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A Unique Carboxyl-terminal Insert Domain in the Hematopoietic-specific, GTPase-deficient Rho GTPase RhoH Regulates Post-translational Processing*

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Background: RhoH is required for T cell development and non-coding RhoH mutations are found in B-cell lymphoma.
Results: The C-terminal insert domain regulates RhoH protein stability via chaperone-mediated autophagy.
Conclusion: Although the insert domain is required for lysosomal uptake, it is dispensable for T cell development.
Significance: Targeting the insert domain may permit alteration of RhoH protein levels without impairing vital protein functions.

RhoH is a hematopoietic-specific, GTPase-deficient member of the Rho GTPase family that was first identified as a hypermutable gene in human B lineage lymphomas. RhoH remains in a constitutively active state and thus its effects are regulated by expression levels or post-translational modifications. Similar to other small GTPases, intracellular localization of RhoH is dependent upon the conserved "C*AAX*" box and surrounding sequences within the carboxyl (C) terminus. However, RhoH also contains a unique C-terminal "insert" domain of yet undetermined function. RhoH serves as adaptor molecule in T cell receptor signaling and RhoH expression correlates with the unfavorable prognostic marker ZAP70 in human chronic lymphocytic leukemia. Disease progression is attenuated in a *Rhoh*$^{-/-}$ mouse model of chronic lymphocytic leukemia and treatment of primary human chronic lymphocytic leukemia cells with Lenalidomide results in reduced RhoH protein levels. Thus, RhoH is a potential therapeutic target in B cell malignancies. In the current studies, we demonstrate that deletion of the insert domain (LFSINE) results in significant cytoplasmic protein accumulation. Using inhibitors of degradation pathways, we show that LFSINE regulates lysosomal RhoH uptake and degradation via chaperone-mediated autophagy. Whereas the C-terminal prenylation site is critical for ZAP70 interaction, subcellular localization and rescue of the *Rhoh*$^{-/-}$ T cell defect *in vivo*, the insert domain appears dispensable for these functions. Taken together, our findings suggest that the insert domain regulates protein stability and activity without otherwise affecting RhoH function.

Rho GTPases are molecular switches linking environmental signals to cell survival, cell cycle regulation, gene expression, and cytoskeleton reorganization (1–3). RhoH is a hematopoietic-specific Rho GTPase that was initially identified as an oncogene fusion partner and target for somatic hypermutation in various B cell malignancies (4–7). We and others have demonstrated that RhoH is critical for T cell function through regulating T cell receptor (TCR)[2] complex formation at the immune synapse (8–11). Whereas loss of *Rhoh* results in a profound T cell deficiency in a genetic knock-out mouse model, the effect of *Rhoh* knock-out on B cell development in these mice is modest (12). However, genetic deletion of *Rhoh* in a murine model of B cell chronic lymphocytic leukemia (CLL) results in significantly delayed disease progression despite T cell immunodeficiency (12, 13). The RhoH expression level is also correlated with the poor prognostic marker of aberrant ZAP70 expression in human CLL cells (12). This is of particular interest as RhoH mutations found in B cell lymphoma affect non-coding, presumably regulatory regions suggesting that RhoH protein levels may be critical for the survival of malignant B cells (7, 14, 15). We previously demonstrated that RhoH is involved in spatiotemporal regulation and activation of Rac and RhoA GTPases in CLL cells (13). Thus, lack of RhoH blocks migration and access of CLL cells to supportive cells of the microenvironment that appear important for survival of these cells (13). We have also demonstrated that *ex vivo* and *in vivo* Lenalidomide treatment is associated with decreased RhoH protein levels in human CLL cells (13). These observations suggest a potential therapeutic benefit of targeting RhoH expression in B cell malignancies. However, given the requirement of RhoH in TCR signaling, a major aim will be to retain T cell function at the same time. Therefore a better understanding of the functional RhoH protein domains appears mandatory.

RhoH is a constitutive active, GTP-bound member of the family of atypical Rho GTPases of the Rnd3 family (4, 16–18).

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[2] The abbreviations used are: TCR, T cell receptor; CLL, chronic lymphocytic leukemia; CMA, chaperone-mediated autophagy; PE, phycoerythrin; CHX, cycloheximide; GA, geldanamycin; EGFP, enhanced green fluorescent fusion protein; TRITC, tetramethylrhodamine isothiocyanate; LAMP, lysosome-associated membrane protein type; APC, allophycocyanin.

Unlike in oncogenic Ras, mutations of coding sequences of Rho GTPases have infrequently been reported in human cancers, whereas alterations in protein levels have been demonstrated for several Rho GTPases in solid tumors (19–21) and leukemic cells (22, 23). Due to its constitutively active state, RhoH activity appears to be mainly determined by the protein level and post-translational modifications (4, 7, 17, 24). In this regard, we have previously demonstrated the functional importance of phosphorylation of an immunoreceptor tyrosine-based activation motif-like sequence, unique in RhoH among all Rho GTPases, as one mechanism of regulation (8).

Cellular protein levels can be modulated by altering protein stability. It has been recently demonstrated that binding of thalidomide to cereblon (CRBN) inhibits the E3 ubiquitin ligase complex involved in proteosome-dependent protein degradation (25), suggesting that ubiquitination may be an important target of some immunomodulatory drugs. Interestingly, RhoH contains a unique insert domain (LFSINE) in its C-terminal region between the polybasic domain and prenylation site, the function of which is still largely unknown. Here we investigated the mechanism of RhoH protein stability. We demonstrate that RhoH can be degraded via the LFSINE domain by chaperone-mediated autophagy (CMA) in lymphoid cell lines. However, the LFSINE domain does not affect RhoH function in normal T and B cell development. This suggests a potential drug target for modulation of RhoH protein levels in malignant cells.

EXPERIMENTAL PROCEDURES

Rhoh$^{-/-}$ *Mice*—The generation and characterization of the T cell and B cell phenotype of the *Rhoh*$^{-/-}$ mouse model has been described previously (8, 12, 26). After generation, 129Sv mice have in addition been backcrossed to a C57BL/6J background. All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston (Boston, MA).

Antibodies and Reagents—The following fluorescence labeled antibodies were used for flow cytometry analysis: B220-allophycocyanin (APC)(RA3–6B2), CD3ϵ-PE (145–2C11), CD4-PE-Cy7 (RM4–5), CD8a-APC(53–6.7), CD45.2-APC-Cy7 (104) (all from BD Biosciences), and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). For immunoblotting and immunoprecipitation experiments the following antibodies were used: anti-HA antibody (6E2) (Cell Signaling Technology), anti-HA high affinity (3F10) (Roche Applied Science), anti-ZAP70 (99F2) (Cell Signaling Technology), anti-ZAP70 (1E7.2) (Upstate), anti-RACK1 antibody (D59D5) (Cell Signaling Technology), anti-Flotillin-2 antibody (C42A3) (Cell Signaling Technology), and anti-β-actin (AC-15) (Sigma) as well as protein A/G plus-agarose immunoprecipitation agent (sc-2003, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The RhoH antibody has previously been described (27). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Cell Signaling Technology) were used as secondary antibodies for detection. To assess protein stability cycloheximide (Sigma), AICAR (Sigma), MG132 (Sigma), geldanamycin (AG Scientific Inc., San Diego, CA) (28–30), ammonium chloride (Sigma), chloroquine (Sigma) (31, 32), and FTI-277 (Sigma) were used.

The following antibodies and reagents were used for Lin$^-$Sca1$^+$ckit$^+$ (LSK) cell labeling and sorting. Dynabeads® Biotin Binder (Invitrogen), 7-aminoactinomycin D (Invitrogen), CD16/CD32 (Fcγ III/II Receptor) clone 2.4G2 (BD Pharmingen™), phycoerythrin (PE)-streptavidin (eBioscience, San Diego, CA), (APC) anti-mouse CD117 (c-Kit) (eBioscience), (FITC) anti-mouse Sca-1 (eBioscience, San Diego, CA), and the biotinylated antibodies: CD5 (Ly-1, 53–7.3) biotin (BD Pharmingen™, San Jose, CA), CD8a (Ly-2, 53–6.7) biotin (BD Pharmingen), CD45R/B220 (RA3–6B2) biotin (BD Pharmingen), CD11b (M1/70) biotin (BD Pharmingen), TER-119/erythroid cell (Ly-76, TER-119) biotin (BD Pharmingen), and Ly-6G and Ly-6C (RB6–8C5) biotin (BD Pharmingen).

