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The Transmembrane E3 Ligase GRAIL Ubiquitinates and Degrades CD83 on CD4 T Cells

Leon L. Su, Hideyuki Iwai, Jack T. Lin, and C. Garrison Fathman

Ubiquitination of eukaryotic proteins regulates a broad range of cellular processes, including T cell activation and tolerance. We have previously demonstrated that GRAIL (gene related to anergy in lymphocytes), a transmembrane RING finger ubiquitin E3 ligase, initially described as induced during the induction of CD4 T cell anergy, is also expressed in resting CD4 T cells. In this study, we show that GRAIL can down-modulate the expression of CD83 (previously described as a cell surface marker for mature dendritic cells) on CD4 T cells. GRAIL-mediated down-modulation of CD83 is dependent on an intact GRAIL extracellular protease-associated domain and an enzymatically active cytosolic RING domain, and proceeds via the ubiquitin-dependent 26S proteasome pathway. Ubiquitin modification of lysine residues K168 and K183, but not K192, in the cytoplasmic domain of CD83 was shown to be necessary for GRAIL-mediated degradation of CD83. Reduced CD83 surface expression levels were seen both on anergized CD4 T cells and following GRAIL expression by retroviral transduction, whereas GRAIL knock-down by RNA interference in CD4 T cells resulted in elevated CD83 levels. Furthermore, CD83 expression on CD4 T cells contributes to T cell activation as a costimulatory molecule. This study supports the novel mechanism of ubiquitination by GRAIL, identifies CD83 as a substrate of GRAIL, and ascribes a role for CD83 in CD4 T cell activation. The Journal of Immunology, 2009, 183: 438–444.

Ubiquitination is an evolutionarily conserved process that covalently attaches polyubiquitin chains to target proteins. This modification can result in proteolytic degradation as well as nonproteolytic outcomes that regulate a broad range of critical cellular functions, including regulation of transcription and protein trafficking. Attachment of ubiquitin to target proteins occurs through a highly organized process involving the sequential actions of different classes of modifying enzymes (1). The first step in this process involves an ATP-dependent attachment of ubiquitin to the ubiquitin-activating enzyme (E1). Next, the thiol ester-linked ubiquitin is transferred from the E1 enzyme to a cysteine residue in an ubiquitin-conjugating enzyme (E2). In the last step of the conjugation process, the E2 enzyme, together with ubiquitin-protein ligase (E3) transfers ubiquitin to target proteins, where a stable isopeptide bond is formed between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. The E3 ligase is the central determinant of specificity in the substrate conjugation process; however, mapping specific target lysine sites or consensus ubiquitination motifs on target proteins has been a challenge.

GRAIL (gene related to anergy in lymphocytes; rnf128) was initially identified in a differential display screen of cDNA obtained from anergized T cell clones (2), and subsequent studies demonstrated that GRAIL was a critical element in the induction of T cell anergy in Ag-specific murine CD4+ T cell clones in vitro and in OVA-tolerized DO.11 mice in vivo (3). More recent studies have demonstrated that GRAIL is expressed in resting CD4 T cells and is degraded upon activation (4). Structure-function studies have characterized GRAIL as a type I transmembrane single subunit ubiquitin E3 ligase protein with a cytosolic zinc-binding RING finger domain and a luminal or extracellular protease-associated (PA) domain. In CD4 T cells, GRAIL has been shown to modulate expression of CD40L, a critical costimulatory molecule required for T cell activation (5), and to regulate the RhoA signaling pathway by ubiquitination of RhoGDI (6).

The ubiquitination process has been adopted by viruses to evade host antiviral immune responses. HSV-1 has been shown to infect human mature dendritic cells (DCs) by down-regulating the surface molecule CD83 via an ubiquitin-dependent 26S proteasome pathway (7). The degradation of CD83 was found to be dependent on the immediate-early gene product, ICP0 of HSV-1. ICP0 contains a RING finger domain highly homologous to that of GRAIL. Thus we asked whether CD83 might serve as a substrate for GRAIL.

Materials and Methods

Cell culture, plasmids, and transfections

HEK293 cells were cultured in DMEM supplemented with 10% FCS and antibiotics (Invitrogen). Wild-type murine CD83 cDNA, provided by A. Steinkasserer (University Hospital Erlangen, Erlangen, Germany), was subcloned into the pEF1-mycHis plasmid (Invitrogen). CD83 point mutants were generated using QuickChange Site-Directed Mutagenesis Kit (Stratagene). Murine GRAIL (rnf128) and its mutants were cloned into a bicistronic internal ribosome entry site (IRES) enhanced GFP (eGFP) vector. All transfections were performed using Lipofectamine 2000 (Invitrogen).

