Cloning and Preliminary Characterization of a Calcium-binding Protein Closely Related to Nucleolin on the Apical Surface of Inner Medullary Collecting Duct Cells*

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Calcium stone crystal attachment to the urinary epithelium plays an essential role in the development of kidney stones by allowing small crystals to be retained in the kidney until they become macroscopic. We among others have described attachment of stone crystals to cultured renal epithelia (Wiessner, J. H., Kleinman, J. G., Blumenthal, S. S., Garancis, J. C., and Mandel, G. S. (1987) J. Urol. 138, 640–643). To isolate protein(s) that may participate in crystal attachment, apical membranes of cultured renal inner medullary collecting duct were biotinylated, the cells were lysed with detergent, the lysate was subjected to hydroxyapatite chromatography, and fractions were incubated with calcium oxalate monohydrate. Electrophoresis of material solubilized from the crystals showed several selectively adsorbed protein bands. A 110-kDa band stained positively for biotin and for glycosides and bound 45Ca. The amino acid sequence of this band was determined to be that of a protein closely related to rat nucleolin (nucleolin-related protein; NRP). NRP was cloned and sequenced and was 83% homologous with the previously sequenced nucleolar protein nucleolin. Using temperature-induced phase partitioning with Triton X-114, NRP was associated with both the insoluble membrane skeleton pellet and the soluble aqueous phase but not the soluble detergent phase. This association with the membrane skeleton was increased in the presence of calcium. Thus, NRP is associated with the apical membrane skeletal elements in a calcium-dependent fashion. The physiological role of NRP remains to be determined; however, a pathophysiological role may be that of mediating the attachment to the renal tubular epithelium of calcium stone crystals.

Crystals of materials that comprise kidney stones must be retained within the kidney to provide a nidus for development of the mature kidney stone. It is unlikely that single crystals could grow fast enough or produce large enough aggregates as they traverse the nephron to become lodged in the terminal collecting ducts on the basis of size alone (2, 3). We have demonstrated attachment of stone crystals to renal epithelia in vitro, including COM, the most common stone constituent, as well as calcium oxalate dihydrate and hydroxyapatite (HA). We have proposed that apical membrane glycoproteins in renal tubules may play a role in attachment of calcium oxalate and calcium phosphates to the epithelium, thereby providing a mechanism for retention.

In the present study, we analyzed surface proteins from rat inner medullary collecting duct (IMCD) cells for their ability to bind ionic calcium, calcium oxalate crystals, and HA with high affinity. We describe a novel cell surface glycoprotein, NRP, closely related to nucleolin and propose that this protein plays an important role in crystal attachment in kidney stone disease. Nucleolin, the major nucleolar phosphoprotein, has a molecular mass of about 110 kDa and is considered to be a transcriptional factor for preribosomal RNA synthesis. Initially, it was localized to a dense region of the nucleolus, but later it was also found in cytoplasm. Nucleolin or a protein closely related to it has been demonstrated in association with the plasma membranes of some cells, where it functions as a receptor for lipoproteins, viruses, extracellular matrix, growth factors, and other molecules (4–9). Calcium-dependent properties of nucleolin or proteins related to it have not been described. In the present study, we examine the influence of calcium on the distribution of NRP.

MATERIALS AND METHODS
Cell Cultures—IMCD cell cultures originally obtained from Dr. John Schwartz (Boston University Medical School) were cultured as described previously (10). These are continuously passaged cells from enzymatically disaggregated papillae of Harlan Sprague Dawley rats. Cultures from up to the 12th passage were used in the present work. Cells were cultured in serum-free Ham’s F-12 and Delbecco’s medium with 1% fetal bovine serum (Life Technologies, Inc.) supplemented with 5 µg/ml transferrin, 6 µg/ml insulin, 0.018 µg/ml hydrocortisone, 0.0017 µg/ml selenium, 30 µg/ml penicillin G, and 50 µg/ml streptomycin sulfate.

Antibodies—NRP was detected by Western blot analysis using a polyclonal anti-nucleolin antiserum produced against purified nucleolin extracted from 3T3-F442A cells and kindly supplied by Dr. Raymond Petryshyn (Children’s Medical Center, Washington, D. C.) (11). Antibody against Grb2 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

45Ca Overlay Assay (12)—Netrocellulose membranes to which proteins to be tested for calcium binding properties were transferred were soaked for 1 h in a solution containing 60 mM KCl, 5 mM MgCl2, and 10 mM imidazole-HCl at pH 6.8, with several changes of solution. This was followed by a 10-min incubation in the same solution containing 1 mCi/liter 45Ca. After incubation, membranes were washed with H2O to remove excess 45Ca. Protein bands that bound 45Ca were detected by autoradiography.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF151373.

