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Stress-Regulated Targeting of the NKG2D Ligand Mult1 by a Membrane-Associated RING-CH Family E3 Ligase

Timothy J. Nice, Weiwon Deng, Laurent Cos coy, and David H. Raulet

NKG2D is a stimulatory receptor expressed by NK cells and some T cell subsets. Expression of the self-encoded ligands for NKG2D is presumably tightly regulated to prevent autoimmune disorders while allowing detection of infected cells and developing tumors. The NKG2D ligand Mult1 is regulated at multiple levels, with a final layer of regulation controlling protein stability. In this article, we report that Mult1 cell-surface expression was prevented by two closely related E3 ubiquitin ligases membrane-associated RING-CH (MARCH)4 and MARCH9, members of an E3 family that regulates other immunologically active proteins. Lysines within the cytoplasmic domain of Mult1 were essential for this repression by MARCH4 or MARCH9. Downregulation of Mult1 by MARCH9 was reversed by heat-shock treatment, which resulted in the dissociation of the two proteins and increased the amount of Mult1 at the cell surface. These results identify Mult1 as a target for the MARCH family of E3 ligases and show that induction of Mult1 in response to heat shock is due to regulated association with its E3 ligases. The Journal of Immunology, 2010, 185: 5369-5376.

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Abbreviations used in this paper: C-M4, C1498-MARCH4 cells; C-M9, C1498-MARCH9 cells; C-MARCH9; C-MARCH19; C-MARCH19 cells transduced with mouse MARCH9; ctrl, untreated; HA, hemagglutinin; HS, heat shock; LAK, lymphokine activated killer; M9, MARCH9; MARCH, membrane-associated RING-CH; siRNA, small interfering RNA; TM, transmembrane; WT, wild-type.

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Mult1 expression at the cell surface in a manner that depends on lysine residues in the Mult1 cytoplasmic domain and that can be reversed by heat shocking the cells.

Materials and Methods

Cells and heat shock

All cells were cultured in complete DMEM, consisting of DMEM (Life Technologies, Carlsbad, CA), 10% FCS (Omega Scientific, Tarzana, CA), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 0.2 mg/ml glutamine (Sigma-Aldrich, St. Louis, MO), 10 μg/ml gentamicin sulfate (Lonza, Basel, Switzerland), 20 mM HEPES (Fisher BioReagents, Fair Lawn, NJ), and 50 μM 2-ME (EMD Chemicals, Gibbstown, NJ). Heat shock was performed by placing cells in a 45°C water bath for 30 min, followed by a 4-h incubation at 37°C. Dead cells were excluded from flow cytometry analysis through staining with 7-aminoactinomycin D.

Cytotoxicity assay

Lymphokine-activated killer (LAK) cells were prepared by culturing splenocytes for 4–5 d in 1000 U/ml IL-2. LAK cells were cocultured with 51Cr-labeled target cells in a standard 4-h [51Cr]-release assay (20).

Abs

Mult1 Ab (clone 237104; R&D Systems, Minneapolis, MN), H60a Ab (clone 205326; R&D Systems), hemagglutinin (HA) Ab (HA.11; Covance, Princeton, NJ), PE-conjugated goat F(ab′)2 fragment to rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA), PE-conjugated goat Ab to mouse IgG1 (Southern BioTechnology Associates, Birmingham, AL), PE-conjugated MHC class I (H-2Dβ) Ab (clone 28-14-8; eBioscience), and PE-conjugated MHC class I (H-2Dβ) Ab (clone 28-14-8; eBioscience, San Diego, CA), PE-conjugated H-2Kb Ab (clone AF6-88.5; eBioscience), and PE-conjugated goat Ab (clone 205326; R&D Systems), hemagglutinin (HA) Ab (HA.11; Covance, Princeton, NJ), PE-conjugated goat F(ab′)2 fragment to rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA), PE-conjugated goat Ab to mouse IgG1 (Southern BioTechnology Associates, Birmingham, AL), PE-conjugated MHC class I (H-2Dβ) Ab (clone 28-14-8; eBioscience, San Diego, CA), PE-conjugated H-2Kb Ab (clone AF6-88.5; eBioscience), and PE-conjugated ICAM-1 (clone YN1/1.7.4; eBioscience) were used for flow-cytometric analysis. Mult1 Ab (clone 1D6; kind gift of S. Jonic, University of Rijeka, Rijeka, Croatia) was used for immunoprecipitation and Western blotting. Ubiquitin Ab (clone P4D1; Santa Cruz Biotechnology, Santa Cruz, CA), α-tubulin Ab (Calbiochem, San Diego, CA), myc Ab (clone 9E10; eBioscience), HA Ab (HA.11; Covance), HRP-conjugated goat anti-mouse IgG (Pierce, Rockford, IL), and peroxidase-conjugated goat anti-mouse L chain (Jackson Immunoresearch Laboratories) were used for Western blots.

