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NXP-1, a Human Protein Related to Rad21/Scc1/Mcd1, Is a Component of the Nuclear Matrix

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Nuclear matrix is a complex intranuclear network supposed to be involved in the various nuclear functions. In order to identify the nuclear matrix proteins, we isolated a cDNA clone from a human placenta cDNA library. This clone was partially represented a known cDNA clone HA1237. HA1237 encoded a 631-amino-acid peptide, which we designated NXP-1. NXP-1 was related to yeast Rad21/Scc1/Mcd1, *Xenopus* XRAD21, and mouse PW29, and identical with HR21spA isolated from a human testis cDNA library. We developed a polyclonal antibody to the purified NXP-1 bacterially expressed as a fusion protein with GST. Western blot analysis with anti-NXP-1 polyclonal antibody showed nuclear matrix localization of NXP-1 in HeLa cells. Indirect immunofluorescence staining also showed nuclear and nuclear matrix localization of the NXP-1. Results of *in vitro* binding assays employing nuclear matrix preparations indicated that the N-terminal region (16–128 amino acid) of NXP-1 has an important role in nuclear matrix distribution.

Key Words: **nucleus; nuclear matrix; nuclear scaffold; Rad; DNA repair; cohesin.**

It is becoming clear that the cell nucleus contains a highly organized fibrogranullar network. Over the past 20 years many investigations have been devoted to identifying the nuclear network. Insoluble structures have been obtained from nuclei in vitro by different techniques and are variously referred to as nuclear matrix [1], nucleoskeleton [2] or nuclear scaffold [3]. The nuclear matrix is defined as the structure that remains after salt extraction and nuclease treatment. It consists of a nuclear lamina, a residual nucleolus, and an internal non-chromatin fibrogranullar matrix. The nuclear matrix has been implicated in a variety of nuclear functions, DNA replication and repair, mRNA transcription and processing, and attachment of supercoiled DNA loops [4–7].

An important function of the nuclear matrix has been described to be the repair of DNA damage. Major questions were whether repair events are confined to the nuclear matrix compartment, and how they are distributed in DNA loops associated with the nuclear matrix. Previous works have suggested that association of the DNA with the matrix can influence the repair function of ultraviolet (UV) light-induced DNA-breaks and the sensitivity of mammalian cells to ionizing radiation [8–11]. These results support the notion that the nuclear matrix is an important site for the assembly of an efficient repair complex. But there is no evidence that DNA repairing enzymes, Rad protein family members, associate with the nuclear matrix.

We searched the components of the nuclear matrix and named a newly identified nuclear matrix protein NXP-1, where NXP stands for nuclear matrix protein. NXP-1 is related to yeast Rad21 which is involved in the repair of ionizing radiation-induced DNA double-strand breaks [12, 13]. HR21spA [14], which is identical with NXP-1, and PW29 [15], a mouse version of NXP-1, were isolated from a human testis cDNA library and mouse F9 cDNA library, respectively. Recently, it was reported that, yeast Rad21/Scc1/Mcd1 [16, 17] and XRAD21 [18], a *Xenopus* homolog of NXP-1, involved in sister chromatid cohesion in the eukaryote cell cycle. However, little is known about the intranuclear distribution and physiological function of the mammalian NXP-1/HR21/PW29 in the cell cycle. In this study, we report the association of NXP-1 with interphase nuclear matrix and requirement of the N-terminal region for the interaction.

Abbreviations used: NXP-1, nuclear matrix protein 1; GST, glutathione *S*-transferase; PMSF, phenylmethanesulfonyl fluoride; HRP, horseradish peroxidase; VRC, vanadyl ribonucleoside complex; FITC, fluorescein isothiocyanate.

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EXPERIMENTAL PROCEDURES

Cell culture and immunostaining were carried as described previously [19]. In entire experiments, HeLa cells were grown asynchronously in F12 medium containing 10% fetal calf serum at 37°C.

Purified rat liver nuclei (10 mg) were mixed with complete Freund's adjuvant and injected to BALB/c mouse six times with 2-week intervals. After immunization, the mouse was bled and the antiserum was used for immunoscreening of cDNA library. Two distinct cDNA clones, H2 and H4, were cloned by conventional immunoscreening of 1.6×10^4 independent clones of a λgt 11 human placenta cDNA library. Inserts that were released by *Eco*RI digestion were subcloned into the vector pBluescript II KS(+) (Stratagene Cloning Systems, La Jolla, CA) and were further analyzed by sequencing. Homology search was performed using BLAST programs with GenBank and EMBL databases.

