Engaged and Bystander T Cell Receptors Are Down-modulated by Different Endocytotic Pathways* 

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T cell antigen receptor (TCR) engagement by stimulatory antibodies or its major histocompatibility complex-antigen ligand results in its down-modulation from the cell surface, a phenomenon that is thought to play a role in T cell desensitization. However, TCR engagement results in the down-modulation not only of the engaged receptors but also of non-engaged bystander TCRs. We have investigated the mechanisms that mediate the down-modulation of engaged and bystander receptors and show that co-modulation of the bystander TCRs requires protein-tyrosine kinase activity and is mediated by clathrin-coated pits. In contrast, the down-modulation of engaged TCRs is independent of protein-tyrosine kinases and clathrin pits, suggesting that this process is mediated by an alternate mechanism. Indeed, down-modulation of engaged TCRs appears to depend upon lipid rafts, because cholesterol depletion with methyl-β-cyclodextrin completely blocks this process. Thus, two independent pathways of internalization are involved in TCR down-modulation and act differentially on directly engaged and bystander receptors. Finally, we propose that although both mechanisms coexist, the predominance of one or the other mechanisms will depend on the dose of ligand.

Regulation of TCR1 expression at the plasma membrane is an important means of calibrating the T cell response to its MHC-antigen peptide (MHCp) ligands. At rest, the TCR is continuously internalized and recycled at the T cell surface, a cycle that is dependent on a dileucine endocytosis motif present in the CD3γ subunit (1–5). The significance of the constitutive internalization-recycling process is unknown, although it could certainly add plasticity to the topological distribution of the TCR. Indeed, this may facilitate the rapid transport of TCRs to the immune synapse via recycling endosomes when an appropriate antigen-presenting cell is encountered (6). Furthermore, the endocytosis of the TCR is increased, and TCR recycling is reduced upon T cell activation leading to the down-modulation of the TCR at the cell surface. This is in part because of the phosphorylation of a serine residue upstream of the dileucine motif in CD3γ, which improves the presentation of the dileucine signal to the endocytic machinery. As a result, the dileucine motif binds to AP-2 that in turn recruits the clathrin-dependent endocytic machinery leading to the internalization of the TCR (7). However, although the serine-dependent dileucine motif of CD3γ is required for phorbol ester-induced TCR down-regulation, it is not necessary for TCR down-regulation induced by anti-CD3 antibodies or by antigen-presenting cells bearing the appropriate MHCp. Hence, it appears that other endocytic signals may be involved in these cases (8–11).

It remains unclear what the mechanisms are that underlie TCR down-modulation. Some studies suggest that TCR down-modulation is mediated by an increase in internalization and degradation (9, 12), whereas others indicate that TCR down-regulation is the result of the increased degradation of the internalized receptors (13). Whichever the case, TCR degradation through a combination of lysosome- and proteasome-dependent mechanisms does appear to occur (13, 14).

The contribution of TCR signaling to TCR internalization and down-modulation is also unclear. Some studies suggest that the TCR internalization and down-modulation are protein-tyrosine kinase (PTK)-dependent processes that ultimately result in the degradation of the TCR (5, 15–17). However, in other studies PTK inhibitors did not affect TCR down-modulation (18, 19).

By studying the T cells expressing two distinct TCRs, it was concluded that only TCRs directly triggered by the appropriate MHCp are down-regulated (20–22). However, other studies have demonstrated that TCR engagement by antigen, superantigen, or anti-TCR antibodies not only led to the down-regulation of directly triggered TCRs but also to the co-modulation of a proportion of non-engaged (bystander) TCRs (23–28).

We found previously that bystander TCRs are modulated by a mechanism that requires the activation of PTKs and protein kinase C by the engaged TCR (28). Furthermore, it was recently shown that in contrast to the internalization of engaged TCR, the modulation of bystander TCRs is highly dependent on protein kinase C and the dileucine endocytosis motif of CD3γ (23). In contrast the engaged TCR is down-modulated by a mechanism independent of PTKs. We have further investigated the mechanisms that mediate the internalization of engaged and co-modulated TCRs and found that although the bystander TCR is internalized by a clathrin-dependent process, internalization of the engaged TCR is lipid-raft-dependent.

