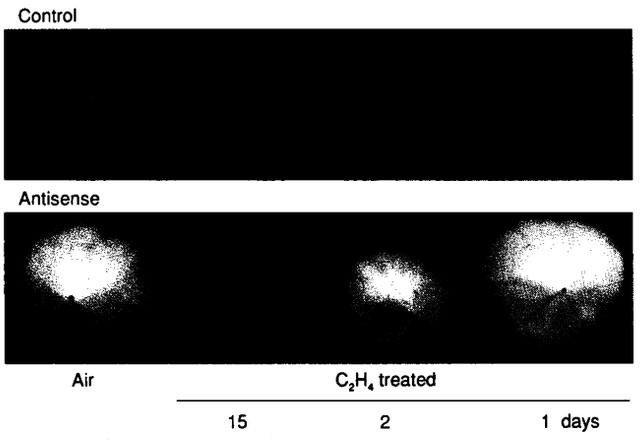

Fig. 5. Effect of C_2H_4 treatment on the antisense phenotype. Mature green fruits from control and homozygous antisense plants were harvested 49 days after pollination and treated with air for 15 days or with 10 μl of C_2H_4 per liter of air for 1, 2, or 15 days and then returned to air (*25*). Fruits were photographed on day 16 after harvesting.

malian hormone receptors are both transcriptional activators and Zn^{2+} metalloproteins (*22*). Expression of antisense RNA to ACC synthase may ameliorate losses due to overripening of fruits and vegetables during transportation or because of lack of refrigeration.

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11. Fruit pigments were extracted with methylene chloride and separated by high-performance liquid chromatography on a Microsorb C_{18} reverse-phase column (Rainin Instruments, Emeryville, CA). The lycopene peak was quantified with the use of external standards with absorbance readings taken at 450 nm. The lycopene content in micrograms per gram of fruit mass of the fruits including those shown in Fig. 3 was the following: control in air at 50 days (green), <0.02; control in air at 59 days (red), 19; control in C_3H_6 at 59 days (red), 25; antisense in air at 57 days (green), <0.02; antisense in air at 70 days (yellow), <0.02; and antisense in C_3H_6 at 70 days (red), 17. The numbers are the average of three measurements.
12. The compressibility was measured in pounds per square inch with a 5/16-inch Magness-Taylor puncture probe (D. Bellauf Manufacturing, Washington, DC). The compressibility data was the following: control in air at 50 days (green), 184; control in air at 59 days (red), 52; control in C_3H_6 at 59 days (red), 52; antisense in air at 57 days (green), 196; antisense in air at 70 days (yellow), 156; and antisense in C_3H_6 at 70 days (red), 52. The numbers are the average of four measurements.
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18. The lycopene content in micrograms per gram of fruit mass of the fruits shown in Fig. 5 is as follows: control green fruit at 49 days, <0.02; control in air for 15 days, 38; control in C_2H_4 for 15 days, 76; control in C_2H_4 for 2 days, 35; control in C_2H_4 for 1 day, 32; antisense green fruit at 49 days, <0.02; antisense in air for 15 days, <0.02; antisense in C_2H_4 for 15 days, 29; antisense in C_2H_4 for 2 days, 1.1; and antisense in C_2H_4 for 1 day, 0.03. The numbers are the average of two measurements.
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23. We ligated the 1789-bp insert of the cDNA clone ptACC2 (*9*) into the Sma I site of pUC19 to yield ptACC2A. A 302-bp CaMV 35*S* promoter fragment was isolated from Hind III–digested pJ024d [D. W. Ow *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4870 (1987)]. The ends were filled with Klenow polymerase and digested with Bam HI [J. Sambrook *et al.*, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. The promoter fragment was inserted into ptACC2A digested with Xba I, made blunt-ended with Klenow polymerase, and digested with Bam HI. The resulting plasmid ptACC2B contains the 302-bp CaMV 35*S* promoter, followed by the tACC2 ACC synthase cDNA in an antisense orientation. The 2.1-kb antisense gene (CaMV-tACC2) was excised by partial Sac I and complete Sal I digestion and ligated into pBI101 vector (Clontech, Palo Alto, CA), which yields pP035 (Fig. 1A). The pP035 plasmid was transferred from *Escherichia coli* DH5α to *Agrobacterium tumefaciens* LBA4404 by triparental mating with *E. coli* HB101 harboring pRK2013. The tomato cultivar VF36 was transformed with 14-day-old cotyledons as described [S. McCormick *et al.*, *Plant Cell Rep.* **5**, 81 (1986)].
24. Genomic DNA was extracted from frozen leaf tissue, digested with Eco RI and Pst I, and hybridized with a ^{32}P-labeled 657-bp Sac I–Hind III fragment from the fourth exon of the *LE-ACC2* gene (4642 to 5299 bp) (*9*).
25. Fruits were placed in 4-liter glass jars and flushed with a stream of water-saturated air with or without 1000 μl of C_3H_6 or 10 μl of C_2H_4 per liter of air. We monitored C_2H_4 production by sealing individual fruits in glass containers of known volume for 1 hour. Gas samples (1 ml) were analyzed with a Hewlett-Packard gas chromatograph.
26. Total nucleic acids were extracted from tomato fruit and analyzed as described (*9*). Sense and antisense ACC synthase RNA were detected with strand-specific ^{32}P-labeled riboprobes transcribed by T7 polymerase containing the tACC2 cDNA (*9*). The washing was at 75°C instead of 60°C. Other hybridization probes were a random-primed, ^{32}P-labeled, 1.2-kb Eco RI fragment of tACC4 (*9*); a 1.6-kb Eco RI fragment of a PG cDNA (*15*); and a 0.9-kb fragment obtained with the polymerase chain reaction on the *TOM13* cDNA (228 to 1122 bp) (*14*).
27. This paper is dedicated to G. G. Laties and J. E. Varner on their retirement as professors of biology at the University of California at Los Angeles and Washington University, respectively. We thank M. Johnston, L. Giudice, and R. A. Wells for help with the manuscript; B. Baker and S. McCormick for seeds of the cultivar VF36; K. Corr and M. Boylan for technical advice; and J. Fink, N. Shen, K. Fong, N. Mahoney, A. Spelletich, and B. Lamb for technical and greenhouse assistance. Supported by grants from the NSF (DCB-8645952, DCB-8819129, DCB-8916286), the NIH (GM-35447), and the United States Department of Agriculture (5835-23410-D002) (to A.T.).

