bottom of the smaller half of the specimen (Fig. 1, a and b). This object differs in appearance and density from that of the enclosing matrix and surrounding eggshells and resembles an embryo in an early stage of development (*16, 17*). It is rare, but not unusual, for soft organic matter to be preserved through diagenesis (for example, jellyfish, worms, tendons) (*18*). Moreover, the inferred early-stage embryo in the fossil egg is comparable in size to what would be found in modern reptile eggs before oviposition. Most reptiles, except turtles (*19*), lay eggs containing embryos whose development is about equivalent to a 48-hour chick embryo.

The presence of a pathological eggshell and the inverted eggshell curvature in the hingelike area on the Jurassic egg indicate that the egg was retained beyond normal oviposition, possibly as a result of traumatic events leading to the death of the mother. It is not known how long it takes for pathological shell to form; however, the mother must be alive for this process. In such double-layered eggs the embryo may still develop for a short time but will ultimately suffocate for lack of oxygen caused by misalignment of the pore canals (*20*). Because no other egg has been found, it is possible that the mother was disturbed during oviposition and had no chance to lay this last egg. The oviducal position of the egg and burial with the gravid female would also explain the fine preservation of the egg and the enclosing eggshell. The egg may have been fractured and opened during the death of the mother, and the oviduct may have held the two halves together until preservation by sediments.

Whole dinosaur eggs, pathological eggshell, and embryonic remains are extremely rare, especially before the Cretaceous (*21, 22*). However, with an eggshell structure that has a new type of pore canal and unidentifiable embryonic remains, we are as yet unable to link the specimen to any of the dinosaurs of the Cleveland-Lloyd fauna. We may, however, exclude the sauropods as they have a totally different eggshell structure (*23*). Reliable taxonomic referral, at this time, can only be based on the presence of hatchlings or embryos of known identity or an established fossil record of a structural type that has been clearly identified with a taxon.

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24. We thank M. Philbrick for photography; the doctors and technicians of the American Fork Hospital and St. Benedicts Image Center for CAT scanning; the technicians of Columbia Scientific Inc. and Dynamic Digital Displays for three-dimensional images; and Pixar for three-dimensional animation. Supported by Brigham Young University, Colorado University Museum, and Whitehall Foundation, Inc.

27 September 1988; accepted 6 February 1989

Mutations in a Protein Kinase C Homolog Confer Phorbol Ester Resistance on *Caenorhabditis elegans*

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The *tpa-1* gene mediates the action of tumor-promoting phorbol esters in the nematode *Caenorhabditis elegans*. A genomic fragment that constitutes a portion of the *tpa-1* gene was cloned by Tc*1* transposon tagging and was used as a probe to screen a nematode complementary DNA library. One of the isolated complementary DNA clones had a nucleotide sequence that predicts a polypeptide of 526 amino acids. The predicted amino acid sequence revealed that the predicted *tpa-1* protein sequence is highly similar to protein kinase C molecules from various animals, including man.

PHORBOL ESTERS SUCH AS 12-O-TETradecanoyl phorbol-13-acetate (TPA) and phorbol-12,13-didecanoate (PDD) are potent tumor promoters and cause characteristic responses in many in vivo and in vitro biological systems (*1*). These compounds induce severe disturbances in the behavior and growth of the soil nematode *Caenorhabditis elegans* (*2*). The effects are specific, since phorbol esters that are not tumor-promoting do not appear to cause any disturbances in *C. elegans*. This relation between tumor promotion and effects on the nematode has led to the suggestion that *C. elegans* has a cellular component similar to that which mediates tumor promotion (*3*). To identify this cellular component and clarify the mechanism of action of phorbol esters, we have isolated and analyzed TPA-resistant mutants that show little TPA-induced phenotypic change. We have also investigated the gene *tpa-1*IV, defined by the mutants, which is involved in the action of TPA (*4*). Here, we report the molecular cloning of the *tpa-1* gene by Tc*1* transposon tagging (*5*) and its sequence analysis.

