T Cell Antigen Receptor Ubiquitination Is a Consequence of Receptor-mediated Tyrosine Kinase Activation*

(Received for publication, October 19, 1995, and in revised form, December 11, 1995)

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Engagement of the T cell antigen receptor results in both its phosphorylation and its ubiquitination. T cell antigen receptor ubiquitination was evaluated in J urkat, a well characterized human T leukemia cell line. Treatment of cells with the tyrosine kinase inhibitor herbimycin A resulted in an inhibition of receptor ubiquitination. Consistent with this, pervanadate, which increases cellular tyrosine phosphorylation, enhanced receptor ubiquitination. A requirement for receptor-mediated tyrosine kinase activity for ubiquitination was confirmed in cells lacking the tyrosine kinase p56lck and also in cells that are defective in expression of CD45, a tyrosine phosphatase that regulates the activity of p56lck. The need for tyrosine kinase activation for ubiquitination was not bypassed by directly activating protein kinase C and stimulating endocytosis of receptors. These observations establish ubiquitination of the T cell antigen receptor as a tyrosine kinase-dependent manifestation of transmembrane signaling and suggest a role for tyrosine phosphorylation in the ligand-dependent ubiquitination of mammalian transmembrane receptors.

For many transmembrane receptors, including the multisubunit TCR,\(^3\) signaling is initiated by ligand-induced aggregation (1, 2). The earliest obligate intracellular event following TCR aggregation is the activation of the src-family protein tyrosine kinases, Lck (p56lck) and Fyn (p59fyn). Lck and/or Fyn phosphorylate TCR subunits resulting in the association of a third tyrosine kinase, ZAP-70 (70-kDa ε-associated protein), with the TCR and to subsequent activation events (3–5). CD45, a tyrosine phosphatase that dephosphorylates key regulatory residues on Lck and Fyn, is also implicated in the initiation of TCR-mediated signaling (6).

TCRs consist of six different polypeptides, these include the antigen-recognition element, in most cells an αβ heterodimer, and a set of invariant signal transducing subunits. The invariant subunits include CD3-δ, -ε, and -γ and the structurally distinct TCR-ζ subunit, which exists within the TCR as a disulfide-linked homodimer (4). The minimal signal transducing element of the TCR is the immunoreceptor tyrosine-based activation motif (ITAM) (7). ζ monomers have three ITAMs, and each CD3 subunit has one. ITAMs include two tyrosine residues 10 or 11 amino acids apart that are potential phosphorylation sites. The ζ subunit is a particularly prominent substrate for tyrosine phosphorylation; up to 5% of ζ subunits are phosphorylated on multiple tyrosines upon TCR engagement (8–11).

In addition to being a substrate for tyrosine phosphorylation when cross-linked by antibody or mitogen (11), TCRs also are ubiquitinated. The covalent modification of proteins with chains of ubiquitin, a highly conserved 76-amino acid polypeptide, plays a central role in the targeting of abnormal proteins and a number of regulatory cytosolic and nuclear proteins for degradation in the 26 S proteasome (12–16). Ubiquitination occurs via a multi-enzyme process involving families of enzymes termed E1–E3. E1 (ubiquitin-activating enzyme) is involved in the ATP-dependent charging of ubiquitin. The high energy thiol-ester bond between E1 and ubiquitin is transferred to an E2 (ubiquitin-conjugating enzyme), E2s either by themselves or in conjunction with E3s (ubiquitin protein ligases), transfer ubiquitin monomers or mult ubiquitin chains to target proteins, where isopeptide linkages are formed with lysine residues.

The signals that lead to ubiquitination of most naturally occurring substrates are unknown. The N-end rule established a relationship between the N-terminal amino acid of certain proteins and susceptibility to ubiquitination (14). For the cyclins and cjun, specific internal polypeptide sequences have been implicated in targeting for ubiquitination (17, 18), and for cyclins as well as IκBα, serine phosphorylation also plays a role in this process (19–22).

The TCR is distinguished from most ubiquitination substrates by its long half-life and by being ubiquitinated in response to a specific external stimulus. TCR ubiquitination occurs on multiple subunits and on multiple intracellular lysines, with mono- and mult ubiquitinlated species detectable (23). As with tyrosine phosphorylation, the ζ subunit is the most prominent substrate for this modification, likely due to the nine intracellular lysines in each ζ monomer, compared with two or three for each of the other invariant subunits. Fundamental to understanding TCR ubiquitination is a determination of the events that couple receptor engagement to this modification. Using J urkat (24), a well characterized human T leukemia cell line, we address the relationship between receptor occupancy and ubiquitination. Our findings establish a relationship between receptor ubiquitination and early signaling events mediated by TCR engagement and the activation of protein tyrosine kinases.

