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Signals and Pathways Regulating Nucleolar Retention of Novel Putative Nucleolar GTPase NGP-1(GNL-2)

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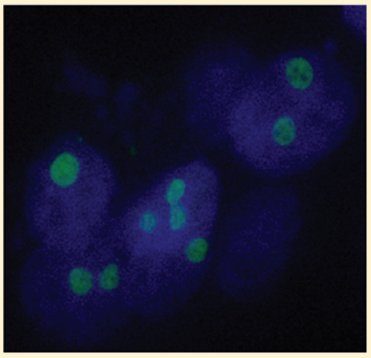
ABSTRACT: NGP-1(GNL-2) is a putative GTPase, overexpressed in breast carcinoma and localized in the nucleolus. NGP-1 belongs to the MMR1-HSR1 family of large GTPases that are emerging as crucial coordinators of signaling cascades in different cellular compartments. The members of this family share very closely related G-domains, but the signals and pathways regulating their subcellular localization and their functional relevance remain unknown. To improve our understanding of the nuclear transport mechanism of NGP-1, we have identified two nucleolar localization signals (NoLS) that are independently shown to translocate NGP-1 as well the heterologous protein to the nucleolus. Site-specific mutagenesis and immunofluorescence studies suggest that the tandem repeats of positively charged amino acids are critical for NGP-1 NoLS function. Interestingly, amino-terminal (NGP-1^{1-100}) and carboxyl-terminal (NGP-$1^{661-731}$) signals independently interact with receptors importin-β and importin-α, respectively. This investigation, for the first time, provides evidence that the interaction of importin-α with C-terminal NoLS (NGP-$1^{661-731}$) was able to target the heterologous protein to the nucleolar compartment. Structural modeling analysis and alanine scanning mutagenesis of conserved G-domains suggest that G4 and G5 motifs are critical for GTP binding of NGP-1 and further show that the nucleolar localization of NGP-1 is regulated by a GTP gating-mediated mechanism. In addition, our data suggest that an ongoing transcription is essential for efficient localization of NGP-1 to the nucleolus. We have observed a high level of NGP-1 expression in the mitogen-activated primary human peripheral blood mononuclear cells (hPBMC) as well as in human fetal brain-derived neural precursor cells (hNPCs) in comparison to cells undergoing differentiation. Overall, the results suggest that multiple mechanisms are involved in the localization of NGP-1 to the nucleolus for the regulation of nucleolar function in cell growth and proliferation.



The nucleolus is a very prominent membrane-free subnuclear organelle popularly known as the "ribosome factory" of the cell.[1−4] Apart from its conventional role in rRNA processing and ribosome biogenesis, it is also recognized as a central player in dynamic signal transduction pathways. Some of the nontraditional roles of the nucleolus include stress response, protein degradation, signal recognition particle assembly, cell cycle regulation, and telomere maintenance.[5−12] The nucleolus is emerging as a major regulator of cell growth and proliferation via modulation of dynamic compartmentalization of cell cycle regulators like p53, ARF, and MDM2.[13−16] Recent reports suggest that an efficient localization of GTP-binding proteins to the nucleolar compartment is critical for the growth and proliferation of cancer and stem cells.[17−22] GTP-binding proteins act as molecular switches in signal transduction pathways by their innate ability to switch between on and off states via GTP binding and/or a hydrolysis mechanism.[23−26]

Compartmentalization is a well-documented form of predicting and defining the functions of the proteins.[27−29] The constant exchange of molecules between the nucleus and the cytoplasm occurs through aqueous channels spanned across the nuclear envelope formed by large multiprotein assemblies called nuclear pore complexes (NPC). The exchange of molecules into and out of the nucleus is now established as a crucial process for regulating cell cycle and other signaling pathways.[27,28] Molecules with masses greater than 35−45 kDa are transported across the nuclear membrane via signal-mediated energy-dependent pathways by interacting with soluble transport receptors such as importins and exportins. In general, the proteins destined to be imported into the nucleus carry defined signal sequences, most often rich in stretches of positively charged amino acids, known as the nuclear localization signals (NLSs). The proteins containing these signal sequences are specifically recognized directly by