High salt isolation of nuclear matrix was carried out essentially as described by He *et al.* [20]. After a wash with PBS, cells were extracted with cytoskeletal buffer (CSK; 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, supplemented with leupeptin, aprotinin, and pepstatin (1 μg/ml each), 1 mM PMSF, 1 mM DTT, 20 mM vanadyl ribonucleoside complex (VRC), and 0.5% (vol/vol) Triton X-100). After 3 min at 4°C, the cytoskeletal frameworks were separated from soluble proteins by centrifugation at 5,000 × g for 3 min. Extraction buffer (CSK buffer containing 250 mM ammonium sulfate) was added to the samples, and the mixture was incubated for 5 min at 4°C, and then centrifuged. Chromatin was solubilized in DNA digestion buffer, same as CSK buffer except for containing 200–500 U/ml of RNase-free DNase I, for 40 min at 37°C. This treatment removed all the DNA and histones from the nucleus. The pellet was further washed with the digestion buffer, and then centrifuged. The remaining pellet was considered the nuclear matrix. In some experiments, 5 mM $CaCl_2$ was substituted for 1 mM EGTA in the CSK, Extraction and DNase I digestion buffers.

For in situ extraction, cells grown on coverslips were washed three times with ice-cold PBS and then treated with ice-cold CSK buffer for 5 min, followed by extraction buffer for 5 min and DNase I digestion buffer for 40 min. The cells were washed with the digestion buffer and then fixed in 4% paraformaldehyde in PBS for 15 min.

The recombinant fusion protein lacking amino-terminal region of NXP-1 (denoted pGST-442–631) was generated by digestion of pGST-NXP-1 with *Cla*I and *Bam*HI, followed by filling-in and religation. Purified recombinant GST-fusion proteins of NXP-1(442–631) were used to generate polyclonal antiserum.

For the filter binding assays, we prepared additional fusion protein constructs in a pGEX-2T-derived plasmid (pGEX-2TK). pGEX-2TK-442–631 was generated by ligation of the fragment excised from pGST-442–631 into pGEX-2TK vector. pGEX-2TK-16–128 and pGEX-2TK-128–442 were generated in similar ways. Approximately 1–5 mg of fusion proteins was added to 1 ml of glutathione-Sepharose beads and then washed twice in kinase buffer (20 mM Tris pH 7.5, 12 mM $MgCl_2$, 1 mM DTT). Labeling reactions were carried out in the kinase buffer, containing 50 μCi of (γ-^{32}P)ATP (3000 Ci/mmol) (Amersham Pharmacia Biotech Inc.) using protein kinase (A kinase) catalytic subunit (P2645 Sigma Chemical Co.) at 4°C for 30 min. The reactions were terminated by the addition of 10 mM sodium phosphate (pH 8.0), 10 mM sodium pyrophosphate, 10 mM EDTA and 1 mg/ml BSA. The elution was performed with 30 mM glutathione in the GST binding buffer. For estimation of labeling efficiency, small aliquots of the reaction mixture were counted, fractionated on 15% polyacrylamide gels and visualized by autoradiography. GST-2TK has a single phosphorylation site by A kinase. According to the protein motif search, a phosphorylation site by A kinase and several phosphorylation sites by other protein kinases were found in the peptide sequence of NXP-1. However, NXP-1 fused with normal GST, which has no phosphorylation site by the A kinase, was not significantly phosphorylated by the kinase (data not shown). Therefore, GST-2TK fusions of truncated NXP-1 mutants were considered to be labeled in the GST-2TK region.

^{32}P-labeled GST-2TK, GST-2TK-16–128, GST-2TK-128–442 and GST-2TK-442–631 were incubated with isolated nuclei or nuclear matrix from 3Y1 and HeLa cells in 50 mM Tris-HCl (pH 7.5) at 4°C for 30 min. The nuclei and nuclear matrix were centrifuged and washed twice with 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl. The binding of ^{32}P-labeled peptides to the nuclei or nuclear matrix was assayed using SDS-PAGE and autoradiography.

