tion to cannabinoids is dosage (*1*). Low doses of HU-210 abolish the behavioral response to novelty and inhibit the HPA stress response, whereas higher doses, particularly under conditions of novelty, have the opposite effect (*12*), as in the test described here. Comparative analysis of the patterns of Fos expression in the withdrawal and short-term HU-210 treatment conditions demonstrated an overlap as well as a dissociation of affected brain regions (Table 2), implicating the involvement of different neural substrates in the anxiety-like response induced by a single high dose of cannabinoid as opposed to withdrawal from long-term cannabinoid exposure. In the central amygdala, Fos expression appeared dispersed after a single injection of HU-210, whereas after antagonist-induced withdrawal Fos-positive nuclei were densely distributed (Fig. 2). In the BNST, immunopositive cells were found in a more medial-anterior gradient during cannabinoid withdrawal, whereas Fos activation was more prominent in the lateral dorsal region after short-term cannabinoid exposure (Table 2). In the hypothalamus, the PVN exhibited less Fos immunoreactivity during cannabinoid withdrawal compared with the effects of a single treatment with cannabinoid agonist. Thus, HPA activation after a single exposure to HU-210 in drug-naïve rats appears to be mediated directly by the PVN, whereas the increase in plasma corticosterone concentrations during withdrawal may involve activation of the central amygdala, transmitted to the PVN through its direct connections or by the BNST, which, in turn, may also activate the PVN. Because both the central amygdala and PVN are thought to be involved in anxiety-like behavioral responses to stress (*13, 14*), these observations suggest that the balance between the contributions of both structures after acute cannabinoid treatment or antagonist-induced withdrawal may result in the particular behavioral reactivity to the novelty condition in the defensive withdrawal test.

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Receptor-Mediated Activation of a MAP Kinase in Pathogen Defense of Plants

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Parsley cells recognize the fungal plant pathogen *Phytophthora sojae* through a plasma membrane receptor. A pathogen-derived oligopeptide elicitor binds to this receptor and thereby stimulates a multicomponent defense response through sequential activation of ion channels and an oxidative burst. An elicitor-responsive mitogen-activated protein (MAP) kinase was identified that acts downstream of the ion channels but independently or upstream of the oxidative burst. Upon receptor-mediated activation, the MAP kinase is translocated to the nucleus where it might interact with transcription factors that induce expression of defense genes.

Plants react to pathogen attack with a variety of defense responses, including transcriptional activation of defense genes, accumulation of phytoalexins and pathogen-related (PR) proteins, and impregnation of the cell wall with phenolic substances and specific proteins (*1*). Infection of parsley leaves with spores from the soybean pathogen *Phytophthora sojae* leads to small necrotic lesions resulting from hypersensitive cell death, incorporation of phenolic compounds into, and apposition of callose onto, cell walls at the infection site, as well as local and systemic activation of defense-related genes and secretion of fouranocoumarin phytoalexins into the infection droplet (*2, 3*). Cultured parsley cells show most of these defense reactions when treated with elicitor preparations from the fungus and have been used as a model system to study the plant-pathogen interactions (*4–7*). An extracellular 42-kD fungal glycoprotein was identified in these preparations as the principal elicitor of the multicomponent defense response in parsley cells (*6*). An oligopeptide fragment of 13 amino acids in length (Pep13) within this glycoprotein is necessary and sufficient to induce the same reactions as the intact glycoprotein (*7, 8*). Pep13 specifically interacts with a plasma membrane target site in the plant and initiates a signal transduction cascade leading to the transient activation of plant defense genes and the accumulation of phytoalexins (*7*).

Elicitor signal transduction in parsley cells involves Ca^{2+}-dependent transient changes in protein phosphorylation, suggesting the participation of protein kinases in defense gene activation (*9*). To detect specific protein kinases that catalyze such reactions, we treated cultured parsley cells with Pep25, a larger fragment of the elicitor that includes the Pep13 sequence and induced an identical response but was more stable in the culture medium than Pep13 (*7*). A protein kinase that phosphorylated myelin basic protein (MBP) was activated within 5 min after elicitor treatment (Fig. 1A). From its relative mobility on SDS-polyacrylamide gels, the apparent molecular mass of this enzyme was estimated to be ~45 kD, similar to that of known plant mitogen-activated protein (MAP) kinases (*10–15*).

