cell surface molecule that can recognize this domain.

The observation that only those CTLp's and Thp's that have been signaled through their antigen-specific surface receptors are killed by exposure to an antibody recognizing all lymphocytes (anti-α3) may prove useful for establishing tolerance in the adult animal to a particular antigen.

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11 December 1990; accepted 15 March 1991

CREB: A Ca^{2+}-Regulated Transcription Factor Phosphorylated by Calmodulin-Dependent Kinases

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The mechanism by which Ca^{2+} mediates gene induction in response to membrane depolarization was investigated. The adenosine 3′,5′-monophosphate (cAMP) response element–binding protein (CREB) was shown to function as a Ca^{2+}-regulated transcription factor and as a substrate for depolarization-activated Ca^{2+}-calmodulin–dependent protein kinases (CaM kinases) I and II. CREB residue Ser^{133} was the major site of phosphorylation by the CaM kinases in vitro and of phosphorylation after membrane depolarization in vivo. Mutation of Ser^{133} impaired the ability of CREB to respond to Ca^{2+}. These results suggest that CaM kinases may transduce electrical signals to the nucleus and that CREB functions to integrate Ca^{2+} and cAMP signals.

DNA ELEMENTS THAT CONTAIN THE consensus sequence TGACGTCA were originally identified as cAMP response elements (CREs) in neuropeptide genes (*1*), but recent studies in neuronal cell lines have mapped inducibility by membrane depolarization and increased intracellular Ca^{2+} concentrations to similar sequences in the c-*fos* (*2–4*) and proenkephalin genes (*5*). Depolarization of PC12 pheochromocytoma cells is correlated with rapid phosphorylation of the CRE-binding protein CREB on amino acid residue Ser^{133} (*4*). Phosphorylation of Ser^{133} stimulates the ability of CREB to activate gene transcription (*6*), suggesting that CREB may mediate transcriptional induction by Ca^{2+} as well as by cAMP. However, because multiple CRE-binding proteins exist (*7, 8*), distinct members of this family may separately confer Ca^{2+} and cAMP inducibility on the CRE (*4*). To determine whether CREB functions as a Ca^{2+}-activated transcription factor and to test if phosphorylation of Ser^{133} is important for this activation, we have targeted CREB to a different DNA regulatory sequence by fusing it to the DNA-binding and dimerization domain of the yeast transcriptional activator GAL4 (*9*) (Fig. 1). The use of a reporter gene that contains GAL4 binding sites allowed a spe-

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cific functional analysis of CREB without interference from endogenous CRE-binding proteins.

When cotransfected into rat PC12 cells with a human c-*fos* reporter gene that contained one GAL4 binding site (pAF42G_1), the GAL4-CREB fusion construct conferred Ca^{2+} inducibility (Fig. 1B). In the absence of stimulation, no transcripts were detected from transfected or endogenous c-*fos* genes. When the intracellular Ca^{2+} concentration was increased by K^+ depolarization or by treatment with the Ca^{2+} ionophore A23187, the major Ca^{2+}-inducible transcript corresponded to correctly initiated human c-*fos* (c-fos^H) mRNA. The GAL4-CREB fusion protein conferred a level of induction comparable to that observed in depolarized PC12 cells transfected with a reporter gene containing a CRE (Fig. 1B).

The Ca^{2+}-dependent induction of c-fos^H by GAL4-CREB required the GAL4 binding site and was dependent on the CREB moiety of the GAL4-CREB fusion protein (Fig. 1B). The GAL4 DNA-binding domain either by itself (*10*) or fused to an acidic activating region (GAL4-B17) (*11*) was unable to confer Ca^{2+} inducibility. As expected, the GAL4-CREB fusion also conferred cAMP-inducible expression on the GAL4 binding site (Fig. 1B) (*12*). The levels of inducibility with Ca^{2+} and cAMP were similar, suggesting that CREB functions as a transcriptional regulator that is responsive to both second messengers. In contrast, GAL4-CREB did not mediate transcriptional activation by the protein kinase C activator 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Fig. 1B).

