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T Cell Antigen Receptor–Mediated Activation of Phospholipase C Requires Tyrosine Phosphorylation

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Triggering of the antigen-specific T cell receptor–CD3 complex (TCR-CD3) stimulates a rapid phospholipase C–mediated hydrolysis of inositol phospholipids, resulting in the production of second messengers and in T cell activation and proliferation. The role of tyrosine phosphorylation in these events was investigated with a tyrosine protein kinase (TPK) inhibitor, genistein. At doses that inhibited TPK activity and tyrosine phosphorylation of the TCR ζ subunit, but not phospholipase C activity, genistein prevented TCR-CD3–mediated phospholipase C activation, interleukin-2 receptor expression, and T cell proliferation. These findings indicate that tyrosine phosphorylation is an early and critical event that most likely precedes, and is a prerequisite for, inositol phospholipid breakdown during receptor-mediated T cell activation.

ACTIVATION OF T LYMPHOCYTES BY ligands of the TCR-CD3 complex is associated with phosphoinositide-specific phospholipase C (PI-PLC)–mediated breakdown of inositol phospholipids; this gives rise to two second messengers, namely, inositol phosphates and diacylglycerol (*1*). The resulting increase in intracellular Ca^{2+} concentration and translocation of protein kinase C (PKC) to the plasma membrane are thought to initiate the cascade of biochemical events leading to activation of T cell effector functions and clonal expansion (*2, 3*). Triggering of the TCR-CD3 complex also induces tyrosine phosphorylation of a number of proteins (*4*), including the ζ subunit of this complex (*5*). This implies a role for a TPK in T cell activation. Although this tyrosine phosphorylation appears to be independent of inositol phospholipid hydrolysis (*6, 7*) and activation of PKC (*8*), the exact relationship between these two pathways and their relative contribution to the overall process of T cell activation remain unknown. In particular, it is not known whether the stimulation of PI-PLC and the subsequent production of inositol phospholipid–derived second messengers require a tyrosine phosphorylation event.

We have studied the role of tyrosine phosphorylation in signal transduction through the TCR-CD3 complex with the use of a TPK inhibitor, genistein. This isoflavone compound was found to specifically inhibit the epidermal growth factor receptor, $pp60^{c\text{-}src}$, and $pp110^{gag\text{-}fes}$ TPKs, whereas it had marginal effects on several serine and threonine kinases (*9*). To determine whether genistein inhibits TPKs in T lymphocytes, we tested it in three different assays. (i) We measured total TPK activity of T lymphocyte membranes that had been incubated for 10 min with various concentrations of genistein. The drug inhibited the kinase activity in a dose-dependent manner, with 50% inhibition being achieved at 30 μg/ml (Fig. 1A). (ii) The effect of genistein on the enzymatic activity of $pp56^{lck}$, a T cell–specific TPK (*10*), was evaluated by measuring the ability of $pp56^{lck}$ to phosphorylate a synthetic peptide substrate (Fig. 1B) or to become autophosphorylated (Fig. 1C). Autophosphorylation correlates with activity toward exogenous substrates (*10*). Both phosphorylation reactions were similarly inhibited in a dose-dependent manner with an inhibition constant (K_i) of 10 μg/ml. (iii) The effects of genistein on tyrosine phosphorylation in intact cells was assessed by measuring phytohemagglutinin (PHA)-induced TCR ζ chain phosphorylation (Fig. 2). T cell mitogens, antigen, and antibodies to the antigen receptor stimulate a TPK that phosphorylates the ζ chain on tyrosine residues (*5*). Tyrosine phosphorylation of ζ was readily induced by PHA (Fig. 2). However, when T cells were incubated for 10 min with genistein before PHA addition, no phosphorylation of TCR ζ was detected (Fig. 2). At a concentration (30 μg/ml) that completely blocked mitogen-induced TCR ζ phosphorylation (Fig. 2), genistein had no effect on phorbol ester–induced translocation of PKC to the plasma membrane in the same cells and only minimally inhibited (<25%) the phosphorylation of CD3 γ or ε subunits, or both, on serine and threonine residues (*11*).

