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Necdin Interacts With the Ribonucleoprotein hnRNP U in the Nuclear Matrix

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Abstract Necdin is expressed predominantly in terminally differentiated neurons, and its ectopic expression suppresses cell proliferation. We screened a cDNA library from neurally differentiated embryonal carcinoma P19 cells for necdin-binding proteins by the yeast two-hybrid assay. One of the positive clones contained cDNA encoding a carboxyl-terminal portion of heterogeneous nuclear ribonucleoprotein U (hnRNP U), a nuclear matrix-associated protein that interacts with chromosomal DNA. We isolated cDNA encoding full-length mouse hnRNP U to analyze its physical and functional interactions with necdin. The necdin-binding site of hnRNP U was located near a carboxyl-terminal region that mediated the association between hnRNP U and the nuclear matrix. In postmitotic neurons, endogenously expressed necdin and hnRNP U were detected in the nuclear matrix and formed a stable complex. Ectopically expressed necdin was concentrated in the nucleoli, but co-expressed hnRNP U recruited necdin to the nucleoplasmic compartment of the nuclear matrix. Furthermore, under the same conditions necdin and hnRNP U co-operatively suppressed the colony formation of transfected SAOS-2 cells. These results suggest that necdin suppresses cell proliferation through its interaction with hnRNP U in the specific subnuclear structure. J. Cell. Biochem. 84: 545–555, 2002.

Key words: terminal differentiation; growth arrest; nuclear matrix; Prader-Willi syndrome; postmitotic neurons

Terminally differentiated cells such as neurons and skeletal muscle cells withdraw from the cell cycle and enter the postmitotic state. The permanent arrest of cell division is the most fundamental feature displayed by these postmitotic cells. However, little is known about the molecular mechanisms whereby these cells remain in permanent mitotic quiescence. The murine embryonal carcinoma P19 cells differentiate into postmitotic neurons in response to retinoic acid treatment [McBurney et al., 1988]. We have isolated a 325 amino acid residue protein encoded in a cDNA sequence from a subtraction library of neurally differentiated P19 cells and termed this protein necdin [Maruyama et al., 1991]. The necdin gene is expressed in postmitotic neurons but not in transformed cells [Aizawa et al., 1992; Uetsuki et al., 1996]. The necdin gene is expressed in almost all postmitotic neurons in central and peripheral nervous tissues of mice, although its expression levels vary among neuronal cell types. Besides postmitotic neurons, the necdin gene is expressed in skeletal muscles [Taniguchi et al., 2000], cartilage, and brown fat [Gerard et al., 1999], all of which contain postmitotic cell populations.

The human necdin gene NDN is located on chromosome 15q11-q12, a region deleted in the Prader-Willi syndrome (PWS) [Jay et al., 1997; MacDonald and Wevrick, 1997; Nakada et al., 1998]. PWS is a neurogenetic disorder related to genomic imprinting and its major symptoms such as feeding problems, gross obesity, and hypogonadism are consistent with a hypotha-

Abbreviations used: GFP, green fluorescent protein; MAP2, microtubule associated protein 2, MBP, maltose binding protein; PWS, Prader-Willi syndrome; Rb, retinoblastoma protein.

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lamic defect. NDN is maternally imprinted and transcribed only from the paternal allele [Jay et al., 1997; MacDonald and Wevrick, 1997; Sutcliffe et al., 1997]. Necdin is not expressed in cells prepared from PWS patients whose chromosome 15q11.2-q12 region in the paternal allele is deleted. Necdin is abundant in postmitotic neurons in the brain stem and hypothalamus of mice [Aizawa et al., 1992; Uetsuki et al., 1996]. Disruption of the mouse necdin gene results in early postnatal lethality [Gerard et al., 1999], a reduction in specific hypothalamic neurons and behavioral alterations [Muscatelli et al., 2000], which are reminiscent of human PWS. These observations suggest that necdin plays a key role in differentiation and development of subsets of neurons in the brain, especially in the hypothalamus.

Ectopic expression of necdin suppresses the growth of proliferative cells [Hayashi et al., 1995]. Furthermore, necdin interacts with E2F1, a key transcription factor that controls cell cycle progression, and represses E2F-driven transcription [Taniura et al., 1998]. Necdin also binds to p53 and represses p53-dependent transcription of the p21 promoter but does not counteract p53-induced growth suppression [Taniura et al., 1999]. Thus, necdin is a unique growth suppressor that targets both E2F1 and p53. Besides these transcription factors, necdin binds to NEFA and nucleobindin, both of which are calcium-binding proteins involved in intracellular calcium homeostasis [Taniguchi et al., 2000]. These findings suggest that necdin is a multifunctional protein that targets various factors involved in the regulation of cell proliferation and survival.

We report here that necdin physically and functionally interacts with hnRNP U, which is localized to the nuclear matrix [Dreyfuss et al., 1988; Kiledjian and Dreyfuss, 1992; Romig et al., 1992]. We determined both the necdin-binding and nuclear matrix-attachment regions of hnRNP U. Furthermore, we demonstrate that hnRNP U recruits necdin to the nucleoplasmic compartment of the nuclear matrix, and that hnRNP U and necdin cooperatively suppress cell growth.

MATERIALS AND METHODS

Yeast Two-Hybrid Assays

Double-stranded cDNA was generated from poly(A) RNA isolated from neurally differentiated P19 cells using a cDNA synthesis kit (Amersham-Pharmacia Biotech) and inserted into GAL4 activation domain vector pGAD424 (Clontech). The mouse necdin cDNA corresponding to amino acids 102–325 was cloned into GAL4 DNA binding domain vector pGBT9 (Clontech). pGBT9 and pGAD424 were introduced into yeast strain SFY526, and the clones were selected on leucine (Leu−) and tryptophan (Trp−) selection plates. Plasmid DNAs from β-galactosidase-positive colonies were recovered in bacteria strain DH5α and sequenced. Full-length hnRNP U cDNA was cloned from a λgt10 cDNA library of neurally differentiated P19 cells [Maruyama et al., 1991].