Gel electrophoresis and transfer of proteins to nitrocellulose membranes were carried out and the filters were blocked for 2 h in blocking solution (phosphate-buffered saline containing 2% bovine serum albumin and 0.1% Triton X-100). Filters were incubated with ^{32}P-labeled GST-2TK and GST-2TK-16–128 as described by Kaelin *et al.* [21]. Blots were then washed four times for 15 min each with PBS containing 0.1% Triton X-100, and subjected to autoradiography.

RESULTS AND DISCUSSION

In an attempt to identify structural components of the cell nucleus, rat liver nuclei were prepared and used as immunogens to generate polyclonal antibodies. The antibodies were used to screen a human placenta λgt11 cDNA expression library. Two positive clones, H2 and H4, were isolated from 1.6×10^4 clones. Based on sequence analysis, H4 (1.5 kb) was identified as a partial cDNA of hnRNP M protein. H2 consisted of 350 nucleotides and was identical with a part of an EST clone registered with the name HA1237 (GenBank/EMBL Accession Number: D38551).

HA1237 was one of 40 new genes (KIAA 0041–KIAA 0080) obtained by analysis of randomly sampled cDNA clones from the human immature myeloid cell line KG1. It contains 3647 bp of cDNA sequence and an open reading frame encoding a protein of 631 amino-acid residues, starting at nucleotide position 185. We named the protein encoded by HA1237, NXP-1 (nuclear matrix protein-1). We identified a mouse homolog of NXP-1, PW29, by homology search against the GenBank database. The nucleotide and amino acid homologies were 97% and 94%, respectively, between PW29 and NXP-1. PW29 protein was purified from the mouse F9 cells with a pokeweed-agglutinin column by Yu *et al.* [15], and the cDNA was cloned from mouse F9 cDNA library. However, the intracellular localization of PW29 was not determined. McKay *et al.* [14] isolated HR21spA (HR21), identical to NXP-1, from a human testis cDNA library. The database analysis indicated that NXP-1 was related to yeast Rad21 (GenBank/EMBL Accession Number: M96437), a double strand break-repairing enzyme [12], and the homology scores were 23% for total amino acid sequence overall and 58% for the amino-terminal 100 amino acids.

In this study, we developed polyclonal antibodies against purified NXP-1 which was bacterially expressed as a fusion protein with GST. Western blot analysis with anti-NXP-1(442–631) antibodies was carried out to determine the subcellular localization of

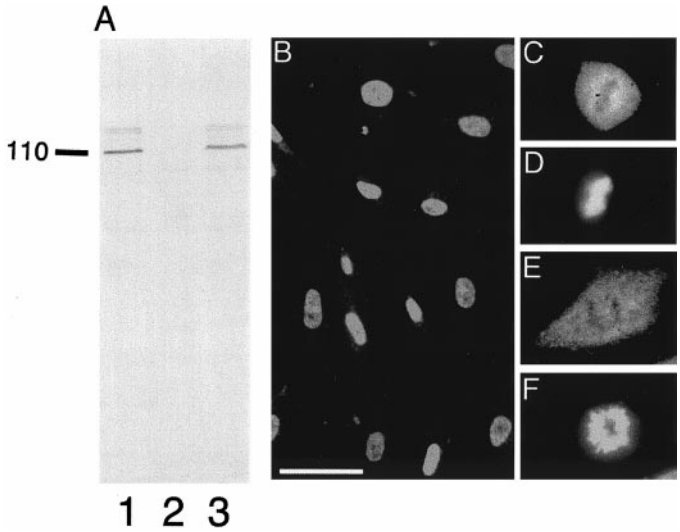

FIG. 1. Immunochemical analysis of HeLa cells. (A) Total cell lysate, cytoplasmic fraction and nuclear fraction were prepared from HeLa cells and subjected to Western blot analysis with anti-NXP-1(442–631) antibody. The lanes were loaded with samples derived from the same amounts of cells (50 μg protein) as follows: lane 1, whole cell lysate; lane 2, postnuclear supernatant; lane 3, purified nuclei. Sizes of the bands as estimated by comparison with the bands of molecular mass markers are indicated in kDa. (B, C, E) Indirect immunofluorescent labeling of HeLa cells, detected by the anti-NXP-1 antibody. HeLa cells were fixed with 4% paraformaldehyde in PBS and incubated with the 1st antibody. Subsequently, cells were incubated with FITC-labeled anti-rabbit IgG antibody. Bar, 70 μm. (D, F) same fields with C and E, staining with Hoechst, respectively.

