Human Mob Proteins Regulate the NDR1 and NDR2 Serine-Threonine Kinases*

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Human NDR1 (nuclear Dbf2-related) is a widely expressed nuclear serine-threonine kinase that has been implicated in cell proliferation and/or tumor progression. Here we present molecular characterization of the human NDR2 serine-threonine kinase, which shares ~87% sequence identity with NDR1. NDR2 is expressed in most human tissues with the highest expression in the thymus. In contrast to NDR1, NDR2 is excluded from the nucleus and exhibits a punctate cytoplasmic distribution. The differential localization of NDR1 and NDR2 suggests that each kinase may serve distinct functions. Thus, to identify proteins that interact with NDR1 or NDR2, epitope-tagged kinases were immunoprecipitated from Jurkat T-cells. Two uncharacterized proteins that are homologous to the Saccharomyces cerevisiae kinase regulators Mob1 and Mob2 were identified. We demonstrate that NDR1 and NDR2 partially colocalize with human Mob2 in HeLa cells and confirm the NDR-Mob interactions in cell extracts. Interestingly, NDR1 and NDR2 form stable complexes with Mob2, and this association dramatically stimulates NDR1 and NDR2 catalytic activity. In summary, this work identifies a unique class of human kinase-activating subunits that may be functionally analogous to cyclins.

Mammalian genomes encode two highly related serine-threonine kinases, NDR1 (nuclear Dbf2-related) and NDR2. Human NDR1 (GenBank accession number NP_059202) was named based on its homology to the yeast Dbf2 kinase and characterized as a nuclear serine-threonine kinase expressed in all human tissues with the exception of the brain (1). Human NDR1 and Saccharomyces cerevisiae Dbf2 activity can be dramatically stimulated by okadaic acid, suggesting that the NDR family of kinases require phosphorylation for proper activation (2, 3). Indeed, Ca2+ inducible NDR1 autophosphorylation as well as phosphorylation by an unidentified upstream kinase (4).

NDR1 activity is also regulated by interaction with Ca2+/S100 calcium-binding proteins (5).

Although its precise function(s) remain unclear, evidence suggests that NDR1 may be involved in cell proliferation and/or tumor progression. First, NDR1 is hyperactivated in several S100B-positive melanoma cell lines (5). Given that S100B is overexpressed in greater than 80% of metastatic melanomas (6), NDR1 activity may influence tumor metastatic potential. Second, microdissection and gene expression profiling reveal that NDR1 mRNA is up-regulated 1.9-fold in high versus low risk ductal carcinoma in situ (7). Despite these intriguing observations, NDR1 has no known substrates and has not been implicated in any signal transduction pathway.

Substrate identification has undoubtedly been hampered by the seemingly low enzymatic activity of NDR1. Indeed, NDR1 expressed in either Escherichia coli or COS-1 cells is unable to transphosphorylate "generic" kinase substrates such as histone H1, myelin basic protein (MBP), casein, or phosphovitin (1). Nevertheless, purified human NDR1 possesses catalytic activity. By screening a small peptide library, Hemnings and colleagues (8) identify a short peptide (KKRNRRLSVA) that serves as a NDR1 substrate. Taken together, these data suggest that either NDR1 has a very stringent substrate specificity or we have insufficient biological insight to purify active preparations of NDR1.

Mammalian genomes encode a related kinase, NDR2 (GenBank accession number NP_055815), which is ~87% identical to NDR1 at the amino acid level (4, 8). Currently, the NDR2 kinase remains uncharacterized. However, a recent retroviral insertional mutagenesis study found that the murine NDR2 gene (also known as serine-threonine kinase 38-like) was disrupted in two independent B-cell lymphomas (9). This result suggests that NDR2 may also be involved in tumor initiation or progression. Given their potential involvement in cancer, we sought to characterize the human NDR1 and NDR2 kinases. In this study, we identified and characterized two new NDR1 and NDR2 binding partners, Mob1B and Mob2.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa and 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 5 units/ml penicillin, and 5 μg/ml streptomycin. HeLa cells were transiently transfected using FuGENE 6 transfection reagent (Roche Applied Science) as described previously (10). Fluorescence microscopy and indirect immunofluorescence microscopy were performed on a DeltaVision platform (Applied Precision Inc.) as described previously (10). 293T cells were transiently transfected with calcium phosphate. Jurkat T-cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and penicillin/streptomycin.

