Calcium/Calmodulin Regulates Ubiquitination of the Ubiquitin-specific Protease TRE17/USP6*

The TRE17 (USP6/TRE-2) oncogene induces tumorigenesis in both humans and mice. However, little is known regarding its regulation or mechanism of transformation. TRE17 encodes a TBC (Tre-2/Bub2/Cdc16)/Rab GTPase-activating protein homology domain at its N terminus and a ubiquitin-specific protease at its C terminus. In the current study, we identified the ubiquitous calcium (Ca2+)-binding protein calmodulin (CaM) as a novel binding partner for TRE17. CaM bound directly to TRE17 in a Ca2+-dependent manner both in vitro and in vivo. The CaM-binding site was mapped to two hydrophobic motifs near the C terminus of the TBC domain. Point mutations within these motifs significantly reduced the interaction of TRE17 with CaM. We further found that TRE17 is mono-ubiquitinated and promotes its own deubiquitination in vivo. CaM binding-deficient mutants of TRE17 exhibited significantly reduced monoubiquitination, suggesting that binding of Ca2+/CaM to TRE17 promotes this modification. Consistent with this notion, treatment of cells with the CaM inhibitor W7 reduced levels of TRE17 monoubiquitination. Interestingly, the calcium ionophore A23187 induced accumulation of a polyubiquitinated TRE17 species. The effect of A23187 was attenuated in CaM binding-deficient mutants of TRE17. Taken together, these studies indicate a role for Ca2+/CaM in regulating ubiquitination through direct interaction with TRE17.

The covalent modification of proteins by ubiquitination regulates diverse cellular processes such as cell cycle progression, apoptosis, gene expression, and membrane trafficking (1–3). Proteins can be modified by the addition of a chain of multiple ubiquitin molecules (polyubiquitination) or a single ubiquitin (monoubiquitination). The most extensively studied function of ubiquitination is its role in targeting proteins for degradation by the 26 S proteasome. This requires appending of a chain of at least four ubiquitin molecules to a substrate. In recent years, monoubiquitin has been shown to elicit numerous nondegradative functions, including endocytosis, lysosomal targeting, DNA repair, histone regulation and gene expression, and viral budding (4, 5).

Ubiquitination occurs through a mechanism requiring the sequential activity of three enzymes. First, a ubiquitin-activating enzyme (E1) activates the C-terminal glycine of ubiquitin. Next, an ubiquitin-conjugating enzyme (E2) transfers the activated ubiquitin to the substrate, which is bound to a ubiquitin ligase (E3). E3 enzymes fall into two classes, the RING type and the HECT type. With RING-type ligases, ubiquitin is transferred directly from the E2 to the substrate, with the ligase serving as an adaptor to bridge their interaction. In contrast, ubiquitin is covalently linked to HECT ligases through a thiol ester intermediate before transfer to the substrate. A hierarchical organization of these enzymes exists, such that the human genome encodes a single E1 but at least 50 E2 enzymes and ~1000 E3 ligases (1, 3). This diversity of E3 ligases allows exquisite specificity of protein ubiquitination.

Equally important in the regulation of cellular ubiquitination are deubiquitinating enzymes (DUBs), which catalyze the removal of ubiquitin from substrates as well as disassemble ubiquitin chains to replenish intracellular pools (6, 7). Five subclasses of DUBs have been described, the largest and most diverse being the USP (ubiquitin-specific protease) or UBP (ubiquitin-specific processing protease) subclass (hereafter referred to as USP). USPs contain two highly conserved short motifs termed the Cys and His boxes, which encompass key catalytic residues. USPs are believed to target specific protein substrates. Ubiquitin C-terminal hydrolases (UCH) constitute the second class of DUBs and are believed generally to function nonspecifically in the cleavage of free ubiquitin chains. Finally, recent studies have identified the OTU, J Josephin, and JAMM/MPN+ domains as three additional families of deubiquitinating enzymes (6, 7).

