Treatment of PC12 Cells with Nerve Growth Factor Induces Proteasomal Degradation of T-cadherin That Requires Tyrosine Phosphorylation of Its Cadherin Domain

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T-cadherin (T-Cad), a unique member of the cadherin family, is transcribed in a variety of cells. Its expression is regulated at transcriptional level by Dnmt3b and post-translational level by the proteasomal pathway in these cells. Here, we show that T-cadherin expression is also regulated at transcriptional level by DNA methyltransferase 3b (Dnmt3b) during nerve growth factor (NGF) treatment, underscore the significance of its transcriptional repression mediated by Dnmt3b and post-translational degradation through the proteasomal pathway. These data, together with the inhibitory role of T-Cad in neurite outgrowth of PC12 cells upon NGF treatment, underscore the significance of its stringent regulation during this differentiation process.

This article has been withdrawn by the authors. In June 2017, the Journal raised questions about Figs. 1B, 2C, 3A, 3C, 3E, 4C, 5A, 5C, 6B, 7A, and 7D. The original data and originally submitted figures were not available for evaluation. The authors were able to locate some data for repeat experiments conducted at the time of the original work, which they state support the conclusions of the paper, and offered to prepare new figures based on that data. However, the Journal declined that offer, a decision with which the authors disagree. The authors state that any errors in the construction of figures in the paper do not alter the scientific conclusions of the work, and they stand by the reproducibility of the experimental data and the conclusions of the paper.

T-Cad gene is silenced by promoter hypermethylation in a variety of cancers (10, 11), and is associated with poor prognosis of cervical cancer. We have recently identified T-Cad as a novel target of DNA methyltransferase 3b (Dnmt3b) in PC12 cells (4). Dnmt3b represses T-Cad expression that requires histone deacetylation but is independent of de novo DNA methylation (4). Furthermore, ectopic expression of T-Cad inhibits NGF-induced neurite outgrowth, which emphasizes the negative role of T-Cad in this differentiation process.

Ubiquitin–proteasome system is a key player that regulates cellular protein level (for review, see Refs. 12 and 13). It requires multiple steps of enzyme catalysis involving E1, E2, and E3. In this ATP-dependent process involving a series of reactions, ubiquitin is transferred to a lysine residue of the target polypeptide to form a polyubiquitinated protein, which is degraded by the proteasome.

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the 26 S proteasome. This sequential action of enzymes allows different layers of regulation and precise co-operation among components of the protein complex. The substrate specificity of the proteasomal degradation resides in the E3 ligase that recognizes and catalyzes the addition of ubiquitin moiety to the substrate. One of the E3 ligase complexes, the anaphase-promoting complex/cyclosome (APC/C) is coupled to cell cycle progression by targeting different cyclins and cell cycle-regulated proteins to degradation. The activity and substrate specificity of this complex was determined during cell cycle phase transition by interaction of APC/C with Cdc20 in early mitosis and Cdh1 (CDC20 homolog 1) in anaphase and G1. Its substrates include cyclins (e.g. cyclin B) and other cell cycle regulators (for review, see Refs. 12 and 13) as well as cell cycle-regulated proteins such as Dnmt1 (14). The SCF (Skp-Culin-F box protein) complexes contain a core ubiquitin ligase composed of Cul1/Cdc53, Skp1, the Ring finger protein Rbx1/Roc1/Hrt1, and a member of the F-box family of proteins (for review, see Ref. 15). The SCF ubiquitin ligase also has wide spectrum of substrates including IκB, Sic1, G1 cyclins, and other cell cycle-related proteins (16). The Cbf1 family composes single protein Ring finger E3 ligases. These family of proteins are coupled to tyrosine kinase signaling especially in the immune system (17). Ubiquitin E3 ligases exhibit substrate specificity, which bestow the cells different layers of regulation of gene expression upon distinct stimuli.

Here we show that T-Cad is also regulated at the post-translational level by degradation through the ubiquitin-proteasome system, which requires tyrosine phosphorylation of Cdh1 (E3) ligase. These results suggest that T-Cad is regulated at both RNA and protein levels, which contribute to NGF-induced neurite outgrowth.

