-PKC-δ (C-17) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phospho-PKC (pan) and anti-PKD/PKC-μ Abs were from New England Biolabs (Beverly, MA). Anti-actin mAb (MAB 1501) was from Chemicon (Temecula, CA). Anti-mouse I-A^k mAb directed against the β chain (10.2.16, mouse IgG2b) was purified from ascitic fluid. Goat anti-mouse (GAM) IgG was from Southern Biotechnology (Birmingham, AL). Polyclonal anti-caveolin Ab was from Transduction Laboratories (Lexington, KY). HRP-conjugated anti-rabbit Ig and anti-mouse Ig were from DAKO (Glostrup, Denmark). Mouse IgG2b isotype control Ig was from Caltag Laboratories (Burlingame, CA). HRP-conjugated cholera toxin B subunit, anti-β-tubulin mAb (TUB 2.1), methyl-β-cyclodextrin (MβCD), Triton X-100 and normal mouse serum were from Sigma (St Louis, MO). FITC-conjugated anti-rabbit IgG was from Jackson Immunoresearch (West Grove, PA). Alexa Fluor488-conjugated goat anti-mouse Ig and phalloidin were from Molecular Probes (Eugene, OR). Latex beads (6 μm, cat. N#7312) were from Polysciences (Warrington, PA). Calphostin C and cytochalasin D were from Calbiochem (Merck Eurolab, Fontenay-sous-Bois, France).

Cell culture

The SaI sarcoma is a methylcholanthrene-induced murine sarcoma of A/J (H-2$K^kA^kD^d$) strain mice. The SaI cell line was maintained in culture in DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% decomplemented FCS, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine at 37°C in 5% CO_2.

SaI cells were tri-transfected with A_α^k and A_β^k MHC class II cDNA plus pSV2neo vector DNA by the calcium phosphate method (called SaI/A^k transfectants) (Ostrand-Rosenberg et al., 1990). I-A^k molecules, prematurely truncated for the C-terminal 12 and 10 amino acids, respectively, are expressed in SaI cells (SaI/A^k tr), as previously described (Ostrand-Rosenberg et al., 1991). The transfected SaI/A^k and SaI/A^k tr tumor cells were maintained in culture in IMDM Glutamax-I (Gibco-BRL, Rockville, MD) supplemented as described for SaI and maintained under selection by treatment with 400 μg/ml geneticin (Gibco-BRL, Rockville, MD) for one week per month.

Sample preparation

Cell treatment and stimulation

Cells were stimulated with either 10 μg/ml goat anti-mouse Ab (GAM, isotype control) or 10 μg/ml 10.2.16 mAb crosslinked with 10 μg/ml GAM Ab for different times at 37°C. DIG disruption was carried out by treating cells with the cholesterol-sequestering agent MβCD (10 mM MβCD for 15 minutes at 37°C) prior to Ab stimulation. Neither cell viability nor surface MHC class II expression levels were affected by this treatment. Where indicated, SaI/A^k were pretreated at 37°C with either the broad-spectrum PKC inhibitor calphostin C (50 nM, 30 minutes), the actin polymerization inhibitor cytochalasin D (10 μM, 60 minutes) or an equivalent volume of the appropriate diluent.

Preparation of total cell lysates (TCLs)

Cells (5×10^6) were washed in cold PBS and then lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM PMSF, 10 mM NaF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 4 μg/ml aprotinin) on ice for 30 minutes. Following centrifugation at 14,400 ***g***, 4°C for 20 minutes to pellet cellular debris, nuclei and large insoluble material, the protein concentration of the supernatants was determined using the Bradford assay (Bio-Rad Laboratories). TCLs were immunoblotted to detect total I-A^k expression in SaI/A^k and SaI/A^k tr.

Preparation of DIGs

DIGs were isolated as described by Xavier et al. (Xavier et al., 1998). Cells (30×10^6) were washed twice and incubated in 350 μl of MBS-T buffer [25 mM morpholinoethanesulfonic acid (MES), 150 mM NaCl pH 6.5, 1 mM Na_3VO_4, 1 mM PMSF, 10 mM NaF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 4 μg/ml aprotinin and 0.5% Triton X-100] for 30 minutes on ice. Lysates were ultracentrifuged after mixing with an equal volume of 85% sucrose (w/v) in MBS at the bottom of a polycarbonate ultracentrifuge tube (Beckman Instruments, Palo Alto, CA), which was then overlaid with 2 ml of 35% sucrose followed by 1 ml of 5% sucrose in MBS containing 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF and 1 μg/ml aprotinin. After centrifugation at 200,000 ***g*** for 18 hours at 4°C in a SW55Ti rotor (Beckman Instruments), eight fractions of 500 μl were collected from the top to the bottom of the tube. Fractions 3 and 4 correspond to the DIG-containing 5/35% sucrose interface.

