trogens are removed, resulting in enhanced osteoclast development in the marrow, which is responsible for the increased bone resorption and hence the loss of bone.

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19 February 1992; accepted 11 May 1992

Expression of a Ryanodine Receptor–Ca^{2+} Channel That Is Regulated by TGF-β

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Ryanodine receptors (RyRs) are intracellular channels that release calcium ions from the sarcoplasmic reticulum (SR) in response to either plasma membrane depolarization (in skeletal muscle) or increases in the concentration of intracellular free Ca^{2+} (in the heart). A gene (β4) encoding a ryanodine receptor (similar to, but distinct from, the muscle RyRs) was identified. The β4 gene was expressed in all tissues investigated, with the exception of heart. Treatment of mink lung epithelial cells (Mv1Lu) with transforming growth factor beta (TGF-β) induced expression of the β4 gene together with the release of Ca^{2+} in response to ryanodine (but not in response to caffeine, the other drug active on muscle RyRs). This ryanodine receptor may be important in the regulation of intracellular Ca^{2+} homeostasis.

Extracellular stimuli, such as binding of hormones and neurotransmitters, cause a sustained increase in the concentration of intracellular free Ca^{2+} $[Ca^{2+}]_i$, which is mediated by the activation of channels that release Ca^{2+} from intracellular stores (*1*). One class of intracellular Ca^{2+} channel, the inositol trisphosphate ($InsP_3$)–operated Ca^{2+} channel (or $InsP_3$ receptor), is present in most cells (*2, 3*). A second class of intracellular Ca^{2+} channel, the ryanodine receptor, is sensitive to the plant alkaloids ryanodine (Ry) and caffeine and is present in the SR of striated muscle (*4*) as well as in smooth muscle, neurons, eggs, epithelial cells, and secretory cells (*5–13*). The two classes of intracellular Ca^{2+} channels are either present together in a single compartment or segregated, at least partially, in two distinct Ca^{2+} stores (*6, 7, 9, 14*) that may cooperate in complex processes such as the generation of Ca^{2+} oscillations and the propagation of Ca^{2+} waves (*2, 15, 16*). Other tissues and cell lines are not responsive to caffeine, the usual probe for RyRs.

To isolate genes whose expression is regulated by transforming growth factor beta (TGF-β) (*17*), we differentially screened a cDNA library prepared from TGF-β–treated mink lung epithelial cells (Mv1Lu) with the use of cDNA probes from either TGF-β–treated or untreated cells (*18*). One such cDNA clone (β4) was identified and further characterized. The amount of β4 mRNA that was expressed increased as early as 1 hour after the addition of TGF-β (*19*), and after 15 to 24 hours of continuous treatment had increased 30-fold (Fig. 1A). Constitutive and TGF-β–induced expression of β4 mRNA was observed in various cell lines. Induction of β4 mRNA by TGF-β was also observed in pPVU-O 1.5.3 cells (*20*), an Mv1Lu clone transformed with SV40 large T (LT) antigen (Fig. 1B). These cells no longer responded to the antiproliferative effect of TGF-β, but the induction of extracellular matrix proteins and nuclear transcription factors such as jun-B was maintained (*20*), which suggests that TGF-β induction of β4 mRNA levels was not a consequence of its effects on growth.

Treatment of cells with cycloheximide and TGF-β caused accumulation of greater amounts of β4 mRNA than treatment with either agent alone, whereas treatment with 2-aminopurine (2-AP), an inhibitor of serine-threonine kinases (*21*), abolished the effect of TGF-β, and treatment with phorbol myristate acetate left it unchanged (Fig. 1C). Therefore, induction of β4 mRNA (i) appeared to be a primary response to TGF-β because it did not require synthesis of new proteins, and (ii) appeared to be mediated by, or sensitive to, protein kinase or kinases that are different from protein kinase C but responsive to 2-AP. Results of nuclear run-on assays performed on nuclei isolated from control and TGF-β–treated cells indicated that TGF-β stimulated transcription of the β4 gene (Fig. 1D). The β4 mRNA was expressed in several tissues, including those of the spleen, lung, and kidney, but not of the heart (Fig. 1E).