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A 32-kD GTP-Binding Protein Associated with the CD4-p56^{lck} and CD8-p56^{lck} T Cell Receptor Complexes

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The guanosine triphosphate (GTP)–binding proteins include signal-transducing heterotrimeric G proteins (for example, G_s, G_i), smaller GTP-binding proteins that function in protein sorting, and the oncogenic protein p21^{ras}. The T cell receptor complexes CD4-p56^{lck} and CD8-p56^{lck} were found to include a 32- to 33-kilodalton phosphoprotein (p32) that was recognized by an antiserum to a consensus GTP-binding region in G proteins. Immunoprecipitated CD4 and CD8 complexes bound GTP and hydrolyzed it to guanosine diphosphate (GDP). The p32 protein was covalently linked to [α-^{32}P]GTP by ultraviolet photoaffinity labeling. These results demonstrate an interaction between T cell receptor complexes and an intracellular GTP-binding protein.

GTP-BINDING PROTEINS AFFECT the enzymatic activity of adenyl cyclase, retinal cyclic guanosine monophosphate phosphodiesterase (GMP),

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and phospholipase C (*1, 2*). Activation of GTP-binding proteins in T cells by nonhydrolyzable GTP analogs induces phosphoinositol turnover, suggesting that GTP-binding proteins may function in T cell activation (*3*). Ultraviolet cross-linking of [α-^{32}P]GTP to T cell proteins reveals ten GTP-binding proteins, including ones of 30 and 34 kD (*4*).

To determine if the CD4-p56^{lck} and CD8-p56^{lck} complexes (*5*) contained a GTP-binding protein, we used an antiserum (anti–G protein) to the consensus GTP-binding region (Gly-(X)$_4$-Gly-Lys) of the heterotrimeric G protein G_z (*6*) that recognizes α subunits of the heterotrimeric G proteins G_s, G_i, G_z, G_o, and transducin.

Immune complexes were isolated from [γ-^{32}P]adenosine triphosphate (ATP)–labeled membranes of the human T cell line HPB-ALL. Antibodies to CD4 (anti-CD4) and CD8 (anti-CD8) precipitated a phosphorylated 55- to 60-kD protein corresponding to p56^{lck}, a 32- to 33-kD doublet (p32), and several higher molecular weight bands (Fig. 1). Anti–G protein precipitated two prominent bands of 56 kD and 32 to 33 kD (Fig. 1), suggesting that a molecule recognized by this antiserum is associated with CD4-p56^{lck} and CD8-p56^{lck}. Occasionally, other less prominent bands of 40 to 85 kD were coprecipitated; these may represent other G proteins or G protein–associated proteins. Similar patterns of immunoprecipitated bands were observed from normal human thymocytes and peripheral blood lymphocytes (PBL) (Fig. 1).