NXP-1 in 3Y1 and HeLa cells (Fig. 1A). A protein of 110 kDa was readily detected in the nuclear fraction as well as total cell lysate from HeLa cells (Fig. 1). The observed molecular weight is similar with 90–110 kDa of Rad21, 120 kDa of XRAD21 and 120 kDa of PW29. However, these apparent molecular weights are not consistent with the predicted molecular weight (70 kDa) for NXP-1, Rad21, XRAD21, and PW29. Yeast Rad21 has phosphorylated versions giving bands of apparently larger size on SDS-PAGE [13]. Yu *et al.* suggested that the difference is explained by unusual electrophoretic behavior due to the presence of highly charged segments in the PW29 polypeptide [15].

NXP-1 distributed throughout the nucleus (Fig. 1B) without nucleolus in the interphase cells. McKay *et al.* reported that mRNA content of hHR21 was decreased in G1 phase and increased in S phase and reached maximum in G2/M phase in synchronized HeLa cells [14]. However, the protein content of hHR21 during the cell cycle has not been determined. In this report, we determined the expression and distribution of NXP-1 protein in HeLa cells using specific antibody. In Fig. 1B, we observed different intensities of nuclear immunostaining from cell to cell, which were considered to be due to mixed culture of cells in the G1 phase and S/G2 phase.

By double detection of immunofluorescence and Hoechst-stained chromatin, NXP-1 distributed entire cytoplasm without the core region of metaphase and anaphase chromatin (Fig. 1C–1F). These findings are consistent with the release of cohesin complex from the mitotic chromatin [18].

The subnuclear distribution of endogenous NXP-1 in interphase nucleus was further characterized in HeLa cells. Cells were collected from the tissue culture plates and nuclei were isolated. We further extracted the nuclear pellet sequentially with buffers containing Triton X-100, ammonium sulfate, and DNase I, finally obtaining the insoluble nuclear matrix. Western blot analysis of these fractions using anti-NXP-1(442–631) for HeLa (Fig. 2A) cells indicated that NXP-1 is a nuclear matrix protein. Release of NXP-1 from the nuclear matrix into the supernatants was apparently reduced when 5 mM Ca^{2+} was added as a substitute for 1 mM EGTA to the CSK, Extraction and DNase I digestion buffers. Ca^{2+} possibly promoted binding of NXP-1 to the nuclear matrix, because Ca^{2+} binding with PW29 was reported [15]. It is also possible that the absence of NXP-1 from the supernatants is due to degradation by a soluble Ca^{2+}-activated protease. In

