Separable Portions of the CD2 Cytoplasmic Domain Involved in Signaling and Ligand Avidity Regulation

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Summary

Effective T cell immune responses require the molecular interplay between adhesive and signaling events mediated by the T cell receptor for antigen (TCR) and other cell surface coreceptor molecules. In this report, we have distinguished between the role of regulated adhesion and transmembrane signaling in coreceptor function using the T cell glycoprotein CD2. By binding its ligands on antigen-presenting cell (APC), CD2 serves both to initiate signal transduction events and to promote cellular adhesion. Furthermore, the avidity of CD2 for one ligand, CD58 (LFA-3), is regulated by TCR signaling. We have expressed wild type CD2 and a series of mutated CD2 molecules in an antigen-specific murine T cell hybridoma. Structure–function studies using these stably transfected cell lines identify two structurally and functionally distinct regions of the 116 amino acid (aa) cytoplasmic domain. One region is required for CD2-mediated signal transduction, and a separate COOH-terminal 21 aa portion is required for CD2 activity regulation. Cell lines expressing CD2 molecules lacking the cytoplasmic segment required for CD2-initiated IL-2 production retain the ability to upregulate CD2 avidity. Conversely, cell lines expressing CD2 mutants lacking the cytoplasmic segment required for avidity regulation retain the ability to initiate CD2-specific signaling. In antigen-specific T cell responses, basal binding of CD2 to its ligands enhances antigen responsiveness only minimally, whereas regulated avidity and transmembrane signaling are both required for optimal coreceptor function. Taken together, these studies demonstrate the independent contributions of regulated adhesion and intracellular signaling in CD2 coreceptor function.

While the TCR confers recognition of peptide antigens in the context of MHC proteins, several other T cell proteins contribute to T function by transducing biochemical signals or by augmenting cellular adhesion (1). CD2 is a T cell glycoprotein that plays a dual role in T cell activation. Through interactions with its ligands CD58 (LFA-3), CD59, and CD48 (reviewed in reference 2), CD2 initiates intracellular signaling cascades and enhances intercellular adhesion to APC. T cell activation is triggered by certain pairs of mAb directed against different CD2 epitopes (3), and the interaction of CD58 with CD2 enhances antigen-specific T activation (4). CD2 and CD11a/CD18 (LFA-1) provide the T cell with two pathways for regulating cellular adhesion (1, 5). Occupancy of the TCR has been shown to upregulate the avidity of both CD2 (6) and LFA-1 (7) for their respective ligands, albeit through distinct signaling pathways (7, 8).

Although the importance of signaling and adhesion is well established, the relative contributions of these processes to T cell coreceptor function and antigen responsiveness have been difficult to assess. Here we have used CD2 as a model system to dissect these functions. Expression of human wild type and mutant CD2 molecules in an antigen-specific mu-

rine T cell hybridoma has permitted the identification of distinct portions of the CD2 molecule required for CD2-mediated signaling and TCR-induced changes in CD2 avidity. Functional studies using cell lines expressing these CD2 mutants revealed that signaling and adhesion independently contribute to CD2 function.

Materials and Methods

Cell Lines, Cell Culture, mAbs, and Other Reagents. The anti-CD2 mAbs 9-1 (9), $T11_2$ and $T11_3$ (3), and the hamster anti–mouse CD3 ϵ chain mAb 145-2C11 (10) were the kind gifts of S. Y. Yang and B. Dupont (Memorial Sloan-Kettering, New York), E. L. Reinherz and S. F. Schlossman (Dana-Farber Cancer Institute, Boston, MA), and J. Bluestone (University of Chicago, Chicago, IL), respectively.

The generation of the murine hybridoma, By155.16, has been described (11). Hybridoma cell lines were grown in RPMI 1640 (Mediatech, Inc., Herndon, VA) supplemented as described (4, 6) and with either 10% heat-inactivated FCS (complete medium) or 10% iron-supplemented bovine serum (both from Sigma Chemical Co., St. Louis, MO). Daudi cells were grown in complete medium containing 20% FCS.