MATERIALS AND METHODS

Cell Lines and Antibodies—Cell lines were maintained in complete medium containing RPMI 1640 (Biofluids) and 8% fetal calf serum (25). The cell line J CaM1.6 (26) was obtained from the American Tissue
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RESULTS

TCR Ubiquitination Is Correlated with Tyrosine Phosphorylation—To determine whether events distal to TCR aggregation are required for ubiquitination, Jurkat cells were incubated at 37 °C for 10 min in the absence or the presence of the TCR agonist OKT3 (Fig. 1A, first two lanes). This monoclonal anti-CD3+ antibody stimulates tyrosine phosphorylation of ζ (33). Detergent-soluble lysates were immunoprecipitated with anti-CD3 together with a polyclonal anti-ζ antisera. This combination was used because human ζ dissociates from other TCR components in Triton X-100 (34, 35). Immunoblotting with anti-phosphotyrosine revealed the anti-CD3-dependent increase in the 21-kDa form of phosphorylated ζ (Fig. 1A, lower panel, compare first two lanes). When evaluated by immunoblotting with anti-ubiquitin, a number of anti-CD3-dependent ubiquitinated TCR species were detected (Fig. 1A, upper panel). The most prominent of these represent di-, tri-, and tetra-ubiquitinated forms of ζ, which migrate at 32, 40, and 48 kDa, respectively. The 48-kDa species is often not well visualized, due to poor recognition by polyclonal anti-ubiquitin. In addition to ζ, other TCR components are also ubiquitinated in response to receptor ligation (11) and contribute to the overall increase in density above 40 kDa. To determine whether tyrosine kinase activation is required for ubiquitination, Jurkat cells were treated with herbimycin A. This tyrosine kinase inhibitor enhances the degradation of src family tyrosine kinases such as Lck and Fyn (36). Herbimycin A at 3 μM did not affect viability or receptor levels (not shown) but resulted in a marked diminution in antibody-induced ζ phosphorylation (Fig. 1A, lower panel). When receptor ubiquitination was evaluated, it was similarly inhibited by this agent (Fig. 1A, upper panel). Incubation of cells with 0.3 μM herbimycin A, which did not block phosphorylation, had no effect on TCR ubiquitination.

The herbimycin A results suggest a relationship between TCR ubiquitination and TCR-mediated tyrosine kinase activation. To further assess this relationship, the effects of pervanadate on TCR ubiquitination were evaluated. Pervanadate increases global cellular tyrosine phosphorylation by inhibiting protein tyrosine phosphatases and has been shown to mimic...
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TCR-mediated signaling (37–40). When Jurkat cells were pre-treated with pervanadate, a dramatic increase in total cellular tyrosine phosphorylation was observed in whole cell lysates, treated with pervanadate, adramaticincreaseintotalcellular TCR-mediated signaling (37–40). When Jurkat cells were pre-treated cells, the TCRs were specifically immunoprecipitated from pervanadate-gigistic with regard to previously greater than that achieved with anti-CD3 (OKT3) cross-mutant form of this enzyme expressed in JCaM1.6 (26) are indicated by this figure, prior to lysis and immunoprecipitation as described for Fig. 1. Immunoblotting was carried out with either anti-ubiquitin or anti-phosphotyrosine as indicated. A, anti-Lck immunoblot of Triton X-100-soluble lysates showing restoration of wild type Lck expression in J Lck.H5 and J Lck.B3 stable transfectants of JCaM1.6.22. The positions of wild type Lck and a band corresponding to an inactive mutant form of this enzyme expressed in JCaM1.6 (26) are indicated by arrows. Lysates from 1 × 10⁶ cells were loaded in each lane. C, TCRs from Jurkat and stable transfectants of JCaM1.6.22 with Lck were evaluated as in A.

The tyrosine kinase activation plays a crucial role in TCR signaling also requires the tyrosine phosphatase CD45. This TCR-associated transmembrane phosphatase is believed to function in the initiation of signaling by dephosphorylating key regulatory residues on Lck and Fyn (6). CD45-deficient cells are defective in signaling and do not exhibit the normal increase in TCR phosphorylation seen in response to receptor occupancy (27, 46, 47). In such a Jurkat variant (J45.01), no ligand-induced TCR ubiquitination was detected (Fig. 4A), despite comparable levels of surface TCR (not shown) and total cellular ζ (Fig. 4B, lower panel). As with the Lck negative cells, PMA and ionomycin did not result in ubiquitination (not shown). Consistent with the requirement for tyrosine kinase activation for ubiquitination, treatment of wild type Jurkat cells with PMA and/or ionomycin in the absence of TCR engagement resulted in neither TCR ubiquitination nor tyrosine phosphorylation (not shown).