Cloning of MARCH cDNA

The human MARCH proteins were amplified from EST clones purchased from the American Type Culture Collection (Manassas, VA) and subcloned into pcDNA-HA. Mouse MARCH4 and MARCH9 were amplified from mouse fibroblast cDNA using the following primers: BamHI-M4F, 5′-CTGAACCATCACAACGGCATG-3′, and HindIII-M9R3, 5′-AGCAGCTCTACTGGAGTCACC-3′. The human MARCH proteins were the same MARCH proteins reported to downregulate MHC class I molecules (9).

Quantitative RT-PCR

Total RNA was isolated using TRIzol LS (Invitrogen, Carlsbad, CA), followed by digestion of contaminating DNA using DNA-free (Ambion, Austin, TX), according to the manufacturer’s protocol. RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and the resulting cDNA was used for quantitative PCR. Duplicate or triplicate amplification mixtures were prepared with 0.01–1 μg cDNA, SYBR GreenER SuperMix (Invitrogen), and 200 nM forward and reverse primers and cycled using the ABI 7300 real-time PCR system. Cycling parameters used were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. The following primers were used: Mult1-5′, 5′-CAATGTCTCTGTCCTCGGAA-3′; Mult1-3′, 5′-CTGAACATGCTCTGGAC-3′; MARCH4-5′, 5′-CTCGGAAGTACCCAGTGGAC-3′; MARCH4-3′, 5′-CTCGGAAGTACCCAGTGGAC-3′; MARCH9-5′, 5′-ATGGACGGTGAACGGTA-3′; MARCH9-3′, 5′-ATGGACGGTGAACGGTA-3′. Relative levels of MARCH4 and MARCH9 were determined using the relative quantitation method.

Retroviral constructs and transduction of cells

FLAG or HA-tagged Mult1 was cloned into the pMSCV2.2-ires-GFP retroviral vector (22). Myc-tagged MARCH4 and MARCH9 were subcloned into pMSCV-ires-Thy1.1. Mutants of Mult1 were made, as described (7). Retroviral supernatants were generated by cotransfecting 293T cells with plasmids encoding VSV gag/pol and env and pMSCV retroviral constructs using Lipofectamine 2000 (Invitrogen). Culture supernatants collected 48 h after transfection were added directly to actively proliferating cells, and transduced cells were sorted based on GFP or Thy1.1 expression.

Immunoprecipitation and Western blotting

Lysates were prepared by resuspending cell pellets in commounoprecipitation buffer (0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris pH 7.4, 10% glycerol). Protease inhibitor mixture tablets (Roche, Basel, Switzerland) were added to the buffer immediately before use. When assessing ubiquitination of Mult1, 200 nM n-ethylmaleimide was included in the lysis buffer. Cells were lysed at 4°C for 30 min and cleared by centrifugation at 16,000 × g for 15 min. Lysates were precomplexed with protein G PLUS agarose beads (Santa Cruz Biotechnology). Precomplex lysates were incubated with 1 μg Mult1 Ab (ID6) for 1 h, followed by incubation with protein G agarose beads overnight. Beads were washed three times in lysis buffer and boiled in SDS sample buffer for separation by SDS-PAGE. Samples resolved by SDS-PAGE were transferred to nitrocellulose membranes. Membranes were blocked in 5% milk and incubated with primary Abs, followed by HRP-coupled secondary Abs. Following incubation with Western lightning chemiluminescence reagent (PerkinElmer, Wellesley, MA), membranes were exposed to film and developed.