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**EXPERIMENTAL PROCEDURES**

**Cells**—The cell line CH1β/pRex TTγ (TTγ clone) is a Jurkat transfected that has been described previously and that expresses the TCRα and β chains (Vβ3.1) of HA 1.7, a T cell clone specific for influenza hemagglutinin peptide 307–319 (27). This cell line was additionally transduced with a chimeric gene encompassing the extracellular and membrane domains of the human IL-2 receptor α chain (Tac) fused to the cytoplasmic tail of the CD3ζ chain (29). The cell line Jk.scTCRβ (clone 2D10) was obtained by transfection of Jurkat with the expression vector pSRα-scTCRVβ.

**Antibodies and Chemicals**—The mouse monoclonal antibody directed against human CD3, OKT3, was obtained from Ortho Diagnostics. The anti-Tac antibodies, MAR 108 and TP1/6 were generously provided by Drs. M. López-Botet and F. Sánchez-Madrid, respectively (Hospital de la Princesa, Madrid, Spain). The CD3ζ-specific antisera, Ab 448, was generated by immunization of a New Zealand rabbit with a synthetic peptide corresponding to the amino acid sequence 109–132 of human CD3ζ coupled to keyhole limpet hemocyanin. The anti-clathrin heavy chain antibody was purchased from Transduction Laboratories. The fluorescein isothiocyanate-conjugated antibodies specific for mouse IgG and fluorescein isothiocyanate-conjugated streptavidin were purchased from Southern Biotechnology, and the fluorescein isothiocyanate-conjugated antibodies specific for mouse Vβ8 (F23.1) were purchased from Pharmingen. The antibody specific for Vβ3, Jovi, was a kind gift of Dr. M. Owen (Cancer Research). PP2 was purchased from Calbiochem, methyl-β-cyclodextrin was purchased from Sigma, and nitrophenyl phosphate (NPP)-bovine serum albumin was purchased from Biosearch Technologies.

**DNA Constructs**—To generate the GFP-dynamin and GFP-dynamin K44A constructs, the 3.2-kb fragment from the pUHD10-3-Dyn and pUHD10-3-Dyn K44A vectors (30) were subcloned into the plZRIRES/gfp vector (31).

**Down-regulation Experiments**—Antibody-coated plates were generated by incubating plastic 96-well plates (Costar) overnight at 37 °C with 100 μl/well of the appropriate antibody (25 μg/ml) in phosphate-buffered saline, and they were washed with phosphate-buffered saline before use. Using radiochemical techniques, the total bound antibody under these conditions was estimated as 20 ng/well. Cells (10^6/well) were incubated with the desired antibodies in culture medium at 37 °C for the times indicated. The cells were then collected, transferred to ice-cold PBS-buffered saline, and washed twice. Subsequently, the cells were stained with the stimulating antibody, followed by a phycoerythrin-conjugated secondary antibody. In co-modulation studies, the cells were stained with the specific antibody labeled with biotin followed by streptavidin-phycocerythrin. The cells were ultimately analyzed in a FACSCalibur flow cytometer (BD Biosciences), and the mean fluorescence intensity was measured at each point.

**Inhibition of Clathrin-mediated Endocytosis**—Potassium depletion was carried out as described previously (32). Briefly, the cells were washed and resuspended in hypotonic medium (Dulbecco’s modified Eagle’s medium:water, 1:1), and after 5 min at 37 °C the cells were washed in medium without K⁺ (100 mM NaCl, 50 mM HEPES, pH 7.4) and then resuspended in the same medium with 1 mg/ml bovine serum albumin. Cells were incubated at 37 °C in wells previously coated with the desired antibodies for the times indicated. For acid treatment, the cells were washed and resuspended in RPMI supplemented with 2.5g/liter glucose, 10% fetal bovine serum, and 10 mM acetic acid, as described (33). The cells were then incubated at 37 °C in wells previously coated with the desired antibodies for the times indicated.