Western blot and immunoprecipitation analysis

Whole cell extracts were prepared by washing the cells with ice-cold PBS, then resuspending the pellet in ice-cold lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% Brij96, 1 mM EDTA, 1 mM NaF, 1 mM β-glycerophosphate with freshly added protease inhibitor mixture, and...
Results

GRAIL down-modulation of CD83 requires intact GRAIL PA and RING domains

The RING domain of ubiquitin E3 ligases is a specialized type of zinc finger of 40 to 60 residues known to bind two atoms of zinc. This domain is defined by the cross-brace motif of C-X3-C-X9–34-C-X1–37-H-X2–37-(N/C/H)–X2–C-X4–48-C-X3–C. The similarity in RING domain sequence of ICP0 and GRAIL is shown in Fig 1A, in which the conserved cysteine or histidine residues shared between ICP0 and GRAIL that form the zinc-binding sites are boxed. The RING domain of GRAIL forms a C3H2C3-type (RING-H2 finger), a slight variation of the C3HC4-type of RING found in ICP0.

GRAIL is a type I transmembrane protein that contains an extracellular or luminal PA domain that is separated from the cytosolic RING finger domain by a membrane-spanning sequence. Initially, to determine whether GRAIL could modulate CD83 expression, we transiently expressed CD83 with wild-type GRAIL in non-CD83-expressing HEK293 cells using a bicistronic vector with eGFP under control of an IRES. Transfection with the empty vector, encoding only eGFP, was used as a control. FACS analysis 36 h post-transfection showed a reduction in CD83 surface expression in GRAIL-eGFP positive cells compared with transfected vector control cells (Fig 1B). We found that the decrease in CD83 surface expression was dependent on a functional GRAIL-RING
domain, as an enzymatically inactive mutant of GRAIL bearing two histidine to arginine substitutions (H2N2) in the RING domain abrogated the reduction in CD83 expression. Similar to the H2N2 RING mutant, a GRAIL mutant lacking the N terminus PA domain (ΔPA) was also unable to down-modulate CD83 levels in HEK293 cells.

We next asked if the down-modulation of CD83 surface expression was a consequence of decreased protein expression, and did not represent a defect in CD83 transport to the cell surface or retention of CD83 in cytoplasmic vesicles. In the presence of wild-type V5 epitope-tagged GRAIL, the protein level of Myc-tagged CD83 was determined by immunoprecipitation (ip) with anti-V5 Ab, followed by Western blot (wb) probed with anti-His Ab (top left), or immunoprecipitation with anti-myc Ab, followed by Western blot probed with anti-V5 Ab (top right). Blots were reprobed with appropriate Ab to show presence of immunoprecipitated protein (bottom).

CD83 is a GRAIL E3 substrate

**FIGURE 2.** N-terminal PA domain of GRAIL facilitates CD83 binding. A, HEK293 cells were transfected with CD83 myc/His-tagged vector (1.0 μg) along with wild-type and mutant GRAIL-V5-tagged vectors (1.0 μg each). Thirty-six hours post-transfection, interaction between GRAIL and CD83 was determined by immunoprecipitation (ip) with anti-V5 Ab, followed by Western blot (wb) probed with anti-His Ab (top left), or immunoprecipitation with anti-myc Ab, followed by Western blot probed with anti-V5 Ab (top right). Blots were reprobed with appropriate Ab to show presence of immunoprecipitated protein (bottom). B, HEK293 cells were transfected with 0.5 μg of ICOS-myc-tagged plasmid, along with vector or GRAIL-V5 tagged plasmid (1.0 μg each). Protein levels were analyzed as described in Fig. 1C. V, Vector; GR, GRAIL; H2, H2N2 GRAIL; ΔPA, N-terminal PA deletion.

**FIGURE 3.** GRAIL-mediated degradation of CD83 proceeds via an ubiquitin-mediated process. HEK293 cells were transfected with 0.5 μg of CD83-myc, 0.5 μg of Flag-Ubiquitin, and either 1.0 μg of vector, GRAIL, or H2N2 plasmid, as indicated. Before lysis preparation, cells were either left untreated or treated with MG132 (25 μM) for 2 h. To eliminate the possibility of protein coprecipitation in the samples, lysates were boiled in lysis buffer containing 1% SDS for 5 min before a 10-fold dilution and subsequent immunoprecipitation (ip) using anti-myc Abs. The blot was probed with anti-Flag-HRP to detect the polyubiquitinated CD83 species (top). The blot was reprobed with anti-myc to show CD83 immunoprecipitation levels (bottom). wb, Western blot; ip, immunoprecipitation.