In Vitro Binding Assays

cDNAs encoding various hnRNP U fragments were generated by polymerase chain reaction (PCR) and subcloned into pMAL C2 (New England Biolabs) to make maltose binding protein (MBP) fusion proteins. The MBP-hnRNP U fusion proteins were affinity-purified using amylose resin [Taniura et al., 1998]. The recombinant polyhistidine (His)-tagged necdin protein was produced in Sf9 insect cells [Taniura et al., 1999]. Purified MBP-hnRNP U fusion protein immobilized on amylose resin (5 μl) was incubated with His-tagged necdin protein (100 ng) in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA at 4°C for 30 min, and bound proteins were eluted with 20 mM maltose. Eluted proteins were separated by SDS–PAGE, immunoblotted with anti-necdin antibody C2 [Maruyama et al., 1991], and detected with peroxidase-conjugated anti-rabbit IgG (Cappel) using chemiluminescence method (Renaissance, NEN).

Cell Transfection and Immunocytochemistry

To construct expression vectors for Myc-tagged hnRNP U deletion mutants (UΔN, UΔ1–123; UΔND, UΔ613–684; UΔC, UΔ616–800; UΔGT, UΔ712–800; UC, U614–800), mutated cDNA were generated by PCR using full-length hnRNP U (UF, U1–800) cDNA as a template, inserted directionally in 6 × Myc tag pBluescript (a gift from Dr. M. W. McBurney, University of Ottawa), and cloned into pRc/CMV expression vector (Invitrogen). pRc/CMV vectors expressing hnRNP U, deletion mutants and full-length necdin (pRc-Ndn) [Taniura et al., 1998] were transfected to SAOS-2 cells

grown on glass coverslips by calcium phosphate method. Cells were fixed 48 h after transfection in 4% formaldehyde (pH 7.4) for 20 min at 4°C, permeabilized in methanol for 20 min at room temperature, and incubated for 1 h at room temperature with anti-Myc antibody (9E10), anti-necdin antibody NC243 [Niinobe et al., 2000] or anti-lamin B antibody (SC6216, Santa Cruz Biotechnology). For detection of intranuclear protein translocation, SAOS-2 cells grown in 35-mm dishes were transfected with expression vectors encoding the full-length necdin cloned in pEGFP N1 (Clontech) (GFP-necdin) (0.8 μg) and Myc-tagged full-length hnRNP U and its deletion mutants (3.2 μg each). Total amount of plasmids was adjusted to 4 μg per 35-mm dish by adding empty pRc/CMV. Cells were labeled with antibodies for fibrillarin (AFB01, Cytoskeleton) and Myc (9E10), and observed by fluorescence microscope (BX 50-34-FLAD 1, Olympus). Images were processed using Adobe Photoshop 5.0 software.

Nuclear Matrix Preparation

The nuclear matrix was prepared by the sequential extraction method as described [He et al., 1990]. Cells grown on coverslips were sequentially treated in situ with cytoskeleton buffer (CSK) containing 10 mM Pipes (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM vanadyl ribonucleoside complex, 1 × protease inhibitor cocktail (Complete, Roche Molecular Biochemicals), and 1 mM PMSF for 3 min at 4°C. Chromatin was solubilized by DNA digestion with 25 U/ml RNase free DNase (RQ1, Life Technologies) in CSK buffer for 50 min at 30°C. The cells were treated three times with 0.25 M ammonium sulfate for 10 min at room temperature, and further extracted three times with 2 M NaCl in CSK buffer for 5 min at room temperature. The remnants were fixed and immunostained. For immunoblot analysis, suspended cells were treated with the above solutions, and centrifuged at 600*g* for 3 min between treatments. Volumetric equivalents (one-fifth of the total volumes) were electrophoresed and immunoblotted with antibodies to Myc, necdin (NC243), hnRNP U (HUT), and lamin B. HUT was raised in rabbit against purified MBP-hnRNP U (amino acids 614–800) fusion protein. The integrated densities of immunoreactive bands were measured using NIH Image software (version 1.61).

P19 Cell Culture

P19 cells were cultured in minimum essential medium-alpha (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Life Technologies). P19 cells were induced to differentiate by retinoic acid treatment for 3 days, and cultured in serum-free medium (Opti-MEM I, Life Technologies) for 4 days to enrich neuronal population [Aizawa et al., 1992]. Neuronal nuclear and cytoplasmic fractions were prepared by the small-scale extraction method [Schreiber et al., 1989]. Lysates (20 μg protein/lane) were separated by SDS–PAGE and detected with NC243 and HUT. The nuclear matrix was prepared from neuron-enriched P19 cell cultures (P19 neurons) as described above. The whole cell and nuclear matrix were labeled with NC243, HUT, a monoclonal antibody against microtube associated protein 2 (MAP2) (MAB378, Chemicon International), and anti-lamin B antibody.

Immunoprecipitation and Immunoaffinity Purification

SAOS-2 cells were transfected with pRc-necdin and the expression vector for Myc-UF (U1-800) or Myc-UΔND (UΔ613–684) (2 μg each per 35-mm dish). Cells were harvested 48 h after transfection, homogenized in CSK buffer, and centrifuged at 10,000*g* for 10 min to remove soluble proteins. Remaining pellets were solubilized in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, and 0.1% SDS. After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant (200 μg protein) was incubated for 2 h at 4°C with anti-Myc antibody or antibody C2. The complexes were precipitated with Protein A-sepharose (Amersham Pharmacia Biotech) and eluted with SDS–PAGE buffer. For detection of endogenous complexes of necdin and hnRNP U, nuclear extracts were prepared from P19 neurons and applied to HiTrap NHS-activated affinity column (Amersham Pharmacia Biotech) (400 μg protein to 1 ml column volume) coupled with IgG (1 mg) of C2, HUT or non-immune rabbit serum. The bound proteins were eluted with 0.1 M glycine-HCl (pH 2.5). The eluted fraction was precipitated with 10% trichloroacetic acid, rinsed with cold acetone, separated by

SDS–PAGE, and immunoblotted with NC243 and HUT.