To determine whether the elicitor-activated protein kinase might belong to the class of MAP kinases, we incubated the same cell extracts used for activity assays with three different antisera—M7, M11, and M14—that were raised against synthetic peptides representing the COOH-terminal 10 amino acids of the alfalfa MMK4, MMK2, and MMK3 MAP kinases, respectively (*12*). Elicitor treatment exclusively activated a protein kinase that was immunoprecipitated by the M7 antiserum (Fig. 1B). The similarity of the activation kinetics in the kinase and immunoprecipitation assays indicate that elicitor treatment activates a specific MAP kinase pathway in parsley cells.

Because the M7 antiserum specifically recognized the elicitor-responsive MAP kinase from parsley, a radiolabeled fragment of the alfalfa *MMK4* gene was used to screen a cDNA library prepared from RNA isolated from cultured parsley cells. A 1.6-kb cDNA fragment was isolated that contained an open reading frame of 1113 nucleotides potentially encoding a protein of 371 amino acids and a molecular mass of 43 kD. The deduced amino acid sequence is most similar to those of the MAP kinases from *Arabidopsis* (MPK3, 83%) (*11*), alfalfa (MMK4, 81%) (*12*), and tobacco (WIPK, 83%) (*13*). The overall structure of the parsley, tobacco, *Arabidopsis*, and alfalfa kinases is highly conserved (Fig. 2). DNA gel blot analysis of parsley cells with the radiolabeled kinase cDNA fragment under high-stringency hybridization conditions revealed the parsley kinase to be present as a single-copy gene (*16*). RNA gel blot analysis of cultured parsley cells with radiola-

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beled fragments, containing either the coding region or the 3′-untranslated region of the kinase cDNA, showed a severalfold increase of kinase transcript levels within 30 min after elicitor treatment (*16*). When the parsley cDNA was expressed as a fusion protein with glutathione-S-transferase (GST) in *Escherichia coli*, the recombinant protein catalyzed its autophosphorylation and phosphorylated MBP. In immunoblots, the GST-MAP kinase fusion protein was exclusively recognized by the M7 antiserum that recognized the elicitor-responsive protein kinase from cultured parsley cells. In contrast, the M11 and M14 antisera did not decorate the parsley kinase fusion protein. These results suggest that the cDNA isolated from parsley cells indeed encodes the elicitor-activated kinase detected in the activity and immunocomplex assays, which we therefore denote ERM kinase for elicitor-responsive MAP kinase.

To investigate whether Pep13 activates this kinase through the same receptor that is used for the induction of the other defense responses, we determined ERM kinase activation upon treatment of parsley cells with four different but structurally related elicitor oligopeptides. Pep13 and Pep25, both corresponding to the wild-type sequence of the 42-kD *P. sojae* glycoprotein elicitor, activated the elicitor-responsive MAP kinase in an identical manner (Figs. 1 and 3). Two Pep13 derivatives in which the second ($Pep13A_2$) or the fifth ($Pep13A_5$) amino acid had been replaced by alanine did not activate ERM kinase, whereas a derivative with an alanine substitution in position 12 ($Pep13A_{12}$) was as active as Pep13 and Pep25 (Fig. 3). These results correlate well with binding and elicitor studies (*7*) with the same Pep13 derivatives, which showed that $Pep13A_{12}$ competes with binding of Pep13 to its receptor and elicits a normal pattern of defense reactions. In contrast, $Pep13A_2$ and $Pep13A_5$ were inactive in both assays, indicating that the Pep13 receptor that initiates the multicomponent defense response is also engaged in ERM kinase activation.