Generation of CD2 Mutants. CD2-S326A, CD2-N327A, CD2-N327D, and CD2-ΔT227 have been described (4, 6). The CD2 mutants 264DE, 262AADE, 260AAAADE, Δ260-65, and Δ274-279 were produced by site-directed mutagenesis using the Muta-Gene kit (Bio-Rad, Richmond, CA). The anti-sense oligonucleotides used for mutagenesis were: 264DE: 5'AGGTGCCTGGGACTCATCAC-CAGGTGGTGG 3'; 262AADE: 5'CTGGGACTCATCAGCAGC-TGGTGGAGGAGG 3'; 260AAAADE: 5'CTCATCAGCAGC-TGCTGCAGGAGGATGTTG 3'; Δ260-265: 5'AGGTGCCTG-GGAAGGAGGATGTTG 3'; Δ274-279: 5' CTGGTGCTGAAC-GGGACGATGACT 3'; Δ228-287: 5' AGCTACTCTGTGAGG-CCTTGTCTCCAGCTC 3'; Δ227-307: 5' TGGCTGAACTCG-AGGTGTCTCCAGCTCCTC 3'; Δ227-323: 5' TTAATTAGA-GGAAGGGGATGTCTCCAGCTCCTC 3'. Δ260+ Δ274 was produced by subjecting Δ260-265 to a second round of mutagenesis. Each mutant was subcloned into the pFNeo expression vector (12), and the nucleotides encoding the cytoplasmic domain were sequenced. Δ227-307 and Δ227-323 were generated using the method of Deng and Nickoloff (13). Δ228-287 was produced by introducing a StuI site in the cytoplasmic domain at position 228, digesting the resulting construct with StuI creating two DNA species, and closing the larger piece with T4 DNA ligase.

Transfection, Selection, and Analysis of IL-2 Production of CD2 Mutant-expressing Cell Lines. Wild type (CD2-XH14) and mutant CD2 constructs were introduced into By155.16, and G418-resistant cell lines were obtained as described (6, 12). For each CD2 mutant, at least four representative cell lines were selected based on CD2 and TCR surface expression (8), and functional testing was performed within 8 wk of isolation. Measurement of IL-2 production by T cell hybridomas was performed using the IL-2–dependent cell line CTLL-20 as described (8, 14).

The expression of mutated CD2 by transfected cell lines was confirmed by performing PCR using 1×10^6 cells lysed in 30 ml PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.35, 0.45% NP-40, 0.45% Tween-20) in the presence of 100 mg/ml proteinase K (Sigma Chemical Co.) for 1 h at 65°C. 5 ml of this reaction, an oligonucleotide primer corresponding to aa positions 191-198 of the CD2 transmembrane region (5' GGCATATGTG-GAGGAGGCAGCCTC 3'), and a primer (5' GGCTGAGTTC-GAAGATGCAGAGGT 3') corresponding to the 3' untranslated region of CD2 were amplified by PCR. The resulting DNA fragment was sequenced using the femtomole cycle sequencing kit (Promega Corp., Madison, WI).

Microtiter Plate Binding Assay. The binding of immunoaffinity purified CD58 (15) to polystyrene plates was confirmed by using purified anti-CD58 mAb TS2/9 in an ELISA assay specific for mouse Ig (Vector Labs., Inc., Burlingame, CA). Binding assays were performed as described (8, 14) and were repeated between three and seven times to confirm reproducibility.

Results

Production of CD2 Cytoplasmic Mutant-expressing Cell Lines. To identify the regions of the cytoplasmic domain required for CD2-mediated signaling and avidity regulation, we constructed a series of CD2 cytoplasmic mutants (Fig. 1). These CD2 mutants were subcloned into the expression vector pFNeo and introduced by electroporation into the HLA-DR-specific murine T cell hybridoma, By155.16 (11). Previous studies have revealed that transfection of human CD2 into this hybridoma results in cell lines that produce IL-2 in response to pairs of anti-CD2 mAbs and that show en-
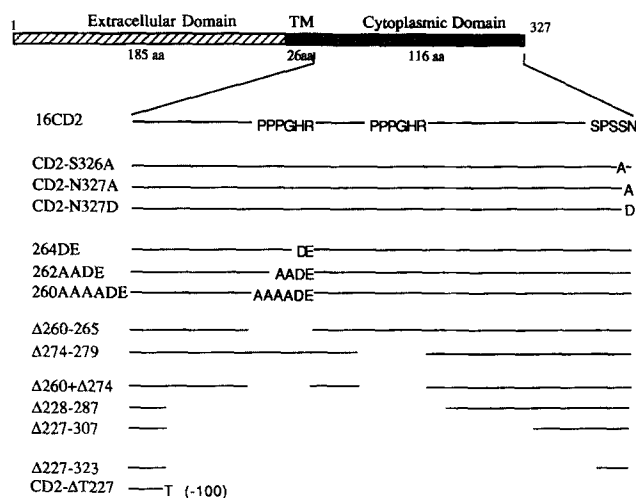