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Surface Biotinylation of Membrane Proteins—IMCD cells growing on 100-mm diameter plastic plates were rinsed in PBS with 1 mm CaCl₂ and 1 mm MgCl₂ and were cooled for 10 min in a refrigerator. Then they were incubated with 0.5 mg/ml NHS-SS-biotin or sulfo-NHS-biotin (Pierce), 3 ml/plate, for 1 h at 4 °C with constant agitation. To remove unreacted biotin, cells were washed with phosphate-buffered saline containing 0.1 M glycine. Cells were then lysed in a 50 mM HEPES, pH 7.5, buffer containing 150 mM NaCl; 1.5 mM MgCl₂; 1 mM EGTA; 10% glycerol; 1% Triton X-100; the protease inhibitors leupeptin, aprotinin, and pepstatin (all at 10 μg/ml); and phenylmethylsulfonyl fluoride (100 μg/ml). This mixture was incubated at 4 °C for 15 min and centrifuged at 12,000 × g for 15 min. The supernatant was collected and further purified as indicated below.

Microsome Fraction Preparation—Cultured IMCD cells were scraped from plates, pelleted by centrifugation, washed with phosphate-buffered saline, and then resuspended in 50 mM HEPES buffer at pH 7.4, containing 1 mM EGTA, 9 g/liter sucrose, and the above mentioned protease inhibitors. Homogenization was done using a Dounce tissue grinder so as to preserve intact nuclei. The suspension was centrifuged at 1000 × g for 3 min, and the supernatant was collected. The resulting pellet was solubilized in SDS-sample buffer or washed with phosphate-buffered saline, pH 7.4, containing protease inhibitors.

Nucleolar Fraction Preparation—Cells were washed with phosphate-buffered saline and lysed with 1% Triton X-100 lysis buffer, as described above. The suspension was centrifuged at 1000 × g for 5 min, then the supernatant discarded, and the pellet was washed several times with the same buffer. The washed pellet was suspended in a nucleolar extraction buffer using 10–15 strokes of a Dounce homogenizer. The buffer contained protease inhibitors as described previously, 100 mM KCl, 5 mM EGTA, 0.5 mM dithiothreitol, 1 mM Na₃VO₄, and 10 mM HEPES at pH 7.4 (as described in Ref. 13 with modifications). Then particulate material was removed by centrifugation, and the supernatant was subjected to SDS-PAGE.

Temperature-induced Phase Separation of Membrane-associated Proteins, Intrinsic Membrane Proteins, and Proteins Associated with Membrane Skeleton (14)—Washed membranes were resuspended and incubated on ice for 5 min in 0.15 M NaCl containing 1% Triton X-114 and 10 mM Tris-HCl at pH 7.4. The solubilized membranes were incubated at 20 °C for 10 min and centrifuged at 3,000 × g for 3 min. The detergent phase was extracted three additional times with 10 volumes of the same buffer, containing 0.06% Triton X-114 and precipitated with 20 volumes of acetone at −20 °C. The aqueous phase was extracted three more times with Triton X-114 to a concentration of 2%. The insoluble pellet, representing the membrane skeleton fraction, was boiled with sample buffer, containing β-mercaptoethanol.

Detection of Glycoproteins, Biotin, and Antibodies—Total carbohydrates were separated by liquid chromatography using a BioScale CHT-I HA column (Bio-Rad). The column was loaded with the clarified lysate and then washed with PBS, pH 6.8, until base-line OD was reached. The bound proteins were eluted with 5 column volumes of PB at pH 6.8, using a linear gradient from 0 to 500 mM NaCl. The eluate was collected in 0.5-ml fractions; fractions 28–30 were pooled, exchanged into an AU (15) and incubated overnight with 10 mg of COM crystals in presence of protease inhibitors. The crystals with the adsorbed proteins were washed five times with AU buffer and solubilized in 0.5 M EDTA. This solution was extensively dialyzed against water and lyophilized. The resulting proteins were electrophoresed on 5–15% gradient polyacrylamide gel, electroblotted to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and stained with Amido Black staining solution (Sigma). Peptide purification for microsequencing was done according to a published protocol (16). Amino terminus microsequence-
Properties of NRP as a Calcium-binding Protein—To investigate further the nature of association of NRP with plasma membrane, we performed experiments with Triton X-114 temperature-induced phase partitioning. This technique can be applied for separation of integral and peripheral membrane proteins (14). Because of the calcium-binding property of NRP, the effect of calcium on NRP membrane attachment was investigated. A microsome pellet was prepared as described earlier, and is supported by data reported by others (19). To determine whether surface-associated NRP is the same or different from the nucleolar shuttle protein of these cells, low speed Triton X-100-insoluble pellet consisting predominantly of whole nuclei was washed several times, extracted, and precipitated with COM crystals. The crystal-associated proteins were solubilized and subjected to PAGE, and a 110-kDa band was sequenced. The first 20 amino acids were identical to those found in surface-associated NRP.