Binding of Pep13 to its receptor induces phytoalexin synthesis, defense gene activation, in vivo phosphorylation of proteins, and the production of an oxidative burst, which all depend on the integrity of specific ion channels that mediate rapid ion fluxes across the cell membrane in response to elicitor (*7*, *9*, *17*, *18*). To investigate whether ERM kinase activation also depended on the activity of these ion channels, we incubated parsley cells with the ion channel blocker, anthracene-9-carboxylate (A9C), which inhibits the elicitor-stimulated ion fluxes, thereby blocking all subsequent defense responses (*17*). Under these condi-

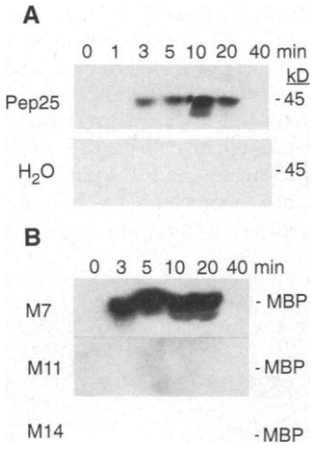

Fig. 1. A specific MAP kinase is activated by elicitor. Suspension cultured parsley cells were treated with the synthetic peptide elicitor, Pep25 (175 nM), or water alone. Cell extracts were prepared at 0, 1, 3, 5, 10, 20, and 40 min after initiation of elicitor treatment in extraction buffer [25 mM tris-HCl (pH 7.5), 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% NP-40, 15 mM *p*-nitrophenylphosphate, 60 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptine and aprotinin]. After centrifugation at 100,000*g* for 1 hour, the cleared supernatant was used. (**A**) In-gel protein kinase assay. Each lane contained 20 μg of total protein from cell extracts, which was separated by SDS–polyacrylamide gel electrophoresis (PAGE). MBP (0.5 mg/ml) was used as a substrate and was polymerized in the polyacrylamide gel. After protein renaturation, the kinase reactions were carried out in the gel with [γ-^{32}P]adenosine 5′-triphospate (ATP) as described (*12*). (**B**) Immunoprecipitation of an elicitor-responsive MAP kinase. Cell extracts containing 100 μg of total protein were immunoprecipitated with 5 μg of protein A–purified M7, M11, and M14 antibodies that were produced against synthetic peptides encoding the COOH-terminal 10 amino acids of the alfalfa MMK4, MMK2, and MMK3 MAP kinases, respectively (*12*). The immunoprecipitated proteins were washed three times with wash buffer I (20 mM tris-HCl, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100), once with the same buffer but containing 1 M NaCl, and once with kinase buffer [20 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT]. Kinase reactions of the immunoprecipitated proteins were performed in 15 μl of kinase buffer containing MBP (0.5 mg/ml), 0.1 mM ATP, and 2 μCi of [γ-^{32}P]ATP at room temperature for 30 min. The reactions were stopped by the addition of SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

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                10                  20                  30                  40          I       50
ERMK  MANP----GD GQYTDFPAIQ THGGQFIQYN IFGNLFQVTK KYRPPIMPIG
WIPK  MADANMGAGG GQFPDFPSVL THGGQYVQFD IFGNFFEITT KYRPPIMPIG
MPK3  -----MNTGG GQYTDFPAVD THGGQFISYD IFGSLFEITS KYRPPIIPIG
MMK4  MARV----NQ NGVAEFPAVQ THGGQFVQYN VFGNLFEVTA KYRPPIMPIG

                60                  70          II      80                  90         III     100
ERMK  RGAYGIVCSI MNTETNEMVA VKKIANAFDN YMDAKRTLRE IKLLRHLDHE
WIPK  RGAYGIVCSV LNTELNEMVA VKKIANAFDI YMDAKRTLRE IKLLRHLDHE
MPK3  RGAYGIVCSV LDTETNELVA MKKIANAFDN HMDAKRTLRE IKLLRHLDHE
MMK4  RGAYGIVCSL LNTETNELVA VKKIANAFDN HMDAKRTLRE IKLLRHLDHE