MATERIALS AND METHODS

ZLLL (MG132), chloroquine, genistein, 4610 and anti-FLAG M2 antibody were purchased from Sigma and lactacystin was obtained from Toronto Research Chemicals, Inc. T-Cadherin antibodies (sc-7940) were from Santa Cruz Biotechnology. P(Tyr) antibodies (py20 and py99) were from Santa Cruz Biotechnology. Ku-70 (N3H10) antibody was from Upstate Biotechnology. Mouse monoclonal antibody to phosphotyrosine (polyclonal) (sc-7941) was from Santa Cruz Biotechnology. Rabbit anti-DDK antibody were purchased from Sigma and lactacystin was obtained from Toronto Research Chemicals, Inc. T-Cad-FLAG expressing PC12 cells untreated or treated with NGF for 2 days were washed in serum- and phosphate-free medium and incubated in the same medium for 1 h, followed by incubation with [32P]orthophosphate (0.5 mCi/ml) (MP Biochemicals) in phosphate-free medium containing dialyzed serum (fetal bovine serum or horse serum) for 4 h. The cells were washed with Tris-buffered saline, lysed in RIPA buffer, and subjected to immunoprecipitation with anti-FLAG antibody. The proteins pulled down were separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to autoradiography using PhosphorImager analysis and Western blot analysis with anti-FLAG antibody. 32P-Signal was quantified using ImageQuant software (GE Healthcare).

Pulse-Chase Experiment

This experiment was done as described earlier (14). T-Cadflag expressing PC12 cells either untreated or treated with NGF for 48 h were labeled with [35S]methionine (1 μCi/ml) (MP Biochemicals) for 1 h in methionine-free medium were washed with phosphate-buffered saline followed by incubation in the complete RPMI medium containing 2 mM methionine. The cell were harvested at 0, 2, 4, and 6 h and equal amounts of protein (250 μg) extracted in RIPA buffer from each group was immunoprecipitated with anti-FLAG antibody. More proteins (500 μg of NGF-treated) were used from NGF-treated cells to make a comparable 0-h T-Cad level. The precipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose...
membrane, and subjected to PhosphorImager analysis. The blot was also subjected to Western blot analysis with the same antibody. $^{35}$S-Signal was quantified using ImageQuant software.

**Ubiquitination in Vivo followed by Co-immunoprecipitation**

Whole cell extracts from T-Cad expressing cells or vector-transfected PC12 cells were immunoprecipitated with anti-FLAG or anti-ubiquitin antibody (Santa Cruz) as described (14). The immune complex eluted in SDS-loading buffer was divided into two equal parts, separated in parallel, transferred to nitrocellulose membrane, and subjected to Western blot analysis with anti-FLAG or anti-ubiquitin antibody.

**Subcellular Fractionation**

T-Cadflag-expressing cells treated with NGF in the presence or absence of ZLLL were homogenized in buffer containing Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM EDTA), 2 mM dithiothreitol, protease inhibitor mixture (Sigma), and centrifuged at 2,000 × g for 5 min to remove intact cells and nuclei. The supernatant was centrifuged at 100,000 × g for 90 min. The pellet (membrane) and supernatant (cytosol) fractions were then subjected to SDS-PAGE and Western blot analysis. Cells that are not treated with NGF were used as controls. Indirect immunofluorescence assay was performed as described (4, 18).

**RESULTS**

**NGF-induced Differentiation of PC12 Cells Causes Rapid Decline in T-Cad Protein Level**—We have previously shown that ectopic expression of T-Cad impedes NGF-induced neurite outgrowth in PC12 cells (4). To study the relationship between T-Cad levels and NGF-mediated neuronal differentiation, we studied the expression profile of T-Cad during this process. Real-time reverse transcriptase-PCR analysis showed that NGF exposure had no significant effect on T-Cad mRNA level after 24 h, which was reduced by only ∼40% after 48 h (Fig. 1A). In contrast, T-Cad protein levels rapidly decreased by ∼70% within the first 24 h and nearly 90% by 36 h (Fig. 1B and C). These data suggested to us that T-Cad expression is regulated post-transcriptionally at the early stage of neurite outgrowth. Like the endogenous T-Cad protein, ectopic T-Cadflag also rapidly decreased with time (by ∼55% and 85% at 36 and 48 h, respectively) in PC12 cells in response to NGF treatment (Fig. 1B and C). The relatively slow rate of depletion of the recombinant protein was probably due to its higher level compared with the endogenous protein. Transcription of ectopic T-Cadflag from the cytomegalovirus promoter was not suppressed by NGF (data not shown). Therefore, decreased levels of T-Cadflag upon NGF treatment further supports the notion that T-Cad is regulated at the protein level in the initial stages of NGF response.

