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Early Human T Cell Activation Events With Engagement of Surface MHC Class II

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Abstract Major histocompatibility complex (MHC) class II are expressed on most activated human lymphocytes. They direct antigen presentation events in dendritic cells and B cells (collectively called antigen presenting cells), but the role for MHC class II in human T cells is not well understood. To understand the role of surface MHC class II and to identify the molecules involved in signaling, we have defined the early activation sequence in T cells when MHC class II are engaged by a specific antibody. Specifically, we have characterized the involvement of phosphotyrosine kinases, phospholipase C (PLC), and Ca^{2+} mobilization. With the engagement by either whole anti-class II antibody or its Fab fragments, the enzymatic activity of $p56^{lck}$ and ZAP-70 increased, but there was no increase in $p59^{fyn}$ activity. In addition, the intracellular free Ca^{2+} increased, which was due to enhanced influx and not to the mobilization of intracytoplasmic Ca^{2+}. These events did not require cross-linking because they were not significantly augmented by the addition of antispecies antibody. The coimmunoprecipitation of tyrosine phosphorylated PLC-γ1 with surface MHC class II suggested that PLC-γ1 could be recruited to MHC class II after engagement. These results show the complexities of the early signals transduced by the engagement of surface MHC class II on T cells. J. Cell. Biochem. 70:346–353, 1998. © 1998 Wiley-Liss, Inc.

Key words: MHC class II; T-helper cells; phosphotyrosine kinase; phospholipase C-γ1

Among the species, only activated human T lymphocytes express major histocompatibility complex (MHC) class II on the cell surface [Pantaleo et al., 1993], but their contribution to the regulation of the immune response is not completely characterized. Because T cells do not possess an antigen capture mechanism comparable to surface IgM on B cells, it is unlikely that the main function of their surface MHC class II involves antigen presentation. In an antigen presenting cell (APC), MHC class II molecules bind an antigenic fragment of a foreign peptide in an intracellular compartment for transport to the surface, where the complex is recognized by the T cell receptor (TCR) from $CD4^+$ T cells [Amigorena et al., 1994, 1995; Germain, 1994; Qiu et al., 1994]. In addition, MHC class II molecules transduce signals to activate APC when MHC class II are cross-linked by a specific antibody [St. Pierre et al., 1989], which is thought to mimic TCR binding [Faassen and Pierce, 1995]. The activation sequence, which results in the increased expression of costimulatory and adhesion molecules, enables the cooperation between T cells and B cells in their response to an immunogen [Clark and Ledbetter, 1994].

The anti-class II cross-linking involved a cascade of protein tyrosine phosphorylation, phosphatidylinositol turnover, and an increase in cytosolic Ca^{2+} in human B and T cells [Lane et al., 1990; Mooney et al., 1990; Odum et al., 1991a,b]. In B cells, the MHC class II β-chain cytoplasmic domain transduced the stimulus from the cross-linking of MHC class II [Wade et al., 1991] to initiate the tyrosine kinase-dependent activation of phospholipase C [Mooney et al., 1990; Cambier et al., 1991; Odum et al., 1991a,b; Wade et al., 1991]. However, it was not clear whether phospholipase C (PLC) formed a complex with surface MHC class II with engage-

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ment of class II. Despite the involvement of these activation events, the engagement of MHC class II by a soluble specific antibody leads to negative signaling, whereas immobilized antibody usually leads to positive signaling [Moretta et al., 1982; Vaickus et al., 1989; Racioppi et al., 1990]. In addition, early T-cell activation genes such as c-myc and c-fos were not affected by anti-class II monoclonal antibody (mAb), whereas late activation genes such as c-myb and N-ras were strongly diminished [Racioppi et al., 1990].

We have characterized the early intracellular events that associated with the engagement of MHC class II on T cells. MHC class II signaling involved phosphotyrosine kinases (PTKs), PLC-γ1, and Ca^{2+} influx. There seemed to be no requirement for cross-linking, at least during the immediate time period after the engagement of surface MHC class II, probably because the naturally occurring form of surface MHC class II was already a dimer of dimers.