As tyrosine phosphorylation has been implicated in the regulation of cell growth (*12*), we examined the effects of genistein on mitogen-induced activation of T cells. T cells stimulated with PHA or with OKT3, a monoclonal antibody to CD3, in the presence of genistein failed to undergo the blast transformation typical of activated T cells. Instead, the cells remained as small, round, resting cells (*13*). Likewise, genistein inhibited mitogen-stimulated induction of the interleukin-2 (IL-2) receptor α subunit (p55) and prevented the increase in ornithine decarboxylase activity (Table 1) normally seen during blast transformation (*2, 14*). Moreover, genistein inhibited, in a dose-dependent manner, the PHA- or OKT3-induced proliferation of human T cells (Table 1). This inhibitory effect did not result from drug toxicity; the viability of the cells was not significantly affected by culture in the presence of genistein for 3 days. Furthermore, when human blood lymphocytes were treated with genistein (up to 30 μg/ml) for 20 hours, washed, and then cultured in the presence of OKT3 or PHA, no decrease in proliferation was observed (*13*). These results suggest that genistein is

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nontoxic, but blocks T cell activation at an early point in mitogenesis, apparently preventing transition from the quiescent (G_0) to the prereplicative (G_1) phase of the cell cycle. However, we cannot exclude the possibility that genistein also inhibits later events. Indeed, genistein also inhibited T cell proliferation produced by a combination of phorbol ester plus Ca^{2+} ionophore (*13*). This effect may reflect the requirement for an IL-2 receptor–associated TPK during IL-2–mediated T cell proliferation (*15*). This possibility is supported by our finding that genistein inhibited the response of PHA-activated T cell blasts to recombinant IL-2 (*13*).

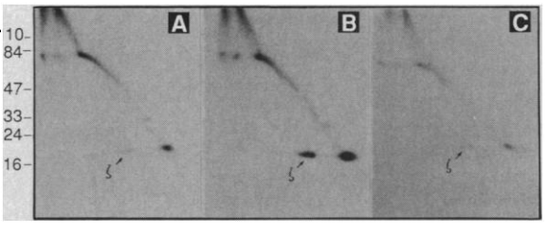

Fig. 2. Inhibition of PHA-induced tyrosine phosphorylation of the TCR ζ subunit in intact human T cells by genistein. (**A**) No stimulus. (**B**) Cells were stimulated with PHA (10 μg/ml) for 15 min. (**C**) Cells were treated with genistein (30 μg/ml) for 10 min and then stimulated with PHA (10 μg/ml) for 15 min. T cells (5×10^7) were metabolically labeled with [^{32}P]-orthophosphate (0.2 mCi/ml) for 2 hours, treated with or without genistein and PHA, washed, and then lysed in PBS containing 0.5% Triton X-100, 1 m*M* Na_3VO_4, and protease inhibitors. The TCR ζ subunit was immunoprecipitated with a specific rabbit antiserum (No. 390) and the precipitates were analyzed by two-dimensional (nonreducing-reducing) SDS-PAGE and autoradiography. The position of TCR ζ is indicated. A protein on the diagonal with similar size might represent TCR ζ that was reduced during electrophoresis in the first dimension or other phosphorylated subunits of CD3 with a similar size, such as δ or ε. Markers are in kilodaltons.

Because TCR-CD3–mediated T cell activation is associated with PI-PLC–mediated production of the second messengers, inositol 1,4,5-triphosphate and diacylglycerol, we ascertained the effect of genistein on this signaling pathway. In agreement with inhibition at a proximal site, we found that genistein, at doses similar to those that blocked TPK activity, also prevented the enhanced formation of inositol phosphates in T lymphocytes stimulated with PHA (Fig. 3A) or antibodies to CD3 (Fig. 3B). At genistein concentration of 30 μg/ml, the inhibition was essentially complete. This was also seen when the production of inositol 1,4,5-trisphosphate was measured separately. Radioactive counts recovered in the inositol 1,4,5-triphosphate fraction, eluted from [^{3}H]inositol-labeled cells with 1.6*M* ammonium formate and 0.1*M* formic acid, were 87 cpm in unstimulated cells, 191 cpm (120% increase) in mitogen-stimulated cells, and 77 cpm in cells stimulated with mitogen in the presence of 30 μg/ml genistein. In contrast, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), an inhibitor of PKC and cyclic nucleotide-dependent protein kinases (*16*), failed to inhibit inositol phosphate formation (and TPK activity) at a concentration (30 μg/ml) that completely blocked the enzymatic activity of PKC (*13*).

