The T cell antigen receptor ζ chain and other T cell antigen receptor components are ubiquitinated on receptor occupancy. A systematic mutagenesis of the ζ subunit was undertaken to determine the sites of ubiquitination. Ubiquitination was found to occur in the cytoplasmic domain of ζ with multiple lysines serving as sites for mono- and polyubiquitination. The mutation of all potential sites of ubiquitination did not inhibit receptor tyrosine phosphorylation or the ubiquitination of other T cell antigen receptor subunits. Lysines introduced into nonnative positions in the ζ molecule were also able to serve as sites for ubiquitination. These findings demonstrate that once a T cell antigen receptor is targeted for ubiquitination, there is little specificity with regard to the lysine residues that are modified.

The T cell antigen receptor (TCR)\(^1\) consists of dimeric antigen recognition elements and a set of four invariant signaling molecules that include the ζ subunit and CD3-δ, -ε, and -γ (1, 2). TCR occupancy leads to the activation of one or more nonreceptor protein tyrosine kinases and eventually to T cell activation (3, 4). Tyrosine-based activation motifs, found in three copies in ζ and one copy in each of the CD3 components, constitute the minimal signal-transducing unit of the TCR (5–7). Consistent with this, single-chain structures having the intracytoplasmic domains of either ζ or CD3-ε are capable of activating T cells independent of other TCR subunits (8–11). In addition to serving to activate tyrosine kinases, the invariant TCR components are themselves substrates for tyrosine phosphorylation, with ζ being a particularly prominent and well-studied substrate (12–15).

TCR occupancy also results in the covalent linkage of ζ and other invariant components to ubiquitin (16). Ubiquitin is a 76-amino acid polypeptide that is covalently linked to proteins through the formation of isopeptide bonds between the carboxyl terminus of ubiquitin and the e-amino groups of lysines. Ubiquitination has been extensively characterized as a signal leading to the degradation of cytosolic proteins (17–19); indeed several short-lived regulatory proteins have been demonstrated to be degraded after ubiquitination (20–22). The generation of polyubiquitin chains bound to a specific lysine is considered an obligatory event in ubiquitin-mediated degradation of cytosolic proteins (17, 18). The addition of ubiquitin monomers and chains occurs as the result of a multienzyme process involving enzymes termed E1–E3 (17–19). According to current models developed primarily in yeast and in vitro systems, the E3 enzymes recognize target proteins by virtue of exposed NH\(_2\)-termini, with the ubiquitination of targets dependent on the nature of the first amino acid, a concept known as the N-end rule (18). Ubiquitination has been implicated in a number of cellular processes, including the biogenesis of ribosomes and peroxisomes, DNA repair, and the regulation of the cell cycle (19). Recently a yeast ubiquitinating enzyme has been found to be homologous to a product of the human oncogene tre-2 (24, 25).

Several membrane proteins have been found to be constitutively monoubiquitinated (26–29); in contrast, TCR ubiquitination is dependent on receptor occupancy (16). Ligand-dependent ubiquitination has also been found for the platelet-derived growth factor receptor and for the Fc receptor for immunoglobulin ε (Fcε) (30–32). As all of these receptor molecules, with the exception of one of the Fcε subunits (33), have their amino termini oriented outside the cell, models for ubiquitination of cytosolic proteins that involve direct recognition of amino termini by cytosolic E3 enzymes do not apply.

Although artificial ubiquitin substrates have been studied in yeast, little is known regarding the requirements for ubiquitination of naturally occurring mammalian substrates. The ability to detect ubiquitinated ζ allows activation-dependent ubiquitination of a mammalian transmembrane protein to be evaluated. To determine whether ubiquitination occurs on the extracellular or intracytoplasmic domain and whether specific lysine residues are targeted for ubiquitination, we have undertaken a systematic mutagenesis of the TCR-ζ subunit.

