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WC1 proteins, which are specifically expressed by bovine γδ T cells from a gene array containing 13 members, are part of the scavenger receptor cysteine-rich family. WC1 cytoplasmic domains contain multiple tyrosines, one of which is required to be phosphorylated for TCR coreceptor activity, and a dileucine endocytosis motif. Like the TCR coreceptor CD4, WC1 is endocytosed in response to PMA. Because WC1 endocytosis may play a role in the activation of γδ T cells, we examined WC1 endocytosis in the adherent cell 293T and Jurkat T cell lines using a fusion protein of extracellular CD4 and the transmembrane and cytoplasmic domain of WC1. Individual mutation of the two leucine residues of the endocytic dileucine motif in the WC1 cytoplasmic domain significantly reduced PMA-induced endocytosis in both cell types and enhanced IL-2 production stimulated by cocross-linking of CD3/TCR and CD4/WC1 in Jurkat cells, suggesting that the sustained membrane coligation of CD3/TCR with WC1 caused by a decrease in endocytosis increases T cell activation. Mutation of two serines upstream of the endocytic dileucine motif affected endocytosis only in adherent 293T cells. Although the two upstream serines were not required for WC1 endocytosis in Jurkat cells, the pan–protein kinase C inhibitor Gö6983 blocked endocytosis of CD4/WC1, and mutation of the upstream serines in WC1 inhibited IL-2 production stimulated by cocross-linking of CD3/TCR and CD4/WC1. These studies provide insights into the signaling of WC1 gene arrays that are present in most mammals and play critical roles in γδ T cell responses to bacterial pathogens. The Journal of Immunology, 2015, 194: 000–000.

Because bacterial products ligate the γδ TCR and activate γδ T cells (35), our hypothesis is that WC1 and the γδ TCR are coligated by bacterial products, leading to activation of the γδ T cells and en-
endoctyosis of γ8 TCR/CD3 and WC1, similar to the endocytosis of αβ TCR/CD3 and CD4. The cytoplasmic domains of WC1 proteins are similar to those of CD3γ, CD3δ, and CD4, containing two consensus [DE]XXX[LIM] dileucine motifs, multiple serines, and two to four YXXφ tyrosine-based motifs (20, 23). We showed previously that mutation of the tyrosine of one of the YXXφ tyrosine-based motifs in CD3γ does not affect phorbol ester–induced endocytosis, suggesting that the dileucine motifs may instead regulate WC1 phorbol ester–induced endocytosis (22). In this study, we investigate the requirement for two serines and the membrane proximal dileucine motif in phorbol ester–induced WC1 endocytosis in both HEK-293T cells and Jurkat T cells.

Materials and Methods

Cell culture

HEK-293T cells were cultured in IMDM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FCS and 50 μg/ml gentamicin. Jurkat T cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS and 20 μg/ml gentamicin, 10 μM 2-ME, 10 mM HEPES (pH 7.2), 1 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acid (Invitrogen).

CD4/WC1-3 constructs and mutagenesis

CD4/WC1-3 double-serine mutants (CD4/WC1-3 S13A/S14A) and two individual leucine mutants (CD4/WC1-3 L30D and CD4/WC1-3 L31D) were generated by site-directed mutagenesis using the QuiChange II Site-Directed Mutagenesis Kit (Stratagene, Wilmington, DE) and CD4/WC1-3 in pBk-CMV as a template. The primer sequences were as follows: CD4/WC1-3 S13A/S14A forward 5′-GCAGGAGGCGGCGCTTGAGCTCCC-3′ and CD4/WC1-3 S13A/S14A reverse 5′-AGCCATCTTATAGGCGGCTTACCTTGACACCCAGAAGGTAATCGAGCTTAC-3′; CD4/WC1-3 L30D forward 5′-GAGGAGCTAGTACGGAGAGGACACCAGCAGAAGGAGG-3′ and CD4/WC1-3 L30D reverse 5′-CCTTCCTTCTGGGTCAGATCGTG-3′; CD4/WC1-3 L31D forward 5′-GCCAGCCTCCTTGCGGTGTCG-3′ and CD4/WC1-3 L31D reverse 5′-CAGACCTTCTCCTGCGGTGTCG-3′. The PCR reactions were 20 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 7 min. All constructs were confirmed by sequencing. CD4/WC1-3 mutants were excised from pBk-CMV using SpeI and ClaI and ligated into the lentiviral pLIGEaW vector (22).