Transfection of small interfering RNA oligonucleotides

Relative levels of MARCH4 and MARCH9 were determined using the primers MARCH4-9.5′, 5′-CAGTGCGACGCCCCAT-3′ and MARCH4-9.3′, 5′-ACTTCTCAGGCTGT-3′, followed by digestion with Sall, which selectively digests MARCH4 amplicons. Fifty thousand fibroblasts were transfected with 100 pmol small interfering RNA (siRNA) oligonucleotides using Lipofectamine 2000. The following oligonucleotides were used: GFP-22 (Qiagen, Valencia, CA), control oligonucleotide (Invitrogen), and three presynthesized MARCH9 oligonucleotides (Invitrogen). Seventy-two hours later, cells were lifted and used for analysis of Mult1 surface expression and MARCH9 quantitative PCR.

Results

Screen of human MARCH proteins identifies MARCH4 and MARCH9 as Mult1 E3 ligases

The nine mammalian MARCH proteins that were originally described can be distinguished based on the number of TM domains they possess. MARCH1, MARCH2, MARCH3, MARCH4, MARCH8, and MARCH9 have two TM and are most closely related to the viral homologs. Plasmids encoding HA-Tagged versions of these two-TM MARCH proteins cloned from human cDNA were used to screen for the ability to regulate Mult1 expression. 293T cells were transiently cotransfected with individual human MARCH plasmids or control plasmid, together with a plasmid encoding Mult1 and IRES-GFP. Cells were stained 24 h later with Mult1 Abs, and transfected cells were selectively analyzed by gating on GFP-expressing cells. Strikingly, cotransfection of Mult1 with either of two highly related family members (human MARCH4 and human MARCH9) prevented Mult1 cell-surface expression (Fig. 1A). This effect was specific to human MARCH4 and human MARCH9, because no other MARCH family members that were tested (MARCH6, MARCH7, MARCH10 and MARCH11 were not examined) caused notable variations in Mult1 expression compared with control cells. Interestingly, these two proteins were the same MARCH proteins reported to downregulate MHC class I molecules (9).

To determine the role of the Mult1 cytoplasmic domain in MARCH9-mediated downregulation, 293T cells were cotransfected with Mult1 cytoplasmic domain mutants and human MARCH9 (Fig. 1B). Human MARCH9 strongly inhibited cell-
results were obtained in two additional experiments. Lysed in coimmunoprecipitation buffer, followed by immunoprecipitation of Mult1 and detection of FLAG, HA, or ubiquitin by Western blotting. Similar indicating comparable Mult1 gene expression. Shaded graphs represent staining of cells transfected with a plasmid encoding IRES-GFP only.

Expression of GFP, which is encoded by the same bicistronic mRNA as Mult1, was similar in all of the samples, indicating comparable Mult1 gene expression. Shaded graphs represent staining of cells transfected with a plasmid encoding IRES-GFP only. C. Cells were lysed in coimmunoprecipitation buffer, followed by immunoprecipitation of Mult1 and detection of FLAG, HA, or ubiquitin by Western blotting. Similar results were obtained in two additional experiments.

surface expression of a Mult1 truncation mutant lacking aa 253–334 (the Δ239 mutant), showing that most of the cytoplasmic domain, containing four cytoplasmic lysine residues (K278, K280, K310, and K311), was not necessary for MARCH9-mediated downregulation. In contrast, human MARCH9 cotransfection did not impair cell-surface expression of a Mult1 truncation mutant (the Δ239 mutant) lacking a slightly larger segment, comprising amino acids 239–334, including two additional cytoplasmic lysine residues (K240 and K241) compared with the Δ253 mutant. The role of the cytoplasmic lysine residues was confirmed by the demonstration that the KR mutant, a full-length Mult1 in which six cytoplasmic lysine residues were substituted with arginine residues, was only slightly downregulated by human MARCH9. The marginal downregulation of the KR mutant by human MARCH9 was reproducible, however, suggesting that nonlysine residues in the cytoplasmic domain may be suboptimal targets for MARCH9-mediated ubiquitination, as was demonstrated in the case of ubiquitination targeted by the other MARCH family proteins, MIR1, MK3, and MARCH2 (23, 24). Together, these results show that the Mult1 cytoplasmic domain is critical for downregulation by MARCH9 and that lysines within this domain are required for this regulation.