The 2413-nucleotide sequence of clone β4 contained an open reading frame of 641 amino acids followed, after a TAA stop codon, by an untranslated region and a short polyadenylated [poly(A)$^+$] tail. The amino acid sequence of the protein deduced from the sequence of clone β4 was similar to that of the COOH-terminal sequences of human and rabbit RyRs. Two forms of the RyR, skeletal and cardiac, were cloned and are encoded by two different genes (*4*). In Northern (RNA) blots containing poly(A)$^+$ RNA (10 μg) isolated from TGF-β–treated Mv1Lu cells, clone β4 hybridized to a low-abundance mRNA of about 16 kb (*19*), a size similar to those observed for muscle RyR mRNA. The β4 clone thus appeared to be a partial cDNA that encoded a sequence with similarity to the COOH-terminus of skeletal and cardiac RyRs, including the last 7 (M4 to M10) of the 12 putative membrane-spanning regions of these proteins (Fig. 2). The β4

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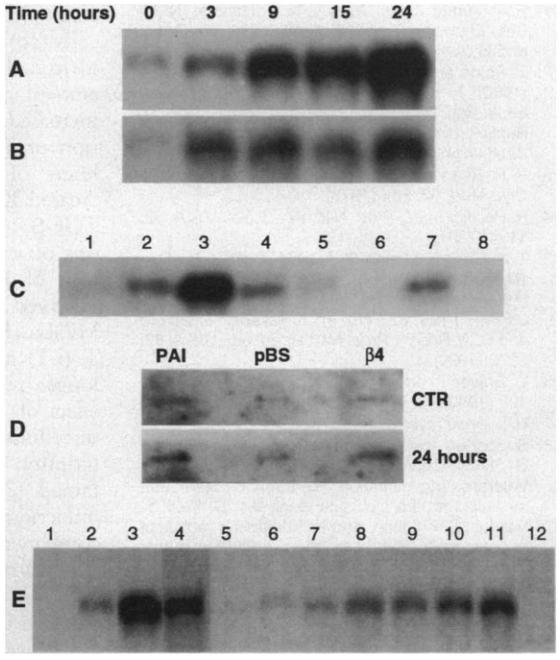

Fig. 1. Expression of β4 mRNA. (**A**) Time course of β4 mRNA induction in Mv1Lu cells treated with TGF-β. Actively growing cells were treated with TGF-β (1 ng/ml) (porcine TGF-β1, R&D Systems, Minneapolis, Minnesota) and RNA was extracted at the indicated time and analyzed by RNase protection assay (*23*). (**B**) Analysis of β4 mRNA expression in SV40 LT antigen–transformed pPVU-O 1.5.3 cells. (**C**) Expression of β4 mRNA in Mv1Lu cells treated for 6 hours with either TGF-β (1 ng/ml), cycloheximide (CHX) (10 μg/ml), 2-aminopurine (2-AP) (10 mM), phorbol ministate acetate (TPA) (100 ng/ml), or combinations of these drugs as indicated. Lane 1, control (CTR); lane 2, TGF-β; lane 3, TGF-β plus CHX; lane 4, CHX; lane 5, 2-AP; lane 6, TGF-β plus 2-AP; lane 7, TGF-β plus TPA; lane 8, TPA. (**D**) Nuclear run-on transcription assay of β4 gene expression in untreated and TGF-β–treated cells. PAI (plasminogen activator inhibitor type I gene), pBS (Bluescript vector, as control). (**E**) Expression of clone β4 mRNA in mink tissues. Lane 1, tRNA; lane 2, Mv1Lu at time zero; lane 3, Mv1Lu after 9 hours; lane 4, skeletal muscle; lane 5, liver; lane 6, kidney; lane 7, lung; lane 8, stomach; lane 9, spleen; lane 10, ileum; lane 11, jejunum; and lane 12, heart.

protein shared an overall sequence identity of 57 and 61% with the skeletal and cardiac RyRs, respectively. The similarity was greater in the most COOH-terminal part that includes the putative transmembrane domains 7 to 10 but decreased in the region that corresponds to the transmembrane domains M4 to M6 (where the skeletal and cardiac proteins also diverge). In particular, the β4 protein appears to lack a sequence corresponding to the putative transmembrane domain M5 that is conserved in the other two receptors (*4*). The similarity of the β4 sequence to skeletal and cardiac RyR sequences suggests that it may encode a new RyR.