Transduction of Cell Lines—HA-tagged C-terminal deleted or mutant versions of *RHOH* were generated (Fig. 1, *A* and *B*) and cloned into a murine stem cell virus-based retroviral vector coexpressing yellow fluorescent protein (YFP) or as enhanced green fluorescent fusion protein (EGFP), as previously described (8, 10, 33). Recombinant retroviruses were generated using Phoenix-gp cells (34). Briefly, 8 μg of retroviral vector plasmid DNA, 10 μg of Moloney leukemia virus gag-pol plasmid, and 3 μg of galv or RD114 envelope plasmid were cotransfected into Phoenix cells using the $CaPO_4$ coprecipitation method (Invitrogen). Retroviral supernatants were collected every 12 hour (h). Titer of recombinant retrovirus was determined by infecting HT1080 cells using serial dilution (35). The human cell line Jurkat was infected with a multiplicity of infection of 5 on fibronectin-coated plates (CH-296, Takara-Bio, Otsu, Japan), with the high-titer retrovirus supernatant. Subsequently YFP$^+$ cells were sorted and used for biochemical analysis.

Tissue Culture—To assess protein stability and protein degradation, Jurkat T cells were transduced with high titer retroviral supernatant of wtRhoH or mutant RhoH$^{\Delta CT}$, RhoH$^{\Delta PR}$, and RhoH$^{\Delta LFSINE}$ expressing constructs (Fig. 1*B*). Thereafter YFP$^+$-transduced cells were sorted and cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin (100 IE/ml). To assess protein stability cells were incubated for a time course of 0, 1, 3, and 6 hour (h) with the protein synthesis inhibitor cycloheximide (50 μM CHX). Subsequently RhoH protein degradation was assessed over time by immunoblot analysis using anti-HA antibody. β-Actin was included as loading control and reference. To further investigate pathways involved in RhoH protein degradation, cells were next treated for 6 h with a variety of proteosomal inhibitors (30 μM MG132, 30 μM MG115, 50 μM ALLN), a caspase inhibitor (30 μM), and AICAR (1 mM), which is known to inhibit AMP-activated protein kinase as well as nonspecific macromolecule degradation via autophagy (36, 37). Thereafter inhibition of RhoH protein degradation was determined by immunoblot in comparison to vehicle-treated control samples. Most short-lived proteins are degraded via a proteosomal pathway following ubiquitination (38). However, ubiquitination also targets proteins to endocytic compartments and degradation in the lysosomes (39, 40). Chaperone-mediated autophagy (CMA) comprises an alternative degradation pathway that depends on the interaction of the target protein with chaperone molecules such as heat shock proteins (Hsp) and subsequent targeting of the substrate proteins to the lysosomal

membrane (41, 42). In most proteins this is mediated via a specific recognition sequence (KFERQ) in the substrate protein (43). We next treated Jurkat cells expressing HA-tagged wtRhoH or RhoH$^{\Delta LFSINE}$ for a time course of 0, 3, and 6 h with geldanamycin (5 μM GA), an ansamycin derivative benzoquinone compound that specifically inhibits the essential ATPase activity of Hsp90 needed for its function in proper folding, stability, and function of target proteins (30). To further investigate the CMA degradation pathway, cells were next treated with the lysosomal inhibitor ammonium chloride (30 mM) or chloroquine (10 μM), GA, or a combination of both for 6 h and again RhoH protein levels were detected by immunoblot.

Immunoblotting—For immunoblotting and immunoprecipitation assays, cells were lysed in Mg^{2+} lysis/wash buffer (Upstate Biotechnology) containing 8% glycerol, phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate) and complete protease inhibitor mixture (Roche Applied Science) on ice. HA-tagged RhoH protein was assessed after separation on 12% SDS-polyacrylamide gels and transfer to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using anti-HA antibody in a 1:1,000 dilution. β-Actin was determined as a loading control for total protein levels using anti-β-actin antibody in a 1:10,000 dilution. HRP-coupled anti-mouse or anti-rabbit antibodies at a dilution of 1:2000 and LumiGlo chemiluminescent substrate (Cell Signaling Technology) were used for detection. To determine intracellular protein localization, sorted YFP-positive cells were fractionated into the cytosolic fraction, detergent-soluble membrane fraction, and detergent-insoluble membrane fraction as described previously (10). Briefly, YFP^+ cells were lysed by sonication in ice-cold buffer A (250 mM sucrose, 20 mM Tris, pH 7.8, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, and complete protease inhibitor mixture (Roche)). Particulate membrane-containing (P100) and cytoplasm-containing (S100) fractions were separated by centrifugation at 100,000 $\times$ *g* for 30 min. Membrane fractions were solubilized with Mg^{2+} lysis/wash buffer (Upstate Biotechnology) and separated by additional centrifugation for 30 min at 100,000 $\times$ *g*. 0.5% SDS was used to extract the detergent-insoluble cytoskeleton-containing fraction. Equal volumes of each fraction were resolved on a SDS-polyacrylamide gel and immunoblotted with anti-HA antibody to assess the RhoH protein. Thus, identical equivalents of each fraction were used to compare subcellular localization of RhoH mutants. In addition anti-RACK1 and anti-Flotillin-2 staining were utilized to confirm the purity of each cellular fraction.

Immunoprecipitation—Cellular lysates (1 $\times$ 10 e6 cells/100 μl) were immunoprecipitated, using anti-HA (3F10, 100 ng, Roche) or anti-ZAP70 (1E7.2, 1 μg, Upstate Biotechnology) antibodies and protein A/G-agarose immunoprecipitation reagents (Santa Cruz Biotechnology Inc.). The immunoprecipitated mixture or 10 μl of total protein was separated on a 12% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were probed with anti-HA or anti-ZAP70 antibodies as indicated in each experiment.

Immunofluorescence Staining—For immunofluorescence staining Jurkat T cells were transduced with retroviral supernatant of EGFP-fused versions of wtRhoH or mutant RhoH. Cells were then layered onto poly-L-lysine coverslides and fixed using BD Cytofix/Cytoperm (BD Biosciences) solution. Thereafter cells were stained with rhodamine (TRITC)-labeled phalloidin (Invitrogen) and finally sealed with DAPI containing mounting medium. XYZ series fluorescence images were captured with a PerkinElmer UltraView Vox Spinning Disc confocal microscope (Nikon Inc., Melville, NY) equipped with a $\times$60 objective lens and ultraview molecular laser, connected to a Hamamatsu C9100-50 camera. Images were analyzed for intracellular localization of RhoH protein using Volocity software (PerkinElmer Life Sciences).

Mouse Bone Marrow Cell Transduction and Transplantation—Mouse bone marrow cells were transduced with retroviral supernatant, as described previously (44, 45). To this end EGFP-fused wtRhoH or C-terminal-deleted mutants of RhoH subcloned into a murine stem cell virus-based retroviral vector were used (8). In brief, $Lin^-Sca1^+ckit^+$ bone marrow (LSK) cells from C57BL/6 *WT* or C57BL/6 *Rhoh*$^{-/-}$ mice were sorted, stimulated, and infected on fibronectin-coated plates (CH-296, Takara-Bio, Otsu, Japan), with the high-titer retrovirus supernatant generated in Phoenix-gp cells. Two days after transduction, the percentage of $EGFP^+$ cells was determined by flow cytometry and equal numbers of transduced cells were transplanted by intravenous injection into lethally irradiated (11.5 gray with a 137 Cs irradiator) B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ recipient mice. Two and three months after transplantation, white blood cell counts were obtained and the percentage of $CD45.2^+/EGFP^+$ T and B cells in the peripheral blood of the animals was determined by flow cytometry.

Flow Cytometric Analysis—Blood samples were obtained by retroorbital puncture at 2 and 3 months after transplantation. Before flow staining, red blood cells were lysed for 10 min at room temperature by incubation in BD PharmLyse (BD Biosciences). Cell suspensions were then incubated for 30 minutes (min) in the dark with a 1:100 dilution of the appropriate antibodies at 4 °C. DAPI staining was used for exclusion of dead cells. Subsequently, cells were washed to remove excessive unbound antibody, resuspended in phosphate-buffered saline (PBS) with 2% FCS, and finally analyzed with an LSRII cytometer using the FACSDiva software program (BD Bioscience). $CD45.2^+$, $EGFP^+$ T cell subsets were identified by CD3, CD4, and CD8 staining; B cells were defined by B220 staining.

