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Cell density-dependent nuclear/cytoplasmic localization of NORPEG (RAI14) protein ☆

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Abstract

NORPEG (RAI14), a developmentally regulated gene induced by retinoic acid, encodes a 980 amino acid (aa) residue protein containing six ankyrin repeats and a long coiled-coil domain [Kutty et al., J. Biol. Chem. 276 (2001), pp. 2831–2840]. We have expressed aa residues 1–287 of NORPEG and used the recombinant protein to produce an anti-NORPEG polyclonal antibody. Confocal immunofluorescence analysis showed that the subcellular localization of NORPEG in retinal pigment epithelial (ARPE-19) cells varies with cell density, with predominantly nuclear localization in nonconfluent cells, but a cytoplasmic localization, reminiscent of cytoskeleton, in confluent cultures. Interestingly, an evolutionarily conserved putative monopartite nuclear localization signal (P^{270}KKRKAP^{276}) was identified by analyzing the sequences of NORPEG and its orthologs. GFP-NORPEG (2–287 aa), a fusion protein containing this signal, was indeed localized to nuclei when expressed in ARPE-19 or COS-7 cells. Deletion and mutation analysis indicated that the identified nuclear localization sequence is indispensable for nuclear targeting.
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Keywords: Nuclear/cytoplasmic localization; Nuclear localization signal; NORPEG; RAI14; Retinal pigment epithelium; Immunofluorescence; Retinoic acid; Nucleolin; Ankyrin repeats

NORPEG (novel retinal pigment epithelial cell gene, *RAI14*) is a retinoic acid regulated gene that we originally characterized from the human retinal pigment epithelial (RPE) cell line ARPE-19 [1]. This gene containing 18 exons and spanning a 176 kb region is localized to the 5p13.3-p13.2 region of human chromosome 5. The *NORPEG* gene encodes two transcripts (~3 and ~5 kb in size). It is expressed in many human tissues, but predominantly in placenta and testes. The sequence deduced for the NORPEG protein from its cDNA sequence contains 980 amino acid residues with a predicted molecular mass of 110 kDa [1]. An alternatively spliced transcript of the *NORPEG* gene is reported to encode a minor variant containing 983 amino acid residues with differences at the N-terminal region [2]. *Norpeg (Rai14)*, the mouse ortholog, is developmentally regulated and encodes a protein consisting of 979 amino acid residues [1]. The mouse Norpeg protein shows 91% sequence similarity to the human NORPEG protein. Peng et al. [3] have independently discovered this protein and named it Ankycorbin based on its observed association with the actin cytoskeleton. Both human NORPEG and

☆ *Abbreviations: NORPEG*, novel retinal pigment epithelial cell gene; *RAI14*, retinoic acid induced 14; RPE, retinal pigment epithelium; aa, amino acid; NLS, nuclear localization signal; NES, nuclear export signal; GFP, green fluorescent protein.

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mouse Norpeg proteins contain 6 ankyrin repeats towards the N-terminal region and a long coiled-coil domain towards the C-terminus. The functional role of the NORPEG protein is not yet known.

The present study was aimed at characterizing the protein encoded by the NORPEG gene. We have expressed the N-terminal region (amino acid residues 1–287) of NORPEG as a HIS tag fusion protein in *Escherichia coli* and used it to produce a highly specific anti-NORPEG polyclonal antibody. Using this antibody, a cell density-dependent subcellular localization of the native NORPEG was observed in ARPE-19 cells by confocal immunofluorescence analysis. A nuclear localization was observed in non-confluent cells whereas a cytoplasmic localization occurred in confluent cells. Examination of the amino acid sequences of NORPEG revealed the presence of nuclear localization signals, a nuclear export signal, and a binding site for 14-3-3 protein. An evolutionarily conserved putative monopartite nuclear localization signal (P^{270}KKRKAP^{276} in human NORPEG) was found to be functional by its ability to target the green fluorescent protein (GFP)-fusion protein, GFP-NORPEG (2–287 aa), to nuclei, specifically to nucleoli, when expressed in ARPE-19 or COS-7 cells.

Materials and methods

Plasmid construction. Total RNA was isolated from ARPE-19 cells with RNAzol B (Tel-Test, Inc.) and fractionated on an oligo(dT) cellulose column. The Poly(A)$^+$ RNA fraction thus obtained was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and the primer, 5′-AGAGCTCTCCTGTGCTGA (corresponds to 3465–3448 bp of *NORPEG* cDNA). The reaction mixture was treated with RNase and an aliquot was subjected to polymerase chain reaction (PCR) with high fidelity Pfu Turbo DNA polymerase (Stratagene) employing 5′-GTGGAGCAGCCAGCTGGGTC and 5′-CATCAGGACCAGACCTC as sense and antisense primers, respectively. The amplification product, corresponding to 16–3327 bp of *NORPEG* cDNA, was cloned into pCR-Blunt vector (Invitrogen). This plasmid DNA was used as a template for PCR with primers 5′-GATCTCGAGTGAAGAGCTTGAAAGCGAAG and 5′-TATACCCGGGTTAATCACTCAACTGGGTAGG, containing the restriction enzyme sites *Xho*I and *Xma*I, respectively (extra nucleotides added to create restriction sites and stop codon are underlined). The sequence of the PCR product, when verified by DNA sequencing, was found to correspond to bases 113–972 of *NORPEG* cDNA sequence (Accession No. AF155135) that we reported earlier, except that we found base C (instead of A) at position 926. Further sequencing and database analysis including human EST sequences confirmed that the correct base at position 926 is indeed A. Thus, it is necessary to change Thr^{272} in the previously reported NORPEG protein sequence to Lys^{272} [1]. This corrected NORPEG sequence is used in the present study. The PCR product was digested with the indicated restriction enzymes and cloned into phrGFP-N1 mammalian expression vector (Stratagene) to generate GFP-NORPEG (2–287 aa). The sequence of the plasmid insert was verified by dideoxy termination sequencing. This plasmid construct encodes a GFP-fusion protein containing amino acid residues 2–287 of NORPEG. The GFP-NORPEG (2–980 aa) construct was made similarly using primers 5′-GATCTCGAGTGAAGAGCTTGAAAGCGAAG and 5′-ATACCCGGGTTACTTCTTTTGAGACTGGTT (extra nucleotides added to create restriction sites are underlined).