Plasmids—The full-length NDR1 and NDR2 open reading frames (ORFs) were amplified by PCR from cDNA prepared from total HeLa RNA. The NDR1 ORF was cloned into the pECMV-FLAG (10) or pEGFP-C1 vector (Clontech) to generate pFLAG-NDR1 and pGFP-NDR2 ORFs.
NDR1, respectively. Similarly, the NDR2 ORF was cloned into the pECMV-FLAG or pEGFP-C1 vector to generate pFLAG-NDR2 and pGFP-NDR2. Kinase-dead (KD) derivatives were generated by PCR using the QuickChange mutagenesis kit (Stratagene) with oligonucleotides that change the invariant Lys to Ala (K118A for NDR1, K119A for NDR2). Retroviral vectors were also constructed by cloning FLAG-NDR1 or FLAG-NDR2 ORFs into the murine stem cell virus (MSCV) vector pMSCV-puro (Clontech), generating pMSCV-FLAG-NDR1 and pMSCV-FLAG-NDR2. Retroviruses were packaged as described previously (11). Full-length Mob1B and Mob2 ORFs were amplified by PCR from cDNA prepared from Jurkat total RNA and inserted into pEGFP-C1 to generate pGFP-Mob1B and pGFP-Mob2B, respectively.

**Northern and Western Blot Analyses**—To generate unique NDR1 and NDR2 ORFs, 3′- and 5′-untranslated regions (UTRs) at both ends of each ORF were gel-purified and labeled with a Rediprime II labeling system (Amerham Biosciences). Radiolabeled probes were hybridized to a First-Choice Northern Human Blot I membrane (Ambion) in UltraHybr (Ambion), washed to a stringency of 0.2× SSC, 0.1% SDS at 42 °C, and exposed to a PhosphorImager (Amerham Biosciences). Western blot analysis was performed as described previously (11) using monoclonal anti-FLAG M2 (Sigma), monoclonal anti-NDR1 (BD Transduction Laboratories), monoclonal anti-p75/LEDGF (BD Transduction Laboratories), or polyclonal anti-GFP. BenchMark™ protein ladder (Invitrogen) was used as a molecular weight standard for SDS-PAGE and Western blot analysis.

**Identification of NDR1- and NDR2-interacting Proteins**—Jurkat T cells were infected with MSCV, MSCV-FLAG-NDR1, or MSCV-FLAG-NDR2 and expanded in the presence of 1 μg/ml puromycin. Whole-cell extracts were prepared in immunoprecipitation buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.5% Triton X-100, 5% glycerol, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate containing Complete protease inhibitors (Roche Applied Science) and clarified by ultracentrifugation. Samples were resolved by 12% SDS-PAGE, stained with Simply Blue (Invitrogen). The immunoprecipitated protein was much more consistent with Bab71443. Alignments were formatted with the ESPript tool (16).

**RESULTS**

**Tissue Expression and Subcellular Localization of NDR1 and NDR2**—The human NDR1 and NDR2 kinases are ~87% identical at the amino acid level (Fig. 1A). Most of the nonidentical residues cluster at the N and C termini of the enzymes, and these differences are highly conserved in the murine NDR1 and NDR2 sequences. Residues known to regulate NDR1 activity including Thr-74, Ser-281, and Thr-444 (reviewed in Ref. 8) are conserved in NDR2 and correspond to NDR2 residues Thr-75, Ser-282, and Thr-445 (Fig. 1B). The NDR1 S100B-binding domain (residues 62–86) (5) contains one conservative and one nonconservative change in NDR2 (residues 63–87), whereas the NDR1 nuclear localization signal (residues 265–276) (1) contains a single conservative change in NDR2 (residues 266–277).