The requirement for lysine residues within the cytoplasmic domain strongly suggested that the downregulation of Mult1 by MARCH9 involved ubiquitination. To confirm that Mult1 is ubiquitinated by MARCH4 and MARCH9, Mult1 was immunoprecipitated from transfected 293T cells, followed by detection of ubiquitin by Western blotting. High m.w. ubiquitin species were evident in immunoprecipitates from cells cotransfected with human MARCH4 or human MARCH9 and wild-type (WT) Mult1 but were notably less abundant when the KR or Δ239 Mut Mult1 mutants were expressed (Fig. 1C). The fact that these ubiquitinated species were dependent on lysines in the Mult1 cytoplasmic domain, were absent in the vector-transfected cells, and were larger than Mult1 (55 kDa), strongly suggested that they represent ubiquitinylated Mult1. Surprisingly, although WT but not KR Mult1 was ubiquitinated and depleted from the cell surface in the presence of human MARCH4/9, there was no apparent decrease in total WT Mult1 protein levels seen by Western blot (Fig. 1C). This finding suggests that the downregulation of Mult1 in the presence of MARCH4/9 in 293T cells is due to sequestration in an intracellular compartment.

It remained possible that the effect of MARCH4/9 on Mult1 expression was indirect, so coimmunoprecipitation of the two proteins was performed to measure the extent of their physical interaction. Mult1 was immunoprecipitated from cotransfected cells, followed by Western blotting to detect HA-tagged human MARCH proteins (Fig. 1C). Human MARCH4 and human MARCH9 were coimmunoprecipitated with Mult1, showing that a physical interaction does occur. The interaction of Mult1 with the human MARCH proteins did not depend on the presence of lysines in the Mult1 cytoplasmic domain, because human MARCH4 and human MARCH9 were coimmunoprecipitated with the KR mutant to the same extent as with WT Mult1. In contrast, human MARCH4/9 failed to coimmunoprecipitate appreciably with the Δ239 mutant of Mult1, indicating that the cytoplasmic domain, independent of the cytoplasmic lysine residues, might be important for allowing interaction with MARCH4 or MARCH9. Together, these data show that MARCH4 and MARCH9 interact with Mult1 in a manner dependent on the Mult1 cytoplasmic domain, leading to its ubiquitination and reduced surface expression.

Mouse MARCH4/9 downregulate endogenous Mult1

Although the MARCH proteins are highly conserved between humans and mice, it was important to verify the results obtained with the human proteins using mouse MARCH4/9 in a more physiological experimental setting. Mouse MARCH4/9 were amplified from cDNA, N-terminally tagged with the myc epitope, and cloned into a MSCV retroviral expression vector. Retroviral transduction was used because it allowed stable ectopic expression at lower levels than transient transfection, thus minimizing potential nonspecific effects of forced expression. Two cell lines (C1498 and WEHI 7.1) were chosen for ectopic expression of mouse MARCH4/9 because they express endogenous Mult1.

FIGURE 1. Screen of human MARCH proteins identifies MARCH4 and MARCH9 as Mult1 E3 ligases. 293T cells were cotransfected with a plasmid encoding WT Mult1-IRES-GFP and a plasmid encoding the indicated human MARCH proteins. A and B. GFP+ cells were analyzed 24 h after transfection by flow cytometry for Mult1 expression. Expression of GFP, which is encoded by the same bicistronic mRNA as Mult1, was similar in all of the samples, indicating comparable Mult1 gene expression. Shaded graphs represent staining of cells transfected with a plasmid encoding IRES-GFP only. C. Cells were lysed in coimmunoprecipitation buffer, followed by immunoprecipitation of Mult1 and detection of FLAG, HA, or ubiquitin by Western blotting. Similar results were obtained in two additional experiments.
Stable expression of mouse MARCH4 or MARCH9 in either of these cell lines led to decreased cell-surface expression of endogenous Mult1 (Fig. 2A, 2B), confirming that the mouse MARCH proteins downregulate Mult1. The downregulation of Mult1 was specific, because there was little or no downregulation of a distinct TM NKG2D ligand (H60a) that is normally expressed by WEHI 7.1 cells (Fig. 2C). In contrast, the endogenous Db class I MHC molecule was partially downregulated by mouse MARCH9 in C1498 cells, consistent with reports that transfected human MARCH4/9 downregulate MHC class I and ICAM-1 in human cells (Fig. 2D) (9, 12). In contrast, mouse MARCH9 failed to downregulate ICAM-1 or a distinct MHC I molecule, Kb, in transfected C1498 cells, suggesting selectivity in the effects of mouse MARCH proteins compared with human MARCH proteins (Fig. 2D). Although the possibility remains that mouse MARCH9 regulates other TM molecules not examined in this study in a more dramatic fashion, these results clearly show that targeting for downregulation by mouse MARCH9 is quite specific, and Mult1 is one of its targets.