Cloning and Sequencing of NRP—mRNA was prepared from IMCD cells and used as a template for first strand cDNA synthesis. The integrity of the mRNA was checked by Northern hybridization using a glyceraldehyde-3-phosphate dehydrogenase probe. cDNA was reverse transcribed from this mRNA using either universal hexameric primer or with T18 primer supplied with the kit. The NRP cDNA was amplified with an N-terminal oligonucleotide primer derived from amino acid sequence of the isolated NRP with the addition of an initial methionine and the C-terminal primer 5'-TTATCACAAGTTGTCTTCT. This latter, antisense primer was constructed based on the observation that all known nucleolins from different species have identical sequences at their 3' end. Following amplification, the 2.1-kilobase pair DNA fragment was purified and cloned into PCR 2.1 cloning vector (Invitrogen). Three clones obtained from independent cDNA-synthesis reactions were sequenced, and the consensus sequence is presented here (Fig. 6). An identical product was amplified and cloned from RNA extracted from papillas of Harlan Sprague Dawley rats (from which the IMCD cells were derived) using NRP-specific primers.

Western analysis was performed using a polyclonal antiserum against nucleolin and will be referred to hereafter as NRP (Fig. 2). We examined whether NRP would also react with anti-nucleolin polyclonal antibody produced against purified nucleolin. These antibodies yielded an excellent signal with NRP (Fig. 2).

Cellular Localization of NRP—To verify the surface localization of NRP, surface proteins of cultured IMCD were labeled with sulfo-NHS-biotin and precipitated from column fractions with COM crystals as described above. Western blotting shows that NRP has been completely precipitated as well as the corresponding biotinylated band (Fig. 5A). The specificity of surface biotinylation using this protocol is demonstrated by the failure to biotinylate Grb2, a cytosolic protein that associates with growth factor-related tyrosine kinase receptors (Fig. 5B), and is supported by data reported by others (19). To determine whether surface-associated NRP is the same or different from the nucleolar shuttle protein of these cells, low speed Triton X-100-insoluble pellet consisting predominantly of whole nuclei was washed several times, extracted, and precipitated with COM crystals. The crystal-associated proteins were solubilized and subjected to PAGE, and a 110-kDa band was sequenced. The first 20 amino acids were identical to those found in surface-associated NRP.

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Properties of NRP as a Calcium-binding Protein—To investigate further the nature of association of NRP with plasma membrane, we performed experiments with Triton X-114 temperature-induced phase partitioning. This technique can be applied for separation of integral and peripheral membrane proteins (14). Because of the calcium-binding property of NRP, the effect of calcium on NRP membrane attachment was investigated. A microsome pellet was prepared as described earlier, resuspended in TBS with 0 or 5 mM CaCl2, and subjected to fractionation with Triton X-114. Three fractions were obtained:
authentic Grb2 are marked with arrowheads was used for Western analysis (27). Supernatant (1) and dissolved crystals (2) were subjected to SDS-PAGE and blotted to nitrocellulose. Biotin detection (Bt) as indicated in the legend to Fig. 1 and Western analysis with anti-nucleolin polyclonal antiserum (NRP West) were performed. B, immunoprecipitation with polyclonal Grb2 antiserum after surface biotinylation. Biotinylated proteins were detected as indicated above, and the same Grb2 antiserum was used for Western analysis (Grb2 West). The positions of NRP and authentic Grb2 are marked with arrowheads.