In striated muscle, eggs, neurons, and other secretory cells, RyRs are intracellular channels that release Ca^{2+} from stores in response to increased $[Ca^{2+}]_i$ (Ca^{2+}-induced Ca^{2+} release) (*4–7*). These RyRs are activated by caffeine (which apparently sensitizes these channels to the stimulatory effect of $[Ca^{2+}]_i$) and bind Ry with a high affinity, which locks them in an open, low-conductance state (*1, 5, 16*).

At variance with the results in striated muscle, smooth muscle, neurons, eggs, and secretory cells (*5–13*), neither control nor TGF-β–pretreated Mv1Lu cells released intracellular Ca^{2+} in response to caffeine (administered at concentrations ranging from 2 to 90 mM). Nor was any effect of caffeine detected after various treatments that modify Ca^{2+} homeostasis in the cells, such as incubation for 5 min without or with Ca^{2+} (up to 10 mM) in the medium or prestimulation with 0.5% serum a few minutes before treatment with caffeine (*19*).

Treatment (up to 20 min) with Ry (100 to 200 μM) also failed to induce any changes in $[Ca^{2+}]_i$ in control or TGF-β–treated cells, which confirms that the effect of Ry is use-dependent, that is, requires activation of the RyR function to be established (*1, 4–6*). Treatment with a suboptimal concentration of the $InsP_3$-mobilizing nonapeptide bradykinin (Bk, 0.05 μM) to increase $[Ca^{2+}]_i$ and consequently activate Ry binding to RyR (*1, 4–6*) revealed the presence of a Ry-sensitive Ca^{2+} channel in TGF-β–treated cells. Treatment with Ry (200 μM) for 10 min yielded an increase in the size and especially in the duration of the $[Ca^{2+}]_i$ transient induced by Bk as compared with that in Ry-untreated cells (Fig. 3B). The subsequent decreased response to thapsigargin (Tg) (0.1 μM), an SR and endoplasmic reticulum Ca^{2+}–adenosine triphosphatase inhibitor (Fig. 3B) (*6, 7*), indicated that the Ry plus Bk-induced $[Ca^{2+}]_i$ increase in TGF-β–treated cells was caused by release of Ca^{2+} from intracellular stores. In contrast, Ry had no effect in TGF-β–untreated cells (Fig. 3A).

To verify that TGF-β–treated cells express a functional RyR, we investigated Ca^{2+}-store depletion according to the two-