The observed effect of genistein could result from either direct or indirect inhibition of PI-PLC. To address this question, we added genistein directly to a PLC enzymatic assay and found that the formation of [^{3}H]inositol 1,4,5-trisphosphate from labelled phosphatidylinositol 4,5-bisphosphate was not affected even at a genistein concentration of 30 μg/ml (Fig. 4). This result was apparent with both membrane-bound and cytosolic PI-PLC activity (Fig. 4). Thus, inhibition of inositol phospholipid turnover in mitogen-stimulated T cells is not due to direct inhibition of PI-PLC. The most plausible explanation for our findings is that genistein blocks an early and critical tyrosine phosphorylation event required for activation of PI-PLC by TCR-CD3 triggering, thereby preventing inositol phospholipid hydrolysis and consequent cell activation. This is consistent with the findings that tyrosine phosphorylation can occur in the absence of detectable inositol phospholipid breakdown (*6, 7*) and that one of the earliest measurable biochemical changes in TCR-CD3–triggered T cells is tyrosine phosphorylation of a 135-kD substrate (*17*). This

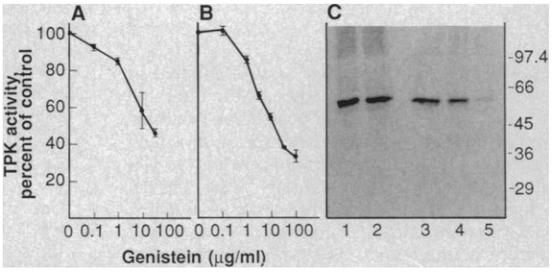

Fig. 1. Dose-dependent inhibition of tyrosine kinase activity by genistein. (**A**) Total membrane-bound T cell tyrosine kinase activity. (**B**) Tyrosine kinase activity of pp56^{lck}. (**C**) Autophosphorylation of pp56^{lck} in murine thymoma LSTRA cell membranes in the presence of 2% dimethyl sulfoxide (DMSO) (lane 1), no addition (lane 2), or genistein at a concentration of 10 μg/ml (lane 3), 30 μg/ml (lane 4), or 100 μg/ml (lane 5). Human mononuclear cells (~80% T cells) were isolated by gradient centrifugation on Histopaque 1077 (Sigma) from the blood of healthy volunteers. Total tyrosine kinase activity of lymphocyte membranes and the tyrosine kinase activity of pp56^{lck} immunoprecipitated with OKT4 (a monoclonal antibody to CD4), were measured as described (*26*). The membranes or immunoprecipitates were incubated for 10 min at 0°C in different concentrations of genistein dissolved in DMSO [final concentration 2% (v/v)], mixed with assay buffer containing saturating concentrations (10 m*M*) of a tyrosine-containing synthetic peptide substrate and γ-^{32}P–labeled adenosine triphosphate (ATP) (5 to 10 Ci/mmol), and incubated at 30°C for 2 min. Phosphorylated peptide was separated from radioactive ATP on phosphocellulose paper and quantitated by liquid scintillation counting. The data shown represent mean and range from a single experiment (duplicate determinations). Similar data were obtained in three separate experiments. LSTRA cells, known to overexpress pp56^{lck} (*10*), were kept in logarithmic growth in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μ*M* β-mercaptoethanol. Autophosphorylation of pp56^{lck} in LSTRA membranes was carried out as described (*26*). Membrane protein (20 to 40 μg) was incubated with 0.2 μ*M* [γ-^{32}P]ATP (800 Ci/mmol) in 50 μl of 25 m*M* Hepes (*p*H 7.3), 5 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, 2.5 m*M* β-mercaptoethanol, and 100 μ*M* Na_3VO_4 for 30 s at 30°C. The reaction was stopped by addition of SDS sample buffer and analyzed by SDS-PAGE (10% gel). Markers are in kilodaltons.

