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20. We made these measurements using a four-probe method to avoid errors due to the resistance of the

electrical contacts (silver epoxy) to the gold films. In addition to the possibility of practical applications of these experiments, careful measurement of resistivity may allow an estimation of the degree of atomic (gold-gold) contact between the two surfaces.

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Thymocyte Expression of RAG-1 and RAG-2: Termination by T Cell Receptor Cross-Linking

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The expression of the V(D)J [variable (diversity) joining elements] recombination activating genes, RAG-1 and RAG-2, has been examined during T cell development in the thymus. In situ hybridization to intact thymus and RNA blot analysis of isolated thymic subpopulations separated on the basis of T cell receptor (TCR) expression demonstrated that both TCR⁻ and TCR⁺ cortical thymocytes express RAG-1 and RAG-2 messenger RNA's. Within the TCR⁺ population, RAG expression was observed in immature CD4⁺CD8⁺ (double positive) cells, but not in the more mature CD4⁺CD8⁻ or CD4⁻CD8⁺ (single positive) subpopulations. Thus, although cortical thymocytes that bear TCR on their surface continue to express RAG-1 and RAG-2, it appears that the expression of both genes is normally terminated during subsequent thymic maturation. Since thymocyte maturation in vivo is thought to be regulated through the interaction of the TCR complex with self major histocompatibility complex (MHC) antigens, these data suggest that signals transduced by the TCR complex might result in the termination of RAG expression. Consistent with this hypothesis, thymocyte TCR cross-linking in vitro led to rapid termination of RAG-1 and RAG-2 expression, whereas cross-linking of other T cell surface antigens such as CD4, CD8, or HLA class I had no effect.

DURING DEVELOPMENT, THE THYMIC rudiment is seeded by bone marrow-derived stem cells that have not yet undergone TCR gene rearrangement (1). As thymic cells mature, each cell assembles the genes that encode a TCR α - β or γ - δ heterodimer through the recombination of individual variable (V), diversity (D), and joining (J) germline elements for each chain (2). This assembly process is known as V(D)J recombination. In general, mature T cells express only a single TCR (3). It is the specificity of the TCR that appears to determine the fate of the thymocyte as it undergoes thymic selection (4). Recently, two genes that appear to encode components of the V(D)J recombinase, RAG-1 and RAG-2, have been cloned (5). Coexpression of RAG-1 and RAG-2 is both necessary and sufficient to induce V(D)J recombination in fibroblasts, and their

expression in lymphoid cell lines correlates precisely with the presence of V(D)J recombinase activity (5, 6). These findings suggested that the regulation of RAG-1 and RAG-2 expression might contribute to the control of TCR recombination during lymphoid ontogeny. To explore this possibility, we have examined RAG expression in lymphoid cells developing in the thymus.

The intrathymic expression of RAG-1 and RAG-2 mRNA transcripts was analyzed by in situ hybridization (Fig. 1). Sections of thymus from a 32-day-old mouse were hybridized with RAG-1 antisense (Fig. 1B) or RAG-2 antisense (Fig. 1C) RNA probes. Control probes included RAG-1 sense and RAG-2 sense (Fig. 1D) RNA probes. Both the RAG-1 and RAG-2 transcripts were detected throughout the cortex although the RAG-1 hybridization was consistently more intense. The stronger signal for RAG-1 compared to RAG-2 seen by in situ analysis was also seen in RNA (Northern) blots hybridized with cDNA probes of roughly comparable size labeled to equal specific activities (see below). A demarcation of RAG-1 and RAG-2 expression was observed between the thymic cortex and medulla (Fig. 1, E and F). Little or no specific

antisense hybridization was seen with RAG-1 or RAG-2 in the medulla. The extensive specific hybridization for both RAG-1 and RAG-2 in the cortex was surprising in that a large percentage of cortical thymocytes express TCR on their surface (2), an indication that they have already carried out productive V(D)J recombination. This suggested that productive TCR gene rearrangement and surface TCR expression was by itself not sufficient to terminate RAG expression.

