Biochemical characterization of mouse brain necdin

Etsuko MARUYAMA*

Department of Molecular Neurobiology, Tokyo Metropolitan Institute for Neuroscience, 2–6 Musashidai, Fuchu, Tokyo 183, Japan

Necdin is a protein encoded by neural differentiation-specific mRNA derived from embryonal carcinoma cells (P19). Necdin of mouse brain was characterized by Western blotting and silver-staining analysis by using affinity purified antibodies to 17 synthetic peptides of deduced C-terminal amino acids. Necdin exhibits a molecular mass of 51 kDa on SDS/PAGE, and is localized in the S1 and S2 nucleosomal fractions. Sonicated necdin is found in all fractions of Sephacryl S-300 gel filtration chromatography, with a peak at 700 kDa. Necdin is released on micrococcal nuclease digestion, which is essential for electrophoretic migration on acetic acid/urea/Triton gels, suggesting that it could be a DNA-binding protein. Nucleosomal necdin shows two peaks at approx. 10 S and approx. 20 S on sucrose gradient centrifugation in the presence of 0.6 M NaCl, and a single peak in the presence of 2.0 M NaCl. Necdin forms a huge complex through chemical cross-linking with glutaraldehyde or dimethyl sulphate. The silver-staining intensity of the 51 kDa band corresponds to the decrease in the immuno-staining in a reagent concentration-dependent manner. Necdin binds tightly to a double-stranded DNA affinity chromatography column, and can be eluted from it with 2.0 M NaCl after washing with 0.6 M NaCl (approx. 100 ng per ml of gel). This purified necdin exhibits a pI of 9.1 on isoelectric focusing. The nucleosomal necdin complex (> 200 kDa) was adsorbed on an organomercurial agarose affinity chromatography column and was eluted with 10 mM DTT, revealing that necdin is possibly involved in the transactive nucleosomal complex. These data show that necdin is a nuclear basic DNA-binding protein that associates with other molecules to regulate transcriptionally active genes and nuclear function.

INTRODUCTION

In the cell cycle of neurons, the new cell cycle, which comprises the consecutive phases G1, S, G2 and M, cannot be initiated except in primary neuron cells. To investigate the mechanisms underlying its initiation and repression during neuronal cell differentiation it is essential to identify the genes involved. The P19 mouse embryonal carcinoma cell line, which differentiates into neuronal cells on exposure to retinoic acid, is a suitable model system with which to analyse the regulation of neuronal differentiation. Thus necdin, which is specifically expressed in differentiated neurons, was found as a retinoic acid-inducible neuronal differentiated protein in P19 cells. Its cDNA encodes a 37 kDa protein consisting of 325 amino acid residues, and the mRNA was expressed in brain but not in non-neuronal tissues [1]. Further immunohistochemical studies showed that necdin is a nuclear protein expressed in differentiated mouse brain cells [2]. Proteins localized in the nuclei of neuronal cells are of special interest because a higher-order nucleoprotein complex, which seems to proceed with alteration of DNA structures, mediates a variety of DNA metabolic events such as replication, transcription and recombination. Recently, K. Takagi and K. Yoshikawa (unpublished work), using an *in situ* hybridization technique, found that necdin mRNA is located in post-mitotic neuron cells, and concluded that necdin is useful as a marker of differentiated brain cells, especially in an early stage of neurogenesis. Hence necdin is likely to function in neurons, yet it lacks any known motifs that would confer a biochemical role. All trials, such as cloning of stable transfectants and isolation from a bacterial expression vector, were unsuccessful, and consequently I used mouse brain for analysis of the necdin molecule as a first step towards understanding its role. Proteins that are important in the neuronal differentiation of P19 cells are beginning to be identified, including retinoic acid receptors and the transcription factors Oct-3 and Brn-2. Here I report studies on subcellular localization, characterization by affinity chromatography and biochemical analysis of the necdin protein band by comparison of silver-staining and immunostaining, and shed light on its nuclear function related to transcriptionally active genes.

MATERIALS AND METHODS

Chemicals

Micrococcal nuclease was from Funakoshi, Pefablock SC from Boehringer, and poly(vinylidene difluoride) (PVDF) membranes were from Applied Biosystems. Chemiluminescence detection materials, including goat anti-rabbit IgG conjugated to alkaline phosphatase, and a chemiluminescence substrate, were from Tropix (Bradford, MA, U.S.A.). DNA affinity cellulose (single strand and double strand) was purchased from Pharmacia LKB Biotechnology Inc., and organomercurial agarose (Affi-Gel 501) from Bio-Rad.