**MARCH9 expression inhibits lysis by NK cells**

To determine whether MARCH proteins regulate the susceptibility of target cells to NK-mediated lysis, transduced C1498 cells were used as target cells for activated NK cells (LAK cells). C1498 cells transduced with mouse MARCH4 or mouse MARCH9 were killed significantly less well by LAK cells than by parental C1498 cells (Fig. 3A). A greater reduction in killing was observed with cells transduced with mouse MARCH9 compared with mouse MARCH4, which correlated with a greater reduction in Mult1 in the former cells (Fig. 3A). However, although mouse MARCH9-transduced cells were nearly devoid of cell-surface Mult1, the cells retained partial sensitivity to LAK cell killing, presumably as a result of the presence of additional stimulatory ligands for NK cells on C1498 cells that are not downregulated by MARCH9.

To determine the component of the reduced killing that was due to downregulation of Mult1, we compared killing by LAK cells prepared from WT or NKG2D knockout mice. Parental C1498 cells were killed significantly, although modestly, less well by NKG2D-deficient LAK cells than by WT LAK cells. Because C1498 cells express Mult1, but not other NKG2D ligands (data not shown), these data suggested that Mult1 contributed significantly to NK killing of C1498 cells but that ligands for other activating receptors also played a large role, as was true of most target cell lines studied elsewhere (20). In contrast, C1498-MARCH9 cells were killed equally well by WT or NKG2D-deficient LAK cells, showing that removal of Mult1 from the cell surface prevented NKG2D-dependent killing. However, it was notable that the reduction in killing accomplished by MARCH9 transduction was greater than the reduction in killing of parental C1498 cells resulting from NKG2D deficiency. Therefore, it is likely that MARCH9 transduction downregulated ligands for other activating NKRs, in addition to downregulating Mult1. The identity of the other affected ligands in our analysis remains unclear, but there are numerous NK-activating ligands, some of which may remain to be identified. The affected ligands are probably not ICAM-1, CD48 (ligand for 2B4), or CD155 (ligand for DNAM-1), because we did not observe ICAM-1 downregulation in MARCH9-transduced cells (Fig. 2D) and did not detect CD48 or CD155 expression on C1498 cells (data not shown). The possibility that NK stimulatory and inhibitory ligands other than Mult1 are also targets of MARCH9 was suggested by published data (9, 12, 15). Together, these data show that MARCH9-mediated downregulation of Mult1 and probably other activating ligands on C1498 cells inhibits functional recognition by NK cells.

**MARCH9 and regulation of Mult1 by heat shock**

In some cell types, including WEHI 7.1, a fibroblast cell line, and a subset of primary thymocytes, expression of Mult1 was shown to be inducible by heat shock (Fig. 4) (7). In contrast, WT C1498 cells normally express somewhat higher levels of cell-surface Mult1; no increase was observed after the cells were
subjected to heat shock (Fig. 4). It is not known why Mult1 is not regulated by heat shock in C1498 cells, but transduction of mouse MARCH9 into C1498 cells greatly reduced the high basal level of Mult1, raising the possibility that MARCH9 restored a missing component in the pathway regulating Mult1. Indeed, when mouse MARCH9-transduced C1498 cells were heat shocked, substantial levels of Mult1 were restored to the cell surface, whereas little change in Mult1 expression was seen on parental C1498 cells (Fig. 4). These data suggest that the parental C1498 cells lack an E3 ligase for Mult1, and this defect is rescued by ectopic expression of MARCH9.