As alluded to above, USPs constitute the largest family of DUBs, with over 80 members predicted in humans. Recent work has implicated USPs in numerous processes, including eye development, cell cycle progression, apoptosis, immune signaling, gene silencing, and neurological disease (6, 7). However, in only a handful of cases have in vivo substrates been identified. Several well documented examples include fat facets (which targets liquid facets), HAUSP (which targets p53) (8), the CYLD tumor suppressor (which targets TRAF2) (9–11), and Ubp8 (which targets histone H2B) (12, 13).

Our laboratory has focused on the cellular functions of the USP TRE17 (transfection recombined on chromosome 17; also referred to as USP6 and tre-2). TRE17 was originally identified as an oncogene, based on its ability to induce transformation of murine fibroblasts (14). Recent work has revealed that TRE17 also induces neoplastic growth in humans. The TRE17 locus was found to be a recurring target of chromosomal translocation in an osseous neoplasm termed aneurysmal

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*This work was supported by Public Health Service Grant CA-81415 from NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; USP, ubiquitin-specific protease; GAP, GTPase-activating protein; CaM, calmodulin; Ub, ubiquitin; DUB, deubiquitinating enzyme; UCH, ubiquitin-specific processing protease; UCH, ubiquitin C-terminal hydrolase; GST, glutathione S-transferase; HA, hemagglutinin; MBP, maltose-binding protein; NEM, N-ethylmaleimide; BPATa, 1, 2-bis(aminophenoxyethane-N,N,N',N' tetraacetic acid.
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Bone cyst (15, 16). Five different fusion partners of TRE17 were identified in aneurysmal bone cysts, and in each case "promoter swapping" occurred, leading to aberrantly high expression of TRE17 (15, 17). Despite strong evidence for the role of TRE17 in neoplastic growth in vivo, the mechanism by which it induces transformation remains unknown.

TRE17 encodes a TBC (tre-2/bub2/cdc16)/Rab GTPase-activating protein (GAP) homology domain at its N terminus and a USP domain at its C terminus. Our recent work showed that the TBC domain of TRE17 targets the Arf6 GTPase, which regulates plasma membrane-endosomal recycling and actin remodeling (18). However, TRE17 does not function as a GAP for Arf6. Rather, TRE17 binds directly to Arf6 via its TBC domain and appears to promote recycling of endocytic vesicles to the plasma membrane. Targets of the TRE17 USP domain have not been identified. Furthermore, it is not known whether TRE17 USP activity or effects on Arf6-dependent trafficking are required for transformation.

In the current study, we sought to identify mechanisms of TRE17 regulation. We report herein that the ubiquitous calcium (Ca\(^{2+}\))-binding protein calmodulin (CaM) is a Ca\(^{2+}\)-dependent binding partner of TRE17. We further show that TRE17 is mono- and polyubiquitinated and that its association with CaM promotes these modifications. Our work thus reveals a role for Ca\(^{2+}\)/CaM in regulating ubiquitination through direct interaction with TRE17.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin and streptomycin, and GlutaMax (Invitrogen). Cultures were maintained at 37 °C in 5% CO\(_2\). HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions.

**Plasmids**—Human calmodulin 2 cDNA was isolated by reverse transcription-PCR from total HeLa RNA, and its sequence was confirmed by direct automated sequencing. The entire open reading frame was described previously (18, 19). Point mutants HA-T17(447)/L311D, HA-T17(447)/V306D, and HA-T17(447)/R15A were generated by overlap extension PCR. HA-T17(447)/L311D/pcDNA3, harboring internal deletions of residues 447–506, 447–305, and 447–201, were generated from HA-T17(447)/pcDNA3, and HA-T17(447)/R15A/pcDNA3 was generated by direct automated sequencing. The entire open reading frame was confirmed by direct automated sequencing. The entire open reading frame was described previously and were generously provided by Dr. Mark Hochstrasser (Yale University) (20). Mutation of cysteine 541 to serine in HA-T17(447)/pcDNA3 was added to lysis and wash buffers where indicated. Samples were boiled, fractionated by SDS-PAGE, subjected to immunoblotting, and detected by enhanced chemiluminescence (Amersham Biosciences).

