Thy-1.2 (30-H12) or with biotinylated MAb to I-A^d (AMS-32.1) (PharMingen) followed by fluorescein isothiocyanate–conjugated streptavidin, and analyzed by flow cytometry.
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24. Microtiter wells (Immulon 2; Dynatech) were coated with KLH or a 0.5% octyl glucoside extract of SRBC ghosts. Wells were blocked for 1 hour with sample diluent (Genetic Systems, Seattle) and then washed with phosphate-buffered saline that contained 0.05% Tween 20. Sera were serially diluted in sample diluent and incubated with coated wells for 1 hour at 23°C. Wells were washed, and Ab binding was detected by addition of horseradish peroxidase–conjugated Abs to murine IgG1 (Southern Biotech, Birmingham, AL), then detected with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Genetic Systems). Absorbances at 450 nm (A_{450}) were recorded on microtiter plate reader (Genetic Systems). Titers were determined from dilution curves as the dilution required to give a value for A_{450} of five times background.
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Activation-Induced Ubiquitination of the T Cell Antigen Receptor

Cristina Cenciarelli, Damon Hou, Kou-Ching Hsu, Barbara L. Rellahan, David L. Wiest, Harry T. Smith, Victor A. Fried, Allan M. Weissman*

The ζ subunit of the T cell antigen receptor (TCR) exists primarily as a disulfide-linked homodimer. This receptor subunit is important in TCR-mediated signal transduction and is a substrate for a TCR-activated protein tyrosine kinase. The ζ chain was found to undergo ubiquitination in response to receptor engagement. This posttranslational modification occurred in normal T cells and tumor lines. Both nonphosphorylated and phosphorylated ζ molecules were modified, and at least one other TCR subunit, CD3 δ, was also ubiquitinated after activation of the receptor. These findings suggest an expanded role for ubiquitination in transmembrane receptor function.

The TCR is a multicomponent transmembrane receptor that consists of clonally derived heterodimeric antigen recognition elements and a set of invariant subunits. These invariant subunits include the members of the CD3 complex (δ, ε, and γ) and the ζ subunit (*1*). The ζ subunit exists primarily as a disulfide-linked homodimer consisting of two 16-kD monomers (*2*, *3*). A subset of ζ chains undergoes multiple tyrosine phosphorylations upon receptor stimulation such that the predominant form migrates with an apparent molecular size of 21 kD in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*4*–*7*).

To evaluate activation-dependent changes in the migration of ζ, we incubated the T cell hybridoma 2B4 (*8*) in the presence or absence of an activating monoclonal antibody to the CD3 ε subunit of the TCR, 2C11 (*9*). TCRs were immunoprecipitated, resolved on two-dimensional nonreducing-reducing SDS-PAGE (diagonal gels), and immunoblotted with antibodies directed to ζ. In unactivated cells (Fig. 1A), the ζ homodimer appeared as a prominent 16-kD species that migrated at 32 kD before reduction. After activation (Fig. 1B), the 21-kD form of phosphorylated ζ was seen directly above ζ as described (*4*, *5*). Unexpectedly, a number of additional immunoreactive species of 24, 32, and 40 kD were also observed in a variety of disulfide-linked combinations. Less prominent forms with apparent molecular sizes of approximately 27 kD under reducing conditions were also observed.

These larger immunoreactive species were further characterized on two-dimensional gels in which nonequilibrium pH gradient electrophoresis (NEPHGE) under reducing conditions was followed by SDS-PAGE in the second dimension (NEPHGE-PAGE) (Fig. 1, C and D). This demonstrated the activation-specific appearance of a ladder-like group of species above ζ, which corresponded to the 24-, 32-, and 40-kD forms seen on the diagonal gels. At each of these molecular sizes, the proteins were homogeneous with regard to isoelectric point (pI). The 21-kD phosphorylated form of ζ migrated with a more acidic pI. A 27-kD activation-specific acidic form was also seen migrating above the 21-kD form of phosphorylated ζ. On longer exposures of the autoradiograms (*10*), other spots were seen above the 27-kD species, which gave the appearance of a second, less intense ladder-like set of species that converged with the more prominent forms toward a neutral pH. This suggested the modification of ζ and, to a lesser extent, phosphorylated ζ with multimers of a neutral 8-kD protein.