To confirm that the recombinant T-Cadflag was authentic T-Cad, we pulled down the ectopic protein expressed in PC12 cells with anti-FLAG antibody followed by Western blot analysis with T-Cad antibody. The result showed that ectopic T-Cad immunoprecipitated by anti-FLAG antibody was indeed T-Cad (Fig. 1D). Lack of T-Cad precipitation in vector-transfected cells further supports the notion that T-Cad is regulated at the protein level in the initial stages of NGF response.

**FIGURE 1.** T-Cad protein is rapidly degraded upon NGF treatment. **A**, total RNA isolated from cells untreated or treated with NGF for 12 to 48 h were used for real-time quantitative reverse transcriptase-PCR analysis with T-Cad and 18 S rRNA primers. The ratio of T-Cad mRNA to 18 S rRNA in the absence of NGF treatment was assigned the value of 100. B, parental PC12 cells or cells expressing ectopic T-Cadflag were treated with NGF for 12 to 48 h. Whole cell extracts were subjected to Western blot analysis with anti-T-Cad or anti-FLAG antibody and the signal was detected by ECL™ after incubation with horse-radish peroxidase-conjugated anti-mouse secondary antibodies. Ku-70 or the nonspecific protein band (NS) was used as loading control. NS was used for normalization because the amount of proteins loaded in cells expressing T-Cadflag (30 μg) was too low to detect housekeeping genes. C, quantitative representation of the T-Cad level at different time points after NGF treatment. T-Cad level before NGF treatment was assigned a value of 100. The results are mean of three independent experiments ± S.E. D, extracts (50 μg of protein) from vector-transfected or T-Cad expressing cells were used for immunoprecipitation analysis with anti-FLAG antibody followed by Western blot analysis with anti-T-Cad antibody to demonstrate that the identity of the recombinant protein is T-Cad.
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affected cells showed the specificity of the anti-FLAG antibody. Anti-FLAG antibody showed specificity in precipitating and detecting ectopic T-Cad, whereas T-Cad antibody could not efficiently immunoprecipitate T-Cad. We, therefore, used anti-FLAG antibody in subsequent experiments to analyze T-Cad expression PC12 cells.

NGF Treatment Facilitates Proteasomal Degradation of T-Cad in PC12 Cells—To determine whether NGF-induced down-regulation of T-Cad is a translational or post-translational event, we determined its half-life using a pulse-chase experiment. T-Cad expressing PC12 cells in the presence or absence of NGF were incubated with [35S]methionine for 1 h to label the newly synthesized proteins followed by chase with excess unlabeled methionine for different time periods. As expected, [35S] signal in T-Cad pulled down by anti-FLAG antibody decreased with time during the chase period indicating that T-Cad turned over rapidly in NGF-treated cells (Fig. 2, A, top panel, lanes 4–8, and B). In contrast, [35S] signal in control cells was more stable during the chase period indicating a longer half-life (Fig. 2A, top panel, lanes 1–4). The half-life of T-Cad was ~2 h in NGF-treated cells (Fig. 2B). Western blot analysis showed that the total T-Cad level increased slightly with time especially in the 4- and 6-h cells during chase periods, in both groups (Fig. 2A, middle panel, compare lanes 3, 4, and 7, 8 with 1 and 5, respectively). This is perhaps due to its rapid synthesis in cells growing in the complete medium followed with regular sera instead of dialyzed sera used for immunoprecipitating with [35S] methionine. These results altogether suggest that NGF induces rapid degradation of T-Cad.

We next monitored the effect of NGF on endogenous T-Cad in PC12 cells. Because T-Cad in immunoblot analysis we used cycloheximide (CHX) to study the degradation of presynthesized T-Cad degradation. We confirmed protein biosynthesis in PC12 cells (Fig. 2C, left panel, lane 1). Like T-Cadflag, endogenous T-Cad also turned over at a faster rate in differentiated PC12 cells (Fig. 2, C and D), whereas the level of T-Cad remained in control cells were not significantly altered during this time period. This data shows that like T-Cadflag, endogenous T-Cad is rapidly degraded in the presence of NGF.