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the transport receptor, importin-β, or through an adapter, usually importin-α, and facilitate nuclear translocation. Although many NLSs have been identified, the following three types of NLSs have been characterized in detail so far. Classical NLS, which encompasses a stretch of positively charged lysine residues, was originally identified in SV40 T antigen and interacts with importin-α.[30,31] An arginine-rich NLS, identified in HIV Rev, directly binds to importin-β without importin-α.[32,33] Glycine-rich NLS (M9 NLS), initially identified in proteins involved in mRNA transport,[34−36] interacts with transportin, which is closely related to importin-β. Once the cargo binds to its respective receptor, it will then be imported into the nucleus in a Ran-GTP-dependent manner.[30−37] Many arginine-rich motifs are implicated in protein nuclear/nucleolar transport, and a consensus sequence for nucleolar targeting is yet to emerge. Because the nucleolus is not separated from the rest of the nucleus by a membrane, it is likely that mechanisms of localization of protein to this subnuclear compartment may follow a different paradigm than those operating to sequester proteins to membrane-bound organelles.

The MMR1-HSR1 family of GTP-binding proteins is highly conserved from prokaryotes to highly evolved mammals and humans.[38] They have a unique G-domain organization in which G-motifs are circularly permuted where the G5 and G4 domains are at the N-terminal region of the protein in contrast to the regular G-domain organization that follows from the N-terminal G1 to the C-terminal G5.[26,30] We have previously shown that MMR1-HSR1 family orthologue Grn1 from fission yeast and human GNL3L are important for growth and proliferation by regulating nucleolar pre-rRNA processing and ribosome biogenesis.[18] The growth defect caused by Δ*Grn1* in fission yeast is efficiently complemented by human GNL3L,[18] suggesting the importance of nucleolar putative GTPases for cell proliferation. NGP-1 was recently identified as a breast cancer autoantigen and was shown to primarily localize in the nucleolus and may play a critical role in regulating nucleolar function.[39] The current hypothesis is that an alteration of rRNA processing and ribosome biogenesis in the nucleolar compartment may play an important role in cell proliferation during tumor development. The role of the newly discovered GTPase, NGP-1, in nucleolar function remains unknown.

As a first step in understanding the molecular basis for NGP-1 function and the mechanism(s) by which it is transported into the nucleolar compartment in mammalian cells, we have generated panels of amino- and carboxyl-terminal deletion as well as site-specific mutants to facilitate the dissection of the molecule into functional domains. Nuclear/nucleolar targeting activity was found to reside in both amino-terminal (residues 1−100) and carboxyl-terminal (residues 661−731) regions of NGP-1. In addition, in vitro protein−protein interaction analysis demonstrated that NGP-1 NoLSs interacted with importin-α and importin-β independently for efficient nucleolar translocation. Our data suggest that the GTP-driven cycling mechanism regulates retention of NGP-1 in the nucleolus. Finally, the high levels of NGP-1 expression in actively proliferating mitogen-activated human PBMCs as well as neural precursor cells in contrast to differentiating cells suggest that NGP-1 may be essential for maintaining active cell proliferation.