Quick Change II Site Directed Mutagenesis Kit (Stratagene) was used to generate other plasmid constructs from GFP-NORPEG (2–287 aa). The oligonucleotides (sense) employed for making the plasmid constructs, GFP-NORPEG (2–287/232–280 aa deleted), GFP-NORPEG (2–287/7–264 aa deleted), and GFP-NORPEG (2–287 aa/K^{271}KRK^{274} → A^{271}AAA^{274} mutated), were 5′-TTCTCTTGGATACAATGCCAGTCCTACCCAGTTGAGT, 5′-GTGAAGAGCTTGAAAGCGGAAAGAAGTGGAACTCCAA, and 5′-AGAAAGAAGTGGAACTCCAGCTGCTGCTGCTGCTCCACCACCTCCTAT, respectively.

Protein expression and antibody production. The amino acid residues 1–287 (with the exception that Lys^2 was changed into Glu^2 in order to introduce a restriction enzyme site for cloning) of the human NORPEG protein were expressed as a C-terminal HIS tag fusion protein in *E. coli* using pET28 expression vector (Novagen). The recombinant protein was purified using Ni–NTA–agarose (Qiagen). The purity of the protein was assessed by SDS–polyacrylamide gel electrophoresis and Coomassie blue R-250 staining.

The purified HIS tag fusion protein was used as an antigen to raise antibodies in rabbits (Covance Research Laboratory). The anti-serum was purified by immunoaffinity chromatography using the antigen immobilized to cyanogen bromide activated agarose. The immunoreactivity against HIS tag was removed by repeated passing of the antibody preparation over a column containing HIS tag peptide immobilized to agarose beads.

Cell culture. Human retinal pigment epithelial cells (ARPE-19), a rapidly growing human RPE cell line, were grown on 100 mm culture dishes at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)–nutrient mixture F12 (50:50), supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) as previously described [1,4]. COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Immunohistochemistry. Immunohistochemistry was performed by QualTek Molecular Laboratories. Briefly, four-micron human placenta sections were deparaffinized, subjected to 20 min of steam heat induced epitope recovery, and then treated with anti-NORPEG antibody. The immunoreactivity was visualized using a conjugated horseradish peroxidase based avidin-biotin system with 3,3′-diaminobenzidine as substrate. The sections were then counterstained with hematoxylin and photographed using a Nikon E800 microscope.

Transfection, immunofluorescence analysis, and confocal microscopy. ARPE-19 or COS-7 cells were transfected with plasmid constructs encoding GFP fusion proteins using Effectene reagent from Qiagen. Briefly, cells were grown on coverslips to ~80% confluency before transfecting them with plasmid constructs. The GFP fluorescence was analyzed 48 h following transfection. Cells were briefly fixed in 4% formaldehyde in phosphate buffer and washed. Cell nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole) staining. Fluorescence analysis was performed using a Leica SP2 confocal microscope (Leica Microsystems, Inc.). Nucleolin, a marker for nucleoli, was immunostained using a monoclonal anti-nucleolin antibody (Leinco Technologies, Inc) as the primary antibody and Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) as the secondary antibody. ARPE-19 cells grown on Lab-Tek chamber slides (Nalge Nunc International) were fixed with 4% formaldehyde in phosphate buffer and immunostained using the anti-NORPEG antibody (16 μg/ml) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Cell nuclei were stained with DAPI while actin was stained with Alexa Fluor 568 phalloidin (Invitrogen).

Western blot analysis and immunoprecipitation. SDS–polyacrylamide gel electrophoresis was carried out using NUPAGE Bis–Tris gels and MOPS–SDS running buffer (Invitrogen). The protein bands from the gels were then electroblotted onto nitrocellulose membranes. The blots were probed for immunoreactivity with the affinity-purified anti-NORPEG antibody using the ECL Western blotting Detection Kit (Amersham Biosciences). Immunoprecipitation was performed employing protein A agarose and the samples obtained were analyzed by immunoblotting.

Bioinformatics. CD-Search [5] was used to detect protein domains and sequence similarities to classified protein families. PSORT II was used to search for nuclear localization signals and to predict the subcellular localization of NORPEG [6]. Positions of likely kinase target sites were determined by the CBS Prediction Servers at http://www.cbs.dtu.dk/

(Technical University of Denmark, Lyngby, Denmark). Other motifs were visually scanned, using published consensus sequences. Potential functions and biological pathways for NORPEG were suggested by searching Gene Ontology at http://www.informatics.jax.org (Mouse Genome Database, Mouse Genome Informatics Web Site, The Jackson Laboratory, Bar Harbor, Maine). Sequence manipulations were performed with the DNAStar suite of programs (DNASTAR, Inc., Madison, WI). Multiple sequence alignments were produced using BioEdit [7].