Figure 1. Schematic representation of CD2 cytoplasmic domain mutants. The wild type CD2 molecule (16CD2) is shown at the top. Single aa substitutions of CD2 are named for the wild type aa, its position in the CD2 sequence, and the substituted aa. Multiple aa substitutions are named by the position of the first substitution followed by the substituted aa. Internal deletions are named for the aa joined after deletion of the intervening aa.

hanced antigen responsiveness when cocultured with APC coexpressing HLA-DR, CD58, and CD59 (4, 6, 16). Cell lines expressing essentially equivalent levels of both the TCR and each mutated CD2 molecule were chosen for further analysis (data not shown). To confirm that each of these cell lines expressed the correct mutated form of CD2, the nucleotides encoding the cytoplasmic domain of CD2 were amplified by PCR and sequenced (data not shown).

Identification of a Cytoplasmic Segment Required for CD2-mediated IL-2 Production. Stimulation of each CD2 mutant-expressing cell line via the TCR-CD3 complex with immobilized anti-CD3 mAb resulted in the production of similar amounts of IL-2 (Fig. 2 a). In contrast, stimulation of these cell lines with a combination of anti-CD2 mAbs ($T11_2$ plus $T11_3$) revealed three levels of CD2-mediated responses. Cell lines expressing the wild type (CD2-XH) and COOH-terminal cytoplasmic point mutants of CD2 (CD2-S326A, CD2-N327A, and CD2-N327D) each produced comparable amounts of IL-2, 100–150-fold over background (Fig. 2 b). Similarly, cell lines (264DE, 262AADE, and 260AAA-ADE) expressing cytoplasmic aa substitution mutants of the first PPPGHR motif, previously suggested to be necessary for CD2 signaling (4, 15, 17–19), produced equivalent amounts of IL-2 as cell lines expressing wild type CD2 when matched for responsiveness to anti-CD3 mAb stimulation (Fig. 2 b). In contrast, deletion of either of the first (Δ260-265) or second (Δ274-279) PPPGHR motif resulted in a 10–30-fold reduction in IL-2 production upon anti-CD2 mAb treatment as compared to wild type CD2. Deletion of both repeats (Δ260+ Δ274) or larger internal deletions (Δ228-287, Δ227-307, and Δ227-323) resulted in a complete loss of CD2-initiated IL-2 production (Fig. 2 b). A similar pattern of re-
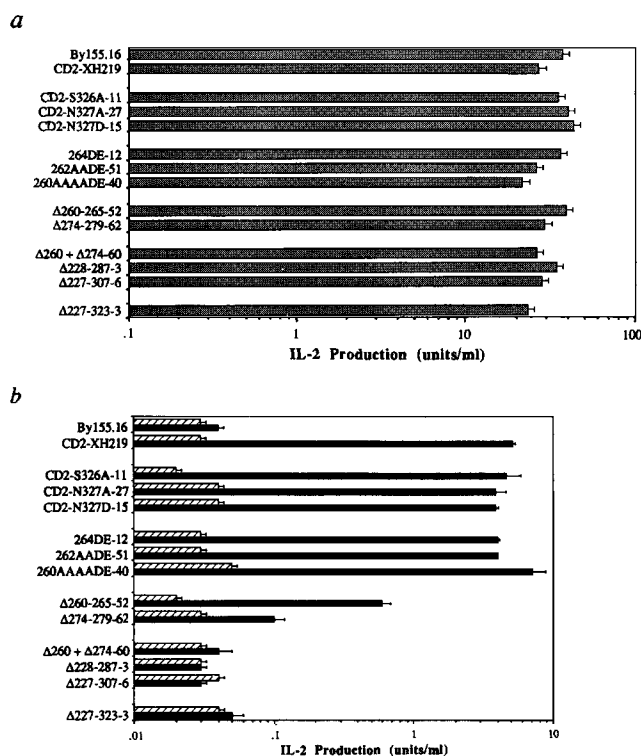
Figure 2. Stimulation of murine T cell hybridomas by anti-CD3 or anti-CD2 mAbs. (a) IL-2 production by murine T cell hybridomas after stimulation with plate-bound anti-CD3 mAb (145-2C11). 145-2C11 was plated at a concentration of 10 ng/ml. (b) IL-2 production of murine T cell hybridomas after stimulation with T11$_2$ and T11$_3$ (1:1,000 dilution of ascites). Data is reported as mean ± SE in U/ml of human rIL-2. In (a) incubation of cell lines in the absence of mAb resulted in the production of <0.05 U/ml of IL-2. The experiments shown are representative of at least two independent determinations. For each mutant-expressing cell line at least four independently derived cell lines were tested, each giving similar results.