Normal murine splenocytes were next analyzed. Freshly isolated splenocytes (Fig. 2A) and cells that had been incubated at 37°C for

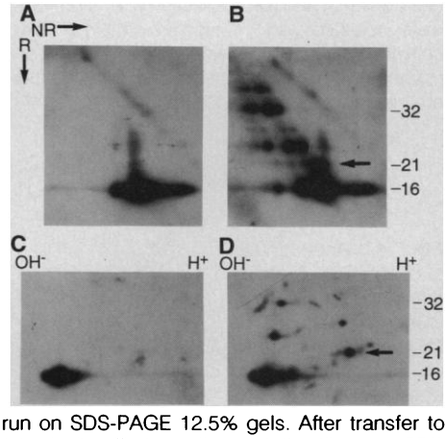

Fig. 1. Activation-induced modifications of ζ in 2B4 hybridoma cells. The 2B4 cells (10^8 cells per condition) and LK cells (an Fc receptor–bearing B cell hybridoma) (*20*) were incubated at 37°C for 45 min in the absence (**A** and **C**) or presence (**B** and **D**) of 2C11 as described (*7*). Cells were chilled to 4°C with phosphate-buffered saline and phosphatase inhibitors (*21*). Cell pellets were lysed in lysis buffer that contained Triton X-100, protease and phosphatase inhibitors (*21*), and postnuclear supernatants immunoprecipitated with a monoclonal antibody directed against the α subunit on 2B4 cells, A2B4-2 (*2*, *22*). Immunoprecipitates were separated under nonreducing conditions in SDS-PAGE tube gels (10.5%) (A and B) or run in NEPHGE tubes (*2*) with a pH range from 3 to 10 (C and D). After equilibration, all tubes were run on SDS-PAGE 12.5% gels. After transfer to nitrocellulose membranes, proteins were immunoblotted with affinity-purified anti-ζ antibodies (551) and detected by ^{125}I-labeled protein A (ICN) (*23*). The 21-kD form of phosphorylated ζ is indicated by arrows (B and D). The spot directly above the arrow in (D) is a hyperphosphorylated form of ζ. Antiserum 551 was raised to a peptide that corresponded to amino acids 151 to 164 of murine ζ (*23*) and does not recognize the η alternative splice of ζ. The diagonal gels (A and B) and the NEPHGE gels (C and D) are from separate experiments. NR, nonreducing; R, reducing. Molecular size markers are indicated to the right in kilodaltons.

C. Cenciarelli, D. Hou, K.-C. Hsu, B. L. Rellahan, D. L. Wiest, A. M. Weissman, Experimental Immunology Branch Division of Cancer Biology, Diagnosis, and Centers/National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.
H. T. Smith and V. A. Fried, Department of Cell Biology and Anatomy, New York Medical College, Valhalla, NY 10595.
*To whom correspondence should be addressed.

1 hour (Fig. 2B) had large amounts of phosphorylated ζ without detectable amounts of the larger immunoreactive structures. Receptor engagement with 2C11 at 37°C for 1 hour (Fig. 2C) resulted in the appearance of the 24- and 32-kD immunoreactive forms and fainter 40-kD forms. In other experiments, small baseline amounts of these species were detected, perhaps as a result of in vivo antigen exposure. The specificity of immunoprecipitation was assessed in concanavalin A (Con A)–stimulated splenocytes. An identical pattern was seen with both anti-ζ (Fig. 2E) and 2C11 (Fig. 2F) immunoprecipitations but not with rabbit immunoglobulin (Fig. 2D). Thus, all of the immunoreactive forms of ζ are associated with other TCR components.