To identify the proteolytic pathway involved in the rapid turnover of T-Cad, we treated the cells with lactacystin, a specific proteasomal inhibitor, in the presence or absence of NGF. T-Cad protein level was significantly elevated in both untreated (−NGF) and NGF treated (+NGF) cells after treatment with lactacystin for 24 h (Fig. 3A and B). The recovery of T-Cad protein was, however, higher (~2.7-fold) in NGF-treated cells compared with the control cells (1.8-fold). Treatment of cells with chloroquine, a lysosomal protease inhibitor, did not affect T-Cad level (Fig. 3, C and D). To confirm further that T-Cad is indeed degraded through the proteasomal pathway, the cells were treated with another potent proteasomal inhibitor, ZLLL. Pretreatment with ZLLL not only blocked T-Cad depletion but also elevated its level compared with control cells that are not exposed to NGF (Fig. 3E, compare lanes 5, 7 and 9 with lane 1). In fact, T-Cad accumulated linearly with time when protein degradation was blocked by ZLLL (Fig. 3, E and F, y = 7x + 79.53, RSQ = 0.98). These data, taken together, suggest that T-Cad is degraded by the proteasomal pathway, which is further enhanced by NGF treatment.

T-Cad Is Ubiquitinated in PC12 Cells—Ubiquitination marks proteins for proteasomal degradation. It also serves as a signal for the endocytosis of membrane-bound proteins (19). To determine whether T-Cad is ubiquitinated in vivo, cell extracts were prepared in buffer containing 1% SDS followed by heat inactivation of deubiquitinating enzymes. The cell extract was diluted and immunoprecipitated with anti-FLAG (for FLAG-tagged T-Cad) or anti-ubiquitin antibody followed by Western
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confirmed the involvement of proteasome in the turnover of T-Cad under normal physiological conditions. As expected, ubiquitin antibody also pulled down many other ubiquitinated proteins (Fig. 4B, lanes 17–20). T-Cad was not detected in the vector-transfected cells after immunoprecipitation with either anti-FLAG (Fig. 4B, lanes 3, 4, 13, and 14) or anti-ubiquitin (Fig. 4B, lanes 7 and 8) antibody, which confirmed the specificity of immunoprecipitation analysis.

Ubiquitinated T-Cad Accumulates on the Plasma Membrane of PC12 Cells When Its Degradation Is Blocked by ZLLL—T-Cad is linked to the plasma membrane through the glycosylphosphatidylinositol anchor. To determine whether membrane-bound T-Cad is ubiquitinated or it needs to be internalized to the cytosol for ubiquitination and subsequent degradation, S-100 extracts from T-Cad expressing cells either untreated or treated with +ZLLL were fractionated to membrane and cytosolic fractions. T-Cad was detected only in the membrane fractions, which was elevated markedly in ZLLL-treated cells (Fig. 4C, lanes 1, 3, and 5, 7). High molecular weight polyubiquitinated T-Cad was detected only in the membrane fraction of PC12 cells upon inhibition of proteasomal degradation especially after NGF treatment. These results suggest that the membrane-bound T-Cad is preferentially polyubiquitinated.
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FIGURE 5. Cdh1 enhances proteasomal degradation of T-Cad. A, T-Cad was co-transfected with the vector or substrate recognition component of different E3 ligases such as Cbl family of proteins (Cbl-b or Cbl-c), APC/C ligase components (Cdh1-HA or Cdc20-HA), or SCF-E3 ligase complex (Cullin1) into PC12 cells. Whole cell extracts from these cells after transfection were used for Western blot analysis with anti-FLAG (to detect T-Cad) or anti-β-tubulin antibody. B, quantitative representation of the T-Cad level in PC12 cells transfected with various vectors (for review, see Refs. 12, 13, and 20). The substrate specificity of proteasome system involves multiple steps of enzyme catalysis that mediate degradation of proteins by the ubiquitin-proteasome system (for review, see Refs. 12, 13, and 20). The substrate specificity of proteasome system involves multiple steps of enzyme catalysis—Degradation of proteins by the ubiquitin-proteasome system involves multiple steps of enzyme catalysis—Degradation of proteins by the ubiquitin-proteasome system involves multiple steps of enzyme catalysis that mediate degradation of proteins by the ubiquitin-proteasome system (for review, see Refs. 12, 13, and 20). The substrate specificity of proteasome system involves multiple steps of enzyme catalysis that mediate degradation of proteins by the ubiquitin-proteasome system involves multiple steps of enzyme catalysis that mediate degradation of proteins by the ubiquitin-proteasome system. We used Western blot analysis with anti-FLAG to detect T-Cad and anti-β-tubulin as the control. C, the level of T-Cad in vector-transfected cells was taken as 100. D, T-Cad was co-transfected with 1 to 3 μg of the vector or substrate recognition component of different E3 ligase complexes (Cdh1-HA or Cdc20-HA), or SCF-E3 ligase complex (Cullin1) into PC12 cells. Whole cell extracts from these cells after transfection were used for Western blot analysis with anti-FLAG to detect T-Cad and anti-β-tubulin as the control. The level of T-Cad was normalized to that of β-tubulin. The level of T-Cad transfected with Cdh1, Cdc20, or Cdc20 plus Cullin1 was significantly increased compared with only 4% retention after NGF treatment (Fig. 5, panels 7 and 8, and C). Additional domain deletion of preceding cadherin domains also increased the level of T-Cad. E, Western blot analysis of the C-terminal region and glycosylphosphatidylinositol anchoring site was also targeted to the membrane (Fig. 6, panels I–III). Interestingly, the T-Cad mutant (ΔCD2–5) that lacks the N-terminal region preceding cadherin domains was destabilized in PC12 cells and NGF treatment augmented the process further (Fig. 6, B, lanes 13–15, and C). This enhanced degradation is probably due to the inability to localize the truncated protein to the membrane and rapid degradation of the misfolded protein. In contrast, deletion of cadherin domains 2–5, leaving the signal peptide intact (ΔCD2–5), significantly stabilized T-Cad (Fig. 6, B, compare lanes 4–6 to lanes 1–3, and C). No marked differences in the degradation of this mutant were observed before and after NGF treatment (Fig. 6, B and C). Interestingly, deletion of CD3–5 (entire CD3 and CD4 and part of CD5) also facilitated degradation of T-Cad, which was more pronounced after NGF treatment (Fig. 6, B, lanes 10–12, and C). These results suggest that CD2 of T-Cad and the association of T-Cad with the membrane are essential for its NGF-mediated degradation.