Figure 3. Binding of murine hybridomas to CD58. Cell lines were stimulated with no stimulus (ϕ, striped bars), 10 μg/ml anti-CD3 mAb 145-2C11 (black bars), or 50 ng/ml PMA (white bars) for 10 min and plated onto immunoaffinity purified CD58. The percentage of cells specifically bound after 30 min is shown (mean ± SE for four independent experiments). Four to six cell lines of each CD2-mutant expressing cell line were tested.

sponsiveness was observed when these CD2 mutants were tested for their ability to increase intracellular cAMP levels after stimulation with an anti-CD2 mAb (data not shown). These observations demonstrate that CD2 signal transduction requires the integrity of at least one of the two PPPGHR cytoplasmic motifs located between aa residues 260 and 279.

The Cytoplasmic Portions Required for CD2-mediated Signaling and TCR-induced Regulation of CD2 Avidity Are Distinct. The ability of these CD2 mutants to bind immunoaffinity purified CD58 under resting and activated conditions was then determined using an in vitro binding assay. CD2⁻ hybridomas did not bind CD58 under any conditions (Fig. 3). In contrast, each CD2-expressing hybridoma bound CD58 equivalently with basal binding ranging between 6 and 12%. As reported previously (6), anti-CD3 mAb or PMA stimulation of hybridomas expressing wild type CD2 or a single aa substitution of CD2 at position 326 (CD2-S326A) resulted in a two- to threefold enhancement of binding to CD58 (Fig. 3). Single aa substitutions of the COOH-terminal asparagine (CD2-N327A or CD2-N327D) completely abrogated anti-CD3- or PMA-induced regulation of CD2 avidity, without
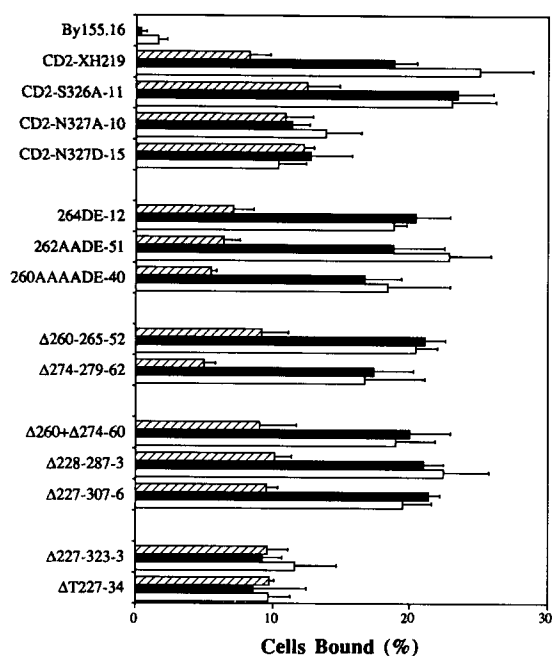
affecting the ability of these cell lines to signal via CD2 (Fig. 3 and Reference 6).