MATERIALS AND METHODS

Antibodies and Reagents

An anti-MHC class II, 9.3F10 (IgG2a) [Van Voorhis et al., 1983], was purified by protein G agarose (Pharmacia Biotech, Uppsala, Sweden) affinity column chromatography from the culture supernatant of HB 180 hybridoma (ATCC, Rockville, MD). The mAb was eluted with pH 3.6 Na acetate buffer and immediately neutralized to pH 7.2. Anti-PLC-γ1 mAb, 4G10 mAb against phosphotyrosine, and polyclonal antisera against human $p56^{lck}$, $p59^{fyn}$, and ZAP-70 were purchased from UB Inc (Lake Placid, NY). Tetanus toxoid (TT) was a gift from Wyeth-Ayers (Marietta, PA). Goat anti-mouse immunoglobulin (GAMIg) was purchased from Jackson Immuno-Research Labs (West Grove, PA) and was used at 25 μg/ml.

Fab Preparation

Anti-class II mAb was digested by papain, as described elsewhere [Good et al., 1980]. Briefly, 1–5 mg/ml of mAb were digested in freshly prepared papain in 0.01 M EDTA, 0.06 M 2-ME, 0.05 M cysteine-HCl, pH 6.2, at 37°C for 4–6 h. Digestion was terminated by the addition of 75 mM iodoacetamide at room temperature for 30 min. Whole IgG and Fc portions were depleted by protein A/G. Digested products were separated by Sephadex G 100 (Pharmacia Biotech) chromatography and assayed by sodium dodecylsulfate polyacrylamide get electrophoresis (SDS-PAGE) under nonreducing conditions.

Preparation and Maintenance of T-Cell Lines and Clones Specific for TT

Jurkat cell lines were obtained from ATCC and cultured in RPMI 10% fetal calf serum. Peripheral blood T cells were obtained from peripheral blood mononuclear cells (PBMCs) of donors with informed consent by adherence to plastic culture dish and by nylon wool selection. The starting cell population was usually 10% MHC class II^+ and 97% $CD3^+$ by flow cytometry (results not shown). Human T-cell clones (TCCs) specific for TT were obtained by the limiting dilution technique. Briefly, PBMC were isolated from heparinized blood of a recently vaccinated donor (HLA-DR5/DR w8) after the removal of monocytes by adherence to a plastic culture dish. T-cell lines are established by culturing nonadherent, 10^6 PBMCs/ml in 20 μg/ml TT for 7–10 days in complete medium (RPMI 1640, 2 mM L-glutamine and 10 mM HEPES), followed by 7–10 days of incubation with 15 units/ml human rIL-2. The T cells were isolated by centrifugation with lymphocyte separation medium. The cells were restimulated with TT and irradiated PBMC. TT-specific T clones were obtained by limiting dilution from TT-specific T cell lines and stimulated by autologous PBMC presentation of whole TT.

Immunoprecipitation

Five million cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% digitonin, aprotinin, 1 mM phenyl methyl sulfonyl fluoride, 1 mM Na orthovanadate), and postnuclear supernatant was subjected to immunoprecipitation with 1–2 μg of specific antibody, as described previously [Hubert et al., 1993]. Immunoprecipitated proteins were recovered by incubation with 100 μl of protein A (PA)–Sepharose beads at 4°C overnight and washed three times in lysis buffer. The proteins were eluted, dissolved by 2 min of boiling in SDS-sample buffer, and analyzed by 10% SDS-PAGE.

The coimmunoprecipitation experiment was done as described elsewhere [Sieh et al., 1994].

Briefly, anti-class II mAb 9.3F10 was incubated with live TCC at 37°C for 4 min, washed, and lysed in lysis buffer. Cell lysate was centrifuged at 100,000g for 30 min, and postnuclear membranes were incubated with PA-Sepharose beads (Sigma, St. Louis, MO) for 16 h at 4°C. Immunoprecipitated proteins were separated by gel electrophoresis. Protein bands were blotted onto nitrocellulose membrane and detected by a specific antibody in Western blot (WB) assays.

WB Analysis

Protein bands from SDS-PAGE were transferred to nitrocellulose membrane and detected by a specific antibody or an antispecies antibody covalently linked to horseradish peroxidase if the primary antibody was not labeled. After the membrane was incubated with ECL (Amersham, Arlington Heights, IL), bands were visualized on Kodak XOmat AR film (Rochester, NY).

