The T-cell antigen receptor (TCR) is a hetero-oligomeric membrane complex composed of at least seven transmembrane polypeptide chains that has served as a model for the assembly and degradation of integral membrane proteins in the endoplasmic reticulum (ER). Unassembled TCR chains fail to mature to the Golgi apparatus and are rapidly degraded by a non-lysosomal pathway that has been proposed to be autonomous to the ER. In these studies we show that the "ER degradation" pathway that has been proposed to be a type I membrane protein containing a short N-linked oligosaccharides and the N-terminal signal peptide. The susceptibility of this 28-kDa species to extravesicular protease indicates that it is not protected by the ER membrane and, hence, cytoplasmic. These data suggest a model in which TCR chains that are translocated across the membrane, core-glycosylated, but fail to assemble are dislocated back to the cytoplasm for degradation by cytoplasmic proteasomes. Our data also suggest that covalent modification of TCR chains with ubiquitin is not required for its degradation.

The T-cell antigen receptor (TCR) is a type I membrane protein containing a short (5-amino acid) cytoplasmic domain and a 223-residue extracellular domain that has four potential sites for N-glycosylation. Mature TCRs on the surface of the antigen-specific T-cell hybridoma line 2B4 migrates as a broad 42–44-kDa band (2–4). However, when expressed in the absence of other TCR subunits, TCRs are synthesized as a 38-kDa core-glycosylated precursor that is sensitive to digestion with endoglycosidase H and is rapidly degraded with a half-time of ~50 min (5, 6). This degradation process is not affected by inhibitors of autophagy, lysosomal proteolysis, or ER-Golgi traffic. Moreover, TCRa chains in these cells are localized to the "ER region" by immunofluorescence and electron microscopy (5). Together, these studies have led to the conclusion that TCRa degradation occurs at a site "within or closely associated with the ER" (5). However, efforts to identify ER-specific proteases that participate in TCRa degradation have been unsuccessful.

Several recent reports have suggested a role for the proteasome in the ER degradation of some membrane or luminal proteins (reviewed in Refs. 7 and 8). For example, misfolded cystic fibrosis transmembrane conductance regulator (CFTR) molecules that fail to exit the ER are rapidly degraded by a process that requires covalent modification with ubiquitin and is blocked by lactacystin, a specific proteasome inhibitor (9). Degradation of other ER-restricted proteins including mutant human α1-antitrypsin (10), yeast carboxypeptidase Y (11), and MHC class I heavy chains (12, 13) has also recently been shown to require proteasome activity. How these proteins, which are sequestered within the ER lumen, are recognized and delivered to the cytoplasmic proteasome complex is unknown.

In this paper we have examined the role of the ubiquitin-proteasome pathway in the ER degradation of newly synthesized TCRa chains. Our data suggest a model in which TCRa chains are first translocated into the ER, cleaved by signal peptidase, and N-glycosylated with core high mannose glycans. These chains are subsequently exported back to the cytoplasmic face of the ER, where they are deglycosylated and delivered to the proteasome for degradation. Moreover, our data suggest that ubiquitination of TCRa is not required for this process.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**HEK293 cells were grown and transiently transfected by calcium phosphate precipitation as described previously (14). In some experiments, N-acetyl-l-leucyl-l-leucyl-l-norleucinal (ALLN), calpain inhibitor I, Calbiochem) or lactacystin (a kind gift from S. Omura, Kitasato Institute, Tokyo) at indicated concentrations was added to the fresh media. The cDNA corresponding to 2B4 TCRa (15) in pCMI8 was a kind gift from J. Bonifacino (National Institutes of Health).

**Site-directed Mutagenesis—**The 11 lysine residues in TCRa were mutated to arginine by 8 sequential rounds of polymerase chain reaction-based “megaprimer” mutagenesis (16). The mutant construct (KαR) was verified by sequence analysis, which revealed the presence of an additional mutation of Phe154 to Tyr. Functional comparison with a KαR lacking this additional mutation showed that this conservative change does not influence either the kinetics of degradation or its sensitivity to proteasome inhibitors.

