populations (Fig. 3B). Therefore both α and β chains were down-regulated in $CD8^+\alpha_T$ but not in $CD8^+\alpha_E$ cells. This suggests that the induction of anergy in T cells (which is associated with down-regulation of the TCR and its coreceptors) probably requires a critical threshold of receptor occupancy, as has been described for mature B cells (*22*): Transgenic α_E cells, the expansion of which is also antigen-dependent, do not down-regulate their receptors (Fig. 3B).

Our results indicate that binding of the transgenic TCR to male antigen has different consequences in the thymus and periphery. Immature thymocytes die without cell division (*23*), whereas mature T cells proliferate vigorously at first but then either disappear or down-regulate their receptors and so become refractory to antigenic stimulation. The latter mechanism may be one of many by which peripheral T cells can be "silenced" and may be similar to that which has been described for mature B cells (*22*)—which can also down-regulate surface immunoglobulin expression and become unreactive. The differential behavior of thymocytes and mature T cells with regard to confrontation of self antigens lends support to the hypothesis of Ledenberg (*23*), which proposes different consequences of antigenic stimulation in immature versus mature lymphocytes. Nevertheless, our experiments also suggest that elimination of self-reactive clones can also occur in the periphery as a result of encounters between mature T cells and antigen. In contrast to the thymus, this cell elimination is not sudden, and its cause, as well as its mechanism, needs further investigation.

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Split Anergy in a $CD8^+$ T Cell: Receptor-Dependent Cytolysis in the Absence of Interleukin-2 Production

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Engagement of the antigen-specific receptor (TCR) of $CD4^+$ T lymphocytes without a second (costimulatory) signal prevents the subsequent production of interleukin-2 (IL-2) by these cells. Because IL-2 is a key immunoregulatory lymphokine and is also produced by a subset of $CD8^+$ T cells that are able to kill target cells, the effect of engaging the TCR of one such clone in the absence of costimulatory signals was examined. The capacity for TCR-dependent IL-2 production was lost, indicating comparable costimulator-dependent signaling requirements for IL-2 production in $CD4^+$ and $CD8^+$ T cells. However, TCR-mediated cytotoxicity was not impaired, implying that costimulation is required for only certain TCR-dependent effector functions.

THE ESTABLISHMENT OF A SELF-TOLERANT state among T cells is essential to the development of a useful immune system that responds to foreign antigens without undesirable self-reactivity. Defects in self-tolerance result in autoimmune disease, and knowledge about basic mechanisms of tolerance induction may prove beneficial for treatment or prevention of such diseases, as well as for efforts aimed at increasing the acceptance of histoincompatible tissue grafts. Two distinct mechanisms of T cell tolerance have been defined and studied at the clonal level: deletion, which is the result of programmed cell death and occurs predominantly during intrathymic maturation of T cells (*1*); and anergy, a state of viable T cells that is characterized by diminished or absent lymphokine secretion as a result of TCR engagement (*2*). Anergy can be induced in differentiated, mature $CD4^+$ type 1 (T_H1) T cell clones (*3*) by exposure to complexes of antigen and appropriate major histocompatibility complex (MHC) molecules in the absence of certain uncharacterized "costimulatory" signals on the antigen presenting cell (APC) (*4*). This anergy is most apparent as a lack of both IL-2 production and clonal proliferation after subsequent exposure to antigen and fully competent APCs. A subset of murine $CD8^+$ T cells can secrete IL-2, and previous studies of this population indicated that adherent cells provided antigen-unspecific, costimulatory signals that were essential for optimal proliferation and secretion of IL-2 in response to alloantigenic spleen cells (*5*). To explore whether this lack of response in the absence of costimulation might be associated with induction of a state of anergy, we isolated a class I ($H\text{-}2D^b$)–restricted, IL-2 producing, cytotoxic $CD8^+$ clone, termed GX1 (*6*), from the lymph node cells of a C57Bl/6 (B6) mouse that had been immunized with trypsin-digested chicken ovalbumin (T-OVA) (*7*).

When APCs are chemically fixed, they cannot stimulate IL-2 secretion or the proliferation of T_H1 $CD4^+$ T cells (*8, 9*). To determine if the $CD8^+$ clone GX1 had a similar response pattern, we exposed GX1

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cells to T-OVA in the presence of either irradiated, unfixed B6 splenic accessory cells, or irradiated, paraformaldehyde-fixed splenic cells (Table 1). Fixation of accessory cells prevented antigen-induced proliferation. The fixed accessory cells were not nonspecifically inhibitory or toxic, because the GX1 cells proliferated in the presence of fixed cells plus IL-2.