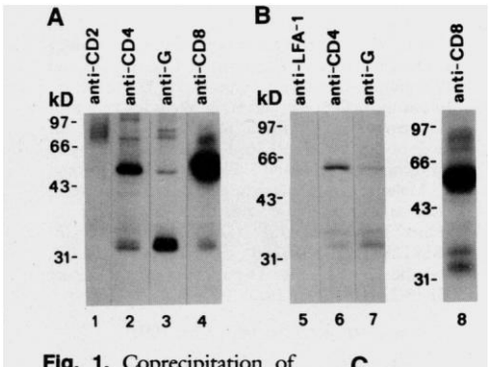

Fig. 1. Coprecipitation of p32 with the CD4-p56^{lck} and CD8-p56^{lck} complexes. Membranes from HPB-ALL cells (**A**), thymocytes (**B**), or PBLs (**C**) (8×10^7 cells per milliliter) were prepared (*11*) and labeled in 100 µl of 25 mM Hepes (pH 7.2), 3.8 mM $MnCl_2$, 3.7 mM $MgCl_2$, and 60 µCi of [γ-^{32}P]ATP (3000 Ci/mmol, NEN) for 12 min at 25°C. Membranes were solubilized in 20 mM tris (pH 8.15), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and NP-40 (1%). The lysate was incubated with antibody and BSA (1 mg per sample) at 4°C for 1 hour, and 50 µl of a suspension (1:1) of protein A–Sepharose (Pharmacia LKB) in lysis buffer containing NP-40 (1%) was added. The sample was rotated for 30 min and washed twice with lysis buffer containing NP-40 (1%). The antibodies used were 19thy 5D7 (anti-CD4) (*14*), 2T8-5H7 (anti-CD8) (*15*), P-960 [anti–G protein (anti-G)] (*6*), anti-Lck (*16*), Ras10 (anti-Ras) (*17*), 2F12 (anti-CD11a) (*18*), T112 (anti-CD2) (*19*), and rabbit antibody to mouse IgG (Dako).

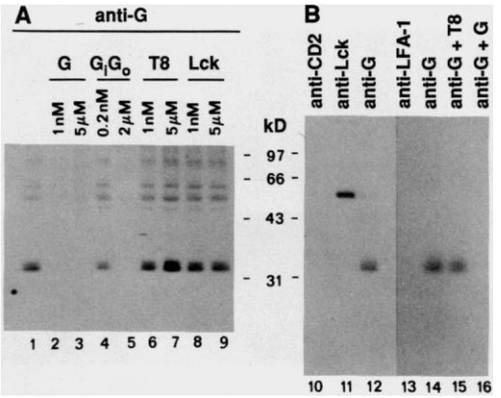

Fig. 2. Precipitation of p32 and associated molecules by anti–G protein (anti-G). Precipitation was blocked by the peptide used to generate the antiserum (G) and by a mixture of G_i and G_o (**A**). The p32 protein immunoprecipitated with anti-CD4 was reprecipitated by anti–G protein (**B**). Immunoprecipitations were done as described in Fig. 1; peptides were added during incubation with the antibody. T8 peptide, ArgArgValCysLysCysProArgProValValLysSer; Lck peptide, GluLeuTyrGlnLeuMetArgLeuCysTrpLysGluArg. For reprecipitations (*5*), anti-CD4 immunoprecipitates were boiled for 5 min in 30 µl of a solution of SDS (5%). The supernatant was filtered with an Amicon concentrator (Centricon 30), diluted in lysis buffer containing NP-40 (1%), and immunoprecipitated.

Recognition of p32 and associated proteins by anti–G protein was blocked by the peptide used to generate the antiserum, and by purified G_i and G_o, which contain the consensus GTP-binding domain (Fig. 2). Peptides corresponding to portions of CD8 and p56^{lck} did not block recognition (Fig. 2).

To determine if the p32 associated with CD4 was directly recognized by anti–G protein, anti-CD4 immunoprecipitates were denatured in SDS and reprecipitated with anti–G protein. Of the proteins present in the anti-CD4 immunoprecipitate, only p32 was recognized by anti–G protein (Fig. 2). Furthermore, recognition of p32 was blocked by the peptide used to generate the antiserum but not by an irrelevant peptide (Fig. 2).

To determine if the 32-kD proteins precipitated by antibody to p56^{lck} (anti-lck), anti-CD4, and anti–G protein were identical, the immunoprecipitated proteins were analyzed by two-dimensional SDS–polyacrylamide gel electrophoresis (PAGE). Anti-CD4, anti-lck, and anti–G protein precipitated a 32- to 33-kD protein that resolved at an identical isoelectric point (*p*I) of 5.0 to 5.2 (Fig. 3). Likewise, the 55- to 60-kD protein (p56^{lck}) coprecipitated by anti-CD4 migrated at the same *p*I as the corresponding protein precipitated by anti–G protein.