Cell-surface expression of the MHC class I molecule D\textsuperscript{b} was diminished by transduction of mouse MARCH9 in C1498 cells, so it seems to be a target of MARCH9. Strikingly, however, D\textsuperscript{b}, unlike Mult1, was not induced by heat shock in mouse MARCH9-transduced C1498 cells. In fact, for unknown reasons, heat shock caused a reduction in D\textsuperscript{b} levels at the cell surface in mouse MARCH9-transduced and parental C1498 cells (Fig. 4). Taken together, these data indicate that Mult1 downregulation by MARCH9 is reduced following heat shock, but MARCH9-mediated downregulation of other proteins, such as D\textsuperscript{b}, is not. The results suggest that heat shock-dependent regulation targets Mult1 rather than targeting the MARCH9 E3 ligase or other targets of MARCH9.

A dynamic association of Mult1 with MARCH9 underlies its regulation

The fact that D\textsuperscript{b} is not induced by heat shock in mouse MARCH9-transduced cells suggested that a global change in MARCH9 activity might not be the cause of Mult1 regulation. To test this possibility, RNA and protein levels were analyzed before and after heat shock. As expected, transcript levels for mouse MARCH9 were higher in mouse MARCH9-transduced cells compared with parental C1498 cells, but there was not a significant reduction in transcript levels following heat shock (Fig. 5A). Similarly, the transduced mouse MARCH9 protein, detected by Western blotting with Myc epitope Abs, was not noticeably depleted following heat shock (Fig. 5B). These data argue strongly against the possibility that heat shock stimulates Mult1 expression by reducing MARCH9 expression.

An alternative explanation for Mult1 regulation is that the interaction between Mult1 and MARCH9 is reduced or prevented following heat shock, thus preventing targeting of Mult1. To test this hypothesis, the extent of interaction between myc-tagged mouse MARCH9 and Mult1 was determined in lysates from C1498 cells, before or after heat shock. Similar amounts of Mult1 were detected in the immunoprecipitates, but the amount of mouse MARCH9 coimmunoprecipitated was substantially reduced (~5-fold) in heat-shocked cells (Fig. 5C), quantification not shown). This indicates that the association of mouse MARCH9 with Mult1 was reduced following heat shock. The reduced interaction of Mult1 and mouse MARCH9 correlated with the increased display of Mult1 on the cell surface (Fig. 4, top panels). These data are consistent with a dynamic Mult1–MARCH9 interaction and, because MARCH9 levels are not regulated by heat shock, suggest that a heat shock-sensitive event controls the extent of this interaction.

Analysis of endogenous MARCH9

Although transduction of mouse MARCH4 and mouse MARCH9 clearly resulted in suppression of Mult1, it was important to investigate the role of the endogenous proteins. No Abs against mouse MARCH4 or MARCH9 are available, so analysis of endogenous expression was limited to transcript levels. To determine the tissue distribution of these MARCH family members, cDNA from a panel of mouse tissues and cells was analyzed by quantitative RT-PCR for the presence of MARCH4 and MARCH9 transcripts (Fig. 6A). Transcripts for MARCH9 were represented...
in a broad range of tissues, but MARCH4 transcripts were more selectively expressed, most notably in the brain and lung. The tissue distribution observed was similar to published analysis of human MARCH4/9 expression (9), suggesting similar roles for these proteins in mice and humans.

To more directly address the relative expression level of MARCH9 and MARCH4, PCR primers were designed that amplified a homologous region of these highly related transcripts, including a distinguishing SalI restriction enzyme site that is unique to MARCH4. SalI digestion of the PCR products was used to estimate the relative abundance of transcripts encoding MARCH4 (cleaved) versus MARCH9 (uncleaved). An established mouse fibroblast line (5) was selected for this more detailed analysis because this line had been well characterized in terms of Mult1 regulation. The PCR product from fibroblast cDNA was only visible as uncleaved product, indicating that MARCH9 is the only transcript present in appreciable quantities in these cells (Supplemental Fig. 1). Therefore, MARCH9 could be specifically studied in these cells without the complication of appreciable coexpression of MARCH4.