To confirm that the expression of RAG-1 and RAG-2 by TCR⁺ cortical thymocytes was a general feature of mammalian T cell development, we investigated human thymocyte subpopulations (Fig. 2A). After productive TCR gene rearrangement, the resulting TCR heterodimer is expressed on the cell surface in close association with several other invariant polypeptides, and forms a structure referred to as the TCR-CD3 complex (7). Like that of the mouse, the human thymic cortex contains both TCR-CD3⁻ cells, which have not completed productive TCR gene rearrangement, and immature TCR-CD3⁺ cells that have undergone successful V(D)J recombination (1). As expected, RAG-1 and RAG-2 were co-expressed at high levels in unseparated human thymocytes (Fig. 2B). Cell fractionation revealed that both RAG-1 and RAG-2 transcripts were expressed in TCR-CD3⁻ cells, as would be expected if these cells are undergoing V(D)J recombination. In addition, as was predicted from the in situ hybridization analyses, RAG-1 and RAG-2 were also expressed in TCR-CD3⁺ cells (Fig. 2C).

Immature cortical TCR-CD3⁺ thymocytes express both the CD4 and CD8 accessory molecules. Thus, they are frequently referred to as "double positive" cells. About 90% of TCR-CD3⁺ cells in human thymic tissue are double positive cells, but the majority of double positive thymocytes die in the thymus before further maturation (8). The continued maturation of a small number of double positive cells is apparently dependent on the ability of their TCR-CD3 complex to interact with a self-MHC class I or II molecule expressed on thymic epithelial cells (Fig. 2A) (4). Such an interaction, although poorly understood, results in cell survival and "positive selection" and ensures that the T cell response to foreign antigen will be restricted by self-MHC molecules. If positive selection occurs as a result of TCR interaction with a class I MHC molecule, thymocytes differentiate to "single positive" cells that express only the class I-restricting antigen CD8 and lose CD4 expression. In contrast, if selection occurs through interaction with an MHC class II antigen, the

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single positive cells retain the expression of the CD4 antigen and lose CD8 expression. Mature single positive thymocytes (TCR-CD3⁺CD4⁺CD8⁻ or TCR-CD3⁺CD4⁻CD8⁺) are found in the thymic medulla, and also in smaller numbers in the inner

cortex (9). As found with the in situ hybridization experiments, the subpopulation of TCR-CD3⁺ cells that expressed CD4 but not CD8 (that is CD4⁺CD8⁻ single positive) expressed neither RAG-1 nor RAG-2 (Fig. 2C). The reciprocal population of

TCR-CD3⁺ single positive cells (CD4⁻CD8⁺) also failed to show detectable RAG-1 or RAG-2 transcripts (10).

The foregoing demonstrate that the surface expression of a TCR-CD3 complex is not by itself sufficient to terminate RAG mRNA expression in vivo since TCR-CD3⁺CD4⁺CD8⁺ (double positive) thymocytes expressed both RAG-1 and RAG-2. However, the finding that neither RAG-1 nor RAG-2 transcripts were detectable in single positive thymocytes indicated that expression of these genes was lost in cells at this stage of maturation. As progression to the TCR-CD3⁺CD4 or CD8 single positive stage is thought to require TCR engagement by self-MHC molecules on thymic epithelial cells (4), it seemed possible that TCR cross-linking in vitro might result in the termination of RAG expression in TCR-CD3⁺ thymocytes. A monoclonal antibody (MAb) to CD3 (anti-CD3) attached to tissue culture dishes was used in culture to induce uniform cross-linking of the TCR-CD3 complex on the surface of unfractionated thymocytes. This resulted in significant down-regulation of the steady-state mRNA of both RAG-1 and RAG-2 (Fig. 3A). Most of the RAG-1 and RAG-2 loss occurred within 2 hours after stimulation, and the mRNA continued to decline over the 20-hour culture period. In addition to demonstrating that TCR-CD3 cross-linking dramatically reduces RAG-1 and RAG-2 expression, these data confirm that RAG-1 and RAG-2 were being expressed in TCR⁺ cells. The TCR⁻ thymocytes would not be responsive to treatment with the monoclonal antibody to anti-CD3 since CD3 is not expressed on the surface of a thymocyte in the absence of a TCR heterodimer (7).