To tag *tpa-1* with transposon Tc*1*, we isolated spontaneous TPA-resistant mutants of the strains NJ82 and RW7097 (*6*) that could grow in TPA (1 μg/ml). The phenotypes of spontaneous mutants exemplified by MJ566 (*k532*), isolated from RW7097 (Fig. 1), were similar to those obtained with ethylmethane sulfonate (EMS)–induced *tpa-1* mutants (*4*).

Both the complementation test (Fig. 1, D and I) of MJ566 (*k532*) against the EMS-induced *tpa-1* standard reference mutant MJ500 [*tpa-1(k501)*IV] and three-factor crosses (7) indicated that MJ566 harbors the *k532* mutation in *tpa-1*. The *tpa-1* mutants MJ562 (*k529*), MJ564 (*k531*), and MJ566 (*k532*) were used for our analysis; the first two were independently isolated from the mutator strain NJ82, and had a phenotype similar to that of RW7097-derived MJ566 (*k532*).

For molecular analysis, MJ562 (*k529*) was ten times outcrossed to the wild-type N2 and N2-derived *tpa-1*IV–linked standard genetic markers (*8*). This outcrossed mutant was designated MJ563 (*k530*). The Tc*1* polymorphic pattern in Southern analysis of the genomic DNA from MJ563, N2, and

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the standard genetic markers revealed novel 3.2-kb and 3.4-kb bands in the Hind III digest of the MJ563 DNA (Fig. 2A). Three-factor crosses with *dpy-9* and *unc-17* as genetic markers mapped the 3.2-kb band at less than 0.9 map units from the *tpa-1* locus, whereas the 3.4-kb band was located 1.8 map units from *tpa-1*.

Excision of the 1.6-kb Tc*1* fragment by Eco RV digestion (*9*) of the 3.2-kb fragment resulted in the recovery of a 1.6-kb Tc*1* flanking sequence. This 1.6-kb flanking sequence hybridized to the 2.4-kb Hind III fragment in the parent strain NJ82 and in N2 and the standard genetic markers. Theoretically, the insertion of the 1.6-kb Tc*1* transposon into the 2.4-kb fragment should result in a 4-kb fragment instead of the 3.2-kb molecule that we found. This inconsistency results from the repeated outcrosses of the original isolate MJ562 (*k529*), which we performed to obtain MJ563 (*k530*); these outcrosses must have been accompanied by a chromosomal rearrangement that generated the 3.2-kb fragment. Indeed, the 1.6-kb sequence derived from MJ563 (*k530*) hybridized to a 4-kb Hind III digest of the MJ562 (*k529*) DNA. Here it is also important that the size shift of the hybridizing fragment from 2.4 to 4 kb was associated with acquisition of TPA resistance. This association was also true with MJ564 (*k531*). The clear association between the size shift and TPA resistance was demonstrated with the mutant MJ566 (*k532*) in which the expected size shift from 2.4 to 4 kb and vice versa correlated with acquisition and loss of TPA resistance, respectively (Fig. 2B). These results indicate that the 2.4-kb fragment constitutes at least a portion of the *tpa-1* gene.

The 1.6-kb Tc*1* flanking sequence was used to screen a *C. elegans* cDNA library (*10*) for a cDNA derived from a *tpa-1* gene transcript. One of the positive clones, designated cDNA-1, contained a 1.8-kb insert, which hybridized to the same 2.4-kb Hind III fragment as that recognized by the 1.6-kb flanking sequence probe. The sequence of this insert (*11*) dictated a part of a putative open reading frame with the stop codon TGA at position 1581 that can encode a protein of 526 amino acid residues (*12*). The 5′ region of the mRNA corresponding to cDNA-1 probably extends beyond the 5′ end of this cDNA, since there is no potential initiation codon at this end.