**Monitoring of TRE17 Ubiquitination in Vivo**—HeLa cells were transfected with the various TRE17 alleles and then treated with W7 (100 μM for 40 min) or A23187 (40 μM in the presence of 1 μM extracellular CaCl\(_2\) for 1 h) as indicated. Cells were lysed in (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl\(_2\), 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, 10% glycerol, and 10 mM N-ethylmaleimide plus protease inhibitors). Lysates were immunoprecipitated with anti-HA matrix (Roche Applied Science), washed four times in the same buffer, and then fractionated by SDS-PAGE, subjected to immunoblotting, and detected by enhanced chemiluminescence (Amersham Biosciences).

**RESULTS**

CaM Binds Directly to TRE17 in a Ca\(^{2+}\)-dependent Manner—To identify its binding partners using yeast two-hybrid analysis. Two splice variants of TRE17 have been described, which give rise to the TRE17(long) and TRE17(onco) isoforms (Fig. 1). The DOA4— (Y8), and TRE17(long)/pGEX-KG plasmids have been described previously and were generously provided by Dr. Mark Hochstrasser (Yale University) (20). Mutation of cysteine 541 to serine in TRE17(onco) was achieved with QuikChange (Stratagene, La Jolla, CA) using the following primers: 5' -CAACCTGGGAAAC-ACT-AGTTTCATGAACGTCAAGCATCC-3' (sense) and 5'-GGATTGCTT-GAGTTCATGAAA-CTATGTGTTTCCACAGTTG (antisense).

**Pull-down and Co-immunoprecipitation Assays**—HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Cells were lysed in CaM lysis buffer (phosphate-buffered saline, 500 μM BAPTA, 0.1% Triton X-100, 1 mM dithiothreitol, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) supplemented with the indicated free CaCl\(_2\) concentration. Concentrations of CaCl\(_2\) used to give the desired free Ca\(^{2+}\) concentrations were estimated using MaxChelator software (C. Patton, Stanford University, CA). Free Ca\(^{2+}\) concentrations in the lysis buffers were confirmed using a Fura-2-based fluorescence measurement according to the manufacturer’s (Calbiochem) instructions. Cells were solubilized on ice for 10 min and then pelleted at 16,000 × g for 10 min at 4 °C in a microcentrifuge. An aliquot of the clarified supernatant was removed for direct immunoblotting. The remainder was incubated with glutathione-Sepharose (Amersham Biosciences), CaM-agarose (Sigma), or anti-HA (Roche Applied Science) beads as indicated for 4 h at 4 °C with constant mixing. Beads were washed four times in CaM wash buffer (phosphate-buffered saline, 500 μM BAPTA, 0.01% Triton X-100, 1 mM dithiothreitol, protease inhibitors) supplemented with the appropriate CaCl\(_2\) concentration. N'-Ethylmaleimide (NE) was added to lysis and wash buffers where indicated. Samples were boiled, fractionated by SDS-PAGE, subjected to immunoblotting, and detected by enhanced chemiluminescence (Amersham Biosciences).

**For direct binding of TRE17 to CaM, T17(447) was purified as an MBP fusion from E. coli C60/ in vivo**—Human calmodulin 2 cDNA was isolated by reverse transcription-PCR from total HeLa RNA, and its sequence was confirmed by direct automated sequencing. The entire open reading frame was subcloned into the mammalian expression vector pEBG to generate a CaM BAPTA, 0.1% Triton X-100, 1 mM dithiothreitol, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) supplemented with the indicated free CaCl\(_2\) concentration. Concentrations of CaCl\(_2\) used to give the desired free Ca\(^{2+}\) concentrations were estimated using MaxChelator software (C. Patton, Stanford University, CA). Free Ca\(^{2+}\) concentrations in the lysis buffers were confirmed using a Fura-2-based fluorescence measurement according to the manufacturer’s (Calbiochem) instructions. Cells were solubilized on ice for 10 min and then pelleted at 16,000 × g for 10 min at 4 °C in a microcentrifuge. An aliquot of the clarified supernatant was removed for direct immunoblotting. The remainder was incubated with glutathione-Sepharose (Amersham Biosciences), CaM-agarose (Sigma), or anti-HA (Roche Applied Science) beads as indicated for 4 h at 4 °C with constant mixing. Beads were washed four times in CaM wash buffer (phosphate-buffered saline, 500 μM BAPTA, 0.01% Triton X-100, 1 mM dithiothreitol, protease inhibitors) supplemented with the appropriate CaCl\(_2\) concentration. N'-Ethylmaleimide (NE) was added to lysis and wash buffers where indicated. Samples were boiled, fractionated by SDS-PAGE, subjected to immunoblotting, and detected by enhanced chemiluminescence (Amersham Biosciences).