Measurement of Intracellular Ca^{2+} ($[Ca^{2+}]_i$) in Cell Suspension

$[Ca^{2+}]_i$ was measured in cells loaded with the fluorescent calcium indicator fura-2 AM (CalBiochem, San Diego, CA), as described previously [Malgaroli et al., 1987]. Twenty million cells per milliliter were incubated for 30 min at 37°C in 5 μM fura-2 AM in RPM1 1640 containing 1% fetal bovine serum. Cells were washed and spun in a microcentrifuge. Immediately before experiments, cells were resuspended to 2 ml at 3 × 106/ml in a medium containing 130 mM NaCl, 10 mM HEPES, 5mM KCl, 1 mM $MgCl^2$, 0.44 mM KH^2PO^4, 4.2 mM $NaHCO^3$, 0.4 mM $CaCl^2$, and 10 mM glucose, pH 7.4. Twenty-five micrograms per milliliter of anti-class II mAb or its Fab fragment were added to cell suspension. $[Ca^{2+}]_i$ levels were measured by a Perkin-Elmer LS 50B luminescence spectrometer (Oak Brook, IL) at two excitation wave lengths, 340 nm and 380 nm, and an emission wave length of 510 nm after calibration according to Grynkiewicz et al. [1985].

Assay for PTK Activity

Human $p56^{lck}$, $p59^{fyn}$, and ZAP-70 were immunoprecipitated from cell lysate (5 × 10^6 cells/immunoprecipitation) by specific antibody PA-Sepharose immunoabsorbent beads. Normal mouse IgG was used as a negative control. The positive control was anti-CD4 for $p56^{lck}$, W6–32 anti-MHC class I mAb for ZAP 70 [Skov et al., 1997], phorbol 12-myristate 13 acetate (PMA) 50 ng/ml and phytohemagglutinin (PHA) 2 μg/ml treatment for 1 h (PMA + PHA) for $p59^{fyn}$. PTK activity was assayed by the SignaTECT™ Protein Tyrosine Kinase Assay System kit (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, two biotinylated substrate peptides were reacted with immunoprecipitated PTK in the presence of excess [γ^{32}P]-ATP. The excess free [γ^{32}P] ATP was removed by washing, and the reaction was spotted onto a SAM^{2}™ Biotin Capture Membrane. The membrane was counted in a beta counter gated for [γ^{32}-P].

RESULTS

Effects of the Engagement by Anti-Class II mAb and Fab on the Activity of PTKs

To define the effects of the engagement of surface MHC class II on early activation events, we determined the enzymatic activities of the PTK $p56^{lck}$, ZAP-70, and $p59^{fyn}$ in T cells after incubation with 9.3F10 mAb and its Fab for 5 min. T cells were lysed, and postnuclear cell lysate was immunoprecipitated with anti-$p56^{lck}$, anti-ZAP-70, and anti-$p59^{fyn}$. Immunoprecipitated PTKs were assayed for their ability to phosphorylate tyrosine residues from substrate peptides. Peripheral T cells preincubated with either 9.3F10 or its Fab fragments showed, as compared with cells incubated with normal mouse IgG, an increase in activity of $p56^{lck}$ (Fig. 1A) and ZAP-70 (Fig. 1B) but not of $p59^{fyn}$ (Fig. 1C). These increases in PTK activity, however, were lower than those associated with anti-CD4, anti-MHC class I, and the treatment of cells with PMA + PHA. Cross-linking by goat anti-mouse IgG did not significantly augment PTK activity in cells treated with 9.3F10 or its fragments but caused marked increase in cells treated with anti-CD4 (Fig. 1A).

PLC-γ1 Coimmunoprecipitated With Surface MHC Class II

Because PTKs have been involved in transducing signals from the stimulated TCR, CD4, and CD8 [Chan et al., 1992; Ley et al., 1994], we wanted to determine whether any of the PTK or PLC-γ1 formed a complex with MHC class II. Antibodies specific to $p56^{lck}$, $p59^{fyn}$, ZAP-70, and PLC-γ1 were used in WB analysis of proteins