To gain insight into NDR2 biology, we initially characterized its tissue distribution and subcellular localization. Northern blot analysis revealed that NDR2, similar to NDR1 (1), was expressed in most human tissues and was particularly abundant in the thymus (Fig. 1B). We next cloned the full-length NDR1 and NDR2 ORFs into expression vectors encoding an N-terminal FLAG or GFP tag. HeLa cells were transiently transfected with these expression vectors, and NDR1 and NDR2 subcellular localization was monitored by fluorescence microscopy or indirect immunofluorescence microscopy. FLAG-NDR1 and GFP-NDR2 proteins had accumulated in the nucleus but were also detected in the cytoplasm (Fig. 1C). In contrast, FLAG-NDR2 and GFP-NDR2 proteins were predominantly excluded from the nucleus and exhibited a punctate cytoplasmic distribution (Fig. 1C). Hemmings and colleagues (1) have previously characterized NDR1 amino acid residues 265–276 (KKRAETWKNRR) as a nuclear localization signal, because the deletion of this basic peptide had mislocalized hemagglutinin-NDR1 to the cytoplasm in transiently transfected COS-1 cells (1). Because NDR2 contains only a single conservative mutation in this region (KKN to KKN) (see Fig. 1A), it is intriguing that NDR2 predominantly accumulated in the cytoplasm. Incubation of transiently transfected cells with leptomycin B, a specific Crm1-dependent nuclear export inhibitor, did not alter the intracellular distribution of either kinase, suggesting that differences in nuclear import/export kinetics were not likely to explain their differential compartmentalization at steady state (data not shown). Finally, we compared the localization of epitope-tagged NDR1 to endogenous NDR1. Biochemical fractionation and Western blotting indicated that endogenous NDR1 was localized to both the nucleus and the cytoplasm (Fig. 1C, lower panel), which is consistent with our FLAG-NDR1 and GFP-NDR1 analyses.

**Mob Proteins Comimmunoprecipitate with NDR1 and NDR2**—To identify proteins that interact with NDR1 and NDR2, we generated Jurkat T-cell lines stably expressing FLAG-NDR1 or FLAG-NDR2. Following affinity purification, eluted samples were resolved by SDS-PAGE (Fig. 2A). Specific bands were...
excised from the gel, and the indicated proteins were identified by a combination of peptide mass fingerprinting using MALDI-TOF MS and mass spectrometric sequencing using MALDI-TOF/TOF MS/MS. We identified the 95-kDa band detected in NDR1 immunoprecipitations as Hsp90 (GenBank™ accession number P07900) and Hsp90 (GenBank™ accession number NP_031381). However, these proteins were also identified in Mock samples, suggesting that Hsp90 partially adhere nonspecifically to the anti-FLAG resin under these conditions.

Aside from Hsp90, we identified the 30-kDa band coimmunoprecipitating with both NDR1 and NDR2 as Mob2 (Fig. 2A) and the 26-kDa band coimmunoprecipitating with NDR2 as Mob1B. Of note, the 26-kDa band is not visible on the scanned Simply Blue-stained gel (top panel) but was readily...
Mob2 Colocalizes with NDR1 and NDR2—We first sought to characterize the localization of Mob1B, the ~26-kDa protein that was faintly detected in NDR2 immunoprecipitations (Fig. 2A). To this end, the full-length Mob1B ORF was cloned into an expression vector in-frame with an N-terminal GFP tag. GFP-Mob1B localization was then examined by fluorescence microscopy in transiently transfected HeLa cells. Mob1B exhibited observable following silver staining (bottom panel). A sequence analysis demonstrated that the identified proteins are homologous to the S. cerevisiae Mob1 and Mob2 proteins (Fig. 2B).