A computer search for homologous proteins with the amino acid sequence deduced from cDNA-1 detected the highest homology to protein kinase C (PKC) molecules of various animals. The PKC family contains multiple members with a similar primary structure (*13–17*); each member is composed of a single polypeptide with two major functional domains, an NH_2-terminal regulatory domain and a COOH-terminal kinase domain. The regulatory domain contains two conserved regions, designated C1

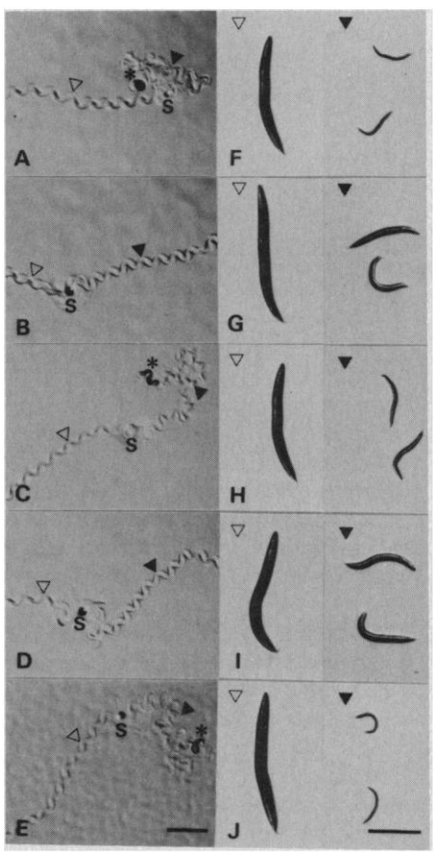

Fig. 1. Behavior and growth of spontaneous TPA-resistant mutants represented by MJ566 (*k532*). (**A** to **E**) Behavior and (**F** to **J**) growth of *C. elegans*, treated or untreated with TPA (1 μg/ml). The animals in (A) to (E) were treated at the L4 stage for 12 hours at 20°C; those in (F) to (J) from the L1 stage for 4 days at 20°C, a time long enough for L1 animals to become gravid adults. The results were typical of those found in $n \geq 5$ similar experiments. (A and F) RW7097, +/+TPA-sensitive control from which MJ566 was derived. The treated animals developed uncoordinated locomotion (left an abnormal track on a bacterial lawn), were arrested at no later than the L3 stage by size, and produced no offspring. (B and G) MJ566, *k532/k532* homozygote for the TPA-resistant mutation *k532*. The treated animals moved normally (left a normal track). They also grew gravid, although they grew slower and smaller in the presence of TPA than in its absence. (C and H) *dpy-5fer-1/++;k532/+* heterozygote. Treated heterozygotes developed uncoordinated locomotion indistinguishable from that of +/+ sensitive controls and produced very few offspring. (**D** and **I**) *dpy-5fer-1/++;k532/k501* heterozygote for *k532* and the *tpa-1* reference mutation *k501*. The treated heterozygotes behaved and grew just as did their parents MJ566 (*k532*) and MJ500 (*k501*), indicating that the *k532* and *k501* mutations are in the same gene *tpa-1*. (E and J) *dpy-5fer-1/++;+/+* TPA-sensitive control. The result was identical to that shown in (A) and (F). ▼, TPA-treated animals; ▽, untreated animals; S, starting point; asterisks, animals that developed uncoordinated locomotion after TPA treatment. Scale bars: (A to E) 1.0 mm; (F to J) 0.5 mm. Locomotor behavior was examined by placing TPA-treated or untreated *C. elegans* on bacterial lawns on agar plates and allowing them to leave tracks [TPA-sensitive animals develop uncoordinated locomotion within 60 min of treatment (*2*)]. Growth was observed by culturing animals on agar plates with or without TPA (*4*). Genetic construction for the tested animals and complementation test against the *tpa-1* standard reference mutant MJ500 (*k501*) were carried out as reported (*4*).