To examine whether the loss of RAG expression in response to TCR-CD3 cross-linking was a specific effect, unfractionated thymocytes were stimulated with immobilized monoclonal antibodies to TCR-CD3, CD4, CD8, or HLA class I molecules (Fig. 3B). Only TCR-CD3 cross-linking induced a significant decrease in RAG expression. No reproducible effect was seen after cross-linking CD4, CD8, or HLA class I in three separate experiments. These findings suggest that termination of RAG expression requires specific signals, and that these can be provided through the TCR-CD3 complex. Identical results were also obtained with the same monoclonal antibodies in soluble form.

Several additional experiments were performed to confirm our findings. Under some circumstances TCR-CD3 stimulation of thymocytes can induce programmed cell death by apoptosis (11). We therefore excluded the possibility that the cessation of RAG-1 and

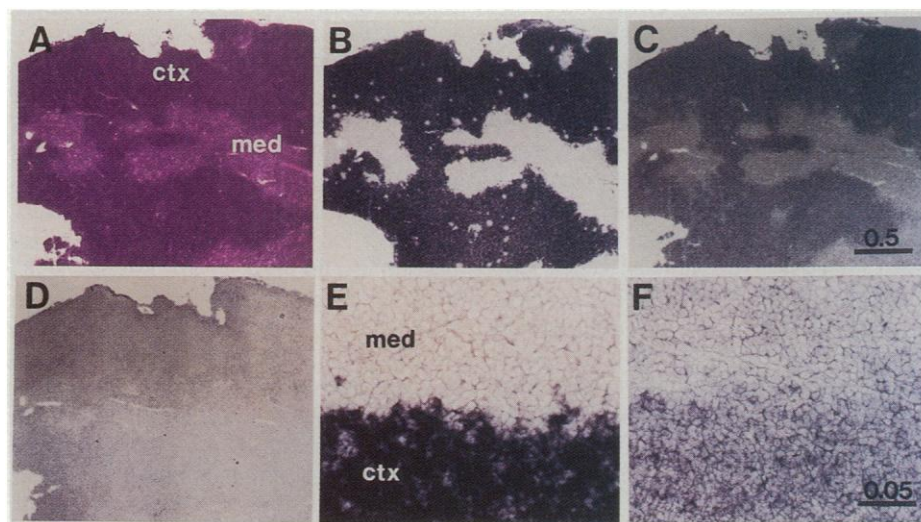
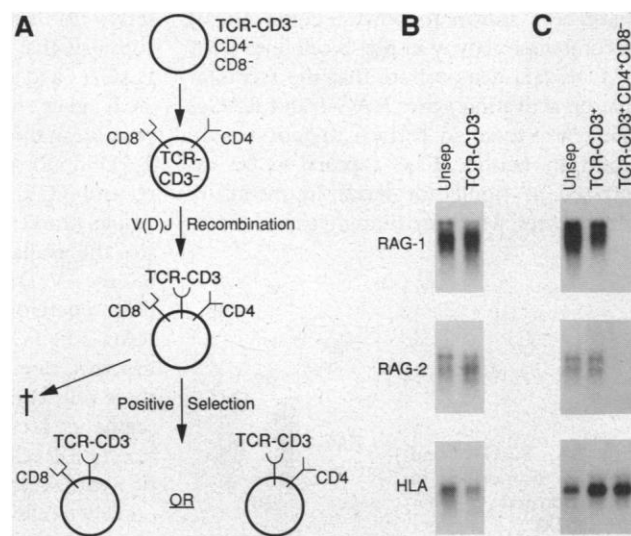