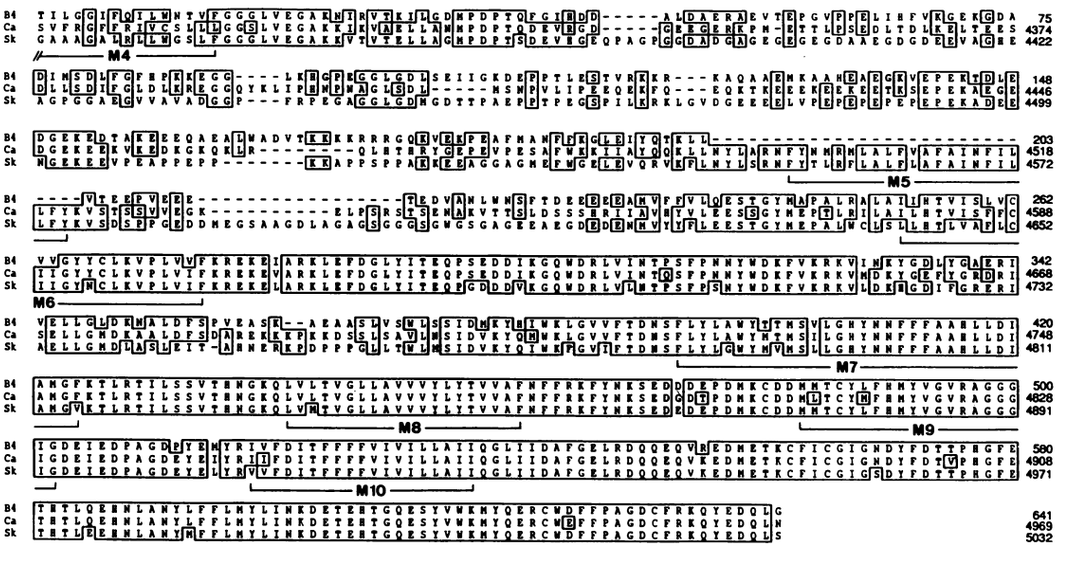

Fig. 2. Sequence alignment of the protein encoded by the cDNA clone β4 and the same region from the rabbit skeletal and cardiac RyR proteins. Conserved amino acids are boxed. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Alignment was obtained with the use of the GCG sequence analysis software.

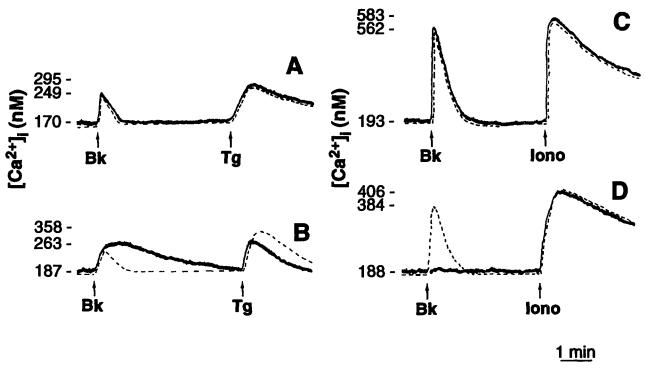

Fig. 3. Expression of a functional Ry-sensitive Ca^{2+} channel in Mv1Lu cells after treatment with TGF-β. (**A** and **C**) Cells cultured under standard conditions. (**B** and **D**) Cells pretreated for 26 to 28 hours with TGF-β (2 ng/ml). The direct effect of Ry (200 μM) followed by suboptimal Bk (0.05 μM) on Ca^{2+} release in TGF-β–treated and untreated cells is shown in (A) and (B). Continuous and dashed lines refer to traces recorded with and without Ry, respectively. Thapsigargin (0.1 μM) was added where indicated. Effect of Ry (10 μM) plus serum (0.5%) pretreatment on the Bk (1 μM) response is shown in (C) and (D). In the first part of the experiment (not shown in the figure) the cells, pretreated for 10 min with or without Ry (continuous and dashed lines, respectively), were exposed to serum (*24*). The cells were then switched to the Ca^{2+}-free medium and treated with Bk, followed by Iono (0.5 μM) where indicated (*6, 24*). Similar results were obtained when Bk was administered in the first, and serum (or Tg, 0.1 μM) in the second step of the protocol (*19*).

step protocol previously described (6). Untreated cells and cells treated with TGF-β were incubated for 10 min, with or without Ry (10 μM), in Ca^{2+}-containing medium and then with serum for a further 10 min to increase $[Ca^{2+}]_i$ and thus facilitate Ry binding to the activated RyR. The cells were then stimulated with an optimal concentration of Bk (1 μM) in Ca^{2+}-free medium. Pretreatment of the cells with Ry (continuous line) prevented the increase in $[Ca^{2+}]_i$ induced by Bk (Fig. 3D) or Tg (*19*) in TGF-β–pretreated cells but not in control cells (Fig. 3C). This inhibition of the response to Bk was not due to the toxicity of Ry because the response of the cells to the Ca^{2+} ionophore ionomycin was normal (*5–7*) (Fig. 3D).

The effect of Ry on Ca^{2+} release induced by Bk indicates that, as observed in other cells (*6, 7, 9*), at least a fraction of the RyR induced by TGF-β in Mv1Lu cells is localized to the same Ca^{2+} store as the $InsP_3$ receptor. Under these conditions, binding of Ry to the TGF-β–induced RyR locks the channel in an open, low-conductance state and depletes this store, which is then unable to discharge Ca^{2+} through the InsP3 receptor after stimulation with Bk (*1, 2, 6, 7*).