              IV 110                120           V     130                 140                 150
ERMK  NVIARTDVIP PPLRREFTDV YIATELMDTD LHQIIRSNQG LSEEHCQYFL
WIPK  NVIGLRDVIP PPLRREFSDV YIATELMDTD LHQIIRSNQG LSEDHCQYFM
MPK3  NIIAIRDVVP PPLRRQFSDV YISTELMDTD LHQIIRSNQS LSEEHCQYFL
MMK4  NVIGLRDVIP PPLRREFNDV YITTELMDTD LHQIIRSNQN LSDEHCQYFL

              160                 170           VI  180         VII             200
ERMK  YQLLRGLKYI HSANIIHRDL KPSNLLLNAN CDLKICDFGL ARHNTDDEFM
WIPK  YQLLRGLKYI HSANVLHRDL KPSNLLVNAN CDLKICDFGL ARPNIENENM
MPK3  YQLLRGLKYI HSANIIHRDL KPSNLLLNAN CDLKICDFGL ARPTSENDFM
MMK4  YQILRGLRYI HSANIIHRDL KPSNLLLNAN CDLKIIDFGL ARPTMESDFM

          * *     210  VIII   220                 230 IX      240                 250
ERMK  TEYVVTRWYR APELLLNSSD YTAAIDVWSV GCIYMELMNR KPLFAGKDHV
WIPK  TEYVVTRWYR APELLLNSSD YTAAIDVWSV GCIFMELMNR KPLFGGKDHV
MPK3  TEYVVTRWYR APELLLNSSD YTAAIDVWSV GCIFMELMNR KPLFPGKDHV
MMK4  TEYVVTRWYR APELLLNSSD YTSAIDVWSV GCIFMELMNK KPLFPGKDHV

              260 X               270                 280                 290                 300
ERMK  HQMRLLTELL GSPTEADLGF VRNEDAKRFI LQLPRHPRQP LRQLYPQVHP
WIPK  HQIRLLTELL GTPTEADLGF LQNEDAKRYI RQLPQHPRQQ LAEVFPHVNP
MPK3  HQMRLLTELL GTPTESDLGF THNEDAKRYI RQLPNFPRQP LAKLFSHVNP
MMK4  HQMRLLTELL GTPTDADVGL VKNDDARRYI RQLPQYPRQP LNRVFPHVHP

              310     XI  320                 330                 340                 350
ERMK  LAIDLIDKML TFDPSKRITV EEALAHPYLA RLHDIADEPI CTKPFSFEFE
WIPK  LAIDLVDKML TFDPTRRITV EEALDHPYLA KLHDAGDEPI CPVPFSFDFE
MPK3  MAIDLVDRML TFDPNRRITV EQALNHQYLA KLHDPNDEPI CQKPFSFEFE
MMK4  LAIDLVDKML TIDPTRRITV EEALAHPYLE KLHDVADEPI CMEPFSFEFE

              360                 370
ERMK  TAHLGEEQIK DMIYQEALAF NPDCA
WIPK  QQGIGEEQIK DMIYQEALSL NPEYA
MPK3  QQPLDEEQIK EMIYQEAIAL NPTYG
MMK4  QQHLDEEQIK EMIYREALAL NPEYA
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Fig. 2. Primary structure of an elicitor-responsive MAP (ERM) kinase from parsley. The nucleotide and predicted amino acid sequence of the ERM kinase has been deposited with GenBank, DNA Data Base Japan, and European Molecular Biology Laboratory databases (accession number Y12875). The primary sequence of ERM kinase was deduced from the sequence of a cDNA clone isolated by standard methods (*28*) from a parsley cDNA library constructed in the λ-ZAP vector (Stratagene), by use of a 1.1-kb random primed ^{32}P-labeled DNA probe (megaprime labeling kit, Amersham), representing the near full-length open reading frame of MMK4 (*12*). The positive clone thus isolated is aligned with its closest homologs, MPK3 from *Arabidopsis thaliana* (*11*), MMK4 from *Medicago sativa* (*12*), and WIPK from *Nicotiana tabacum* (*13*). Shaded areas represent identical sequences. Roman numerals indicate kinase subdomains (*29*). Conserved phosphorylation sites are marked with an asterisk.