Fig. 1. RAG-1 and RAG-2 in situ hybridization of sections of the murine thymus. (A) Adjacent tissue sections from a mouse thymus (age 32 days) were stained with hematoxylin-eosin (ctx, thymic cortex; med, thymic medulla) or processed for in situ hybridization (23) with the following RNA probes: (B) RAG-1 antisense, (C) RAG-2 antisense, or (D) control with RAG-2 sense (the control with RAG-1 sense probe gave an identical result). Specific RAG-1 and RAG-2 antisense hybridization (as revealed by the dark staining regions) is confined to the thymic cortex. (A to D) Photographed at the same magnification; the calibration bar in (C) represents 0.5 mm. (E and F) The cortical-medullary boundary in sections hybridized with RAG-1 or RAG-2 antisense riboprobes, respectively (bar, 0.05 mm).

Fig. 2. Expression of RAG-1 and RAG-2 in thymocyte subpopulations. (A) Schematic outline of T cell ontogeny in the thymus. T cell precursors that seed the thymus have not undergone rearrangement of their T cell receptors. Within the thymic cortex they begin to express both CD4 and CD8 and undergo V(D)J recombination of their TCR genes. After productive TCR gene rearrangement, thymocytes express TCR-CD3 on the surface as well as both the CD4 and CD8 molecules. Those TCR-CD3⁺CD4⁺CD8⁺ ("double positive") thymocytes that undergo positive selection as a result of a TCR-MHC interaction differentiate to single positive cells, either TCR-CD3⁺CD4⁺CD8⁻ or TCR-CD3⁺CD4⁻CD8⁺. The TCR-CD3⁺ thymocytes that are not positively selected die in the thymus. (B) Northern blot of RNA isolated from unseparated (Unsep) and TCR-CD3⁺ thymocytes hybridized with RAG-1, RAG-2, and HLA probes. (C) Northern blot of RNA isolated from unseparated (Unsep) thymocytes, TCR-CD3⁺ thymocytes, and TCR-CD3⁺CD4⁺CD8⁻ "single positive" thymocytes hybridized with RAG-1, RAG-2 and HLA probes. Purified populations of thymocytes were isolated (24), and total cellular RNA was extracted after cell lysis in guanidinium isothiocyanate after which Northern blots were prepared with equalized RNA samples for subpopulations (25). RAG-1 transcripts were eight to ten times more abundant than RAG-2 transcripts. For example, the times of exposures in (B) are 16 hours for RAG-1, and 48 hours for RAG-2. This difference in RNA abundance presumably explains the difference in signal to noise ratio observed with in situ hybridization of RAG-1 and RAG-2 probes.



RAG-2 expression observed in vitro was simply the result of cell death induced by anti-CD3. To this end thymocytes were stimulated in the presence of interleukin-2 (IL-2), which blocks anti-CD3-induced cell death (12) and supports the proliferation of human double positive thymocytes stimulated by TCR-CD3 cross-linking (13). Down-regulation of RAG-1 and RAG-2 expression by anti-CD3 stimulation was not affected by the addition of IL-2 to the culture media (14), and cell counts revealed no significant difference in cell recovery after 6 hours of culture in medium alone, or after stimulation with anti-CD3 plus IL-2 (95.0 ± 2.2 compared to $94.1 \pm 2.2\%$). Viability in both populations exceeded 99% as assessed by trypan blue exclusion. We conclude that the large decrease in RAG expression after treatment of unfractionated or TCR-CD3⁺ cells with anti-CD3 was not due to cell death.