Because GAL4-CREB contains the leucine zipper dimerization motif (*13, 14*), endogenous PC12 proteins that contain leucine zippers might interact with GAL4-CREB and cause the observed transcriptional activation. To test this possibility, we constructed a GAL4-CREB mutant in which the leucine zipper was deleted but the GAL4 DNA-binding and dimerization domains remained intact (GAL4-CREBΔLZ) (Fig. 1A). GAL4-CREBΔLZ conferred both Ca^{2+} and cAMP inducibility on the GAL4 reporter construct (Figs. 1B and 2); however, the magnitudes of Ca^{2+} and cAMP induction were reduced. Similarly, GAL4-CREB proteins with other point mutations or small deletions within the CREB leucine zipper region that prevent dimerization (*14*) also confer Ca^{2+} and cAMP inducibility, but with reduced efficiency (*10*). The leucine zipper may be important for folding the CREB dimer into an optimal conformation for transcriptional activation. Alternatively, a portion of the Ca^{2+} and cAMP responses seen with GAL4-CREB may be due to dimerization with endogenous CREB proteins.

To determine whether phosphorylation of CREB is functionally important for depolarization- and Ca^{2+}-dependent activation of transcription, we mutated the major site of CREB phosphorylation in depolarized PC12 cells (Ser^{133}) to alanine. This mutation (M1) reduced by ~80% the ability of GAL4-CREBΔLZ to mediate transcriptional activation by either membrane depolarization or cAMP (Fig. 2). The M1 mutation does not affect the stability of CREB or its nuclear localization (*6*). These results suggest that Ca^{2+}-dependent phosphorylation of Ser^{133} is important for CREB activation by membrane depolarization. However, mutation of Ser^{133} did not completely abolish the ability of GAL4-CREBΔLZ to confer either Ca^{2+} or cAMP inducibility. The basis of this residual activity is unclear (*15*).

The transducing kinase that couples the depolarization-induced Ca^{2+} signal to the phosphorylation and activation of CREB may be a Ca^{2+}-calmodulin–dependent protein kinase (CaM kinase). CaM kinases are activated when the intracellular Ca^{2+} concentration is increased after membrane depolarization (*16, 17*), and induction of c-*fos* by depolarization is specifically inhibited by calmodulin antagonists (*18*). CaM kinases I and II have wide tissue distributions but are especially abundant in neural tissues (*17, 19*). CaM kinase II has been implicated in diverse regulatory processes in the nervous system, including long-term potentiation (LTP) (*20*). The CaM kinase I substrates synapsin I and protein III are both neuron-specific proteins that are phosphorylated on a single common amino acid residue by both cAMP-dependent protein kinase (PKA) and CaM kinase I (*17, 21*). The fact that a common residue in CREB (Ser^{133}) is phosphorylated in vivo in response to either Ca^{2+} or cAMP suggested the possibility that CaM kinase I may mediate Ca^{2+}-dependent phosphorylation of CREB.

We tested whether purified CREB can serve as a substrate in vitro for CaM kinase I or II. Two major CRE-binding polypeptides of 43 and 38 kD were purified by DNA-affinity chromatography from extracts