**Immunoprecipitation and Western Blot Analysis**—Cells were collected, washed, resuspended at 3 x 10^6 cells/ml of RPMI supplemented with 10 mM HEPES, pH 7.4, and warmed at 37 °C for 10 min for each time point. Afterward, anti-CD3 was added for the periods specified before the cells were lysed in 1 ml of Brij 96 lysis buffer containing protease and phosphatase inhibitors (0.33% Brij 96, 140 mM NaCl, 10 mM Tris-HCl, pH 7.8, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, and 20 mM sodium fluoride). Immunoprecipitation and immunoblotting were performed as described previously (34).

**RESULTS**

The Nature and Dose of the Ligand Determines the Sensitivity of Antibody-induced TCR Down-modulation to PTK Inhibitors—Conflicting results have been obtained from different studies on the TPK dependence of antibody-induced TCR down-modulation (15–19). One reason for such variation could be the use of soluble or immobilized (plate-bound) antibodies. We analyzed the effect of the Src kinase inhibitor PP2 on TCR down-modulation induced by stimulation of Jurkat T cells with different doses of soluble or plate-bound anti-CD3 antibodies. The dose-response curves in the absence of PP2 were very different when soluble or immobilized anti-CD3 was used (Fig. 1A). In the presence of soluble anti-CD3, TCR down-modulation was observed at concentrations as low as 0.1 μg/ml, and it steadily increased in response to increasing antibody concentrations. When plate-bound antibody was used, TCR down-modulation was not observed until the antibody concentration reached 1 μg/ml and above. The response to increasing concentrations of immobilized anti-CD3 was abrupt, resulting in a shift from none to almost complete down-modulation with a 10-fold increase in antibody concentration (Fig. 1A).

The effect of PTK inhibition by PP2 was also dependent on the way the stimulus was presented. Although TCR down-modulation induced by soluble anti-CD3 was partly inhibited at all antibody concentrations, the effect on TCR down-modulation induced by a plate-bound antibody was strongly dependent on the dose of the antibody (Fig. 1A). At relatively low concentrations (1–3 μg/ml) TCR down-modulation was completely inhibited by PP2, whereas at high concentrations (10–30 μg/ml) TCR down-modulation was refractory to PTK inhibition. At the dose used, PP2 completely blocked TCR signaling, even at high doses of stimulating antibody, as measured by the tyrosine phosphorylation of total proteins and the induction of the CD69 activation marker (data not shown), suggesting that PTKs were completely inhibited. Therefore these results suggest that PTK-dependent and -independent mechanisms mediate the antibody-induced down-regulation of the TCR. Moreover, the preponderance of one or the other depends on the way the antibody is presented (soluble or immobilized) as well as on the dose of antibody.

At certain concentrations of plate-bound antibody, TCR engagement led to its down-modulation by a PTK-independent mechanism. A feature that characterized this mechanism was that the stimulated cells shift abruptly from a non-modulated to a fully modulated state (clearly seen in Fig. 1B). In the absence of PP2, stimulation with increasing concentrations of soluble anti-CD3 induced a gradual decrease of TCR expression on the cell surface. However, when stimulated with a high concentration of plate-bound anti-CD3 (10 μg/ml) two cell populations were seen to exist, one in which the TCR was not down-modulated (Fig. 1B, population a) together with another in which TCR down-modulation was virtually complete (population b). In contrast, stimulation with a small amount of immobilized anti-CD3 (1 μg/ml) induced only a small decrease in TCR expression in the whole T cell population (Fig. 1B). Incubation with PP2 completely prevented the decrease in TCR intensity in cells stimulated with low doses of immobilized anti-CD3 (or with soluble anti-CD3) but did not alter the discontinuous down-modulation induced by high doses of immobilized anti-CD3.