Statistical Analysis—Statistical differences were determined by Student's *t* test or Mann Whitney U test using the IBM SPSS Statistics 21 program. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Deletion of the Insert Domain Significantly Increases RhoH Protein Stability Contributing to Its Cytoplasmic Accumulation—Previous data has suggested that post-translational regulation of RhoH determines its protein level and cellular function(s) (8, 10). Other members of the atypical Rho GTPases of the Rnd3 family are resistant to guanosine nucleotide dissociation inhibitor (GDI)-mediated sequestration in the cytoplasm and membrane localization appears to be constitutive in nature (3, 7, 17). The polybasic domain and prenylation site at the C terminus of RhoH have been shown to regulate membrane localization and

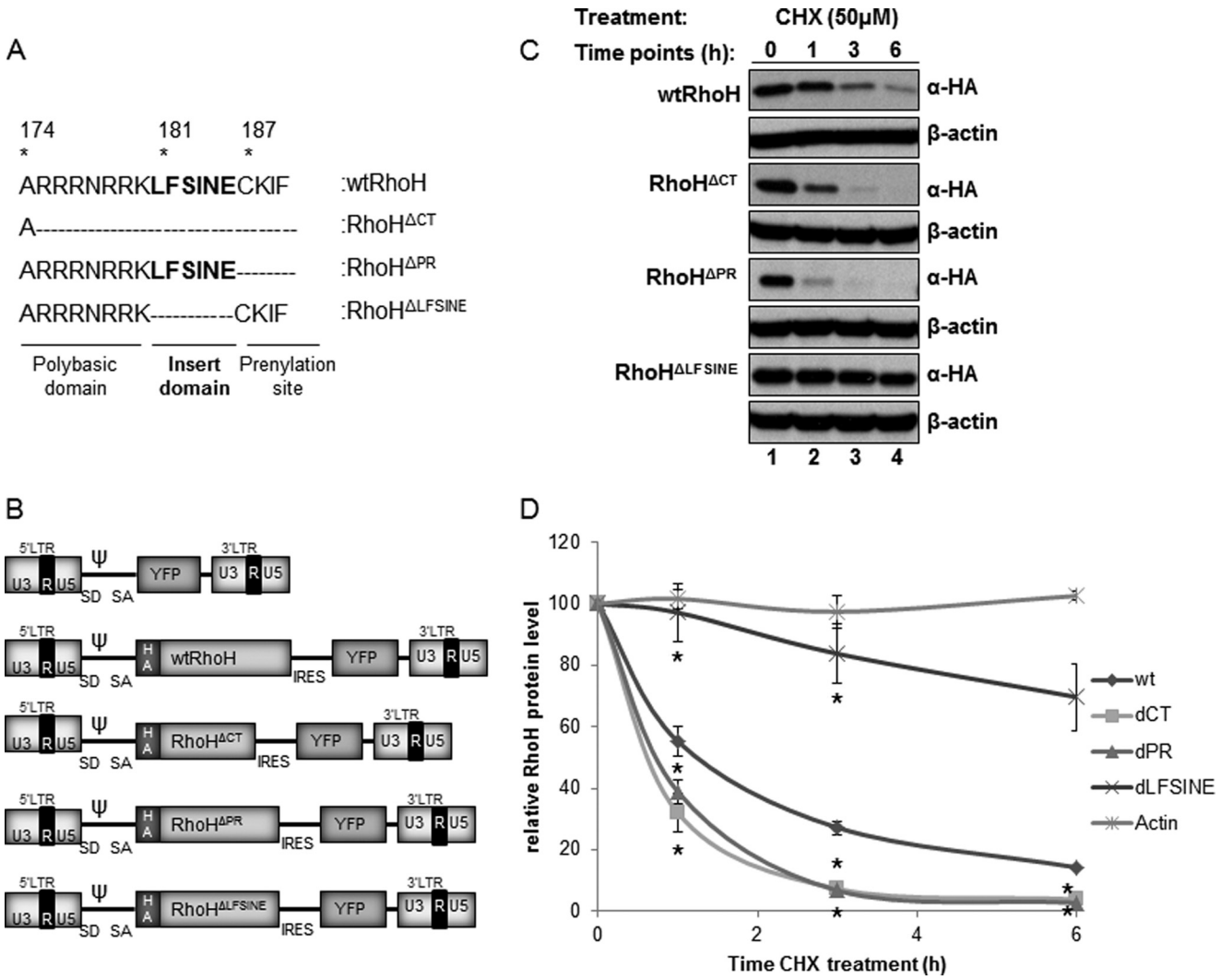

FIGURE 1. **Insert domain mutation of RhoH results in significantly increased protein stability.** *A,* schematic diagram of the C terminus of RhoH showing three mutants used in analysis compared with the wt sequence. The *numbers* denote amino acid positions within the RhoH protein sequence marked by *asterisks* (*). *B,* design of retrovirus vectors coexpressing HA-tagged versions of RhoH mutants and YFP utilized for *in vitro* studies. The positions of the RhoH cDNA and mutants is shown. *LTR*, long terminal repeats; *SD*, splice donor; *SA*, splice acceptor; *ψ*, packaging sequence. *C,* HA-tagged wtRhoH, RhoH$^{\Delta CT}$, RhoH$^{\Delta PR}$, or RhoH$^{\Delta LFSINE}$ mutant-expressing Jurkat T cells were treated with CHX for the indicated time points and HA-tagged RhoH protein levels analyzed following immunoblotting with anti-HA antibody. *β*-Actin was used as a loading control. Shown is a representative immunoblot. *D,* quantification of HA-RhoH in relationship to *β*-actin over time. ∗, *β*-actin; ◇, wtRhoH; □, RhoH$^{\Delta CT}$; Δ, RhoH$^{\Delta PR}$; ×, RhoH$^{\Delta LFSINE}$. Shown is the densitometric quantification (mean ± S.E.) of repeat experiments performed three times; *, $p < 0.05$; Student's *t* test.

protein function in TCR signaling (8, 10, 33). RhoH possesses an additional, unique insert sequence (LFSINE) between these domains, the function of which has thus far not been characterized. To assess the functional role of these domains on protein stability and localization in more detail, C-terminal deleted RhoH mutants were designed and cloned into retroviral vectors (Fig. 1, *A* and *B*). Given the well documented, vital function of RhoH in TCR signaling and T cell development we first studied the role of the C-terminal RhoH sequences in Jurkat T cells. To this end, Jurkat cells were transduced with these HA-tagged wt or C-terminal-deleted RhoH mutants. To analyze the effect of the mutated domains on protein stability, the transduced cells were then treated with CHX and RhoH levels were determined over time by immunoblot. wtRhoH was characterized by a protein half-life of less than 3 h (Fig. 1, *C* and *D*), which is similar to published data on the half-lives of other atypical Rho GTPases such as RhoB (32). Loss of either the prenylation site or polybasic domain (RhoH$^{\Delta CT}$ and RhoH$^{\Delta PR}$) reduced RhoH protein half-life by ~50%. Interestingly, concomitant treatment of wtRhoH transduced Jurkat cells with CHX and a farnesyltransferase inhibitor (20 μM, FTI-277), which inhibits the lipid modification required for membrane localization of the cognate protein, had a similar effect and reduced RhoH protein stability (data not shown). However, deletion of the insert domain (RhoH$^{\Delta LFSINE}$) substantially enhanced protein stability compared with wtRhoH (Fig. 1, *C* and *D*). Protein levels of the RhoH$^{\Delta LFSINE}$ mutant were not affected by CHX treatment for up to 6 h, suggesting that LFSINE has a negative regulatory role in RhoH protein degradation.

Proteosomal Pathways Regulate Degradation of C-terminal Deleted Mutants but Do Not Affect wtRhoH and RhoH$^{\Delta LFSINE}$ Protein Degradation—Many short lived cytoplasmic proteins become tagged with ubiquitin and then degraded by proteosomes (46, 47). Moreover, it has been demonstrated that monoubiquiti-