MATERIALS AND METHODS

Plasmid Construction. To create the mammalian expression vector for NGP-1, we amplified the entire NGP-1 coding sequence from the HeLa cDNA library (Clontech) via polymerase chain reaction (PCR) using appropriate primers (Table 1). The PCR-amplified products were digested with HindIII and EcoRV and cloned into the pcDNA3 vector (Invitrogen Life Technologies) as fusion proteins with enhanced Green Fluorescent Protein (eGFP). The plasmids encoding amino acid residues 1−600, 1−100, 101−600, 101−731, 601−731, and 661−731 of NGP-1 were generated by amplifying cDNA fragments corresponding to the respective coding regions and then subcloned into pcDNA3 as eGFP fusion proteins. The primers used for PCR amplification to generate NGP-1 mutant constructs are listed in Table 1. The numbers represent the amino acid residues included in the amplified DNA fragments. GFP fusion proteins containing HIV-1 Rev-NLS and SV40 large T-antigen NLS (SV40-NLS) were constructed by fusing the primer sequences (Table 1) corresponding to Rev-NLS or SV40-NLS at the N-terminus of GFP. Briefly, the appropriate primer sequences representing Rev-NLS or SV40-NLS were synthesized. Equal amounts of corresponding forward and reverse primers were mixed, incubated at 94 °C for 1 min, and allowed to anneal by being slowly cooled. Annealed primers were fused in-frame with the C-terminus of GFP using EcoRI and XhoI restriction enzymes. All constructs were sequenced to verify the correct reading frame.

To create various NGP-1 site-specific G-domain mutants (G1m−G5m) and the amino- and carboxyl-terminal basic domain mutants, we synthesized appropriate primers (Table 1) and exchanged the specific amino acids by Quick-change mutagenesis according to the manufacturer's instructions (Stratagene). To test the signal-mediated nuclear transport, we generated DsRed−NGP-1^{1-100}−GFP fusion protein (70 kDa), which exceeds the passive diffusion limit of the nuclear pore complex. Briefly, the NGP-1^{1-100}−GFP protein was PCR amplified and fused in frame with the C-terminus of DsRed using KpnI and XhoI restriction enzymes. GFP was directly fused with the C-terminus of DsRed to create the DsRed−GFP protein as a control. All the constructs were sequenced to verify the integrity of each clone.

Cell Culture, Transfection, and Western Blotting. Cos-7, HeLa, and 293Tcells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (FBS). The infection−transfection protocol for the *Vaccinia* virus expression system was as described previously.[40] Briefly, Cos-7 cells were grown to 90% confluence on 60 mm diameter plates, infected for 1 h at 37 °C with vTF7-3, a *Vaccinia* virus expressing T7-RNA polymerase, at a multiplicity of infection of 10, and then transfected with wild-type or relevant NGP-1 mutant constructs using Lipofectine (Invitrogen Life Technologies). After 12−16 h, the transfected cells were solubilized in loading buffer [62.5 mM Tris-HCl (pH 6.8), 0.2% SDS, 5% 2-mercaptoethanol, and 10% glycerol] and separated via sodium dodecyl sulfate−10% polyacrylamide gel electrophoresis (SDS−10%PAGE). Following electrophoresis, proteins were transferred to a Hybond-P membrane (Amersham Pharmacia) and probed with the monoclonal anti-GFP antibody (Santa Cruz) at 1:1000 dilutions. Protein-bound antibodies were probed with horseradish peroxidase (HRP)-conjugated specific secondary antibodies (1:3000 dilution) and developed using the enhanced chemiluminescence-plus detection system (Amersham Pharmacia). To determine the stability of NGP-1−GFP fusion protein, we transfected GFP and NGP-1-GFP into 293T cells and treated them