The costimulatory signals could be provided separately and independently of the antigen-MHC complex (Table 2): allogeneic B10.A spleen cells restored GX1 proliferative responses to antigen. GX1 cells also proliferated in response to antigen in the presence of irradiated B10.A cells but in the absence of syngeneic spleen cells. Given that GX1 cells express H-2D^b (*6*), these latter results indicate that GX1 cells can present T-OVA to each other via their D^b molecules—as has been described for other peptide-specific cytotoxic T cell clones (*10*)—but they lack the ability to provide the costimulatory signals to themselves.

The lack of a proliferative response by GX1 cells after stimulation with T-OVA and fixed accessory cells was likely attributable to a lack of IL-2 production, because no IL-2 activity was detected in the supernatants of such cultures (Table 3). Secretion of interleukin-3 (IL-3) and interferon-γ (IFN-γ) was reduced, though not eliminated, as has been observed with CD4$^+$ T$_H$1 clones (*11*). This similarity between the CD8$^+$ cytotoxic T cell clone GX1 and CD4$^+$ T$_H$1 clones in requiring fixation-sensitive accessory cell functions for optimal proliferation and IL-2 secretion suggested that GX1 cells might enter a state of sustained unresponsiveness (anergy) after exposure to peptide-MHC complexes in the absence of costimulation. GX1 cells were therefore exposed to fixed B6 spleen cells plus T-OVA for 2 days, and then tested in functional assays with living target cells or APCs (Table 4). GX1 cells that were first cultured with T-OVA in the presence of fixed APCs did not proliferate in response to subsequent stimulation with antigen plus irradiated accessory cells. These otherwise unresponsive cells were nevertheless able to proliferate in IL-2–containing medium, indicating that they were fully viable and had not developed a G$_1$-S cell cycle block, as T cell hybridomas do after receptor occupancy (*12*). These same cells that did not proliferate when stimulated through their antigen receptors could lyse EL4 cells in an antigen-specific manner and could kill P815 cells in a redirected lysis assay in the presence of an antibody to CD3 (Fig. 1). The lysis of the EL4 targets in the presence of T-OVA exceeded that seen with GX1 cells not preexposed to fixed cells plus antigen. This greater cytotoxicity indicates that the capacity for cytolysis that we observed under conditions suitable for induction of anergy at the level of IL-2 production was not simply a residual response that had been partially but not fully down-regulated, as with IFN-γ and IL-3 (*13*). We considered the possibility that the failure of anergized GX1 cells to proliferate was due to their enhanced cytolytic potential, and thus to rapid cytolysis of accessory cells. However, first culturing GX1 cells with T-OVA plus irradiated accessory cells likewise enhanced cytotoxicity. Nonetheless, GX1 cells cultured in such a manner proliferated in response to a second antigenic challenge (*14*). Thus, enhanced cytolysis did not account for proliferative anergy.

Current concepts of T cell anergy derive from investigations of class II MHC–restricted, CD4$^+$ T$_H$1 T cell clones (*2*) or from the analysis of CD4$^+$ T cells that express specific TCR V$_\beta$ segments and which participate in responses to class II MHC–associated ligands such as Mls (*15*). Our data indicate that this emerging model of proliferative anergy can be extended to at

Table 1. Failure of GX1 cells to proliferate in response to T-OVA in the presence of paraformaldehyde-fixed B6 spleen cells. GX1 cells (2×10^4 cells per microwell) were stimulated with IL-2–containing rat concanavalin A supernatant (CAS) or with various amounts of T-OVA in the presence or absence of 1×10^6 paraformaldehyde-fixed (0.5% paraformaldehyde for 20 min) or irradiated (2000 R) B6 spleen cells. [^{3}H]Thymidine was added after 48 hours, and cultures were harvested for liquid scintillation counting after a 16-hour [^{3}H]thymidine pulse. In the absence of CAS or T-OVA, [^{3}H]thymidine uptake was $\leq 0.5 \times 10^3$ cpm. Data are representative of four experiments.