Results

Expression of the N-terminal 287 amino acid residues of human NORPEG as a HIS tag fusion protein and the generation of an anti-NORPEG antibody

The first 287 amino acid residues of NORPEG were expressed as a HIS tag fusion protein using a bacterial protein expression system. The ~32 kDa fusion protein was purified to homogeneity using Ni–NTA–agarose (Fig. 1A). The expressed protein was used to generate anti-NORPEG antibodies in rabbits, and the affinity-purified antibody preparation showed immunoreactivity, as expected, towards the antigen when analyzed by Western blotting (Fig. 1B). This figure also shows that the antibody specifically reacted with a ~110 kDa (size predicted for NORPEG) protein present in ARPE-19 cell extracts. The ~110 kDa protein was also immunoprecipitated from the cell extracts with the anti-NORPEG antibody preparation (Fig. 1C). In addition, the antibody preparation was characterized by immunohistochemistry. Human placenta sections showed intense immunoreactivity, especially in trophoblasts (Fig. 1D). No immunoreactivity was observed when normal rabbit IgG was substituted for the antibody. The immunoreactivity appears to be specific since it was effectively abolished when the antibody was pretreated with the antigen.

Cell density-dependent nuclear/cytoplasmic localization of NORPEG

The anti-NORPEG antibody was used to verify the presence of NORPEG protein in ARPE-19 cells by immunofluorescence, and further, to examine the subcellular distribution of NORPEG. As shown in Fig. 2, the confluent and nonconfluent cultures of the cells exhibited strikingly different patterns of NORPEG localization. The confluent cells showed the localization of NORPEG in cytoplasm reminiscent of actin cytoskeleton. However, in nonconfluent cells, the NORPEG was mainly localized to nuclei.

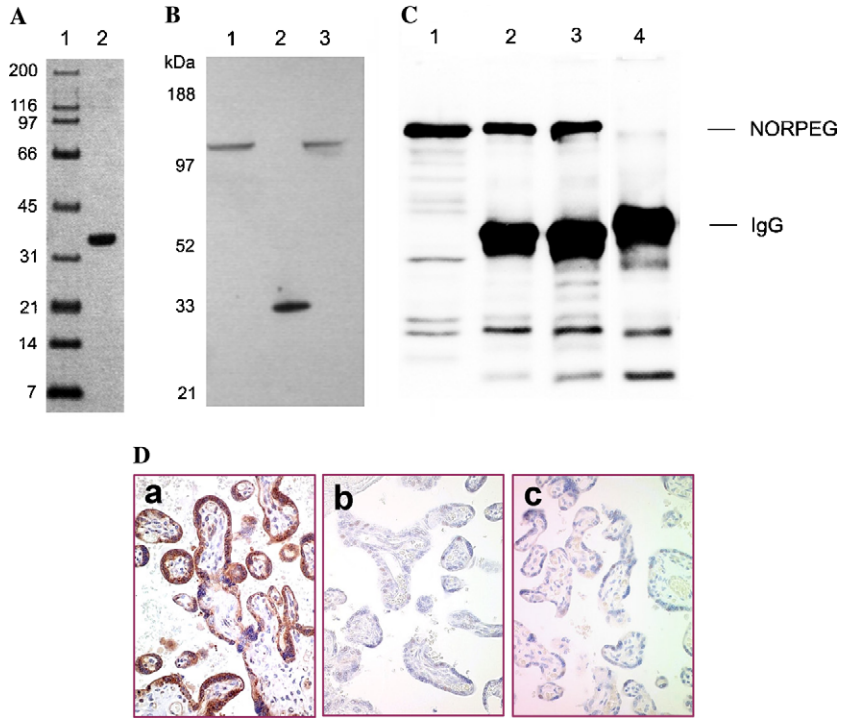

Fig. 1. Expression of NORPEG (1–287 aa), anti-NORPEG antibody production, and characterization of native NORPEG protein. (A) The cDNA encoding NORPEG (1–287 aa) was cloned into pET expression vector. The HIS tag fusion protein was expressed in *E. coli*, purified, and analyzed by SDS–PAGE. Lane 1, molecular size markers; lane 2, purified NORPEG fusion protein. (B) Western immunoblot analysis using anti-NORPEG antibody. Lanes 1 and 3, ARPE-19 cell extracts; lane 2, expressed NORPEG (2–287 aa) protein used to produce antibody. The antibody preparation shows immunoreactivity to a ~110 kDa protein band (expected size for NORPEG) present in the ARPE-19 cell extracts. (C) Immunoprecipitation analysis using anti-NORPEG antibody. ARPE-19 cell extracts prior to immunoprecipitation (lane 1) as well as immunoprecipitates obtained using 2.5 μg anti-NORPEG antibody (lane 2), 5 μg anti-NORPEG antibody (lane 3), and 2.5 μg rabbit IgG (lane 4) were analyzed by Western immunoblotting. Immunoreactive bands corresponding to NORPEG and IgG are indicated. (D) Immunohistochemical analysis using anti-NORPEG antibody. Human placenta showed intense immunoreactivity (brown staining) (a). The immunoreactivity was not observed when the antibody was substituted with normal rabbit IgG (b). The immunoreactivity was effectively blocked when anti-NORPEG antibody was absorbed with the antigen (c).