Table 1. Primers Used for the Construction of NGP-1 Variants

primer[a]	sequence (5′−3′)
NGP HindII+	TGCGACAAGCTTGCCGCCACCATGGTGAAGCCCAAGTACAAA
NGP EcoRV−	CACCATGATATCCTGCTTTTGTCTGAATTTTTTGCGTTTGTG
NGP 101 HIndIII+	ATCCTAAAGCTTGCCGCCACCATGACAGTTATGAAGGATCCATAC
NGP 100 EcoRV−	GTTAGGGATATCATCCATTTCCTCTTGAAATTT
NGP 600−	ATCGATGATATCCAGTGCTTTAATAACGGCTTT
NGP H3 601+	ATCTGCAAGCTTGCCGCCACCATGGATGAGAAGATTGCCAAATAT
NGP H3 661+	ATCTGCAAGCTTGCCGCCACCATGGCACAAAGGGAAGAGGAACAG
NGP G5m+	CAAGTTCTTGATGCTGCTGCTGCAATGGGTACTCGTTCC
NGP G5m−	GGAACGAGTACCCATTGCAGCAGCAGCATCAAGAACTTG
NGP G2m+	GTGGCTCCCATTGCAGCTGAAGCAAAGGTCTGGCAGTAT
NGP G2m−	ATACTGCCAGACCTTTGCTTCAGCTGCAATGGGAGCCAC
NGP G3m+	TTCCTGATTGACTGTGCAGCTGTGGTTTACCCCTCTG
NGP G3m−	CAGAGGGGTAAACCACAGCTGCACAGTCAATCAGGAA
NGP D258A+	CTTAACAAATGTGCTCTTGTTCCAACC
NGP D258A−	GGTTGGAACAAGAGCACATTTGTTAAG
NGP K323A+	CCAAATGTTGGCGCTAGCTCTGTGATA
NGP K323A−	TATCACAGAGCTAGCGCCAACATTTGG
NGP S324A+	CCAAATGTTGGCAAGGCTTCTGTGATAAATAC
NGP S324A−	GTATTTATCACAGAAGCCTTGCCAACATTTGG
NGP S325A+	GTTGGCAAGAGCGCTGTGATAAATACA
NGP S325A−	TGTATTTATCACAGCGCTCTTGCCAAC
NGP R33,35A+	GGAGGCCAAAACATGGCCGACGCCGCCACCATCCGGCGC
NGP R33,35A−	GCGCCGGATGGTGGCGGCGTCGGCCATGTTTTGGCCTCC
NGP R39,40A+	GACCGGGCCACCATCGCCGCCCTGAATATGTATAGG
NGP R39,40A−	CCTATACATATTCAGGGCGGCGATGGTGGCCCGGTC
NGP R49,50A+	GTATAGGCAAAAGGAGGCCGCCAACAGTCGTGGTAAAA
NGP R49,50A−	TTTTACCACGACTGTTGGCGGCCTCCTTTTGCCTATAC
NGP R682-4A+	GCGCTTACATCAAAAGAAGCCGCCGCCGCAGTACGACAGCAACG
NGP R682-4A−	CGTTGCTGTCGTACTGCGGCGGCGGCTTCTTTTGATGTAAGCGC
NGP K711-3A+	GAAAAATAGGAACAGGAACGCCGCCGCCACCAATGACTCAGAGGG
NGP K711-3A−	CCCTCTGAGTCATTGGTGGCGGCGGCGTTCCTGTTCCTATTTTTC
NGP P467,471A+	CCACCCAATGCAGAGGCACTTGTGGCCGCTCAGCTTCTACCCTCC
NGP P467,471A−	GGAGGGTAGAAGCTGAGCGGCCACAAGTGCCTCTGCATTGGGTGG
NGP KKR+	GAGCACCTTCCAAAAAGGGAGCTGCTGCAAAGGCACAAAGGGAAGAGG
NGP KKR−	CCTCTTCCCTTTGTGCCTTTGCAGCAGCTCCCTTTTTGGAAGGTGCTC
NGP KRK+	TCAGAGGGACAGAAACACGCAGCTGCAAAATTCAGACAAAAGCAG
NGP KRK−	CTGCTTTTGTCTGAATTTTGCAGCTGCGTGTTTCTGTCCCTCTGA
NGP 50+	CTAATAAAGCTTGCCGCCACCATGAACAGTCGTGGTAAAATAATTAAACCC
NGP 50−	CTAATAGATATCACCACGACTGTTCCTGCGCTCCTT
Rev NLS+	AATTCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGATAGC
Rev NLS−	GCTATCTCCACCTTCTTCTTCTATTCCTTCGGGCCTGTCGGAATT
SV40 NLS+	AATTCACACCCCCAAAGAAAAAGAGAAAGTAGAAGACCCATAGC
SV40 NLS−	GCTATGGGTCTTCTACTTTCTCTTTTTCTTTGGGGGTGTGAATT

[a] Plus for forward and minus for reverse.

with the translational inhibitor cycloheximide (CHX) 24 h after transfection. Cells were collected at various time periods after CHX treatment and equal amounts of proteins resolved via SDS−10% PAGE followed by Western blot analysis using the anti-GFP antibody as described above.