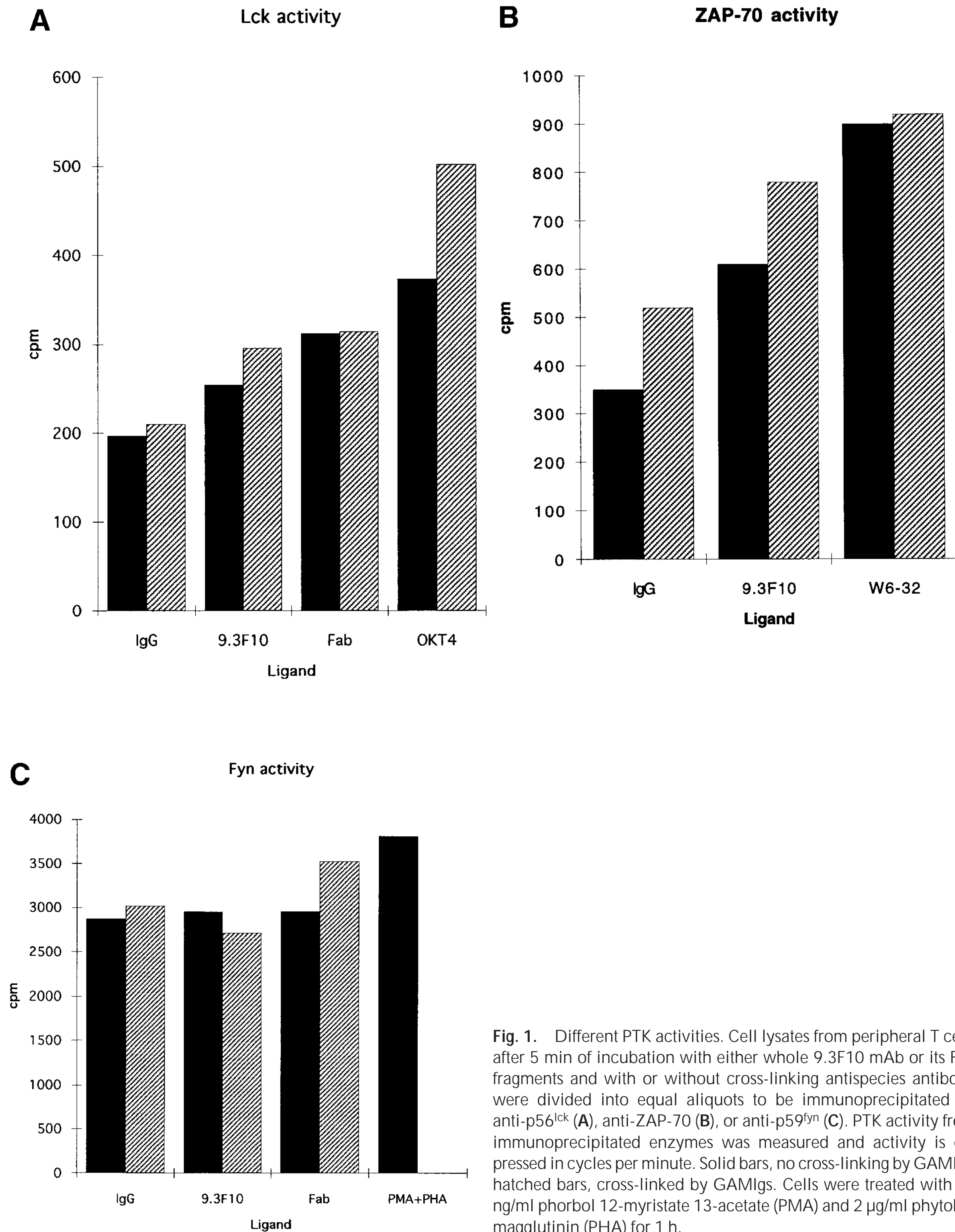

Fig. 1. Different PTK activities. Cell lysates from peripheral T cells after 5 min of incubation with either whole 9.3F10 mAb or its Fab fragments and with or without cross-linking antispecies antibody were divided into equal aliquots to be immunoprecipitated by anti-p56^{lck} (**A**), anti-ZAP-70 (**B**), or anti-p59^{fyn} (**C**). PTK activity from immunoprecipitated enzymes was measured and activity is expressed in cycles per minute. Solid bars, no cross-linking by GAMIgs; hatched bars, cross-linked by GAMIgs. Cells were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 2 μg/ml phytohemagglutinin (PHA) for 1 h.

coimmunoprecipitated with surface MHC class II on activated T cells. In the Jurkat T cell line and in activated human T cells, only PLC-γ1 coimmunoprecipitated with surface MHC class II. In addition, the coimmunoprecipitated PLC-γ1 from activated T cells was tyrosine phosphorylated (Fig. 2).

In contrast, we found no association between surface MHC class II and p56^{lck}, p59^{fyn}, or ZAP-70 by this technique (results not shown).