TCR Ubiquitination Requires the Regulatory Tyrosine Phosphatase CD45—In addition to tyrosine kinases, TCR-mediated signaling also requires the tyrosine phosphatase CD45. This TCR-associated transmembrane phosphatase is believed to function in the initiation of signaling by dephosphorylating key regulatory residues on Lck and Fyn (6). CD45-deficient cells are defective in signaling and do not exhibit the normal increase in TCR phosphorylation seen in response to receptor occupancy (27, 46, 47). In such a Jurkat variant (J45.01), no ligand-induced TCR ubiquitination was detected (Fig. 4A), despite comparable levels of surface TCR (not shown) and total cellular ζ (Fig. 4B, lower panel). As with the Lck negative cells, PMA and ionomycin did not result in ubiquitination (not shown). As expected, when these cells were reconstituted with CD45 (J45.LB3.3) (28), anti-CD3-dependent TCR ubiquitination was easily detected (Fig. 4B, upper panel). This establishes that TCR ubiquitination is not only dependent on tyrosine kinases but also on the presence of a tyrosine phosphatase that regulates kinase activity.

DISCUSSION

This study establishes that TCR ubiquitination requires intact coupling to tyrosine kinase activation and is not simply the...
consequence of the recognition of aggregated TCR cytoplasmic domains by E2/E3 enzymes. This requirement is not bypassed by stimulating serine phosphorylation and internalization of engaged receptors. Pervanadate, which results in an acute increase in tyrosine phosphorylation, is sufficient to result in detectable levels of TCR ubiquitination, even in the absence of receptor ligation. Thus, an acute increase in tyrosine phosphorylation, independent of receptor occupancy, appears to be sufficient to result in ubiquitination. However, the finding that the level of ubiquitination seen with pervanadate is consistently less than that found with receptor occupancy suggests that specific signals generated in response to TCR engagement may be important in stimulating a maximal level of TCR ubiquitination. These signals may be a direct manifestation of TCR oligomerization or perhaps reflect a different temporal order or pattern of phosphorylation induced when tyrosine kinases are activated by TCR ligation.

The platelet-derived growth factor receptor and c-kit (the stem cell factor receptor) are tyrosine kinase-containing members of the growth factor family of receptors that are ubiquitinated in response to their cognate ligands (48, 49). In the case of platelet-derived growth factor receptor, mutation of autophosphorylation sites correlates with decreased ligand-dependent ubiquitination (50), and for c-kit, the tyrosine kinase inhibitor genistein results in decreased ligand-dependent ubiquitination (49). Several other mammalian transmembrane receptors that either signal by coupling to tyrosine kinase activation (51) or that contain intrinsic tyrosine kinase activity (52) are ubiquitinated in an occupancy-dependent manner. Taken together with our findings, these observations suggest that tyrosine phosphorylation likely plays an important role in ligand-dependent ubiquitination of a number of mammalian transmembrane receptors.

One means by which receptor ubiquitination might occur in response to tyrosine kinase activation is by the phosphorylation of E2/E3 enzymes with resultant changes in their associations and/or activities. In fact, in one case, the in vitro tyrosine phosphorylation of an E2 was found to correlate with enhanced ubiquitination (53). Alternatively, receptors might be ubiquitinated by E2/E3 enzymes that contain sites that bind phosphotyrosine, such as is seen with SH2 domains, although no enzymes fitting this description have thus far been identified.

The function of ubiquitination in the biology of transmembrane receptors remains to be elucidated. It has generally been assumed that transmembrane receptors are degraded in lysosomes. However, to be exposed to lysosomal proteases, intracytosolic receptor domains would first need to be engulfed in autophagocytic vesicles. Alternatively, the intracytosolic domains of receptors could be degraded, at least in part, in a ubiquitin-dependent fashion in the 26 S proteasome. This could be viewed as a protective mechanism, insuring the degradation of the signaling domains of activated receptors. Although re-
results obtained with the platelet-derived growth factor receptor are suggestive (50), for no transmembrane receptor has a causal relationship between ligand-induced ubiquitination and proteasomal degradation been established. The recent finding of herbimycin A-induced ubiquitination and proteasomal degradation of the insulin-like growth factor receptor, while not addressing the issue of ligand-dependent ubiquitination, demonstrates that transmembrane receptors may, in fact, be degraded by proteasomes (54).

Regardless of the fate of ubiquitinated receptors, the steric effects of ubiquitin moieties branching off of the intracytoplasmic tails of receptors would be expected to impact negatively on intracellular associations with signaling molecules and between receptors. For the TCR, Fyn, ZAP-70, and CD45 are between receptors. For the TCR, Fyn, ZAP-70, and CD45 are shown to be phosphatase substrates for the protein tyrosine kinase Fyn, and ZAP-70 is a substrate for the protein tyrosine phosphatase CD45 (69). Thus, whether or not ubiquitination is a major factor in ligand-dependent receptor degradation, it is likely that this modification represents a means of modulating the function of activated transmembrane receptors.

Acknowledgments—We thank J. D. Ashwell, U. D’Oro, B. J. Druker, G. A. Koretzky, S. Rois, and L. E. Samelson for reagents; A. Weiss for suggesting the use of CAM1.6. and J. D. Ashwell, J. J. O’Shea, R. K. Ribaudo, and J. D. Weissman for critical review of this manuscript.

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