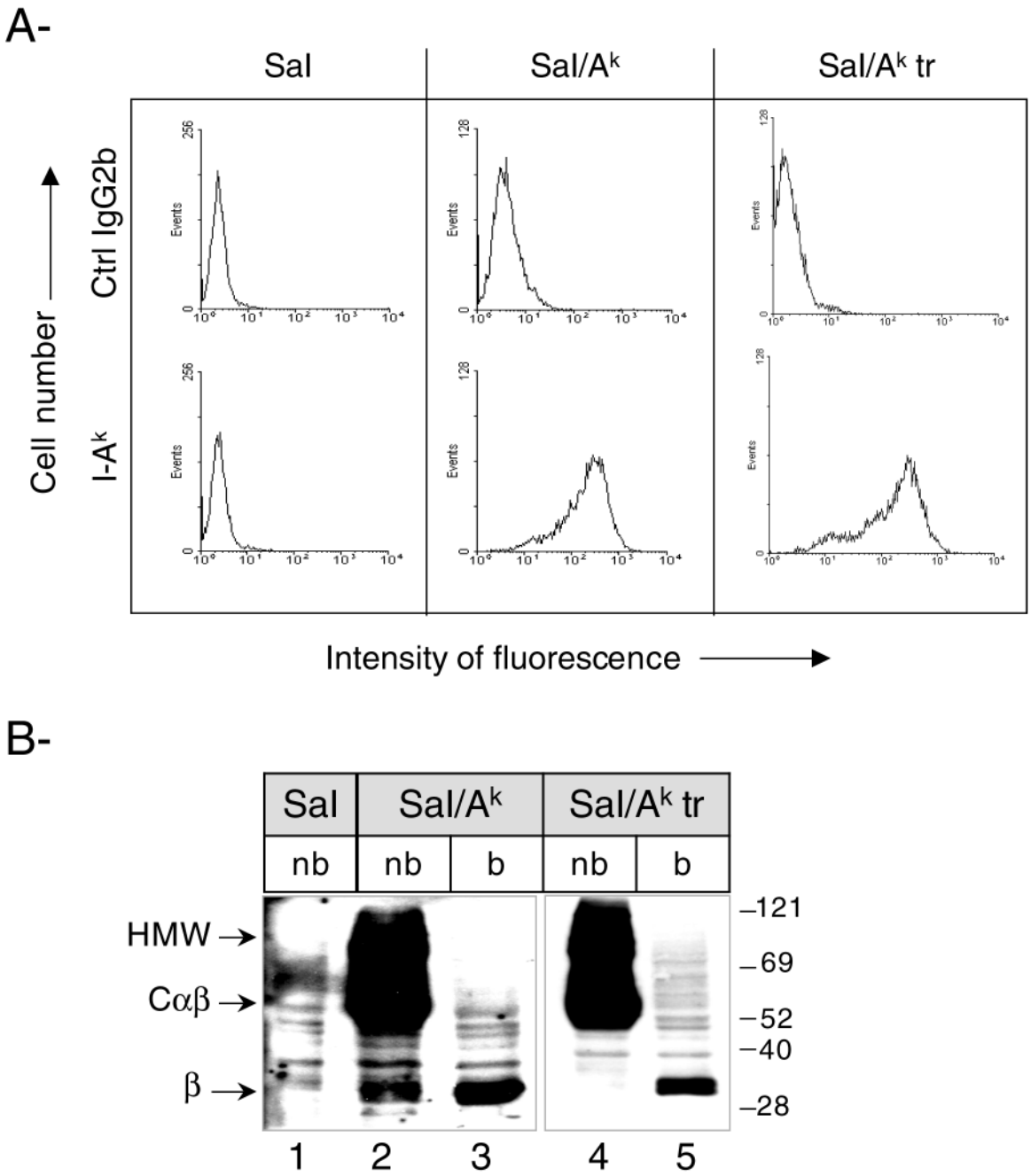


Fig. 1. Analysis of MHC class II molecules in SaI/A^k cells. (A) Surface expression of I-A^k molecules on SaI, SaI/A^k and SaI/A^k tr cells was determined by FACS analysis. Binding of I-A^k mAb and the isotype control Ig (IgG_{2b}) is shown. (B) Peptide-associated MHC class II αβ compact heterodimers (Cαβ), high-molecular-weight (HMW) complexes and free β chains were detected by western blotting on total cell lysates from SaI, SaI/A^k and SaI/A^k tr cells. Lysates were incubated in Laemmli sample buffer for 30 minutes at room temperature (nb, lanes 1, 2) or for 10 minutes at 95°C (b, lanes 3 and 5). Samples were migrated on a 10% SDS-PAGE gel before transfer to a PVDF membrane and immunoblotting with 10.2.16 mAb.

Western blotting

10-15 μg of TCLs or 15 μl of each sucrose fraction were boiled in Laemmli sample buffer and proteins were separated on a 10% SDS-PAGE (15% for GM1 detection). After electrophoresis, the proteins were transferred onto PVDF (AP Biotech, Buckinghamshire, UK) for 1 hour at 100 V. The membranes were blocked for 1 hour at room temperature in PBS/0.1% Tween 20 (PBS-T) containing 5% nonfat dry milk. The blots were then probed for 1 hour with primary antibody diluted in blocking buffer. After two washes in PBS, the blots were incubated for 1 hour with HRP-conjugated secondary Ab. Bands were visualized using the ECL system (AP Biotech). Arbitrary quantification of the bands corresponding to PKC-α and phospho-PKC was carried out using the NIH Image based Scion software (Scion Corporation, Frederick, ML).

SDS-stability assay

MHC class II heterodimers are stabilized by peptide binding and resist dissociation after incubation in SDS-detergent at room temperature.

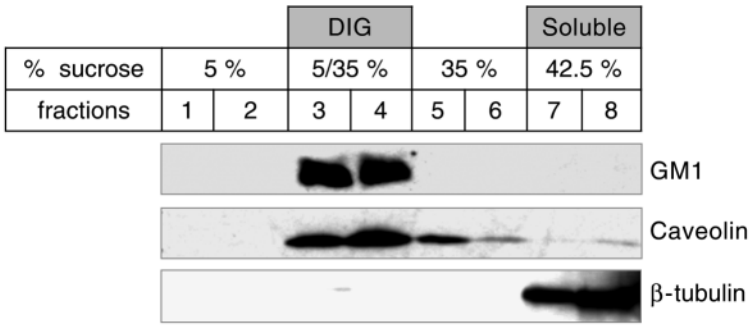


Fig. 2. DIGs are characterized by their GM1 and caveolin content. DIGs were characterized in SaI/A^k cells by examining the localization of the typically DIG residents glycolipid GM1 and caveolin compared with the typically non-DIG-localizing protein β-tubulin in the sucrose gradient. Cell lysates were fractionated on sucrose gradient and all fractions were immunoblotted with the respective Abs. DIG fractions correspond to fractions 3 and 4, whereas the soluble fractions correspond to fractions 7 and 8.

Heating to 95°C dissociates the heterodimer into free α and β chains. Samples were incubated either at room temperature for 30 minutes or heated at 95°C for 10 minutes under reducing conditions and loaded onto a 10% SDS-PAGE. Membranes were blocked with PBS-T/5% milk for 1 hour, washed twice in PBS-T and incubated with 10.2.16 mAb at 1 μg/ml in PBS-T/2% milk for 1 hour. After two washes in PBS, the blots were incubated for 1 hour with HRP-conjugated anti-mouse secondary Ab. I-A^k was detected by electrochemiluminescence (ECL).