Mult1 in fibroblasts is maintained at low levels under normal conditions as a result of ubiquitination and degradation. To determine whether the endogenous MARCH9 in these cells was responsible for low cell-surface expression, siRNA oligonucleotides targeting MARCH9 were used to deplete MARCH9 transcripts. Nontransduced fibroblasts or fibroblasts ectopically expressing WT Mult1 and GFP were transfected with GFP oligonucleotides, negative control oligonucleotides, or three different MARCH9 oligonucleotides. Although the GFP oligonucleotides efficiently inhibited GFP expression in $\approx80\%$ of cells, demonstrating the high efficiency of transfection, none of the three MARCH9 oligonucleotides used for knockdown resulted in appreciable increases in endogenous or ectopic Mult1 expression (Fig. 6B). Analysis of RNA levels in these cells showed that the MARCH9 oligonucleotides were effective, reducing transcript amounts by 70–95% (Fig. 6C). Also, transfection of MARCH9 siRNA oligonucleotides into 3T3 cells ectopically expressing MARCH9 effectively restored Mult1 surface expression, further illustrating their effectiveness (Supplemental Fig. 2). To ensure that there were no off-target effects on Mult1 that could confound interpretation of the results, Mult1 transcripts were also quantified. Although one of the oligonucleotides (M9 #3) seemed to reduce Mult1 RNA levels in this and some other experiments, the MARCH9 oligonucleotide that resulted in the best specific knockdown (M9 #1) had no effect on Mult1 transcript amounts (Fig. 6C). It is unlikely that endogenous MARCH4 was responsible for downregulating Mult1 in the absence of MARCH9, because these cells expressed MARCH4 mRNA levels below the limit of detection (Fig. 6A). Moreover, no additional effect was observed when MARCH4 oligonucleotides were cotransfected with the MARCH9 oligonucleotides (T.J. Nice and D.H. Raulet, unpublished observations). The results argue that MARCH9 and MARCH4 are not necessary for the basal suppression of Mult1 expression in these cells, but they may carry out this function redundantly with another E3 ligase.

**Discussion**

The MARCH E3 ligases and their viral homologs are a recently described protein family. These proteins are distinguished from other RING domain-containing E3 ligases by their unique configuration of cysteines and a histidine. Interestingly, they are all TM proteins with at least two membrane-spanning segments, and most of their ubiquitination targets are TM proteins. Of the MARCH-targeted proteins that have been identified, most are TM mole-

![FIGURE 6. Analysis of endogenous MARCH9. A, RNA from the indicated cells and tissues was used for MARCH4 or MARCH9 quantitative RT-PCR, and relative quantity was determined by normalization to RPS29. An asterisk (*) indicates that transcript amounts were below the limit of detection. B, Nontransduced fibroblasts or fibroblasts expressing HA-tagged Mult1 were transfected with control oligonucleotides (solid line) or the indicated siRNA oligonucleotides (dashed line). Expression of endogenous Mult1, GFP, and HA-Mult1 was analyzed 72 h after transfection. C, RNA was extracted from the siRNA-transfected fibroblasts (B) for quantitative PCR analysis, and relative levels of Mult1 and MARCH9 transcripts were determined by normalizing to RPS29. Similar results were obtained in two additional experiments. M9, MARCH9.](http://www.jimmunol.org/)

NKG2D ligand regulation by a MARCH family E3 ligase
cules within the immune system. Our results demonstrate that ectopic expression of two members of this family (MARCH4 and MARCH9) prevent the expression of Mult1, adding this NKG2D ligand to the list of MARCH-regulated proteins. Our data support the notion that endogenous MARCH proteins, particularly the two-TM MARCH proteins, play numerous roles in the regulation of immune functions by regulating the cell-surface display of proteins that activate immune responses.

MIR2, a homolog of the MARCH proteins encoded by KSHV, was recently shown to downregulate the human NKG2D ligands MICA and MICB (19). Because it is believed that MIR2 originated as a human MARCH gene that was captured by KSHV, it is plausible that human MARCH proteins may also regulate human NKG2D ligands. However, we failed to observe MICA or MICB downregulation in Jurkat cells transfected with any of the human two-TM MARCH cDNAs (T.J. Nice and D.H. Raulet, unpublished data). Hence, MIR2 and endogenous human MARCH proteins may have diverged in their capacity to target MICA/B. It remains possible that MICA or MICB is regulated by one of the MARCH proteins not tested in this study or that MARCH proteins regulate a member of the distinct ULBP family of NKG2D ligands. Alternatively, Jurkat cells may be subject to constitutive stress signals that prevent targeting of MICA/B by transfected MARCH proteins.