Cell Proliferation Assays

Colony formation assay was carried out using SAOS-2 cells as described previously [Taniura et al., 1999]. SAOS-2 cells grown in 35-mm dishes were transfected with pRc-necdin (0.8 μg) in combination with pRc-Myc-UF (3.2 μg), pRc-Myc-UΔND (3.2 μg), or pRc-Myc-UΔGT (3.2 μg) by the calcium phosphate method. Total amount of plasmids was adjusted to 4 μg/dish by adding empty pRc/CMV. G418 (500 μg/ml) was added to the culture medium 48 h after transfection. The cells were incubated for 10 days, fixed, and stained with 0.4% crystal violet. The integrated densities of stained colonies were measured using NIH-Image software. BrdU incorporation was analyzed as described [Zhang et al., 2000]. SAOS-2 cells grown in 35-mm dishes were transfected with 0.8 μg of pMSCV-puro (Clontech) and 3.2 μg of expression vectors, and cells were selected 24 h after transfection with puromycin (1.25 μg/ml). After selection for 48 h, cells were incubated in the presence of 10 μM BrdU for 4 h, and fixed with 70% ethanol containing 20 mM glycine-HCl (pH 2.0) for 30 min at −20°C. BrdU and nuclear DNA were labeled with a monoclonal anti-BrdU antibody (BMC9313) (Roche Molecular Biochemicals) and 3.3 μM Hoechst 33342, respectively. Cells carrying BrdU-positive nuclei were counted and statistical significance was tested using *t* test.

RESULTS

Necdin Interacts With hnRNP U

We screened a cDNA library (4×10^5 transformants) of P19 neurons by the yeast two-hybrid assay using NH_2-terminally truncated necdin (amino acids 102–325) as a bait and isolated two positive clones. One of the positive clones encoded a sequence homologous to the COOH-terminus of human hnRNP U [Kiledjian and Dreyfuss, 1992]. The other clone encodes a partial sequence of NEFA as reported previously [Taniguchi et al., 2000]. A full-length mouse hnRNP U cDNA clone was isolated from a λgt10 cDNA library of P19 neurons using cloned cDNA as a hybridization probe. The cDNA sequence contains an open reading frame encoding an 800-amino acid residue protein that completely matches the sequence reported previously [Matsui et al., 1999]. We determined

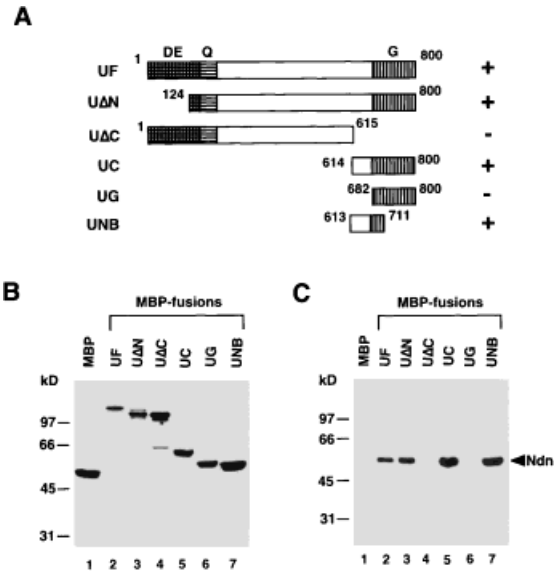


Fig. 1. In vitro binding assay for interaction between necdin and hnRNP U. **A**: Diagram of hnRNP U deletion mutants. Full-length hnRNP U (UF, U1–800) and its deletion mutants (UΔN, U124–800; UΔC, U1–615; UC, U614–800; UG, U682–800; UNB, U613–711) were produced as MBP-fusion proteins and used for in vitro binding assay. **B**: Expression of MBP-hnRNP U fusion proteins. Purified MBP fusion proteins of hnRNP U deletion mutants (MBP-fusions) were separated by SDS–PAGE and stained with Coomassie Brilliant Blue. Molecular size markers in kDa are at the left. **C**: In vitro binding assay. Purified MBP fusion proteins were immobilized on amylose resin and incubated with His-tagged necdin. Bound His-tagged necdin (Ndn) was eluted by maltose and immunoblotted with antibody C2. Results (+,−) are shown in (A).

the necdin-binding domain of hnRNP U using hnRNP U and its deletion mutants fused to MBP by in vitro binding assay (Fig. 1A). The fusion proteins purified from bacterial lysates had predicted sizes of polypeptides (Fig. 1B, lanes 2–7). MBP-UF (U1-800) and the NH_2-terminal deletion mutant UΔN (UΔ1–123) interacted with necdin (Fig. 1C, lanes 2 and 3), whereas the COOH-terminal deletion mutant UΔC (UΔ616-800) had no necdin binding activity (Fig. 1C, lane 4), suggesting that the COOH-terminal domain holds the binding activity. We then synthesized the COOH-terminal fragment (UC, U614–800) and the COOH-terminal G-rich fragment (UG, U682–800). UC bound to necdin but UG was unable to interact (Fig. 1C, lanes 5 and 6), suggesting that an NH_2-terminal region of UC contains the necdin binding site. The necdin binding region was narrowed down to amino acids 613–711 of hnRNP U (UNB) by this in vitro binding assay (Fig. 1C, lane 7).