**Deubiquitination Assays**—Deubiquitinating activity of TRE17 alleles was measured as described previously (20). Briefly, Ub-Met-β-galactosidase/pACYC184 was co-transformed with the specified GST fusion construct into E. coli MC1061. Positive clones were grown overnight and then incubated with 200 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The culture was pelleted, resuspended in sample buffer, fractionated by SDS-PAGE, and then immunoblotted with anti-β-galactosidase (Rockland, Gilbertsville, PA). The DOA4— (Y8), and TRE17(long)/pGEX-KG plasmids have been described previously and were generously provided by Dr. Mark Hochstrasser (Yale University) (20). Mutation of cysteine 541 to serine in TRE17(onco) was achieved with QuikChange (Stratagene, La Jolla, CA) using the following primers: 5' -CAACCTGGGAAAC-ACT-AGTTTCATGAACGTCAAGCATCC-3' (sense) and 5'-GGATTGCTT-GAGTTCATGAAA-CTATGTGTTTCCACAGTTG (antisense).

**Monitoring of TRE17 Ubiquitination in Vivo**—HeLa cells were transfected with the various TRE17 alleles and then treated with W7 (100 μM for 40 min) or A23187 (40 μM in the presence of 1 mM extracellular CaCl\(_2\) for 1 h) as indicated. Cells were lysed in (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl\(_2\), 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, 10% glycerol, and 10 mM N-ethylmaleimide plus protease inhibitors). Lysates were immunoprecipitated with anti-HA matrix (Roche Applied Science), washed four times in the same buffer, and then immunoblotted with anti-Ub or anti-HA.
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TRE17(long) and TRE17(onco) to CaM (Fig. 1B). Binding was not significantly enhanced when Ca\(^{2+}\) was raised further to 100 \(\mu M\) (data not shown). To delineate the site of interaction, we tested a C-terminal truncation mutant that encodes the first 447 amino acids of TRE17 (T17(447)) (see Fig. 1A). T17(447) also bound to CaM in the presence of 1 \(\mu M\), but not 100 \(nM\), Ca\(^{2+}\) (Fig. 1B). Together, these data reveal that TRE17 binds to CaM in a Ca\(^{2+}\)-dependent manner and that this interaction is regulated by physiologically relevant changes in Ca\(^{2+}\) levels. Furthermore, the N-terminal 447 amino acids of TRE17 are sufficient to mediate this interaction.

We next examined whether TRE17-CaM interaction was direct by using recombinant purified proteins. MBP-tagged T17(447) was used, as recombinant TRE17(onco) and TRE17(long) could not be purified from E. coli in sufficient quantities. Purified MBP-T17(447) was incubated with CaM or control agarose beads. As seen in Fig. 1C, MBP-T17(447) but not MBP was specifically retained on CaM beads in the presence of 1 \(\mu M\) Ca\(^{2+}\). As described above, association was significantly reduced when Ca\(^{2+}\) was lowered to 100 \(nM\). These results confirm that TRE17 binds directly to CaM in a Ca\(^{2+}\)-dependent manner.