Stable expression of chimeric CD4/WC1-3 in Jurkat T cells

Lentiviruses were packaged by first plating 8 × 10⁶ HEK-293T cells in 60-mm dishes. Twenty-four hours later, cells were transfected with 2 μg DNA (containing 1 μg pLIGEaW, 0.25 μg pCMV-VSVG, and 0.75 μg pCMV-ΔN9, and 6 μl FuGENE HD (Roche, Palo Alto, CA). Lentiviral supernatants were harvested at 48–72 h and harvested at 48 and 72 h posttransfection and supplemented with 10 μM 2-ME, 10 mM HEPES (pH 7.2), 1 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acid (Invitrogen).

Flow cytometry analysis

HEK-293T or Jurkat T cells (2 × 5(308,275),(344,291)) in 0.5% BSA-PBS were fixed and permeabilized with cytofix/cytofluor (BD Biosciences, San Jose, CA) at 4°C for 20 min. Cells were then permeabilized in ice-cold 90% methanol for 20 min before incubating with anti-human CD4 Ab. Quantification of CD4 molecules was done using Quantum Simply Cellular calibration beads (Bangs Laboratories, Fishers, IN), according to the manufacturer’s instructions.

Phospho-epitope mapping of CD4/WC1-3

CD4/WC1-3 S13A/S14A, CD4/WC1-3 L30D, and CD4/WC1-3 L31D constructs were transiently expressed in HEK-293T cells. After 24 h, cells were washed and phosphorylated by treating transfected cells with the PKC activator PMA for 1 min, and 68°C for 7 min. All constructs were confirmed by sequencing. CD4/WC1-3 mutants were excised from pBk-CMV using SpeI and ClaI and ligated into the lentiviral pLIGEaW vector (22).

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PhMA-induced endocytosis in HEK-293T cells and Jurkat T cells

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lentiviruses encoding wild-type and mutant CD4/WC1 proteins. The lentiviral vector used encodes both GFP and CD4/WC1 sequences, allowing equalization of lentiviral DNA integrations into the genome by flow cytometric sorting on the GFP reporter. Normally, GFP expression tracks CD4/WC1 expression; however, after sorting for equivalent GFP expression, both of the leucine mutants had higher levels of protein on the cell surface compared with either the wild-type CD4/WC1 or serine mutant (Fig. 2A, 2B), although the ratio of cell surface protein/cytoplasmic CD4/WC1 protein was the same in all cells. This indicates that mutation of the dileucine motif leads to