Flow cytometry analysis

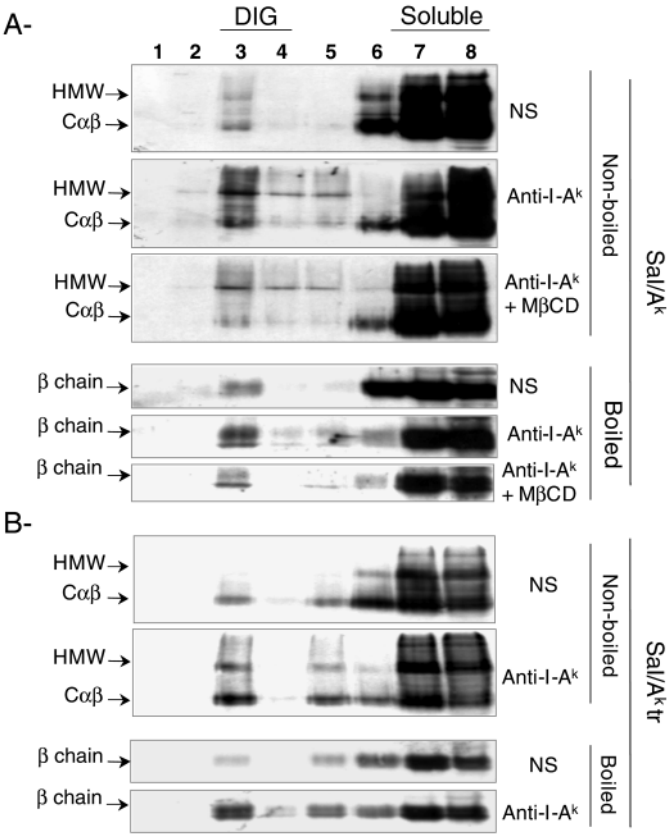
Cells were washed once in PBS, resuspended in PBS containing 1% FCS and 0.1% sodium azide and incubated with either control isotype (mouse IgG$_{2b}$) or the I-A^k-specific mAb (10.2.16) for 15 minutes. Cells were washed in PBS/SVF/azide and then incubated with Alexa Fluor488-conjugated goat anti-mouse Ig for 15 minutes on ice in the dark. After washing, cells were resuspended in PBS containing 5 μg/ml propidium iodide and analyzed by flow cytometry on a FACScan (Becton Dickinson). Dead cells were excluded based on FSC/SSC profile and propidium iodide staining. Histograms were constructed based on analysis of 10,000 cells.

Preparation of antibody-coated latex beads

Latex beads were coated with either irrelevant mouse Igs or 10.2.16 antibody according to the manufacturer's recommendations. 100 μg of purified Ig was allowed to bind to 0.2 ml beads pre-treated with 0.1 M borate buffer, pH 8.5. Beads were washed and resuspended in storage buffer (PBS pH 7.4, 10 mg/ml BSA, 0.1% sodium azide, 5% glycerol) at 4°C.

Confocal microscopy

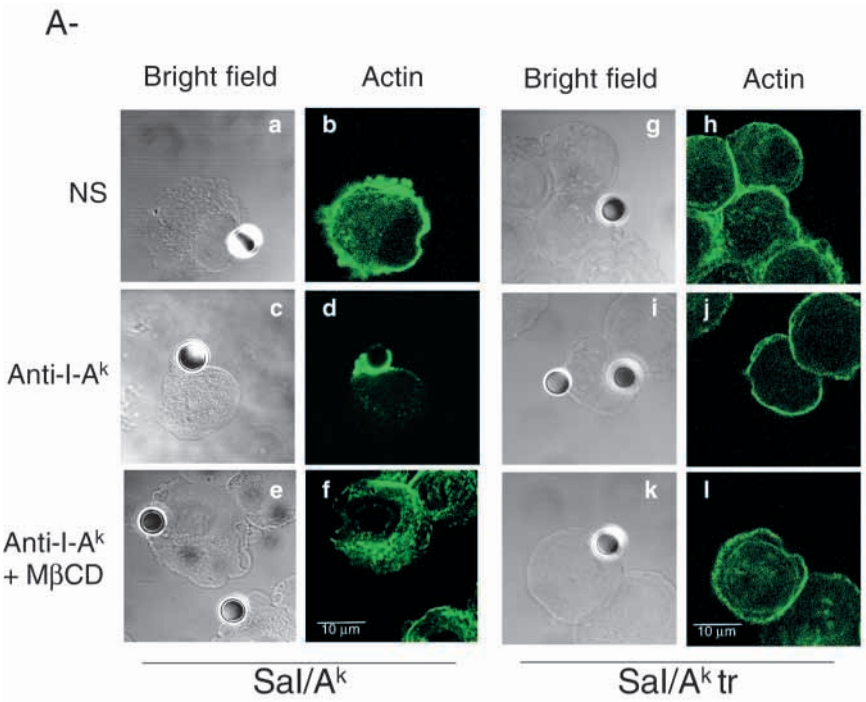
Cells were stimulated with either 10.2.16-coated beads or irrelevant mouse Ig-coated beads for 30 minutes at 37°C at a ratio of one bead per cell. Where indicated, cells were pretreated with either MβCD (10 mM, 15 minutes), calphostin C (50 nM, 30 minutes) or cytochalasin D (10 μM, 60 minutes) prior to stimulation. The cells were then cytospun and air-dried for 20 minutes before fixation in cold methanol for the PKC-α staining or in 2.5% paraformaldehyde (PFA)/PBS followed by permeabilization with 0.1% Triton X-100 for the actin staining. Cells fixed with PFA were incubated with 50 mM NH_4Cl in PBS. Cells were washed in PBS and labeled with primary antibody (polyclonal anti-PKC-α Ab) or with Alexa Fluor488-labeled



C-

	% DIG-associated MHC class II	
	Sal/A^k	Sal/A^k tr
NS	10.7	7.3
Anti-I-A^k	21	22
Anti-I-A^k + MβCD	8	nd

Fig. 3. MHC class II engagement induces the recruitment of I-A^k and I-A^k tr molecules into DIGs. The distribution of I-A^k and I-A^k tr molecules through the sucrose gradient and its modulation in response to I-A^k stimulation was analyzed by western blotting. (A) SaI/A^k cells were pretreated or not with MβCD for 15 minutes, and stimulated or not (nonstimulated, NS) via I-A^k for 15 minutes before lysis and fractionation on sucrose gradient. Fractions were incubated in Laemmli buffer for 30 minutes at room temperature (non-boiled) or for 10 minutes at 95°C (boiled). Samples were migrated on 10% SDS-PAGE gels, transferred to PVDF and immunoblotted with 10.2.16 mAb. Cαβ, compact αβ dimers of MHC class II molecules; HMW, high-molecular-weight complexes; β chain, free β chains. (B) SaI/A^k tr cells were stimulated or not via I-A^k, lysed and fractionated as above, followed by immunoblotting with 10.2.16 mAb. (C) Immunoblots of free β chains (boiled condition) of I-A^k and I-A^k tr were scanned and each fraction was quantified by densitometry. The results are expressed as percentage of DIG-associated MHC class II. Representative data from one of three typical experiments is shown.



