Physical and Functional Interactions of Neuronal Growth Suppressor Necdin with p53*

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Necdin is expressed in virtually all postmitotic neurons, and ectopic expression of this protein suppresses cell proliferation. Necdin, like the retinoblastoma protein, interacts with cell cycle promoting proteins such as simian virus 40 large T antigen, adenovirus E1A, and the transcription factor E2F1. Here we demonstrate that necdin interacts with the tumor suppressor protein p53 as well. The yeast two-hybrid and in vitro binding analyses revealed that necdin bound to a narrow region (amino acids 35–62) located between the MDM2-binding site and the proline-rich region in the amino-terminal domain of p53. The electrophoretic mobility shift assay showed that necdin supershifted a complex between p53 and its binding DNA, implying that the p53-necdin complex is competent for DNA binding. In p53-deficient osteosarcoma SAOS-2 cells, necdin markedly suppressed p53-dependent activation of the p21/WAF promoter. Necdin and p53 inhibited cell growth in an additive manner as assessed by the colony formation of SAOS-2 cells, suggesting that necdin does not affect p53-mediated growth suppression. On the other hand, necdin inhibited p53-induced apoptosis of osteosarcoma U2OS cells. Thus, necdin can be a growth suppressor that targets p53 and modulates its biological functions in postmitotic neurons.

In the vertebrate central nervous system, neurons withdraw from the cell cycle immediately after differentiation from their proliferative precursors, termed neuroepithelial stem cells. Differentiated neurons are absolutely incompetent to divide even in the presence of extracellular stimuli that promote cell cycle progression of proliferative cells. Therefore, the permanent arrest of the cell cycle is the most fundamental feature displayed by differentiated neurons. However, little is known about the molecular mechanism whereby neurons exit from the cell cycle and remain quiescent all of their lives. In proliferative cells, the cell cycle is strictly controlled by various regulatory proteins. Among them, E2F1 is a principal transcription factor that controls cell cycle progression of dividing cells (reviewed in Ref. 1). In the G1 phase, E2F1 is inactivated by interacting with the transactivation domain of the tumor suppressor p53 (11). Moreover, necdin interacts with the transcription factor E2F1 and suppresses E2F1-dependent transcription (11). These characteristics of necdin resemble those of Rb, although these proteins are structurally dissimilar.

Both necdin and Rb bind to the COOH-terminal transactivation domain of E2F1 (11). This transactivation domain is also a target of MDM2, a cellular oncoprotein product that binds to SV40 large T antigen and adenovirus E1A (11). Although MDM2, unlike necdin, enhances E2F1-mediated transcriptional activation (13). Furthermore, MDM2 interacts with the transactivation domain of the tumor suppressor p53 and represses the p53-driven transcriptional activity (14, 15). There is a substantial degree of homology between the E2F1 and p53 activation domains (13, 14), suggesting a conservation of binding sites for specific proteins. These findings prompted us to examine whether necdin also interacts with p53.

In this study, we demonstrate that necdin does form a specific complex with p53 to modulate p53-mediated biological functions. We have used the yeast two-hybrid system and in vitro binding analyses to map the necdin-binding region on p53. Using cultured cells that are often adopted for functional analyses of p53, we have examined the effects of necdin on p53-mediated transcriptional activation, growth arrest, and apoptosis. The present results implicate that necdin is a novel type of growth suppressor that targets both p53 and E2F1.
Experimental Procedures

Yeast Two-hybrid Assay—GAL4 DNA-binding domain vector (pGBT9) and GAL4 activation domain vector (pGAD424) were purchased from CLONTECH. cDNAs encoding various p53 subsequences were generated from full-length p53 cDNA (a gift from Dr. T. Akiyama, University of Tokyo) by polymerase chain reaction using specific primers and inserted into pGAD424. These vectors were introduced along with pGBT9 harboring neddin cDNAs (11) into Saccharomyces cerevisiae SFY526. Transformants were grown on leucine- and tryptophan-deficient synthetic dropout medium plates, and the colony lift filter assay for β-galactosidase activity was carried out as recommended by CLONTECH. The reaction was evaluated, and the results were separated into four ranks with the time for the appearance of blue colonies at 30 °C, ++, less than 2 h; +, 2–6 h; −, >6 h; −, remaining white over 12 h.