Preparation of antibodies

Antisera were made against rabbits by using synthetic peptides (residues 58–72 for the N-terminal region, and residues 309–325 for the C-terminal region) as previously reported [2], and kindly provided by Dr. K. Yoshikawa (Osaka University). The

Abbreviations used: DMS, dimethyl sulphate; IEF, isoelectric focusing; PVDF, poly(vinylidene difluoride).

* Present address: Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, 2–6 Musashidai, Fuchu, Tokyo 183, Japan.

polyclonal antisera were purified by peptide-conjugated affinity chromatography, and the specificity of the antibody was examined by competition with the synthetic peptides.

Western blotting analysis

For SDS/PAGE, 3–16 % or 8 % (w/v) gels were used. Proteins were transferred electrophoretically to PVDF membranes by the semi-dry blotting method. The membranes were blocked with PBS containing 5 % (w/v) non-fat dry milk and 0.1 % Tween-20 (Tween/PBS) for 1 h at room temperature, and then incubated for 12 h at 4 °C with a 1:2000 dilution of the affinity-purified antibody in Tween/PBS. The filters were washed three times for 15 min in Tween/PBS, and then incubated with a 1:3000 dilution of the goat anti-rabbit alkaline phosphatase-conjugated second antibody in Tween/PBS for 1 h at room temperature. A chemiluminsecence kit (Boehringer) was used to reveal the bands as described by the manufacturer, using disodium 3-(4-methoxyspirol{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1.^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) as a substrate. X-ray films were exposed for periods from 30 min to 18 h. Protein concentrations were determined by a Protein assay or DC Protein assay (Bio-Rad).

Isolation and nuclease digestion of mouse brain nuclei

Nuclei were isolated from fresh brains (postnatal day 6 or day 25) by the method of [3] with minor modifications. All procedures were performed at 4 °C unless otherwise stated. The brains (5 g) were homogenized with a Dounce homogenizer in 50 ml of buffer A (0.32 M sucrose containing 25 mM triethanolamine, 5 mM sodium butyrate, 25 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM spermidine and 0.5 mM Pefablock SC, pH 7.4). To obtain a nuclear S fraction, the freshly isolated nuclei were washed in solution A at 4 °C by centrifugation at 1000 ***g*** for 10 min and then suspended in the same buffer at a concentration of about 3 mg/ml (as DNA). The nuclei were preincubated at 37 °C for 5 min with 10 mM $CaCl_2$ and then 20 units of micrococcal nuclease per mg of DNA were added. After incubation for 10 min at 37 °C, the sample was cooled on ice. After 10 min on ice, the sample was centrifuged for 10 min at 13000 ***g*** at 4 °C and the supernatant (S1 fraction) collected. After the pellet (0.25 ml) had been dispersed in a 2 mM EDTA solution (pH 7.4), additional EDTA solution was added to give a final concentration of about 4 mg/ml, and solubilization of the sample was continued by suction into a pipette. After 10 min on ice, a supernatant and a pellet were obtained by centrifugation (S2 and nuclear matrix fractions respectively). A freshly prepared sample were needed for biochemical characterization of necdin.

Gel-filtration chromatography and sucrose gradient centrifugation

Isolated brain nuclei necdin was solubilized by sonication, with five 10 s bursts in a sonicator (Tomy Co.), and soluble and insoluble protein fractions were separated by centrifugation for 20 min at 20000 ***g***. The supernatant (300 μg of protein) was applied to a Sephacryl S-300 chromatography column (1.2 cm × 70 cm), equilibrated with 50 mM Tris/HCl, pH 7.4, and containing 0.3 M NaCl when SDS was also contained in the buffer. Proteins were fractionated at 0.2 ml/min.

For sucrose-gradient centrifugation analysis of the S fraction, the protein (0.2 ml; 200 μg in 50 mM Tris/HCl, pH 7.5) was layered on top of a 12 ml 5–25 %(w/w) sucrose gradient in 50 mM Tris/HCl (pH 7.5), and then centrifuged at 250000 ***g*** ($R_{max.}$) at 4 °C for 14 h in the RPS 40T rotor from Hitachi Koki Co. The gradients were fractionated, from bottom to top, into eight 1.4 ml aliquots, each of which was assayed by Western blotting and silver-staining.

Affinity chromatography

The protein extracted by sonication was loaded onto a double-stranded or single-stranded calf thymus DNA–cellulose column at room temperature, and the bound proteins were then eluted with buffer containing 10 mM Tris/HCl (pH 7.5) and 0.3 M NaCl, with increasing concentrations of NaCl. The flowthrough and eluted fractions were precipitated with 25 % (w/v) trichloroacetic acid followed by washing with ethanol and diethyl ether, and then analysed by Western blotting.