In peripheral T cells, cross-linking of the TCR-CD3 complex induces the activation of phospholipase C, which liberates diacylglycerol and inositol tris-phosphate from the cell membrane. These two second messengers in

turn lead to the membrane translocation of protein kinase C and the release of intracellular calcium stores, respectively (15). The combination of phorbol myristate acetate (PMA) plus ionomycin mimics these events by stimulating protein kinase C translocation and intracellular calcium release directly. Treatment of unfractionated thymocytes with PMA plus ionomycin led to the loss of detectable RAG-1 and RAG-2 mRNA within 6 hours of stimulation (Fig. 4). This decrease in RAG expression was more complete than when the same cells were treated with monoclonal anti-CD3, suggesting that perhaps this combination of mitogens was able to down-regulate RAG expression in TCR-CD3⁺ thymocytes which were unaffected by the monoclonal anti-CD3. When purified TCR-CD3⁺ thymocytes were treated with PMA plus ionomycin, RAG expression was dramatically reduced (Fig. 4). Thus, TCR-CD3⁺ thymocytes are competent to down-regulate RAG expression in response to agents that directly activate protein kinase C and increase intracellular calcium levels, despite the lack of surface TCR expression. These data are consistent with the hypothesis that RAG mRNA expression is maintained in the developing TCR-CD3⁺ thymocyte in part because the surface component of the signal transduction system is absent, such that one or both of these second messenger systems cannot be induced. Similar signal transduction events may also regulate RAG-1 and RAG-2 in B cells since PMA and ionomycin treatment have been shown to down-regulate V(D)J recombinase activity in pre-B cell lines (16).

Our data demonstrate that the recombination activating genes RAG-1 and RAG-2 are coexpressed in cortical thymocytes. In contrast, neither gene appears to be expressed at significant levels in medullary thymocytes, which are thought to have com-

pleted TCR-dependent selection processes. In isolated thymocyte subpopulations, we found that TCR⁺CD4⁺CD8⁺ double positive thymocytes expressed both RAG-1 and RAG-2, while TCR⁺CD4⁺CD8⁻ or TCR⁺CD4⁻CD8⁺ single positive cells did not express detectable amounts of mRNA from either gene. This suggests that physiologic termination of RAG expression at the mRNA level occurs during the process of thymic differentiation. Furthermore, cross-linking the TCR-CD3 complex of immature thymocytes in vitro led to the rapid loss of RAG-1 and RAG-2 expression. If the signal transduction events initiated by in vitro cross-linking of TCR-CD3 with monoclonal anti-CD3 correctly mimic signal transduction events that occur during TCR-MHC interactions in the thymus (17), then TCR-CD3 stimulation by either the positive or negative selection process might lead to the termination of RAG expression in vivo.

Together, our data are consistent with the following model of thymocyte development. Coexpression of RAG-1 and RAG-2 in immature thymocytes serves to activate TCR gene rearrangement. When productive ("in frame") rearrangements are achieved the TCR-CD3 complex is expressed on the cell surface. However, surface expression of the TCR-CD3 receptor does not by itself affect RAG expression. During the TCR-dependent selection processes that result in MHC restriction, engagement of the TCR-CD3 complex by an MHC-bearing stromal cell serves to stimulate a signal transduction pathway that leads to the termination of RAG-1 and RAG-2 expression. Further TCR gene recombination would not be possible in the absence of RAG-1 and RAG-2. Undoubtedly, other mechanisms may control TCR gene recombination. For example, transcriptional regulators may control the availability of TCR gene segments to the V(D)J recombination machinery (18). Therefore, expression of RAG-1 and RAG-2 in TCR-CD3⁺ double-positive thymocytes does not necessarily imply that these cells continue to rearrange TCR gene segments. However, the recent characterization of a T cell clone with surface expression of two distinct TCR's (19), as well as data on other cells expressing mRNA for two α (20) or β (21) chain transcripts, are consistent with the occurrence of recombination of TCR genes in the presence of a functionally rearranged allele of the same gene. In this regard, recombination of the endogenous α locus can occur in the presence of a rearranged α TCR transgene (22).