Association of TRE17 with Endogenous CaM in Mammalian Cells—To determine whether interaction of TRE17 with CaM occurs in vivo, epitope-tagged forms of the proteins were transfected into HeLa cells. CaM was expressed as a GST fusion together with HA-tagged TRE17(long), which is the isoform endogenously expressed in HeLa cells (Ref. 18 and data not shown). Lysates were pulled down with glutathione-Sepharose beads and then probed with anti-HA to detect associated TRE17. As seen in Fig. 2A, TRE17(long) bound to GST-CaM but not GST alone. T17(447) also co-precipitated specifically with GST-CaM (Fig. 2A).

We next wished to confirm whether TRE17 associates with endogenous CaM. HeLa cells were transfected with HA-tagged TRE17(long), TRE17(onco), T17(447), or control vector. Cells were lysed in the presence of 1 \(\mu M\) Ca\(^{2+}\), immunoprecipitated using anti-HA matrix, and then probed with anti-CaM antibody. Endogenous CaM specifically co-immunoprecipitated with TRE17(long) and TRE17(onco) (Fig. 2B). Again, the N-terminal 447 amino acids of TRE17 were sufficient to mediate interaction, confirming our in vitro binding analysis. Similar experiments could not be performed for endogenous TRE17, because anti-TRE17 antibodies were not sufficiently sensitive to detect the endogenous protein. These experiments nevertheless demonstrate that TRE17 peptides associate with endogenous CaM.

Mapping of the CaM-binding Site in TRE17—Multiple CaM-binding consensus sites have been defined, including 1-10, 1-14, and IQ motifs (28). There are two subtypes (1-5-10 and 1-10) of the 1-10 motif and three subtypes (1-5-8-14, 1-8-14, and 1-14) of the 1-14 motif in which the indicated positions are occupied by hydrophobic amino acids. IQ motifs consist of (I/L)QXXX(K/R), where the first position is isoleucine or leucine, the second is glutamine, the last is basic, and X is any amino acid (28). Within T17(447), which is sufficient to bind CaM, there are one IQ, one 1-8-14, and two 1-5-10 motifs (Fig. 3A). To determine whether any of these motifs mediates binding to CaM, a series of nested deletions was generated and co-expressed with GST-CaM in HeLa cells. Glutathione-Sepharose pull-downs were performed, and association of TRE17 mutants was monitored by anti-HA immunoblotting. Although T17(447) bound strongly to CaM, a construct containing the first 325 amino acids (T17(325)) exhibited greatly reduced binding (Fig. 3B). Mutants encoding the N-terminal 201 or 305 amino acids failed to associate with GST-CaM (Fig. 3B). These data suggest that the CaM-binding site resides at or near amino acids 305–325, which comprises the two 1-5-10 motifs (Fig. 3A).

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To pinpoint the site of interaction, internal deletion mutants were generated in the context of T17(447). As shown in Fig. 4A, deletion of amino acids 305–311 or 306–328 abolished binding of T17(447) to GST-CaM. Thus, both of the 1-5-10 motifs could potentially contribute to CaM binding. This was confirmed through analysis of point mutants in which the conserved hydrophobic residues were mutated. Mutation of the first 1-5-10 motif (T17(447)/V306D,L311D) severely inhibited interaction of T17(447) to CaM (Fig. 4B). Similarly, two independent mutants of the second 1-5-10 motif, T17(447)/F328E and T17(447)/L319D,W320D, were also significantly compromised in binding (Fig. 4B). We further confirmed that mutation of this motif within the context of full-length TRE17(onco) and TRE17(long) inhibited CaM binding (Onco/F328E and Long/F328E, Fig. 4C). In contrast, mutation of the putative IQ motif (mutant R15A, Fig. 4B) had no effect on binding of T17(447) to CaM. Taken together, these results indicate that optimal binding to CaM requires both 1-5-10 motifs of TRE17.