hnRNP U and Necdin Associates With the Neuronal Nuclear Matrix

We next examined the distribution of necdin and hnRNP U to the nuclear matrix of P19-derived neurons, which express both proteins. We first prepared a specific antibody (HUT) against MBP-hnRNP U (U614–800) fusion protein. This antibody recognized exogenous Myc-tagged hnRNP U and endogenous ~120 kDa hnRNP U proteins in SAOS-2 cells (Fig. 2A). Endogenous ~45 kDa necdin was detected in both the cytoplasmic and nuclear fractions of P19 neurons, whereas endogenous hnRNP U was localized exclusively to the nucleus (Fig. 2B). We determined endogenous levels of necdin and hnRNP U in the nuclear matrix prepared from neurons by immunoblotting (Fig. 2C). The Triton X-100 extractable fraction contained ~70% of the total amount of necdin, whereas ~10% was associated with the nuclear matrix. On the other hand, ~30% of the total amount of hnRNP U was detected in the nuclear matrix. Lamin B, one of the major component of the nuclear matrix, was found exclusively in the nuclear matrix fraction. Both necdin and hnRNP U were localized to the nuclei of differentiated neurons, which express the neuronal marker MAP2 (Fig. 2D, a–d). Necdin was also distributed in the neuronal cytoplasm (Fig. 2D, a). The immunocytochemical analysis of in situ extracted nuclear matrix revealed that both necdin and hnRNP U were concentrated in intranuclear speckles throughout the nucleoplasm (Fig. 2D, e,f). Lamin B, a nuclear matrix marker, was localized to the nuclear lamina (Fig. 2D, g). These results suggest that both necdin and hnRNP U are associated with the nuclear matrix of neurons.

Formation of Complexes Between Necdin and hnRNP U

To analyze the complex formation in vivo between necdin and hnRNP U, we carried out immunoprecipitation of nuclear extracts prepared from transfected SAOS-2 cells (Fig. 3A). Necdin was co-precipitated with Myc-hnRNP U by anti-Myc antibody, but not with Myc-UΔND (UΔ613–684) that lacks the necdin-binding domain (Fig. 3A, left). Conversely, Myc-hnRNP U was co-immunoprecipitated with necdin by anti-necdin antibody (Fig. 3A, right). To detect endogenous complexes between necdin and hnRNP U, nuclear extracts prepared from P19 neurons were applied to the immunoaffinity columns coupled with IgGs of anti-hnRNP U and anti-necdin sera, and bound proteins were detected by immunoblotting (Fig. 3B). Proteins bound to anti-necdin IgG contained both ~45 kDa necdin and ~120 kDa hnRNP U (Fig. 3B, lanes 2 and 4). Furthermore, proteins that bound to HUT IgG contained both

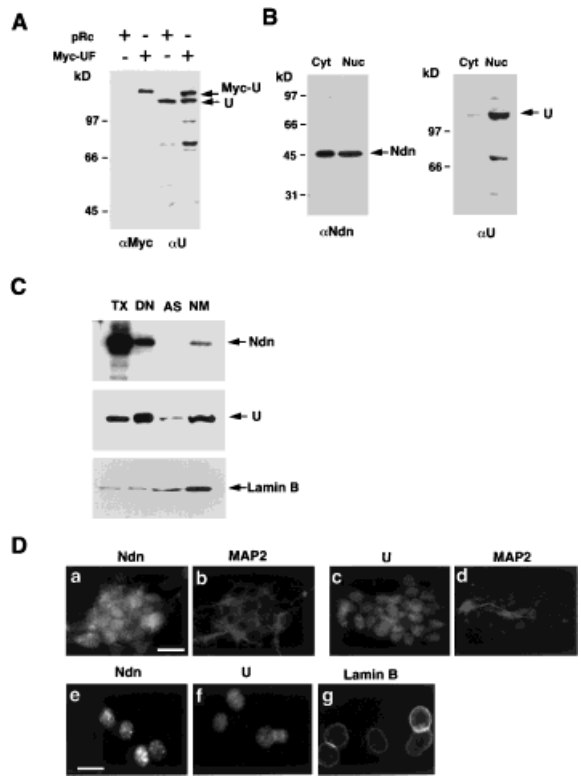


Fig. 2. Association of hnRNP U and necdin with neuronal nuclear matrix. **A**: Specificity of anti-hnRNP U antibody HUT. Cell lysates were prepared from SAOS-2 cells transfected with pRc/CMV (pRc) or pRc-Myc-hnRNP U (Myc-UF). Exogenous Myc-tagged hnRNP U (Myc-U) and endogenous hnRNP U (U) proteins were detected by immunoblotting with anti-Myc antibody (αMyc) or HUT (αU). **B**: Nuclear localization of necdin and hnRNP U. Necdin (Ndn) and hnRNP U (U) in the cytoplasmic (Cyt) and nuclear (Nuc) fractions of P19 neurons were detected by immunoblotting with NC243 (αNdn) and HUT (αU). **C**: Association of necdin and hnRNP U with neuronal nuclear matrix. The nuclear matrix was prepared from suspended P19 neurons by the sequential extraction procedure. Necdin (Ndn), hnRNP U (U), and lamin B in extracted fractions were detected by immunoblotting with NC243, HUT and, anti-lamin B antibody, respectively. **Lanes** (samples extracted or digested with): **TX**, 0.5% Triton X 100; **DN**, 25 U/ml DNase; **AS**, 0.25 M ammonium sulfate; **NM**, nuclear matrix fraction after with 2 M NaCl. **D**: Fluorescence immunocytochemistry. P19 neurons were labeled with NC243 for necdin (Ndn) (**a**) or with HUT for hnRNP U (U) (**c**) in combination with anti-MAP2 antibody for MAP2 (**b,d**). The nuclear matrix was prepared in situ and labeled for necdin (Ndn) (**e**), hnRNP U (U) (**f**), and lamin B (**g**). Scale bars (in a for a–d), 20 μm; (in e for e–g), 10 μm.