To examine the function of p32, we measured GTP binding and hydrolysis of GTP

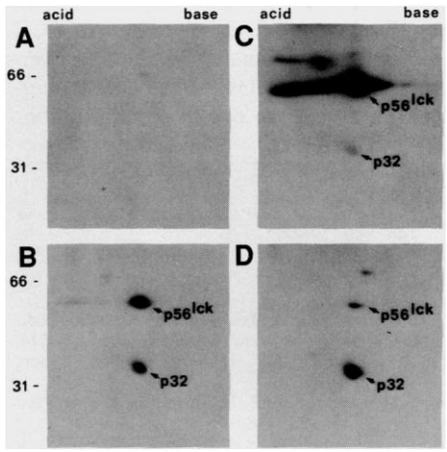

Fig. 3. Two-dimensional SDS-PAGE analysis of immunoprecipitates formed by anti-CD2 (**A**), anti-CD4 (**B**), anti-lck (**C**), and anti–G protein (**D**). Immunoprecipitates of [γ-^{32}P]ATP–labeled proteins from HPB-ALL cells were prepared as described (Fig. 1). Two-dimensional gels were run as described (*20*). Acidic and basic ends of the isoelectric focusing gel are indicated.

to GDP by the immunoprecipitated proteins. Both anti-CD4 and anti-CD8 immunoprecipitates bound GTP (Fig. 4A). Immunoprecipitates with antibody to CD11a (Fig. 4A), antibody to CD2, and rabbit antibody to mouse immunoglobulin G (IgG) (*7*) showed no binding of GTP. The amounts of GTP binding and GTP hydrolysis detected in anti-CD4 and anti-CD8 immunoprecipitates increased over time. The ratio of bound GDP to total bound nucleotide remained approximately constant during the 60-min incubation. Samples of the supernatant contained low amounts of unbound GDP, averaging 9% of unbound nucleotide. A much higher proportion of GDP (60 to 70% of bound nucleotide) was associated with CD4 and CD8 immunoprecipitates, indicating that GTP hydrolysis is specific to CD4 and CD8.

An excess of unlabeled GTP blocked all [α-^{32}P]GTP binding; uridine triphosphate (UTP) and cytidine triphosphate (CTP) inhibited to a lesser degree, demonstrating specificity of nucleotide binding in immunoprecipitates with anti-CD4, anti-CD8, and antibody to p21^{ras} (anti-Ras) (Fig. 4B). Hydrolysis of GTP was Mg^{2+}-dependent (7).

Low amounts of GTP binding were detected in anti–G protein immunoprecipitates (7), which is not surprising because that antiserum reacts with a region crucial to GTP binding (8). A 4000-fold molar excess of the ATP analog 5′-adenylimidodiphosphate (AMP-PNP) was included in all GTPase assays, to prevent binding and hydrolysis of GTP by protein kinases that primarily utilize ATP, such as p56^{lck}. Anti-lck immunoprecipitates (7) bound low amounts of GTP and contained low amounts of phosphorylated p32 (Fig. 3).

Ultraviolet irradiation of guanine nucleotide–binding proteins forms covalent linkages between those proteins and bound guanine nucleotide (4). Proteins from T cell membranes were cross-linked with [α-^{32}P]GTP and separated by two-dimensional SDS-PAGE. One of the T cell proteins migrated at the same molecular weight and isoelectric point as ^{32}P-labeled p32 from an anti-CD4 immunoprecipitate. This [α-^{32}P]GTP–labeled molecule was coprecipitated by anti-CD4, demonstrating that p32 associated with CD4 binds GTP (Fig. 4C).

Receptors with tyrosine kinase activity such as the epidermal growth factor receptor have been functionally linked to G proteins (9). Our results demonstrate that T cell receptors with associated kinase activity interact with a GTP-binding protein, which can only be definitively identified by cloning of p32. Aggregation of CD4 or CD8 with T cell receptor–CD3 (10, 11) and association of p56^{lck} with CD8 or CD4 (12) are correlated with proliferation of T cells. These proliferative effects may be mediated by both p56^{lck} and p32. The interaction of p56^{lck} with p32 may be similar to the functional dependence of src on p21^{ras} (13).