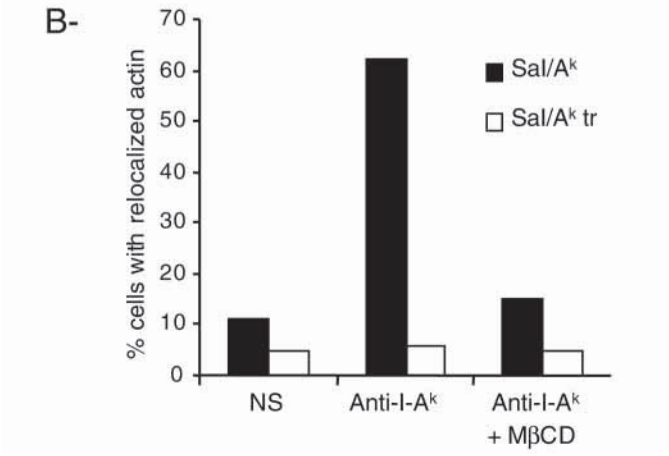


Fig. 4. I-A^k mediated actin reorganization is DIG dependent and requires the intracytoplasmic domains. (A) SaI/A^k and SaI/A^k tr cells were stimulated with 10.2.16 mAb-coated beads (Anti-I-A^k) or irrelevant mouse Ig-coated beads (nonstimulated, NS) at a ratio of one bead per cell for 30 minutes. Cells were cytospun, fixed and permeabilized. Polymerized actin was detected with Alexa488-phalloidin. The bright-field images of single cell/bead conjugates (a,c,e,g,i,k) and the corresponding fluorescent image of the actin cytoskeleton are presented (b,d,f,h,j,l). Polarization of the actin network was observed only at the site of SaI/A^k cell/10.2.16-coated bead contact (d) and was prevented by pre-treatment with MβCD (f). By contrast, no reorganization of the actin cytoskeleton was detected after engagement of SaI/A^k tr cells with 10.2.16-coated beads (j). (B) The percentage of cells undergoing actin reorganization was determined. At least 200 cell-bead interactions from each condition were scored and cells in which actin relocalization had occurred (to at least the same degree as shown in A, panel d) were counted as positive events. Data from one of three typical experiments is shown.

phalloidin for 1 hour. FITC-conjugated anti-rabbit Ab was used to detect PKC-α. Slides were washed in PBS and mounted with Vectashield (VectorLab, Burlingame, CA). Images were acquired with a Zeiss LSM-510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss Axiovert 100M (plan Apochromat 63X 1.40NA oil immersion objective). The Alexa Fluor488 and FITC fluorophores were excited at 488 nm. To avoid selection bias, a minimum of 200 randomly selected cell-bead interactions were examined per slide. The results are expressed as percentages of cells having undergone striking relocalization of the target protein (such as shown in Figs 4, 6).

Results

Surface expression and conformation of the MHC class II molecules

The murine MHC class II$^-$ sarcoma (SaI) transfected with I-A^k α and β chains (SaI/A^k) or with I-A^k α and β chains truncated in the intracytoplasmic tails (SaI/A^k tr) were analyzed by FACS analysis to examine the surface MHC class II expression. SaI cells were used as a negative control. SaI/A^k and SaI/A^k tr cells expressed equivalent levels of I-A^k molecules at the surface (Fig. 1A). The conformation and structure of MHC class II molecules were analyzed by a SDS-stability assay to determine whether I-A^k truncation altered the conformation or the stability of the αβ heterodimer. Peptide-loaded SDS-stable I-A^k complexes were detected by immunoblotting of non-boiled samples. The 10.2.16 mAb directed against I-A^k molecules detects both compact αβ dimers (Cαβ, 55-60 kDa), and high-molecular-weight complexes (HMW, >100 kDa) in non-boiled TCLs from SaI/A^k and SaI/A^k tr cells (Fig. 1B, lanes 2 and 4). Dissociation of SDS-stable complexes by boiling led to the appearance of free β chains (Fig. 1B, lanes 3 and 5). Compact αβ dimer expression was comparable in lysates expressing wild-type or truncated molecules. The non-I-A^k-expressing wild-type sarcoma is shown for comparison (Fig. 1B, lane 1).

Characterization of DIGs

SaI/A^k cells were lysed and fractionated on a sucrose gradient. The GM1, caveolin and β-tubulin content in the eight collected fractions were analyzed by western blotting to identify the DIG fraction. GM1 ganglioside is a glycolipid localizing in DIGs that binds the β subunit of cholera toxin. Caveolin has been described as a DIG-resident protein, whereas β-tubulin typically resides outside of DIGs (Cheng et al., 1999; Harder and Simons, 1997). Fig. 2 shows the expression of GM1 and caveolin in fractions 3 and 4, corresponding to the 5/35% sucrose interface and therefore identifies these low-density fractions as the DIG-containing fractions (Xavier et al., 1998). By contrast, β-tubulin was exclusively expressed in fractions 7 and 8, which correspond to fractions containing high-density

detergent-soluble material. The distribution of DIG and non-DIG markers was identical in SaI/A^k tr and was unchanged by I-A^k-mediated stimulation of either cell type (data not shown).

I-A^k engagement induces MHC class II recruitment to DIGs independently of the intracytoplasmic domains

In order to determine whether MHC class II molecules expressed in this sarcoma were constitutively present in DIGs, and whether such a localization could be modified by I-A^k engagement, we screened the sucrose gradient fractions for I-A^k molecules both in nonstimulated and I-A^k-stimulated SaI/A^k and SaI/A^k tr cells.

I-A^k was weakly expressed in the DIG fraction in nonstimulated SaI/A^k cells (Fig. 3A) under boiled or non-boiled conditions, and the percentage of DIG-associated MHC class II was estimated as 10.7% (Fig. 3C). MHC class II was enriched in fraction 3 following I-A^k engagement, indicating recruitment to DIGs via I-A^k stimulation (Fig. 3A). The recruited MHC class II molecules were present both as compact αβ dimers (55-60 kDa) and HMW complexes (>100 kDa). In boiled samples, the increased expression of the β chain in fraction 3 confirmed the recruitment of MHC class II molecules to DIGs via I-A^k (Fig. 3A). The percentage of DIG-associated MHC class II upon I-A^k engagement was estimated as 21% (Fig. 3C). Disruption of DIG integrity by cholesterol depletion using MβCD abrogated the recruitment of I-A^k to DIGs (Fig. 3A) so that only 8% of MHC class II localized to DIGs under these conditions (Fig. 3C). The detergent-insoluble sediment migrating to the bottom of the gradient contained only trace amounts of I-A^k and therefore was not further analyzed (data not shown).

SaI/A^k tr cells were analyzed under identical conditions (boiled and non-boiled). I-A^k was weakly expressed (7.3% of total MHC class II) in the DIG fractions of nonstimulated cells (Fig. 3B, lane 3). Recruitment of truncated I-A^k to DIGs via I-A^k stimulation was observed and the increased expression in fraction 3 corresponded to 22% of the total MHC class II (Fig. 3C), demonstrating that the truncation of the intracytoplasmic domains of the MHC class II molecules does not impair the recruitment of compact αβ dimers to DIGs.