To determine whether NGP-1 directly interacts with importin-β in the absence of importin-α, we immunodepleted importin-α from the cell lysates and used them for protein−protein interaction assays. Briefly, HeLa cells were grown to 90% confluence on 60 mm diameter plates, infected for 1 h at 37 °C with vTF7-3, a *Vaccinia* virus expressing T7-RNA polymerase, and transfected with NGP-1^{1-100}−GFP or GFP constructs as described above. After 12−16 h post-transfection, cell lysates were prepared, and expression of NGP-1^{1-100}−GFP and GFP constructs was assessed by Western blot analysis using the anti-GFP antibody as described above. Equal amounts of lysates were used to immuno-deplete the importin-α by immunoprecipitation using anti-importin-α antibodies (Santa Cruz) followed by Western blotting using

anti-importin-α antibody to confirm that importin-α is depleted from the cell lysates. The importin-α-depleted lysates containing equal amounts of NGP-1^{1-100}−GFP or GFP were mixed with GST−importin-β or GST bound glutathione Sepharose beads, and the binding reaction was conducted as described below. Bound proteins were resolved via SDS−10% PAGE followed by Western blot analysis using anti-GFP antibodies.

Fluorescence Microscopy. To assess the subcellular localization, Cos-7 or HeLa cells in chamber culture slides (BD Biosciences) were infected with vTF7-3 followed by transfection with NGP-1 expression plasmids using Lipofectine as described above. After 12−16 h, cells were fixed with 3% paraformaldehyde in PBS for 10 min and were probed with the anti-nucleolin antibody (Upstate Biotechnology) followed by goat anti-mouse Alexa Fluor 594 (Molecular Probes) to visualize nucleoli and mounted with 4,6-diamidino-2-phenylindole (DAPI) to stain for nuclei (Vector laboratories). Subcellular localization was assessed with an upright Nikon E800 microscope, and images were recorded using a Nikon DXM1200 camera. Images were acquired using Image Pro-plus 4.5 (Media Cybernetics). Adobe Photoshop 5.0 was used for image processing.

Protein Interaction Assays. GST−importin-α and GST−importin-β expression constructs were obtained from T. Sekimoto (Osaka University, Osaka, Japan), and the fusion proteins were expressed and purified as described previously.[45] Briefly, importin-α and importin-β expression vectors were transformed into *Escherichia coli* M15 containing Rep4 cells and grown at 37 °C in ampicillin-containing medium to an optical density of ∼0.9 at 600 nm prior to induction for 16 h at 18 °C with 1 mM IPTG. Cells were harvested and suspended in 1× *E. coli* lysis buffer and disrupted by vigorous sonication in ice. Glutathione Sepharose 4B beads (Amersham Pharmacia) were washed three times with ice-cold PBS, and the mixtures were rocked for 60 min at 4 °C followed by four washes of protein-bound beads with ice-cold PBS. Bound proteins were eluted in elution buffer [5 mM reduced glutathione in 50 mM Tris-HCl (pH 7.4)], and the integrity of fusion proteins was analyzed by SDS−10% PAGE followed by staining with Coomassie blue.