In accordance with Walker's method [4], necdin was applied to an organomercurial affinity column. EDTA was added to the recovered S fraction to 5 mM and the nucleosomes (500 μg) were loaded on 1 cm × 1 cm columns of Affi-Gel 501 at a flow rate of 20 ml/h. Before organomercurial affinity chromatography, mini-prep Sephacryl S-300 (Pharmacia LKB Biotechnology Inc.) was used to separate nucleosomes from smaller chromatin fragments. After being loaded, the column was washed with buffer B (10 mM Tris/Cl, pH 7.5, 25 mM KCl, 25 mM NaCl, 5 mM sodium butyrate, 5 mM EDTA, 0.5 mM Pefablock SC) to elute the unbound nucleosomes. After extensive washing with buffer B containing 0.5 M NaCl (20 vol.), proteins were eluted with 10 mM DTT.

Chemical cross-linking

The cross-linking reaction was initiated by the addition of glutaraldehyde (0.02–2 mM) or dimethyl suberimidate (Pierce) (0.5–10 mM) to the necdin sample by the methods of Baler et al. [5] and Mirzabekov et al. [6] respectively. The reaction was performed at room temperature, and to stop the reaction the cross-linking was quenched by the addition of 1M lysine to a concentration of 0.1M. The cross-linked species were analysed by electrophoresis on 3–16 % (w/v) gradient gels (TEFCO Co).

Electrophoresis

Acetic acid/urea/Triton gel electrophoresis was performed by the method of Terrell and Johnson [7] with gels containing 1 % (w/v) Triton X-100. Two-dimensional gel electrophoresis (NEPHGE) was performed by the method of O'Farrell et al. [8]. The protein sample for isoelectric focusing (IEF) was prepared by addition to an equal volume of IEF sample buffer containing 2 % (w/v) Nonidet P-40, 2 % (v/v) Cervarite (pH 3–11) (Cerva Inc.), 10 % (v/v) glycerol and 1 % (w/v) Methyl Red, and separated on IEF gel from TEFCO as described by the manufacturer. For two-dimensional gel electrophoresis the samples were at first analysed by IEF on 4.5 % polyacrylamide vertical slab gels containing 8.3 M urea, 2 % Cervarite and 1 % Nonidet P-40, and then a cut strip was put on the second gel by using the NEPHGE system [8]. The cathode buffer was 0.02 M sodium hydroxide and the anode buffer 0.01 M phosphoric acid.

Reproducibility

The experiments were repeated at least three times for all the figures, and similar results were obtained.

RESULTS AND DISCUSSION

Western blotting and subcellular localization

First, Western blotting was performed on the whole homogenate, P1 fraction, mitochondrial fraction and nuclear S fraction.

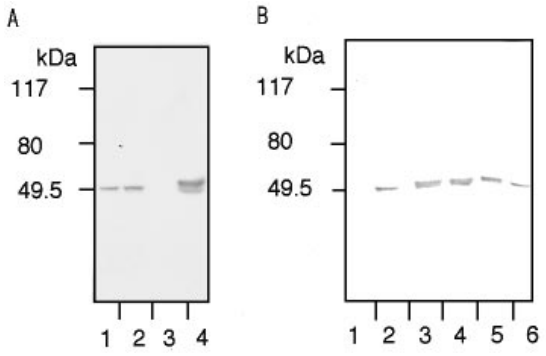

Figure 1 Subcellular localization of necdin

Equal amounts of cytoplasmic and nuclear protein (50 μg) were used for immunoblotting. The nuclear fraction was digested with micrococcal nuclease as described in the Materials and methods section. The S1 fraction is a soluble nucleosomal fraction enriched with mononucleosomes, and the S2 fraction consists of mono- and poly-nucleosomes. (**A**) Lane 1, whole homogenate; lane 2, P1 fraction; lane 3, mitochondrial fraction; lane 4, nucleosomal S fraction. (**B**) Lane 1, 10 000 g supernatant; lane 2, whole homogenate; lane 3, whole nuclear fraction; lane 4, S1 fraction; lane 5, S2 fraction; lane 6, nuclear matrix fraction.