**TRE17 Is Monoubiquitinated and Promotes Its Own Deubiquitination in Vivo**—During our immunoblotting analysis, we noted that in addition to peptides of the predicted sizes, both TRE17(onco) and T17(447) gave rise to a species that migrated ~8 kDa larger (see Figs. 1–4). Notably, this slower migrating product was significantly reduced in all CaM binding-deficient mutants of TRE17 (Fig. 4B). Based on its relative molecular mass, we predicted that this band might represent a monoubiquitinated form of TRE17. To test this hypothesis, HeLa cells expressing HA-TRE17(onco) or -T17(447) were lysed in the presence of NEM to inhibit the activity of cellular deubiquitinating enzymes. Lysates were immunoprecipitated with anti-HA, then immunoblotted with anti-Ub or anti-HA antibodies. The slower migrating species of both TRE17(onco) and T17(447) reacted strongly with the anti-Ub antibody (Fig. 5A, arrows). This signal was specific, as a highly expressed control protein, HA-tagged Akt, showed minimal cross-reactivity with the anti-Ub antibody (Fig. 5A).

In contrast to TRE17(onco) and T17(447), ubiquitination of TRE17(long) was not readily detected (Fig. 5B). Because TRE17(long) is catalytically active as a ubiquitin-specific protease, but TRE17(onco) and T17(447) are not (20), we explored the possibility that TRE17(long) might promote its own deubiquitination. If this were true, inactivation of its USP domain should lead to increased monoubiquitination of TRE17(long). We therefore generated a point mutation in which the highly conserved cysteine residue (Cys-541) required for USP activity was replaced with serine (denoted TRE17(long)/USP mutant). This mutation ablated USP activity, GST-TRE17(long) wild type or TRE17(long)/USP mutant is catalytically inactive, we next examined its ubiquitination status in mammalian
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Agents That Regulate Ca\(^{2+}\)/CaM Signaling Modulate Ubiquitination of TRE17—As mentioned above, all of the CaM binding-deficient mutants of TRE17 appeared to have significantly reduced levels of monoubiquitination (Fig. 4B). This raised the possibility that binding of Ca\(^{2+}\)/CaM to TRE17 might promote its ubiquitination. To further explore this possibility, we examined the effects of Ca\(^{2+}\)/CaM regulatory agents on the ubiquitination of TRE17. Treatment of HeLa cells with the CaM antagonist W7 led to a significant decrease in monoubiquitination of HA-TRE17(onco) and HA-T17(447) (Fig. 6A), indicating that this modification requires endogenous CaM activity. Interestingly, treatment of cells with the calcium ionophore A23187 reproducibly induced the accumulation of a higher molecular weight product of TRE17 that was polyubiquitinated (Fig. 6A). Anti-HA immunoblotting of the anti-HA immunoprecipitates confirmed that this band was TRE17 itself and not an associated ubiquitinated protein (Fig. 6B). This was strongly suggested that TRE17(long) promotes its own deubiquitination in vivo.

The experiment described in Fig. 6B also allowed us to ascertain the effect of TRE17 ubiquitination on its association with CaM. As shown in Fig. 6B, the monoubiquitinated but not polyubiquitinated form of T17(447) retained the ability to bind CaM.

CaM Binding-deficient Mutants of TRE17 Exhibit Reduced Ubiquitination—As a complementary approach to examining the role of Ca\(^{2+}\)/CaM in TRE17 ubiquitination, CaM binding-deficient F328E mutants were analyzed further. As seen in Fig. 7, basal and A23187-induced levels of mono- and polyubiquitination were significantly attenuated in the F328E mutants of TRE17(onco) and T17(447). The effects of A23187 and W7 were not completely abrogated, however, consistent with the observation that these mutants exhibited residual binding to CaM (Fig. 4B). These data further support a role for Ca\(^{2+}\)/CaM in promoting TRE17 ubiquitination and indicate that direct interaction between TRE17 and CaM is required.

DISCUSSION

Our studies identify Ca\(^{2+}\)/CaM as a novel regulator of TRE17. Interaction of CaM is mediated by two 1-5-10-type motifs within TRE17. Point mutations in either of these motifs significantly attenuates binding to CaM. Furthermore, through combined analysis of CaM binding-deficient TRE17 mutants and pharmacological agents that modulate Ca\(^{2+}\)/CaM signaling, we show that binding of Ca\(^{2+}\)/CaM promotes...
monoubiquitination of TRE17. Finally, our results indicate that TRE17 promotes its own deubiquitination in vivo.