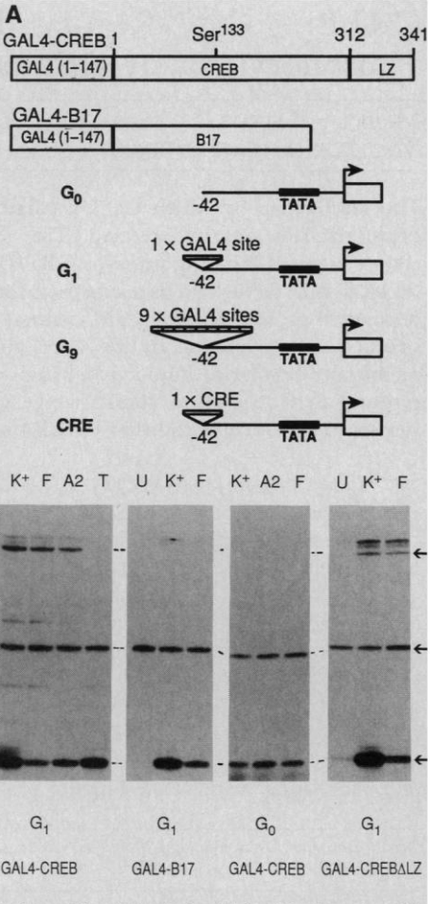

Fig. 1. Analysis of GAL4-CREB fusions by cotransfection assay. (**A**) Structure of GAL4-CREB fusions and reporter genes (*27*). GAL4-B17 contains an acidic activating region (*11*) fused to GAL4 (1 to 147), the DNA-binding domain of GAL4 (*9*). GAL4-CREB contains the complete CREB protein (341 amino acids) fused to the COOH-terminus of GAL4 (1 to 147). GAL4-CREBΔLZ lacks the COOH-terminal 29 amino acids of GAL4-CREB, including the leucine repeat dimerization motif (LZ). GAL4-CREBΔLZM1 contains the Ser^{133} to Ala mutation. The constructs pAF42G_1 (G_1) and pAF42G_9 (G_9) contain, respectively, one and nine GAL4 binding sites inserted at position −42 in the core promoter construct pAF42 (G_0), which encodes the human c-*fos* gene (c-fos^H) (*4*). In pAF42CRE (CRE), the consensus CRE is inserted at position −42. (**B**) Ca^{2+} and cAMP inducibility of reporter genes with GAL4-CREB constructs. GAL4-CREB fusion constructs (5 μg) were transiently cotransfected into PC12 cells (*3, 4*) with reporter constructs pAF42G_0 (G_0) or pAF42G_1 (G_1) (20 μg), as indicated. CRE (20 μg) was transfected alone because the CRE can interact with endogenous CREB. An α-globin expression plasmid (pSVα-1; 3 μg) was used as a control for transfection efficiency. Cells were left unstimulated (U) or were stimulated as indicated by 60 mM K^+ and 10 mM Ca^{2+} (K^+), A23187 (10 μg/ml) (A2), or 10 μM forskolin (F) for 60 min or by TPA (0.3 μg/ml) (T) for 30 min. Correctly initiated transcripts from the transfected reporter gene (c-fos^H), the endogenous rat gene (c-fos^R), and the internal control gene (globin), were assayed by ribonuclease (RNase) protection (*3, 4*) and are indicated. Bands above c-fos^H represent incorrectly initiated transcripts (*28*).

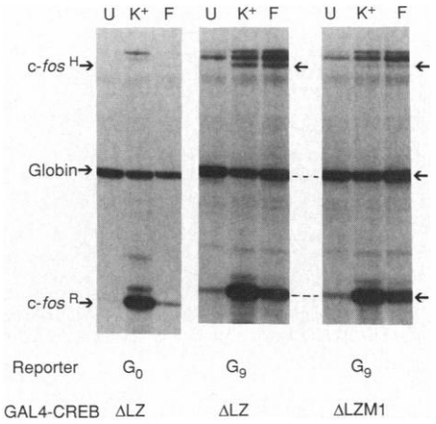

Fig. 2. Effect of the Ser^{133} to Ala mutation on GAL4-CREBΔLZ. Transient transfection assays were performed as in Fig. 1 with GAL4-CREBΔLZ or GAL4-CREBΔLZM1 and reporter constructs $pAF42G_0$ or $pAF42G_9$ as indicated. Bands that represent correctly initiated transcripts are indicated as in Fig. 1.