In Vitro Binding Assay—Various fragments of p53 cDNA were subcloned into pMALC2 (New England Biolabs) to make maltose-binding protein (MBP) fusion proteins. The MBP fusion proteins were affinity-purified with amylose resin as recommended by New England Biolabs. Full-length neddin cDNA was subcloned into a baculovirus transfer vector (pBlueBacHis2/2B) (Invitrogen) for expression as a His-tagged fusion protein. The transfected vector and a wild-type baculovirus DNA (Bac-N-Blue AcMNPV) were introduced into Sf21 insect cells to obtain AcMNPV-Ndn. The recombinant His-tagged neddin expressed in AcMNPV-Ndn-infected Sf9 insect cells was purified using ProBond metal-binding resin (Invitrogen). MBP-p53 fusion proteins (1 μg) bound to amylose resin (5 μl) was incubated with purified His-tagged neddin (100 ng) at 4 °C for 30 min in 0.5 ml of binding buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA). The resin was washed twice with binding buffer, and protein bound were eluted with 20 mM maltose. His-tagged neddin was separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membrane (Millipore) by electrophoretic transfer, and detected with an anti-necdin antibody (C2) (1:1000) (7) and peroxidase-conjugated anti-rabbit IgG (Cappel) using chemiluminescence detection methods (17) and incubated in a reaction mixture (20 μl) containing 50 mM EDTA at 4 °C.

Electrophoretic Mobility Shift Assay—The oligonucleotide probe containing the p53-binding site (5′-TACAGAACATGTCTAAGCATGCT-GGGG-3′) was labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase. cDNAs encoding Myc tag p53 proteins were cloned into pRc/CMV (Invitrogen) for expression of Myc-tagged p53 (amino acids 1–393) (pRc-Myc-p53), its NH2-terminal deletion mutant p53 (amino acids 55–393) (pRc-Myc-p53 (55–393)), and p53 (amino acids 55–393) (pRc-Myc-p53 (55–393)). The Myc tag was added to the p53 subsequences using a 6 × Myc tag plasmid (a gift from Dr. M. W. McBurney, University of Ottawa). The expression vectors were transfected into SAOS-2 cells by the calcium phosphate method (16). Nuclear extracts were prepared 48 h after transfection.

Reporter Assay for p53-driven Transactivation—A 2.4-kilobase fragment of human p21/WAF1 promoter (18) (a gift from Dr. T. Akiyama, University of Tokyo) was subcloned into the HindIII site of the luciferase reporter vector pGL2-basic (Promega). To construct the expression vectors for Myc-p53 (amino acids 1–37) (pRc-Myc-p53 (1–37)) and Myc-p53 (amino acids 35–62) (pRc-Myc-p53 (35–62)), respective p53 cDNA inserts in pMALC2 were subcloned into the Myc tag plasmid and then into pRc/CMV. The p53 expression vectors in combination with the expression vector for neddin (amino acids 1–325) (pRc-neddin) (11) or neddin (amino acids 110–325) (pRc-neddinAN) (11) were transfected into ~70% confluent SAOS-2 cells in 35-mm dishes by the calcium phosphate method (16). Transfectants were harvested 36 h after transfection, and luciferase activities were measured with a luminometer (Lumat LB9501, Berthold) by using a reagent kit (Toyo Ink, Tokyo). Transfection efficiency was normalized with cotransfected LacZ reporter plasmid (pRc-LacZ) (11).