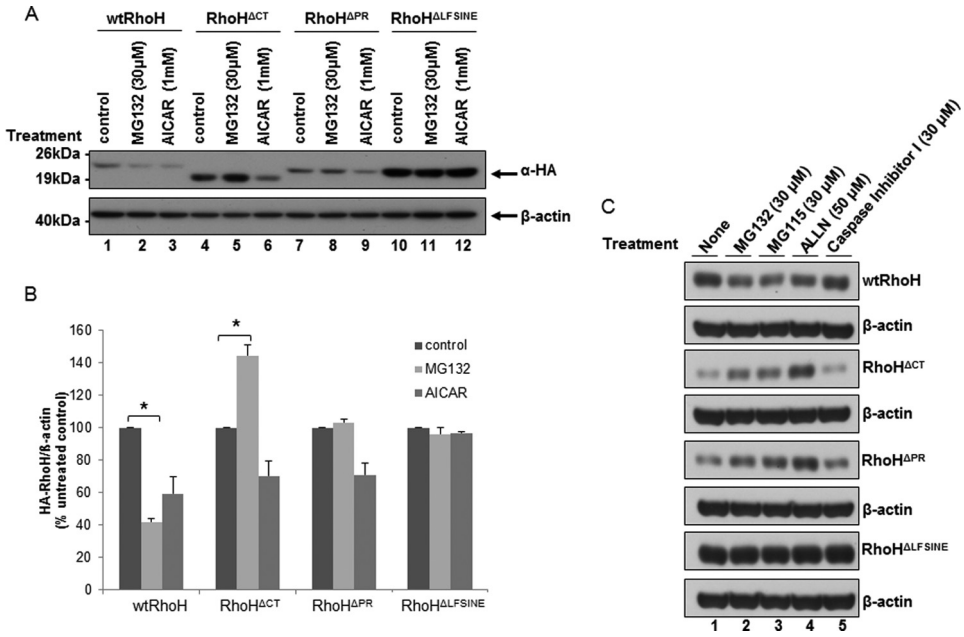

FIGURE 2. **Inhibition of the proteosome degradation pathway prolongs half-life of the carboxyl terminus and prenylation site-deleted mutants but not wt or insert-deleted RhoH.** *A,* HA-tagged wtRhoH, RhoH$^{\Delta CT}$, RhoH$^{\Delta PR}$, or RhoH$^{\Delta LFSINE}$-expressing Jurkat T cells were incubated with the proteosomal inhibitor MG132 and an inhibitor of macroautophagy, AICAR, for 6 h and then levels of HA-RhoH and β-actin (loading control) were analyzed by immunoblotting. RhoH was detected using anti-HA antibody. Shown is a representative experiment. *B*, densitometric measurement of the respective immunoblot bands indicating the relationship of the RhoH mutant protein to β-actin loading control expressed as the percentage of untreated control. Shown is the densitometric quantification (mean $\pm$ S.E.) of three independent repeat experiments; *, $p < 0.05$, Student's *t* test. *C,* transduced Jurkat T cells were incubated with additional proteosomal inhibitors MG115, ALLN, or a caspase inhibitor besides MG132 (at the indicated concentrations) for 6 h and then levels of HA-RhoH and β-actin (loading control) were analyzed by immunoblotting. RhoH was detected using anti-HA antibody.

nation of membrane proteins regulate not only their stability, but also influences protein-protein interactions, protein activity, and subcellular localization (41). Ubiquitination targets these proteins to endocytic compartments and subsequent degradation in lysosomes (40). As prenylation site or polybasic domain-deleted RhoH mutants demonstrated significantly shortened protein half-lives, we next investigated the molecular mechanism by which RhoH$^{\Delta PR}$ and RhoH$^{\Delta CT}$ mutants were degraded so quickly. MG132 treatment enhanced protein levels of C terminus-deleted RhoH mutants in Jurkat T cells at 6 h (Fig. 2, *A*, *lane 5,* and *B*), whereas this treatment had little or modest effects on prenylation site-deleted RhoH$^{\Delta PR}$ (Fig. 2*A*, *lane 8*, and *B*) and no effect on RhoH$^{\Delta LFSINE}$ protein levels (Fig. 2, *A*, *lane 11*, and *B*). Surprisingly, MG132 treatment was associated with a significant decrease in wtRhoH protein (Fig. 2, *A*, *lane 2,* and *B*) suggesting involvement and activation of alternate protein degradation pathways after drug treatment. The effects of other proteosome inhibitors such as ALLN and MG115 were similar, increasing the protein levels of the C-terminal-deleted RhoH$^{\Delta CT}$ mutant but more clearly increasing the levels of the RhoH$^{\Delta PR}$ mutant (Fig. 2*C, lanes 2–4*). Caspase inhibitors did not protect RhoH proteins from degradation (Fig. 2*C*, *lane 5*). None of these proteasomal inhibitors increased wtRhoH levels. These findings suggest that improper localization and protein folding in prenylation site-modified and C-terminal-deleted RhoH mutants activate proteasome-dependent protein degradation.

It has been demonstrated that after T cell receptor activation RhoH becomes degraded within a protein complex via macroautophagy involving a lysosomal pathway in T cells but not in activated B cells (48). To test if this degradation pathway is involved in non-TCR activated cells, we also treated transduced Jurkat cells with AICAR, an inhibitor of AMP-activated protein kinase and nonspecific macromolecule degradation autophagy (36). AICAR had no protective effect on wtRhoH or C-terminal deleted RhoH proteins in non-activated Jurkat T cells (Fig. 2, *A*, *lane 3*, *6*, *9* and *12*, and *B*), suggesting that these proteins are not degraded within a protein complex in non-TCR-activated lymphocytes. In summary, these data indicate that prenylation site and C-terminal-deleted RhoH mutants become degraded via a proteosomal pathway, indicating that C-terminal sequences of RhoH protein are critical for regulation of protein trafficking and targeting to certain cellular compartments involved in protein degradation.

Inhibition of the Chaperone-mediated Autophagy Pathway Prolongs Half-life of wtRhoH—To further elucidate the regulation of wtRhoH protein levels and the effect of the insert domain on protein degradation, an alternative and more selective lysosomal protein degradation pathway was investigated. CMA depends on the interaction of the target protein with chaperone molecules such as heat shock proteins (Hsp) and heat shock cognate protein 70 (Hsc70) and subsequent targeting of the substrate proteins to the lysosomal membrane regulating its uptake via lysosome-associated membrane protein type 2A (LAMP-2A) receptor (41–43). In most cases this is mediated via a specific recognition sequence (KFERQ) in the substrate protein (42). We next examined whether this autophagy pathway was involved in the lysosomal degradation of RhoH protein. GA specifically inhibits ATPase activity needed for normal Hsp90 function and accelerates degradation of client proteins (30).