The presence of 24-, 32-, and 40-kD species suggested the modification of ζ by ubiquitin, a neutral 8-kD protein. One or multiple ubiquitin molecules can be added to target molecules, in most cases by the formation of isopeptide bonds through the ε amino groups of lysine residues (*11, 12*). Ubiquitination targets abnormal proteins for degradation (*11, 12*) and regulates the amount of several rapidly metabolized proteins (*13*). Ubiquitinated proteins have been associated with the cytoskeleton, particularly in the neurofibrillary tangles of Alzheimer's disease (*14, 15*), and there is evidence that ubiquitination participates in a number of other cellular processes (*12*). Sequencing of the MEL-14 leukocyte homing receptor and the receptors for platelet-derived growth factor (PDGF) and growth hormone has revealed covalent modification with ubiquitin (*16*).

To establish whether the activation-induced modification of ζ was a result of the addition of ubiquitin, we resolved anti-TCR immunoprecipitates from 2B4 cells on NEPHGE-PAGE gels and immunoblotted them with a mixture of monoclonal antibodies to ubiquitin (*14, 17*). Activation resulted in the appearance of a group of nonpolymorphic species of 24, 32, 40, and 48 kD (Fig. 3, A and B). When stripped of antibodies to ubiquitin and reprobed with anti-ζ (Fig. 3, C and D), the same proteins were recognized,

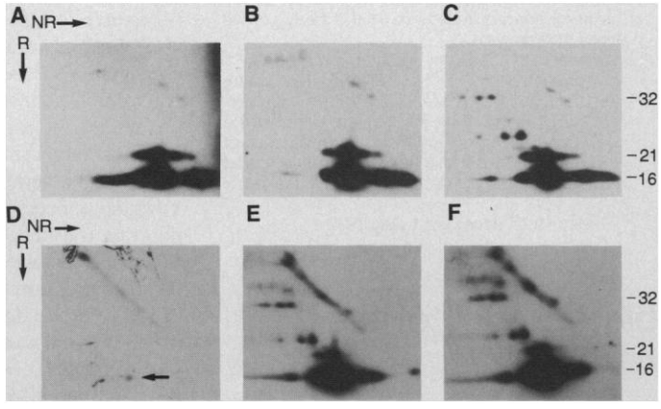

Fig. 2. Characterization of immunoreactive forms of ζ in murine splenocytes. Splenocytes from 8-week-old C57Bl/6 mice (four spleens per condition) were analyzed directly after lysis of red blood cells (**A**) or cultured for 1 hour at 37°C in the absence (**B**) or presence (**C**) of 2C11. Cells were lysed and immunoprecipitated with 2C11 prebound to protein A–Sepharose (Pierce, Rockford, Illinois). In (**D**) through (**F**), splenocytes were stimulated with Con A (5 μg/ml) for 48 hours. After lysis as in Fig. 1, immunoprecipitation was carried out with normal rabbit immunoglobulin (D), with affinity-purified anti-ζ 527 (raised against amino acids 132 to 144 of ζ) (E), or with 2C11 (F). Immunoprecipitates were resolved on diagonal gels and immunoblotted with anti-ζ 551 as in Fig. 1. A small amount of the ζ homodimer is seen with the nonspecific immunoprecipitation (D) and is indicated by an arrow. NR, nonreducing; R, reducing. Molecular size markers are indicated to the right in kilodaltons.

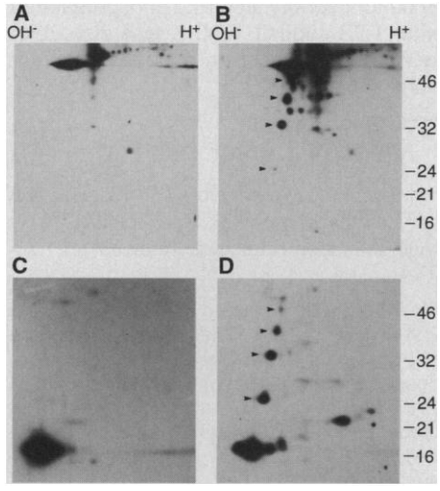

Fig. 3. Anti-ubiquitin and anti-ζ blotting of TCR immunoprecipitates. The 2B4 cells (10^8 cells per condition) and LK cells were incubated at 37°C for 45 min in the absence (**A**) or presence (**B**) of 2C11. Lysates were immunoprecipitated with A2B4-2 and resolved by NEPHGE-PAGE (12.5% slab gels). Proteins were transferred to Immobilon (Millipore), immunoblotted with a mixture of anti-ubiquitin monoclonal antibodies as described (*14, 17*), and detected with enhanced chemiluminescence (Amersham). The blots in (A) and (B) were stripped and reblotted with affinity-purified anti-ζ 551 [(**C**) and (**D**), respectively], and proteins were detected with ^{125}I-labeled protein A. The activation-induced species are indicated with arrowheads in (B) and (D). Molecular size markers are indicated to the right in kilodaltons.