Cell Growth Assay—The colony formation assay using SAOS-2 cells was carried out as described previously (11). SAOS-2 cells grown in 60-mm dishes were transfected with pRc-Myc-p53 (5 μg), pRc-neddin (5 μg), or both (5 μg each) by the calcium phosphate method (16). G418-resistant colonies were added to the culture medium 48 h after transfection. The cells were incubated for 14 days, fixed with 10% acetate/10% ethanol for 15 min to visualize the colonies. For bromodeoxyuridine (BrdUrd) labeling, the 293 cells grown on coverslips in 35-mm dishes were transfected with pRc-LacZ (0.5 μg) and combinations of pRc-Myc-p53 (0.8 μg), pRc-neddin (1.6 μg), and pRc-neddinAN (1.6 μg). The empty vector pRc/CMV was added to equalize the amounts of transfected DNA (4 μg assay). BrdUrd was added to the medium (final concentration, 10 μM) 36 h after transfection, and the cells were fixed 2 h later with 70% ethanol containing 20 mM glycine-HCl (pH 2.0) for 25 min at 20 °C. BrdUrd and β-galactosidase were detected by fluorescence immunocytochemistry with an anti-BrdUrd monoclonal antibody (1200) (Roche Molecular Biochemicals) and an anti-β-galactosidase polyclonal antibody (1:2000) (Chemicon). BrdUrd and β-galactosidase were visualized with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (1:500) (Cappel) and rhodamine B-conjugated goat anti-rabbit immunoglobulin (1:500) (Cappel), respectively, using a fluorescence microscope (BX 50–34–FLAD 1, Olympus). BrdUrd-positive cells among 100 β-galactosidase-positive cells were counted.

Assay for p53-dependent Apoptosis—U2OS cells (~70% confluence) grown on coverslips in 35-mm dishes and transfected with pRc-Myc-p53 and/or pRc-neddin. The cells were fixed at 48, 60, and 72 h with 4% formaldehyde (pH 7.4) for 20 min at 4 °C and treated with methanol for 20 min at 20 °C and incubated with the anti-Myc antibody (1:10) and the anti-neddin antibody C2 (1:1000) for 1 h at 20 °C. Myc-tagged p53 and neddin were visualized with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin and rhodamine B-conjugated anti-rabbit immunoglobulin, respectively. For Hoechst dye staining, the fixed cells were treated with 3.3 μM Hoechst 33342 for 15 min at 20 °C and observed with the fluorescence microscope. p53-immunopositive cells in 10 nonadjacent fields (total area, 10 mm2) were counted. Statistical significance was tested using Student’s t test.

Results

Necdin Interacts with the Transactivation Domain of p53—We first examined whether neddin interacts with p53 by the yeast two-hybrid assay. Because the NH2-terminal sequences of neddin fused to GAL4 DNA-binding protein stimulate the transcription even in the absence of GAL4 activation fusion proteins (11), we used an NH2-terminally truncated form of neddin (amino acids 83–325) as a DNA-binding fusion protein in this assay. As shown in Fig. 1, full-length p53 (amino acids 1–393) strongly interacted with neddin (amino acids 83–325). Because the transactivation domain of p53 is located at the NH2 terminus, NH2-terminally truncated p53 mutants were tested for neddin binding activities. An MD2-binding site-deleted mutant (amino acids 35–393) still retained the ability to bind to neddin, but further deletions (amino acids 55–393 and 75–393) failed to bind to neddin. These results suggest that neddin binds to the transactivation domain, in which a region from amino acids 35–55 is indispensable.

We then tested the in vitro binding between neddin and p53 (Fig. 2). We prepared a series of p53 deletion mutants as MBF fusion proteins and confirmed that purified MBF-p53 fusion proteins had predicted sizes of polypeptides (Fig. 2A). These MBF-p53 fusion proteins were incubated with His-tagged neddin, and formation of p53-neddin complexes in vitro was examined. As shown in Fig. 2B, both p53 (amino acids 1–393) and p53 (amino acids 35–393) bound to neddin, whereas neither p53 (amino acids 55–393) nor p53 (amino acids 75–393) had neddin.
binding activities. An NH₂-terminal region of p53 (amino acids 1–83), which encompasses the transactivation domain, was competent to interact with necdin. An NH₂-terminal subsequence of p53 (amino acids 1–37), which contains the entire MDM2-binding region, failed to interact with necdin, whereas a region (amino acids 35–62) located between the MDM2-binding site and the proline-rich region retained the necdin binding activity. These data are schematically shown in Fig. 2C.