Intracytoplasmic domains are required for I-A^k-mediated DIG-dependent actin reorganization

Cytoskeletal proteins have been attributed a role in the formation and maintenance of DIGs (Ebert et al., 2000; St Pierre and Watts, 1991). We therefore examined the effect of I-A^k-mediated signals on the actin cytoskeleton. We stimulated SaI/A^k and SaI/A^k tr cells with beads coated with anti-I-A^k mAb and directly examined polymerized actin (F-actin) by staining with phalloidin. In SaI/A^k cells, we observed rearrangements of the actin cytoskeleton revealed by the recruitment of actin to the precise site of I-A^k engagement (Fig. 4A, panel d; 62% of cells with actin relocalization). By contrast, stimulation via truncated I-A^k molecules failed to induce reorganization of the actin cytoskeleton since the distribution of actin was unchanged compared with cells that had been allowed to interact with beads coated with irrelevant mouse Igs (Fig. 4A, panel h versus j; 5% and 5.7% of cells with actin relocalization, respectively). The specificity of the

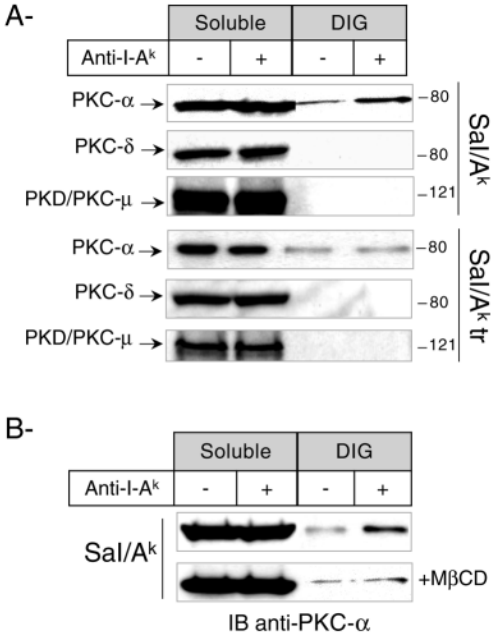


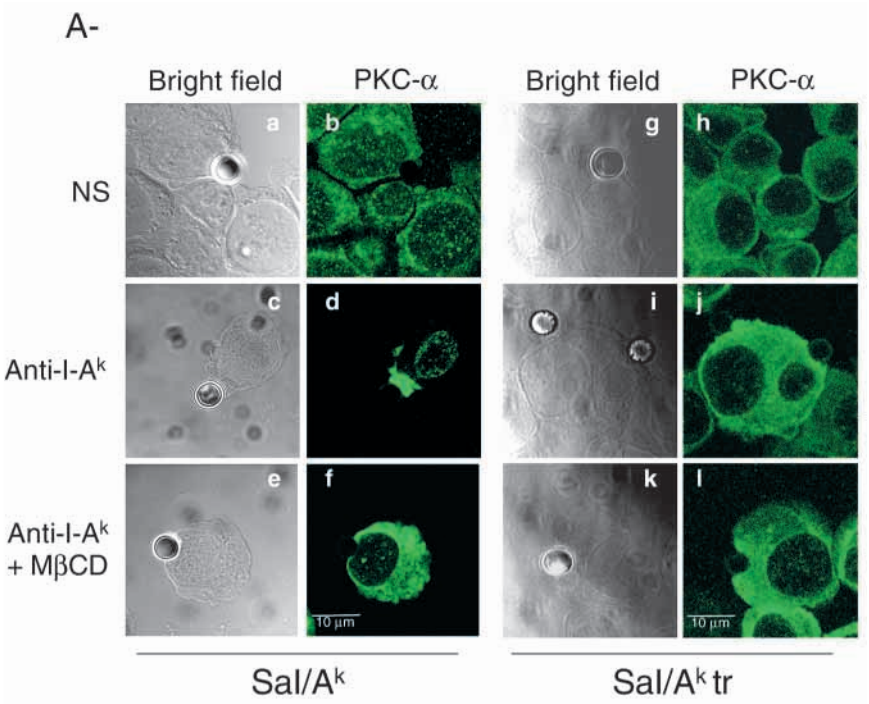
Fig. 5. I-A^k-mediated signaling in SaI/A^k cells induces recruitment of PKC-α into DIGs. (A) Soluble and DIG fractions from SaI/A^k and SaI/A^k tr cells, stimulated or not via I-A^k for 15 minutes, were immunoblotted with anti-PKC-α, -δ, -μ Abs. Only the PKC-α isoenzyme was detected in DIGs isolated from either SaI/A^k or SaI/A^k tr. (B) Cholesterol dependence of PKC-α recruitment was analyzed in SaI/A^k cells pretreated or not with MβCD for 15 minutes and stimulated or not via I-A^k for 15 minutes. Soluble and DIG fractions were immunoblotted (IB) with anti-PKC-α Ab. Pretreatment of SaI/A^k with MβCD completely abrogated PKC-α recruitment to DIGs.

I-A^k-mediated signaling was further confirmed by the absence of actin rearrangement when beads coated with irrelevant mouse Igs were allowed to interact with SaI/A^k (Fig. 4A, panel b; 11% of cells with actin relocalization). In addition, disruption of DIG integrity by MβCD pretreatment of SaI/A^k cells prior to I-A^k stimulation impaired actin reorganization, indicating that this event occurs in a DIG-dependent manner (Fig. 4A, panel f; 15.3% of cells with actin relocalization).

We next examined the impact of I-A^k signaling on the actin content of DIG fractions. Actin was constitutively present in the cholesterol-dependent lipid-rich fractions from SaI/A^k cells and was unchanged by stimulation via I-A^k (data not shown).

PKC-α is recruited to DIGs in response to I-A^k stimulation

The PKC isoenzyme family mediates signals via MHC class II (Brick-Ghannam et al., 1991; Cambier et al., 1987; Rich et al., 1997). SaI/A^k and SaI/A^k tr cells were screened for the expression of four PKC isoforms (α, βII, δ, μ). In both cell lines, PKC-α, δ and μ were readily detected, whereas PKC-βII was absent (data not shown). We therefore studied the distribution of the α, δ and μ PKC isoenzymes in DIGs. We found that only PKC-α was constitutively present in the DIG fractions from SaI/A^k cells and was actively recruited to this fraction by I-A^k stimulation (Fig. 5A). By contrast, while PKC-α was present in the DIG fraction of SaI/A^k tr,