**MATERIALS AND METHODS**

**Cells and Reagents**—2B4.11 is a pigeon cytochrome c-specific T cell hybridoma (34). MA5.8 is a variant of 2B4.11 that expresses no detectable mRNA for ζ (35). The level of surface expression of TCR in this cell line is approximately 5% of the level found in 2B4.11. KL25.5 is an I-E\(^{k}\)-positive murine B lymphoma cell line (36) that expresses receptors for the Fc region of immunoglobulin. 2M.2 cells transfected with a truncated ζ construct (CT106) were a gift from S. Frank and R. Klausner (37).

2C11 is a hamster monoclonal antibody that recognizes an external epitope on the CD3-ε chain (38). A2B4-2 is a mouse monoclonal antibody directed at the variable region of the 2B4 TCR-ε chain (38). Anti-α chain reagents (40) and anti-ubiquitin sera (41) were generated essentially as described.

**Mutagenesis**—Site-specific mutagenesis of cDNAs encoding the ζ molecule was carried out using the transforming mutagenesis kit (Clontech, Palo Alto, CA). Oligonucleotides used for mutagenesis were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer. All mutations were confirmed by dideoxy DNA sequencing using Sequenase version 2 (U. S. Biochemical Corp.). Mutations were carried out on a ζ cDNA cloned into pGEM 7Zf (Promega, Madison WI) or in pFNeo (42).

**Transfections**—All of the mutated cDNAs were cloned into pFNeo for transfection and transformed into DH5α-competent Escherichia coli.
Ubiquitination of TCR-ζ on Multiple Lysines

The murine ζ subunit is translated as a 164-amino acid polypeptide that includes a 21-amino acid co-translationally cleaved leader sequence (43). ζ monomers undergo efficient dimerization to form disulfide-linked homodimers. There are 10 lysines in each mature ζ monomer; one is in the extracellular domain, and the others are distributed in the intracytoplasmic tail (Fig. 1).

To assess the site(s) of ubiquitination, a previously characterized cell expressing a ζ molecule truncated at amino acid 108 (CT108) was evaluated (37). CT108 was generated by transfection into 2M.2, a 2B4.11 variant that expresses low levels of TCR function is not unexpected, as others have demonstrated with no lysines (KR.All) or with only the single extracellular lysine (KR.All-30) were evaluated; cells expressing either KR.All or KR.All-30 supported TCR surface expression at a level comparable with that found with wild type ζ (not shown).

When cells expressing these altered ζ molecules were analyzed by anti-ubiquitin blotting (Fig. 4), no ubiquitinated ζ was detected, while other TCR components were clearly ubiquitinated on activation. This demonstrates that ubiquitination is not occurring on the extracellular amino terminus or on the extracellular lysine but rather on multiple intracellular residues. Despite the lack of ubiquitination, ζ molecules in which all 10 lysines are mutated maintain their ability to function as tyrosine kinase substrates (Fig. 4E). Other parameters of cellular activation including antibody-dependent interleukin-2 production and growth inhibition were similarly unaffected by these mutations (not shown). The lack of effect of these mutations on TCR function is not unexpected, as others have demonstrated.

RESULTS

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FIG. 1. Amino acid sequence of the mature ζ subunit. Numbering begins with the co-translationally cleaved leader sequence (amino acids 1-21). The transmembrane domain is shaded and the lysine residues are in boldface. The position of the amino acid 108 truncation is indicated by an arrow.

FIG. 2. Approximately 10⁶ CT108 cells were incubated with Fe bearing Lk cells in the absence (A) or presence (B) of 2C11 at 37°C for 30 min. 2B4-2 immunoprecipitates were resolved in the first dimension on the basis of charge and in the second dimension by size prior to immunoblotting with affinity-purified antiserum 528. The prominent species migrating at 8 kDa is truncated ζ; the species indicated by arrows in B are ubiquitinated forms.