We have previously reported that the large T antigen, E1A, and E2F1 bind to the central domain of necdin (amino acids 83–292) (11). The p53-binding region on necdin was determined using various necdin deletion mutants in the two-hybrid system (Fig. 3). Both necdin (amino acids 83–325) and necdin (amino acids 102–325) strongly bound to p53, but further NH₂-terminal truncations of necdin (amino acids 110–325, 167–325) failed to interact with p53. Although a COOH-terminally truncated form of necdin (amino acids 83–292) retained the ability to bind to p53, a further truncated form of necdin (amino acids 83–279) had no p53 binding activity. These results suggest that the central region of necdin is indispensable for the interaction with p53. This region coincided with the region required for the interactions with the large T antigen (11). Although p53 and E2F1 share the binding domain on necdin, p53 showed a stronger necdin binding activity than E2F1 (Fig. 3).

Necdin Interacts with p53-DNA Complex—We carried out the electrophoretic mobility shift assay to examine whether necdin affects the ability of p53 to bind to its specific DNA sequence (Fig. 4). SAOS-2 cells transfected with cDNAs for Myc-tagged fusion proteins of p53 (amino acids 1–393), p53 (amino acids 55–393), and p53 (amino acids 75–393) expressed the products of predicted sizes (Fig. 4A). These three p53 species, all of which contain the sequence-specific DNA-binding region, formed complexes with p53 site-carrying DNA (Fig. 4B, lanes 3, 6, and 9). The signals of these p53-containing complexes were competed with excess amounts of the oligonucleotide (data not shown). These major bands were supershifted or diminished in density by the anti-Myc antibody (Fig. 4B, lanes 4, 7, and 10), indicating that shifted complexes contain Myc-tagged p53 proteins. Addition of purified His-tagged necdin protein to the reaction mixture supershifted the complexes containing p53 (amino acids 1–393) and p53 (amino acids 55–393) (Fig. 4C, lanes 2 and 4) but not...
the complex containing p53 (amino acids 75–393) (Fig. 4C, lane 6). These results imply that the necdin-p53 complexes are competent for DNA binding. In this analysis, the NH2-terminally truncated mutant p53 (amino acids 55–393), which failed to interact with necdin in the two-hybrid and in vitro binding assays (Figs. 1 and 2), bound to necdin. It seems likely that a potential binding site present in p53 (amino acids 55–74) is manifested by a conformational change due to p53-DNA complex formation in the mobility shift assay. We were unable to reconstitute the necdin-p53 complex competent for DNA binding by using purified MBP-p53 fusion protein instead of nuclear extracts of p53 cDNA-transfected cells (data not shown), suggesting that additional nuclear factors are required for the complex formation.

**Necdin Represses p53-driven Transcription**—We examined the effect of necdin on p53-driven transactivation using SAOS-2 cells. As shown in Fig. 5A, necdin formed a nuclear complex with Myc-tagged p53 in SAOS-2 cells. Necdin bound to full-length p53 (amino acids 1–393) but not to the NH2-terminally truncated p53 (amino acids 75–393). We then transfected full-length necdin and p53 into SAOS-2 cells with a luciferase reporter vector driven by the p21/WAF1 promoter (18), which contains the p53-binding site. As shown in Fig. 5B, necdin had no appreciable effect on the reporter activity in the absence of co-transfected p53, whereas the p53-stimulated activity (6-fold of the basal activity) was suppressed by full-length necdin in a dose-dependent manner. Necdin (amino acids 110–393), which lacks the p53 binding activity, had no suppressive effect. The suppression of p53-dependent transcriptional activity by necdin was not a result of reduced quantities of p53 protein because co-expressed necdin did not affect the p53 protein level in SAOS-2 cells (data not shown). As shown in Fig. 5C, necdin-induced suppression was partially restored by Myc-tagged p53 (amino acids 35–62), which contains the minimal necdin-binding region but not by Myc-tagged p53 (amino acids 1–37) lacking the necdin-binding region. These results suggest that necdin-induced suppression of p53-driven transcription is mediated through the specific sequence of p53 (amino acids 35–62).