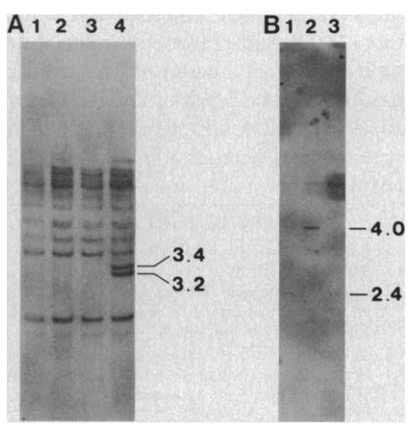

Fig. 2. Identification of genomic DNA related to the *tpa-1* gene. (**A**) Tc*1* polymorphism in the spontaneous *tpa-1* mutant MJ563 (*k530*). DNA samples of N2 (lane 1), *dpy-9(e12)*IV (lane 2), *dpy-9(e12)unc-17(e113)*IV (lane 3), and MJ563 (*k530*)IV (lane 4) were analyzed by DNA hybridization with Tc*1* DNA as a probe. (**B**) Size shift of the 2.4-kb Hind III restriction fragment due to the insertion and the excision of Tc*1*. DNA samples of RW7097 (lane 1), MJ566 (*k532*) (lane 2), and the revertant MJ567 (*k533*) (lane 3) were examined by DNA hybridization with the 1.6-kb Tc*1* flanking sequence as a probe, which was identified in the experiment in (A). Fragment sizes are indicated on the right in kilobases (Hind III–cut λDNA fragments were used as size markers). The TPA-sensitive revertant MJ567 (*k533*) was isolated from the TPA-resistant mutant MJ566 (*k532*), derived from RW7097, by selecting a worm that grew in the absence of but not in the presence of TPA (1 μg/ml). Genomic DNA was extracted as in (*26*). DNA samples were digested with Hind III (Takara Shuzo Co., Kyoto) to completion and subjected to 1% agarose gel electrophoresis (50 μg per lane). DNA blotting was performed as described (*27*). Tc*1* DNA was prepared from pCe2003, a Tc*1*-containing plasmid by Eco RV digestion. Hybridization probes, Tc*1* DNA and the 1.6-kb Tc*1* flanking sequence, were labeled with biotin-11-deoxyuridine triphosphate with a nick-translation kit (BRL). Hybridization was carried out in a solution containing 45% formamide at 42°C. The membranes (Biodyne A, Pall Co., New York) were washed with 2× standard sodium citrate (SSC) and 0.2× SSC at room temperature, and then with 0.16× SSC at 50°C; each SSC solution contained 0.1% SDS. Hybridization bands were detected with streptavidin-alkaline phosphatase complex with a color detection kit (BluGENE, BRL).