Apc/CΔcdh1, the E3 Ligase, Is Required for Proteasomal Degradation of T-Cad—Degradation of proteins by the ubiquitin-proteasome system involves multiple steps of enzyme catalysis (for review, see Refs. 12, 13, and 20). The substrate specificity of the proteasomal degradation resides in the E3 ligase, which recognizes the protein substrate and catalyzes the ubiquitination of specific lysine moieties (21). To identify the E3 ligase that mediates proteasomal turnover of T-Cad we initially focused on the Cbl family of proteins (Cbl-b or Cbl-c) (22), SCF E3 ligase complex (23), Apc/CΔcdh1, and Apc/CΔcd20 E3 ligase complexes (24) that can utilize membrane-associated protein substrates (25–27). For this purpose, T-Cad was co-transfected with expression vectors for Cbl variants (22), or Cdh1, Cdc20 (28), or Cullin1 (29), the polypeptides involved in substrate recognition by these E3 ligase complexes. Western blot analysis showed that relative to the vector-transfected cells, the T-Cad level decreased by ~73% in cells transfected with Cdh1 (Fig. 5, A and B), whereas co-expression of Cdc20, another component of Apc/C ligase, did not exhibit any effect. Surprisingly, over-expression of two of the Cbl family members, Cbl-b and Cbl-c, involved in ubiquitination of the membrane-bound proteins did not affect T-Cad level (Fig. 5, A and B). Similarly, ectopic expression of Cullin 1 (Cul1), the component of SCF complex had no effect on T-Cad level (Fig. 5, A and B). Furthermore, degradation of T-Cad was proportional to the amount of Cdh1 transfected, whereas transfection of Cul1 or Cdc20 had no effect (Fig. 5, C and D). This result reinforces the notion that APC/CΔcdh1 ligase specifically recognizes T-Cad in PC12 cells and induces its proteasomal degradation.

Deletion of Cadherin Extracellular Domain 1 (CD1) Destabilizes T-Cad—To study the molecular signals that are required for proteasomal degradation of T-Cad, we generated different cadherin domain (CD) deletion mutants (Fig. 6A) and determined if these deletions affected the stability of T-Cad. All deletion mutants, except ΔCD1, harbor the N-terminal signal peptide. The level of the wild type T-Cad was reduced with time after CHX exposure in control cells, which was more pronounced in NGF-treated cells (Fig. 6B, upper and lower panels, lanes 1–3, and C). Interestingly, deletion of CD1 (NPS-ΔCD1) destabilized T-Cad in PC12 cells and NGF treatment augmented the process further (Fig. 6B, lanes 7–9). Approximately 25% of the truncated protein was retained after 2 h of CHX treatment in vector-transfected cells compared with only 4% retention after NGF treatment (Fig. 6B, lanes 7 and 8, and C). Additional domain deletion of preceding cadherin domains also increased the level of T-Cad. This result reinforces the notion that Apc/CΔcdh1 ligase specifically recognizes T-Cad in PC12 cells and induces its proteasomal degradation.