**Necdin Has No Inhibitory Effect on p53-induced Growth Suppression**—Both necdin and p53 induce growth suppression of SAOS-2 cells (11, 19). The p21/WAF1 protein is a major effector that mediates p53-induced growth suppression (20), and transcription of the p21/WAF1 gene was suppressed by co-expressed necdin as shown in Fig. 5. Thus, we examined whether the growth suppressive effect of p53 is inhibited by co-expressed necdin in SAOS-2 cells. Both p53 and necdin suppressed the colony formation, and these two proteins in combination reduced the number of colonies in an additive manner (Fig. 6A). In SAOS-2 cells, p53-mediated suppression of colony formation can occur as a result of cell cycle arrest, apoptosis, or both, although it is difficult to differentiate these two phenomena in this system (21). On the other hand, growth of embryonic kidney 293 cells is markedly reduced without inducing apoptosis by overexpressed p53 (22). We thus tested the effect of necdin on p53-induced growth suppression of the 293 cells. The 293 cells were transiently transfected with β-galactosidase-expressing vector and combinations of expression vectors for Myc-tagged full-length p53, full-length necdin, and necdin (amino acids 110–325). The BrdUrd incorporation was analyzed by immunocytochemistry to estimate the cell population in S phase. Both necdin and Myc-p53 reduced the number of BrdUrd-positive cells among β-galactosidase-positive cells, whereas necdin and p53 in combination caused a larger reduction in the number of BrdUrd-positive cells than either protein (Fig. 6B). In contrast, the NH2-terminally truncated necdin (amino acids 110–325), which is incapable of interacting with E2F1, p53, the large T antigen, or E1A (Ref. 11 and Fig. 3), had little or no effect on the cell number in S phase. These results suggest that necdin exerts no inhibitory effect on p53-induced growth arrest, although it suppresses p53-driven transcription.

**Necdin Blocks p53-induced Apoptosis**—Overexpression of p53 in U2OS osteosarcoma cells induces transient cell cycle arrest followed by rapid apoptotic cell death (23). We used this cell line to examine the effect of necdin on p53-induced apoptosis (Fig. 7). U2OS cells were transfected with Myc-p53 alone or in combination with necdin, and morphological changes of p53-expressing cells were examined by fluorescent immunocytochemistry. p53-positive cells were morphologically intact 48 h after transfection and immunoreactive p53 was localized to the...
nuclei (Fig. 7, A and B). Co-expression of necdin had no appreciable effects on the p53-expressing cells. After 60 h, the p53-accumulating cells had abnormal nuclei exhibiting condensation and fragmentation, which are characteristic of apoptosis (Fig. 7, C and D). However, co-expression of necdin blocked the apoptotic changes of p53-positive cells even 72 h after transfection. In these transfectants, both p53 and necdin were present in the nuclei that appeared morphologically intact (Fig. 7, E and F). The protective effect of necdin on p53-inducible apoptosis was quantified by counting the transfectedants having p53-accumulating nuclei (Fig. 7G). The number of p53-positive cells was markedly reduced at 60 and 72 h, but the reduction was significantly restored by co-expressed necdin. These results suggest that necdin inhibits p53-induced apoptosis of U2OS cells.

DISCUSSION

The present study has demonstrated that necdin is a novel type of growth suppressor that interacts with the transactivating domain of p53. Necdin can be placed into a group of p53-interacting proteins such as MDM2 (14, 15), WT-1 (24), Ref-1 (25), p300 (26), and p33ING1 (27), all of which modify p53-mediated biological functions. We found that p53-driven transactivation of the p21/WAF1 promoter was greatly suppressed by necdin (Fig. 6). Furthermore, the necdin-p53 complex was still competent for DNA binding (Fig. 5). These features resemble those of the Rb-E2F1 system. The Rb-E2F1 complex, which is competent for DNA binding, inhibits transcription of various E2F1-responsive genes that are required for DNA replication. By analogy with the Rb-E2F1 system, we
infer that synchronized expression of a battery of genes possessing the p53 site-carrying promoters is controlled by the neclin-p53 complex.

p21/WAF1 is considered as a major mediator through which p53 suppresses cell proliferation (20). Although neclin suppressed p53-dependent stimulation of the p21/WAF1 promoter, neclin had no inhibitory effects on p53-mediated growth suppression of SAOS-2 cells (Figs. 5 and 6). This may be because p53 exerts its growth suppression through a p21/WAF1-independent process in the presence of neclin. However, we cannot rule out the possibility that neclin inhibits growth suppression mediated by p53 that conversely potentiates the growth suppressive effect of neclin. During mouse embryogenesis, the p21/WAF1 gene is expressed during terminal differentiation of skeletal muscles, cartilage, skin, and nasal epithelium, whereas p21/WAF1 mRNA is absent from the embryonic brain and spinal cord (28). Because neclin is highly expressed in postmitotic neurons in the embryonic brain (9), it is possible that neclin substantially suppresses p53-dependent p21/WAF1 expression during neuronal differentiation.