Accessory cells	[^{3}H]Thymidine uptake (mean cpm × 10^{-3})				
	CAS [2% (v/v)]	T-OVA (μg/ml)			
		900	300	100	33
Fixed B6 spleen	30.2	0.6	0.4	0.7	0.6
Irradiated B6 spleen	35.0	19.9	17.1	5.2	1.5
None	32.6				

Table 2. Effect of irradiated third-party spleen cells on the proliferative response of GX1 T cells to T-OVA plus paraformaldehyde-fixed syngeneic spleen cells. GX1 cells (2×10^4 cells per microwell) were stimulated as described in Table, 1, in the presence or absence of 1×10^6 paraformaldehyde-fixed or irradiated (2000 R) B6 spleen cells or 1×10^6 irradiated B10.A spleen cells. [^{3}H]Thymidine was added after 62 hours, and cultures were harvested for liquid scintillation counting 12 hours later. Data are representative of three experiments.

Accessory cells		[^{3}H]Thymidine uptake (mean cpm × 10^{-3})			
Syngeneic (B6)	Third party (B10.A)	T-OVA (μg/ml)			
		900	300	100	0
Irradiated		12.9	3.8	0.8	0.4
Fixed		0.5	0.4	0.3	0.2
Fixed	Irradiated	13.4	1.0	0.2	0.2
	Irradiated	16.5	0.7	0.4	0.4

Table 3. Effect of accessory cell fixation on secretion of lymphokines by GX1 cells. GX1 cells (5×10^4 cells per microwell) were challenged with T-OVA (900 μg/ml) in the presence or absence of 1×10^6 paraformaldehyde-fixed or irradiated (2000 R) syngeneic B6 spleen cells. Culture supernatants were collected after 24 hours and tested in lymphokine bioassays. Lymphokine titers are expressed as the reciprocal of the dilution that yielded a 50% maximal response. IL-3 was measured with the FD.C/1 cell line (*22*). IL-2 was measured with the CTLL-2 cell line (*23*). IFN-γ was measured by its ability to protect L929 fibroblasts from the cytopathic effect of vesicular stomatitis virus. No lymphokine activity was detected in cultures that lacked antigen or GX1 cells. Data are representative of three experiments.

B6 splenic accessory cells	Lymphokine titer		
	IL-3	IFNγ	IL-2
Paraformaldehyde-fixed	20	10	<0.25
Irradiated	150	50	2.5
None	20	40	<0.25

Table 4. After exposure to paraformaldehyde-fixed B6 spleen cells and antigen, clone GX1 failed to proliferate in response to antigen and irradiated APCs. GX1 cells (5×10^5 cells per milliliter) were cultured on day 0 with paraformaldehyde-fixed B6 spleen cells (5×10^6 cells per millilitter) in the presence or absence of T-OVA (900 μg/ml). On day 2, GX1 cells were recovered by centrifugation on Ficoll/Hypaque (relative density, 1.088; Accurate). GX1 cell recovery was 31% of the initial number (day 0) for GX1 cells cultured initially with fixed B6 spleen cells and 22% for GX1 cells cultured with fixed B6 spleen cells plus T-OVA. Averaged over several experiments, the GX1 cell yield from T-OVA–containing cultures was ~50% of the yield from cultures lacking T-OVA. Recovered GX1 cells were stimulated as described in Table 1. [^{3}H]Thymidine was added after 63 hours, and cultures were harvested 6 hours later. Data are representative of three experiments.

Day 2 stimulus	[^{3}H]Thymidine uptake (mean cpm × 10^{-3})	
	GX1 previously exposed to	
	Fixed B6 spleen cells + T-OVA	Fixed B6 spleen cells
Irradiated B6 spleen cells + T-OVA (300 μg/ml)	1.0	105.1
Irradiated B6 spleen cells + T-OVA (60 μg/ml)	0.5	12.4
Irradiated B6 spleen cells + medium	0.4	0.4
Irradiated B6 spleen cells + CAS [2% (v/v)]	12.8	24.4
None	0.5	0.3

least one subset of class I MHC–restricted CD8$^+$ T cells; namely, that capable of autocrine, IL-2–mediated growth. The conditions under which the anergic state is induced appear similar for class I MHC–restricted CD8$^+$, IL-2 producing cells and class II MHC–restricted CD4$^+$ T cells; namely, effective TCR occupancy in the absence of an accessory cell–derived costimulatory signal. As was also observed for the T_H1 model, interleukin-1 (IL-1) could not replace the fixation-sensitive costimulatory function of APCs (*9, 16*). It thus appears that the relation between the TCR, costimulatory signal, and IL-2 gene is similar in T cells of distinct differentiation state. This form of anergy may explain the tolerance of proliferating CD8$^+$ cells specific for class I MHC alloantigen after the intravenous injection of lymphoid cells bearing the antigen (*17*); it may also have clinical relevance in models of posttransfusion transplantation tolerance, given the importance of IL-2–producing CD8$^+$ T cells in allograft rejection (*18*).