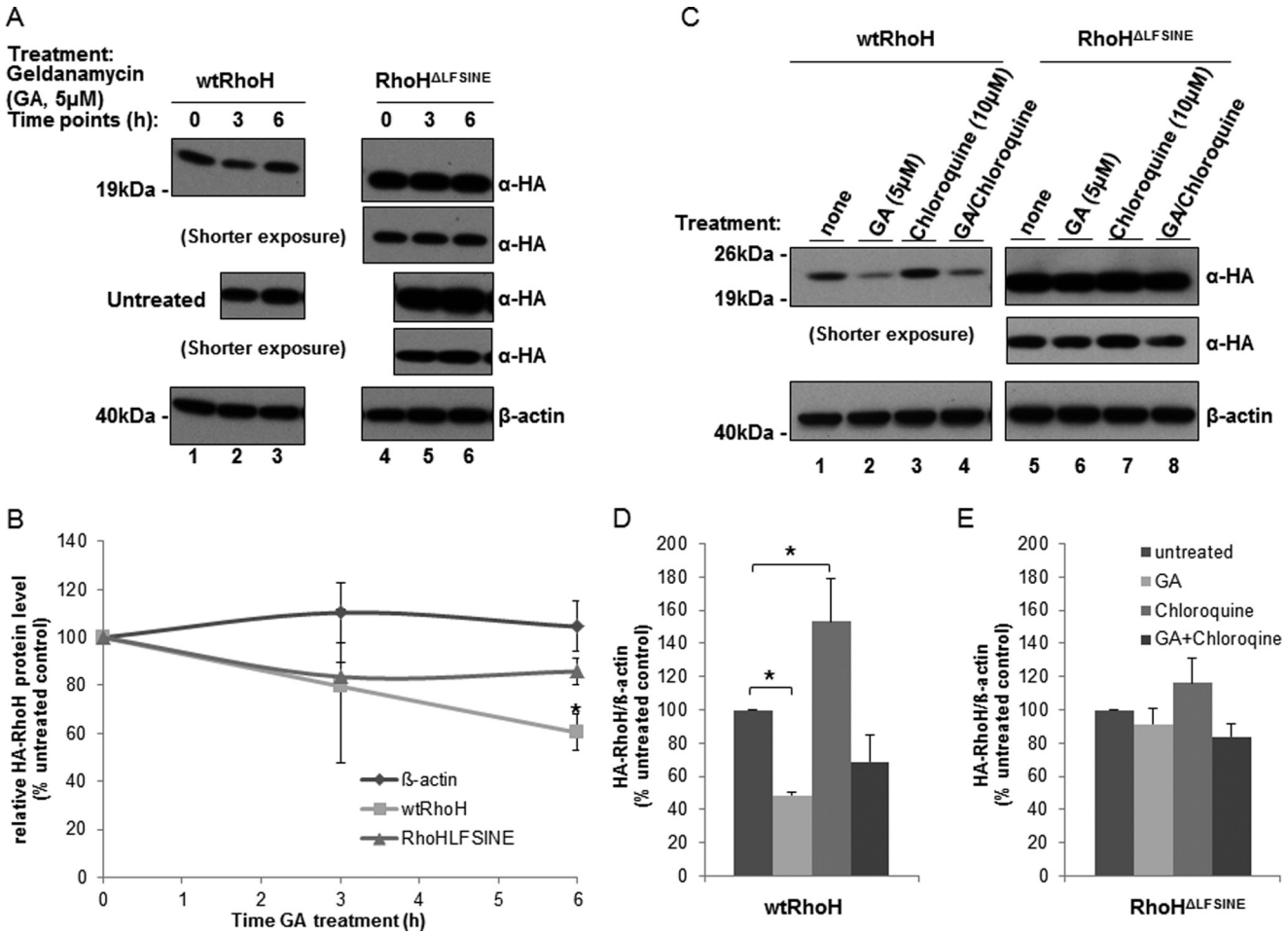

FIGURE 3. **Inhibition of the chaperone-mediated autophagy pathway prolongs the half-life of wtRhoH.** *A,* HA-tagged wtRhoH and RhoH$^{\Delta LFSINE}$-expressing Jurkat T cells were incubated with the Hsp90 inhibitor GA or left untreated for the indicated time points and levels of HA-RhoH and β-actin (internal control) were analyzed by immunoblotting. RhoH was detected using anti-HA antibody. Respective lanes were all run on the same blots. *B,* quantification of HA-RhoH in relationship to β-actin over time relative to untreated control is shown as mean ± S.E. of three experiments; *, $p < 0.05$; Student's *t* test. ◇, β-actin; □, wtRhoH; △, RhoH$^{\Delta LFSINE}$. *C,* HA-tagged wtRhoH and RhoH$^{\Delta LFSINE}$-expressing Jurkat T cells were incubated with geldanamycin and/or chloroquine for 6 h and levels of HA-RhoH (anti-HA antibody) and β-actin (loading control) were analyzed by immunoblotting. Respective lanes were all run on the same blots. *D,* densitometric measurements of immunoblot bands indicating the ratio of the wtRhoH protein to β-actin loading control in relationship to the untreated control sample. *Bar graphs* indicate mean ± S.E. of three independent repeat experiments; *, $p < 0.05$, Student's *t* test one sided. *E,* densitometric measurements indicating the ratio of the RhoH$^{\Delta LFSINE}$ protein to β-actin loading control in relationship to the untreated control sample. *Bar graphs* indicate mean ± S.E. of three independent repeat experiments.

Treatment with GA significantly decreased wtRhoH protein levels compared with untreated controls at 3 and 6 h ($p = 0.02$ at 6 h) but had negligible effect on RhoH$^{\Delta LFSINE}$ protein levels (Fig. 3, *A–C*). This suggests that wtRhoH interacts with chaperone molecules such as Hsp90 that protect it from degradation via the CMA pathway and that reduced RhoH protein levels demonstrated after treatment with GA result from lack of protection from degradation due to disruption of chaperone complexes and activation of CMA. Of note, in *in vivo* biotinylation experiments we have identified members of the heat shock protein family including Hsp90 and Hsp70 as novel interaction partners of RhoH,[3] which is in keeping with our current findings and previous reports (49). The absence of the insert sequence LFSINE appears to retard RhoH degradation via CMA, suggesting that LFSINE may be needed as a recognition signal for lysosomal uptake and degradation.

During CMA, unfolded proteins are recognized by the LAMP-2A receptor on the lysosomal membrane and translocated across the lysosomal membrane for degradation (41–43). We next examined whether wtRhoH degradation was dependent on lysosomal activity. Lysosomal inhibition with chloroquine resulted in a 1.4-fold increase of wtRhoH protein levels in Jurkat T cells ($p = 0.044$), whereas RhoH$^{\Delta LFSINE}$ protein levels were again not affected by chloroquine (Fig. 3, *C–E*). However, GA-mediated wtRhoH degradation was not completely inhibited by co-treatment with chloroquine (Fig. 3, *C*, *lane 4*, and *D*), suggesting that unprotected wtRhoH may also become degraded by proteasomes. As this effect did not occur in RhoH$^{\Delta LFSINE}$ (Fig. 3, *C*, *lane 8*, and *E*), the insert domain may also contain an additional ubiquitination site inducing proteosomal degradation when mislocalized or misfolded (41). To further confirm these findings, HA-wtRhoH-expressing Jurkat cells were next treated with ammonium chloride and leupeptin, which are additional inhibitors of lysosomal degradation or the respective vehicle

[3] A. Mino and D. A. Williams, manuscript in preparation.

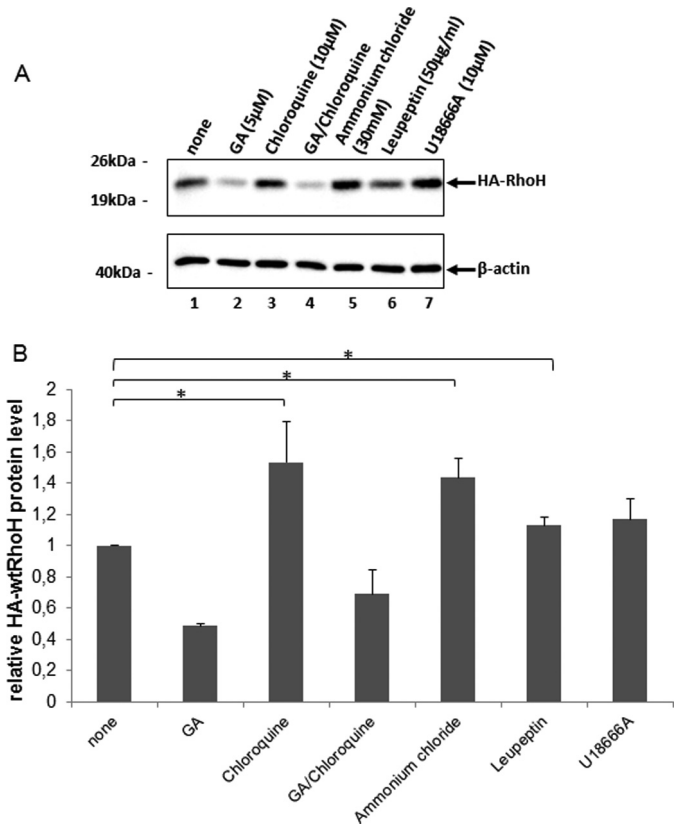

FIGURE 4. **In Jurkat T cells wtRhoH is mainly degraded in lysosomes.** *A*, HA-tagged wtRhoH-expressing Jurkat T cells were incubated for 6 h with vehicle control, lysosomal inhibitors (10 μM chloroquine, 30 mM ammonium chloride, 50 μg/ml leupeptin), and/or GA as well as U18666A (10 μM) to disrupt late endosome lipid dynamics, then cells were lysed and immunoblotted. HA-tagged RhoH was detected by anti-HA-antibody, β-actin was used as loading control. *B*, bar graphs indicate mean ± S.E. of HA-RhoH protein levels in relationship to vehicle control as assessed after densitometric measurement of the respective bands in at least three independent repeat experiments. *, $p < 0.05$, one sided Student's *t* test.

control. wtRhoH protein levels again increased 1.4-fold ($p = 0.012$) and 1.12-fold ($p = 0.032$) (Fig. 4), further suggesting that wtRhoH is degraded via a lysosomal pathway in T cells. In contrast, disruption of late endosome lipid dynamics using 10 μM U18666A did not alter RhoH protein levels ($p = 0.132$) (Fig. 4). These data imply that post-translational degradation of wtRhoH is mainly regulated via CMA. As LAMP-2A receptor-mediated lysosomal protein uptake requires a specific recognition sequence and substrate translocation occurs in a stoichiometric fashion in CMA, the enhanced protein stability of RhoH$^{\Delta LFSINE}$ that is not altered after lysosomal inhibition suggests that LFSINE may contain such an alternative lysosomal recognition sequence. Taken together, these data indicate that wtRhoH becomes degraded mainly via a chaperone-mediated lysosomal pathway in non-activated T lymphocytes.