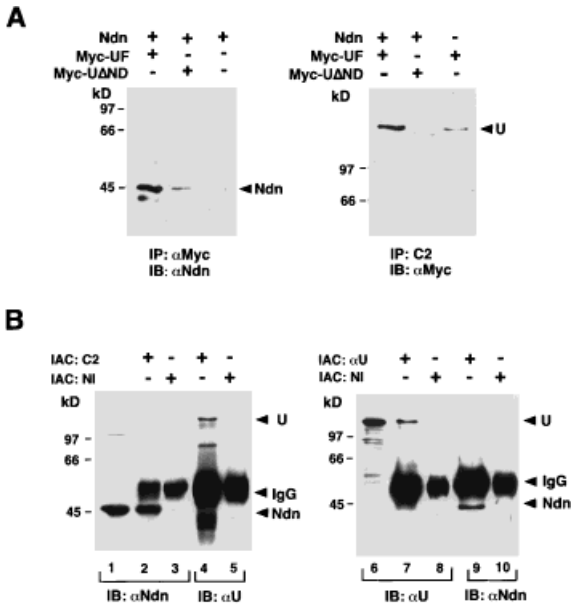


Fig. 3. Interactions in vivo between necdin and hnRNP U. **A**: Immunoprecipitation of the necdin-hnRNP U complex. SAOS-2 cells were transfected with expression vectors for necdin (Ndn), Myc-hnRNP U (Myc-UF) and Myc-hnRNP U Δ613–684 (Myc-UΔND) in combination, and the nuclear extract were immunoprecipitated. **Left**, immunoprecipitation (IP) with anti-Myc antibody (αMyc) and detection (IB) with NC243 (αNdn). **Right**, immunoprecipitation with anti-necdin antibody C2 and detection with anti-Myc antibody. **B**: Detection of endogenous necdin-hnRNP U complexes in neuronal nuclear extracts. Nuclear extracts prepared from P19 neurons were applied to the immunoaffinity column (IAC), and endogenous necdin (Ndn) and hnRNP U (U) in purified samples were detected by immunoblotting. **Left**, immunoaffinity column coupled with anti-necdin IgG (C2) (**lanes 2** and **4**) and detection (IB) with NC243 (αNdn) or HUT (αU). **Right**, immunoaffinity column (IAC) of HUT IgG (αU) (**lanes 7** and **9**) and detection (IB) with HUT (αU) or NC243 (αNdn). **Lanes 1** and **6**, 1/20 of the nuclear extracts; **lanes 3, 5, 8** and **10**, nonimmune IgG (NI) for negative controls. IgG, IgG detached from the immunoaffinity column.

~120 kDa hnRNP U and ~45 kDa necdin (Fig. 3B, lanes 7 and 9). These results suggest that necdin and hnRNP U form stable complexes in neuronal nuclei.

hnRNP U Associates With the Nuclear Matrix Through Its COOH-Terminal Region

To determine the nuclear matrix binding region of hnRNP U, SAOS-2 cells were transfected with cDNAs for Myc-tagged hnRNP U and its deletion mutants, and the nuclear matrix was prepared by in situ sequential extraction. All the hnRNP U mutants contained a putative nuclear localization signal (NLS) (amino acids 218–226) [Kiledjian and Dreyfuss, 1992] were localized to the nuclei by immunocytochemistry (Fig. 4A, a,c,e,g,i). Localization of the mutant UC (U614-800) lacking NLS was also primarily nuclear, but the cytoplasm was weakly stained (Fig. 4A, k). Full-length hnRNP U (UF, U1-800) and the mutants containing the COOH-terminal glycine-rich region UΔN (UΔ1–123), and UC (U614–800) were associated with the nuclear matrix (Fig. 4A, b,d,l). In contrast,

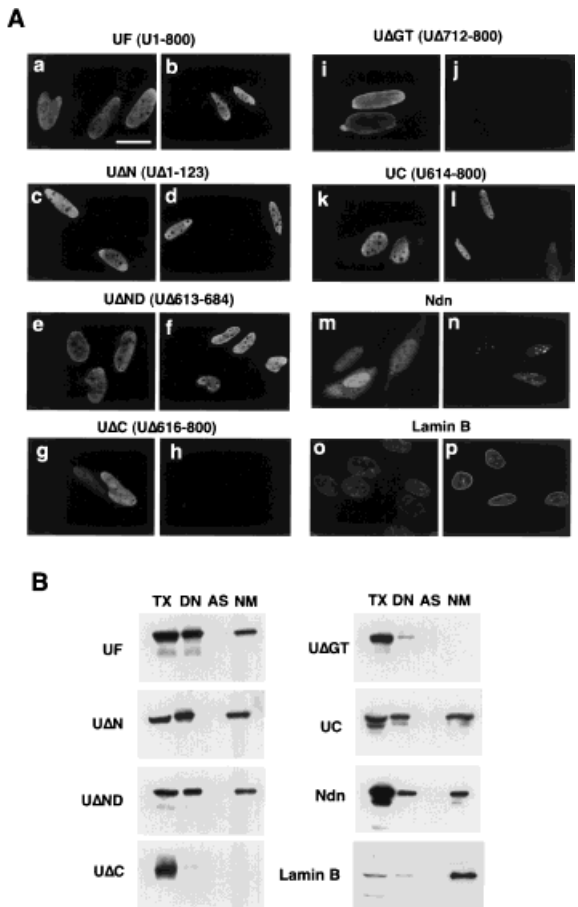


Fig. 4. hnRNP U associates with the nuclear matrix through its COOH-terminal region. **A**: hnRNP U and necdin in the nuclear matrix in situ. SAOS-2 cells were transfected with pRc/CMV vectors expressing Myc-tagged fusions of hnRNP U deletion mutants (UF, U1–800; UΔN, UΔ1–123; UΔND, UΔ613–684; UΔC, UΔ616–800; UΔGT, UΔ712–800; UC, U614–800) and necdin (Ndn). Cells were stained with anti-Myc antibody, NC243 or anti-lamin B antibody (**a, c, e, g, i, k, m**, and **o**). After in situ sequential extraction of the nuclear matrix, cells were stained with the same antibodies (**b, d, f, h, j, l, n**, and **p**). Scale bar (in a for a–p), 20 μm. **B**: Immunoblot analysis. The nuclear matrix was prepared by the sequential extraction from transfected SAOS-2 cells, and volumetric equivalents of the extracted preparations were immunoblotted with anti-Myc antibody, NC243 or anti-lamin B antibody. **Lanes** (samples extracted or digested with): **TX**, 0.5% Triton X 100; **DN**, 25 U/ml DNase; **AS**, 0.25 M ammonium sulfate; **NM**, nuclear matrix fraction after extraction with 2 M NaCl.