Analysis of this IL-2–producing cytotoxic T cell clone also allowed us to study the differential effects of receptor occupancy without costimulation on two distinct sets of TCR-dependent effector responses. In marked contrast to the anergy seen in terms of subsequent IL-2 secretion, such TCR occupancy did not interfere with cytotoxic function of the GX1 clone. This finding indicates that proliferative anergy does not reflect interference with, or down-modulation of, all TCR-mediated signaling activity. GX1 cells thus provide a model for examining the details of the anergic state by providing a readout of events intermediate in the signaling pathways between early biochemical changes—such as an increased intracellular Ca^{2+} concentration, inositol phospholipid hydrolysis, protein kinase C activation (*19*), or changes in protein tyrosine phosphorylation (*20*)—and late events such as nuclear gene activation (*21*).

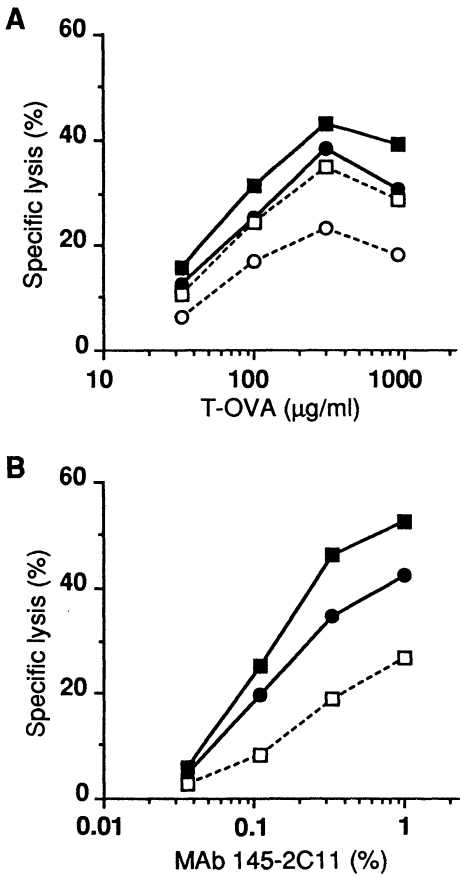

Fig. 1. Cytolytic function of GX1 cells after induction of proliferative anergy. GX1 cells were cultured with paraformaldehyde-fixed syngeneic B6 spleen cells in the presence or absence of T-OVA (900 μg/ml), as described in Table 4. Two days later, GX1 cells were isolated on Ficoll/Hypaque gradients and then incubated in a 4-hour ^{51}Cr release assay with (**A**) H-2^b EL4 targets in the presence of T-OVA or (**B**) H-2^d P815 targets in the presence of monoclonal antibody (MAb) 145-2C11 to CD3 (*24*). GX1 cells were present at effector:target ratios of 5:1 (■, □) or 2.5:1 (●, ○). Closed symbols, GX1 cells previously exposed to T-OVA plus fixed B6 spleen cells (anergized); open symbols, GX1 cells previously exposed to fixed B6 spleen cells only (control). The percent specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%. Maximum release of ^{51}Cr was determined by addition of 2.5% (v/v) Triton X-100.

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Regulation of Phospholipase C–γ1 by Profilin and Tyrosine Phosphorylation

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Epidermal growth factor and platelet-derived growth factor can stimulate the production of the second messenger inositol trisphosphate in responsive cells, but the biochemical pathway for these signaling events has been uncertain because the reactions have not been reconstituted with purified molecules in vitro. A reconstitution is described that requires not only the growth factor, its receptor with tyrosine kinase activity, and the soluble phospholipase C–γ1, but also the small soluble actin-binding protein profilin. Profilin binds to the substrate phosphatidylinositol 4,5-bisphosphate and inhibits its hydrolysis by unphosphorylated phospholipase C–γ1. Phosphorylation of phospholipase C–γ1 by the epidermal growth factor receptor tyrosine kinase overcomes the inhibitory effect of profilin and results in an effective activation of phospholipase C–γ1.