The Insert Domain Is Dispensable for TCR Signaling and ZAP70 Interaction—RhoH is required for proper localization and activation of ZAP70 and LCK in TCR signaling (8–11). Lack of RhoH results in severe T cell deficiency in mice and is associated with milder T cell defects and persistent EV-HPV infections in humans (45). To further examine the role of the C-terminal domains of RhoH in TCR signaling, we next investigated the interaction of mutant RhoH proteins with ZAP70 as well as the intracellular localization of the mutant proteins in Jurkat cells. To this end, HA-tagged wt and C-terminal-deleted RhoH mutants (as shown in Fig. 1, *A* and *B*) were expressed in Jurkat T cells. wtRhoH and RhoH$^{\Delta LFSINE}$ co-immunoprecipitated with ZAP70 using anti-ZAP70 antibody as demonstrated by subsequent immunoblotting with anti-HA antibody for RhoH (Fig. 5*A, upper panels*). There appeared to be a modest, but reproducible increase in RhoH$^{\Delta LFSINE}$ protein immunoprecipitated compared with wtRhoH protein (Fig. 5*A*, *lanes 1*, *2* and *7*, *8*; and data not shown), which may also partially reflect the larger RhoH$^{\Delta LFSINE}$ protein amount present in transduced cells. However, deletion of the carboxyl terminus and prenylation sequence (RhoH$^{\Delta CT}$ and RhoH$^{\Delta PR}$) was associated with complete loss of ZAP70 binding (Fig. 5*A*, *lanes 3*, *4* and *5*, *6*). As interaction of RhoH with ZAP70 and TCR signaling requires the localization of RhoH to the membrane (10), we next performed fractionation of cell lysates obtained from Jurkat cells into cytosolic, detergent-soluble, and detergent-insoluble membrane fractions to determine RhoH protein localization. Initial experiments demonstrated that both HA-tagged wtRhoH and ZAP70 were localized in the cytosolic but also membrane fractions (Fig. 5*B*). Proper fractionation was further assessed by RACK1 and Flotillin-2 staining (Fig. 5*B*). However, prenylation site-deleted RhoH$^{\Delta CT}$ and RhoH$^{\Delta PR}$ protein was associated with significant reduction in membrane localization as determined by immunoblot analysis (Fig. 5*C*, *lanes 5*, *6* and *8*, *9*). Thus, both lack of proper localization and disrupted ZAP70 interaction render these mutants likely non-functional. In contrast, both wtRhoH and RhoH$^{\Delta LFSINE}$ proteins still localized to the membrane fraction (Fig. 5, *C*, *lanes 2*, *3* and *lanes 11*, *12*). Moreover, RhoH$^{\Delta LFSINE}$ accumulated significantly more than wt protein in the cytosolic fraction (Fig. 5*C*, *lanes 1* and *10*). These findings were also confirmed by immunofluorescence experiments in Jurkat cells transduced with EGFP-fused RhoH mutant proteins. As shown in Fig. 5*D*, the prenylation site and C-terminal-deleted mutant proteins were expressed in the nucleus and cytoplasm but did not localize to the membrane. In contrast, wtRhoH and RhoH$^{\Delta LFSINE}$ proteins co-localized with F-actin and appeared to be located in the cell perimeter consistent with membrane localization. Again, the insert-deleted RhoH mutant demonstrated increased accumulation in the cytoplasm, which was not co-localized with F-actin (Fig. 5*D*). These data suggest that the insert domain critically regulates protein stability, but is not required for membrane localization and moreover is dispensable for ZAP70 interaction and TCR function.

The Carboxyl-terminal Insert Domain Is Dispensable for RhoH Function in T Cell Reconstitution—To confirm the functional implications of the C-terminal sequences of RhoH on normal T and B cell development, we performed *in vivo* rescue experiments using *Rhoh*$^{-/-}$ bone marrow cells. We transduced sorted *WT* and *Rhoh*$^{-/-}$ Lin$^-$Sca1$^+$c-kit$^+$ (LSK) cells with retroviral vectors expressing EGFP-fused C-terminal-deleted mutants of RhoH, wtRhoH or empty vector (Figs. 1*A* and 6*A*) and injected the transduced cells into the tail vein of lethally irradiated B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ recipient mice (Fig. 6*A*). Equivalent transduction efficiency was determined by flow cytometry and qualitative protein expression in transduced LSK cells at the time of transplantation was confirmed by

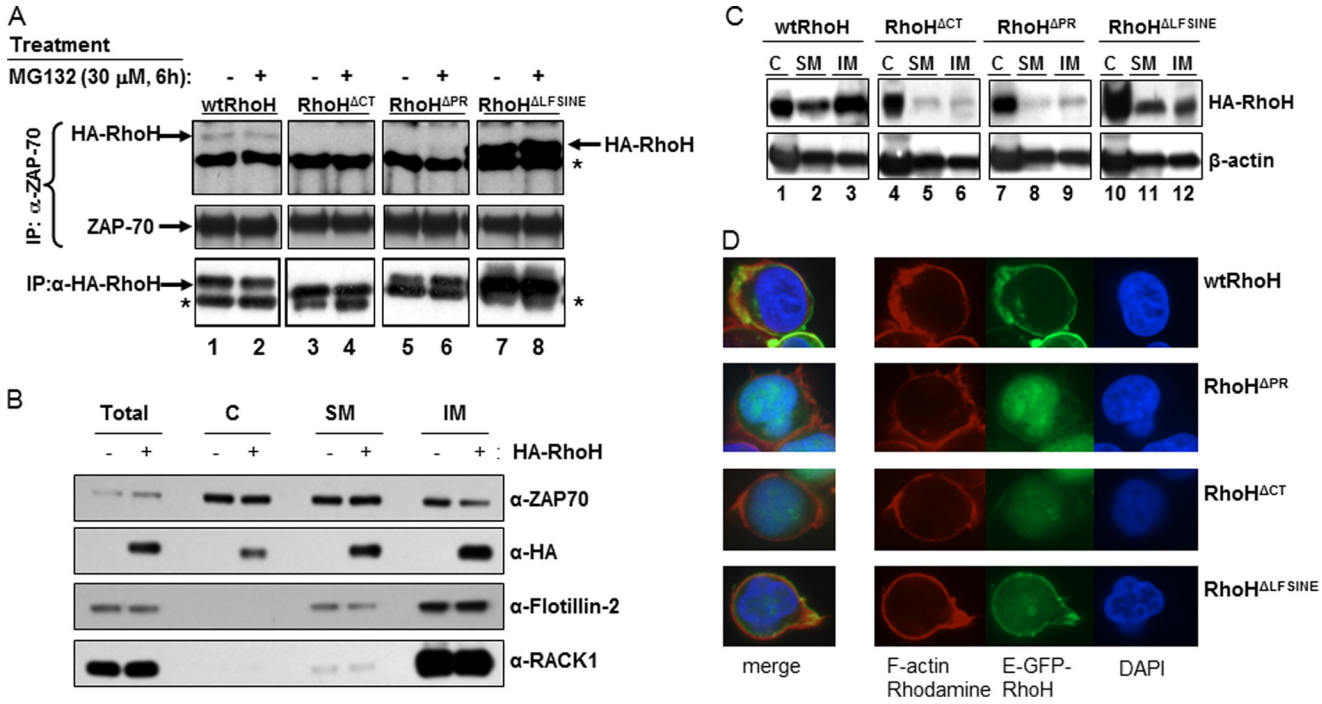

FIGURE 5. **C-terminal sequence of RhoH is critical for interaction of RhoH with ZAP70 and membrane localization.** *A,* HA-tagged wtRhoH, RhoH$^{\Delta CT}$, RhoH$^{\Delta PR}$, or RhoH$^{\Delta LFSINE}$-expressing Jurkat T cells were treated with MG132 for 6 h, then cells were lysed and immunoprecipitation was performed using anti-ZAP-70 antibodies. IP products were analyzed by immunoblotting and co-immunoprecipitated (IP) RhoH detected by anti-HA antibodies. The faster moving IP bands in *lanes 7* and *8* compared with wtRhoH in *lanes 1* and 2 are due to deletion of the insert domain. *Asterisks* (*) denote IgG light chains. Respective lanes were all run on the same blots. *B,* non-transduced or HA-tagged wtRhoH-expressing Jurkat T cells were fractionated into cytosolic (*C*), detergent-soluble membrane (*SM*), and detergent-insoluble membrane (*IM*) fractions. Total lysates and subcellular fractionations were subjected to immunoblot analysis. HA-RhoH was detected with α-HA antibody and ZAP70 by anti-ZAP70 antibody. Staining for the markers Flotillin-2 and RACK1 for lipid rafts and detergent-insoluble cytoskeleton-enriched fractions, respectively, are shown. *C,* HA-tagged wtRhoH, RhoH$^{\Delta CT}$, RhoH$^{\Delta PR}$, or RhoH$^{\Delta LFSINE}$-expressing Jurkat T cells were fractionated into cytosolic, detergent-soluble membrane, and detergent-insoluble membrane fractions and RhoH was detected with α-HA antibody by immunoblotting. Equivalent amounts of each cell fraction have been used to compare subcellular localization of RhoH proteins. Respective lanes were all run on the same blots. *D,* representative confocal images of EGFP-wtRhoH, EGFP-RhoH$^{\Delta CT}$, EGFP-RhoH$^{\Delta PR}$, or EGFP-RhoH$^{\Delta LFSINE}$-expressing Jurkat T cells co-stained for F-actin and DAPI. EGFP-RhoH, *green*; F-actin, *red*; nuclei, *blue*; ×600 magnification. Merged images and single channels are exhibited.