The precise mechanism by which Ca\(^{2+}\)/CaM promotes ubiquitination of TRE17 remains to be determined. The fact that all TRE17 mutants deficient in CaM binding had significantly reduced levels of monoubiquitination indicates that direct interaction between TRE17 and CaM is required. It is important to note that although the CaM binding-deficient mutants encompass nonconservative substitutions, their global conformation was not perturbed as judged by (a) their grossly normal subcellular localization and (b) their ability to associate with known binding partners, such as Arf6, as efficiently as wild-type TRE17.\(^5\) Two possible mechanisms by which Ca\(^{2+}\)/CaM could promote TRE17 ubiquitination are by inhibiting its USP activity or by promoting its interaction with a ubiquitin ligase. Because TRE17(onco) and T17(447) are catalytically inactive but their ubiquitination is decreased by W7 and enhanced by A23187, the former mechanism is unlikely. However, we have been unable to test this directly, as attempts at measuring TRE17 USP activity in vitro have been unsuccessful using recombinant or immunopurified TRE17.\(^6\) It is possible that TRE17 requires association with cellular co-factors for full enzymatic activity. It also remains to be determined whether TRE17 catalyzes its own deubiquitination or functions through an intermediary DUB in vivo.

Thus, it appears more likely that Ca\(^{2+}\)/CaM promotes monoubiquitination of TRE17 by enhancing its interaction with a ubiquitin ligase. Intriguingly, A23187 induced accumulation of a polyubiquitinated form of TRE17. This result touches upon the poorly understood issue of how proteins are selectively modified by mono- versus polyubiquitination. It has been speculated that this switch may be regulated by the stability of the ubiquitin ligase-substrate interaction (29). For example, a ubiquitin ligase that binds only transiently to its substrate would be able to catalyze the transfer of a single ubiquitin moiety before dissociating. In contrast, ubiquitin ligases that bind stably to their substrates via dedicated domains may be able to catalyze multiple rounds of ubiquitin transfer. Notably, we observed that monoubiquitinated TRE17 retained the ability to bind CaM, which might allow the complex to remain associated with the ubiquitin ligase for further chain elongation. In contrast, the polyubiquitinated form of TRE17 was unable to bind CaM, invoking a possible mechanism for the release of CaM and the ligase.

Future work will determine the functional consequences of TRE17 ubiquitination. Monoubiquitination has been linked to multiple cellular responses, including distinct trafficking events such as endocytosis and lysosomal delivery (4, 5). Thus far, we have observed no overt alterations in the steady state localization of CaM binding-deficient mutants of TRE17.\(^5\) Therefore, if monoubiquitination and Ca\(^{2+}\)/CaM do regulate trafficking of TRE17, they likely have a modulatory rather than an essential role, perhaps by affecting the kinetics of trafficking. Based on its estimated relative molecular mass, the polyubiquitinated TRE17 product appears to be modified by at least three ubiquitins. Chains of four ubiquitins have been shown necessary to target proteins to the protea-

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\(^5\) Y. Ye and M. M. Chou, unpublished observations.

\(^6\) A. W. Lau and M. M. Chou, unpublished observations.

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**FIGURE 6. Ca\(^{2+}\)/CaM signaling promotes TRE17 ubiquitination.** A, HeLa cells were transfected with HA-T17(447) or HA-TRE17(onco). Cells were treated with the CaM inhibitor W7 (W) (100 \(\mu\)M for 40 min) or the calcium ionophore A23187 (A) (40 \(\mu\)M in the presence of 1 mM extracellular Ca\(^{2+}\) for 1 h). Extracts were prepared in the presence of NEM and 1 \(\mu\)M Ca\(^{2+}\), immunoprecipitated with anti-HA, and then immunoblotted with anti-HA or anti-Ub antibodies. B, HeLa cells were co-transfected with HA-T17(447) together with GST-CaM (+) or GST vector (−) and then treated with W7 or A23187 as described in A. Extracts were prepared in the presence of NEM and 1 \(\mu\)M Ca\(^{2+}\), pulled down with glutathione-Sepharose beads (GSH Pdn), and then immunoblotted with anti-HA and anti-GST. Mono- and polyubiquitinated forms of TRE17 are indicated with black and gray arrows, respectively.