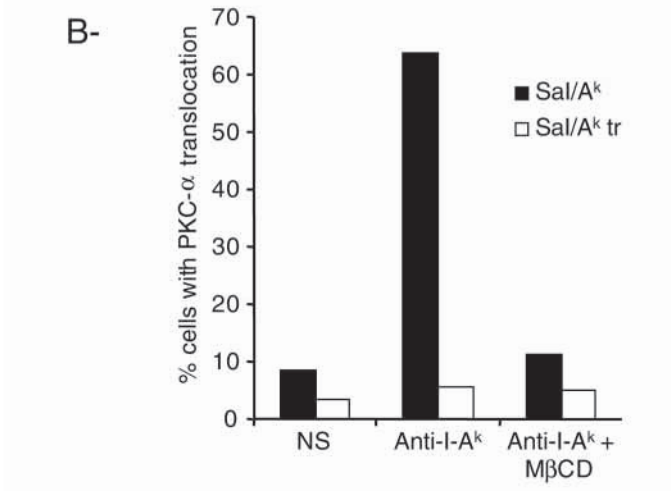


Fig. 6. Truncation of I-A^k prevents DIG-dependent PKC-α translocation to the site of I-A^k engagement. (A) SaI/A^k and SaI/A^k tr cells were incubated with 10.2.16-coated beads (Anti-I-A^k) or irrelevant mouse Ig-coated beads (nonstimulated, NS) for 30 minutes. Cells were cytospun, fixed and stained with anti-PKC-α antibody and revealed with a FITC-labeled secondary antibody. Bright-field images (a,c,e,g,i,k) indicate the site of interaction between the bead and the cell and the corresponding fluorescent images reveal the localization of PKC-α (b,d,f,h,j,l). A single cell-bead conjugate is shown in each panel. Cells pretreated with MβCD prior to I-A^k stimulation (Anti-I-A^k + MβCD) are indicated. (B) The percentage of cells undergoing PKC-α recruitment to the site of I-A^k engagement was determined. At least 200 cell-bead interactions from each condition were examined and cells in which PKC-α recruitment had occurred (to at least the same degree as shown in A, panel d) were counted as positive events. Data from one of three typical experiments is shown.

stimulation via I-A^k tr molecules did not lead to PKC-α recruitment. Neither PKC-δ nor PKC-μ were detected in the lipid-rich fractions of either SaI/A^k or SaI/A^k tr (Fig. 5A). DIG disruption by cholesterol depletion with MβCD reduced the constitutive expression and prevented the recruitment of PKC-α to DIGs of SaI/A^k cells (Fig. 5B). These data demonstrate that I-A^k signaling specifically relocalizes PKC-α to DIGs in SaI/A^k.

DIG-dependent PKC-α translocation to the site of I-A^k engagement requires the intracytoplasmic domains

Confocal microscopy was used to directly examine the impact of the I-A^k signal on PKC-α localization in SaI/A^k and SaI/A^k tr cells. PKC-α was specifically relocalized to the site of I-A^k engagement in comparison with the diffuse localization observed in nonstimulated cells (Fig. 6A, panel b and d; 8.6% and 63.6% of cells with PKC-α translocation, respectively). By contrast, stimulation of SaI/A^k tr did not modify the distribution of PKC-α in comparison with that observed in nonstimulated cells (Fig. 6A, panel h and j; 3.4% and 5.5% of cells with PKC-α translocation, respectively), demonstrating that the intracytoplasmic tails of the I-A^k molecule are required for PKC-α translocation to the site of I-A^k stimulation. Disruption of DIG integrity by MβCD pretreatment of SaI/A^k cells prior to I-A^k stimulation impaired PKC-α translocation, indicating that this event is DIG dependent (Fig. 6A, panel f; 11.4% of cells with PKC-α translocation).

DIG-recruited PKC-α is activated in response to I-A^k stimulation

Activation of PKC can be revealed by phosphorylation of a C-terminal residue (Keranen et al., 1995). We therefore used an Ab directed against PKC phosphorylated at this site to detect PKC activation. Phosphorylated PKC in DIG fractions from SaI/A^k and SaI/A^k tr cells corresponded to a single band with a molecular weight identical to that of PKC-α, and was increased in DIGs only from SaI/A^k cells as a consequence of I-A^k stimulation (Fig. 7A).

A time course analysis of PKC-α recruitment and PKC phosphorylation was performed on DIG fractions from SaI/A^k cells. I-A^k recruitment was characterized in parallel on the same extracts in order to compare the time course of recruitment. Constitutively DIG-localized PKC-α was enriched via I-A^k engagement from 1 minute up to at least 30 minutes as confirmed by densitometry (Fig. 7B). PKC phosphorylation in the DIG fractions increased following the same kinetics as PKC-α recruitment to the DIGs. However, the accumulation of I-A^k in DIGs was visible within 1 minute of stimulation and precedes both PKC-α accumulation and PKC activation (Fig. 7B). Phospho-PKC expression was examined in parallel in the soluble fractions and was not increased. This

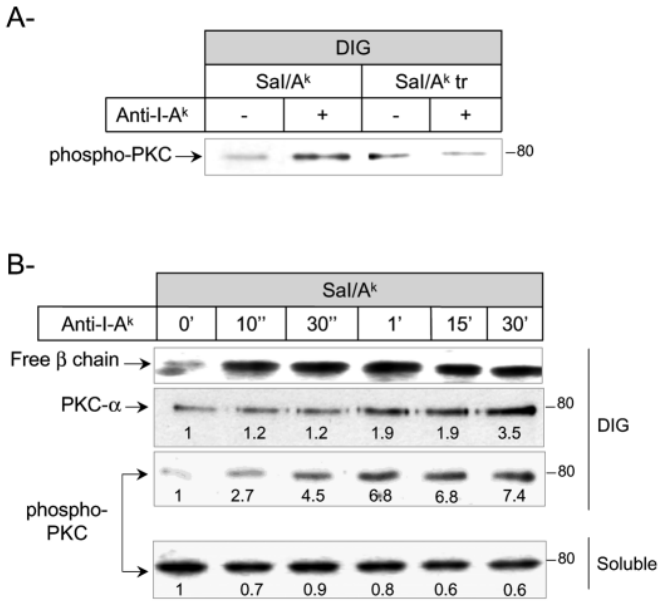


Fig. 7. PKC phosphorylation and time course of recruitment into DIGs. (A) Sal/A^k and Sal/A^k tr cells were stimulated or not via I-A^k for 15 minutes, lysed and fractionated on a sucrose gradient. DIG fractions were migrated on SDS-PAGE, transferred on PVDF and immunoblotted with the specific phospho-PKC Ab. (B) Kinetics of I-A^k recruitment, PKC-α and phospho-PKC recruitment to DIGs were analyzed in Sal/A^k cells. Sal/A^k cells were stimulated via I-A^k for the indicated times, lysed and fractionated on a sucrose gradient. DIG and soluble fractions were immunoblotted with the corresponding Abs for PKC-α and phospho-PKC, and western blots were analyzed by densitometric quantification. Time point 0 is arbitrarily set to 1 in both experiments. The time course of I-A^k recruitment to DIGs was determined by SDS-PAGE under boiled conditions followed by 10.2.16 mAb immunoblotting.

demonstrates that the enhanced expression of phospho-PKC in DIGs is not due to an overall increase in phospho-PKC expression.