Necdin is a protein exhibiting an apparent molecular mass of 51 kDa with a peptide-specific antibody for the C-terminal region (Figure 1A) (although an antibody for the N-terminal region of necdin does not stain any protein) and is localized in the nuclear S fraction. Necdin might be partly modified because a minor band at the lower-side was often detected by using a partly purified sample. Necdin is immunostained throughout brain development, as previously observed in an immunohistological study [2]. Necdin consists of 325 amino acids, so its molecular mass should be 37 kDa [1], but the necdin immunostained band is about 10 kDa heavier than that. This might be due to the abundant basic amino acids (the pI calculated from cDNA is 8.7), as it is known that extensive post-translational modifications or basic amino acid or proline enrichment causes the molecular mass to be higher than deduced. Concerning the specificity of the deduced amino acid sequence, necdin has two regions with extreme acidic and basic pI values, 2.6 (residues 7–65) and 11.5 (residues 117–160) respectively, although the sequence of necdin shows no obvious similarity to that of other known proteins, nor does it at present seem to possess a functional domain. Additionally, proline-rich sequences occur in the former region.

Next, the location of necdin in nuclei was examined because the organization of chromatin in the eukaryotic nucleus is thought to play an important part in the regulation of gene activity. The purified nuclei were treated with micrococcal nuclease, and necdin immunostaining bands of the 10000 g supernatant, whole homogenate and nuclear subcellular fractions, and the S1, S2 and matrix fractions were compared. Linkers in transcribed regions are readily digested with nuclease because distinct structural differences exist between transcriptionally active and inactive DNA linkers. Most of the necdin complex (90%) was released efficiently from isolated nuclei on incubation in the presence of micrococcal nuclease, suggesting that necdin is a DNA-binding protein. No immunostained band was obtained by Western blotting for the cytosolic fraction, necdin being localized in the S1 and S2 nucleosomal fractions and to a smaller extent in matrices (Figure 1B). Therefore necdin may be associated with the transcribed regions of chromatin and may be involved in the regulation of gene expression.

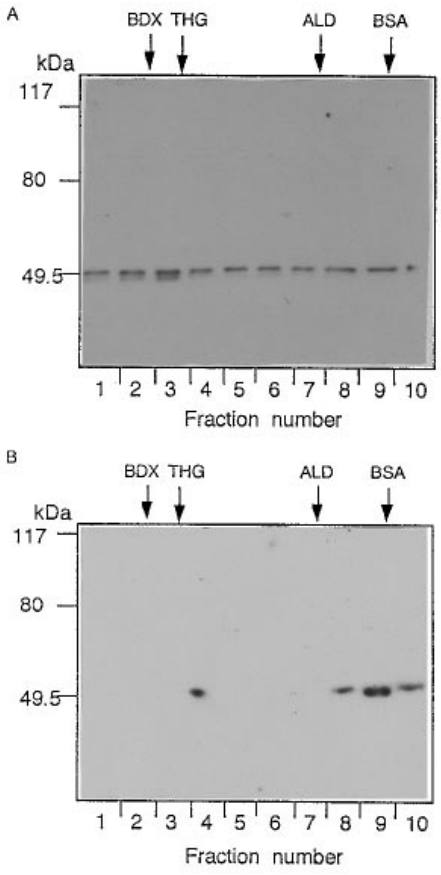

Figure 2 Immunoblotting after separation on Sephacryl S-300 gel chromatography

(**A**) Nuclear sonicated extracts were applied to a column equilibrated with Tris/HCl buffer (pH 7.4) containing 0.3 M NaCl. (**B**) The nuclear fraction that had been boiled with 0.5 % SDS and 2-mercaptoethanol was separated in the presence of 0.5% SDS. Abbreviations: BDX, Dextran Blue; THG, thyroglobulin (670 kDa); ALD, aldolase (158 kDa); BSA, bovine serum albumin (67 kDa).

Gel filtration and sucrose gradient centrifugation

As a biochemical approach, necdin was solubilized with detergents and its native molecular state examined by means of its purification. However, all the trials, such as anionic or cationic ion chromatography of brain extracts, were unsuccessful because of broad elution and extremely low recovery. On gel filtration on Sephacryl S-300, necdin was detected in all fractions in the presence of 0.3 M NaCl (Figure 2A), 0.5% Triton X-100 and 0.3 M NaCl or 0.5% SDS, and the elution peak corresponded to a molecular mass of 700 kDa. However, necdin fractionated at 51 kDa after being boiled in the presence of SDS and 2-mercaptoethanol (Figure 2B). Thus in the native state necdin seems to comprise an assemblage brought about by multiple protein–protein interactions. Consistent with this, a similar pattern for nucleosomal necdin was observed by sucrose gradient centrifugation. Two peaks, at approx. 10 S and approx. 20 S, were observed in the presence of 0.3 and 0.6 M NaCl (Figures 3A and 3C) and necdin was differentially distributed in the various fractions. These results indicate that necdin forms a high-