the COOH-terminal deletion mutants UΔC (UΔ616–800) and UΔGT (UΔ712–800) failed to bind to the matrix (Fig. 4A: h, j). Therefore, it is suggested that the matrix-binding domain of hnRNP U is localized to the COOH-terminal region (amino acids 712-800). hnRNP U lacking the necdin-binding domain UΔND (UΔ613–684), which failed to bind to necdin by co-immunoprecipitation assay, bound to the nuclear matrix (Fig. 4A, f), suggesting that the necdin-binding site and the matrix associating region are distinct. When SAOS-2 cells was transfected with cDNA for necdin, necdin was concentrated in the nucleus (Fig. 4A, m). In the nuclear matrix prepared by in situ extraction, necdin was concentrated in intranuclear speckles and nucleoplasmic staining was also observed (Fig. 4A, n). The distribution pattern of necdin in the nuclear matrix is distinct from that of hnRNP U. Lamin B was concentrated in the nuclear lamina, and a much weaker, diffuse immunoreactivity was detected in the nucleoplasm (Fig. 4A, o,p).

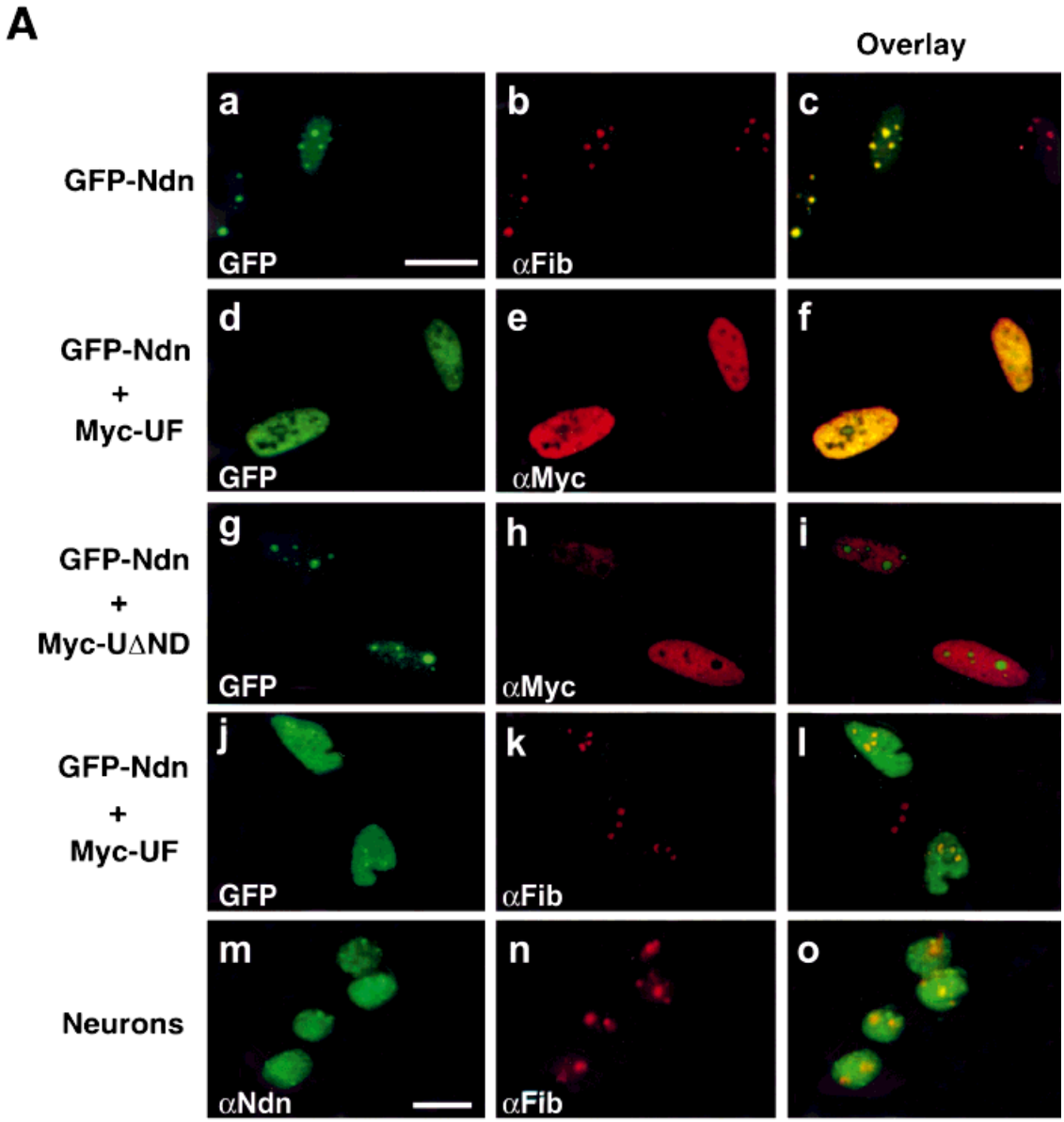
We then examined the association of the deletion mutants of hnRNP U and necdin with the nuclear matrix preparations by immunoblotting (Fig. 4B). UF and the mutants carrying the COOH-terminus (UΔN, UΔND, and UC) were associated with the matrix. UF was distributed in the Triton X-100 extractable fraction (46%), DNase releasable fraction (34%), and the nuclear matrix (20%). In contrast, the mutants lacking the COOH-terminal region (UΔC and UΔGT) were distributed predominantly in the Triton-X 100 extractable fraction. The immunoblot analysis also revealed that ~20% of necdin was associated with the nuclear matrix. Lamin B was found almost exclusively in the nuclear matrix fraction.