tions, Pep13 activation of the ERM kinase was completely inhibited, indicating that ion channel activation was also necessary for this reaction (Fig. 4). Amphotericin B, which mimics elicitor-induced ion fluxes and thereby induces the full set of defense responses (*17*), also activates the ERM kinase in the absence of elicitor (Fig. 4). Activation of ERM kinase (Fig. 4), ion fluxes, and the oxidative burst (*17*) by amphotericin B all occur after a delay of about 30 min. Thus, ERM kinase activation depends on the state of specific ion channels, and activation of these channels is necessary and sufficient for ERM kinase activation as it is for the induction of the other elicitor responses in this system.

The elicitor-stimulated production of reactive oxygen species is thought to be catalyzed by an NADH [nicotinamide adenine dinucleotide (reduced)] or NADPH [nicotinamide adenine dinucleotide phosphate (reduced)] oxidase that is inhibited by diphenylene iodonium (DPI) (*19*). In elicitor-treated parsley cells DPI blocked the oxidative burst, defense gene activation, and phytoalexin accumulation without affecting ion fluxes (*17*). Together with the results from gain-of-function experiments with KO_2, which stimulated phytoalexin production in the absence of elicitor, this placed the oxidative burst downstream of the ion channels within the elicitor signal transduction cascade (*17*). Pep13 activation of the ERM kinase was not inhibited by DPI, indicating that this kinase acts either upstream or independently of the oxidative burst (Fig. 4).

Certain MAP kinases are translocated into the nucleus upon activation, where they may catalyze phosphorylation of transcription factors and thereby regulate gene transcription (*20–22*). The subcellular location of ERM kinase was determined with M7 antiserum in immunofluorescence microscopy before and after treating parsley cells with Pep25 elicitor. Within 3 to 10 min after Pep25 treatment, ERM kinase was translocated into the nucleus (Fig. 5C). Because no nuclear localization signal is present in the ERM kinase, translocation of the activated kinase into the nuclear compartment may be initiated by its interaction with another protein, perhaps a transcription factor. In parsley, several elicitor-responsive genes have been identified and have led to the identification of cis elements and transcription factors that may be involved in mediating pathogen-induced

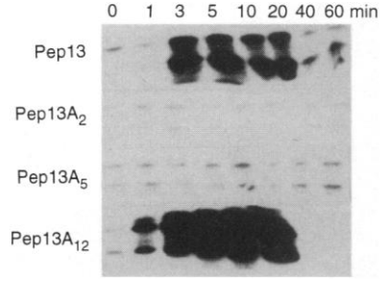

Fig. 3. ERM kinase is exclusively activated by active peptide elicitor. Suspension-cultured parsley cells were treated with the synthetic peptide elicitor Pep13 (50 nM), and with inactive ($Pep13A_2$, 50 nM, and $Pep13A_5$, 50 nM) and active ($Pep13A_{12}$, 50 nM) derivatives. Cell extracts were prepared at 0, 1, 3, 5, 10, 20, 40, and 60 min after elicitor treatment. Cell extracts containing 100 μg of total protein were immunoprecipitated with M7 antibody. The kinase activity of the immunocomplexes was determined by in vitro kinase assays with MBP as substrate as described in Fig. 1B.