MHC class II partitioning to DIGs is independent of MHC class II signaling and does not require integrity of the actin cytoskeleton

Some of the earliest events following MHC class II oligomerization include PKC activation (Cambier et al., 1987) and cytoskeletal reorganization. Disruption of the actin network has been previously shown to inhibit such signaling (St Pierre and Watts, 1991). We examined whether signal transduction was required for recruitment of I-A^k to DIGs. Pretreatment of Sal/A^k cells with the inhibitor of actin polymerization cytochalasin D, and the broad-spectrum PKC inhibitor calphostin C, did not affect recruitment of I-A^k to DIGs induced by I-A^k engagement (Fig. 8A).

The disruption of the cytoskeletal integrity and the inhibition of PKC activation was demonstrated by confocal microscopy. Actin recruitment and PKC-α translocation induced via I-A^k were severely impaired by cytochalasin D and by calphostin C pretreatments, respectively (Fig. 8B,C). Taken together with the relocalization of I-A^k tr to DIGs following engagement by anti-I-A^k mAb (Fig. 3), these data confirm that the recruitment of MHC class II molecules to DIGs is independent of MHC class II-mediated signaling.

Discussion

This study describes the signaling function of MHC class II molecules expressed in a solid tumor and orders the molecular events following engagement of these molecules. This is the first report of MHC class II molecule recruitment and PKC-α activation in a lipid-raft-dependent manner, and reveals that the intracytoplasmic tail is not required for the recruitment of MHC class II molecules to lipid rafts.

Expression of I-A^k in the murine SaI sarcoma was shown to be necessary and sufficient to provoke protection against subsequent inoculation with the wild-type tumor (Ostrand-Rosenberg et al., 1990). The arguments supporting a role for I-A^k signaling in the tumor rejection response are as follows: (1) I-A^k molecules truncated in the intracytoplasmic tail do not provide protection against tumor inoculation (Ostrand-Rosenberg et al., 1991); (2) full-length I-A^k is required for expression of costimulatory molecules by SaI/A^k in vivo (Ostrand-Rosenberg et al., 1996); and (3) SaI/A^k presents cytosolically expressed antigens and therefore potentially presents cytosolic tumor antigens (Armstrong et al., 1997; Armstrong et al., 1998; Qi et al., 2000).

The present data demonstrate that, in addition to its described role in antigen presentation (Armstrong et al., 1997; Armstrong et al., 1998), the MHC class II molecule I-A^k in a murine sarcoma acts as a signal-transducing molecule. I-A^k oligomerization leads to recruitment of MHC class II molecules and PKC-α to DIGs, as well as to PKC-α activation and reorganization of the actin cytoskeleton in a DIG-dependent manner. The proportion of the total cellular protein that localizes to lipid-rich microdomains has been estimated at ~2.5% [(Drevot et al., 2002) and our personal observations]. As such, the recruitment of a given protein to lipid-rich microdomains actually represents a major partitioning to such microdomains. We have quantified the proportion of I-A^k localizing in lipid rafts post-ligand binding as 20%; this figure is consistent with estimations made for diverse immunoreceptors (Anderson et al., 2000; Chung et al., 2001; Drevot et al., 2002). The mechanism of limiting the abundance of a given protein within lipid-rich microdomains has not yet been clarified.

Data from previous studies indicate that signal transduction and antigen presentation via MHC class II molecules can be intimately associated. Nabavi et al. reported that truncation of MHC class II molecules severely impaired PKC activation in B cells and abrogated antigen presentation to certain T-cell clones (Nabavi et al., 1989). The role of the intracytoplasmic tail in signaling via MHC class II molecules was revealed by several studies (Harton et al., 1995; Rich et al., 1997). Truncation of the intracytoplasmic domain of the β chain of I-A^b abrogated cAMP elevation and antigen-dependent Ig production (Harton et al., 1995). Truncation of the β chain of HLA-DR impaired activation of PKC-α and -βII (Rich et al., 1997). The present study demonstrates for the first time that, although the intracytoplasmic domains of the MHC class II molecules are not required for this recruitment to DIGs, they are essential for further PKC-α recruitment to, and activation within, lipid rafts. Activated PKC recruitment to cholesterol-