CELL GROWTH REQUIRES THE COORdinated regulation of multiple biochemical systems including those leading to DNA synthesis and the reorganization of the cytoskeleton. It is remarkable that the whole process can be initiated by the binding of an extracellular factor such as epidermal growth factor (EGF) or platelet derived-growth factor (PDGF) to a single class of membrane receptors. How these receptors are coupled to the biochemical processes they induce is not well understood. For example, it is not clear how binding of EGF to its receptor is coupled to the production of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG).

The EGF receptor (EGFR) is a transmembrane protein with an EGF binding site outside the cell and a tyrosine kinase catalytic domain inside the plasma membrane (*1*). Upon binding EGF, the receptor dimerizes and phosphorylates tyrosine residues on itself and on several effector proteins likely to participate in signal transduction (*1, 2*). Some effector proteins also become physically associated with the receptor (*1, 2*). One such effector is phosphatidylinositide (PI)-specific phospholipase C–γ1 (PLC-γ1), a soluble enzyme in quiescent cells (*3, 4*). This enzyme catalyzes the hydrolysis phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce IP_3 and DAG (*5*). In some cells, EGF and PDGF stimulate the phosphorylation of PLC-γ1 on tyrosine, the binding of PLC-γ1 to the EGFR or to the PDGF receptor (PDGFR), and the production of IP_3 (*3*).

The biochemical connections among these events are not well understood. Most studies have shown that the catalytic activity of purified PLC-γ1 is not altered by tyrosine phosphorylation (*6*). However, in one study PLC-γ1 was activated when phosphorylated on tyrosine by the EGF receptor; in this case immunoprecipitated PLC-γ1 was used and the substrate consisted of PIP_2 in mixed micelles with two detergents (*7*).

The role of profilin, a cytoplasmic actin binding protein, in the PI signaling pathway has been equally mysterious. Profilin from human platelets binds four to five molecules of PIP_2 or phosphatidylinositol 4-monophosphate with relatively high affinity but does not bind other lipids (*8, 9*). Binding of

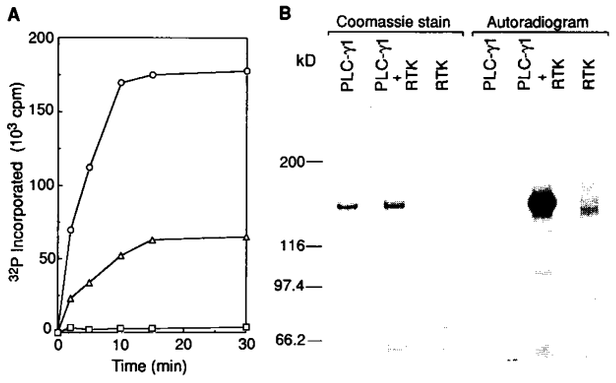

Fig. 1. Phosphorylation of PLC-γ1 by the EGF receptor. (**A**) Time course of phosphorylation by EGFR of PLC-γ1 (○), or of itself in the absence of PLC-γ1 (△). Control samples contained PLC-γ1 but no EGFR (□). The phosphorylation reaction was initiated by addition of ATP. At indicated time points, a 5-μl portion of the reaction mixture was removed and analyzed for phosphoprotein content (*6*). (**B**) Analysis by SDS–polyacrylamide gel electrophoresis (6% gels) and autoradiography of the protein and phosphoprotein content of the reaction mixtures. The lanes contain 30 μl of each reaction mixture, collected 30 min after the initiation of the phosphorylation reaction. Molecular mass markers (in kilodaltons) are indicated. Phosphorylation was performed at 22°C in 60 μl of a solution containing 20 mM Hepes, pH 7.4, 25 mM $MgCl_2$, 4 mM $MnCl_2$, 0.1 mM Na_3VO_4, 100 ng of EGF, and 10 μM [γ-^{32}P]ATP (1.5 Ci/mmol), in the presence of 5 μg of PLC-γ1 and 20 ng of lectin-purified EGFR (*6, 19*). This experiment is representative of three experiments performed with EGFR and four experiments performed with truncated recombinant EGFR (20 ng) corresponding to the cytoplasmic tyrosine kinase domain (*13*).

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