The present study also demonstrated that neclin blocked p53-induced apoptosis (Fig. 7). These characteristics resemble those of the Wilms’ tumor suppressor WT-1. WT-1 binds to p53 and inhibits p53-induced apoptosis without affecting the p53-mediated cell cycle arrest (23). However, WT-1, unlike neclin, shows enhancement of p53-dependent transactivation. On the other hand, apoptosis of HeLa cells mediated by p53 is significantly suppressed by co-expression of Rb, which does not interfere with the transcriptional activity of p53 (29). Thus, neclin may be a novel type of growth suppressor that inhibits both p53-dependent transactivation and apoptosis. The neclin-binding region of p53 was mapped to amino acids 35–55 and 35–62 as determined by the two-hybrid assay and by the in vitro binding assay, respectively. This region resides between the binding site for TATA box-binding protein-associated factor (30, 31) and the proline-rich domain. Recent studies have shown that p53 mutants devoid of the proline-rich domain lack apoptotic activities but can still mediate growth arrest (21, 32), indicating that the proline-rich domain is responsible for p53-mediated apoptosis. Thus, the neclin-binding domain of p53 is appropriately situated to exert negative effects on both transactivation (through the TATA box-binding protein-associated factor-binding site) and apoptosis (through the proline-rich domain).

The neclin gene is expressed in postmitotic neurons but not in proliferative neuroepithelial stem cells (8, 9). Thus, neclin may not modulate p53-mediated cellular responses in the same manner as it does in replicating cells. Although p53 has been implicated in the induction of cell cycle arrest and apoptosis of dividing cells, several lines of evidence suggest that p53 is also involved in differentiation and apoptosis of postmitotic neurons. Infection of cultured cells with a recombinant retrovirus encoding a dominant negative inhibitor of endogenous p53 inhibits neuronal differentiation of PC12 pheochromocytoma cells and protects cultured primary neurons from spontaneous apoptotic death (33). Mice lacking the functional p53 gene have an unusually large number of birth defects such as exencephaly and impaired neural tube closure (34, 35). These findings suggest that p53 plays a regulatory role in directing neurons toward differentiation and death at early stages of neuronal development. Neclin may prevent nascent neurons from p53-mediated apoptosis and keep them viable during neurogenesis. On the other hand, p53 mediates or induces apoptosis of fully differentiated neurons. For example, p53 overexpression by adenovirus-mediated gene delivery induces neuronal death with features characteristic of apoptosis (36). Wild-type (p53+/−) neurons are severely damaged by exposure to excitotoxins such as glutamate and kainate as well as by treatment with the topoisomerase I inhibitor camptothecin, whereas p53-deficient (p53−/−) neurons are resistant to these compounds (37, 38). Because neurons continue to express neclin until late adult stages of mice (8), neclin potentially blocks p53-mediated apoptosis of fully differentiated neurons throughout their lives. We are now studying the functional interactions between neclin and p53 in postmitotic neurons using adenovirus-mediated gene transfer and cultured neurons derived from neclin-deficient mice.

The human neclin gene is localized to chromosome 15q11.2-q12 within the Prader-Willi syndrome (PWS) deletion region.
and contains CpG-rich regulatory sequences in the 5′-end region (39). Recent studies have demonstrated that this gene is maternally imprinted and deleted in PWS (40, 41). Major symptoms of PWS (e.g., hypogonadism, feeding problems, and gross obesity) have been suggested to be hypothalamic in origin. Among discrete brain regions of mice, necdin mRNA is most abundant in the hypothalamus (9). Therefore, it is tempting to speculate that deficiency of necdin gene expression promotes p53-dependent neuronal death in the brain, especially in the hypothalamus, to cause various types of abnormalities seen in PWS. Studies using various neuronal cell lines and necline-deficient mice are currently in progress in our laboratory to clarify physiological and pathological implications of necdin-p53 interactions in neuronal functions.

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