**Immunoblotting, Metabolic Labeling, and Immunoprecipitation—**HEK293 cells transiently transfected with TCRa were processed for immunoblot analysis as described previously (9). Samples were resolved in 11% SDS-polyacrylamide gels and electrophoresed to nitrocellulose. Blots were probed with the appropriate antibody, and immune-reactive bands were detected by enhanced chemiluminescence. Metabolic labeling and immunoprecipitation was carried out as described (14) with the following modifications. Cells were pulse-labeled with 500 μCi/ml [35S]Met/Cys (>1,000 Ci/mmol, NEN Life Science...
from the resulting post-nuclear supernatant were then sedimented in a translocation of TCR intact signal sequence. Together, these results suggest that was carried out on TCR complete media for 14 h. PNGase F (New England Biolabs) digestion produced the accumulation of a novel, detergent-insoluble 28-kDa fraction and also led to its appearance in the detergent-insoluble proteasome inhibitors ALLN or lactacystin increased the proteasome inhibition, the 38-kDa species was completely soluble below), was also detected occasionally. In the absence of proteasome inhibitors, the 38-kDa species was completely soluble and corresponded to partially glycosylated form of the protein (see (5). A minor band with 2-kDa faster mobility, probably corresponding to the signal-cleaved form of TCR migrated primarily as a detergent-soluble 38-kDa band. Asterisk indicates the position of a partially glycosylated form of TCR. B, characterization of 28-kDa TCRa. Cells transiently transfected with TCRa (lanes 3–6) or mock-transfected control cells (lanes 1 and 2) were labeled to steady state with [35S]Met/Cys in the presence of ALLN (20 mM). Cells were lysed with nonionic detergents and separated into soluble (S), insoluble (I), and incomplete, resulting in a ladder of partially glycosylated TCRa chains which accumulated in proteasome-inhibited cells. TCRa was immunoprecipitated from detergent-soluble and insoluble fractions of transfected HEK cells that had been metabolically labeled to steady state with [35S]Met/Cys in the presence of ALLN (Fig. 1B). In this 15% acrylamide gel the lower band of the 28-kDa doublet comigrates with the limit product of PNGase-deglycosylated 38-kDa TCRa, corre-
FIG. 2. TCRα degradation is blocked by proteasome inhibitors. HEK293 cells transfected with TCRα were pulse-labeled for 10 min and chased for the times indicated without protease inhibitor (A) or in the presence of 20 μg/ml ALLN (B) or 50 μM lactacystin (C). Cells were separated into detergent-soluble and insoluble (pellet) fractions as indicated and immunoprecipitated with mAb H28-710. Band intensity was quantified by densitometry and plotted as a percentage of the signal at 0 min. In C, cells transfected with vector (lanes 1 and 4) or TCRα cDNA (lanes 2, 3, 5, and 6) were pulse-labeled for 10 min (P) and chased for 180 min (C). The sizes of partially glycosylated TCRα forms are indicated by the asterisks.

TCRα Chains Are Dislocated from the ER—We used cell fractionation and protease protection to test the possibility that TCRα degradation by proteasomes is associated with its dislocation from the ER to the cytoplasm. Cells were lysed by mechanical disruption, and the post-nuclear supernatant was centrifuged at 100,000 × g. A small amount (<5%) of TCRα (both the 38-kDa and the 28-kDa forms) was recovered in the supernatant, even after a second round of 100,000 × g centrifugation, suggesting that some TCRα had been released to the cytosolic fraction (data not shown). However, the majority of TCRα chains sedimented with the microsomal pellet fraction, suggesting that they are associated with ER membranes or are present as high molecular weight aggregates. To determine the orientation of these TCRα chains with respect to the ER membrane, the microsomal pellet fraction was subjected to digestion with protease K (Fig. 3). The endogenous lumenal proteins BiP and GRP94 were resistant to digestion by protease K in the absence, but not the presence, of detergent. By contrast, the ~10-kDa cytoplasmic tail of calnexin was readily cleaved by the protease indicating that this fraction contained ER vesicles that were sealed and of uniform membrane orientation. Core-glycosylated TCRα in the 100,000 × g pellet was completely protected from protease digestion, confirming that it had been correctly translocated. Strikingly, both bands of the 28-kDa unglycosylated doublet were highly susceptible to protease K digestion, indicating that they must be present on the exterior, i.e. cytoplasmic side of the vesicles. These data strongly suggest that reverse translocation of TCRα from the ER accompanies its degradation by the proteasome.