To determine whether CD2 avidity regulation was a separable function or was dependent upon CD2-initiated IL-2 production, we analyzed the ability of cell lines expressing CD2 signal transduction mutants for their ability to upregulate CD2 avidity. Stimulation of cell lines expressing the CD2 mutants lacking both PPPGHR motifs (Δ260+ Δ274), lacking the aa residues between 228 and 287 (Δ228-287), or lacking the aa between residues 227 and 307 (Δ227-307) resulted in an increase in CD2 adhesiveness comparable to cells expressing wild type CD2 (Fig. 3). These cell lines did not produce any IL-2 after stimulation with anti-CD2 mAbs (Fig. 2 b), but retained the ability to upregulate CD2 avidity after T cell activation, demonstrating that CD2-mediated signaling and the regulation of CD2 avidity were independent functions. Deletion of the cytoplasmic domain between residues 227 and 323 (Δ227-323) or deletion of the COOH-terminal 100 aa (CD2-ΔT227) completely abrogated the ability of cell lines not only to respond to anti-CD2 mAbs but also to upregulate CD2 avidity (Figs. 2 b and 3). These observations demonstrate that the cytoplasmic regions required for CD2 avidity regulation and CD2-mediated signal transduction are distinct and define a 21 aa region required for CD2 avidity regulation.

Avidity Regulation and Transmembrane Signaling Contribute Independently to CD2 Coreceptor Function. The interaction of CD2 with its ligands contributes significantly to the

ability of T lymphocytes to respond to antigen (4, 5, 20). Although specific for HLA-DR, the parental CD2⁻ hybridoma By155.16 responds poorly to stimulation by the HLA-DR⁺, CD58⁺ human B cell line Daudi in the absence of expression of a human coreceptor such as CD2 (6). To determine the contributions of CD2 avidity regulation and signaling to antigen responsiveness, cell lines expressing mutated CD2 molecules were stimulated with Daudi cells, and IL-2 production was measured. Expression of the wild type CD2 (CD2-XH219) or a single aa substitution of CD2 that does not affect either CD2 signaling or avidity regulation (CD2-S326A) resulted in a 40–50-fold enhancement of IL-2 production over the parental CD2⁻ hybridoma (Fig. 4), as described (6, 16).

Cell lines expressing CD2 mutants defective for both avidity regulation and CD2 signaling (e.g., Δ227-323; Fig. 4) enhanced IL-2 production only slightly, two- to threefold over the CD2⁻ hybridoma, confirming that basal binding of CD2 to its ligands contributes to coreceptor function (4, 20). Daudi stimulation of cell lines expressing single aa substitutions of CD2 that were unable to upregulate CD2 avidity (CD2-N327A or CD2-N327D) enhanced antigen responsiveness, but not as well as cell lines expressing wild type CD2 (Fig. 4). These cell lines retained the ability to initiate CD2-dependent signal transduction. Conversely, cell lines expressing CD2 mutants defective in CD2-mediated signaling (Δ228-287 or Δ227-307) but completely functional in their ability to regulate CD2 avidity also exhibited a partial ability to enhance IL-2 production (Fig. 4). Thus, the loss of either CD2 avidity regulation or signaling function both resulted in partial defects in CD2 coreceptor function. These observations define three components of CD2 function that each contribute

to antigen responsiveness: basal ligand binding, regulated ligand avidity, and signal transduction.

Discussion

This report identifies distinct portions of the CD2 cytoplasmic domain that separately regulate intracellular signaling and CD2 avidity for ligand. Basal binding of CD2 to its ligands enhances antigen responsiveness only modestly. Regulated CD2 avidity and CD2 signal transduction both participate in the T cell response to antigen. Thus, coreceptor-mediated adhesion and transmembrane signaling independently contribute to T cell activation.

The repeated motif PPPGHR is involved in coupling CD2 to intracellular signaling pathways. Deletion of either motif resulted in a partial defect in CD2 signaling (Fig. 2 b), consistent with a previous study involving a CD2 deletion mutant truncated between the two motifs (19). Although the substitution of His-Arg at aa 264/265 with Asp-Glu (264DE) has been reported to abrogate CD2-mediated signaling (19), we have observed that this mutant produces essentially equivalent amounts of IL-2 after stimulation with pairs of anti-CD2 mAbs when compared to wild type CD2 in over 50 cell lines derived from two independent transfections. In addition, the ability of the 262AADE and 260AAAADE mutants to respond to stimulation by a pair of anti-CD2 mAbs confirms the results obtained with the 264DE mutant. Simple substitutions within the first repeat (this report) or in the second repeat (19) are thus insufficient to abrogate CD2 signaling and, by inference, coupling to downstream elements.