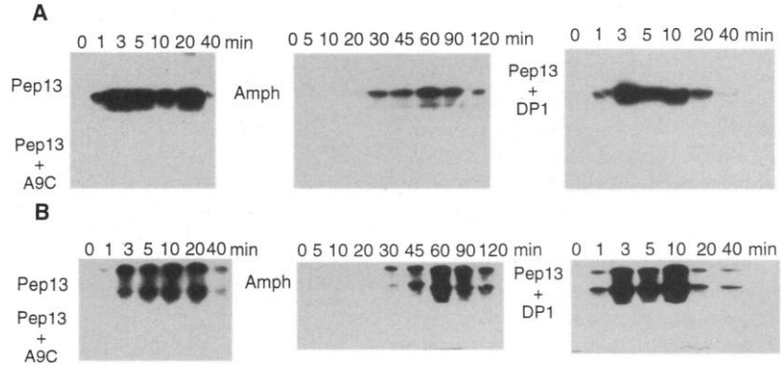

Fig. 4. ERM kinase activation depends on elicitor-stimulated ion-channel activity but not on an oxidative burst. Suspension-cultured parsley cells were preincubated with 100 μM of the ion-channel blocker anthracene-9-carboxylate (A9C), with 50 μM of the polyene antibiotic, amphotericin B (Amph), or with 50 μM of diphenylene iodonium (DPI), an inhibitor of the oxidative burst, followed by addition of Pep13 (50 nM) to A9C- and DPI-treated cells 30 min later. After the indicated periods of treatment the cells were harvested, and total protein was extracted and analyzed by (A) in-gel MBP kinase assays and (B) M7 antibody–precipitated immunocomplex kinase assays as described in Fig. 1.

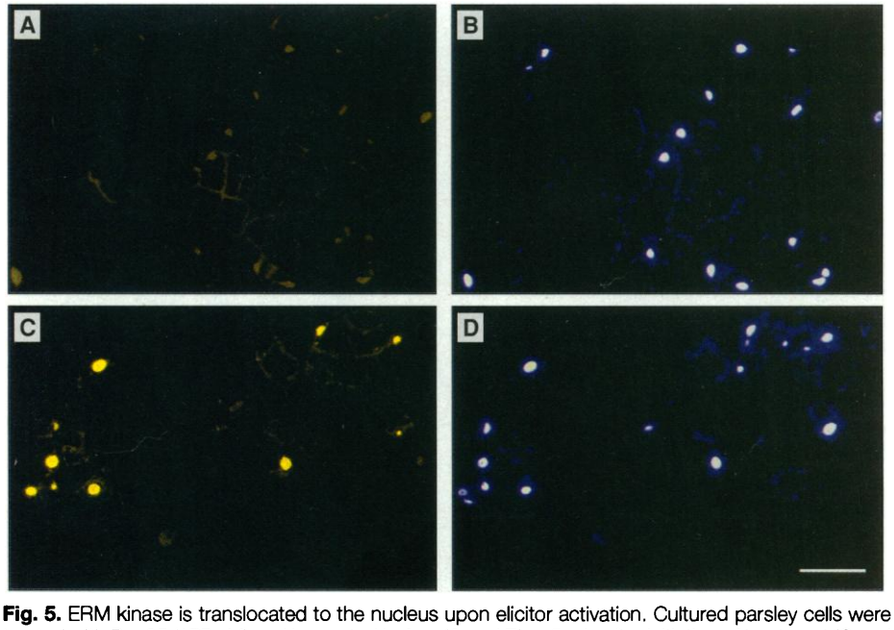

Fig. 5. ERM kinase is translocated to the nucleus upon elicitor activation. Cultured parsley cells were treated with Pep25 (175 nM) and harvested before (**A** and **B**) or 5 min after initiation of treatment (**C** and **D**). Sections (2 μm) across cell clusters were fixed with 4% formaldehyde, embedded in polyethylene glycol (*30*), and either stained with the M7 antiserum (A and C), specifically recognizing ERM kinase, or with 4′,6′-diamidino-2-phenylindole (DAPI) (B and D) to visualize nuclei. Biotinylated secondary antibody, streptavidin–horseradish peroxidase, and fluorescein tyramid reagent were used to visualize the primary antibody bound to ERM kinase according to the manufacterer's instructions (Tyramid Signal Amplification Systems, TSA-Direct-Green, Du Pont, NEN, Boston, Massachusetts). After treatment with Pep25 most nuclei were decorated by the M7 antibody (B), whereas no or little staining was detectable in untreated cells (A), in cells treated with water instead of Pep25, or when the M7 antibody was replaced by preimmune serum. Bar (D), 50 μm.

transcription (*1*, *23*). Although it has not yet been shown that phosphorylation of these transcription factors is responsible for elicitor-induced transcription of PR genes, the elicitor-induced relocation of ERM kinase into the nucleus might link cytosolic signal transduction to nuclear activation of plant defense genes.