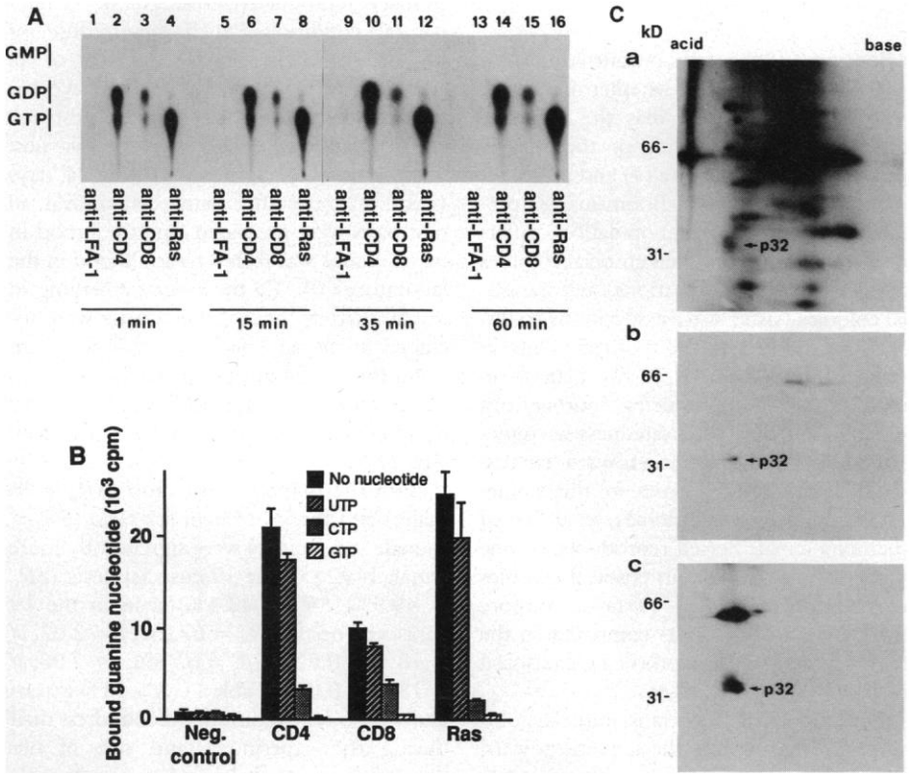

Fig. 4. Binding and hydrolysis of GTP in anti-CD4 and anti-CD8 immunoprecipitates. (**A**) Hydrolysis of bound GTP assayed essentially as described (21). Unlabeled immunoprecipitates were washed in GTP binding buffer (21) containing 0.12 M $(NH_4)_2SO_4$, and incubated in GTP binding buffer containing 25 nM [α-^{32}P]GTP (Amersham, 400 Ci/mmol) and 0.1 mM 5′ AMP-PNP (Sigma). Samples of the supernatant were taken at each time point to monitor hydrolysis of unbound GTP. The Sepharose beads were washed twice in GTP-binding buffer with Triton X-100 (0.01%) (Pierce) and the nucleotides were eluted in a solution of 2 mM EDTA and SDS (0.5%) at 65°C. Eluted nucleotides and supernatant were spotted on polyethyleneimine-cellulose thin-layer chromatography plates (Thomas) and developed in LiCl (0.2 M for 2 min, 1.0 M for 6 min, 1.6 M for remainder). (**B**) Specificity of GTP binding. Nucleotide competition assays were done as in (A), with 0.1 mM unlabeled UTP, CTP, or GTP in the incubation buffer. Sepharose beads were washed and counted in Biofluor (NEN) (n = 3). (**C**) Two-dimensional SDS-PAGE analysis of T cell GTP-binding proteins covalently linked to [α-^{32}P]GTP by ultraviolet photoaffinity labeling. A protein (panel a) was detected with a molecular weight and pI identical to those of ^{32}P-labeled p32, from an anti-CD4 immunoprecipitate (panel c). The [α-^{32}P]GTP–labeled protein p32 was coprecipitated by anti-CD4 (panel b). The 50-kD band was nonspecifically immunoprecipitated, appearing in the negative controls (7). Membranes were made as described in Fig. 1, washed in buffer containing 10 mM tris (pH 7.4), 150 mM NaCl, and 0.12 M $(NH_4)_2SO_4$. GTP was cross-linked to T cell GTP-binding proteins by ultraviolet irradiation as described (4). Whole membranes (from approximately 4×10^6 cells) were solubilized in sample buffer containing NP-40 (3.6%), ampholines (0.09%), 9.4 M urea, and β-mercaptoethanol (0.1%). Membranes (from approximately 9×10^6 cells) were solubilized in lysis buffer containing NP-40 (1%) and immunoprecipitated as described in Fig. 1.

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