**Directly Engaged and Bystander TCRs Are Down-modulated by Different Mechanisms**—Along with others, we have demonstrated that engagement of a TCR with a specific ligand results not only in its own down-modulation but also in that of non-engaged bystander TCRs. We proposed that the differential sensitivity of TCR down-modulation to PP2 inhibition observed upon stimulation with different doses of immobilized anti-CD3 (shown in Fig. 1) might indicate the existence of different mechanisms underlying the down-modulation of directly engaged and bystander TCRs. We determined the sensitivity to PP2 of the down-modulation of engaged and non-engaged TCRs. Jurkat T cells were transfected with an immunoglobulin single chain (scFv)-TCRβ construct in a way that the trans-
fected cell expresses two TCR complexes, one with the endogenous Vβ8-containing TCRβ and the other with the transfected scFv-(and Vβ3)-containing TCRβ. The immunoglobulin single chain fused to the transfected Vβ3-TCRβ confers specificity to the NP hapten, and a stable cell transfectant of scFv(Vβ3)-TCRβ in Jurkat T cells was generated. Stimulation of these cells with NP conjugated to bovine serum albumin induced near maximal down-modulation of the scFv(Vβ3)-containing TCR at a concentration of 30 μg/ml (Fig. 2). The dose-response curve and the sensitivity to PP2 inhibition made the stimulation with NP-bovine serum albumin appear very similar to stimulation with soluble anti-CD3 (Fig. 1A). Furthermore, stimulation with NP-bovine serum albumin induced the co-modulation of the non-engaged Vβ8-containing TCR. Interestingly, the co-modulation of the bystander Vβ8-TCR was completely inhibited by PP2 treatment, whereas down-modulation of the directly engaged TCR was only partially inhibited. Therefore, these results indicate that the bystander non-engaged TCR is down-modulated by a PTK-dependent mechanism, whereas the stimulated TCR is down-regulated by a mixture of PTK-dependent and -independent mechanisms.

**Down-modulation of Directly Engaged and Bystander TCRs Are Differentially Dependent on Endocytosis Mediated by Clathrin-coated Pits**—To further study the mechanisms that

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**Fig. 1. Stimulation with soluble and immobilized anti-CD3 antibody results in PTK-dependent and -independent TCR down-modulation.** A, PP2 sensitivity of TCR down-modulation provoked by soluble and immobilized anti-CD3 antibody. Jurkat T cells were stimulated for 1 h with the soluble anti-CD3 antibody OKT3 or in antibody-coated wells pre-incubated with the OKT3 (immobilized anti-CD3). Down-modulation is shown as the percentage of the mean fluorescence intensity in reference to the mean fluorescence intensity at time 0 (without stimulation). B, flow cytometry histograms show TCR expression after stimulation for 1 h at the concentrations of soluble or immobilized OKT3 indicated (0, 1, and 10 μg/ml). Two populations of cells were observed after stimulation with immobilized OKT3, with down-modulated (b) and with non-down-modulated (a) TCR.
mediate the down-modulation of engaged and bystander TCR, we proceeded to study Jurkat T cells transfected with a CD25(Tac)-CD3ζ chimera (TTζ). This chimera results from appending the cytoplasmic tail of CD3ζ to the extracellular and transmembrane domains of CD25. We preferred to use these cell transfecants instead of the scFv(Vβ3)-TCRβ transfectant, because the TTζ chimera does not associate with the TCR complex (28). On the contrary, it is still possible that a single TCR complex includes two different TCRβs (24, 25, 35). We stimulated clone 4 (a Jurkat clone expressing low levels of TTζ relative to CD3) with an anti-CD3 antibody to study the direct modulation of the TCR complex and the co-modulation of the TTζ chimera. In parallel, clone 65 (a Jurkat clone expressing high levels of TTζ) was stimulated with an anti-Tac antibody to study the direct modulation of the TTζ chimera and the co-modulation of the TCR complex. The stimulation of clone 4 with a high dose of plate-bound anti-CD3 antibody induced both the discontinuous down-modulation of the TCR complex, the “all-or-nothing” effect described in Fig. 1B, and co-modulation of the TTζ chimera (Fig. 3). Conversely, the stimulation of clone 65 with a high dose of anti-Tac resulted in the discontinuous all-or-nothing down-regulation of the TTζ chimera and the co-modulation of the TCR. The stimulation of each clone with the inverse antibody also caused the co-modulation of the bystander receptor, although, probably because of the suboptimal TTζ/TCR ratio, the effect was not so dramatic (data not shown).