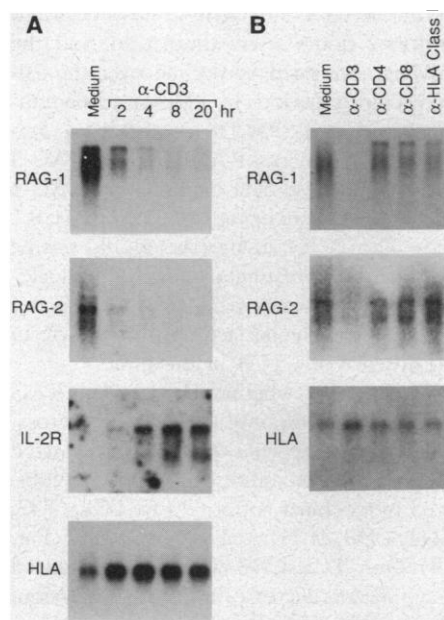
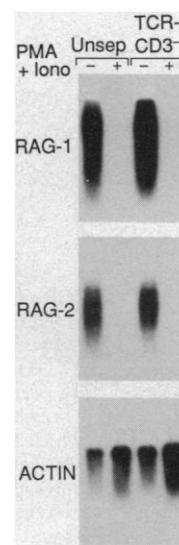


Fig. 3. (A) Loss of RAG-1 and RAG-2 expression after TCR-CD3 stimulation. Unfractionated thymocytes were cultured for 4 hours in medium alone, or stimulated on tissue culture plates coated with a monoclonal antibody to CD3 (α -CD3) (24) and harvested at the time points indicated. RNA was then isolated for Northern blot analysis (25). The IL-2R probe was a 1.9-kb Eco RI-Bam HI fragment (26). (B) RAG expression is not altered by stimulation of CD4, CD8, or HLA class I. Unfractionated thymocytes were cultured for 6 hours in medium alone, or on tissue culture plates coated with the following monoclonal antibodies: anti-CD3 (α -CD3), anti-CD4 (α -CD4), anti-CD8 (α -CD8), or anti-HLA class I (α -HLA class I). The cells were harvested and RNA was extracted for Northern analysis (25).

Fig. 4. RAG-1 and RAG-2 expression in unfractionated and TCR-CD3⁺ thymocytes. Unseparated (Unsep) and TCR-CD3⁺ thymocytes (24) were cultured in medium alone or in medium containing PMA (10 ng/ml) and ionomycin (250 ng/ml) for 6 hours, after which RNA was isolated for Northern blot analysis (25). The actin probe was a 2.0-kb Bam HI fragment (27).



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23. In situ hybridization was performed as described [J. J. M. Chun *et al.*, *Cell* **64**, 189 (1991)] except that probes were labeled with digoxigenin and detected with an antibody to digoxigenin that was conjugated to alkaline phosphatase. Briefly, thymic sections (12 mm thick) were fixed and hybridized with RAG-1 or RAG-2 antisense or sense single-stranded RNA probes produced by in vitro transcription in the presence of digoxigenin-UTP. The RAG-2 antisense and sense probes consisted of nucleotides 1-1966 and 1-2062, respectively, of the RAG-2 cDNA [M. A. Oettinger *et al.*, in (5)]. The RAG-1 probe has been described [J. J. M. Chun *et al.*, *Cell* **64**, 189 (1991)]. In vitro transcription and detection of the probes were carried out with DIG RNA labeling kit and Genius Detection System (Boehringer Mannheim).
24. Thymic tissue was obtained from children under age 3 undergoing corrective cardiac surgery, and a single cell suspension of unseparated cells (Unsep) was prepared from tissue gently pressed through nylon mesh. Cells that were TCR-CD3⁺ were isolated by negative selection with G19-4 (anti-CD3) and goat antibody to mouse immunoglobulin G (on coated magnetic beads) [L. A. Turka, J. A. Ledbetter, K. Lee, C. H. June, C. B. Thompson, *J. Immunol.* **144**,

1646 (1990)]. TCR-CD3⁺ thymocytes were isolated by their adherence to tissue culture plates that were coated with a monoclonal antibody to CD3. Cells were allowed to adhere for 20 minutes, after which non-adherent cells were removed by vigorous washing. Mature single positive thymocytes were isolated by a two-step procedure. First, we negatively selected thymocytes with anti-CD8 MAb (G10.1 MAb) or with anti-CD4 MAb (G17-2) (gift of J. A. Ledbetter) on magnetic beads. The CD3⁺CD4⁺CD8⁻ or CD3⁺CD4⁻CD8⁺ cells were then purified by their adherence to anti-CD3-coated tissue culture plates and were >95% pure.