**FIGURE 1.** Serine and leucine mutations decrease PMA-induced WC1 endocytosis in human embryonic kidney cells. (A) Endocytic dileucine motifs are underlined in CD3γ, CD3δ, CD4, gp130, and wild-type and mutant WC1 cytoplasmic domains. Consensus endocytic motif and mutated amino acids are in bold. Consensus PKC phosphorylation motifs (RXX[ST]; RX[ST]; [ST]F) are indicated with asterisks. (B) HEK-293T cells transfected with constructs encoding CD4/WC1, CD4/WC1S13A/S14A, CD4/WC1L30D, CD4/WC1L31D, or empty vector. Cells were labeled with anti-human CD4 Ab and gated on viable cells. (C) Immunoblot with anti-CD4 or anti-GAPDH Abs of cell lysates from untransfected (NT), empty vector (pBK-CMV), and CD4/WC1 mutant or wild-type (CD4/WC1WT). (D) Transfected 293T cells were treated with DMSO control or PMA, labeled with anti-human CD4 Ab to reveal surface CD4/WC1, and analyzed by flow cytometry. The percentage change in MFI between the DMSO control and PMA-treated cells was calculated [(MFI PMA - MFIControl)]/MFIControl * 100%. The data for each cell type are represented as mean ± SEM for three independent experiments (n = 8). Significant differences between wild-type and mutant cell values are denoted by asterisks, unpaired t test.
increased total protein overall, intracellularly and on the cell surface (Fig. 2C, 2D). To guard against artifacts arising from unequal protein levels between mutants, lentivirally transduced Jurkat cell lines were resorted to equalize CD4/WC1 expression, resulting in lower levels of GFP protein that were reflective of the lower numbers of CD4/WC1 genes in the genomes of CD4/WC1L30D and CD4/WC1L31D compared with CD4/WC1 wild-type and CD4/WC1S13A/S14A (Fig. 3A, 3B). We calculated the number of cell surface CD4/WC1 molecules to verify that the four Jurkat cell lines expressed comparable levels. This analysis also showed that the number of endogenous CD4 molecules expressed by Jurkat T cells is ~14 times lower than that of the transduced CD4/WC1 and, thus, is not a significant contributor to the endocytosed CD4 molecules in these experiments (Fig. 3C). Upon treatment with PMA, the two leucine mutants retained more cell surface molecules compared with wild-type, as seen previously in 293T cells (Figs. 1C, 3D, 3E). However, the serine mutant CD4/WC1S13A/S14A exhibited the same level of decrease in cell surface molecules as did the wild-type CD4/WC1 when expressed in Jurkat T cells (Fig. 3D, 3E), in contrast to what was observed in 293T cells (Fig. 1C). To test whether the PMA-induced decrease in cell surface CD4/WC1 was due to shedding of the extracellular domain of CD4/WC1, we compared cells labeled with anti-CD4 before and after PMA treatment (Fig. 3F). The level of cell-associated CD4/WC1 (surface or internalized) revealed by incubation with anti-CD4 prior to PMA treatment did not differ significantly between the wild-type and any of the mutant CD4/WC1 constructs, indicating that the fluorochrome-labeled anti-CD4 Ab stayed on the surface or was endocytosed rather than being dissociated from the cell. The level of anti-CD4 Ab showed a significant decrease in cells expressing wild-type CD4/WC1 or CD4/WC1S13A/S14A only if they were incubated with anti-CD4 after PMA treatment to reveal retained cell surface CD4/WC1 (Fig. 3F). These results are consistent with PMA-induced endocytosis rather than with the CD4 epitope being shed from the cell surface.

PKC inhibitor reduces endocytosis of CD4/WC1
Because the serine mutant CD4/WC1S13A/S14A reduced PMA-induced endocytosis when it was expressed in 293T cells but not in Jurkat T cells, we investigated whether WC1 endocytosis in Jurkat T cells was independent of serine/threonine phosphorylation by PKC using the inhibitors Gö6983 and Rottlerin (36). The PKC α, β, δ, or ζ inhibitor Gö6983 significantly reduced the PMA-induced endocytosis of wild-type CD4/WC1 (Fig. 4A), as previously reported (22), as well as the serine mutant CD4/WC1S13A/S14A and the leucine mutants CD4/WC1L30D and CD4/WC1L31D (Fig. 4B). In contrast, the PKC θ inhibitor Rottlerin did not inhibit wild-type CD4/WC1 endocytosis, even at the high dose of 10 μM.