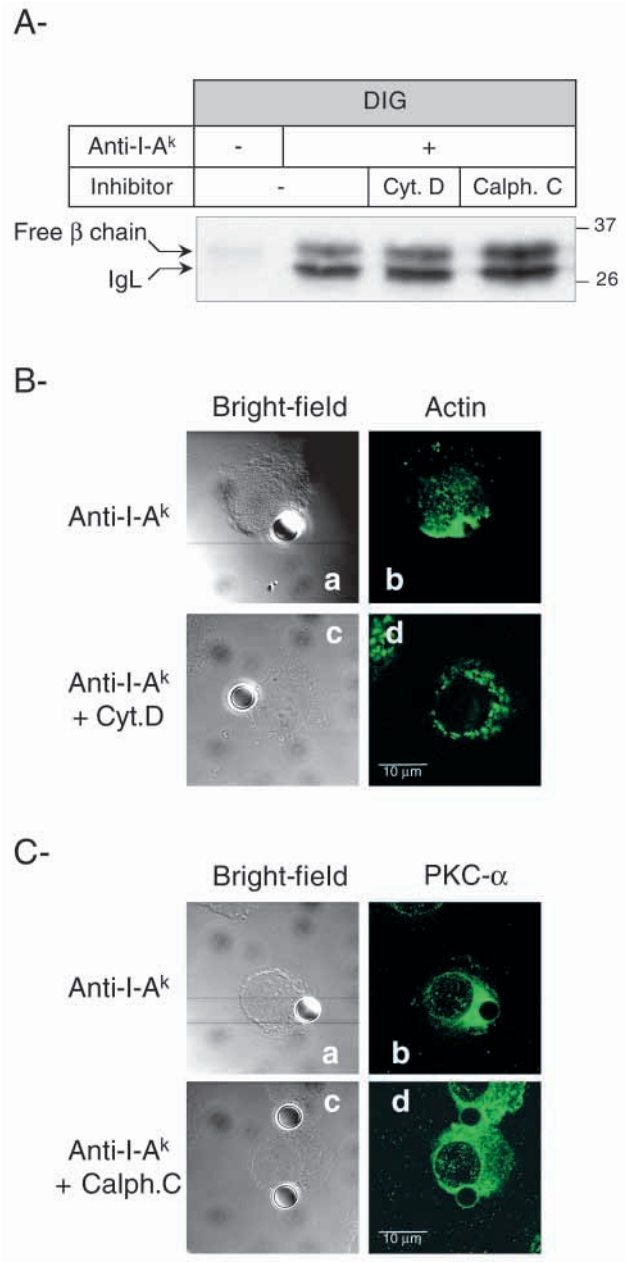


Fig. 8. Recruitment of MHC class II molecules to DIGs is independent of MHC class II-mediated signaling. (A) SaI/A^k cells were pretreated with the inhibitor of actin polymerization cytochalasin D (Cyt.D), or with the PKC inhibitor calphostin C (Calph.C) prior to engagement of I-A^k for 15 minutes at 37°C. I-A^k recruitment to the DIG-containing fraction isolated from SaI/A^k was unchanged in the presence of the above inhibitors as detected by immunoblotting. (B) The effect of cytochalasin D on actin reorganization induced by I-A^k engagement was revealed by confocal microscopy of cell-bead conjugates. Panels a and b represent, respectively, bright-field imaging and Alexa488-phalloidin staining of SaI/A^k cells stimulated with 10.2.16-coated beads. Panels c and d represent, respectively, bright-field imaging and Alexa488-phalloidin staining of SaI/A^k cells pretreated with Cyt.D prior to 10.2.16-coated beads stimulation. (C) The effect of calphostin C on PKC-α recruitment induced by I-A^k engagement was revealed by confocal microscopy of cell-bead conjugates. Panels a and b represent, respectively, bright-field imaging and PKC-α staining of SaI/A^k cells stimulated with 10.2.16-coated beads. Panels c and d represent, respectively, bright-field imaging and PKC-α staining of SaI/A^k cells pretreated with Calph.C prior to 10.2.16-coated beads stimulation.

dependent DIGs is believed to be due to the localization of major PKC substrates within such microdomains. GAP 43, myristoylated C kinase substrates (MARCKS) and CAP 28, collectively termed GMC, are membrane associated by acylation (myristoylation or palmitoylation) and are enriched in Triton X-100-insoluble lipid rafts (Laux et al., 2000). The data presented here shows that signaling is not required for the initial recruitment of I-A^k to lipid microdomains and that signaling commences within DIGs.

The cytoskeletal integrity of the APC has been shown to be important for the efficiency of antigen presentation (Barois et al., 1998; St-Pierre and Watts, 1991). Treatment of the APC with cytochalasin A (which impairs elongation of actin filaments and receptor capping) completely inhibited MHC class II-mediated activation of certain T-cell hybridomas (St Pierre and Watts, 1991). Cytoskeletal reorganization in the APC was therefore proposed to be important for the activation of T cells requiring signals from accessory molecules. This notion is strongly reinforced by the data presented in this study, which demonstrate that the MHC class II-mediated signal specifically reorganizes the APC actin cytoskeleton at the site of I-A^k engagement. Furthermore, lipid raft disruption experiments clearly revealed the DIG dependence of the actin cytoskeleton reorganization. This dynamic reorganization of F-actin at the site of I-A^k stimulation reveals a qualitative modification that was independent of the actin content of DIGs.

MHC class II molecules are generally considered as 60 kDa heterodimers that can be observed under different conformations including: (1) HMW multimers (Roucard et al., 1996); (2) compact dimers, which have bound peptide of high affinity; (3) floppy dimers, which are believed to have 'loosely' bound peptide (Sadegh-Nasseri and Germain, 1991); and (4) empty MHC class II molecules (Ericson et al., 1994; Roucard et al., 1996; Santambrogio et al., 1999). It was therefore important to determine the conformation of the MHC class II molecules recruited to DIGs. The present study reveals that engagement of either I-A^k or I-A^k tr resulted in recruitment of SDS-stable MHC class II molecules to DIGs, both as HMW complexes and as dimers. This model does not allow us to determine whether one or other conformation of I-A^k is preferentially recruited to DIGs since the 10.2.16 Ab recognizes both. However, the difference in the signaling response was not therefore due to the absence of one or other form from SaI/A^k tr.

Anderson et al. reported that DIG disruption impaired antigen presentation under conditions of limited peptide availability (Anderson et al., 2000), whereas Kropshofer et al. did not find that disruption of lipid-rich microdomains perturbed antigen presentation (Kropshofer et al., 2002). However, neither of the previous studies examined the impact of DIG localization on the signaling function of MHC class II. A previous study that did address this point focused on the recruitment and activation of tyrosine kinases after Ab-mediated recruitment of MHC class II to lipid rafts in myeloid APCs (Huby et al., 1999). Our data show that the immediate

recruitment of MHC class II to PKC-α-containing DIGs ensures that signaling can be readily triggered. Inhibition of PKC activation completely abrogated recruitment of PKC-α to the site of I-A^k engagement without perturbing enrichment of I-A^k in the lipid rafts. Signaling is therefore not required for the relocalization of MHC class II to lipid rafts, as our data indicate that it is the localization of the MHC class II molecules in lipid rafts that triggers signaling. This argument is supported by the lack of impact of I-A^k truncation on the I-A^k recruitment to the lipid raft fraction and is similar to the observation that signaling via the B-cell receptor (BCR) originates within lipid microdomains rather than is a prerequisite for BCR relocalization to DIGs (Cheng et al., 2001).

Distribution of MHC class II in lipid-rich microdomains has, as yet, only been described in professional APCs including monocytes, B cells and dendritic cells (Anderson et al., 2000; Huby et al., 1999; Setterblad et al., 2001). A recent study reported the accumulation of MHC class II-peptide complexes in the contact zone formed by APC–T-cell interaction initiated by an agonist MHC class II-peptide complex (Wulfing et al., 2002). The accumulated complexes included non-agonist MHC class II-peptide complexes that were entitled 'accessory ligands' since they were shown to enhance specific T-cell activation. In addition, lipid-rich microdomains were shown to localize to the site of APC–T-cell interaction (Bi et al., 2001). In the present tumor cell model, enrichment of I-A^k molecules in DIGs mediated by I-A^k engagement could reflect an accumulation of 'accessory ligands'. The initiation of intracellular signaling in the sarcoma as a direct consequence of I-A^k recruitment to DIGs could therefore actively contribute to the antitumoral immune response. Furthermore, MHC class II mAbs are currently under study as therapeutic tools for the treatment of lymphoid malignancies (Nagy et al., 2002). The data presented in the current study reveal that the MHC class II antigens should also be considered as potential targets for mAb therapy of solid tumors.

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