immunoblot (data not shown). Two and three months after engraftment, mice that received Rhoh$^{-/-}$ LSK cells transduced with empty vector or the C-terminal-deleted RhoH mutant (RhoH$^{\Delta CT}$) remained deficient of CD3$^+$ T cells (0.1 ± 0.02 and 0.1 ± 0.01 EGFP$^+$ T cells (K/μl), respectively; $n = 4–5$; mean ± S.E.) (Fig. 6*B* and data not shown). In contrast, re-expression of wtRhoH in *Rhoh*$^{-/-}$ cells resulted in a significant rescue of the T lymphopenia comparable with mice transplanted with *WT* BM cells (0.6 ± 0.1 *versus* 0.9 ± 0.1 EGFP$^+$ T cells (K/μl), respectively). Deletion of the insert domain (RhoH$^{\Delta LFSINE}$) was associated with T cell reconstitution similar to that obtained in LSK cells transduced with wtRhoH (0.5 ± 0.1 *versus* 0.6 ± 0.1 EGFP$^+$ T cells (K/μl), respectively) (Fig. 6*B* and data not shown), indicating that the RhoH$^{\Delta LFSINE}$ protein was still functional in T cell development.

Reconstitution of CD4$^+$ T cells was highly dependent on functional RhoH (Fig. 6*C* and data not shown), whereas re-expression of RhoH in *Rhoh*$^{-/-}$ LSK had a significant, but overall lower effect on development of CD8$^+$ T cells (Fig. 6*D* and data not shown). Both wtRhoH and RhoH$^{\Delta LFSINE}$ normalized CD4$^+$ T cell counts compared with engraftment of WT cells. In contrast, animals transplanted with LSK cells expressing RhoH$^{\Delta CT}$ or empty vector remained deficient in CD4$^+$ T cells (Fig. 6*C*). Expression of wtRhoH or RhoH$^{\Delta LFSINE}$ in *Rhoh*$^{-/-}$ LSK also lead to significantly higher CD8$^+$ T cell numbers compared with empty vector or RhoH$^{\Delta CT}$ (Fig. 6*D*). Normal B cell development was overall only mildly affected by lack of RhoH (data not shown). These *in vivo* data confirm our *in vitro* data and suggest that deletion of the insert domain of RhoH results in a protein that remains functional in T cell development and thus reconstitutes TCR signaling, whereas deletion of the C terminus results in lack of RhoH function in T cells.

Overall, our findings provide novel insights in the importance of the C-terminal sequences for post-translational regulation of RhoH protein levels. Given its deregulated expression and function in lymphoid malignancies, targeting the insert domain to modify protein stability may offer new therapeutic approaches in those disease entities characterized by deregulated RhoH expression.

DISCUSSION

RhoH, similar to other Rnd proteins and oncogenic Ras lacks intrinsic GTPase activity and therefore remains in a constitutively active, GTP-bound state (7, 16, 17, 50). Thus, RhoH-dependent cellular functions appear to be regulated by intracellular protein levels (51). RhoH was initially defined as a fusion partner and subject to somatic hypermutation in B cell lymphomas, although the biological relevance of RhoH mutations, which are mainly found in non-coding regions in these diseases, has never been defined (4–6, 14, 15, 52). Genetic studies suggest that RhoH deficiency is associated with minor changes in normal B cell development, whereas deregulated RhoH expres-

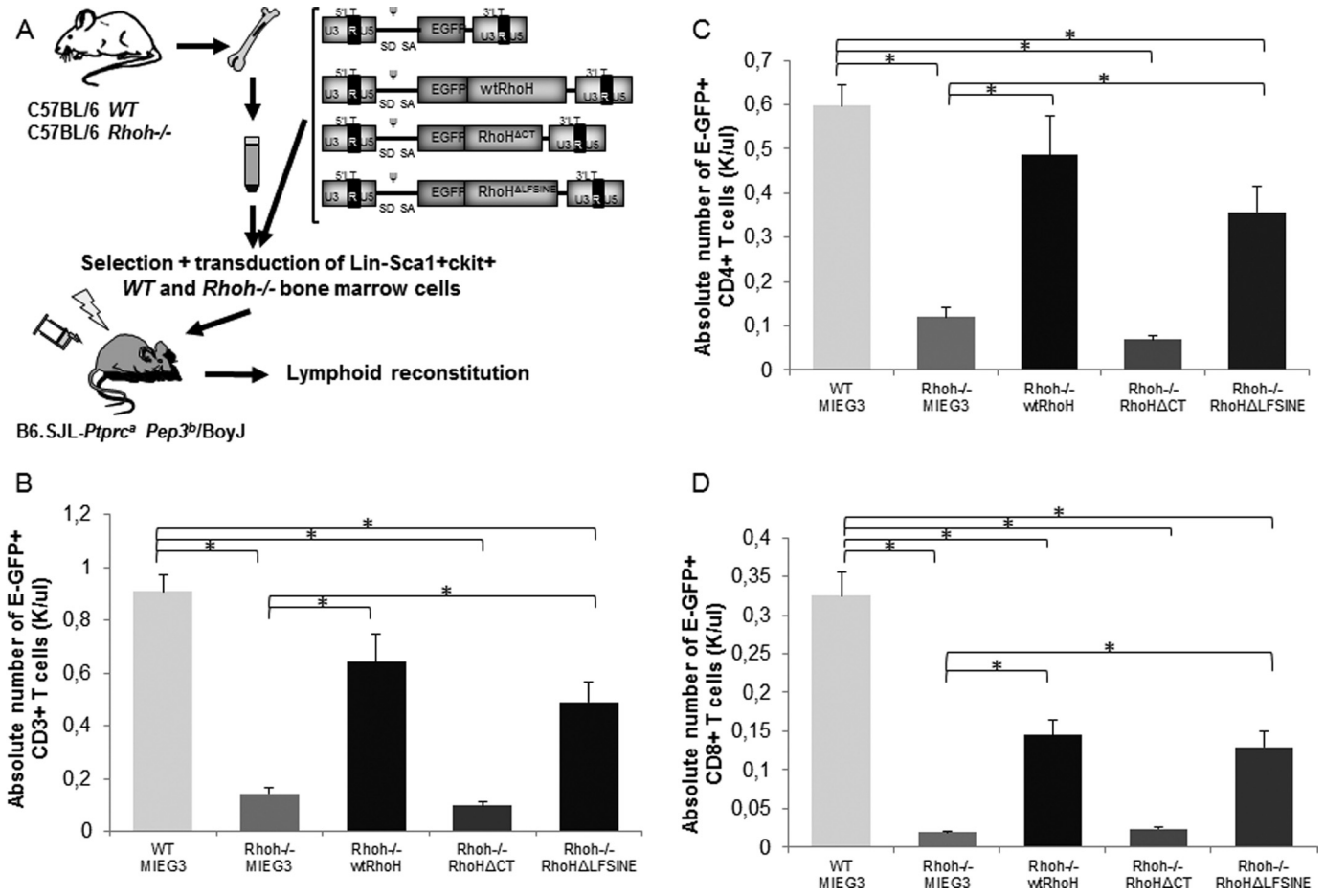

FIGURE 6. **C-terminal sequences of RhoH are critical for T cell reconstitution of *Rhoh*$^{-/-}$ bone marrow cells.** *A,* experimental design of *in vivo* rescue experiments and schematic of retrovirus vectors utilized in this study. *LTR*, long terminal repeats; *SD*, splice donor; *SA*, splice acceptor; *ψ*, packaging sequence. Position of EGFP-fused RhoH cDNA and mutants is shown. *B–D,* flow cytometric analysis showing the absolute number of transduced EGFP$^+$/CD3$^+$ T cells (*B*), EGFP$^+$/CD3$^+$/CD4$^+$ (*C*), and EGFP$^+$/CD3$^+$/CD8$^+$ T (*D*) cells present in the peripheral blood of recipient mice 2 months after transplantation (mean ± S.E., n = 4–5 recipients; *, $p < 0.05$, Student's t test of not equal variance).

sion influences lymphomagenesis and disease progression in CLL (12). Increased RhoH expression levels have been found in human CLL samples in association with high risk features suggesting that deregulated RhoH expression may occur during disease progression (12). We have also demonstrated that in a murine model of CLL, RhoH knock-out leads to delayed disease progression associated with reduced access of malignant cells to protective niches, which contributes to decreased CLL cell survival *in vivo* (12, 13). Furthermore, Lenalidomide, which is effective in treatment of CLL *in vivo* and *in vitro*, results in decreased RhoH expression levels in primary CLL cells (13). Thus, altered expression of RhoH may offer a novel therapeutic approach in B cell malignancies. However, one major concern when applying targeted treatment strategies to alter RhoH levels would be its requirement for TCR function and normal T cell development and any resulting T cell toxicity.