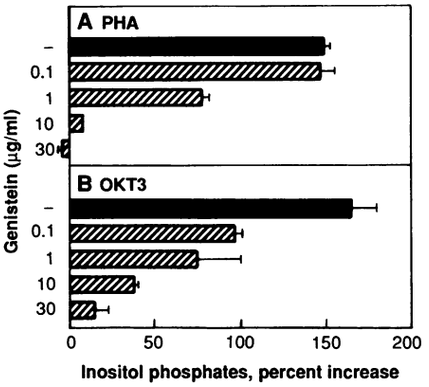

Fig. 3. Inhibition of (**A**) PHA- and (**B**) OKT3-induced inositol phosphate formation in human T cells by genistein. T lymphocytes were incubated with [^{3}H]inositol for 4 to 12 hours in inositol-free medium and then stimulated for 60 min with PHA (5 μg/ml) or OKT3 (1:1000 dilution of ascites fluid). Total inositol phosphates were measured as described (*27*). The data are given as percent increase over unstimulated cells. Unstimulated production of inositol phosphates by 3.3×10^7 cells was 1580 cpm (A) and 300 cpm (B). Data represent mean and range from a single experiment (duplicate determinations). Similar results were obtained in two additional experiments.

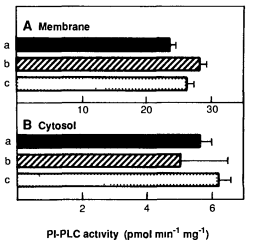

Fig. 4. Effects of genistein on membrane-bound (**A**) and cytosolic (**B**) PI-PLC in vitro. The enzymatic activity was measured as described (*18*), in the presence of no additives (a), 2% DMSO (b), or genistein (30 μg/ml) (c). Briefly, membrane or cytosol preparations of human T lymphocytes were assayed in a final volume of 25 μl containing 20 m*M* sodium phosphate (*p*H 6.8), 40 m*M* KCl, 1 m*M* sodium pyrophosphate, 0.65% octylglucoside, 0.4 m*M* EGTA, 0.8 m*M* $CaCl_2$, and 0.2 m*M* [^{3}H]phosphatidylinositol-4,5-bisphosphate (5 Ci/mol). The mixture was incubated at 37°C for 15 min and the reaction stopped by adding 100 μl of 1% bovine serum albumin, followed by 500 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation and the radioactivity present in 0.5 ml of the supernatant was determined. The counts from a control incubation with no enzyme (buffer only, 200 to 500 cpm) was subtracted from all values. Under the conditions of the assay, the reaction rate was linear with time and amount of protein. Data are mean and range from a single experiment (duplicate determinations). Similar results were obtained in three independent experiments.

phosphorylation precedes inositol phospholipid breakdown and can be detected within 5 to 10 s of receptor occupation.

There are precedents for regulation of PI-PLC by tyrosine phosphorylation; ligand stimulation of some growth factor receptor TPKs, as well as transformation by oncogenic tyrosine kinases, increases the turnover of inositol phospholipids (*18*). Platelet-derived growth factor and epidermal growth factor stimulation of cells expressing the corresponding receptors leads to rapid phosphorylation of the 148-kD γ isozyme of PI-PLC on several tyrosine residues (*19, 20*).

T lymphocytes express several TPKs (*21*) and, as genistein apparently inhibits all tyrosine kinases tested (*9*) (Figs. 1 and 2), our findings do not allow us to determine which TPK mediates the critical tyrosine phosphorylation event leading to PI-PLC activation in T lymphocytes. However, one candidate, $pp56^{lck}$, a TPK found mainly in T cells (*10*), appears to be more sensitive than total T cell membrane TPK (Fig. 1), and may be important in TCR-CD3 signaling (*21*): $pp56^{lck}$ is tightly bound to the cytoplasmic domains of the CD4 and CD8 glycoproteins (*22*), which participate in antigen recognition (*21*), and antibody-mediated cross-linking of CD4 molecules leads to activation of $pp56^{lck}$ and TCR ζ phosphorylation in intact cells (*23*). Another candidate is the $p59^{fyn}$ TPK, the high level expression of which correlates with constitutive TCR ζ phosphorylation in $CD4^-CD8^-$ T cells from mice homozygous for the *lpr* mutation (*24*).