**FIGURE 7. CaM binding-deficient mutant of TRE17 exhibits reduced mono- and polyubiquitination.** HeLa cells expressing HA-T17(447) (A), TRE17(onco) (B), and their CaM binding-deficient mutants (447/F328E and Onco/F328E, respectively) were treated with W7 (W) or A23187 (A) as described in the legend for Fig. 6. Lysates were prepared in the presence of NEM, immunoprecipitated with anti-HA matrix, and then immunoblotted with anti-Ub (right panels). Whole cell lysates (WCL) were also probed directly with anti-HA (left panels). In A, a darker exposure of the anti-HA blot is shown (middle panel) to highlight the polyubiquitinated TRE17 species. Mono- and polyubiquitinated forms of TRE17 are indicated with black and gray arrows, respectively.

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\(^5\) Y. Ye and M. M. Chou, unpublished observations.

\(^6\) A. W. Lau and M. M. Chou, unpublished observations.
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some; thus, it is unlikely that this TRE17 species is targeted for degradation. Accordingly, this peptide accumulates to levels detectable by immunoblotting of whole cell lysates with anti-HA without prior treatment of cells with proteasome inhibitors. It is also notable that TRE17 does not appear as a smear or ladder of bands, as is typical for proteins destined for degradation. Rather, a discrete polyubiquitinated product was observed. Polyubiquitinated TRE17 likely has biochemical and signaling properties that are distinct from the monoubiquitinated form. Indeed, previous work has shown that ubiquitin-interacting motifs, such as the UIM and UBA domains, bind with greater affinity to polyubiquitin chains than to a monoubiquitin (30–32). At present, however, we cannot distinguish whether this TRE17 product is modified at a different number of ubiquitin chains than to a monoubiquitin (30–32). First, it is regulated by alternative splicing to give rise to TRE17(long).

However, our current study suggests that Ca2+ and calmodulin may regulate the subcellular distribution of TRE17. The first step in this regulation occurs through specific subcellular localization of USPs (7). In addition, we are investigating whether polyubiquitination of TRE17(onco) can be induced by agonists that activate calcium signaling. Although monoubiquitination of TRE17(onco) was observed under normal growth conditions, it was difficult to detect the polyubiquitinated form unless cells were challenged with ionophore. This may be because of the low abundance of this species, coupled with the highly localized and transient nature of Ca2+ signaling. Thus, the physiological importance of TRE17 polyubiquitination awaits further study.

The characterization of USPs has expanded rapidly in recent years, and multiple modes of regulation have emerged. The most commonly observed mechanism is transcriptional; various USPs have been shown to be expressed in a tissue- or developmental stage-specific manner or in response to hormonal stimulation (7). Several post-translational mechanisms of USP regulation have also been reported. One level of regulation occurs through specific subcellular localization of USPs (7). In addition, regulation of USP activity by interaction with other proteins has been demonstrated. For example, interaction of USP14 with the regulatory particle of the 26 S protease greatly enhances its deubiquitinating activity (33, 34). In yeast, Ubp3 interacts with Bre5 to deubiquitinate Sec23 (35). TRE17 is subject to regulation on multiple levels. First, it is regulated by alternative splicing to give rise to TRE17(long) and TRE17(onco), only the former of which is catalytically active. Second, the subcellular distribution of TRE17 is regulated by growth factors (18, 19). Our current study suggests that Ca2+/CaM-dependent monoubiquitination may serve as an additional post-translational mechanism to regulate TRE17 function.

Acknowledgments—We thank Dr. Gerd Blobel for critical reading of the manuscript and Dr. David Sacks for useful discussions and CaM antibody.

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