Though equivalent amounts of GRAIL vectors were used for transient expression, the auto-ubiquitinating property of GRAIL resulted in a smaller pool of GRAIL compared with H2N2 (Fig 1C, lane 2). Blots were reprobed for cyclophilin-B (CypB) was used as an internal loading control. C, HEK293 cells were transfected with either CD83 bearing double (K30R), TM, Transmembrane; V, vector; GR, GRAIL.

To further explore the interaction of GRAIL with CD83, Myc/His-tagged CD83 was cotransfected with either full-length, H2N2, or ΔPA GRAIL. Immunoprecipitation studies demonstrated that while full-length and H2N2 GRAIL can both bind CD83, immunoprecipitation of ΔPA GRAIL via its V5 tag did not coprecipitate CD83 (Fig 2A, lane 4). Reciprocally, immunoprecipitation of CD83 via its Myc epitope tag coprecipitated full-length and H2N2 GRAIL (Fig 2A, lanes 6 and 7, respectively), but did not coprecipitate ΔPA GRAIL (Fig 2A, lane 8). These data show that the extracellular PA domain of GRAIL serves as a protein-protein interaction domain and is necessary to facilitate substrate binding to allow the cytosolic or luminal domain of GRAIL to transfer ubiquitin to its substrate.
represents a usual mechanism of E3 ligase substrate capture and recognition in which a single subunit E3 ligase contains a functional recognition domain separated from the catalytic E3 ligase domain by an intervening transmembrane sequence.

A pair of cysteine residues in the extracellular domain of CD83 is in position to permit disulfide bond formation that defines an Ig-like V-set domain, similar to those found in other costimulatory molecules, such as ICOS, CD28, and CTLA-4. We asked whether ICOS, a costimulatory molecule with a similar Ig-like domain, could serve as a target of GRAIL down-modulation. Though ICOS has structural framework similar to CD83, a significant difference between Myc epitope-tagged ICOS protein levels in the presence or absence of wild-type GRAIL (Fig 2B) was not observed. These data imply that regions in addition to the Ig-like domain may be necessary to confer specific GRAIL-substrate interaction. In support of this possibility, crystal structure analysis of CD28 family members (including ICOS) reveals a common ligand binding loop of MYPPPYY (AA 99–104) (8) that is lacking in the extracellular region of CD83.

Anergized CD4 T cells display reduced surface expression of CD83. Ionomycin-anergized CD4 T cells were prepared as described in Fig 3, lane 4, and the H2N2 RING mutant (Fig 3, lane 6). From these results, we conclude that GRAIL down-modulates CD83 via the ubiquitin-dependent 26S proteosome degradation pathway.

CD83 protein degradation proceeds via the 26S proteosome

We next asked whether CD83 is a substrate for GRAIL-mediated ubiquitination. Myc-tagged CD83, together with either vector control, wild-type GRAIL, or the H2N2 GRAIL mutant, were co-expressed in HEK293 cells along with Flag epitope-tagged ubiquitin. To observe the accumulation of ubiquitinated proteins, MG132, a specific 26S proteasome inhibitor, was added for 2 h before cell harvest. Following denaturing immunoprecipitation of epitope-tagged CD83 and probing with anti-Flag Ab, we observed distinctive m.w. laddering in the presence of wild-type GRAIL, indicative of polyubiquitin chain formation (Fig 3, lane 5). The laddering effect was markedly diminished in the presence of vector control (Fig 3, lane 4) and the H2N2 RING mutant (Fig 3, lane 6).