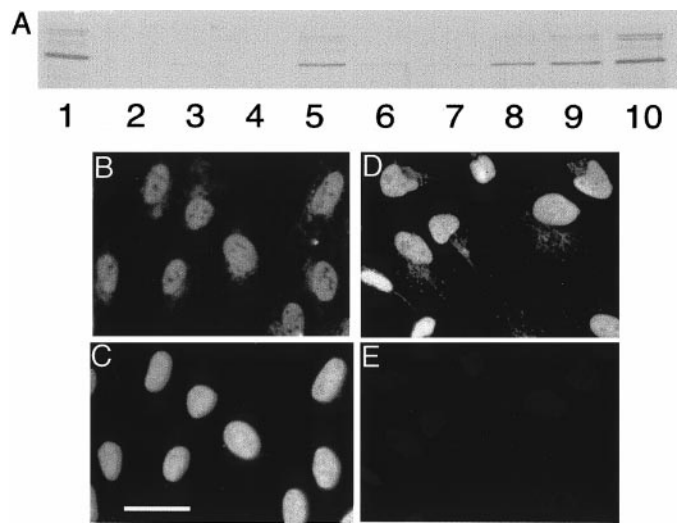

FIG. 2. Immunoblot and staining analysis of nuclear matrix fractions derived from HeLa cells. (A) Cells were sequentially extracted with 0.5% Triton X-100 (CSK), extracted with 0.25 M ammonium sulfate (extraction buffer) and digested with DNase I to remove chromatin (digestion buffer). Extraction and digestion buffer contained 5 mM $CaCl_2$ (lanes 2–5) or 1 mM EGTA (lanes 6–9). The lanes were loaded with nuclear samples derived from 50 μg of HeLa cell nuclei as follows: lanes 1 and 10, whole nuclei; lanes 2 and 6, soluble supernatants of CSK extraction; lanes 3 and 7, soluble supernatants of ammonium sulfate extraction; lanes 4 and 8, soluble supernatants of DNase I digestion; lanes 5 and 9, nuclear matrix. Untreated (B and C) and nuclear matrix (D and E) samples of HeLa cells were fixed with 4% paraformaldehyde in PBS and incubated with anti-NXP-1 antibody. Subsequently, cells were incubated with FITC-labeled anti-rabbit IgG antibody. Hoechst staining for detection of nuclear DNA was carried out on the same coverslips (C and E). DNase I digestion solubilized nuclear DNA totally (E). Bar, 40 μm.

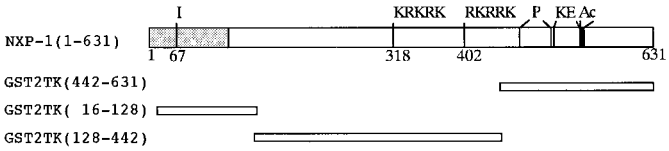

FIG. 3. Schematic representation of NXP-1 deletion mutants. NXP-1(1–631) has a highly conserved region (dotted box), proline rich (P) region, repeating lysine-glutamate (KE) sequence, and 10 consecutive acidic amino acid (Ac) residues. The letter "I" stands for isoleucine at position 67. KRKRK starting at the 318th and RKRRK starting at the 402nd amino acid were assumed to be nuclear localization signal sequences. Fusion constructs were made between various portions of NXP-1 and GST-2TK. The numbers represent amino acid positions within the whole amino acid sequence of NXP-1. GST-2TK encodes the GST attached to a cAMP-dependent protein kinase phosphorylation site at the C-terminus.

situ nuclear matrix treatment and indirect immunofluorescence analysis with anti-NXP-1 antibody were carried out in HeLa cells. NXP-1 distributed throughout the nuclear matrix as it was detected in all regions of untreated nuclei (Fig. 2B and 2D).

To identify the binding domain of NXP-1 for nuclear matrix, phosphorylatable GST-fusion constructs of three truncated mutants of NXP-1 containing the N-terminal (GST-2TK-16–128), internal (GST-2TK-128–442) and C-terminal (GST-2TK-442–631) regions were generated (Fig. 3). GST-2TK-16–128 was specifically co-precipitated with nuclear matrix fractions from HeLa cells (Fig. 4, lane 6). The other two constructs did not reveal a significant association with the

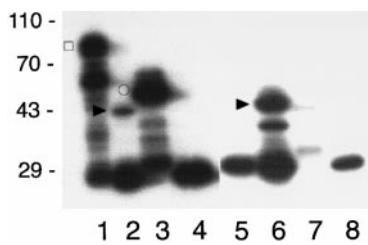

FIG. 4. Coprecipitation with the nuclear matrix. Purified GST-2TK and truncated GST-2TK-NXP-1 mutants were ^{32}P-labeled *in vitro* with protein kinase A, and incubated with isolated nuclear matrix from HeLa cells at 4°C for 30 min. Truncated GST-2TK-NXP-1 mutants used as probes (lanes 1–4) and nuclear matrix-bound GST-2TK-NXP-1 (lanes 5–8) were analyzed by SDS–PAGE and autoradiography. Probe samples (lanes 1–4) represent 0.3% of input as radioactive counts. Lanes 1 and 5, GST-2TK-NXP-1(128–442); lanes 2 and 6, GST-2TK-NXP-1(16–128); lanes 3 and 7, GST-2TK-NXP-1(442–631); lanes 4 and 8, GST-2TK. Positions of molecular mass markers are shown at the left in kilodaltons. Arrowheads and open circle indicate positions of GST-2TK-NXP-1(16–128) and GST-2TK-NXP-1(442–631), respectively. Open square indicates position of GST-2TK-NXP-1(128–442), which is higher than the predicted molecular weight. Since it was detected even in the unphosphorylated sample (data not shown), the peptide was assumed to contribute for lower mobility of NXP-1 protein on SDS–PAGE (Fig. 1A). Higher mobility bands were assumed to be partial degradation products.