**FIGURE 2.** CD4/WC1 dileucine mutants induce surface and cytoplasmic protein accumulation in Jurkat T cells. (A) Jurkat T cells were transduced with CD4/WC1 wild-type, CD4/WC1S13A/S14A, CD4/WC1L30D, or CD4/WC1L31D and sorted for equivalent reporter gene GFP expression. Untransduced Jurkat T cells are also shown. (B) The transduced and sorted populations matched for GFP expression shown in (A) were labeled with anti-human CD4. Untransduced Jurkat cells were labeled with an isotype control Ab. (C) MFI (± SD) of surface and cytoplasmic CD4/WC1 and CD4/WC1 mutants (n = 4). The cytoplasmic levels of CD4/WC1-3 proteins were calculated by subtracting the MFI of cells with only surface staining from the MFI of those with both surface and cytoplasmic staining. (D) Ratio of surface MFI/cytoplasmic MFI of CD4/WC1 and CD4/WC1 mutants. Data are mean ± SD (n = 6).
which inhibits PKC \( \theta, \delta, \) and \( \zeta \) (Fig. 4A) (36). These results suggest that phosphorylation by PKC \( \alpha \) or \( \beta \), but not by PKC \( \theta, \delta, \) or \( \zeta \), is necessary for optimal WC1 endocytosis in Jurkat T cells; additionally, although phosphorylation of S13 or S14 is not required for PMA-induced endocytosis in Jurkat T cells (Fig. 3D, 3E), these results with the PKC inhibitor suggest that PKC phosphorylation of other serines in the WC1 cytoplasmic tail or other proteins is required.

FIGURE 3. Mutations of dileucine motif, but not serines, inhibit PMA-induced endocytosis in Jurkat T cells. (A and B) Transduced Jurkat T cell lines were resorted, gating on the equivalent expression of CD4/WC1 rather than on the equivalent expression of the reporter gene GFP. After sorting, cells were labeled with anti-CD4 Ab (untransduced Jurkat cells; CD4/WC1 wild-type; CD4/WC1S13A/S14A; CD4/WC1L30D; CD4/WC1L31D), analyzed by flow cytometry, and shown as a graph of GFP (A) or CD4 (B) expression. (C) The number of CD4/WC1 molecules expressed by resorted Jurkat T cell lines was determined by quantification of Ag expression in Ab-binding capacity units using cell-sized microspheres with calibrated Ab-binding capacities. The mean value ± SD of each sample is represented (\( n = 3 \)). (D and E) PMA treatment of Jurkat T cells and determination of surface CD4 levels by anti-CD4–allophycocyanin labeling and flow cytometric analysis, followed by gating on live/GFP*/allophycocyanin* cells. The mean ± SD for three independent experiments is shown. (D) Decrease in number of surface CD4 molecules after PMA treatment, as determined in (C). (E) Percentage change in surface CD4 after PMA treatment. (F) Cells labeled with anti-CD4 conjugated with allophycocyanin before or after PMA-induced endocytosis. The mean value ± SD for each sample was calculated as the difference in MFI between control DMSO– and PMA-treated groups and represent two independent experiments (\( n = 6 \)). Significant differences between wild-type and mutant cell lines were determined by an unpaired \( t \) test.
The endocytosis of WC1 is regulated by a dileucine motif

**Discussion**

The endocytosis and signaling of WC1 proteins are of interest to better understand the mechanisms of CCV-mediated endocytosis of transmembrane receptors via dileucine endocytic motifs in general and specifically to gain insights into the signaling capacity of WC1 gene arrays that are present in most mammals (19) and play a critical and determinant role in the γδ T cell response to bacterial pathogens (25, 26, 29). In this study, we correlated the sustained presence of WC1 on the T cell surface caused by mutations in the dileucine endocytic motif that inhibit endocytosis with an increase in IL-2 production when the TCR/CD3 complex was cocross-linked with the CD4/WC1 chimeric molecule. In addition, WC1 dileucine mutations are associated with a higher overall CD4/WC1 protein level per CD4/WC1 gene copy number. The dileucine motif was shown to be a sorting signal for routing gp130 and CLN7 through the multivesicle bodies to the lysosomes (37, 38); thus, the dileucine motif mutants may increase overall CD4/WC1 protein by not routing it to lysosomes for degradation.