MAP kinases were first found in yeast and animals, where they participate in signaling cascades linking plasma membrane receptors that perceive extracellular signals to a variety of cellular response mechanisms (*24*, *25*). The MAP kinases known in plants are activated by environmental stresses and plant hormones (*26*, *27*). Our results demonstrate posttranslational and transcriptional activation of a plant MAP kinase within a signal transduction pathway that mediates the response to a pathogen. Activation of ERM kinase follows input from receptor-regulated ion channels of the plasma membrane and precèdes or parallels the formation of O_2^- radicals, which in turn activate defense genes and phytoalexin synthesis (*17*).

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Differential Requirements for Survival and Proliferation of CD8 Naïve or Memory T Cells

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The requisite molecular interactions for CD8 T cell memory were determined by comparison of monoclonal naïve and memory $CD8^+$ T cells bearing the T cell receptor (TCR) for the HY antigen. Naïve T cells required only the right major histocompatibility complex (MHC) class I–restricting molecule to survive; to expand, they also needed antigen. In contrast, for survival, memory cells did not require the restricting MHC allele, but needed only a nonspecific class I; for expansion the correct class I, but not antigen, was required. Thus, maintenance of CD8 T cell memory still required TCR–MHC class I interactions, but memory T cells may have a lower functional activation threshold that facilitates secondary responses.

The molecular basis of T cell memory remains elusive (*1*, *2*). It is not known if memory responses depend exclusively on an increased frequency of antigen-specific T cells (*3*) or if "memory T cells" with novel biological capacities are generated (*4*). Memory responses have been reported to depend on continuous antigenic stimulation (*5*), but others have observed the persistence of increased frequencies of antigen-specific $CD8^+$ T cells in the apparent absence of antigen (*6*–*8*).

We have investigated the conditions necessary in vivo for the survival and expansion of naïve and memory antigen-specific $CD8^+$ T cells. Because of the degeneracy and redundancy of T cell receptor (TCR) usage in most immune responses, individual clones of antigen-specific T cells "in vivo" cannot be easily examined. T cells may also coexpress different TCRs, and their behavior may be conditioned by nonspecific antigen effects (*1*, *2*). Thus, to characterize the functional properties and the requirements for persistence of memory T cells, we used monoclonal T cell populations.

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Transgenic (Tg) mice bearing a Tg αβ TCR specific for the HY male antigen restricted to major histocompatibility complex (MHC) class I $H\text{-}2D^b$ and deficient in the recombinase gene *RAG2* ($TgRAG2^-$) (*9*) were used to obtain monoclonal populations of $CD8^+$ T cells. In female $TgRAG2^-$ mice, all T cells positively selected in the thymus are $CD8^+$ Tg $TCR\alpha\beta^+$ (Fig. 1A). These cells represent a pure population of naïve T cells, because cross-reactivity with environmental antigens cannot be detected: All these cells are $CD44^-$ and do not divide (*10*, *11*). Studying these cells ex vivo, we could not detect lymphokine mRNAs, but these could be induced after in vitro stimulation with monoclonal antibodies (mAbs) to CD3 (anti-CD3). Virgin T cells constitutively expressed little perforin and FasL mRNAs, which were up-regulated after anti-CD3 stimulation (Fig. 1B) (*12*).

To study the TCR interactions required for the survival or division of naïve CD8 T cells, we compared their fate after transfer into irradiated hosts (*13*) that differed in MHC class I and HY antigen expression. These hosts were C57BL/6 CD8-deficient (*14*) male ($HY^+H\text{-}2^{b+}$) and female ($HY^-H\text{-}2^{b+}$) mice; female $H\text{-}2D^b$-deficient mice (*15*) that lack the MHC class I restriction element of this Tg TCR (*16*) but express other MHC class I molecules including $H\text{-}2K^b$ ($HY^-H\text{-}2D^{b-}$ class I^+); and female class I^- mice defi-