TCRα Degradation Does Not Require Ubiquitination of Lysines—Substrates destined for degradation by the 26 S proteasome are commonly “tagged” by the covalent attachment of multibiquitin chains (20). Inhibition of proteasome function in vitro or in vivo usually induces the accumulation of a significant fraction of highly ubiquitinated proteins, including ER degradation substrates like CFTR (9). As TCRα is a small protein, attachment of even a single ubiquitin moiety (~7 kDa) would result in a readily detectable decrease in gel mobility. In the present study, no such mobility shift was observed in proteasome-inhibited cells (Figs. 1 and 2). However, ubiquitinated TCRα could have been missed if the ubiquitin linkages were labile to cleavage by cellular isopeptidases. To directly test whether TCRα ubiquitination is required for its degradation by the proteasome, we constructed a TCRα mutant (KαR) in which all 11 lysine residues were substituted by arginine. Attachment of ubiquitin to substrates occurs via an isopeptide linkage between a lysine ε-amino group on the substrate and the C-terminal glycine of ubiquitin (21). Cells transfected with KαR were pulse-labeled with [35S]Met/Cys for 10 min, and the KαR protein was immunoprecipitated from both the detergent-soluble and insoluble fractions with anti-TCRα antibody (Fig. 4A). Like wild-type TCRα, KαR was core-glycosylated and rapidly degraded. Remarkably, this degradation was efficiently inhibited by the proteasome inhibitor ALLN, giving rise to the appearance of partially and completely deglycosylated forms in both detergent-soluble and insoluble fractions. KαR degradation was similarly inhibited by 5 μM clasto-lactacystin β-lactone, the active form of lactacystin (22). These data suggest that either ubiquitination of TCRα is not required for its degradation by the proteasome or ubiquitin moieties can be attached to TCRα at alternate non-lysine residue(s). Future studies will be required to distinguish between these two possibilities.

DISCUSSION

Selective proteolysis is the final step in the elaborate network of proofreading and editing processes that have evolved to protect eukaryotic cells against the potentially deleterious consequences of errors that can accrue between genes and proteins. These include alterations in primary sequence due to mutation or to transcriptional and translational errors, as well as the effects of inappropriate spatial and temporal expression. Selective degradation is also required to eliminate unassembled or misassembled subunits of hetero-oligomeric plasma membrane complexes such as the heptameric TCR (4). Eukaryotic cells contain two major proteolytic systems: proteasomes, which are present in the cytoplasm and the nucleus, and lysosomes. In contrast to the lysosome-mediated disposal of mature or partially assembled TCR oligomers, the rapid, nonlysosomal degradation of unassembled TCRα subunits had suggested the existence of a unique degradation system associated with the endoplasmic reticulum (23). However, several recent studies have demonstrated that some misfolded proteins in the ER can...
be degraded by cytoplasmic proteosomes following their re
verse translocation from the ER (7, 8). The data in this paper
demonstrate that unassembled TCRα subunits that have been
biosynthetically translocated into the ER and core-glycosylated
are exported or "dislocated" into the cytoplasm, where they are
deglycosylated and degraded by the proteasome.

TCRα was synthesized in HEK cells as a 38-kDa core-glyco-
sylated precursor that was rapidly degraded. Our data show
that lactacystin and ALLN stabilize this core-glycosylated form
of TCRα, implicating the proteasome in its degradation.
Although the effect of ALLN in stabilizing TCRα has been previ-
ously reported (24), neither its activity against the proteasome
nor its ability to induce the accumulation of dislocated and
deglycosylated forms were recognized at that time. Our data show
that either acute or chronic treatment of TCRα-transfected cells with proteasome inhibitors cause the core-glyco-
sylated 38-kDa TCRα chains to progressively shift to an ~28-kDa
form that also lacks both N-linked oligosaccharides and an
N-terminal signal peptide. As signal peptidase has its active
site at the luminal face of the ER, these data establish that
some TCRα chains must have been at least partially translo-
cated across the ER membrane. The susceptibility of the 28-
kDa species to extravesicular protease indicates that it is not
protected by the ER membrane and, hence, is cytoplasmic.

