Dual Mode of Regulation of Cell Division Cycle 25 A Protein by TRB3

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We have recently demonstrated that TRB3, a novel stress-inducible protein, is an unstable protein regulated by the ubiquitin-proteasome system. The expression level of TRB3 protein is down-regulated by anaphase-promoting complex/cyclosome-cell division cycle division 20 homolog 1 (APC/C^{Cdh1}) through its D-box motif. Here we demonstrate that TRB3 regulates the stability of cell division cycle 25 A (Cdc25A), an essential activator of cyclin dependent kinases (CDKs). The expression level of Cdc25A protein is suppressed by over-expression of TRB3, while knockdown of TRB3 enhances the endogenous Cdc25A expression level. On the other hand, Cdc25A degradation induced by DNA damage is significantly rescued by TRB3. When serine residues in the DSG motif, which is the critical sequences for the degradation of Cdc25A induced by DNA damage, is mutated to alanine (Cdc25ADSG2X), both stimulatory and protective effects of TRB3 on the Cdc25A degradation is disappeared. TRB3 protein interacts with both wild Cdc25A and mutant Cdc25ADSG2X. Expression level of the endogenous TRB3 protein is down-regulated in a genotoxic condition. These results suggest TRB3 is a regulator for adjusting the expression level of Cdc25A both in a normal and a genotoxic conditions.

Key words TRB3; cell division cycle 25 A; DNA damage; degradation; ubiquitin

Cell division cycle 25 A (Cdc25A) is one of the highly conserved dual specificity phosphatases that activate cyclin dependent kinase (Cdk) complexes to regulate the cell cycle progression.2) Cdc25A activates Cdk2–cycin E and Cdk2–cycin A complexes during G1/S transition,3,4) and contributes the G2/M transition by activating Cdk1–cycin B complexes.5) Cdc25A expression is precisely regulated by ubiquitin-mediated proteolysis in both a normal cell cycle and a genotoxic stress condition.6) Cdc25A ubiquitination is mainly mediated by two ubiquitin ligase complexes; anaphase promoting complex/cyclosome-Cdh1 (APC/C^{Cdh1}) for its destruction during mitotic exit and early G1,7) and S-phase kinase-associated protein 1 (Skp1)–Cul1–Fbox-beta-transduction repeat-containing protein (β-TrCP) (SCF^{β-TrCP}) for its proteolysis during G1, S and G2.9) Binding of Cdc25A to APC/C^{Cdh1} is dependent on a KEN box without post-translational modifications,9) while interaction with SCF^{β-TrCP} requires the phosphorylation of serine residues within a so-called DSG motif.10)

Cdc25A plays a role in the checkpoint response to unreplication or DNA damage.6,11) Cdc25A is a phosphorylation target of checkpoint kinases (Chks) and is regulated by Chk kinases in response to DNA damage to degrade via the ubiquitin-proteasome pathway. Cells resistant to degradation of Cdc25A display defects in checkpoint arrest to lead the genomic instability.10)

In Drosophila, reinitiation of mitosis is controlled by regulated expression of Cdc25.12) It has been reported that tribbles, an atypical member of the protein kinase superfamily, acts by specifically inducing degradation of string, one of the Cdc25A orthologs in Drosophila, via the proteasome pathway and delayed G2/M transition.13)

We previously identified TRB3, as one of the human orthologs of tribbles, which induced by endoplasmic reticulum stress and contributed to cell growth inhibition.14) Tribbles and TRB3 contain the classic substrate-binding domains of a protein kinase but not the ATP-binding and kinase-activating domains; therefore, they do not have a kinase activity.15) Recently we have reported that TRB3 is an unstable protein regulated by the ubiquitin-proteasome system.16) The expression level of TRB3 protein is down-regulated by anaphase-promoting complex/cyclosome Cdh1 (APC/C^{Cdh1}) which is a key ubiquitin ligase complex, which regulates the progression of the cell cycle.

In this study, we found that TRB3 interacts with Cdc25A to destabilize its protein in a normal condition, however prevented its degradation in response to DNA damage.