We then examined the role of clathrin-coated pits in the down-modulation of the engaged and non-engaged receptors. We first examined whether we could co-precipitate the engaged and non-engaged receptors with clathrin. The TCR has been shown to undergo AP2- and clathrin-dependent constitutive internalization (7), and accordingly, the TCR co-precipitated with clathrin in non-stimulated cells (Fig. 4A). However, stimulation of clone 4 with a high dose of immobilized anti-CD3 for 15 and 60 min did not increase the TCR that co-precipitated with clathrin, suggesting that down-modulation of the engaged TCR takes place without an increased association with clathrin-coated pits. Interestingly, stimulation of clone 4 with anti-CD3 induced the co-precipitation of clathrin with TTζ, suggesting that stimulation of the TCR could promote recruitment of the bystander receptor to clathrin-coated pits.

To further investigate the dependence of clathrin for TCR down-regulation, we overexpressed a dominant negative mutant of dynamin. This GTPase is necessary to close the neck of the coated pit, and therefore expression of the dominant negative mutant inhibits receptor internalization by preventing the formation of endocytic vesicles (30). The effect of expressing dominant negative dynamin in the down-modulation of engaged and non-engaged receptors was studied by transiently transfecting clone 4 either with the K44A mutant of dynamin 1 using a bicistronic GFP-expressing vector or with wild type dynamin 1. The transfected cells were stimulated with anti-CD3, and the expression of the TCR and TTζ was examined by flow cytometry upon gating on the GFP-positive population.

Expression of the dominant negative dynamin partly inhibited the down-modulation of both the TCR and the bystander TTζ compared with expression of wild type dynamin 1 (Fig. 4B) or to untransfected cells (data not shown).

In addition to its role in clathrin-mediated endocytosis, dynamin may also control the endocytosis of receptors that use a clathrin-independent pathway (36). The most specific and effective method to inhibit clathrin-mediated endocytosis involves the overexpression of a dominant negative Eps15 mutant (37), but this appeared to be toxic in Jurkat cells. We therefore used alternative methods to inhibit clathrin-dependent endocytosis such as the use of an acidic medium and depletion of intracellular potassium, which detach clathrin from the internal side of the plasma membrane (32, 33). When clone 4 was stimulated with a high dose of immobilized anti-CD3, co-modulation of TTζ was completely inhibited by acidification of the medium (Fig. 5A), whereas down-modulation of the engaged TCR remained unaffected. Conversely, upon depletion of intracellular potassium, stimulation with anti-Tac of clone 65 resulted in the inhibition of TCR co-modulation (Fig. 5B). Again, down-modulation of the engaged receptor (TTζ in this experiment) was not affected, except for a slight smoothening of the all-or-nothing effect 30 min after stimulation.

Together, the co-precipitation with clathrin, the inhibition by dominant negative dynamin, and the inhibition by acidification or depletion of intracellular potassium indicate that co-modulation of the bystander receptor is clathrin-dependent. In contrast, down-modulation of the directly engaged TCR appeared to be primarily mediated by a dynamin-sensitive mechanism independent of clathrin.