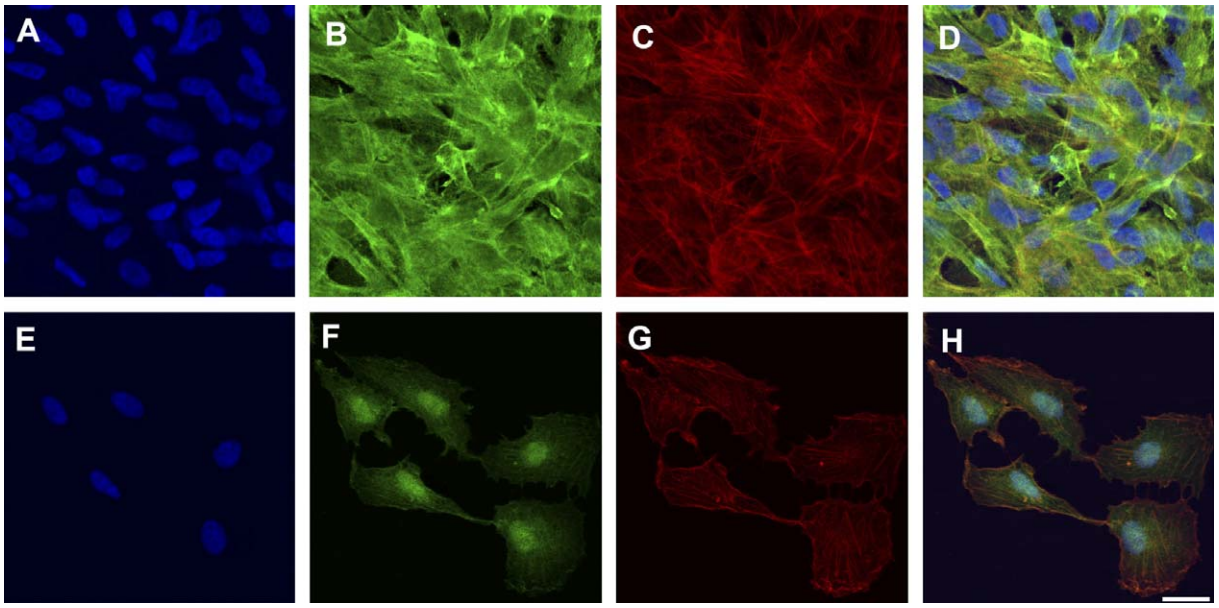

Fig. 2. Immunofluorescence analysis of NORPEG expression in ARPE-19 cells. NORPEG exhibited strikingly different subcellular localization in confluent compared with nonconfluent cultures when ARPE-19 cells were analyzed by immunofluorescence technique using the anti-NORPEG antibody. A cytoplasmic, possibly cytoskeletal, localization is seen in confluent cells (A–D) while a mainly nuclear localization is observed in nonconfluent cells (E–H). (A,E) Blue color indicates DAPI-stained cell nuclei. (B,F) Green color indicates immunoreactivity towards anti-NORPEG antibody. Alexa Fluor 448 conjugated anti-rabbit IgG was used as the secondary antibody. (C,G) Red color indicates actin stained with Alexa Fluor 568 conjugated phalloidin. (D,H) Merged images. Scale bar represents 20 μm.

Analysis of NORPEG protein sequence

As part of the effort to deduce the biological role of NORPEG, a bioinformatics-based analysis of the human protein sequence was conducted to identify protein domains and motifs involved in intermolecular associations. Fig. 3 illustrates these potentially significant features. Regions with structural definitions are named in panel A. These consist of, as noted previously [1], 6 ankyrin repeats at the N-terminal portion of NORPEG, and two lengths of coiled-coils in the C-terminal portion. The coiled-coil regions show significant sequence similarity to the proteins of the SMC (structural maintenance of chromosomes) family. α-Helical areas, as defined by Garnier–Osguthorpe–Robson, are shown in panel B. Panel A also indicates sites matching specific sequence motifs: NORPEG has several nuclear localization signals (NLS) predicted by PSORT II. A monopartite NLS of the pat7 type, consisting of the amino acid residues PKKRKAP, is present at positions 270–276. Not shown are 2 monopartite NLSs of the pat4 type, which overlap this site and a third at positions 663–666. In addition, a bipartite NLS at (sequence KRYAESSSK LEEDKDKK) is found at positions 867–883. Interestingly, a match to a consensus binding site for 14-3-3 proteins (RSITSTP) is at positions 292–298. Binding of 14-3-3 is thought to mask the presence of NLS and nuclear export signals (NES) sites [8]. Finding such a site near the pat7 NLS in NORPEG is consistent with this role. NORPEG also contains the following sequence, L^{352}QAKVASLTL^{361}, which is a perfect match for the NES consensus, given as L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI] [9,10].

The putative pat7 monopartite nuclear localization signal, P^{270}KKRKAP^{276}, is highly conserved among vertebrate NORPEG proteins. The alignment of sequences comprising this region from human, mouse, rat, dog, chicken, and tetradon NORPEG is shown in Fig. 4.

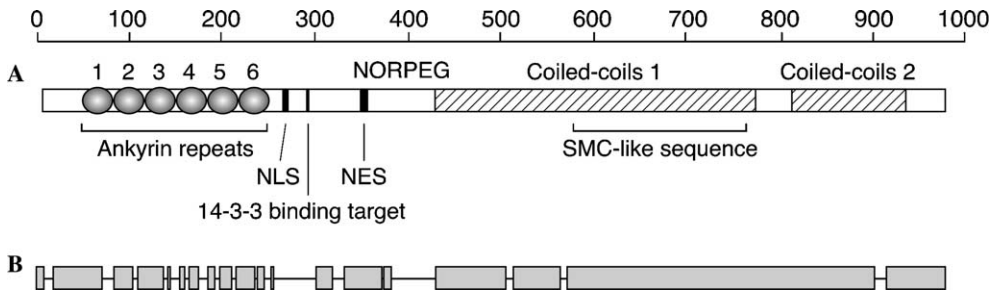

Fig. 3. Schematic representation of structural domains of NORPEG. Relative positions of amino acid residues are indicated on the scale. (A) Structural domains present in NORPEG are: ankyrin repeats region, nuclear localization signal (NLS), binding site for 14-3-3 protein, nuclear export signal (NES), two coiled-coil domains, and structural maintenance of chromosomes (SMC)-like sequence. (B) α-Helical areas present in NORPEG.

Fig. 4. Conservation of nuclear localization signal (NLS) in NORPEG orthologs. The regions of the amino acid sequences surrounding the NLS in NORPEG and its orthologs are aligned, with sequence positions indicated in parentheses. Asterisks denote the residues making up the pat7 monopartite NLS. Residues that are identical in all species are shown in dark background, while the conserved substitutions gray.