FIG. 3. Cells transfected with wild type ζ (A and B) or cells expressing ζ molecules with lysine to arginine mutations at amino acids 54, 88, 89, and 99 (C and D) were incubated in the absence (A and C) or presence (B and D) of 2C11 as in Fig. 2. After immunoprecipitation, samples were resolved on two-dimensional gels and immunoblotted with affinity-purified antiserum 551 (directed against the carboxyl end of ζ). The ladder of species above ζ (16 kDa) shifting toward more acidic pl represents ubiquitinated ζ.
that TCRs without functional ζ cytoplasmic domains are still capable of stimulating interleukin-2 production (44).

In models for ubiquitin-mediated protein degradation, target proteins are ubiquitinated on specific lysines with polyubiquitin chains (18, 45). To determine whether chains of ubiquitin are added to individual lysines, cells expressing ζ molecules with only one lysine at either position 129 or 136 were evaluated (not shown). On receptor engagement, both mono- and diubiquitinated forms of ζ were found but at levels markedly lower than those found in cells expressing wild type ζ. In conjunction with the finding that there are sites of ubiquitination proximal to amino acid 108, this establishes that a minimum of 3 of the 9 intracellular lysines of ζ are ubiquitinated.

To determine if there is specificity with regard to the lysine residues that can be ubiquitinated, 3 consecutive arginines at positions 102–104 of KR.AII were changed to lysines. As demonstrated by anti-ubiquitin immunoblotting (Fig. 5), both mono- and diubiquitinated species were detected after stimulation of cells expressing ζ with lysines in only nonnative positions.

**DISCUSSION**

Our findings establish that multiple intracellular lysines of ζ are modified with one or more ubiquitin moieties and that lysines introduced into nonnative positions similarly serve as substrates for this process. It would appear that once a receptor is targeted for ubiquitination, any available lysine may be ubiquitinated. The findings in the TCR, together with those in the platelet-derived growth factor receptor and the Fce receptor (30–32), establish activation-dependent ubiquitination as a general mechanism whereby receptors are modified. The lack of specificity with regard to lysine residues ubiquitinated, the lack of exposed amino termini, and the requirement for receptor engagement to stimulate ubiquitination all underscore the fact that paradigms generated for the ubiquitination of cytosolic substrates are not necessarily applicable to transmembrane receptors. It remains to be determined whether the enzymes responsible for ubiquitination of the TCR and other receptors are the same ones responsible for catalyzing the ubiquitination of cytosolic proteins. An endoplasmic reticulum membrane-associated ubiquitin-conjugating protein has recently been identified in yeast (46). It will be interesting to know whether analogous proteins exist in mammalian cells and whether they are found associated with the plasma membrane.

The intracellular signals that target particular receptors for ubiquitination remain to be determined. One possibility is that the association of receptors with other intracellular structures such as kinases serves to activate ubiquitin-conjugating enzymes that are localized to cell membranes. Alternatively, it
may be that changes in receptor conformation due to occupancy-induced aggregation make them susceptible to modification. Studies utilizing different means of TCR activation suggest a role for aggregation in the ubiquitination of receptors.2

The fate of receptors modified by ubiquitin remains to be determined. While ubiquitination is associated with the degradation of cytosolic proteins, we have not detected any appreciable increase in the half-life of ζ molecules without lysines relative to wild type ζ.2 As the half-life of wild type ζ is already quite long (12-16 h) and multiple TCR subunits are ubiquitinated, this result is not surprising. It is also possible that ubiquitinated receptors are deubiquitinated and remain intact. If receptors are not degraded after ubiquitination then what purpose does this modification serve? When one considers that the addition of each ubiquitin molecule represents the addition of ~8 kDa of mass, it is easy to envision that the addition of ubiquitin moieties to multiple lysines could significantly affect the ability of receptors to cluster and to physically interact with other intracellular signaling structures. The TCR associates constitutively with one tyrosine kinase (p59fyn) (47, 48) and with another on activation (ZAP-70) (49); it also associates with other signaling molecules including the tyrosine phosphatase CD45 (50). It is tempting to speculate that these noncovalent interactions and the consequences thereof may be affected by TCR ubiquitination.

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REFERENCES


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