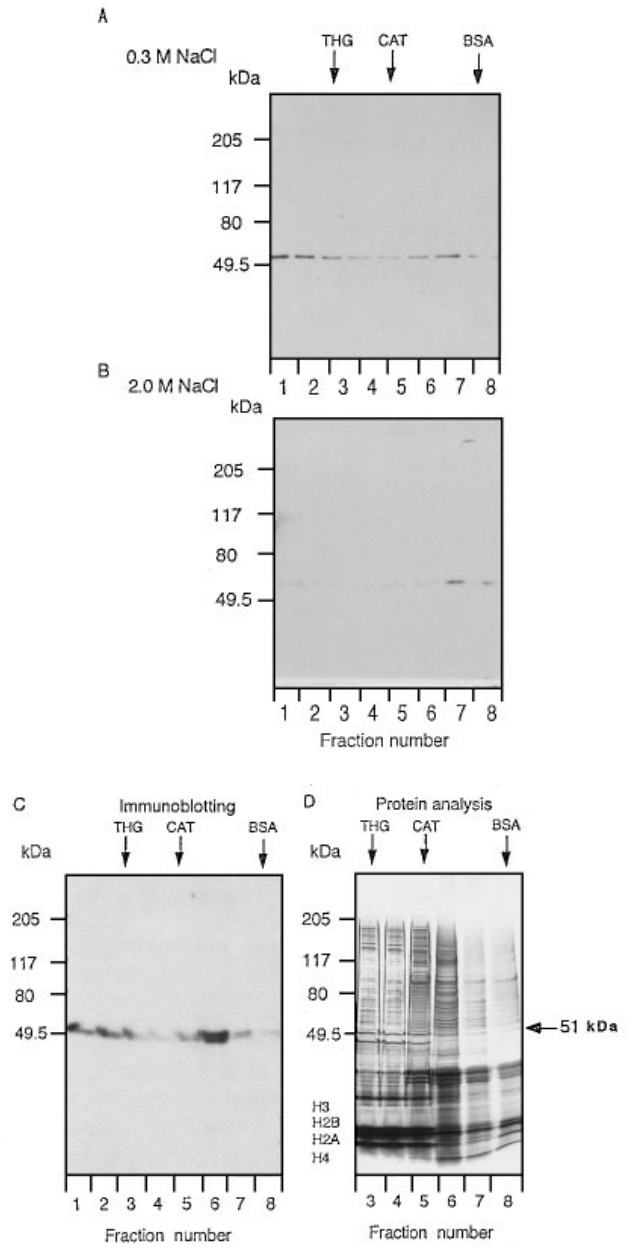

Figure 3 Separation of necdin by sucrose gradient centrifugation

The nucleosomal S fraction was sedimented on a 5–25% sucrose gradient in the presence of 0.3 M NaCl (**A**) or 2 M NaCl (**B**) and fractionated. Each fraction was analysed by SDS/PAGE (3–15% gels) and Western blotting was performed. The immunostaining and protein patterns of sedimentation in the presence of 0.6 M NaCl are shown in (**C**) and (**D**) respectively. Abbreviations: THG, thyroglobulin (20 S); CAT, catalase (11 S); BSA, bovine serum albumin (4.3 S).

molecular-mass complex even in the presence of 0.3 M NaCl or 0.6 M NaCl. In the presence of 2.0 M NaCl necdin fractionated as one major peak sedimenting slightly more rapidly than BSA (Figure 3B), indicating that the necdin complex can dissociate under high salt conditions. Because multimers of nucleosomes can be separated by centrifugation in sucrose gradients, the nucleosomal S fraction was also analysed by silver-staining after sedimentation, as shown in Figure 3D. All fractions contained equimolar amounts of histones H3, H2B, H2A and H4 but not

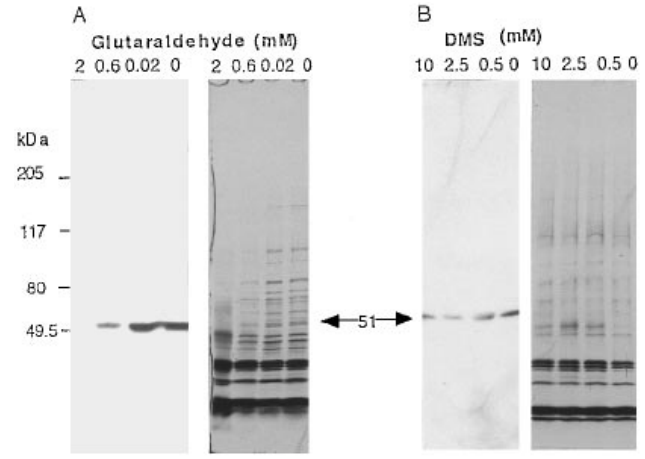

Figure 4 Analysis of necdin bands after chemical cross-linking

Necdin immunobands and protein bands were compared after nucleosomal necdin had been treated with glutaraldehyde (**A**) or DMS (**B**). Silver-stained 51 kDa protein bands were detected in parallel with the intensity of immunostaining with various concentrations of reagents.