Nuclear localization of GFP-NORPEG (2–287 aa)

The ability of the putative nuclear localization signal that we identified to target a protein containing it to cell nuclei was studied by preparing a plasmid construct expressing GFP fusion protein containing residues 2–287 of NORPEG. ARPE-19 cells transfected with this plasmid construct expressed the fusion protein of expected size (~55 kDa) when cell extracts were analyzed by immunoblotting using anti-NORPEG antibody (data not shown). The transfected cells showed green fluorescence in the nuclei, indicating that the expressed fusion protein is localized to this organelle (Fig. 5A). The fluorescence appears to be concentrated in discrete areas of the nucleus. Cells transfected with the plasmid vector alone exhibited fluorescence in the cytoplasm as expected (Fig. 5B). Next, we examined the localization of a GFP fusion protein containing the full length NORPEG protein by transfecting the cells with the GFP-NORPEG (2–980 aa) construct. However, in this case the fusion protein expression was not localized to nuclei as the fluorescence was detected throughout the cytoplasm (Fig. 5C). Transfection of COS-7 cells with these plasmid constructs or vector yielded similar results (Fig. 6). Thus, it appears that the putative nuclear localization signal residing between amino acid residues 2 and 287 of NORPEG is able to target the expressed GFP-fusion protein into cell nuclei. In addition, features present between amino acid residues 287 and 980 appear to hinder nuclear localization.

Expressed GFP-NORPEG (2–287 aa) is concentrated in nucleoli

As noted above, the GFP-fusion protein containing amino acid residues 2-287 appears to be concentrated in discrete areas within the nucleus; these areas appear to resemble nucleoli. Therefore, we performed a co-localization study using an antibody preparation against nucleolin, a marker for nucleoli. COS-7 and ARPE-19 cells expressing GFP-NORPEG (2–287 aa) were immunostained for nucleolin (Fig. 7). The green fluorescence appears to co-localize to certain areas where the immunostaining for nucleolin is strong, suggesting that the expressed fusion protein is concentrated in nucleoli.

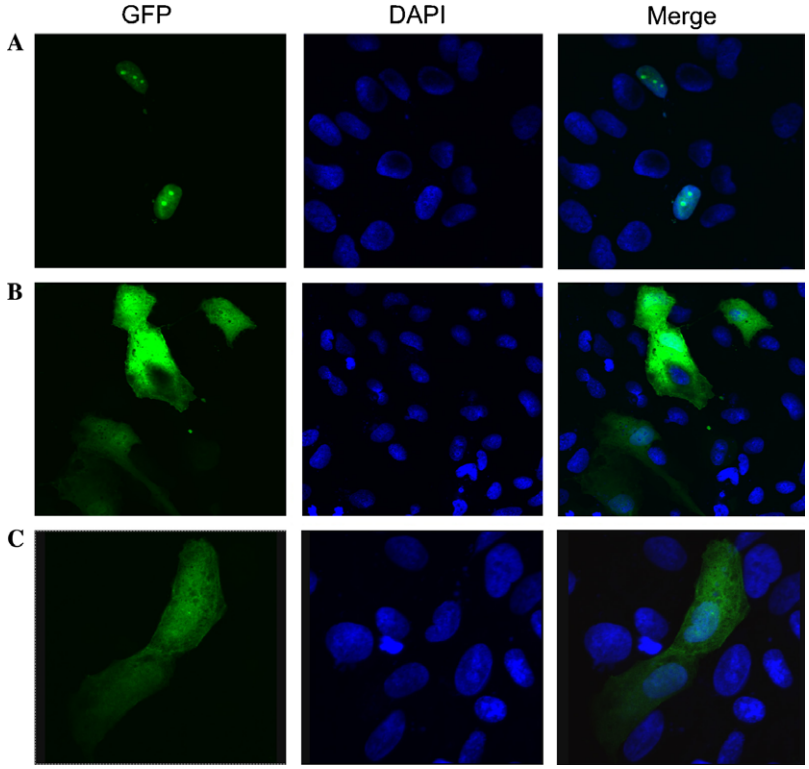

Fig. 5. Expression and nuclear localization of the GFP fusion protein of NORPEG (2–287 aa) in ARPE-19 cells. ARPE-19 cells transfected with GFP fusion protein constructs were analyzed by confocal fluorescence microscopy. The left panel shows GFP fluorescence, the middle panel shows DAPI-stained nuclei, and the right panel shows merged images. (A) Cells transfected with GFP-NORPEG (2–287 aa) construct show fluorescence in discrete pockets in the nuclei. (B) Cells transfected with GFP vector. (C) Cells transfected with GFP-NORPEG (2–980 aa) construct.