By FACS analysis of CD83 surface expression on transduced GFP+ cells we evaluated by FACS analysis at 24 h post-transduction. Percentage of CD83 surface expression on GFP+ cells, denoted by gated region: V, 25.0%; GR, 20.2%; H2, 26.0%. D. Percentage of CD83 surface expression on GRAIL and H2N2-transduced cells, normalized to vector control. Average of three independent experiments. E. Ionomycin-anergized CD4 T cells display increased GRAIL expression. CD4 T cells were prepared as described in A, then either left untreated or treated with ionomycin (1 μM) for 18 h. Lysates were prepared and subjected to SDS-PAGE. Blots were probed with polyclonal GRAIL anti-serum (top) and reprobed with anti-actin (bottom) to ensure equal loading. F. Anergized CD4 T cells display reduced surface expression of CD83. Ionomycin-anergized CD4 T cells were prepared as described in E, then subjected to plate-bound anti-CD3 (0.5 μg/ml) and soluble anti-CD28 (0.5 μg/ml) stimulation for 24 h and CD83 levels were evaluated by FACS. (Stimulated non-ionomycin treated = activated. MFI, 31.5; stimulated ionomycin treated = anergized. MFI, 22.3; isotype control, MFI, 8.8). G. GRAIL RNAi results in increased CD83 expression levels. CD4 T cells were electroporated with GRAIL shRNA vectors (GR RNAi no.1, GR RNAi no.2) or pSiren Luciferase control. Twenty-four hours post-electroporation, cells were either left unstimulated (left) or stimulated with plate-bound anti-CD3 (0.5 μg/ml) and soluble anti-CD28 (0.5 μg/ml) for 24 h (right), then CD83 expression on ZsGreen+ cells were determined by FACS. Percentage of CD83 surface expression on ZsGreen+ cells, denoted by gated region: unstimulated (left): Ctrl, 15.4%; RNAi no.1, 36.0%; RNAi no. 2, 31.1%; stimulated (right): Ctrl, 41.1%; RNAi no. 1, 65.5%; RNAi no. 2. 55.6%.
When coexpressed in HEK293 cells, wild-type GRAIL was able to degrade each of the single lysine to arginine (K → R) CD83 point mutants (Fig 4B), suggesting that there is either no discrimination between the lysine used for GRAIL-mediated ubiquitination, or alternatively, the availability of any two of the three lysines is sufficient for polyubiquitination. To distinguish between these two possibilities, we generated CD83 point mutants bearing double lysine to arginine substitutions. When these mutants were transiently expressed with wild-type GRAIL, we observed degradation of the CD83 mutants bearing the K168/192R or K183/192R point mutations (Fig 4C, lanes 2 and 4, respectively). However, the K168/183R CD83 point mutant was resistant to GRAIL-mediated degradation (Fig 4C, lane 6), indicating a preference for either the K168 or K183 lysine for ubiquitin attachment by GRAIL, while the transmembrane distal lysine residue (K192) is dispensable for ubiquitin-mediated degradation. Finally, we generated the triple lysine to arginine CD83 mutant to confirm that no other residues were involved in GRAIL-mediated degradation of CD83. Consistent with the model, wild-type GRAIL was unable to degrade the K168/183/192R CD83 triple mutant (Fig 4C, lane 8).

**GRAIL down-modulation of CD83 in CD4 T cells**

GRAIL originally was identified to be up-regulated in a differential display of anergic T cell clones; however, endogenous GRAIL has recently been reported to be expressed in resting CD4+ effector T cells (4). In this study, we demonstrate that expression of this endogenous pool of GRAIL protein diminishes upon in vitro TCR activation (anti-CD3/anti-CD28), presumably via auto-ubiquitination and degradation (Fig 5A).

Although CD83 is best studied as a cell surface marker for mature DCs, CD83 has been reportedly expressed on a subset of activated T and B cells (9). To confirm this report, resting CD4 T cells were subjected to TCR activation for the indicated times and stained for inducible CD83 surface expression (Fig 5B). Because CD83 expression inversely correlated with GRAIL protein, we asked whether ectopic expression of GRAIL in CD4 T cells could modulate the expression of CD83. Using a myeloproliferative sarcoma virus-based retroviral vector (pMP71) encoding either wild-type GRAIL or H2N2 GRAIL together with eGFP under control of an IRES, CD4 T cells were transduced. CD83 levels on GFP+ cells were evaluated by FACS analysis 24 h post-transduction.
Following GRAIL transduction, 20% of the CD4 T cells expressed high levels of CD83, compared with 25% and 26% of the vector and H2N2-transduced T cells, respectively (Fig 5C). The percentage of retroviral transduced CD83-expressing cells, normalized to the vector control, is shown in Fig 5D. Hence, the presence of GRAIL reduced the surface levels of CD83 by degrading CD83 in a RING domain-dependent manner. GRAIL and H2N2 transduction of CD4 T cells was verified by immunoblot (data not shown).