FIG. 5. Cell surface localization of NRP compared with cytoplasmic localization of Grb2. A, cultured IMCD cells were surface-biotinylated with membrane-impermeant sulfo-NHS-biotin, and the extract was separated by HA chromatography and incubated with COM. Supernatant (1) and dissolved crystals (2) were subjected to SDS-PAGE and blotted to nitrocellulose. Biotin detection (Bt) as indicated in the legend to Fig. 1 and Western analysis with anti-nucleolin polyclonal antiserum (NRP West) were performed. B, immunoprecipitation with polyclonal Grb2 antiserum after surface biotinylation. Biotinylated proteins were detected as indicated above, and the same Grb2 antiserum was used for Western analysis (Grb2 West). The positions of NRP and authentic Grb2 are marked with arrowheads.

a Triton X-114-insoluble pellet, comprising membrane skeletal elements, a detergent fraction containing solubilized integral membrane proteins, and an aqueous phase with peripheral membrane (also referred to as membrane-associated) proteins partitioned therein. No NRP was detected in the detergent phase; most was found in the aqueous phase, with a significant portion also associated with the membrane skeleton. The amount of insoluble pellet-bound NRP was several times higher in the presence of 5 mM CaCl$_2$ (Fig. 7). A similar observation was made with membrane extraction experiments; 100,000 x g microsome pellet, containing elements of the membrane skeleton together with integral and peripheral membrane proteins, was incubated with TBS buffer alone or in TBS containing 5 mM CaCl$_2$ or 50 mM EDTA. In the presence of CaCl$_2$, all NRP was found to be associated with membranes; without calcium, most of the NRP was washed out with TBS (Fig. 8).

**DISCUSSION**

Crystal attachment to renal tubule cells is thought to be a critical step in kidney stone formation, but the molecules mediating this interaction have not been previously defined. In their observations in BSC-1 cells, Lieske and Toback (20) have suggested that the attachment site for crystals endocytosed by these cells contains an RGDP recognition site and are, thus, integrins or similar molecules. This same group has reported a decline of 80% in crystal adhesion to BSC-1 cells after neuraminidase treatment and a concentration-dependent inhibition of crystal attachment by sialic acid-binding lectin from *T. vulgaris* (21). These data are consistent with their additional observations that coating COM crystals with polyanions or coating of the cells with polycations inhibited crystal attachment to the BSC-1 cells (21, 22). These data suggest that much if not all of the attachment of COM to these cells may be mediated by negative surface charges on apical cell membranes.

Several molecules with anionic components are present on the tubule cell membrane surface. Experiments have been performed to examine lipid perturbations of the apical membranes of cultured IMCD cells in an attempt to determine whether these components of the membrane are responsible for crystal adherence (23). The results suggest that phosphatidylyserine can mediate COM attachment. Membrane glycoproteins also contribute a large number of anionic sites, including backbone sites of sulfation, phosphorylation, or acidic residues, as well as glycosidic side chain sialic acids.

In this study, an avid, relatively specific interaction of membrane glycoproteins and calcium-containing crystals was utilized to select for molecules that may be involved in renal retention of the crystals that comprise kidney stones. Using this strategy, we isolated and identified a glycoprotein related to nucleolin, which we have designated NRP, some of whose properties we have examined.

The amino acid sequences of NRP and rat nucleolin previously cloned (GenBank accession numbers M55015, M55017, M55020, and M55022) exhibit 83% homology (24). This comparison is shown in Fig. 6. Computer analysis of the derived amino acid sequence for NRP shows that it retains three putative RNA-binding regions of rat nucleolin, but potential glycosylation sites are different; NRP has an extra glycosylation site at position 10 and lacks one at position 404. The relative positions of three other proposed glycosylation sites were not changed (320, 483, and 497 for NRP and 321, 481, and 495 for nucleolin). Since we could sequence the first 20 amino acids of NRP without being blocked, it is unlikely that protein is glycosylated at the N terminus. The nuclear localization signal shows differences in two amino acid residues, both occurring at positions that can tolerate point mutations, and some insertions (25). Nucleolin is multiply phosphorylated and has been shown to be a substrate for casein kinase II (26). NRP has 25 potential casein kinase II phosphorylation sites, and rat nucleolin has 22. The extra phosphorylation sites of NRP occur in positions 235, 391, 450, and 457, and one in position 10 is missing. Primer specific only for previously cloned rat nucleolin amplified a product identical to nucleolin from RNA extracted from Harlan Sprague Dawley rat papillas, while the primers specific for NRP also yielded NRP fragments from this source. This suggests that NRP is a new member of the nucleolin family of proteins.