H1. It is difficult to resolve an H1 band because it is known to be heterogeneous, in contrast with the other histones, and to comprise multiple forms that vary between tissues and species. These results mean that the necdin sample behaves in a heteromeric manner in the native state. Hence it has become important to further examine its properties, i.e. how necdin acts or interacts with other substances in nuclei.

Chemical cross-linking

The above gel filtration and sucrose gradient analyses indicated that necdin forms a complex, and so a cross-linking experiment was performed by using glutaraldehyde or dimethyl sulphate (DMS). The necdin immunoband decreased in a concentration-dependent manner (Figures 4A and 4B). Sometimes an immunostained band was detected at the extreme top with the higher concentration of reagent, indicating that native necdin forms a complex of large molecular mass through cross-linking. Unfortunately no associated protein has yet been detected in immunoprecipitation experiments under various conditions. To detect the necdin protein band by SDS/PAGE, the nucleosomal S2 fraction on postnatal day 6 was used, and the bands were compared by immunostaining and silver-staining. The 51 kDa band disappeared in parallel with the decrease in immunostaining in the case of both glutaraldehyde and DMS, and disappeared completely with the highest concentration of glutaraldehyde (Figure 4A). The necdin protein band could be detected only when the sample was prepared before day 6 because after this period another major 50 kDa band appeared and disturbed the detection of the necdin band.

Affinity chromatography

Because nuclease digestion suggested that necdin may interact with DNA of nucleosomes, it was suspected that necdin could bind directly with DNA. Necdin binding ability was analysed by means of DNA affinity chromatogaphy. The patterns of necdin binding by single-stranded DNA affinity chromatography and double-stranded DNA affinity chromatography are evidently

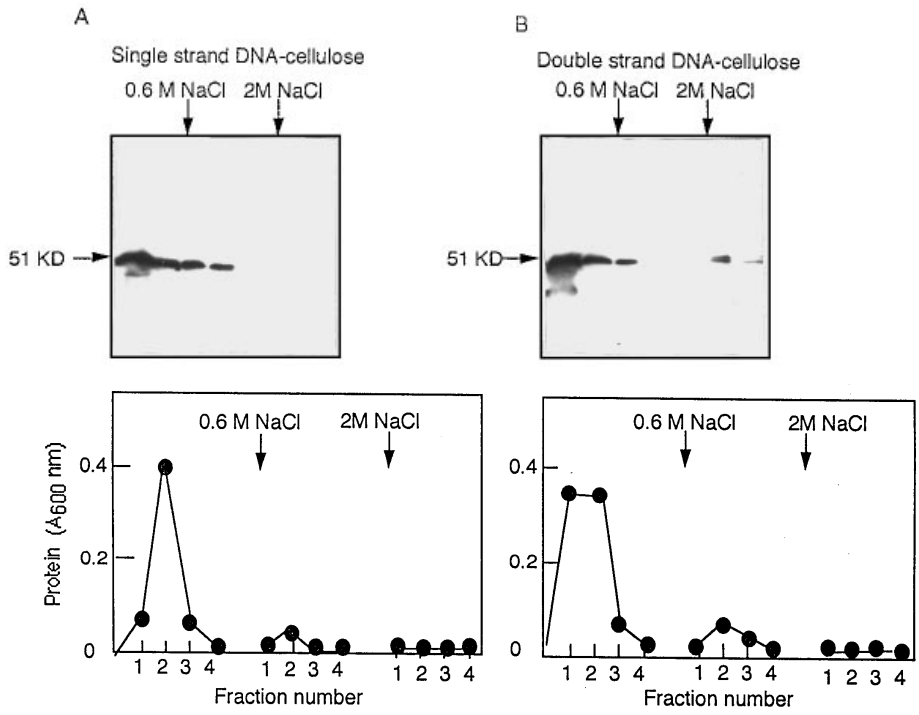

Figure 5 DNA affinity chromatography of mouse brain necdin

Sonicated extracts were applied to a single-stranded (**A**) or double-stranded (**B**) DNA affinity column (0.8 cm × 1.5 cm) and analysed simultaneously. Immunoblotting (upper panel) and protein (lower panel) patterns are shown. Necdin that was bound tightly to a double-stranded DNA affinity column was eluted with 2.0 M NaCl. KD = kDa.