Fibroblast growth factor (FGF) reverses the increased expression of the skeletal RyR in BC3H1 cells that occurs together with myogenic differentiation (*22*). Our results demonstrate that the opposite effect took place: the induction of a Ry-sensitive intracellular Ca^{2+} channel by a growth factor. Whether induction by TGF-β of this novel RyR influences the control of Ca^{2+} homeostasis in TGF-β–treated cells is still unclear. Although our results demonstrate that, in TGF-β–treated Mv1Lu cells, this RyR is coexpressed in the same Ca^{2+} store as the $InsP_3$ receptor, no obvious modification in the size of the store was observed (Fig. 3) (*19*). A possible consequence of the induction of this RyR in Mv1Lu cells could be a change in the regulation and in the kinetics of Ca^{2+} discharge from this store after stimulation of the $InsP_3$ receptor (*1, 2, 15, 16*). It is also possible that a fraction of the expressed RyR is not colocalized with the $InsP_3$ receptor but serves to regulate a distinct Ca^{2+} store (*14*).

The Ry-sensitive Ca^{2+} release was thought to occur only in certain cell types. We report the identification of a novel, broadly expressed RyR that can be induced by TGF-β. We have also identified by polymerase chain reaction two other cDNAs related to, but different from, β4 in hepatoma cells (*19*), which indicates that a family of β4-related Ca^{2+} channels may exist. The data suggest that Ry-sensitive Ca^{2+} channels may participate as extensively as $InsP_3$ receptors in the regulation of intracellular Ca^{2+} signaling.

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18. Complementary DNA was synthesized from poly(A)$^+$ mRNA (5 μg) from TGF-β–treated cells with an oligo(dT) primer [U. Gubler, B. J. Hoffman, *Gene* **25**, 263 (1983)] and was subcloned into Lambda Zap arms (Stratagene, La Jolla, CA). We differentially screened primary phages (1.5×10^5) by preparing two filters (Duralon, Stratagene) from each dish and hybridizing them with cDNA probes prepared from TGF-β–treated and untreated cells. Plaques that preferentially hybridized with the probe from TGF-β–treated cells were isolated and rescreened twice. Inserts were subcloned into the Bluescript pBSSK M13+ vector. The nucleotide sequence of the cDNA clone β4 was determined [F. Sanger, S. Nicklen, A. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)] by sequencing double-stranded DNA at least twice on each strand. We confirmed the sequence by sequencing two other independent isolates.
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23. Antisense RNA labeled with [^{32}P]uridine 5′-triphosphate (UTP) was purified on a sequencing gel (8%). After elution, ^{32}P-labeled transcripts (2.5×10^5 counts per minute) were hybridized overnight at 55°C to total RNA (50 μg) in 30 μl of 40 mM Pipes (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and formamide (80%). The same amount of the probe was hybridized to tRNA (50 μg) as a control. The mixture was treated with ribonuclease (RNase) A (40 μg/ml) and RNase T1 (2 μg/ml) for 1 hour at 33°C. After further treatment at 37°C for 30 min with 10 μl of 20% SDS and 2.5 μl of proteinase K (20 mg/ml), the samples were extracted with phenol-chloroform, precipitated with ethanol, and analyzed on a sequencing gel (8%). A protected fragment of 267 nucleotides, corresponding to the sequence between the unique Nru I and Bst EII sites of clone pβ4.1, was observed after autoradiographic exposure of dried gels. The pPVU-O 1.5.3 cells (*20*), a clone of Mv1Lu cells transfected with the SV40–large T antigen (provided by J. Massagué and M. Laiho), were grown for 2 days and then treated with TGF-β (1 ng/ml). RNA was extracted at the indicated time and β4 mRNA concentrations were evaluated by RNase protection assay. Immediately before the experiments, cells were tested for expression of large T antigen and found, by immunofluorescence with specific monoclonal antibody, to be more than 99% positive. We also tested cells for their responses to the antiproliferative effects of TGF-β by evaluating DNA synthesis with a monoclonal antibody to bromodeoxyuridine, and found them to be unresponsive [V. Sorrentino, R. Pepperkok, R. L. Davis, W. Ansorge, L. Philipson, *Nature* **345**, 813 (1990)]. Nuclei were isolated from untreated cells or cells treated with TGF-β for 24 hours, and newly synthesized RNAs were labeled with [^{32}P]UTP, purified, and hybridized as described [G. Morrone, R. Cortese, V. Sorrentino, *EMBO J.* **8**, 3767 (1989)]. Tissue expression was investigated by RNase protection analysis of total RNA (50 μg)