**Down-modulation of Directly Engaged TCRs Is Dependent on Lipid Rafts—** TCR engagement results in its redistribution into cholesterol- and glycosphingolipid-rich membrane microdomains known as lipid rafts (38, 39). Lipid rafts have been shown to play an important role in T cell activation, because they serve to concentrate important signaling molecules (40). To study the role of lipid rafts on the down-modulation of directly engaged and bystander receptors, we examined the...
DISCUSSION

In this study, we have extended our previous observations showing that TCR engagement results in the down-modulation of bystander TCRs. We have found that stimulation of the TCR with high doses of immobilized anti-CD3 induces its down-modulation by a PTK-independent mechanism, whereas stimulation with low doses of anti-CD3 results in TCR down-modulation by a PTK-dependent mechanism. This contrasts with the effect of soluble anti-CD3, which was PTK-dependent, albeit partially, at all antibody doses analyzed. We propose that antibody engagement of the TCR results in the down-modulation of directly engaged receptors as well as bystander receptors. Furthermore, we showed here that bystander and directly engaged TCRs are down-modulated by different mechanisms. We propose that the down-modulation of the directly engaged TCR is by a PTK-independent mechanism, whereas down-modulation of the bystander TCRs requires PTK-initiated signal spreading.

The differential sensitivity to PP2 of TCR down-modulation by immobilized anti-CD3, and the partial inhibition by PP2 of TCR down-modulation induced with soluble anti-CD3, might reflect the ratios of directly engaged:bystander receptors in each situation. Thus, at low ligand doses most down-modulated TCRs will be bystander TCRs that rather than being directly engaged will have received a signal to down-modulate transmitted by PTKs. This hypothesis is however counterintuitive when the effect of saturating doses (10–30 µg/ml) of soluble anti-CD3 are studied (Fig. 1). At these concentrations of anti-CD3 one should expect that all TCRs are directly engaged. However, only part of the TCR is down-modulated by a PTK-independent mechanism. We propose the hypothesis that at high doses of soluble antibody, monomeric binding predominates (i.e. without cross-linking) thus transforming most antibody-bound TCRs into mere bystander receptors. This hypothesis goes with the idea that direct PTK-independent down-modulation requires strong cross-linking.

In addition to PTKs, we have previously shown that protein kinase C is involved in transmitting the co-modulation signal (28). This finding was recently confirmed and extended when the co-modulation of the bystander TCR was shown to be protein kinase C-dependent and to involve the phosphorylation of the S126DxaxxXL endocytosis motif in CD3γ (23). Additionally, we have now shown that the bystander TCR is down-modu-
lated by a clathrin-mediated mechanism. Furthermore, we
found that TCR triggering induced the association of clathrin
with the bystander TT\textsubscript{H9256} chimaera, suggesting that TCR signal-
ing somehow promotes the association of the bystander recep-
tor with the clathrin machinery.

The clathrin-associated adapter AP-2 has been shown to be
recruited to the CD3\textsubscript{H9253} double leucine motif upon phosphoryla-
tion of Ser-126 (7). Therefore, the sequence of events that
precedes the down-modulation of bystander TCRs seems to
likely involve the activation of PTKs by the engaged TCR,
resulting in protein kinase C activation and the subsequent
phosphorylation of CD3\textsubscript{H9253} in the bystander TCR. Finally, AP-2
and clathrin are recruited to the phosphorylated CD3\textsubscript{H9253}, and the
TCR is endocytosed. Although this sequence of events might
occur, it seems likely to be only part of the picture. We detected
the recruitment of clathrin to the TT\textsubscript{H9256} chimaera that is down-
modulated in a protein kinase C-dependent fashion. However,
the TT\textsubscript{H9256} chimera does not bear protein kinase C phosphoryla-
tion motifs; therefore, additional mechanisms must mediate
the recruitment of the clathrin endocytic machinery to motifs
in the TCR other than the double leucine motif in CD3\textsubscript{H9253}. For
example, these could include the tyrosine-based motifs present
in various copies in the immunoreceptor tyrosine-based activa-
tion motifs of the \( \zeta \) cytosolic region. In addition, it has been
described recently that T cell stimulation with soluble anti-
CD3 antibody induces the Lck-dependent tyrosine phosphory-
ation of the clathrin heavy chain (41), suggesting a possible
mechanism for the transmission of the internalization signal to
bystander TCRs. In this regard, the high sensitivity of TCR
co-modulation to inhibition by M\textsubscript{H9252}CD is in agreement with the
proposed role for lipid rafts, Lck is associated with lipid rafts,
in TCR signaling (42, 43).