CD2 signaling requires coexpression of the TCR (21). Recent studies have suggested that the cytoplasmic portion of the TCR ζ chain (22) plays an important role in CD2 signaling. In addition, CD2 has been shown to coprecipitate with a number of T cell molecules including CD3 ε, CD45, CD5, TCR ζ, p56ˡᶜᵏ, and p59ᶠʸⁿ (reviewed in reference 2). Interestingly, the repeated motif identified here as important for CD2 signaling resembles, but is not identical to, the proline-rich SH3 binding site (23).

Mutational analysis of the COOH-terminal portion of the CD2 molecule has identified a 21-aa segment required for TCR-induced regulation of CD2 avidity. Underscoring its evolutionary importance, this cytoplasmic segment retains the greatest degree of conservation among the human, rat, and mouse CD2 molecules (24). Since CD2 is not a substrate of PKC (25), TCR signaling or PMA treatment likely acts on intracellular molecules that, in turn, interact with this portion of the cytoplasmic domain to effect changes in CD2 avidity.

The mutational analysis of CD2 presented here identifies two distinct portions of the cytoplasmic domain required for CD2 avidity regulation and CD2 signaling. Since these cytoplasmic segments appear to function separately, these domains may interact with different intracellular proteins. A similar division of function has recently been reported for the platelet-derived growth factor receptor, in which separable cytoplasmic SH2 regions have been shown to bind either GAP or PI-3–kinase, and to couple this receptor to distinct sig-
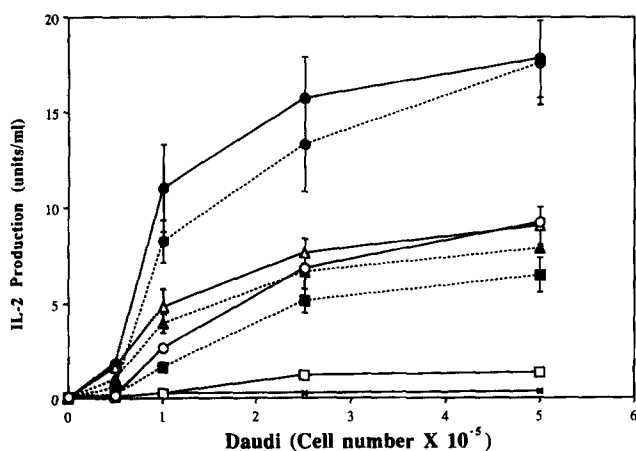

Figure 4. Antigen-specific response of murine T cell hybridomas. CD2⁻ or CD2-expressing murine T cell hybridomas were cocultured with irradiated Daudi cells for 24 h, and the resulting IL-2 quantified. Results are shown as the mean ± SE for each point and are representative of three independent experiments. Cell lines expressing wild type CD2 (—●—), CD2-S326A (···●···), CD2-N327A (—O—), CD2-N327D (—△—), Δ228-287 (···▲···), Δ227-307 (···■···), Δ227-323 (—□—), and no CD2 (X) are shown. For each mutant-expressing cell line, two to four independently derived cell lines were tested, each giving similar results.

naling pathways (26). Further mutagenesis of CD2 as well as overexpression of the cytoplasmic segments defined here may permit the identification of CD2 binding sites with molecules described to coassociate with CD2 as well as with other proteins.

The T cell hybridoma By155.16 requires coreceptor expression for optimal antigen responsiveness. Consequently, this cell line has facilitated comparison of the contributions of CD2 signaling and regulated adhesion to antigen-driven T cell activation. The analysis of CD2 mutants defective in signaling yet fully capable of regulated adhesion, demonstrates that increased CD2 adhesion augments a T cell response to antigen in the absence of CD2-mediated signal transduction. Conversely, CD2 signaling contributes to antigen responsiveness in the absence of CD2 avidity regulation. Both of these functions are required for optimal CD2 coreceptor function. Although these studies have focused on CD2 coreceptor function, it will be interesting to compare the molecular mechanisms of CD2 function with those of other T cell coreceptor molecules. Further analysis of the role of CD2 in T cell activation and adhesion will serve as a useful model system in the understanding of the molecular events involved in T cell activation.

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