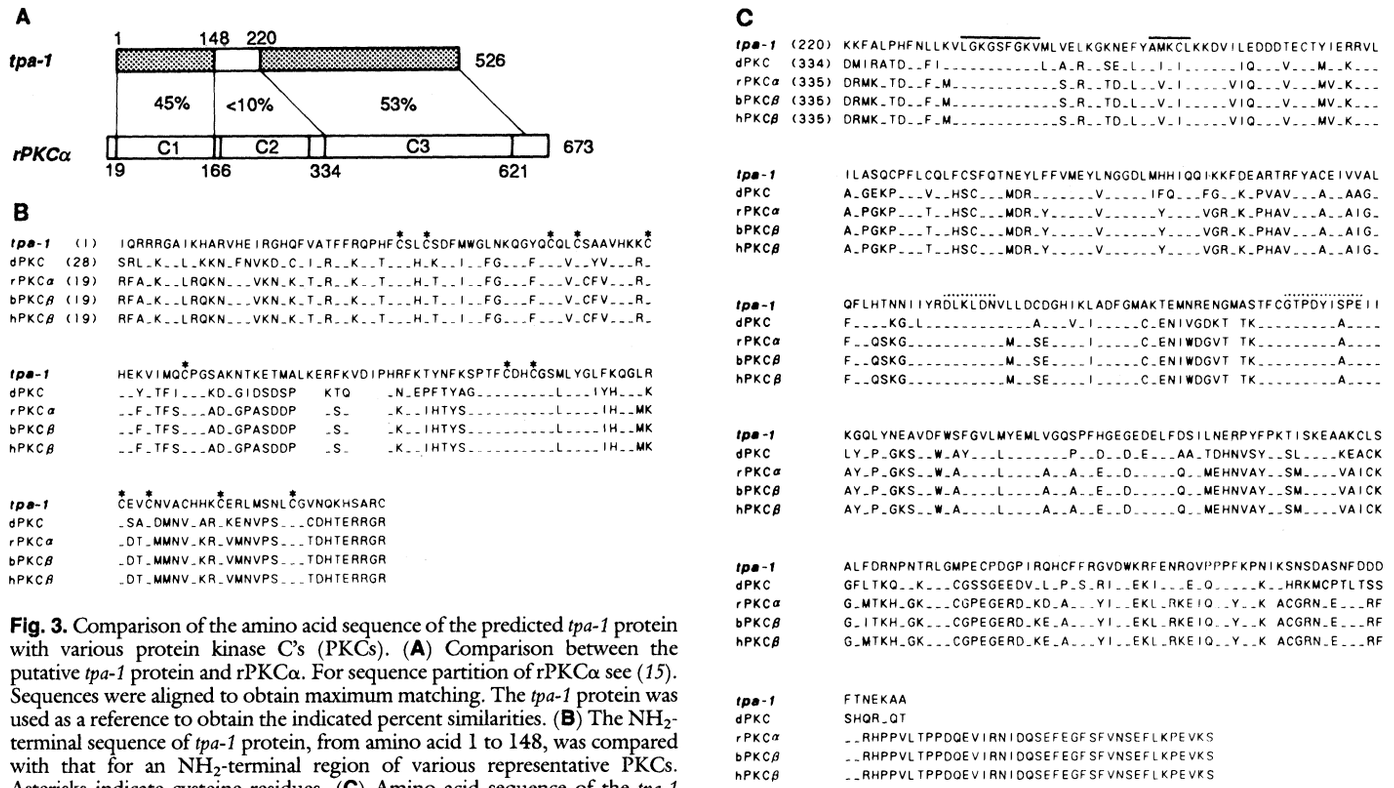

Fig. 3. Comparison of the amino acid sequence of the predicted *tpa-1* protein with various protein kinase C's (PKCs). (**A**) Comparison between the putative *tpa-1* protein and rPKCα. For sequence partition of rPKCα see (*15*). Sequences were aligned to obtain maximum matching. The *tpa-1* protein was used as a reference to obtain the indicated percent similarities. (**B**) The NH_2-terminal sequence of *tpa-1* protein, from amino acid 1 to 148, was compared with that for an NH_2-terminal region of various representative PKCs. Asterisks indicate cysteine residues. (**C**) Amino acid sequence of the *tpa-1* protein from residue 220 onwards was compared with the kinase domain sequence of PKCs. Bold lines on the top of the sequence indicate the characteristic sequence found in an ATP-binding site of various protein kinases (*24*). Broken lines on the sequence indicate the sequences characterized by a serine-threonine kinase (*24*). Residues in various PKCs identical to those in the *tpa-1* protein are indicated by dashes. Insertions are shown as spaces. A number in parentheses represents the residue number from the first amino acid in the figure. dPKC, *Drosophila* PKC (*16*); rPKCα, rabbit PKCα (*15*); bPKCβ, bovine PKCβ (*13*); hPKCβ, human PKCβ (*14*). [The naming of rPKCα (*15*) was later changed to rPKCβ II (*23*).]

and C2, and the kinase domain contains a third conserved region, C3 (Fig. 3A).

In the *tpa-1* protein predicted from cDNA-1, about 45% of the amino acids 1 to 148 are identical to those in the C1 region of other animal PKCs (Fig. 3, A and B). A twice tandemly repeated cysteine-rich sequence of the motif C-X_2-C-X_{13}-C-X_2-C-X_7-C-X_7-C (*18*), which characterizes all known PKCs (*13–17*) and shows resemblance to a putative DNA- and metal-binding finger structure (*19*), is conserved in this region. Also, the *tpa-1* protein contains no obvious regions that may be associated with an EF hand (*20*) or with a lipocortin-type calcium binding consensus sequence (*21*). The region of amino acids 149 to 219 bears no similarity to any region of the reported PKCs (Fig. 3A). The *tpa-1* protein has one notable difference in the regulatory domain; that is, it lacks a sequence corresponding to the C2 region of other PKCs. New members of the PKC family that also lack a C2 region have been reported in rat (*22*) and rabbit (*23*). The evolutionary conservation of *tpa-1*–like molecules implies their importance to animals in general.