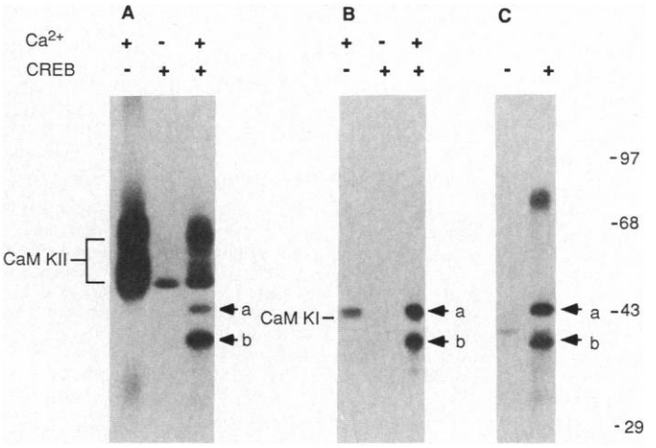

Fig. 3. In vitro phosphorylation of CREB and CREB B by purified protein kinases. (**A**) CaM kinase II. (**B**) CaM kinase I. (**C**) PKA. Reactions were performed with [^{32}P]ATP at 30°C for 5 min, and contained 5 to 10 ng of affinity-purified CREB (+ CREB) (*29*) or an equivalent volume of buffer (− CREB). CaM kinase reactions (*30*) were performed in the presence of Ca^{2+} (+ Ca^{2+}) or in the presence of excess EGTA (− Ca^{2+}). Labeled products were separated on SDS-polyacrylamide gels and visualized by autoradiography. The positions of CREB and CREB B are marked by "a" and "b", respectively. The autophosphorylated products of CaM kinase I (CaM KI) (37 to 42 kD) and CaM kinase II (CaM KII) (51 to 53 kD and 60 kD) are indicated. A diffuse band of ~80 kD in the PKA lane is not consistently seen between different experiments. Positions of molecular size markers are indicated in kilodaltons.

of mouse L cells. The 43-kD protein has the same molecular size as CREB purified from brain or PC12 cells (*22*), comigrates with CREB immunoprecipitated from PC12 cells, and is quantitatively immunoprecipitated by a CREB-specific antiserum (Fig. 3) (*10*). The 38-kD protein (termed CREB B) is likely to be a distinct CREB-related protein (*23*).

Both CREB and CREB B are substrates for CaM kinases I and II in vitro (Fig. 3). After phosphorylation, both CREB and CREB B retain their ability to bind the CRE, as determined by specific retention on a CRE-affinity column (*10*). We performed two-dimensional tryptic phosphopeptide mapping to identify the sites of CREB phosphorylation by CaM kinases I and II. The major tryptic phosphopeptide (site 1) labeled in vitro by CaM kinases I and II comigrated with the CREB tryptic fragment phosphorylated in vivo in response to increased Ca^{2+} (*4*) (Fig. 4). Tryptic peptide 1 contains Ser^{133} as the only Ser or Thr residue (*6, 7*). We therefore conclude that the major in vitro site of CREB phosphorylation by CaM kinases I and II is Ser^{133}. Whether CaM kinases directly phosphorylate CREB on Ser^{133} in vivo remains to be determined. The two-dimensional tryptic maps revealed that in addition to Ser^{133}, CREB was consistently phosphorylated by CaM kinases I and II at several minor sites (Fig. 4), the significance of which is unknown.

We found that CREB is a substrate for PKA, as well as the CaM kinases, and that the major site of phosphorylation by PKA is also Ser^{133} (Fig. 4) (*22*). The ability of both CaM kinase II and PKA to phosphorylate CREB in vitro on the same site has been demonstrated independently by Dash and co-workers (*24*). In addition, they have shown that phosphorylation of this site stimulates the ability of CREB to activate transcription in an in vitro RNA polymerase II–dependent transcription system. The phosphorylation in vitro of a common regulatory site (Ser^{133}) by Ca^{2+}- and cAMP-regulated protein kinases provides a biochemical explanation for how Ca^{2+} and cAMP signaling pathways might converge to activate CREB in vivo.

Analysis of the amino acids that flank Ser^{133} in CREB (7) revealed that this residue lies within a stretch of amino acids the sequence of which is consistent with it being a recognition site for CaM kinases I and II and PKA. The local peptide sequence (-Ser-Arg-Arg-Pro-Ser^{133}-Tyr-Arg-) conforms to the motif -Arg-X-X-Ser(Thr)- (where X represents any amino acid), which is the minimum specificity determinant for CaM kinase II (*19*), as well as to the PKA consensus recognition sequence (-Arg-Arg-X-Ser-) (*25*). Overlap in the phosphorylation sites of CaM kinase II and PKA has been described for other substrates (*26*). Moreover, the Ser residue in synapsin I that is phosphorylated by both CaM kinase I and PKA (*21*) lies within a sequence (Arg-Arg-Arg-Leu-Ser-Asp) that is similar to the CREB Ser^{133} site.

The finding that a regulatory site in CREB is phosphorylated by both Ca^{2+}- and cAMP-dependent protein kinases in vitro provides an explanation for the observation that CREB can mediate a transcriptional response to either Ca^{2+} or cAMP. Thus, the increasingly complex role of the CRE in gene regulation may be a reflection of its importance as a target of multiple signal transduction pathways in the cell.