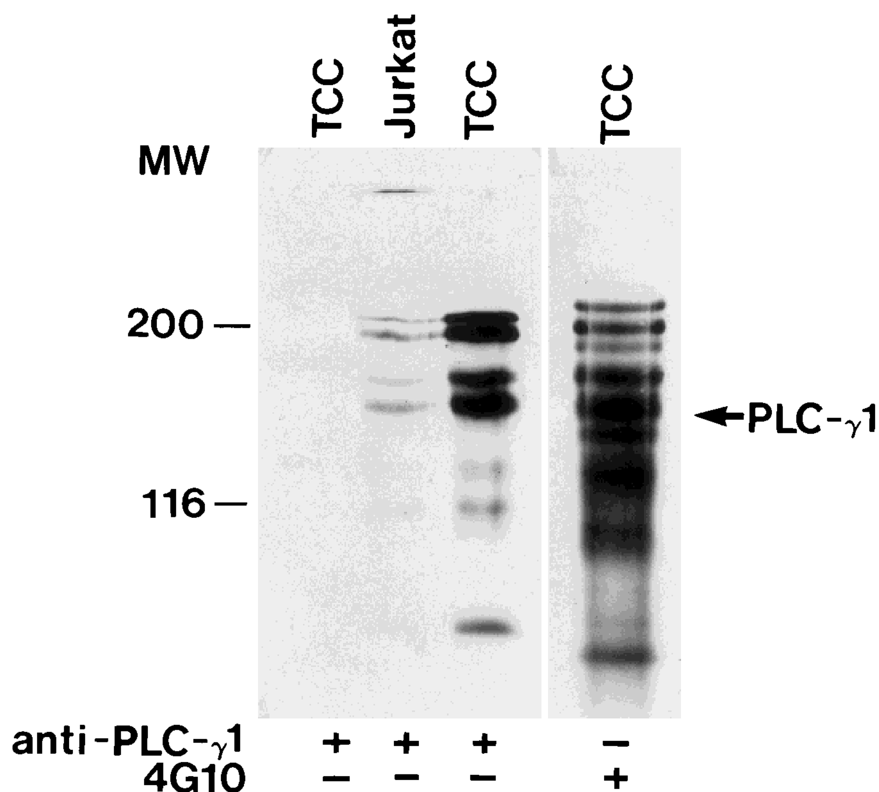

Fig. 2. Tyrosine phosphorylated PLC-γ1 coimmunoprecipitated with surface MHC class II. Jurkat T cells and 18-h PHA-activated TCCs were reacted with 9.3F10 mAb for 4 min and lysed, and immune complex was captured by the addition of PA-Sepharose beads. Immunoprecipitated proteins were separated by SDS-PAGE, blotted to nitrocellulose membrane, and probed with anti-PLC-γ1 mAb or 4G10. MW, molecular weight. **Lane 1:** TCC without 9.3F10. **Lane 2:** Jurkat cells incubated with 9.3F10. **Lane 3:** TCC incubated with 9.3F10. **Lane 4:** TCC incubated with 9.3F10 and Western blotted with 4G10.

Effect of the Engagement of MHC Class II on $[Ca^{2+}]_i$ Flux

To elucidate the pathway of activation that involved PLC-γ1 and other calcium-dependent kinases, we measured the $[Ca^{2+}]_i$ flux in activated human T cells after the engagement of their surface MHC class II. The level of $[Ca^{2+}]_i$ was significantly increased seconds after 9.3F10 and Fab fragments were added to fura-2 charged cells. Anti-CD3-treated cells showed a lesser increase in $[Ca^{2+}]_i$, which was significantly augmented after cross-linking. Cross-linking by GAMIgs did not further increase the $[Ca^{2+}]_i$ levels in cells treated with anti-class II mAb and its Fab fragments (Fig. 3). When culture medium was made without supplemental Ca^{2+} (Ca^{2+}-free medium curve; Fig. 3), similar cell treatments did not involve an augmentation of $[Ca^{2+}]_i$, suggesting that the increase in $[Ca^{2+}]_i$ was due mainly to the influx of calcium.

DISCUSSION

Within a few minutes of an engagement of surface MHC class II on T cells, several early activation events took place. There was an increase in activity of lck and ZAP-70, but fyn activity was unchanged. Phosphorylated PLC-γ1 was in a complex with surface MHC class II, and calcium influx occurred.