hnRNP U Recruits Necdin to Nucleoplasmic Matrix

We then examined whether hnRNP U alters the subnuclear distribution of necdin in the nuclear matrix using a green fluorescent protein (GFP)-necdin fusion (GFP-necdin). GFP-necdin was concentrated in the nucleolar matrix, and the nucleoplasmic matrix was weakly stained as judged by its co-localization with the nucleolar marker fibrillarin (Fig. 5A, a–c). Co-expressed Myc-hnRNP U recruited GFP-necdin to the nucleoplasmic matrix (Fig. 5A, d–f). Myc-hnRNP U lacking the necdin-binding domain (UΔND, UΔ613-684) had much less effects on the translocation of GFP-necdin (Fig. 5A, g–i). GFP-necdin in the presence of co-expressed Myc-hnRNP U was apparently distributed in both the nucleolar and nucleoplasmic matrices (Fig. 5A, j–l). GFP-necdin and Myc-hnRNP U were also accumulated in small nucleoplasmic speckles distinct from the nucleoli (Fig. 5A: d, j). Endogenous necdin was also detected predominantly in the nucleoplasmic matrix and was not particularly concentrated in the nucleolar matrix of postmitotic neurons (Fig. 5A: m–o). In the presence of hnRNP U (Myc-UF), ~80% of the total GFP-necdin positive cells showed the nucleoplasmic distribution pattern, whereas 16 and 4% of them were translocated to the nucleoplasmic matrix in the presence of the hnRNP U mutants defective in necdin-binding (UΔND) and in matrix targeting (UΔGT), respectively (Fig. 5B). These results suggest that both the necdin-binding and nuclear matrix-binding domains of hnRNP U are required for translocation of necdin to the nucleoplasmic matrix.

Necdin and hnRNP U Cooperatively Suppress the Cell Growth

We next examined the effects of necdin and hnRNP U on cell growth by the colony formation assay (Fig. 6A,B). We have previously reported that the expression vector for necdin (10 μg per 60-mm dish) alone induced a marked reduction of colony formation of SAOS-2 cells [Taniura et al., 1998]. We examined cooperative effects of necdin and hnRNP U on cell growth using smaller amounts of expression vectors in the colony formation assay (Fig. 6A,B). Expression vectors for necdin (0.8 μg per 35-mm dish) and hnRNP U (3.2 μg per 35-mm dish) slightly reduced the colony formation of SAOS-2 cells by 25 and 7%, respectively. However, necdin and Myc-hnRNP U in combination caused a marked suppression to $< 10\%$ of the control value. The hnRNP U mutants lacking the necdin binding domain (UΔND, UΔ613–684) and the nuclear matrix binding domain (UΔGT, UΔ712–800) failed to suppress the colony formation in the presence of necdin, suggesting that the growth suppressive effect of hnRNP U is dependent on its abilities to bind to necdin and the nuclear matrix. We also tested the effects of association between necdin and hnRNP U on BrdU incorporation, which represents the S-phase cell population. Necdin and hnRNP U in combination caused a significant reduction in the


A
Overlay
GFP-Ndn
a
GFP
b
αFib
c
GFP-Ndn + Myc-UF
d
GFP
e
αMyc
f
GFP-Ndn + Myc-UΔND
g
GFP
h
αMyc
i
GFP-Ndn + Myc-UF
j
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k
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Neurons
m
αNdn
n
αFib
o
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Nucleoplasmic necdin (%)
100
80
60
40
20
0
*
GFP-Ndn +
pRc
UF
UΔND
UΔGT

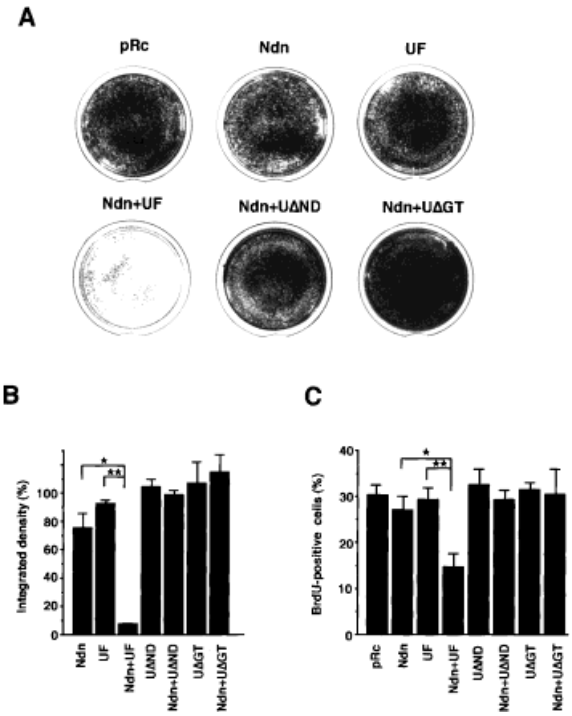

Fig. 6. Cooperation between necdin and hnRNP U in cell growth suppression. **A**: Colony formation assay. SAOS-2 cells were transfected with empty pRc/CMV vector (pRc), pRc-necdin (Ndn) or pRc-Myc-hnRNP U (UF). pRc-necdin was transfected in combination with pRc-Myc-hnRNP U (Ndn + UF), pRc-Myc-hnRNP UΔ613–684 (Ndn + UΔND) or pRc-Myc-hnRNP UΔ712-800 (Ndn + UΔGT). Cells were cultured in the presence of G418 for 10 days and the drug-resistant colonies were stained with crystal violet. **B**: Quantification of the colony formation. Integrated densities of stained colonies of transfected SAOS-2 cells are presented. Each value represents the mean ± SEM (n = 3) (the value of pRc = 100%). * and **, significant at < 0.02 and < 0.01, respectively. **C**: BrdU incorporation assay. SAOS-2 cells were transfected with expression vectors and pMSCV-puro, and puromycin-resistant transfectants were labeled with BrdU. BrdU-positive cells were counted among > 100 cells. Each value represents the mean ± SEM (n = 3). * and **, significant at < 0.05 and < 0.01, respectively.

number of BrdU positive cells (Fig. 6C). In contrast, the mutants UΔND (UΔ613–684) and UΔGT (UΔ712–800) in the presence of necdin had no effect on the S-phase cell number. These results suggest that necdin and hnRNP U cooperatively suppress replicative cell growth.