Our data thus show that tyrosine kinase activity is needed for TCR-CD3–induced activation of PI-PLC and subsequent T cell activation. The TCR-CD3 complex resembles the TPK family of growth factor receptors in that binding of ligand to the receptor complex first stimulates an associated TPK, which, in turn, activates PI-PLC and initiates the cascade of events leading to mitogenesis. Although the TCR-CD3 complex itself lacks TPK activity, a mechanism that enables the receptor complex to mobilize a TPK upon antigen presentation appears to have evolved in T cells.

Note added in proof: After submission of this manuscript, it has come to our attention that similar findings were obtained with a different TPK inhibitor, herbimycin (*25*).

Table 1. Inhibition of T cell activation by genistein. Human blood lymphocytes were cultured in RPMI 1640 plus 5% fetal bovine serum (1×10^6/ml) in the absence or presence of the indicated mitogens [PHA (1 μg/ml); OKT3 (1:1000 dilution of ascites fluid)]. The activity of ornithine decarboxylase (ODC) in cytosolic extracts was measured 18 hours after mitogen addition, as described (*14*). Genistein did not directly inhibit the enzymatic activity of ODC at concentrations as high as 100 μg/ml (*13*). We measured expression of the IL-2 receptor α chain (Tac) after 48 hours by staining the cells with antibody to Tac (1:2000 dilution of ascites fluid) and fluorescein isothiocyanate–conjugated rabbit antibody to mouse immunoglobulin G and by analyzing the cells in a fluorescence-activated cell sorter. [^{3}H]thymidine incorporation in triplicate microtiter cultures (2×10^5 cells per well) was measured at 72 hours after a 6-hour terminal pulse with 1 μCi of [^{3}H]thymidine. ND, not determined.

Stimulus	Genistein (μg/ml)	ODC activity (pmol/hour/mg protein)	Tac-positive cells (%)	[^{3}H]Thymidine incorporation (cpm × 10^{-3})
None		80 ± 10	13	0.8 ± 0.1
PHA		663 ± 56	88	36.0 ± 1.9
PHA	0.3	ND	86	35.7 ± 0.6
PHA	3	ND	76	22.1 ± 1.1
PHA	30	0 ± 0	30	0.5 ± 0.1
None		ND	2	0.8 ± 0.1
OKT3		ND	81	28.8 ± 4.5
OKT3	0.3	ND	70	21.8 ± 2.3
OKT3	3	ND	54	6.2 ± 0.4
OKT3	30	ND	1	1.4 ± 0.4

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Limit of T Cell Tolerance to Self Proteins by Peptide Presentation

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Cytotoxic T lymphocytes (CTLs) recognize foreign peptides bound to major histocompatibility complex (MHC) class I molecules. MHC molecules can also bind endogenous self peptides, to which T cells are tolerant. Normal mice contained CTLs specific for self peptides that were from proteins of ubiquitous or tissue-restricted expression. In vivo, these endogenous self peptides are not naturally presented in sufficient density by somatic cells expressing MHC class I molecules. They can, however, be presented if added exogenously. Thus, our data imply that CTLs are only tolerant of those endogenous self peptide sequences that are presented by MHC class I–positive cells in a physiological manner.

SELF TOLERANCE IN THE IMMUNE system is established by elimination of self-reactive T lymphocytes during thymic differentiation (*1*). The self antigens involved in this negative selection are combinations of self MHC molecules and other self molecules, since T cells specific for combinations of self molecules and foreign MHC are not negatively selected (*2*). These experiments confirmed the hypothesis (*3*) that antigen recognition during establishment of self tolerance follows the same rules as those that apply to the recognition of foreign antigen in the mature immune system. CTLs were found to recognize specific peptide-defined epitopes of cellular proteins, for example, of viral origin, in the context of MHC class I molecules (*4*). Not all possible peptide sequences from a given endogenous cellular protein are actually presented in sufficient density (*5, 6*). Several possible reasons have been put forward to account for this limitation, including specificity of intracellular proteases, problems in peptide-MHC interaction, or self peptides competing for the relevant binding sites. These limitations in peptide presentation have led to the postulate that T cells need not be tolerant of those autologous peptide sequences that are not physiologically presented by MHC-expressing cells in sufficient density if the above hypothesis on establishment of self tolerance is valid (*6, 7*). Thus, nonphysiologic combinations of self MHC and self peptides would not induce negative selection. Our data show this postulate to be correct.