extracted from the indicated tissues of a young mink, as described (*21*).

24. TGF-β–treated or untreated subconfluent Mv1Lu cells were detached from petri dishes by treatment with trypsin (0.05%) and 0.02 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and allowed to recover in growth medium supplemented with 10 mM Hepes (pH 7.4) with gentle swirling for at least 1 hour at 37°C. After washing, the cells were resuspended in complete KRH medium [125 mM NaCl, 5 mM KCl, 12 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 10 mM Hepes (NaOH buffer, pH 7.4)] loaded with fura-2, and analyzed in a Perkin-Elmer LS-5B fluorimeter as described (*5–7*). Cells ($5 \pm 1 \times 10^5$) suspended in complete KRH medium were first incubated for 10 min, with or without ryanodine (10 μM) (dashed or continuous lines, respectively). Cells in Fig. 3, A and B, were then switched to a Ca^{2+}-free KRH medium by addition of excess EGTA (3 mM) and challenged with Bk (0.05 μM) and with Tg (0.1 μM) where indicated (*6, 7*). Cells in Fig. 3, C and D, were treated with fetal calf serum (0.5%) for 10 min to induce, by means of a large increase in $[Ca^{2+}]_i$, the activation of the RyR and promote the effect of the alkaloid. The cells were collected by centrifugation, washed, and resuspended in the same Ca^{2+}-containing KRH medium, and incubation was continued for 15 min to assure refilling of unaffected Ca^{2+} pools and recovery from serum stimulation. Excess EGTA (3 mM) was then added, followed 2 min later by the addition of Bk (1 μM) and then 3 to 4 min later by the addition of Iono (0.5 μM), where indicated. Data represent the results of eight to ten experiments. The $[Ca^{2+}]_i$ calibration values are shown on the left-hand side of the traces.

25. We thank L. Philipson for support, J. Tooze, G. Draetta, and S. Courtneidge for discussions and critical reading of this manuscript, and J. Meldolesi for comments on the manuscript. G.G. was supported by a postdoctoral fellowship from AIRC.

14 February 1992; accepted 1 May 1992

No Requirement for p56^{lck} in the Antigen-Stimulated Clonal Deletion of Thymocytes

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Activation of protein-tyrosine kinases (PTKs) is required for signal transduction during T cell activation, although the pathway used during thymic selection is unknown. An in vitro system was established in which T cell receptor transgenic thymocytes underwent clonal deletion in response to peptide antigen. The effects of two PTK-specific inhibitors, herbimycin A and genistein, on the clonal deletion of immature thymocytes and the activation of mature thymocytes were examined. Clonal deletion occurred while T cell activation was inhibited and when no p56^{lck} activity was evident. Thus, p56^{lck} is not required for the antigen-stimulated step of clonal deletion of immature thymocytes, and negative selection proceeds via a distinct pathway.