**Fig. 2. Identification of NDR1- and NDR2-interacting proteins.** A, extracts were prepared from Jurkat T-cells infected with an empty retroviral cassette (Mock) or retroviral vectors encoding FLAG-NDR1 (NDR1) or FLAG-NDR2 (NDR2) and immunoprecipitated with anti-FLAG M2 affinity resin. Bound proteins were eluted with the FLAG peptide, resolved by SDS-PAGE, and stained with Simply Blue. The position of molecular mass markers in kDa is shown. The position of FLAG-NDR1 (N1) and FLAG-NDR2 (N2) are indicated as well as the coimmunoprecipitating Mob2 and Mob1B proteins. The ~26-kDa Mob1B protein coimmunoprecipitated with NDR2 is not visible on the scanned Simply Blue-stained gel (top panel) but was readily observable following silver staining (bottom panel). Aside from the Mob proteins, Hsp90α and Hsp90β were detected in the NDR1 immunoprecipitation. However, Hsp90α and Hsp90β were also detected in the Mock sample, indicating that these proteins partially adhere nonspecifically to the anti-FLAG resin under these conditions. B, multiple sequence alignment of human (h) Mob1B and Mob2 and the scMob1 and scMob2 proteins. Identical residues are shown in white font with a red background, conserved residues are shown in red, whereas nonconserved residues are shown in black. Gaps are indicated by dots.
both nuclear and diffuse cytoplasmic distribution when expressed alone (Fig. 3A). Coexpression of NDR1 (Fig. 3, B–D) or NDR2 (Fig. 3, E–G) did not noticeably alter the distribution of Mob1B. Consistent with our earlier observations (Fig. 1C), NDR1 localized predominantly to the nucleus with less prominent cytoplasmic speckles (Fig. 3C). NDR2 exhibited heterogeneous cellular distribution with predominantly cytoplasmic speckles (Fig. 3F). Although merged images suggest that Mob1B and NDR1 (Fig. 3D) or NDR2 (Fig. 3G) may have partially colocalized, interpretation of these results is difficult given the pan-cellular distribution of Mob1B.

We next performed a similar localization analysis with Mob2, the ~30-kDa protein that coimmunoprecipitated with both NDR1 and NDR2 (Fig. 2A). When expressed alone, Mob2 accumulated within the nucleus but was also observed within the cytoplasm (Fig. 3H). Coexpression of Mob2 and NDR1 (Fig. 3, I–K) or NDR2 (Fig. 3, L–N) dramatically altered Mob2 distribution. Under these conditions, Mob2 was mostly cytoplasmic with strong perinuclear staining (Fig. 3, I and L). Likewise, NDR1 exhibited reduced nuclear accumulation and more prominent cytoplasmic speckles (Fig. 3J), whereas NDR2 remained mostly cytoplasmic (Fig. 3M). The merged image demonstrates that NDR1 and Mob2 colocalized within the cytoplasm, particularly within perinuclear regions (Fig. 3K). Likewise, NDR2 and Mob2 colocalized predominantly in perinuclear regions but also throughout the cytoplasm (Fig. 3N). Thus, coexpression of NDR1 and Mob2 results in NDR1 redistribution from the nucleus (Figs. 1C and 3C) to the cytoplasm (Fig. 3J). Furthermore, NDR1 or NDR2 coexpression results in Mob2 relocalization to perinuclear regions (Fig. 3, compare I and L with H).

Differential Binding of NDR Kinases to Mob1B and Mob2—The above analysis demonstrates that NDR1 and NDR2 colocalize with Mob2 in human cell lines. To extend these findings, we purified FLAG-NDR1 or FLAG-NDR2 from 293T cells following cotransfection with plasmids encoding GFP, GFP-Mob1B, or GFP-Mob2. Whole cell extracts were analyzed by anti-FLAG (Fig. 4A) and anti-GFP (Fig. 4B) Western blot to ensure equivalent expression of the respective FLAG and GFP fusion proteins. Following anti-FLAG affinity purification, a significant amount of Mob2 was coeluted with NDR1 and NDR2 (Fig. 4D). Mob1B also was coeluted with NDR2 (Fig. 4D) and, to a lesser extent, with NDR1 (see longer exposure in Fig. 4E). These results confirm that NDR1 and NDR2 bind both Mob1B and Mob2. Interestingly, the kinases appear to bind Mob2 more tightly than Mob1B in cell extracts, a finding consistent with our microscopy studies (Fig. 3). We also observed that the Mob1B-NDR2 interaction is significantly weaker than the Mob1B-NDR1 interaction, a finding consistent with our initial immunoprecipitations (Fig. 2A).

Subsequently, we performed in vitro kinase reactions to examine the functional consequence of Mob1B and Mob2 association with NDR1 and NDR2. Mob2 dramatically stimulated autophosphorylation of NDR2 and, to an apparently lesser extent, NDR1 (Fig. 4F). The size of the lower band is consistent with GFP-Mob2 phosphorylation of NDR2 (Fig. 4D).