Next, we wished to determine whether modulating endogenous levels of GRAIL can affect the levels of CD83 expression on T cells. Sustained calcium mobilization has been shown to induce anergy and increase GRAIL levels in CD4 T cells (10, 11). Increased GRAIL expression in ionomycin (1 μM) treated cells was verified by immunoblot (Fig 5E). To determine whether this method of induced T cell unresponsiveness influences CD83 expression levels, CD4 T cells were treated with ionomycin (1 μM) for 18 h before TCR stimulation. Cells treated with ionomycin displayed reduced CD83 surface expression compared with TCR-stimulated non-ionomycin-treated cells (Fig 5F). To further investigate the regulation of CD83 by GRAIL, CD4 T cells were subjected to GRAIL knockdown by RNAi. CD83 levels dramatically increased on GRAIL RNAi-treated ZsGreen+ cells (RNAi no. 1: 36%; RNAi no. 2: 31%) compared with ZsGreen-containing control (Ctrl) vector electroporated cells (Ctrl: 15%) (Fig 5G, left). This difference in CD83 levels is also observed upon TCR stimulation (Ctrl: 41%; RNAi no. 1: 65%; RNAi no. 2: 55%) (Fig 5G, right). These data indicate that CD83 does indeed serve as a target of GRAIL degradation in CD4 T cells. CD83 expression on CD4 T cells is important for T cell proliferation and function.

We used RNAi technology to assess the functional importance of CD83 expression in peripheral CD4 T cells. The mRNA CD83 sequences targeted by the designed sense/antisense RNAi oligonucleotides are listed in Fig. 6A. CD4 T cells were isolated from DO11.10 OVA-specific TCR transgenic mice and electroporated with either a Luciferase ZsGreen-containing vector control-transduced T cells (RNAi no. 1: 65%; RNAi no. 2: 55%) (Fig 5G, right). These data indicate that CD83 does indeed serve as a target of GRAIL degradation in CD4 T cells. CD83 expression on CD4 T cells is important for T cell proliferation and function.

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preferential target substrate sites is still unresolved, but may depend on orientation or topology of substrate binding or association of other accessory molecules, such as OTU-1 and USP8 (15).

CD83 was originally defined as a specific marker for activated DCs, and has been described as mediating intercellular interaction between DCs and T cells. Subsequent studies have shown CD83 up-regulation in subsets of hematopoietic cells, including naturally occurring CD4+CD25+ regulatory T cells (16), activated B cells, a subset of activated NK cells, and activated macrophages (9, 16–18). In this study, we show robust up-regulated expression of CD83, correlating inversely with GRAIL levels, on murine CD4 T cells following TCR engagement. Forced ectopic GRAIL expression, as well as induced GRAIL expression by ionomycin treatment, resulted in diminished CD83 levels, whereas GRAIL knockdown in murine CD4 T cells resulted in increased CD83 surface expression. These data conclusively demonstrate GRAIL-mediated regulation of CD83 expression on CD4 T cells.

In vivo mouse models have provided evidence of a vital role for CD83 function in regulating T cell development and modulating immune responses. CD83−/− mice displayed impaired T cell development and reduced numbers of peripheral CD4+ T cells (19). The requirement for CD83 on thymic epithelial cells for proper thymic selection and egress from the thymus may explain these observations. Alternatively, T cells from the CD83−/− mice may harbor T cell-intrinsic defects that affect development, as these cells exhibit decreased cell survival in an adoptive transfer model (9). Our data corroborate the notion that CD83 is functionally relevant on peripheral CD4 T cells. By specifically knocking down CD83 in peripheral CD4 T cells, we show in an Ag-specific coculture system that impaired CD83 expression results in decreased IL-2 production and reduced proliferative capacity of these cells. These data are consistent with previous reports that T cells isolated from CD83 transgenic mice exhibited an activated phenotype, producing increased amounts of IL-2 (20). Interestingly, in our studies, reduction of CD83 expression on T cells also resulted in a significant reduction of IL-17 production. A soluble form of CD83 has been shown to inhibit Ag-specific T cell activation and prevent murine experimental autoimmune encephalomyelitis (21), presumably by interfering with the CD83:CD83 ligand interaction. Because IL-17 has been found to drive differentiation of pathogenic Th-17 cells in experimental autoimmune encephalomyelitis, CD83 may serve as an attractive target for development of disease intervention therapy. In conclusion, these observations illustrate the importance of CD83 in APC: T interactions, and suggest that CD83 may represent a novel molecular target for GRAIL function in peripheral CD4 T cells.

Disclosures

The authors have no financial conflict of interest.

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