The amino acid sequence of the *tpa-1* protein from residues 220 to 526 exhibits substantial homology to the kinase domain of various other protein kinases, such as adenosine 3′,5′-monophosphate (cAMP)– and guanosine 3′,5′-monophosphate (cGMP)–dependent protein kinases (40% identical in each case). It shares, however, the highest homology (53 to 63%) to PKCs (Fig. 3, A and C). The *C. elegans* protein in this region also contains the sequences LGXGXXGXV (amino acids 233 to 241) and AXKXL (amino acids 254 to 258), both of which are assumed characteristic of an adenosine triphosphate (ATP) binding site of protein kinases (*18, 24*). The sequences DLKLDN (amino acids 351 to 356) and GTPDYISPE (amino acids 389 to 397) suggest that the *tpa-1* protein is a serine-threonine kinase like other known PKCs (*18, 24*).

Our results indicate that the *tpa-1* gene codes for a protein similar to PKCs of other animals. We previously demonstrated (*4*) that mutations in the *tpa-1* gene correct specific behavioral and developmental disturbances induced by tumor-promoting phorbol esters. These results are consistent with the idea that these compounds exert their biological effects through interaction with PKC (*25*). Possibly, the phorbol esters interact with the *tpa-1* gene product to induce the observed phenotypes in *C. elegans*. Whether the induced phenotypes reflect a regulatory role for the *tpa-1* protein in behavior and growth of the animal remains to be seen.

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7. Y. Tabuse and J. Miwa, unpublished data.
8. Outcrossing of NJ82-derived MJ562 to N2 should

reduce the Tc1 copy number and also make the MJ562 Tc1 profile in the genome similar to that for N2. The standard genetic markers used for outcrossing were the *tpa-1*–linked genes *dpy-9(e12)*IV and *dpy-9(e12)unc-17(e113)*IV.
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19 August 1988; accepted 19 December 1988

In Vivo Modulation of Cytolytic Activity and Thy-1 Expression in TCR-γδ$^+$ Intraepithelial Lymphocytes

LEO LEFRANCOIS AND THOMAS GOODMAN

Although the functional aspects of the αβ T cell antigen receptor (TCR) found on most peripheral T cells are well described, the function of the γδ TCR remains unclear. Murine intraepithelial lymphocytes (IEL) of the small intestine are CD8$^+$, express the γδ TCR, and are constitutively lytic. Fresh IEL from germ-free mice had no lytic activity. Moreover, whereas IEL from normal mice are 30 to 50 percent Thy-1$^+$, IEL from germ-free did not express Thy-1. Acclimation of germ-free mice to nonsterile conditions resulted in the generation of Thy-1$^+$ IEL and induction of lytic activity. Thus CD8$^+$ TCR-γδ IEL were regulated by externally derived stimuli via a specific functional interaction between IEL and gut-associated antigens.