MHC class II molecules, which are capable of transducing signal through its transmembranous β-chain domain [St. Pierre et al., 1989], belong to the Ig supergene family. Other members such as CD4, CD8, FcR, sIg, and TCR have been associated with src-type PTKs lck, fyn, and ZAP-70 [Rudd et al., 1996]. Neither PTK nor PLC, however, has been directly associated with MHC class II. The src-family PTKs play major roles in T-cell activation [reviewed in Chan et al., 1994]. Fyn associates with the TCR ζ chain through its N-terminal region [Samel-

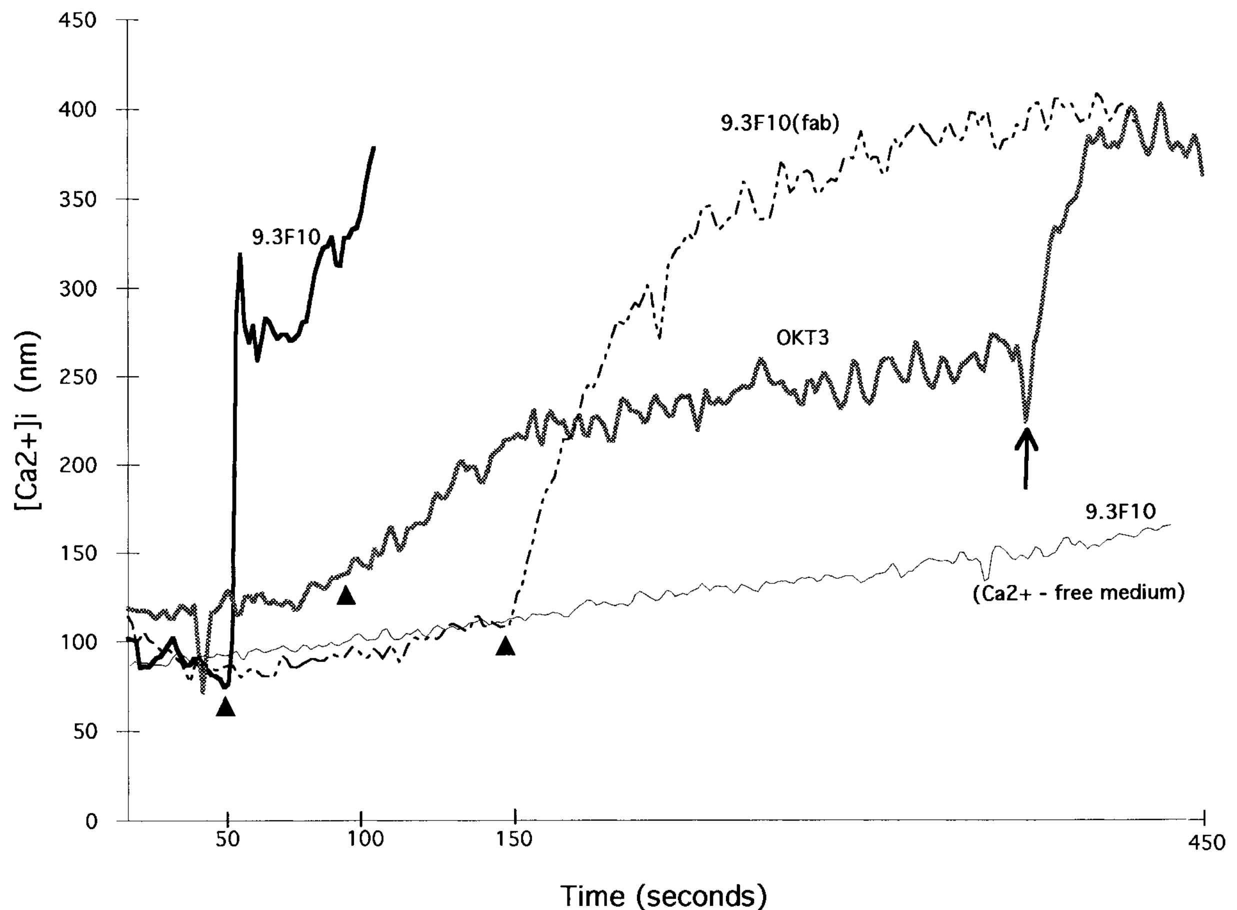

Fig. 3. Intracellular free calcium was increased after surface engagement of MHC class II. $[Ca^{2+}]_i$ concentration was significantly increased in cells incubated with 9.3F10 and its Fab fragments. The addition of goat anti-mouse IgGs to cross-link further increased $[Ca^{2+}]_i$ in cells incubated with OKT3 but not in cells incubated with anti-MHC class II antibodies. There was no rise in $[Ca^{2+}]_i$ when calcium-free medium was used. Arrowheads indicate the time an antibody was added to cell suspension; arrow indicates the time GAMIgs were added for cross-linking. (Ca^{2+}-free medium) denotes the $[Ca^{2+}]_i$ level in the experiment when using medium without Ca^{2+}: 9.3F10 was added at 50 s and GAMIgs were added at 150 s.