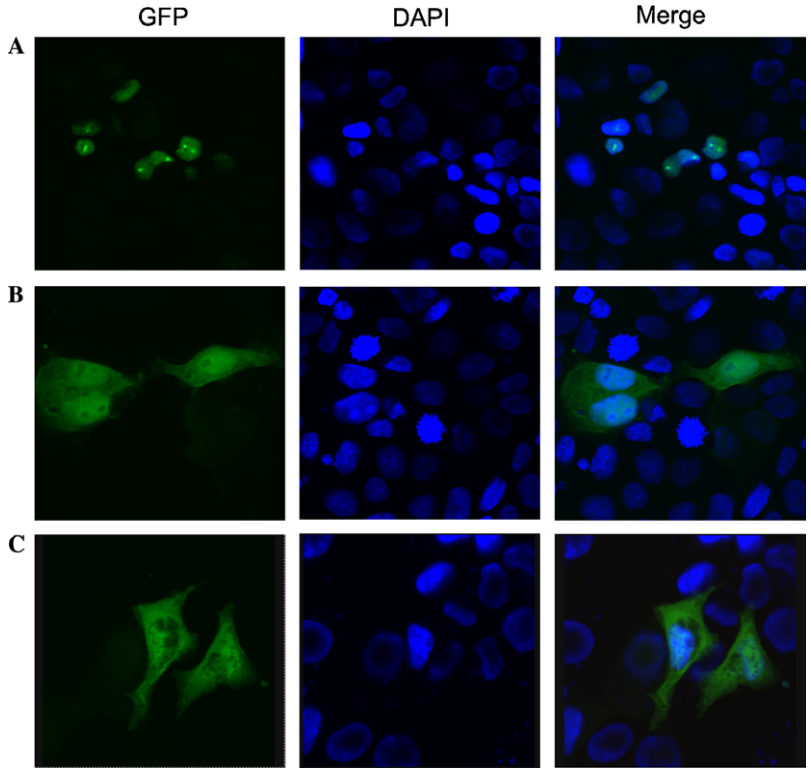

Fig. 6. Expression and nuclear localization of the GFP fusion protein of NORPEG (2–287 aa) in COS-7 cells. COS-7 cells transfected with GFP fusion protein constructs were analyzed by confocal fluorescence microscopy. The left panel shows GFP fluorescence, the middle panel shows DAPI-stained nuclei, and the right panel shows merged images. (A) Cells transfected with GFP-NORPEG (2–287 aa) construct show fluorescence in discrete pockets in the nuclei. (B) Cells transfected with GFP vector. (C) Cells transfected with GFP-NORPEG (2–980 aa) construct.

Dissection of the nuclear localization signal

We investigated the importance of the putative nuclear localization signal, P^{270}KKRKAP^{276}, in targeting GFP-NORPEG (2–287 aa) to cell nuclei. Deletions or mutations were introduced into the original GFP-NORPEG (2–287 aa) plasmid construct by in vitro mutagenesis. The subcellular localization of GFP-fusion protein in ARPE-19 cells transfected with these constructs is shown in Fig. 8. Cells transfected with vector showed fluorescence in the whole cell (Fig. 8A) while those transfected with the original construct (Fig. 8B) showed fluorescence in nuclei, as expected. Removal of the putative nuclear localization signal (P^{270}KKRKAP^{276}) by deleting amino acid residues 232–280 from the original construct disrupted the nuclear localization (Fig. 8C). In contrast, the deletion of most of the amino acid residues (2–264), but not the putative nuclear localization signal, did not affect the nuclear localization (Fig. 8D). Thus, it appears that an effective nuclear localization signal resides between amino residues 265 and 280. The importance of the basic amino acid residues K^{271}KRK^{274} in nuclear targeting of the expressed protein is evident from the following experiment. The mutation of these basic residues in the original construct to A^{271}AAA^{274} effectively disrupted the nuclear localization (Fig. 8E).

Discussion

The human *NORPEG* (*RAI14*) gene, regulated by retinoic acid and during development, encodes a 980 amino acid residue protein [1]. The NORPEG protein contains an ankyrin repeats region towards the N-terminus and a long coiled-coil domain towards the C-terminus. In the present study, we developed an antibody to NORPEG and used it to characterize the NORPEG protein naturally expressed in the RPE cell line, ARPE-19. First, we expressed amino acid residues 1–287 of the predicted NORPEG sequence as a HIS tag fusion protein, and the purified protein was used to produce a polyclonal anti-NORPEG antibody in rabbits. The affinity-purified antibody specifically reacted with a ~110 kDa protein present in ARPE-19 cell extracts, consistent with the predicted molecular size of the NORPEG. Immunohistochemical analysis of human placenta sections showed that this antibody is able to specifically react to NORPEG. The anti-NORPEG antibody that we developed appears to be a useful reagent for studying NORPEG expression in human cells or tissues due to its specificity. The fact that NORPEG sequence is highly conserved in vertebrates suggests that this antibody could be employed in studies using other species too.

Protein expression studies have indicated that NORPEG could be associated with the cytoskeleton [1]. The