25. Northern blots were prepared from equalized samples of total cellular RNA. Each filter was hybridized with RAG-1, RAG-2, and HLA probes. The RAG-1 probe was a 0.9-kb Xho I-Hind III fragment corresponding to bases 2272 to 3180 [D. G. Schatz, M. A. Oettinger, D. Baltimore, *Cell* **59**, 1035 (1989)], and the HLA probe was a 1.4-kb Pst I fragment isolated from pHLA-B7 [A. K. Sood, D.

Pereira, S. M. Weissman, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 616 (1981)]. The RAG-2 probe was a 0.64-kb PCR product amplified from human genomic DNA with PCR primers corresponding to murine bases 825 to 845 and 1431 to 1459 in the sense and antisense strands, respectively [M. A. Oettinger, D. G. Schatz, C. Gorka, D. Baltimore, *Science* **248**, 1517 (1990)]. The sizes of the RAG-1 and RAG-2 mRNA species detected was comparable to those previously observed in pre-B and pre-T cell lines.

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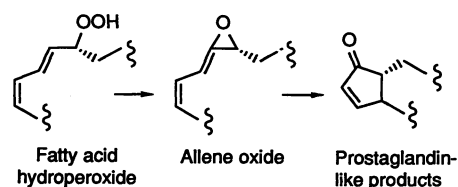
19 February 1991; accepted 29 May 1991

Purification of an Allene Oxide Synthase and Identification of the Enzyme as a Cytochrome P-450

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Fatty acid hydroperoxides (lipoxygenase products) are metabolized to allene oxides by a type of dehydrase that has been detected in plants, corals, and starfish oocytes. The allene oxides are unstable epoxide precursors of more complex products such as jasmonic acid, the plant growth hormone. Characterization of the dehydrase enzyme of flaxseed revealed that it is a 55-kilodalton hemoprotein. The spectral characteristics of this dehydrase revealed it to be a cytochrome P-450. It operates with the remarkable activity of ≥ 1000 turnovers per second. The results establish a new catalytic activity for a cytochrome P-450 and illustrate the cooperation of different oxygenases in pathways of fatty acid metabolism.

ALLENE OXIDES ARE REACTIVE EPOXIDES that are prone to nucleophilic substitution and intramolecular rearrangements (1). The first allene oxides to be synthesized were small molecules designed with hydrophobic substituents to protect the epoxyene grouping from hydrolysis (2). Allene oxides have also been detected as natural products of lipid hydroperoxide metabolism (3). These allene epoxides are fleeting intermediates in the conversion of specific lipoxygenase products to more stable end products that include several prostaglandin-like molecules.



Biosynthesis of allene oxides involves an enzyme-catalyzed dehydration of the lipid

hydroperoxide. The enzymes are selective for the position of the hydroperoxide group and the enantiomeric configuration (3, 4). We describe the purification of an allene oxide synthase (or hydroperoxide dehydrase) and its characterization as a member of the cytochrome P-450 family of hemoproteins.

As a source of enzyme, we used an acetone powder of flaxseed, a classic preparation of this type of hydroperoxide-metabolizing activity (5). Allene oxide synthase activity in fresh tissues is found in the microsomal fraction (6), but the acetone wash removes the microsomal lipids, and subsequent aqueous extraction of the acetone powder leaves the protein in a 100,000g supernatant. After initial ammonium sulfate fractionation of this extract, we examined the ability of the "redissolved" activity to pass through a standard 0.22- μ m filter. Using this simple test in conjunction with a spectroscopic assay of enzyme activity, we established the efficiency of various detergents to effect complete solubilization. Use of 0.1 to 0.25% of the nonionic detergent Emulgen 911 (Kao-Atlas, Tokyo) not only allowed the free passage of the enzyme through the microfilters, it increased the

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