MATRIALS AND METHODS

Reagents Dulbecco’s modified Eagle’s medium, anti-β-actin monoclonal antibody (AC-15), and anti-FLAG monoclonal antibody (M2) were purchased from Sigma. Fetal bovine serum was from HyClone (Logan, UT, U.S.A.). MG132 was obtained from Peptide Institute (Osaka, Japan). Anti-Cdc25A polyclonal antibody (M-191) was from Santa Cruz (Santa Cruz, CA, U.S.A.). Anti-Myc monoclonal antibody (9E10) and anti-HA monoclonal antibody (12CA5) were from Roche (Indianapolis, IN, U.S.A.). Anti-green fluorescent protein (GFP) monoclonal antibody (JL8) was from Clontech (Mountain View, CA, U.S.A.). Anti-p21 monoclonal antibody (95-345) was from Upstate (Lake Placid, NY, U.S.A.). The antisera against human TRB3 was prepared as described previously.15)

Cell Culture The embryonic kidney cell line 293, the human cervical carcinoma cell line HeLa and human melanoma cell line A375 were cultured as described previously.15)

Construction of Expression Plasmids The plasmids

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pCMV5-Flag-TRB3 was constructed as described previously. The plasmids pCMV5B-Flag-Cdc25A, pCMV5B-Myc-Cdc25A, pCMV5B-Flag-Cdc25A(1-260) lacking amino acids (aa) 261-525, pCMV5B-Flag-Cdc25A(261-525) lacking aa1-260, pCMV5B-Flag-Cdc25A(331-460) lacking aa1-330 and 461-525, pCMV5B-Flag-Cdc25A(461-525) lacking aa1-460 or pCMV5B-Flag-Cdc25A(331-525) lacking aa1-330, pCMV5B-Flag-Cdc25A(D8GX), replacing Ser82 and Ser88 with Ala, pCMV5B-Flag-Cdc25A(KEN2mt), replacing Lys141, Glu142 and Asn143 with Ala were generated by polymerase chain reaction (PCR). pMT-123 (HA-Ub) was kindly provided by Dr. D. Bohmann (University of Rochester Medical Center). All constructs were verified by sequencing.

**Immunoprecipitation and Western Blot Analysis**

Cells were transiently transfected and treated as described in the Figure Legends. The lysates were subjected to immunoprecipitation, and 1—2% of the lysate or co-immunoprecipitates was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (5—12.5%), transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with the antibodies indicated in the Figure Legends. The immunoreactive proteins were visualized using ECL (Amersham Bioscience) or Immobilon (Millipore) Western blotting detection reagents, and light emission was quantified with a LAS1000 lumino image analyzer (FUJI, Japan).

**RNA Interference**

Double stranded RNA duplexes corresponding to human TRB3 was obtained from Dharmacon Inc. (Chicago, IL, U.S.A.).

**Transfection**

293 and HeLa cells were transected using the Chen-Okayama method as described previously. For RNA interference, HeLa cells were transected using a lipofection method with Lipofectamine 2000 (Invitrogen).

**RESULTS**

**TRB3 Negatively and Positively Regulates the Stability of Cdc25A Protein**

To determine the TRB3 effect on the Cdc25A stability, we first examined the co-transfection experiment in HeLa cells. Over-expression of TRB3 resulted in decreased Cdc25A steady-state protein levels (Fig. 1A, lane 3), which accompanied by accumulation of polyubiquitin-reactive signals in the Cdc25A immune-complexes (Fig. 1B). We also investigated whether TRB3 depletion affects the Cdc25A protein stability. As shown in Fig. 1C, endogenous TRB3 silencing by small interfering RNA (siRNA) in HeLa cells resulted in increased endogenous Cdc25A steady-state levels. Similar result has been observed when endogenous TRB3 was knock-downed in HepG2 cells. Cdc25A is one of the well-known targets of the DNA damage to induce cell cycle arrest. The expression level of Cdc25A protein is remarkably decreased, when cells are treated with doxorubicin, an anticancer drug that leads to DNA double-strand breaks (Fig. 1A, lane 5). Strikingly, TRB3 rescues the breakdown of Cdc25A protein induced in response to DNA damage (lane 6). These results indicate that TRB3 regulates the Cdc25A stability negatively in a normal condition and positively under the genotoxic stress.