Fig. 3. NDR1 and NDR2 colocalize with Mob2. HeLa cells transiently transfected with pGFP-Mob1B alone (A) or cotransfected with pGFP-Mob1B and pFLAG-NDR1 (B–D) or pFLAG-NDR2 (E–G) were analyzed by fluorescence and indirect immunofluorescence microscopy. A representative field of view illustrating Mob1B (A, B, and E), NDR1 (C), and NDR2 (F) localization is shown. The merged images (D and G) demonstrate that NDR1 and NDR2 partially colocalize with Mob1B, yet interpretation of these data is difficult given the pan-cellular distribution of Mob1B. Similarly, HeLa cells transiently transfected with pGFP-Mob2 alone (H) or cotransfected with pGFP-Mob2 and pFLAG-NDR1 (I–K) or pFLAG-NDR2 (L–N) were analyzed by fluorescence and indirect immunofluorescence microscopy. A representative field of view illustrating Mob2 (I, J, and L), NDR1 (J), and NDR2 (M) localization is shown. Note that coexpression of NDR1 or NDR2 with Mob2 results in Mob2 relocalization (compare H with I and L). NDR1 localization also changes upon coexpression (compare C with J). The merged images (K and N) demonstrate that NDR1 and NDR2 partially colocalize with Mob2, particularly in regions surrounding the nucleus.
treated cells with 1 wild-type (WT) or catalytically inactive (KD) FLAG-NDR1 and for transphosphorylation activity. To determine whether NDR1 autophosphorylation, we next tested these preparations containing KD NDR2. Given that significantly more Hsp90 copurified with KD NDR1 (Fig. 5B), the functional significance of the NDR-Mob interactions was analyzed by an in vitro kinase reaction. Autophosphorylation of NDR2 was weakly observed when NDR2 was purified alone or in the presence of GFP-Mob1B, yet was dramatically stimulated in the presence of GFP-Mob2. NDR1 autophosphorylation was only observed in the presence of GFP-Mob2.

\[ \text{GFP-Mob2 plus or minus (-) plasmids encoding FLAG-NDR1 or FLAG-NDR2. Whole cell extracts (WCE) were analyzed by Western blinding using anti-FLAG M2 (A) or anti-GFP antibodies (B). GFP migrated at -30 kDa, whereas full-length GFP-Mob2 and GFP-Mob1B migrated at -55 and -50 kDa, respectively. The smaller GFP-reactive bands represent C-terminal truncations. WCE were subsequently immunoprecipitated with the anti-FLAG affinity matrix. Immunoprecipitated proteins were eluted with the FLAG peptide and analyzed by anti-FLAG (C) and anti-GFP (D and E). Western blotting. GFP-Mob2 was specifically coimmunoprecipitated with FLAG-NDR1 and FLAG-NDR2. GFP-Mob1B was specifically coimmunoprecipitated with FLAG-NDR2 (D) and, to a lesser extent, with FLAG-NDR1 (E). F, the functional significance of the NDR-Mob interactions was analyzed by an in vitro kinase reaction. Autophosphorylation of NDR2 was weakly observed when NDR2 was purified alone or in the presence of GFP-Mob1B, yet was dramatically stimulated in the presence of GFP-Mob2. NDR1 autophosphorylation was only observed in the presence of GFP-Mob2.} \]

\[ \text{NDR1 also phosphorylated MBP, albeit to a lesser extent than NDR2 (Fig. 5B). Interestingly, reactions containing KD NDR1 exhibited significantly more kinase activity than those containing KD NDR2. Given that significantly more Hsp90 copurified with KD NDR1 (Fig. 5B), the background enzymatic activity was probably the result of unknown kinase(s) associated with a subpopulation of Hsp90. Importantly, MBP transphosphorylation was invariably higher following incubation with WT NDR1 than with KD NDR1 (data not shown).} \]

\[ \text{Discussion} \]