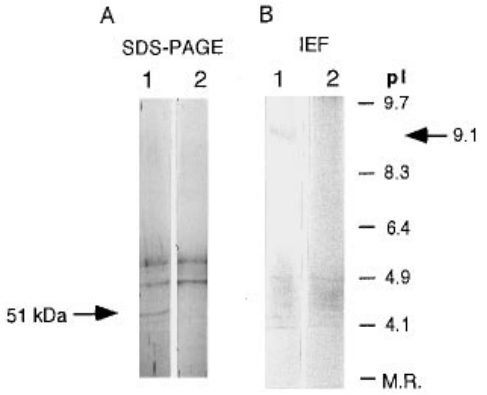

Figure 6 Silver-staining of necdin purified by DNA–cellulose column chromatography

(**A**) Silver-staining after SDS/PAGE. (**B**) Silver-staining after IEF. Lane 1, eluate from a double-stranded DNA–cellulose column with 2.0 M NaCl; lane 2, eluate from a single-stranded DNA–cellulose column. Arrows indicate the molecular mass and pI of the necdin band. M.R., Methyl Red.

different (Figures 5A and 5B). Necdin was eluted by buffer containing 0.6 M NaCl on both columns, but, surprisingly, some necdin bound tightly to the double-stranded DNA column and was eluted by 2 M NaCl. This elution at 2 M NaCl was confirmed for various necdin samples with a gradient of 0.2–2.5 M NaCl. This tight binding to the double-stranded DNA column was also observed with the use of a preparation of neuron-differentiated P19 cells. To determine what kind of molecules bind tightly to necdin on a double-stranded DNA column, the purified protein in 2 M NaCl eluate was analysed after separation and compared by chromatography on both DNA columns. It was demonstrated that necdin itself binds to DNA (approx. 100 ng per ml of gel conjugated with 1.6 mg DNA), and other bands were found to be due to non-specific interactions (Figure 6A). The two bands on both columns are probably non-specific ones because it has been reported that the appearance of multiple bands in the region of 54–68 kDa is caused by 2-mercaptoethanol [9]. These results indicate a possible role of necdin in the regulation of DNA metabolism. In this experiment necdin could also be identified as a protein band on a PVDF membrane by one step-purification, which led to further biochemical analysis (Figure 6B). However, I have not yet obtained necdin in a sufficient quantity for careful characterization of biochemical function and structure.

There are many transcription factors and regulatory proteins with DNA-binding ability, and although the biochemical mechanism by which these proteins act is currently obscure, it is said that the basic region of transcription factors can interact with DNA in co-operation with its acidic region [9a]. The two diverse pI regions of necdin might contribute to its DNA-binding ability. For transcription to function, necdin must interact with some target protein(s) in the transcriptional machinery, such as TATA-binding protein, or it could interact with specific DNA regulatory sequences. A double-stranded DNA-binding protein, the Ku protein, which is expressed ubiquitously, has recently been reported to form a complex with p350 only in the presence of double-stranded DNA to initiate transcription, and the binding of Ku protein to DNA controls the manner of the phosphorylation activity of p350 [10]. This leads to speculation about a similar regulatory role for necdin in the nuclei of neurons.

An organomercurial agarose column retains nucleosomes

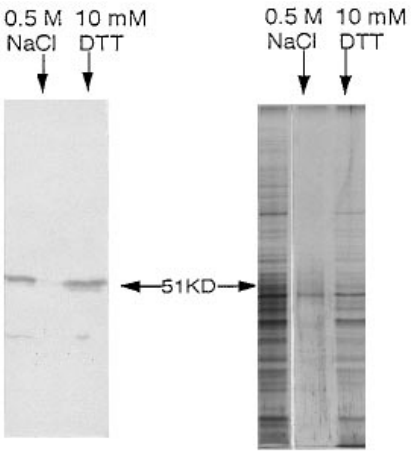

Figure 7 Necdin binding pattern from organomercurial agarose affinity chromatography

The nucleosomal S fraction (postnatal day 7) was applied to an affinity column by the method of Allegra et al. [11], and immunoblotting (left) or silver-staining (right) were performed after SDS/PAGE (8% gel). The left lane in each was a sample before chromatography. Necdin was eluted in a transcriptionally active nucleosomal fraction by 10 mM DTT. KD = kDa.