To investigate whether different CD deletion mutants of T-Cad are targeted to the membrane, the immunofluorescence assay was performed to identify T-Cad flag. As observed earlier (4), wild type T-Cad localized on the membrane (Fig. 6D, panels I–III). Interestingly, the T-Cad mutant (ΔCD2–5) that lacks the C-terminal region and glycosylphosphatidylinositol anchoring site was also targeted to the membrane (Fig. 6D, panels IV–VI). This observation corroborated an earlier finding that the glycosylphosphatidylinositol anchor is not essential for membrane targeting of T-Cad (30), whereas the N-terminal region is required for its proper localization to the membrane. Consistent with this result, the mutant lacking the signal peptide was retained in the cytoplasm (Fig. 6D, panels VII–IX). As expected, all other mutants with intact N and C termini were also confined to the membrane (data not shown).

Next, we identified the domain of T-Cad that is ubiquitinated in vivo. For this purpose, we treated cells ectopically expressing different mutants with NFG followed by ZLLL, the proteasomal
inhibitor, to allow accumulation of polyubiquitinated proteins. The result showed that the high molecular weight proteins indeed accumulated in cells expressing all the mutants upon treatment with ZLLL (Fig. 6E). These polyubiquitinated forms of the highly unstable mutants were markedly elevated (Fig. 6E, Nsp–CD1, Nsp–CD2–5, and Nsp–CD3–5), whereas that of Nsp–CD2–5 was not apparent (Fig. 6E, Nsp–CD2–5). Some ladders were visible in ZLLL-treated Nsp–CD2–5 only when a much higher amount of protein was loaded (data not shown). Interestingly, the levels of the polyubiquitinated form of different proteins (ΔCD1 > Nsp–ΔCD1 > ΔCD3–5 > WT > ΔCD 2–5, Fig. 6E, right panel) correlated inversely with their stability (ΔCD1 < Nsp–ΔCD1 < ΔCD3–5 < WT < ΔCD2–5, Fig. 6C). These results show that multiple lysines in different cadherin domains of T-Cad can be ubiquitinated in vivo.

Tyrosine Phosphorylation Is Essential for T-Cad Degradation—It is known that post-translational modifications of proteins such as phosphorylation act as signatures for their degradation (31–33). Analysis of the T-Cad primary structure revealed several potential tyrosine phosphorylation sites. To determine whether phosphorylation of T-Cad plays a role in its degradation we treated PC12 cells with the general tyrosine kinase inhibitor genistein followed by Western blot analysis with anti-FLAG antibody. The result showed that pretreatment with genistein significantly stabilized T-Cad in NGF-treated cells (Fig. 7A). This result suggests
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The obvious question was whether the phosphorylation status of T-Cad in PC12 cells was altered in response to NGF treatment. To address this issue, the cells were labeled with 

that tyrosine phosphorylation could, at least in part, trigger the degradation of T-Cad in response to NGF.

NGF-induced degradation of T-Cad suggests an inverse relationship between T-Cad level and differentiation state of PC12 cells. NGF-mediated neuronal differentiation is known to

FIGURE 7. Tyrosine phosphorylation and degradation in response to NGF. A, blocked by genistein, a tyrosine kinase (upper panel) or vector-transfected PC12 cells (lower panel) were untreated or treated with NGF for 24 h and collected at 0, 2, and 4 h. Western blot analysis of immunoprecipitated T-Cad with anti-FLAG antibody and autoradiography. B, Western blot analysis of immunoprecipitated T-Cad with phosphotyrosine-specific antibody showed that T-Cad was tyrosine phosphorylated (pY-T-Cadflag) in control PC12 cells, which was significantly increased (9-fold) upon NGF treatment (Fig. 7C). This data showed that increased tyrosine phosphorylation of T-Cad probably causes its faster degradation upon NGF treatment.