There is some evidence for the existence of different nucleolin-related proteins. A membrane-associated form of human nucleolin can be distinguished from the nuclear form by immunoprecipitation with chicken nucleolin-specific antiserum (7), but these authors did not find any difference in amino acid sequences of tryptic peptides derived from the purified 100-kDa protein. Amino acid sequencing of a fructosyllysine-specific binding protein from monocyte cell membranes showed the absence of an initial methionine in comparison with the nucleolin sequence (6). This comparison is shown in Fig. 6. Computer analysis of the amino acid sequence for NRP shows that it retains 83% homology with nucleolin from RNA extracted from IMCD cells, while the primers specific for NRP also yielded NRP fragments from this source. This suggests that NRP is a new member of the nucleolin family of proteins.

The most striking feature of NRP isolated in the current study is long stretches of aspartic and glutamic acid residues, which form two major negatively charged regions of the protein. The calcium-binding properties of NRP can probably be explained by electrostatic interaction of negatively charged surfaces formed by clusters of acidic amino acids and calcium ions that provide areas of partial positive surface charge on the (101) face of CaOx crystals (27). There are other examples of calcium-binding proteins in eukaryotes that contain acidic amino acid domains (28–30). These are generally thought to be involved in calcium storage. Acidic clusters could account for the high capacity and low affinity calcium binding, a common characteristic of all these proteins. Neither NRP nor the pro-
teins mentioned above contain an EF-hand calcium-binding motif. Nucleolin also has a conserved domain structure and contains large acidic clusters toward its N terminus.

NRP interacts in a calcium-dependent manner with components of the membrane skeleton, which may facilitate a calcium-dependent recruitment of NRP to the cell surface. Stabilization of membrane skeleton can be affected by changes of intracellular ion concentration and by removal of the extracellular Ca\(^{2+}\). There are several other examples of calcium-dependent protein-membrane interactions among calcium-binding proteins. Annexin II has an ability to interact in a calcium-dependent fashion with plasma membranes and/or cytoskeletal elements (31). Treatment of cells with the calcium ionophore A23187 has been reported to result in relocation of cytosolic pools of annexins IV and V to the plasma membrane (32). A rise in the level of intracellular Ca\(^{2+}\) leads to the translocation of MRP8 and MRP14, belonging to the S100 protein family, to the plasma membrane and intermediate filaments (33). The interaction of neuronal calcium-binding protein VILIP 1 with the cytoskeleton through actin may cause calcium-dependent recruitment of proteins to the plasma membrane.

**Nucleolin-related Protein on Apical Membrane**

![Cloning of NRP. The consensus nucleotide sequence, shown in lowercase type, was derived from three independent clones produced from sequences amplified with primers NH\(_2\)-(5'-ATGGTGAARCTCGCAAAGGCHG) and COOH-(5'-TTATTCAAACTTCGTCTTCT) using cDNA from IMCD cells. The derived amino acid sequence of NRP is shown in the first row of single letter codes. The second row of single letter codes shows the derived sequence of rat nucleolin. Identical residues in the latter are shown as asterisks. Potential N-glycosylation and serine/threonine phosphorylation sites of NRP are underlined and have gray backgrounds, respectively. The nuclear localization signals of both proteins are shown in boldface type, and the RNA-binding regions are double underlined. Gaps, indicated by dashes, have been inserted to improve alignment.](image-url)

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**FIG. 6.** Cloning of NRP. The consensus nucleotide sequence, shown in *lowercase type*, was derived from three independent clones produced from sequences amplified with primers NH\(_2\)-(5'-ATGGTGAARCTCGCAAAGGCHG) and COOH-(5'-TTATTCAAACTTCGTCTTCT) using cDNA from IMCD cells. The derived amino acid sequence of NRP is shown in the *first row* of single letter codes. The *second row* of single letter codes shows the derived sequence of rat nucleolin. Identical residues in the latter are shown as asterisks. Potential N-glycosylation and serine/threonine phosphorylation sites of NRP are underlined and have gray backgrounds, respectively. The nuclear localization signals of both proteins are shown in *boldface type*, and the RNA-binding regions are double underlined. Gaps, indicated by dashes, have been inserted to improve alignment.
In summary, the electrochemical position of a major band after surface biotinylation of IMCD cells corresponds to the position of NRP. We were also able to selectively precipitate this major biotinylated band with COM crystals. Taken together, these observations lead to conclusion that significant amount of NRP can be found on surface of IMCD cells. The role of NRP on the membrane remains to be determined, but we propose that NRP may mediate attachment of calcium-containing stone crystals to the apical membrane of IMCD cells.

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