Because many growth factor receptors have cytoplasmic tyrosine kinase domains, PTKs are thought to participate in cell growth. The T cell antigen receptor (TCR) complex, which is not itself a PTK, functionally associates with at least two Src-like PTKs, p56^{lck} and p59^{fyn} (*1*). A model has been proposed in which the activation of PTK is the essential first step of biochemical change during T cell activation. Stimulation of the TCR results in the tyrosine phosphorylation of several intracellular substrates within 5 s, whereas the hydrolysis of phosphatidylinositides (PI) does not occur until approximately 30 to 40 s after stimulation (*2*). Furthermore, stimulation of the TCR induces tyrosine phosphorylation of phospholipase C (PLC)–γ1, presumably through the activation of PTKs (*3*). PLC-γ1 catalytic activity itself may be augmented by tyrosine phosphorylation of PLC-γ1 (*4*). The PLC-γ1 catalyzes the breakdown of PI, leading to the generation of inositol trisphosphate (IP_3), which has been implicated in the elevation of intracellular Ca^{2+} levels, and diacylglycerol, a potent activator of protein kinase C. Indeed, treatment of T cells with both phorbol esters (to activate protein kinase C) and Ca^{2+} ionophores (to increase cytosolic Ca^{2+}) can mimic phenotypic changes observed with T cell activation. Two potent PTK-specific inhibitors with distinct modes of action, herbimycin A (*5*) and genistein (*6*), inhibit PI turnover induced by TCR stimulation and result in the failure of T cell activation (*7, 8*). Lastly, p56^{lck} and p59^{fyn} are both required for T cell activation (*9, 10*). Thus, the activation of PTKs is required for signal transduction during T cell activation through the TCR.

Within the thymus, T cells undergo a stringent process of positive and negative selection during development to emerge as self-tolerant, major histocompatibility complex (MHC)–restricted functional cells (*11*). Although the selection process is mediated by the TCR, which also mediates T cell activation, the signal transduction pathway in the thymic selection process is unknown. We investigated whether the signal transduction pathway in the thymic selection of immature T cells is similar to that in T cell activation of mature T cells. Using mice transgenic for a TCR from a T cell hybridoma DO11.10 that recognizes chicken ovalbumin in the context of the MHC class II molecule I-A^d, we showed that exposure of immature thymocytes to self antigen [cOVA(323-339) peptide derived from chicken ovalbumin] induces intrathymic apoptosis in vivo (*12*). Thus, negative selection occurs by apoptosis. With the same TCR transgenic mice, we developed an in vitro clonal deletion model in which both antigen-induced apoptosis of immature CD4$^+$CD8$^+$TCR^{lo} transgenic thymocytes and antigen-induced activation of mature CD4$^+$CD8$^-$ transgenic thymocytes could be observed simultaneously (*13*). A similar in vitro clonal deletion system was reported (*14*). Here, we investigated the signal transduction mechanism of clonal deletion by using pharmacologic agents.

Transgenic thymocytes were cultured with I-A^{d+} antigen-presenting cells in the presence of cOVA(323-339) peptide or cOVA(324-334) peptide (control peptide) with the use of the in vitro system (*13*). The cOVA(324-334) peptide binds to I-A^d (*15*) but does not activate the transgenic TCR (*12*). Three-color analysis of the cultured thymocytes was done with monoclonal antibodies (MAbs) to CD4, CD8, and the transgenic TCR (KJ1-26, a clonotype-specific MAb). The number of mature CD4$^+$CD8$^-$ thymocytes present at the end of 20 hours of culture was similar to the number present at the beginning of culture (Fig. 1, A and B). However, greater than 95% of the immature CD4$^+$CD8$^+$KJ1-26^{lo} thymocytes died after 20 hours of culture in the presence of cOVA(323-339) peptide (Fig. 1, C and D). The presence of cOVA(324-334) control peptide had virtually no effect on clonal deletion or T cell activation. DNA fragmentation was augmented by the cOVA(323-339) peptide as early as 5 hours after culture (Fig. 2), although thymocytes showed background spontaneous DNA fragmentation when cultured in vitro with the cOVA(324-334) peptide. To define the potential role of PTKs in thymocyte clonal deletion and activation, we added either herbimycin A or genistein to the in vitro deletion experiments. Neither herbimycin A nor genistein blocked the clonal deletion of CD4$^+$CD8$^+$KJ1-26^{lo} thymocytes induced by the cOVA(323-339) peptide (Fig. 1, C and D). The DNA fragmentation induced by the cOVA(323-339) peptide was also not inhibited (Fig. 2).

Although no change could be seen in the number of mature CD4$^+$CD8$^-$ thymocytes with or without cOVA(323-339) peptide (Fig. 1, A and B), the addition of cOVA(323-339) peptide for 20 hours acti-

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