**FIG. 4.** TCR engagement induces the association of clathrin to bystander TT\textsubscript{H9256} chimaera. A, clone 4 was stimulated on Petri dishes coated
with 10 \( \mu \)g/ml OKT3 for the times indicated. Cells were collected, lysed, and immunoprecipitated (IP) with anti-CD3 (OKT3) or anti-Tac (MAR108)
antibodies. After SDS-PAGE, immunoblotting was performed with an anti-clathrin heavy chain antibody and then reprobed with anti-Tac antibody
TP1/6. Arrows indicate the positions of clathrin heavy chain and of TT\textsubscript{H9256} as a loading control. B, down-modulation of the engaged and bystander
receptors is inhibited by a dominant negative dynamin-1 construct. Clone 4 cells were transiently transfected either with the K44A mutant of
dynamin 1 in a GFP-expressing vector (Dyn DN) or with wild type dynamin 1 in the same vector (Dyn WT). After stimulation in wells coated with
10 \( \mu \)g/ml OKT3 for the times indicated, TCR (direct modulation) and TT\textsubscript{H9256} (co-modulation) down-modulation was assessed by flow cytometry after
gating the GFP\textsuperscript{+} cells.
Although we have made significant advances in understanding the mechanisms that mediate the co-modulation of the bystander TCR, our knowledge of the mechanism that mediates down-modulation of the directly engaged TCR is more limited. Down-modulation of the engaged TCR is not affected by inhibitors of PTK or protein kinase C or by treatments that inhibit clathrin-dependent endocytosis. Down-modulation of the engaged TCR is ATP- and temperature-dependent, it requires dynamin to close the endocytic vesicle and results in a characteristic all-or-nothing effect. The all-or-nothing effect may suggest the need for a critical number of engaged TCRs to accumulate before producing endocytosis. This accumulation
could be the result of their translocation to lipid rafts. Indeed, we showed in this study that treating cells with a cholesterol-depleting agent, MβCD, can prevent TCR down-modulation. This result strongly suggests that TCR down-modulation involves both clathrin-dependent and lipid raft-dependent mechanisms, as has been proposed for the IL-2 receptor (36). However, our data also indicate that the mechanism for down-modulation of engaged TCRs is PTK-independent. We therefore propose that TCR engagement results in its internalization and down-modulation by a process that involves TCR accumulation in lipid rafts without the participation of signaling components.

Is the PTK-dependent and -independent mechanism model obtained for antibody engagement relevant to MHCp-induced
TCR down-regulation? We previously demonstrated that at high concentrations of MHCp, the TCR is down-regulated by a PTK-independent mechanism, whereas TCR down-modulation was completely abrogated by a PTK inhibitor at low MHCp concentrations (28). Previous studies have generated conflicting results relating to the dependence of TCR down-modulation on PTK (15–19). Although this issues remains unclear, it does seem that PTKs play an important role in routing the endocy-tosed TCR for degradation in the lysosomes (13, 14, 17). Indeed, it has been proposed that TCR engagement induces TCR down-modulation by preventing recycling rather than by increasing internalization above constitutive levels (13). However, high doses of MHCp have similarly been shown to increase TCR internalization (13). Together, the data presented here show that TCR down-regulation is a much more complex process than first thought, involving both directly engaged and by-stander TCRs, which are internalized through distinct endocy-totic pathways.

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