Mouse spleen cells were stimulated with several sources of peptides derived from autologous proteins in a manner that yields primary CTL responses to peptides (*5*). First, C57BL/6 [B6; H-2^b, β_2M^b (β_2-microglobulin allele)] or B10.C-*H-3*^{c} (H-2^b, β_2M^a) spleen cells were stimulated with a synthetic peptide representing amino acids 77 to 89 of β_2M^b, which is expressed in nearly all somatic cells including those thymic resident cells inducing self tolerance, and in T cells themselves. We consistently obtained CTL lines with specificity for the β_2M^b peptide regardless of the β_2M allele expressed by responder cells (Fig. 1). Two representative CTL lines, one expressing β_2M^a, the other β_2M^b (Fig. 1, A and B), did not recognize β_2M^b-expressing EL4 target cells, but they both killed EL4 cells incubated with the stimulating β_2M^b 77–89 peptide. The reactivity pattern of both lines was similar when tested on a panel of β_2M-derived peptides. The β_2M^a 77–89 peptide is hardly recognized or not at all. The β_2M^b 77–89–specific CTL line 041188-5 (β_2M^b) can be more easily blocked with CD8-specific antibody than the β_2M^b-specific CTL line 181B (β_2M^a) (Fig. 1C), which was generated by in vivo immunization (*8*). This supports the suggestion that in vitro–primed, peptide-specific CTLs are of lower affinity than in vivo–primed CTLs (*5*). The P815 targets (H-2^d) incubated with β_2M^b 77–89 are not lysed by 041188-5 CTLs (Fig. 1D), indicating MHC class I–restricted recognition, since neither EL4 nor P815 cells express MHC class II. Thus, normal mice contained CTLs specific for a peptide derived from a ubiquitous autologous protein. These CTLs did not recognize target cells that express that protein.

A second source of autologous peptide was prepared by hydrolysis of hemoglobin, as an example of an abundant protein with a specific tissue distribution. Self tolerance to proteins not synthesized in the thymus is presumably established by presentation of imported protein by thymic antigen-presenting cells. Spleen cells from a B10.129-*H-1*^{b} mouse [H-2^b, Hbb^d (hemoglobin allele)] or from a C57BL/10 mouse (H-2^b, Hbb^s) were stimulated with hydrolyzed hemoglobin of C57BL/10 (Hbb^s). The CTL lines that developed did not distinguish between heterologous and autologous hemoglobin fragments, and they recognized autologous hemoglobin fragments in an antigen-specific and MHC class I–restricted fashion (Fig. 2).

A third source of autologous peptides was prepared by tryptic digest of total liver proteins. B6.K1 (H-2^b) spleen cells were stimulated with such a pool of self peptides. In a parallel culture, B6.K1 spleen cells were stimulated against a pool of peptides derived by similar treatment of allogeneic BALB.HTG (H-2^g) liver proteins. CTL lines derived from these cultures recognized the stimulating peptides in an antigen-specific and MHC class I–restricted fashion (Fig. 3). The line against autologous peptides (709K1-5) cross-reacts to some extent with allogeneic fragments, and vice versa, indicating that some of the determinants recognized by either CTL line are monomorphic between the two mouse strains.

In conclusion, our data show that normal mice contain CTLs reactive with peptide sequences or fragments of ubiquitous or tissue-specific self proteins prepared in a nonphysiologic way, implying that such CTLs are spared from thymic negative selection (*9*). The peptides recognized by our CTL lines may either be not produced at all by normal cells or they may be produced in insufficient quantities to be recognized by

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