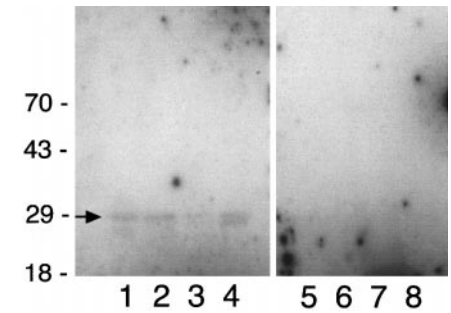

FIG. 5. Filter binding assay. Nuclear matrix proteins were separated by SDS–PAGE and transferred onto membrane as described under Experimental Procedures. Membrane was then incubated with ^{32}P-labeled GST-2TK-NXP-1(16–128) (lanes 1–4) or ^{32}P-labeled GST-2TK (lanes 5–8). Membrane was washed and bands were visualized by autoradiography. Lanes 1 and 5, nuclei from HeLa; lanes 3 and 7, nuclei from 3Y1; lanes 2 and 6, nuclear matrix from HeLa; lanes 4 and 8, nuclear matrix from 3Y1. Arrows indicate the 28-kDa protein detected in all nuclear and nuclear matrix preparations.

nuclear matrix. Recombinant full-length NXP-1 fused with GST-2TK was not available because of poor expression as a soluble form in bacteria.

Filter binding analysis with the truncated fusion proteins as phosphorylated peptide probe indicated that the N-terminus of NXP-1(GST-2TK-16–128) associated with a nuclear matrix protein of 28 kDa (Fig. 5, lanes 1–4). There was no specific binding of GST-2TK (Fig. 5, lanes 5–8), GST-2TK-128–442 and GST-2TK-442–631 (data not shown). The nuclear matrix protein of 28 kDa may serve the association of NXP-1 to nuclear matrix. A possibility remains that the tagging with GST or phosphorylation causes some artificial effect on the binding of NXP-1 to nuclear matrix. However, when expressed in HeLa cells, among the GFP fusion constructs, only the one containing 16–128 amino acids of NXP-1 was localized in the nuclear matrix (data not shown). These results suggest that NXP-1 employs the N-terminal region for binding to the nuclear matrix.

Schizosaccharomyces pombe strains have been used to analyze pathways of spontaneous and double strand break-induced intrachromosomal mitotic recombination. Rad21 and Rad22 are involved in the repair of ionizing radiation-induced DNA damage [22]. Mckay *et al.* [14] reported that mRNA of hHR21spA, which was related to Rad21, expressed in human cells and the level of hHR21spA transcripts was not altered by exposure of normal diploid fibroblasts to 10 Gy ionizing radiation. They suggested possible roles for hHR21spA in V(D)J and meiotic recombination in testis and thymus, respectively, based on the relatively abundant expression of this gene in these organs. Recently, several lines of experiments revealed that the Rad21 is identical with a subunit of cohesin complex, SCC1/MCD1, which is involved in sister chromatid cohesion in mitosis and meiosis [16, 17]. Losada *et al.* [18] re-

ported a *Xenopus* homolog of NXP-1, which was also involved in sister chromatid cohesion. The functional domains for cohesion in the Rad21 family proteins have not been identified yet.

In the present study, we showed that NXP-1 associates with the nuclear matrix through the N-terminal region. Although there has been no report about the subnuclear location of Rad21 and its homologs, Rad21 is also assumed to be attached to the nuclear matrix in yeast based on the greater resemblance of the N-terminal 100 amino acids than other portions between Rad21 and NXP-1. The N-terminal region of yeast Rad21 has an essential function for DNA repair, and hence the mutant which has a single amino acid substitution, Ile67-Thr, performs insufficient DNA repair [12, 13]. These results provide clues as to the physiological function of NXP-1 as well as the significance of the nuclear matrix in DNA repair. There are characteristic amino acid sequences, such as proline-rich sequences, repeating lysine-glutamate sequences, and 10 consecutive acidic amino acids in the carboxyl-terminal region of NXP-1 (Fig. 5), which are not shared by Rad21. These sequences may serve a novel function of NXP-1. It was repeatedly reported that the nuclear matrix, nuclear scaffold, and nucleoskeleton associate with a specific DNA region, such as MAR/SAR [23–26], centromere [27] and telomere [28]. Whether NXP-1 interacts with these DNA regions remains to be studied.

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