RhoGDIs sequester Rho GTPases such as RhoA, RhoB, RhoC, Rac, or Cdc42 in the cytoplasm and are potential substrates of ubiquitin ligases (3). RhoGDI interaction with RhoA suppresses its activation during T cell anergy, suggesting that ubiquitination pathways impact on Rho GTPase activity (53). Schmidt-Mende *et al.* (48) reported that RhoH becomes degraded within a protein complex following TCR activation in T but not B cells. However, little is known to date about the physiological regulation of RhoH protein turnover in unstimulated lymphocytes.

We provide evidence that the wtRhoH protein is characterized by a half-life of less than 3 h. The C-terminal sequences of Rho GTPases contain polybasic, isoprenylation, and palmitoylation sites and respective lipid modifications regulate their subcellular localization (7, 10, 17, 33, 50). As demonstrated in RhoB, these lipid modifications may also be required for uptake into multivesicular bodies and lysosomal degradation of short-lived proteins (32). However, despite the fact that RhoH exhibited a short protein half-life, inhibition of the proteosomal pathway has no protective effect on wtRhoH protein stability, suggesting that the wtRhoH protein is not primarily degraded in proteosomes. Deletion of the C-terminal sequence (RhoH$^{\Delta CT}$) or isoprenylation site (RhoH$^{\Delta PR}$), which results in mislocalized RhoH protein, is associated with rapid proteosomal degradation of the protein. In contrast, deletion of the insert domain, LFSINE (RhoH$^{\Delta LFSINE}$), significantly increases RhoH protein stability and is not affected by inhibition of either proteosomal or lysosomal degradation (Fig. 7). Deletion of the C terminus or prenylation site abolishes membrane localization and diminishes RhoH interaction with ZAP70. In contrast, specific deletion of

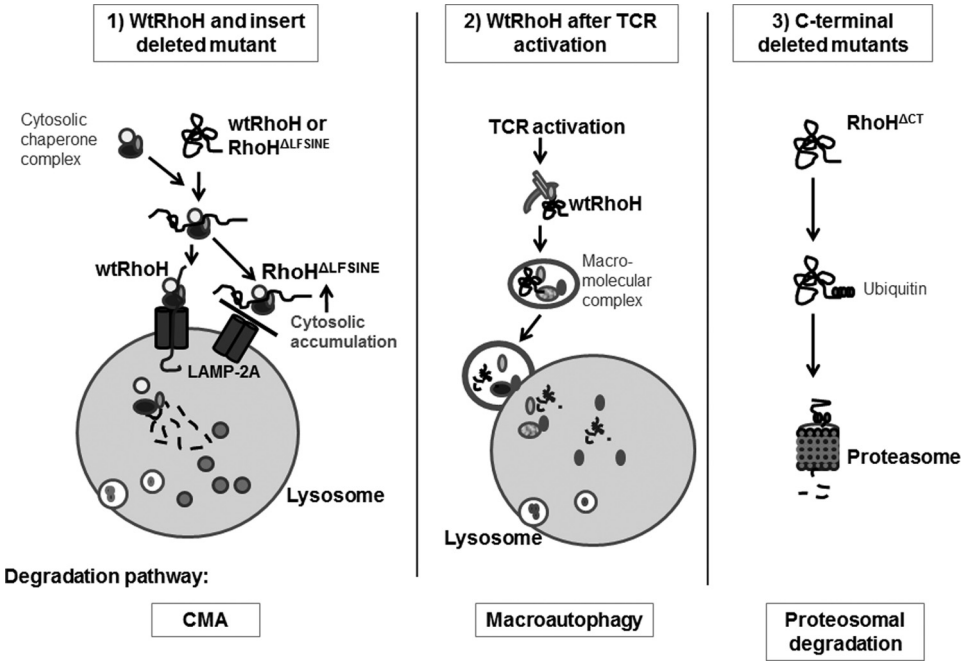

FIGURE 7. **Model of RhoH protein degradation pathways.** *1*, baseline wtRhoH levels are regulated by CMA. Lack of the insert domain LFSINE is associated with cytosolic accumulation of the protein, which is partially reversible by disruption of the interaction with chaperone molecules like heat shock proteins (*e.g.* Hsp90). This suggests that the LFSINE motif serves as a recognition sequence for lysosomal degradation via CMA and cytosolic accumulation occurs due to lack of lysosomal uptake in RhoH$^{\Delta LFSINE}$. *2*, TCR activation results in lysosomal degradation of RhoH within a macromolecular complex as previously shown by Schmidt-Mende *et al. 3,* C-terminal-deleted RhoH mutants RhoH$^{\Delta CT}$ and to a lesser extent RhoH$^{\Delta PR}$ are mislocalized and become mainly degraded via a proteosomal pathway.

the insert domain has no effect on either of these interactions. Furthermore, insert-deleted RhoH functions in a fashion comparable with wtRhoH in *in vivo* T cell rescue experiments further supporting these *in vitro* observations. This suggests that the insert domain may be used to target modifications of RhoH that would change protein levels without affecting its functional role in TCR signaling (8–10).

CMA is a very specific lysosomal degradation pathway where proteins bound to Hsc70-containing chaperone and co-chaperone complexes are recognized by the LAMP-2A receptor via a specific consensus sequence and subsequently degraded in lysosomes (41–43). This degradation pathway appears critical for regulation of wtRhoH homeostasis in non-activated T cells, because wtRhoH accumulates in the cytoplasm after inhibition of lysosomal degradation. Deletion of the insert domain LFSINE inhibits both proteosomal and lysosomal RhoH degradation, indicating that this unique sequence may contain an ubiquitination site. Inhibition of co-chaperone complex formation by treatment with the Hsp90 inhibitor geldanamycin (30) destabilizes the wtRhoH protein, whereas inhibition of the lysosome itself using chloroquine or ammonium chloride delays wtRhoH degradation. However, these treatments have little effect on RhoH$^{\Delta LFSINE}$. These observations suggest that wtRhoH is degraded at least in part via chaperone-mediated autophagy and that the LFSINE sequence is required for lysosomal uptake (Fig. 7). This is of interest, as insights into protein stability and the functional implications of regulatory subunits of RhoH may allow for identification of new drug targets involved in control of protein levels.

Of note, whereas inhibition of lysosomal degradation pathways results in increased wtRhoH protein levels, treatment with proteosomal inhibitors and particularly MG132 significantly reduce the wtRhoH protein in Jurkat cells. Interestingly, interaction and cross-regulation between the proteosomal and lysosomal protein degradation pathways has been demonstrated previously. In neuronal cells pharmacological inhibition of proteosomal activity up-regulates lysosomal enzymes, whereas lysosomal disruptors inhibit both lysosomal and proteosomal degradation. Furthermore, inhibition of lysosomal or proteosomal pathways both lead to up-regulation of Hsc70 levels and induced chaperone-mediated autophagy (39, 40, 54–57). Our studies suggest that CMA is the main mechanism regulating degradation of wtRhoH in non-TCR stimulated T cells. Thus, the negative effect of proteosomal inhibition on wtRhoH protein levels is in keeping with the previous findings that proteosomal inhibition leads to up-regulation of protein degradation via CMA. Lower RhoH protein levels observed after proteosomal inhibition may thus be explained by an increased degradation of wtRhoH via CMA.

Taken together, our studies provide novel insights in the functional importance of the unique C-terminal insert domain of the RhoH protein and its requirement for regulation of protein degradation. We also demonstrate that this sequence is dispensable for RhoH protein interaction with ZAP70 and TCR signaling, as well as for membrane localization *in vitro* and thus for normal T cell development *in vivo*. Overall, we identify a new regulatory element within the RhoH protein that could be utilized to modify RhoH protein levels without impairing vital protein function. This may be of clinical interest in disease entities characterized by deregulated RhoH expression levels, as previously reported in B cell malignancies.

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