THE MUCOSAL IMMUNE SYSTEM IS one of the first lines of defense against bacterial and viral invasion. In the small intestine at least three anatomically distinct lymphoid areas are evident: (i) Peyer's patches, (ii) lamina propria of the villi, and (iii) intraepithelial lymphocytes (IEL) residing between the columnar epithelial cells of the villi. In the mouse, IEL are made up of both Thy-1$^+$ and Thy-1$^-$ CD8$^+$ CD3$^+$ T cells (*1, 2*). This latter subset of T cells has been detected only in this location. The relation of the Thy-1$^-$ IEL to Thy-1$^+$ IEL and to other peripheral T cells is unknown. Recently, we reported that murine CD8$^+$ IEL express heterodimeric T cell receptors (TCRs) composed of γ and δ chains (*3*). Most other murine TCR-γδ–expressing cells described are of the CD4$^-$8$^-$ phenotype and are found in very small numbers in the thymus (*4, 5*), spleen (*6*), or peripheral blood (*5*), or in the skin as dendritic epidermal cells (DEC) (*7, 8*). Most of the CD8$^+$ T cell populations express TCRs composed of α and β chain heterodimers (*9, 10*). In addition, freshly isolated IEL are constitutively lytic, unlike other CD8$^+$ peripheral T cells, implying that in situ activation of IEL occurs under normal circumstances (*3*). However, the origin of the antigens involved in the in vivo activation of IEL has not been determined. Thus, although it is clear that TCR-γδ expressing CD8$^+$ IEL constitute a T cell lineage distinct from other CD8$^+$ T cells, their role in vivo remains unclear.

To determine the nature of the antigens involved in IEL activation, we analyzed IEL from germ-free mice for lytic activity. We used a redirected lysis assay in which a monoclonal antibody (MAb) to CD3 is bound to an Fc receptor–positive target cell to detect all lytic activity regardless of receptor specificity (*11, 12*). The IEL in all experiments were ~95% CD3$^+$8$^+$. In five of five experiments with IEL isolated from a total of 50 Swiss (NIH) germ-free mice virtually no lytic activity was detectable, although the same MAb efficiently directed the lytic activity of a cytolytic T cell clone. Results from a representative experiment are depicted in (Fig. 1). In contrast, IEL from Swiss (NIH) mice reared under standard conditions or IEL from C57BL/6 or BALB/c mice, had cytolytic activity (Table 1) (*13*). The absence of lytic activity in IEL from germ-free mice indicated that environmental antigens were responsible for the in vivo activation of IEL.

We also phenotypically characterized IEL from germ-free mice. Although all CD8$^+$ IEL from germ-free mice expressed CD3, there were few cells with detectable Thy-1 antigen (Fig. 2). However, 35% of IEL from Swiss (NIH) mice were Thy-1$^+$, a percentage similar to our results with IEL from C57BL/6 mice (*3*). Therefore, IEL in the absence of antigenic stimuli in vivo were not cytolytic and did not express Thy-1. If there were a relation between lytic activity and Thy-1 expression in IEL, then removal of Thy-1$^+$ cells from IEL from normal mice should result in the loss of lytic activity. We isolated IEL from C57BL/6 or Swiss (NIH) mice and removed Thy-1$^+$ cells with MAb to Thy-1 and complement. The remaining populations, which were routinely <5% Thy-1$^+$, were assayed for cytolytic activity (Table 1). In all cases, the lytic activity of IEL was reduced to background levels after removal of Thy-1$^+$ cells; thus the lytic activity of IEL resided primarily in the Thy-1$^+$ subset.

To further substantiate the involvement of antigenic stimulation in the generation of Thy-1$^+$ IEL and cytolytic activity, we introduced germ-free mice into our standard mouse colony. After four weeks, IEL from such "acclimated" mice were assayed for cytolytic activity and for Thy-1 expression

Table 1. Lytic activity of IEL after removal of Thy-1$^+$ cells. IEL were treated with antibody to Thy-1, followed by two successive treatments with complement (C). The resulting populations were <5% Thy-1$^+$. Starting populations were 37%, 34%, and 42% Thy-1$^+$ for experiments 1 to 3, respectively. IEL were isolated from C57B1/6J mice (experiments 1 and 2) or from Swiss (NIH) mice (experiment 3). One lytic unit is the number of effector cells required to achieve 20% specific lysis of 2.5×10^3 target cells. Target cells were ^{51}Cr-labeled P815 (DBA/2 mastocytoma) with the addition of 1 μg of anti-CD3 per milliliter. Lytic activity in all cases without the addition of anti-CD3 was negligible.

Experiment	Lytic activity (lytic units per 10^6 cells)	
	C alone	anti–Thy-1 + C
1	9.3	<0.1
2	8.0	<0.1
3	9.2	<0.1

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