**Cdc25A Physically Interacts with TRB3**

As TRB3 is considered to be a regulator of Cdc25A, we next examined whether TRB3 can interact with Cdc25A. Cell extract was prepared from 293 cells co-expressed with Myc-TRB3 and Flag-Cdc25A, followed by immunoprecipitation-Western blot analysis. As shown in Fig. 2A, TRB3 was found to interact with Cdc25A. We next assayed the interaction of various Cdc25A deletion mutants to map the region responsible for TRB3 binding. The deletion mutant with C-terminal half of Cdc25A (aa 261-525) is relatively stable and shown to be interacted with TRB3 (Fig. 2B). Further experiments demonstrated that the region aa 331-460 is crucial in TRB3 binding (Fig. 2C). On the other hand, the N-terminal half region of Cdc25A (aa 1-260) is not detected even in the presence of a
proteasome inhibitor, MG132, so it is hard to consider whether N-terminal region is necessary for TRB3 association from this experiment.

Role of DSG Motif and KEN Box of Cdc25A in Its Down-Regulation by TRB3

Cdc25A is constantly tuned over in cycling cells. Two different ubiquitin ligases (SCFβ-TrCP and APC/C(Cdh1) complex) are known to be involved in Cdc25A turnover, and interaction with these complexes requires specific recognition motifs in Cdc25A. One is DSG motif for SCFβ-TrCP binding, and the other is KEN box for APC/C(Cdh1). To examine the possible involvement of these regions in the unstabilizing effect of TRB3, we determined the effect of mutation of these motifs (Cdc25ADSG2X and Cdc25AKEN2mt). These mutants are quite stable and still interact with TRB3 in 293 cells (Figs. 3A, B). When Myc-TRB3 is co-expressed with Flag-Cdc25A^DSG2X, its expression level remained unaffected compared to that of single transfection (Fig. 3C). Likewise, over-expressed TRB3 did not change the expression level of KEN2 mutated Cdc25A either (Fig. 3D). These results suggest that DSG and KEN motifs in Cdc25A are involved in the TRB3 effect of its unstabilizing.

TRB3 Is Down-Regulated by Genotoxic Stress

We have already shown that TRB3 is markedly induced by endoplasmic reticulum (ER) stress via induction of stress-related transcription factors, activating transcription factor (ATF) and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP). To determine whether the expression
As Cdc25A is an essential activator of cyclin-dependent kinase during normal cell-cycle progression, it is strictly regulated at the protein level, being periodically synthesized and degraded via ubiquitin-proteasome pathway.\(^4,8,19\) Cdc25A is also thought to be one of the proto-oncogenes because of its transformation ability\(^{20}\) and its over-expression in many cancers, both at mRNA and protein levels.\(^{21}\) This deregulated expression may be due to anomalous E2F1/c-Myc transcriptional activity or alternatively to a reduced rate of protein degradation. Our results demonstrated that TRB3 down-regulates the Cdc25A expression level in a normal condition and recovers its degradation induced in response to DNA damage, suggesting that TRB3 could be a crucial regulator of Cdc25A for fine-tuning of its abundance.

In Drosophila, reinitiation of mitosis is regulated by Cdc25 expression level.\(^{12}\) One of the Cdc25 orthologs in Drosophila, string is expressed zygotically in the embryo. It has been reported that tribbles, an atypical member of the protein kinase superfamily, acts by specifically inducing degradation of string via the proteasome pathway and delayed G\(_2/M\) transition.\(^{13}\) Our observation also demonstrates the Cdc25A unstabilizing effect of TRB3, one of the tribbles orthologs at the steady state. The TRB3 action to Cdc25A could be also shown and involved in the oocyte maturation.