son et al., 1990; Samelson and Klausner, 1992; Sarosi et al., 1992], whereas lck associates with CD4 and CD8 through a noncovalent interaction with cysteine residues CXCP of CD4 and CD8 and its N-terminal region [Rudd et al., 1988; Veillette et al., 1988; Turner et al., 1990]. Our data showed that the engagement of MHC class II augmented lck and ZAP-70 but not fyn activity. This discrimination is probably related to specific roles that fyn and lck play in T cells. Both fyn and lck were involved with thymocyte development, but whereas fyn was not necessary for TCR-mediated proliferation [Samelson et al., 1990; Sarosi et al., 1992], lck played an important role in it [Rudd et al., 1988; Veillette et al., 1988]. How do MHC class II molecules associate with PTKs? The cytoplasmic sections of β chain (IYFRNQKGHSGLQPTGFLS) and α chain (FIIKGVRKSNAAERRGPL) [Pinet et al., 1995] do not possess the immunoreceptor tyrosine-based activation motif (ITAM) or the CXCP sequence to allow for the binding to ZAP-70 or lck [Turner et al., 1990; Chan et al., 1994]. The exact mechanism of this is unclear, but we favor one of the following possibilities. First, the SH2 domain of lck and ZAP-70 could bind to the phosphorylated tyrosine of the β chain. Second, MHC class II molecules could form a functional linkage with interleukin-2R (IL-2R) [Odum et al., 1993]. IL2-R signal transduction involved $p56^{lck}$, which associated with the acidic region of IL-2Rb through its kinase domain [Hatakeyama et al., 1991]. Human $p56^{lck}$, once activated, induces the tyrosine phosphorylation of specific substrate such as PLC-γ1 [Jacques et al., 1996]. The SH2 domain of

PLC-γ1 could bind to β chains in a manner similar to its binding to the receptors for epidermal growth factor, platelet-derived growth factor, $p56^{lck}$, and CD3 [Margolis et al., 1990; Kim et al., 1991; Dasgupta et al., 1992; Weber et al., 1992] after autophosphorylation of the receptors [Anderson et al., 1990]. The MHC class II β chain was phosphorylated near the carboxyl terminus [Kaufman and Strominger, 1979] and could bind directly to PLC-γ1. In addition, SH2–SH3 adapter proteins (e.g., Grb-2, Shc, and Crk) could link MHC class II to the PTK, PLC, or Ras signaling pathways [Clark and Brugge, 1995]. The activity of PLC-γ1 mobilizes intracellular Ca^{2+}, which correlated with our results showing an increase in $[Ca^{2+}]_i$ after the engagement of surface MHC class II. However, most of the Ca^{2+} ions seem to have come from the extracellular solution, which is consistent with the observations on calcium fluxes in human activated T cells and Jurkat cells reported by Donnadieu et al. [1992].

The cross-link of MHC class II on the T-cell surface does not seem to be necessary for immediate signal transduction because the increase in PTK activity mediated by monovalent binding was not significantly enhanced by cross-linking. Cross-linking antibody also did not augment the $[Ca^{2+}]_i$ influx associated with the engagement of surface MHC class II. A three-dimensional crystographic study showed that MHC class II existed in dimer of dimers [Brown et al., 1993; Ploegh and Benaroch, 1993]. Our data suggest that surface MHC class II, by existing in dimer-of-dimers form, was already primed to transduce signal and only required monovalent engagement for early activation. MHC class II comprises binding sites for CD4, superantigens, TCR, and antigenic peptides. The engagement of any of these sites by soluble products from lysed pathogens and activated cells may happen more frequently during an immune response to infectious agents. Surface MHC class II on activated T cells may serve as a target for various ligands whose immunoregulatory role could begin with the involvement of specific PTKs, PLC-γ1, and calcium influx.

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