Our data indicate that the majority of dislocated TCRα sed-
iments at relatively low speed and is insoluble in nonionic
detergent. This change in detergent solubility is probably the
result of the formation of high molecular weight aggregates.
TCRα contains an unconventional transmembrane domain
that is interrupted by four polar or potentially charged amino
acids. In the absence of oligomer partners that could shield
these side chains from the hydrophobic core of the lipid bilayer,
these polar residues have a dominant destabilizing influence
over the rest of the molecule (6, 25, 26). It is unlikely, therefore,
that nascent TCRα chains are able to effectively partition from
the hydrophilic environment of the translocon into the lipid
bileayer. At the same time the remaining 16 hydrophobic resi-
dues that constitute the TCRα transmembrane domain are
unlikely to be able to effectively partition into the cytosol and
may facilitate aggregation of the degraded dislocated chains.
It is possible that the inability of this heterodimer transmem-
brane to effectively partition into the lipid bilayer may facili-
tate its dislocation without ever fully dissociating from the translocon.

The data presented in this paper suggest that ubiquitina-
tion of TCRα chains is not required for their degradation by cyto-
plasmic proteasomes. Although the attachment of high molecular
weight ubiquitin polymers has been demonstrated to in-
crease the susceptibility of substrate for degradation by 26 S
proteasome, modification by ubiquitin is neither a necessary
(27, 28) nor a sufficient signal (29) for degradation. The re-
quirements for ubiquitination of membrane and secretory pro-
ducts degraded by the proteasome is also variable. For example,
inhibition of proteasome-mediated degradation of α1-antitryp-
sin (10) or MHC class I heavy chain (13, 30) does not appear to
lead to the accumulation of ubiquitinated forms, although the
lack of an evident ubiquitin "ladder" is not sufficient evidence
upon which to exclude a role for ubiquitin. By contrast, there is
evidence supporting a requirement for substrate ubiquitination
in the degradation of other membrane and secretory proteins
including connexin 43 (31) and CFTR in mammalian cells (9)
and Sec61p (32) and carboxypeptidase Y (11) in yeast.

In the absence of ubiquitination, what signals are used to
target ER degradation substrates to the proteasome? In cyto-
megalovirus-infected cells, two gene products appear to possess
the capacity to induce the dislocation of MHC class I heavy
chains from the ER and accompany them to the proteasome.
We speculate that in non-virus-infected cells such targeting
could be accomplished by direct coupling of proteasomes to the
dislocation apparatus. Possibly, the presence of a misfolded
protein in association with the dislocation apparatus could
provide a signal that would recruit the docking of proteasome.
Such a signal could be transmitted via a transmembrane chaperone
like calnexin, as has been suggested recently (10). For
this model to be true, dislocation of substrate would be predicted
to depend on proteasome activity. In our studies <30%
TCRα was dislocated (as measured by the appearance of deg-
lycosylated chains) after 3 h in the presence of proteasome
inhibitor, even though >75% TCRα would have been degraded
during the same interval in the absence of proteasome inhibi-
tors. Although preliminary, these data suggest that dislocation
of TCRα from the ER may be coupled to the activity of the
proteasome.

Taken together, the data presented above support the con-
clusion that TCRα chains are dislocated from the ER for de-
gradation by cytoplasmic proteasomes. Thus, TCRα joins a
growing number of membrane and secretory proteins which
appear to be disposed of by a process involving dislocation from
the ER and subsequent degradation by cytoplasmic protea-
osomes. Since TCRα has served as a prototype that has largely
defined the process of ER degradation, we propose that the
cytosolic degradation pathway may be the major pathway for
degradation of misfolded or unassembled proteins in the ER.

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manuscript.

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FIG. 4. Degradation of mutant TCRα lacking lycins. HEK293
cells expressing lysine-less TCRα mutant (KαR) were pulse labeled
with [35S]Met/Cys in the absence (A) or presence (B) of ALLN (20
µg/ml), chased for the times indicated, and processed as described in the
legend to Fig. 2.
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