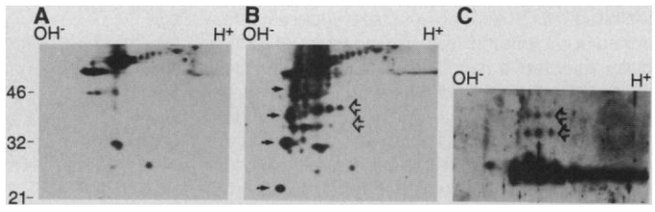

Fig. 4. Anti-ubiquitin and anti-CD3 δ immunoblotting of TCR immunoprecipitates. The 2B4 cells were stimulated as in Fig. 3 in the absence (**A**) or the presence (**B** and **C**) of 2C11. A2B4-2 immunoprecipitates were resolved by NEPHGE-PAGE (10.5% slab gels). Proteins were transferred to Immobilon membranes (A and B) or to nitrocellulose (C). Filters were probed with anti-ubiquitin (A and B) or with polyclonal anti-CD3 δ (C); blots were developed with enhanced chemiluminescence. The species indicated by the closed arrows (B) comigrated with immunoreactive forms of ζ as in Fig. 3. The species indicated by the open arrows (B and C) comigrated. Molecular size markers are indicated to the left in kilodaltons.

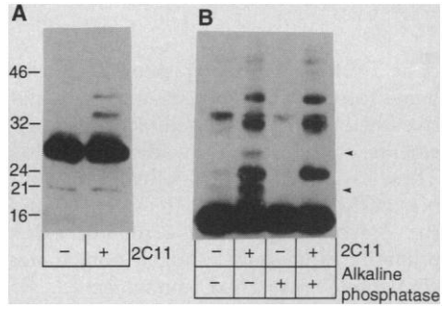

Fig. 5. Gel analysis under reducing conditions of activation-induced species. Samples were immunoblotted with a polyclonal anti-CD3 δ (**A**) or anti-ζ 551 (**B**). In (B), immunoprecipitates were treated overnight either with or without alkaline phosphatase (24 units) (Boehringer Mannheim) before being resolved on SDS-PAGE. The arrowheads indicate the position of the alkaline phosphatase–sensitive species. The constant band at approximately 32 kD in (B) corresponds to a small amount of nonreduced ζ homodimer. The 2B4 cells and LK cells were incubated for 20 min at 37°C. Lysis and immunoprecipitation with A2B4-2 was performed as in Fig. 1; 8×10^7 cells were used for each lane in (A) and 2×10^7 cells were used for each lane in (B). All samples were resolved on SDS-PAGE (12.5%) and detected by enhanced chemiluminescence. Molecular size markers are to the left in kilodaltons.

which demonstrates that ζ was ubiquitinated. Unmodified ζ (16 kD) was not recognized by the anti-ubiquitin reagents.

The presence of additional activation-dependent species on ubiquitin blots (Fig. 3, A and B) suggested the possibility that other TCR components were ubiquitinated. Immunoprecipitated TCRs were resolved on lower percentage polyacrylamide gels and immunoblotted with anti-ubiquitin (Fig. 4, A and B). Spots that varied in pI and migrated at 34 and 42 kD (Fig. 4B) were suggestive of ubiquitination of the 26-kD CD3 δ subunit, which exhibits heterogeneity in pI as a result of variable modifications with sialic acid. A duplicate immunoblot from activated cells was probed with a polyclonal antiserum to CD3 δ (*18*). In addition to the 26-kD δ, two sets of larger molecular size forms were seen (Fig. 4C) that comigrated with the species detected by anti-ubiquitin (Fig. 4B). These larger forms of δ were detected only in activated cells (Fig. 5A). The presence of charge heterogeneity for ubiquitinated δ indicated that these subunits contained sialic acid and therefore had traversed the intermediate Golgi apparatus.