DISCUSSION

Our present study has shown that necdin physically and functionally interacts with hnRNP U. The present in vitro binding assay showed that the region containing residues 613–711 of hnRNP U is required for the interaction with necdin (Fig. 1). The RNA binding region of hnRNP U has previously been mapped to the COOH-terminal G-rich region containing RGG box motifs (amino acids 695–716 of human hnRNP U) [Kiledjian and Dreyfuss, 1992]. Furthermore, hnRNP U has been known as a nuclear matrix-associated protein [Tsutsui et al., 1993; Fackelmayer et al., 1994; Mattern et al., 1996]. The present transfection study revealed that hnRNP U is associated with the nuclear matrix via its COOH-terminus (amino acids 613–800) (Fig. 4). Thus, the COOH-terminal region (amino acids 613–800) of hnRNP U comprises binding domains for necdin, RNA, and the nuclear matrix. Chromosomal DNA is associated with the nuclear matrix via the specific DNA element termed SAR/MAR (scaffold/matrix attachment region). hnRNP U binds to SAR/MAR and may participate in anchoring genomic DNA to the nuclear matrix [Romig et al., 1992; Tsutsui et al., 1993]. The SAR/MAR-specific DNA-binding domain of hnRNP U has been mapped to its NH_2-terminus SAF-Box [Gohring et al., 1997; Kipp et al., 2000]. Thus, hnRNP U may link chromatin and the nuclear matrix via its NH_2-terminus and COOH-terminus, respectively.

Ectopic necdin was concentrated in the nucleoli of transfected SAOS-2 cells after in situ extraction to prepare the nuclear matrix. In contrast, necdin was localized predominantly to the nucleoplasmic matrix in postmitotic neurons and hnRNP U-cotransfected SAOS-2

Fig. 5. hnRNP U recruits necdin to the nucleoplasmic matrix. **A**: Translocation of necdin by co-expressed hnRNP U. SAOS-2 cells were transfected with combinations of expression vectors for GFP-necdin (GFP-Ndn) (**a–l**), Myc-hnRNP U (Myc-UF) (d–f and j–l), and Myc-hnRNP UΔ613–684 (Myc-UΔND) (g–i). The nuclear matrix was prepared in situ, immunostained for fibrillarin (αFib) (b and k, red) or Myc-tag (αMyc) (e and h, red) and observed by fluorescence microscopy. GFP, fluorescence of GFP-necdin fusion protein. The nuclear matrix of P19 neurons (Neurons) (**m–o**) was double-labeled with NC243 (αNdn) (m, green) and anti-fibrillarin antibody (αFib) (n, red). The images are merged for co-localization (overlay). Scale bars (in a for a–l), 20 μm; (in m for m–o), 10 μm. **B**: Quantification of nucleoplasmic necdin translocated by co-expressed hnRNP U. GFP-necdin (GFP-Ndn) was transfected in combination with expression plasmids encoding Myc-hnRNP U (UF), Myc-hnRNP UΔ613–684 (UΔND), Myc-hnRNP UΔ712–800 (UΔGT), or pRc/CMV empty vector (pRc) into SAOS-2 cells. After the nuclear matrix was prepared in situ, cells showing translocated GFP-necdin were scored in GFP positive cells. Each value represents the mean ± SEM (n = 3). *, significant at < 0.05.

cells (Fig. 5). We used the colony formation assay of SAOS-2 cells for elucidating functional roles of the necdin-hnRNP U complex and demonstrated that necdin and hnRNP U cooperate to suppress both cell growth and S phase entry (Fig. 6). The hnRNP U mutants lacking the necdin binding domain (UΔND, UΔ613–684), and the nuclear matrix binding domain (UΔGT, UΔ712–800) neither translocated necdin to the nuclear matrix nor suppressed the cell growth in cooperation with necdin. Recently, it was reported that the NH_2-terminal 45 residues of hnRNP U (SAF-Box) are necessary and sufficient for specific binding to SAR/MAR DNA [Kipp et al., 2000]. We observed that hnRNP UΔN (UΔ1–123) lacking SAF-Box showed neither necdin translocation activity nor growth suppression (data not shown), suggesting that the interaction between necdin/hnRNP U complex and chromatin DNA is necessary for these cooperative functions.

Necdin binds to the viral oncoproteins SV40 large T antigen and adenovirus E1A [Taniura et al., 1998]. The viral oncoprotein selectivity of necdin is consistent with that of retinoblastoma protein (Rb), whose major cellular target is the transcription factor E2F1. Necdin, like Rb, interacts with the transactivation domain of E2F1 and represses E2F1-driven transcription [Taniura et al., 1998]. Rb is associated with the nuclear matrix [Mancini et al., 1994] and interacts with hBRM/hBRG1 proteins [Strober et al., 1996], which are chromatin remodeling factors associated with the nuclear matrix [Reyes et al., 1997]. Rb requires hBRG1 to keep cells in G1 phase of the cell cycle [Dunanief et al., 1994], suggesting that Rb-dependent growth suppression involves chromatin remodeling. The fact that hnRNP U anchors chromosomal DNA to the nuclear matrix leads to the speculation that the necdin-hnRNP U complex induces cell growth suppression through nuclear matrix mediated modifications of chromatin configuration. Further studies on the functional roles of the necdin-hnRNP U complex in the nuclear matrix will provide valuable information about molecular mechanisms underlying permanent growth arrest and terminal differentiation of postmitotic cells.

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