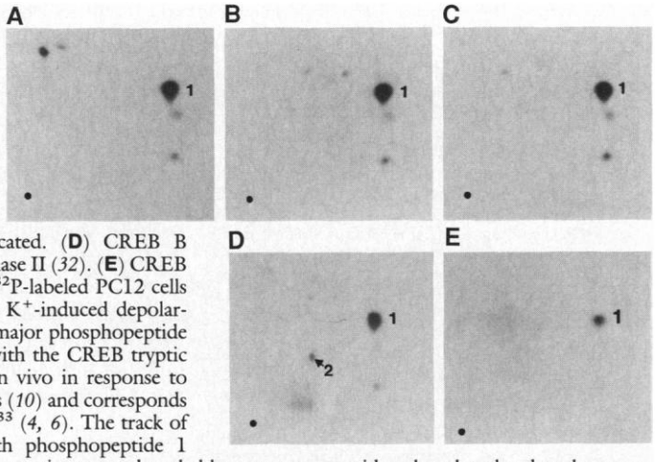

Fig. 4. Sites of CREB phosphorylation by CaM kinase I, CaM kinase II, and PKA. Two-dimensional tryptic phosphopeptide maps (*4, 31*) are shown. CREB was phosphorylated in vitro by (**A**) CaM kinase I, (**B**) CaM kinase II, or (**C**) PKA, as indicated. (**D**) CREB B phosphorylated by CaM kinase II (*32*). (**E**) CREB immunoprecipitated from ^{32}P-labeled PC12 cells that had been subjected to K^+-induced depolarization for 5 min (*4*). The major phosphopeptide (marked "1") comigrates with the CREB tryptic fragment phosphorylated in vivo in response to Ca^{2+} in mixing experiments (*10*) and corresponds to phosphorylation of Ser^{133} (*4, 6*). The track of faint spots trailing beneath phosphopeptide 1 varies in intensity between experiments and probably represents peptides phosphorylated at the same site that have not completely migrated to the major spot 1 position during chromatography. Origins are marked by a black dot. Electrophoresis at pH 1.9 was performed from left (+) to right (−), and chromatography was performed in the ascending direction.

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27. The complete coding region of the rat CREB cDNA (*7*) was amplified by the polymerase chain reaction (PCR) and fused in frame to GAL4 (1 to 147) encoded in the plasmid pSG424 (*9*) to form GAL4-CREB. The Ser^{133} to Ala mutation (M1) was subcloned (as an Aat II–Kpn I restriction fragment) into GAL4-CREB from the plasmid RSV-CREBfM1 (*6*) to generate GAL4-CREBM1. GAL4-CREBΔLZ and GAL4-CREBΔLZM1 were constructed by amplifying the corresponding regions from GAL4-CREB and GAL4-CREBM1 by PCR.
28. The two bands slightly above c-fos^{H} that are also detected in the ribonuclease (RNase) protection assay represent transcripts initiating from within the vector and from a cryptic mRNA start site in the TATA region of the c-*fos* construct (*4*).
29. CREB was purified by DNA-affinity chromatography [J. T. Kadonaga and R. Tjian, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5889 (1986)] on columns (40 μg of DNA per milliliter) prepared from oligonucleotides of the following sequences: CRE column, 5′-CCCG*TGACGTCA*ACAC-3′ and 5′-*TGACGTCA*CGGGGTGT-3′; AP-1 column, 5′-CCCG*TGAGTCA*ACAC-3′ and 5′-*TGACTCA*CGGGGTGT-3′ (consensus elements in italics). Nuclear extracts (200 mg of protein) were prepared from 2×10^{11} mouse L cells by the chloroquine DNA intercalation method [H. Schroter, P. E. Shaw, A. Nordheim, *Nucleic Acids Res.* **15**, 10145 (1987)]. We purified CREB by sequential passage through a serum response element column [R. Treisman, *EMBO J.* **6**, 2711 (1987)], two CRE columns, one AP-1 column to remove AP-1 proteins, and a final CRE column. CRE-binding activity was followed by gel shift assay (*4*). Purified CREB (~0.5 μg, as estimated by in vitro DNA-binding activity) was concentrated in a Centricon ultrafiltration device (Amicon, Danvers, MA) and dialyzed overnight against 10 mM Hepes (pH 7.4) and 10% glycerol.
30. In vitro phosphorylation of CREB by purified rat brain CaM kinase II (1 to 5 μg/ml) was performed in 50 mM Pipes (pH 7), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), calmodulin (5 μg/ml), bovine serum albumin (100 μg/ml), 10 μM adenosine triphosphate (ATP), 1.5 μM [γ-^{32}P]ATP, and either 0.3 mM $CaCl_2$ or 0.2 mM EGTA. In vitro phosphorylation by CaM kinase I was performed as described (*17*) with 1 mM EGTA or 1 mM EGTA and 1.5 mM $CaCl_2$. In vitro phosphorylation by PKA catalytic subunit (20 to 40 μg/ml) (Sigma) was performed in 10 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, and 1.5 μM [γ-^{32}P]ATP.
31. K. Luo, T. R. Hurley, B. M. Sefton, *Oncogene* **5**, 921 (1990).
32. Two-dimensional tryptic analysis of CREB B phosphorylation in vitro by CaM kinase I, CaM kinase II, and PKA reveals that the major phosphopeptide labeled by these enzymes comigrates exactly with phosphopeptide 1 labeled in CREB (Fig. 4) (*10*), suggesting that CREB and CREB B may possess a conserved phosphorylation site. Hai *et al.* (*8*) have identified a protein, ATF1, that contains a sequence that is identical to the CREB peptide that contains Ser^{133}. ATF1 may therefore be identical to CREB B.
33. We thank H. Schulman and N. Waxham for purified CaM kinase II; A. Nairn for CaM kinase I; I. Sadowski for pSG424; M. Carey for pG9E4T; and M. Montminy for antiserum W39 to CREB and RSV-CREBfM1. Supported by NIH grant R01 CA 43855, grant R01 NS 28829 from the National Institute of Neurological Disorders and Stroke, an American Cancer Society Faculty Research Award (FRA-379) (M.E.G.), and a Scholar's Award from the McKnight Endowment Fund for Neuroscience (M.E.G.).