The finding of relatively acidic species, including a 27-kD form above 21-kD phosphorylated ζ (Fig. 1D) (*10*), indicated that ubiquitinated forms of phosphorylated ζ also exist. To confirm this, we incubated immunoprecipitates from activated and unactivated cells with alkaline phosphatase before electrophoresis on SDS-PAGE (Fig. 5B). This resulted in the loss of phosphorylated ζ (21 kD) as well as the loss of the 27-kD species. Thus, it is apparent that this 27-kD form was ubiquitinated phosphorylated ζ.

We estimate that approximately 10% of TCR-associated ζ is ubiquitinated upon activation. On the basis of the distribution of heterodimers on diagonal gels, it would appear that ubiquitinated ζ chains are more likely to be dimerized to other ubiquitinated ζ molecules than to 16-kD ζ. These proteins exist primarily as components of assembled receptors (Fig. 2) (*10*), and their appearance is not a result of redistribution from a detergent insoluble pool (*10*). Human T cells also demonstrate the same activation-induced forms of ubiquitinated ζ (*10*).

In 2B4 cells, both ubiquitinated ζ and phosphorylated ζ appear within 5 min of stimulation and persist for at least 2 hours (*10*). A dichotomy is apparent in freshly isolated normal splenocytes where ubiquitination of ζ increases markedly with receptor engagement, whereas tyrosine phosphorylated ζ is present at maximal levels without stimulation. This may be a result of the in vivo signals that regulate these processes. Alternatively, it may be a manifestation of differences in the half-lives of these modifications or of the receptors that are altered. The activation-induced ubiquitination of the TCR suggests the possibility of a more generalized function of this process in the regulation of transmembrane receptor function. Ubiquitination may serve as a means to down-regulate receptors by targeting them for degradation, or, like phosphorylation, it may affect function by altering signal transducing properties and associations of modified receptors.

Note added in proof: Ligand-induced changes in the ubiquitination of the receptor for PDGF have recently been described (*19*).

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The Skeletal Muscle Chloride Channel in Dominant and Recessive Human Myotonia

Manuela C. Koch, Klaus Steinmeyer, Claudius Lorenz, Kenneth Ricker, Friedrich Wolf, Michael Otto, Barbara Zoll, Frank Lehmann-Horn, Karl-Heinz Grzeschik, Thomas J. Jentsch*

Autosomal recessive generalized myotonia (Becker's disease) (GM) and autosomal dominant myotonia congenita (Thomsen's disease) (MC) are characterized by skeletal muscle stiffness that is a result of muscle membrane hyperexcitability. For both diseases, alterations in muscle chloride or sodium currents or both have been observed. A complementary DNA for a human skeletal muscle chloride channel (*CLC*-1) was cloned, physically localized on chromosome 7, and linked to the T cell receptor β (*TCRB*) locus. Tight linkage of these two loci to GM and MC was found in German families. An unusual restriction site in the *CLC*-1 locus in two GM families identified a mutation associated with that disease, a phenylalanine-to-cysteine substitution in putative transmembrane domain D8. This suggests that different mutations in *CLC*-1 may cause dominant or recessive myotonia.

Autosomal recessive generalized myotonia (GM) is a nondystrophic disorder of skeletal muscle and was clinically separated (*1*) from autosomal dominant myotonia congenita (MC) (*1*, *2*). In GM, myotonic stiffness starts in early childhood in the legs. It progresses for some years, affecting the arms, neck, and facial muscles. After the patient reaches approximately 20 years of age, the disease remains unchanged. In most patients, muscle stiffness is associated with transient weakness after rest. In MC, complaints are similar but more benign.

Muscle stiffness caused by these diseases is a result of repetitive firing of muscle fiber action potentials (myotonic runs) (*3*). Two mechanisms have been proposed to cause the underlying intrinsic muscle hyperexcitability: (i) a decrease in muscle chloride conductance, as found in several human