The effect of the mutation of the upstream serines on endocytosis is cell context dependent. Although most of the studies showing that upstream serines are necessary for clathrin-mediated endocytosis of other important lymphocyte signaling receptors were performed using adherent cells [CD4 in HeLa cells and gp130 in COS-7 African green monkey fibroblast-like cells (14, 37)], mutation of a serine at the L-5 position upstream of the dileucine inhibits PMA-induced endocytosis of CD4 in a murine thymoma cell line (39), as well as endocytosis of CD3γ in a CD3γ− derivative of a Jurkat T cell (40). This could indicate that the WC1 dileucine motif is unique in being serine independent in a T cell line because the upstream serines are farther away from the dileucine motif than are those in CD3γ, CD4, and gp130. In contrast, arguing cocross-linking the serine mutant CD4/WC1S13A/S14A and CD3 suggests that S13 and S14 are required for intracellular signaling events rather than regulating endocytosis. In contrast, the dileucine mutants CD4/WC1L30D and CD4/WC1L31D made more IL-2 than wild-type only with high levels of anti-CD4 Ab (Fig. 5). Mutation of the first leucine (L30D) promoted significantly more IL-2 production at high levels of anti-CD4 Ab than did mutation of the second leucine (L31D; Fig. 5), which correlates with the known strict requirement for leucine at position L0 (15). Taken together, these data suggest that inhibition of WC1 endocytosis by mutation of the dileucine motif results in more surface availability of WC1, leading to sustained activating signals through the TCR/CD3 complex in conjunction with the WC1 coreceptor.
against exceptionalism of the WC1 dileucine motif with regard to the role of its upstream serines is the lack of contacts found between a crystallized AP2 σ2 subunit and a phosphorylated serine in a peptide containing CD4’s dileucine motif (15), implying that CD4 endocytosis also may be independent of those serines.

The fact that the WC1 S13A/S14A mutant decreased IL-2 production when CD4/WC1 and TCR/CD3 complex was co-cross-linked, but did not affect endocytosis, supports the theory that the requirements for WC1 endocytosis are decoupled from WC1-mediated signaling leading to T cell activation and, specifically, that WC1 serine phosphorylation is important for the latter. The results with the PKC inhibitors showing that PKC α or β activity is involved in endocytosis could imply indirect phosphorylation of another substrate, such as an accessory cargo molecule that strengthens the WC1 interaction with AP2, or AP2 itself (41). In support of this hypothesis, it is known that direct CD4 endocytosis induced by PMA treatment is regulated via a dileucine motif in CD4 that then binds directly to the AP2 α- or β subunit; however, endocytosis is also known to be induced indirectly by HIV Nef binding to CD4 and to the AP2 α- or β subunits (9, 42).

Although we did an in-depth study of an archetypal WC1 molecule (known as WC1-3), it is important to note that there are 13 genes coding for WC1 coreceptors, 4 of which differ in their cytoplasmic domains from the other 9 and show increased signaling capacity (23). Nine WC1 cytoplasmic domains are designated as type I, three are designated as type II, and one is designated as type III. Interestingly, one difference shared by the four type II or III molecules is the presence of a membrane proximal dileucine endocytosis motif. Instead of the membrane distal dileucine motif, KESSLV sequence in the one difference shared by the four type II or III molecules is the type II cytoplasmic domains, with the exception of type I WC1-1 and WC1-13 cytoplasmic domains. WC1-1 and WC1-13 contain a membrane distal ESLWLFL sequence rather than ES[F]PWLL. The type III cytoplasmic domain noted above as being associated with increased IL-2 production and an inactive membrane proximal dileucine motif also has an inactive membrane distal dileucine motif, KESSSLV. It is of interest that the type III cytoplasmic domain is able to meet the requirement for a tyrosine phosphorylation event by the phosphorylation of a membrane distal YDDV motif rather than the membrane proximal YEEL motif, which is used in all other WC1 cytoplasmic domains (22, 23). The endocytosis of WC1 containing the type III cytoplasmic domain may be governed by similar compensatory mechanisms. These differences among the various WC1 intracytoplasmic domains are an intriguing glimpse into the differential regulation of transmembrane receptor endocytosis and signaling. Although we used surrogate cell lines and CD4/WC1 chimeric molecules to study the signaling motifs of WC1 in this study, future studies will be directed at evaluating the effect of these differences in primary cells. It will be particularly interesting to determine the ligands for the various WC1 molecules and the implications that their differences in signaling capacity have for the γδ T cell response to various pathogens.

Disclosures

The authors have no financial conflicts of interest.

References