containing transcribed DNA sequences, whereas the compactly beaded nucleosomes of transcriptionally inert genes pass through the column [11]. Walker et al. [4] examined the reason for the selective binding of nucleosomes from transcriptionally active genes to organomercurial agarose columns and presented evidence that histone H3-thiol groups are directly involved in the organomercury binding of transcribed DNA sequences of mammalian cells. The nucleosomal nedcin complex of more than 200 kDa bound on organomercurial agarose affinity chromatography and eluted with DTT (Figure 7, left). No necdin band was found in the flowthrough fraction or the 0.5 M NaCl eluates. The protein band was also revealed on silver-staining, as shown in Figure 7 (right). The necdin band was detected just above a 50 kDa protein band. The necdin complex treated with 8 M urea was not adsorbed on the column, probably because the protein complex was disrupted. These results suggest that necdin is probably involved in a histone octomer comprising a transcriptionally active gene, although it cannot be ruled out that other thiol group(s) that have an affinity for mercury at pH 7.5 are exposed on the necdin complex surface.

Analysis by acetic acid/urea/Triton electrophoresis, two-dimensional electrophoresis and isoelectric focusing

No clear immuno-band was detected by non-reduced SDS/PAGE, so nuclear necdin was analysed by acetic acid/urea/Triton electrophoresis with and without nuclease digestion. Necdin was stained on the top of the well without digestion, but migrated after nuclease digestion (Figure 8), indicating that necdin is involved in a histone–DNA complex. The molecular mass (51 kDa) was confirmed by using cross-linked cytochrome C (monomer, dimer, trimer, tetramer and hexamer) (Oriental Co).

As it was thought that monomeric necdin binds tightly to a double-stranded DNA affinity column (Figure 6A), the necdin pI was estimated with this purified sample. The pI of necdin was determined to be 9.1 by isoelectric focusing (Figure 6B). This is very similar to the theoretical isoelectric point of pH 8.7. In contrast, an immunostained spot was detected at pI 6.2 on two-

Figure 8 Change in necdin mobility with nuclease treatment on acetic acid/urea/Triton gel electrophoresis

Nuclear necdin was analysed by acetic acid/urea/Triton electrophoresis after micrococcal nuclease digestion (+) or without digestion (−). The digested necdin (+) was able to migrate into the gel.

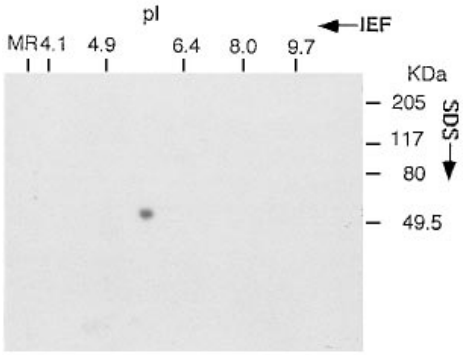

Figure 9 Immunoblotting of necdin after two-dimensional gel electrophoresis

Sonicated extracts were analysed by two-dimensional electrophoresis. At the the end of the run, a gel was washed in SDS/PAGE lysis buffer for 30 min and then equilibrated for 15 min in the blotting buffer for immunoblotting. The pI of unpurified necdin was found to be 6.2.

dimensional gel electrophoresis (NEPHGE system) with a sample extracted by sonication (Figure 9), suggesting that crude necdin forms a complex with acidic molecules such as small DNA fragments in spite of the presence of urea. In fact Figures 2, 3 and 4 show that necdin can form a heterogeneous complex of high molecular mass.

As to the mechanism of nuclear localization, it is uncertain whether necdin is transferred in nuclei through as yet unknown nuclear localization signals or by passive diffusion. Because it was reported [12] that the small protein (approximately 40 kDa) diffused, or was co-translocated in association with other nuclear proteins, I propose that necdin can form a complex by binding with DNA, as soon as it has diffused as a monomeric form through a small opening, to modify the DNA structure.

Immunocytochemistry indicates that necdin could be involved in neural cell formation, and here I have identified biochemically interesting aspects to be explored in detail. As a next step, the importance of necdin in DNA metabolism could be elucidated by analysing the transcriptionally active genes in necdin-binding nucleosomes during neuron differentiation.

In conclusion, it was confirmed that necdin is a polymerized nuclear basic protein enriched in the *trans*-active nucleosomal

fraction of the brain, and may play an important role in neural differentiation through the DNA-binding ability shown in this study.

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