Here, we present molecular characterization of the human NDR2 serine-threonine kinase. Furthermore, we identified a new family of human kinase regulators via their interactions with the NDR1 and NDR2 serine-threonine kinases. Mob2 colocalizes with NDR1 and NDR2 in human cell lines and binds NDR1 and NDR2 in vitro. Importantly, the Mob2 interaction dramatically stimulates NDR1 and NDR2 kinase activity. In contrast to NDR1, which is predominantly nuclear, NDR2 is localized predominantly to the cytoplasm. This observation is intriguing given the extremely high degree of sequence identity between the two enzymes. The punctate cytoplasmic distribution of FLAG-NDR2 and GFP-NDR2 (and to a lesser extent FLAG- and GFP-NDR1) suggests that a population of NDR2 may be restricted to a specific subcellular compartment. Mob1B and Mob2 were identified as NDR1 and NDR2 binding partners. Transient transfection studies demonstrated that
Mob2. Transfected cells were pulsed with FLAG-NDR1 and FLAG-NDR2 expression vectors along with pGFP-plexes. Interestingly, NDR2 is significantly more active autophosphorylation and transphosphorylation of heterologous cally stimulates the kinase activity of NDR1 and NDR2. We visualization of both proteins, and colocalization is mostly observed heterologously expressed proteins. Interestingly, the coexpres lines with the caveat that this analysis was performed with NDR1 and NDR2 partially colocalize with Mob2 in human cell interferes with the interaction. We also demonstrated that Mob2 is salt- and/or detergent-sensitive or that the GFP moiety it is possible that the interaction between NDR1 or NDR2 and genuinely have a higher affinity for Mob2 than Mob1B. Indeed, the kinases. At present, it is unclear whether the NDR kinases and NDR2 and is affinity-purified in a 1:1 stoichiometry with NaCl, 1% Triton X-100). Mob2 tightly associates with NDR1 and FLAG-NDR2. The catalytic activity of NDR1 and NDR2 is demonstrated that Mob2 dramati- accounts for the increased background kinase activity associated with Cbk1 and is important for maintaining polarized growth and 19). Aside from Mps1, scMob1 also binds the Dbf2 serine-ding yeast gene required for cytokinesis and mitotic exit (18, 23). Taken together, our results show that Mob regulation of NDR kinase family members Sid2 and Orb6, respectively (22, 23). The work presented here provides the first documentation of a serine-threonine kinase stimulated by a human Mob homologue. The proteins identified here are homologous to the S. cerevisiae Mob1 and Mob2 proteins (scMob1, scMob2). scMob1 and scMob2 were initially identified via a yeast two-hybrid interaction with the Mps1 serine-threonine kinase (hence, the name Mob, Mps One Binder) (18). MOB1 is an essential budding yeast gene required for cytokinesis and mitotic exit (18, 19). Aside from Mps1, scMob1 also binds the Dbf2 serine-threonine kinase (20) and regulates Dbf2 catalytic activity (3). scMob2 also forms a complex with the NDR family member Cbk1 and is important for maintaining polarized growth and regulation of the daughter cell-specific transcription factor, AceII (21). Interestingly, Schizosaccharomyces pombe Mob1 and Mob2 have been shown to interact with the fission yeast NDR kinase family members Sid2 and Orb6, respectively (22, 23). Taken together, our results show that Mob regulation of NDR activity has been conserved from yeast to humans. It was previously hypothesized that scMob1 may activate Dbf2 by inducing a conformational switch analogous to cyclin-mediated activation of Cdkks (3). Recently, the crystal structure of human Mob1A (BAB14525) was solved (Protein Data Base accession number 1P11) (14). Of note, Mob1A is ~95% identical to Mob1B described here. The Mob1A structure revealed a negatively charged flat surface that is conserved among all of the Mob proteins that probably mediates electrostatic interactions with kinase binding partners (14). Although the structure of Mob1A does not resemble that of cyclins, cocystal studies of Mob-NDR complexes might lend support to the cyclin-Cdk analogy. Regardless of the biophysical mechanism of activa-
tion, Mob proteins are conserved kinase-activating subunits. A sequence analysis demonstrated that the human genome contains seven Mob genes (data not shown). It will be interesting to determine whether other Mob proteins also regulate human NDR1, NDR2, or other members of the NDR kinase family. Given that NDR1, NDR2, and Mob2 have been implicated in melanomas, aggressive ductal carcinomas in situ, B-cell lymphomas, and hepatocellular carcinomas, molecular insight into the functions of the NDR kinases may lead to new therapeutic opportunities for the treatment of human cancers.

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