We have previously showed that TRB3 is markedly induced by various ER stresses.\(^{44}\) However, genotoxic stress was reported to down-regulate TRB3 mRNA expression.\(^{22}\) Consistent with this, we demonstrate that the protein level of Cdc25A is also decreased in response to DNA damage induced by an anticancer drug, doxorubicin. This result indicates that under the genotoxic condition, the suppressive effect of TRB3 on the Cdc25A proteolysis is usually down-regulated due to the reduction of TRB3 expression itself. On the contrary, these finding support the idea that under the various stressful conditions (ER stress, hypoxia, amino acid deprivation, oxidative stress, etc.) to induce the TRB3 expression, Cdc25A protein would be stabilized even under the genotoxic conditions by up-regulated TRB3 and the checkpoint function will be lost, thus resulting in genomic instability and cancer predisposition. Multiple primary human lung, colon, and breast tumors express high levels of TRB3 transcript.\(^{23,24}\) It is possible that the accumulation of Cdc25A protein by TRB3 over-expressed in multiple human tumors and tumor-derived cell lines is involved in the tumorigenesis and malignant alteration of cancer. Further study is necessary to clarify whether over-expressed TRB3 is contributed to tumorigenesis.

We show that TRB3 interacts with the region aa 331-460 of Cdc25A. This region is the part of catalytic domain of Cdc25A, which raises the possibility that TRB3 regulates the phosphatase activity of Cdc25A and its binding ability to CDK/cyclin complexes as well. TRB3 can also associate with KEN or DSG mutants of Cdc25A, indicating that TRB3 might not compete with \(\beta\)-TrCP or Cdh1 for binding to Cdc25A via these motifs. However, the unstabilizing activity of TRB3 to these two Cdc25A mutants are not observed at all, suggesting that these motifs are crucial to facilitate the unstabilizing of Cdc25A protein by TRB3 and that \(\beta\)-TrCP and/or Cdh1 are involved in this effect.

We have previously demonstrated that TRB3 is a short-lived protein and its steady-state level is balanced through proteasome-dependent degradation, which is facilitated by APC/C\(^{Cdh1}\).\(^{16}\) APC/C is a key ubiquitin ligase complex, which regulates the progression of the cell cycle by control the ubiquitination and subsequent degradation of a number of core cell-cycle regulators. As previously mentioned, APC/C\(^{Cdh1}\) also regulates the Cdc25A stability in a normal condition at mitotic exit and in early G\(_1\). Taken together, it is possible that the expression of TRB3 is periodically regulated in cell cycle, resulting the Cdc25A stability is also time-dependently modified.

Important question still remain unsolved. Why TRB3 regulates differently the Cdc25A stability in the cell conditions? A possibility is that the modification mode of Cdc25A protein is different from in a normal condition and under the genotoxic stress. Cdc25A is a well-characterized target of Chk1/Chk2 and other kinase(s) in response to DNA damage.\(^{12}\) Phosphorylation could alter the interaction or response of TRB3 to Cdc25A, resulting in the different action of TRB3 to Cdc25A stability. A second possibility is that TRB3 may differently influence the function of Cdc25A kinase(s) and Cdh1. Steady state level of Cdc25A is mainly controlled by APC/C\(^{Cdh1}\) through its KEN box. In contrast, in the case of the genotoxic condition, SCF\(^{\beta\text{-TrCP}}\) is mainly responsible for the breakdown of Cdc25A phosphorylated by Chk1 and a recently discovered kinase NEK11.\(^{10,25}\) We have previously demonstrated that TRB3 silencing caused the accumulation of Cdc25A and Cdc20, another well-known target of APC/C\(^{Cdh1}\), in HepG2 cells without any change of Cdh1 expression level, suggesting that TRB3 has some influence on the Cdh1 dependent degradation.\(^{15}\) TRB3 is considered as a pseudokinase, which contains the typical substrate-binding domains, but lack the ATP binding and kinase-activation domains.\(^{15}\) It is possible that some kinase(s) or other modifying enzyme(s) cannot recruit to Cdc25A or upstream kinase(s) when they interact with TRB3, and that TRB3 could be an endogenous kinase inhibitor, acting as a decoy kinase-like protein for upstream kinase(s) for Cdc25A phosphorylation.
In summary, this study provides that the cell-cycle activator, Cdc25A is positively and negatively regulated by stress inducible pseudokinase TRB3 at the protein level. Important roles of TRB3 in Cdc25A implicate the additional function of TRB3 in cell cycle regulation besides the recently described roles in the stress response. These results allowed us to investigate the precise role of TRB3 in cell cycle to understand whether TRB3 contributes to deregulation of DNA damage checkpoints and tumorigenesis.

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