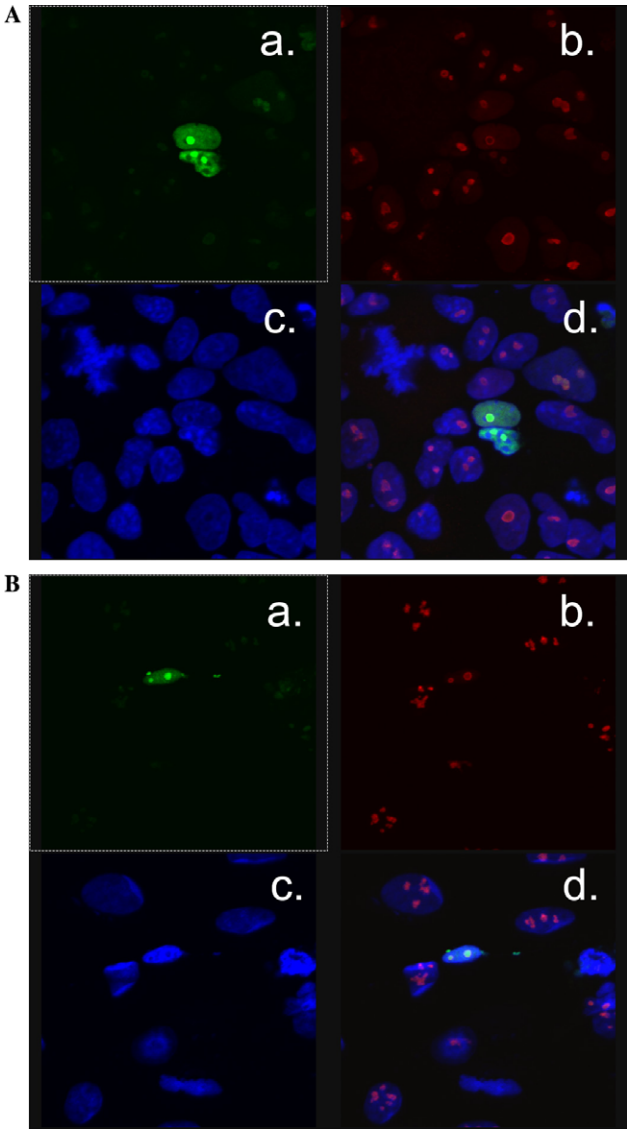

Fig. 7. GFP-NORPEG (2–287 aa) expression appears to be localized to nucleoli. Cells were transfected with GFP-NORPEG (2–287 aa), immunostained for nucleolin (a protein known to be localized to nucleoli), and then analyzed by confocal microscopy. (a), GFP fluorescence; (b), nucleolin immunostaining; (c), DAPI staining of nuclei; and (d), merged image. (A) COS-7 cells. (B) ARPE-19 cells.

mouse ortholog (Ankycorbin, Norpeg) was indeed identified as a protein indirectly associated with actin cytoskeleton [3]. Our results obtained from immunofluorescence analysis of NORPEG in confluent cultures of ARPE-19 cells are in agreement with these reports. The NORPEG immunoreactivity in this case was localized to cytoplasm and showed a close resemblance to actin staining by phalloidin. However, NORPEG exhibited a distinct nuclear localization in nonconfluent cultures of ARPE-19 cells. Cell density-dependent intracellular localization of proteins is not unprecedented. For example, Smad2 in corneal fibroblasts has been reported to have a nuclear localization in nonconfluent cultures and cytoplasmic localization in confluent cultures [11]. Nuclear translocation of Smad2 is regulated by TGF-β and the increased functional expression of TGF-β receptors in nonconfluent fibroblast cells is responsible for the nuclear localization in this case [11]. Numerous other proteins are controlled by cell density; among them are the von Hippel–Lindau tumor suppressor gene product [12], the ERM family of proteins [13], the tight junction protein ZO-2 [14], and aryl hydrocarbon receptor [15]. In the case of NORPEG, the factors regulating the observed nuclear localization of NORPEG in nonconfluent ARPE-19 cells remain to be identified.

Analysis of amino acid sequences of NORPEG and known orthologs showed the presence of a highly conserved putative nuclear localization signal towards the N-terminus. A stretch of basic amino acid residues (P^{270}KKRKAP^{276}) that we identified match the pat7 type of monopartite nuclear localization signal [16]. Lysine-arginine rich nuclear localization sequences are recognized and transported to the nucleus by importin β/importin α complexes [17]. The predicted nuclear localization sequence appears to be functional according to our GFP fusion protein expression studies. A GFP fusion protein, GFP-NORPEG (2–287 aa), containing the nuclear localization sequence, was localized to the cell nuclei when expressed in ARPE-19 or COS-7 cells. Removal of the nuclear localization sequence from this construct by deleting amino acid residues 232–280 disrupted the nuclear localization. However, deletion of amino acid residues 2–264 did not affect the nuclear targeting. Thus, the nuclear localization sequence resides between amino acid residues 265 and 280. The mutation of the lysine-arginine rich residues K^{271}KRK^{274} into A^{271}AAA^{274} adversely affected the nuclear targeting of the fusion protein. Therefore, these lysine-arginine residues form the core of the nuclear localization signal present in NORPEG. It is interesting to note that the KKRK sequence has been reported to be important for the nuclear localization of Bfl-1S protein [18], and a related sequence, KRKK, has been recently shown to constitute a functional nuclear localization signal for the ataxia-telangiectasia mutated (ATM) protein [19].

The nuclear localization signal failed to target the GFP fusion protein containing the full length NORPEG (amino acid residues 2–980) to nuclei when expressed in ARPE-19 or COS-7 cells, indicating greater complexity in the control of NORPEG intracellular localization. Localization of a protein can be regulated through both nuclear localization sequences and nuclear export sequences, and these signals can be made inaccessible by interactions with other proteins such as 14-3-3 or by intramolecular masking [8,20,21]. Indeed, NORPEG possesses additional sequences known to function as regulators of subcellular localization: L^{352}QAKVASLTL^{361} matches the consensus of a nuclear export signal while R^{292}SITSTP^{298} matches a binding site sequence for 14-3-3, a classic masking protein. For example, intramolecular masking of the nuclear localization signal is the reason why the p50 form of transcription factor NF-κB and its p105 precursor are seen in the nucleus and cytoplasm, respectively [22]. Processes such as phosphorylation close to