We reasoned that tyrosine phosphorylation at the CD2 domain may initiate T-Cad degradation because its deletion stabilized the protein (Fig. 6, B and C). It was, therefore, imperative to identify signature tyrosine residue(s) in this domain that may be required for this degradation. We focused on tyrosine 327 in this domain that had the potential to be a phosphorylation target by NetPhos2.0 program (www.cbs.dtu.dk/services/NetPhos), and are predicted to be a potential target of the protein tyrosine kinases by the group-based phosphorylation scoring method. Accordingly, we mutated tyrosine 327, and showed that this mutant was resistant to proteasomal degradation (Fig. 7D). This observation suggests that tyrosine phosphorylation at this site acts as a signal to induce its degradation by proteasomal pathway after NGF treatment. As expected, significantly increased polyubiquitinated forms were detected in Y327F mutant relative to the wild type protein (Fig. 7E).

DISCUSSION

In an earlier study we demonstrated that NGF-induced differentiation of PC12 cells causes up-regulation of DNA methyltransferase 3b and down-regulation of DNA methyltransferase 1 and 3a (18). Depletion of Dnmt3b by small interfering RNA retarded the differentiation process. This observation suggested an important role for Dnmt3b and some of its target genes in the differentiation of PC12 cells to neuronal phenotype. Subsequently, we identified T-Cad as a target of Dnmt3b (4). An interesting finding was the suppression of T-Cad promoter by Dnmt3b independent of its catalytic activity (4). The present study demonstrated that T-Cad is also subjected to proteasomal degradation and that NGF enhances this process to maintain a differentiated phenotype. The rapid turnover of proteins by the ubiquitin-proteasome system provides a tool for the regulation of their function in various cellular processes. Tightly controlled regulation at transcriptional and post-translational levels is likely to be essential for the proper function of T-Cad in NGF-induced PC12 cell differentiation. It correlates well with the function of T-Cad as a negative regulator of axon guidance because its overexpression inhibits neurite outgrowth in PC12 cells (4).
propagate signaling cascades initiated by NGF-receptor tyrosine phosphorylation. At least three observations support a critical role of tyrosine phosphorylation, induced by NGF treatment, in the turnover of T-Cad. First, treatment of cells with tyrosine kinase inhibitor genistein abrogates the degradation process. Second, T-Cad is phosphorylated in PC12 cells and the level of Tyr(P) species increased upon NGF exposure. Third, mutation of tyrosine residue at cadherin domain 2 renders this protein significantly resistant to degradation. There are at least six other potential tyrosine phosphorylation sites in T-Cad. It is conceivable that phosphorylation at one or more of these sites can also trigger its degradation. Genistein is a broad-spectrum inhibitor of tyrosine kinases, many of which have been shown to alter protein turnover by a variety of mechanisms. Hence, we cannot rule out the potential role of other modifications in addition to tyrosine phosphorylation in the turnover of T-Cad. Indeed, it is known that T-Cad is subject to other post-translational modifications such as ADP-ribosylation (34).

The factors involved in the proteasomal degradation of T-Cad deserve comment. E3 ligases exhibit substrate specificity, which bestow the cells different layers of regulation of gene expression in response to distinct stimuli. APC/C(Cdh1) specifically induced degradation of T-Cad, as co-transfection of Cdh1 and T-Cad facilitates its turnover, and the degradation of T-Cad is dependent on the amount of co-transfected Cdh1. It is known that APC/C(Cdh1) is coupled to cell cycle and is required for cell cycle progression by targeting cell cycle control protein (21). This E3 complex is also highly expressed in the mitotic neuron and could play a potential role in axonal growth and patterning (35). The factors involved in the proteasomal degradation of T-Cad coupled with the inhibitory role of APC/C(Cdh1) suggest that T-Cad harbors multiple lysines that can be ubiquitinated in vivo. Unstable T-Cad mutants were significantly more polyubiquitinated than the stable ones. Ubiquitination of different deletion mutants of T-Cad, albeit at different levels, suggests that T-Cad harbors multiple lysines that can be ubiquitinated in vivo (Figs. 6 and 7). The question remains whether the accessibility of different lysine residues by proteasomal machinery accounts for the stability of these different mutants. Finally, the membrane-associated proteins can be internalized by both clathrin-dependent and clathrin-independent pathways (39). It would be of interest to elucidate the mechanism by which T-Cad is internalized and determine whether it is regulated by NGF.

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