20 December 1990; accepted 22 March 1991

Recognition of Self Antigens by Skin-Derived T Cells with Invariant γδ Antigen Receptors

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Thy-1$^+$ dendritic epidermal T cells (dECs) express invariant γδ antigen receptors and are found in intimate contact with keratinocytes in murine epidermis—thus raising the possibility that keratinocytes express a ligand for the antigen receptor of these T cells. Thy-1$^+$ dECs were stimulated to produce lymphokines by interaction with keratinocytes in vitro. This stimulation was mediated through the dEC antigen receptor and did not appear to be restricted by the major histocompatibility complex. Thus, dECs can recognize self antigens and may participate in immune surveillance for cellular damage rather than for foreign antigens.

IN MICE, MOST T CELLS IN THE LYMphoid tissues express diverse antigen receptors that consist of α and β chains and recognize antigens bound to self major histocompatibility complex (MHC) proteins. A minority of lymphoid T cells express clonally diverse γδ T cell receptors (TCRs) (*1*) and recognize a wide array of antigens, including MHC class I, II, and Ib gene products, as well as bacterial heat shock proteins (*2–7*). In contrast to the lymphoid organs, several epithelial tissues contain mostly γδ T cells. The epidermis contains a unique population of cells, the Thy-1$^+$ dECs (*8–10*), which express invariant γδ TCRs composed of $V_\gamma3/J_\gamma1\text{-}C_\gamma1$ and $V_\delta1/D_\delta2/J_\delta2\text{-}C_\delta$ chains (*11, 12*) and have not been found elsewhere in the adult mouse (*13*). Similarly, the intraepithelial T cells of the female reproductive tract and the tongue express γδ TCRs composed of an invariant $V_\gamma4/J_\gamma1\text{-}C_\gamma1$ chain and an invariant δ chain identical to that found in the dECs (*14*). The absence of clonal diversity in the TCRs of these epithelium-associated T cells suggests that they might perform immunological functions that are distinct from those of T cells with clonally diverse antigen receptors. These nondiverse T cells might recognize damage-induced self antigens in a mechanism of trauma signal surveillance (*11, 15*). Here, we provide evidence in support of this concept by demonstrating that dECs can specifically recognize self antigens produced by skin-derived keratinocytes. This information and the physical interactions observed in situ lend support to a potential role of dECs in surveillance of skin keratinocytes in vivo for damage induced by stress, infection, or other means.

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