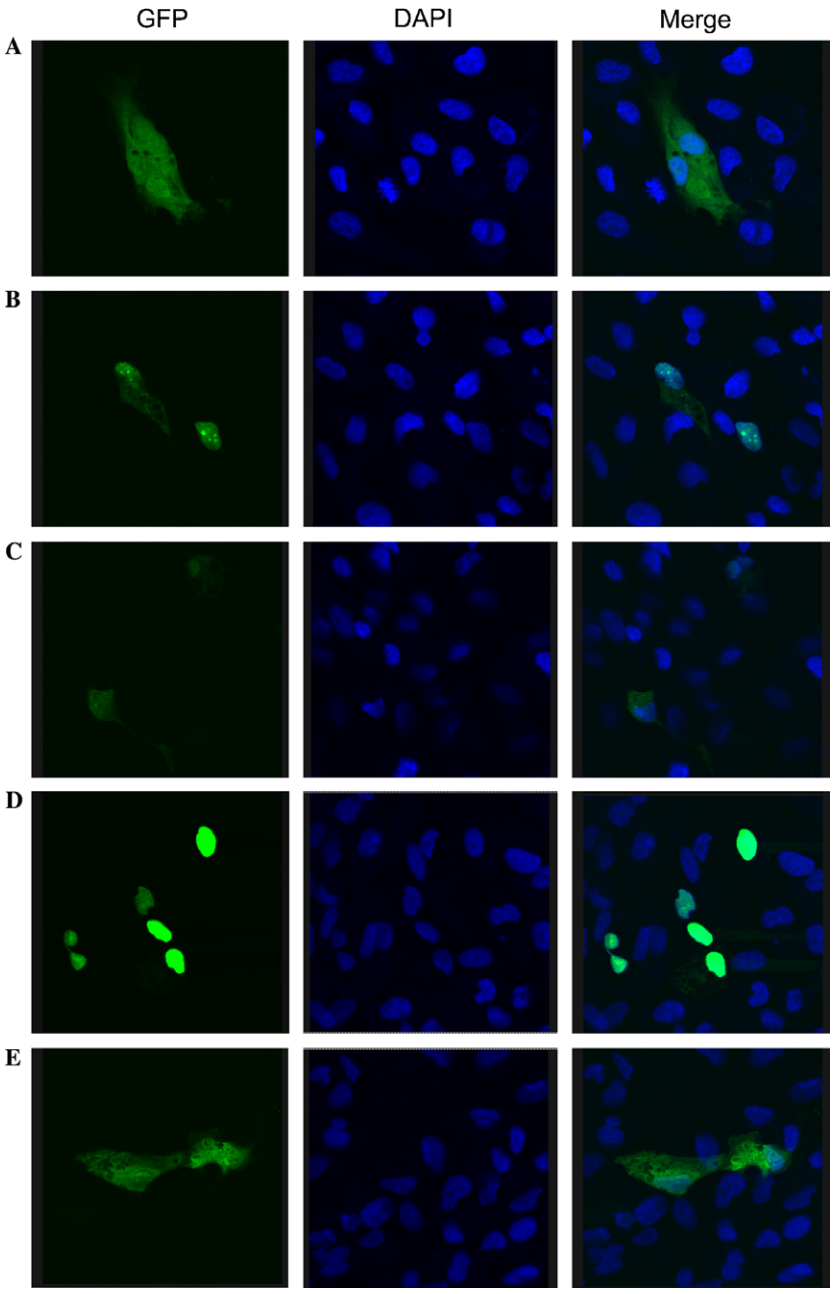

Fig. 8. Characterization of the putative nuclear localization signal. The GFP-NORPEG (2–287 aa) expression construct plasmid was subjected to in vitro mutagenesis to either delete or mutate the putative nuclear localization signal region, P^{270}KKRKAP^{276}. ARPE-19 cells were transfected with these expression constructs and the GFP fluorescence was analyzed by confocal microscopy. GFP fluorescence (left panel), DAPI-stained nuclei (middle panel), and merged images (right panel) are shown. (A) GFP vector. (B) GFP-NORPEG (2–287 aa). (C) GFP-NORPEG (2–287 aa/232–280 aa deleted). This construct lacks the putative nuclear localization signal (P^{270}KKRKAP^{276}). (D) GFP-NORPEG (2–287 aa/ 7–264 aa deleted). This construct contains the nuclear localization signal. (E) GFP-NORPEG (2–287 aa/K^{271}KRK^{274} → A^{271}AAA^{274} mutated).

the signal sequences or disulfide bond formation can also lead to the intramolecular masking [23].

The GFP-NORPEG (2–287 aa) fusion protein, when expressed in ARPE-19 or COS-7 cells, was found to accumulate in nucleoli, the site of ribosomal RNA synthesis, processing, and ribosome maturation [24]. The expressed protein was found to co-localize with nucleolin, a known marker for nucleoli. Nucleolin, an abundant protein of the nucleolus, is involved in many important cellular functions including rRNA maturation, ribosome assembly, and nucleo-cytoplasmic transport [25]. The nuclear localization sequence P^{270}KKRKAP^{276} and basic amino acid residues in its vicinity may play a role in the observed nucleolar localization. A stretch of basic amino acid residues (RKKRKKK) has recently been reported to be sufficient for the nucleolar localization of NF-κB inducing kinase [26]. An arginine-rich domain that can bind rRNA is present in the nucleolar protein p120 [27]. It is also possible that GFP-NORPEG (2–287 aa) binds nucleolin or other nucleolar proteins. The ability to interact with nucleolin is

known to play an important role in the nucleolar localization of telomerase [28].

In conclusion, we have shown that the subcellular localization of NORPEG in cultured retinal pigment epithelial (ARPE-19) cells is dependent upon cell density by confocal immunofluorescence analysis using a polyclonal antibody raised against a HIS tag fusion protein containing N-terminal 287 amino acid residues of the predicted sequence of NORPEG. It is present in the cytoplasm in confluent cultures of ARPE-19 cells, but exhibits a distinct nuclear localization in nonconfluent cultures. An evolutionarily conserved monopartite nuclear localization signal (P^{270}KKRKAP^{276}) was identified by analyzing the sequences of NORPEG and its orthologs; through deletion and mutational analysis of GFP-NORPEG (2–287 aa), we have shown that this sequence is essential for nuclear targeting. The potential role of the nuclear export signal (L^{352}QAKVASLTL^{361}) and the 14-3-3 protein binding site (R^{292}SITSTP^{298}) in regulating the subcellular localization of NORPEG remains to be investigated. Finally, NORPEG belongs to the growing list of proteins having both nuclear and cytoskeletal associations, and whose intracellular localization varies with cell density. These characteristics imply a role in signal transduction in a pathway yet to be determined.

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