The interaction of MARCH4 or MARCH9 with Mult1 was specific in several respects. First, the other MARCH family members failed to appreciably downregulate Mult1 in the transfection experiments (Fig. 1A). Second, stable expression of mouse MARCH9 using retroviral transduction did not downregulate several other immunologically relevant cell-surface proteins, including several potential MARCH targets, such as ICAM-1, H60a, and K. In contrast, the class I molecule D, another potential target, was downregulated, although not to the same extent as Mult1. These demonstrations of specificity argue against a non-specific effect of overexpression. In fact, the level of MARCH9 RNA in transduced C1498 cells (Fig. 5A) was only ~5-fold greater than levels seen in tissues with the most abundant MARCH9 RNA (e.g., brain). Given the likely heterogeneity of expression in a tissue, the level of MARCH9 mRNA in individual cells in the tissue may be substantially higher. These considerations suggest that MARCH9 is not grossly overexpressed in C1498-MARCH9 cells.

Although ectopically expressed MARCH4/9 led to downregulation of Mult1 expression, MARCH4 and MARCH9 are probably not the exclusive E3 ligases for Mult1, because knockdown of MARCH9 in fibroblasts that did not express detectable levels of MARCH4 transcript had no effect on Mult1 expression. The most likely explanation for this observation is redundancy of MARCH4/9 with unidentified cellular E3 ligases. These could be untested MARCH family members or non-MARCH E3 ligases. Notably, MARCH4/9 transcripts were more abundant in several tissues (e.g., brain, lung, and kidney; Fig. 6A) than in the cell lines analyzed, so MARCH4/9 may have a more prominent role in Mult1 regulation in the context of those tissues.

Although expression of MARCH4 or MARCH9 in 293T cells and C1498 cells led to a strong downregulation of Mult1 from the cell surface, Western blotting showed that they caused no (Figs. 1C, 4C), or at most a 2-fold, reduction (T.J. Nice, W. Deng, and D.H. Raulet, unpublished data) in total Mult1 amounts in these cells. These observations suggest that Mult1 downregulation by MARCH4/9 was largely due to sequestration within the cell. MHC class II regulation by MARCH1/8 was also shown to be partially due to sequestration in intracellular compartments, although some degradation was also observed (17). In contrast, downregulation of Mult1 mediated by endogenous E3 ligases in fibroblasts resulted in very substantial reductions in total cellular Mult1 (7). Hence, in distinct circumstances, E3 ligases may prevent Mult1 cell-surface expression by degrading the protein or sequestering it in an intracellular compartment.

Interestingly, Mult1 was induced by heat shock in fibroblasts, where downregulation was not dependent on MARCH9, and in C1498-MARCH9 cells, where MARCH9 is the exclusive relevant E3 ligase. In terms of the mechanism of heat-shock regulation, these findings suggest that Mult1, rather than the E3 ligases, is targeted. This idea is supported by the observations that MARCH9 levels in C1498-MARCH9 cells did not change in response to heat shock (Fig. 5), and heat shock did not induce expression of the distinct MARCH9-targeted protein D (Fig. 4). A motif within the Mult1 cytoplasmic domain may be targeted by this stress response, in a manner that controls the interaction of Mult1 with its E3 ligases. Indeed, the Mult1 cytoplasmic tail is required for physical association with MARCH4/9; therefore, it may also be important for the regulation of their interaction following heat shock. This interaction could be disrupted by the association of a regulatory molecule with the cytoplasmic tail that prevents E3 binding and/or ubiquitination or that targets Mult1 to a compartment inaccessible to the E3 ligase. Alternatively, heat shock may repress a regulator that facilitates E3 binding and/or ubiquitination of Mult1 but not other targets of MARCH4/9. Further analysis is required to identify critical motifs within the cytoplasmic domain and how they regulate E3 ligase binding in response to heat shock.

Disclosures
The authors have no financial conflicts of interest.

References