GFP, full length and various NGP-1 mutants, GFP-SV40 NLS, and GFP-HIV-1 Rev NLS expression plasmids were transfected into Cos-7 cells, and the expression of all the fusion proteins was confirmed by Western blot analysis using anti-GFP antibodies (Santa Cruz) as described above. The binding reaction mixtures comprised equal amounts of cell lysates containing GFP or NGP-1-GFP with GST−importin-α or −importin-β protein bound to 30 μL of glutathione Sepharose beads in a final volume of 300 μL of binding buffer [25 mM HEPES (pH 7.9), 150 mM KCl, 0.1% NP-40, 5% glycerol, 0.5 mM DTT, 0.4 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and aprotinin, leupeptin, and pepstatin (1 μg/mL each)]. After incubation for 4 h at 4 °C, the beads were washed five times with binding buffer. Bound proteins were eluted by being boiled for 5 min in Laemmli dissociation buffer containing SDS. Eluted proteins were resolved via SDS−12% PAGE followed by Western blot analysis using anti-GFP antibodies as described above.

To test the direct interaction between NGP-1 and the nuclear transport receptor, importin-β, HeLa cells were transfected with NGP-1^{1-100}−GFP protein using the *Vaccinia* virus expression system as described above. GFP alone was transfeted as a control. After transfection for 12−16 h, the cell lysates were prepared and equal amounts of cell lysates were used to immunodeplete importin-α using the polyclonal anti-importin-α antibody (Santa Cruz) by immunoprecipitation assay as described elsewhere.[40] Depletion of importin-α from the total cell lysate was assessed by Western blot analysis using the anti-importin-α antibody. Immunodepleted lysate was used for the in vitro GST pull-down assay with GST−importin-β protein followed by Western blot analysis as described above.

GTP Binding Assay. Wild-type NGP-1 and its various G-domain mutants were transfected in Cos-7 or 293T cells and lysed with buffer containing 20 mM HEPES (pH 7.9), 20 mM $MgCl_2$, 300 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.4 mM PMSF, and aprotinin, leupeptin, and pepstatin (1 μg/mL each) and were precleared with Protein A−agarose beads before being incubated with GTP−agarose beads (Innova Biosciences). Bound proteins were resolved via SDS−12% PAGE followed by Western blot analysis using anti-GFP antibodies.

Isolation of Human Fetal Neural Precursor Cells. Primary cultures of human neural stem/precursor cells were prepared as described previously.[46] The human fetal brain samples were collected from elective medical termination of first-trimester pregnancies with informed consent from mother and handled as per protocols approved by the institutional human ethics committee based on guidelines adopted by the Indian Council of Medical Research. Following the isolation, human neural precursor cells (hNPCs) were characterized by using neural precursor cell marker nestin by immunofluorescence and reverse transcription PCR (RT-PCR). The hNPCs were later differentiated into astrocytes by providing predefined medium conditions. Astrocytes were characterized by astrocyte cell-specific marker glial fibrilary acidic protein by immunofluorescence as described previously.[40]

RNA Extraction and RT-PCR. RNA extractions were conducted with the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. The samples were pipetted up and down several times before being loaded onto the column. RNA obtained with this procedure was free of genomic DNA. RNA samples were quantitated with the UV spectrophotometer, and 1 μg of total RNA was used for RT-PCR with 100 pM oligo-dT and 200 units of superscript-II (Invitrogen Life Technologies) in a total volume of 10 μL according to the manufacturer's instructions. Semiquantitative PCRs for NGP-1 were conducted using forward and reverse primers (25 pM each), 1 μL of the cDNA, and 2.5 units of Taq polymerase (MBI-Fermentas) in total reaction volume of 25 μL for 20 cycles. β-Actin was used as an internal control to determine that an equal amount of cDNA was used in each PCR.

RESULTS

NGP-1 Is an Evolutionarily Conserved GTP-Binding Protein. NGP-1 was initially identified in human ductal breast carcinoma as a nucleolar putative GTPase and encodes a polypeptide of 731 amino acids with a predicted molecular mass of 80 kDa. Recent studies suggest that NGP-1 was ubiquitously expressed at a high level in testis and muscle.[39] NGP-1 contains at least five G-domain consensus motifs, designated as G1-, G2-, G3-, G4-, and G5-like sequences that define a G-protein (Figure 1A,B). A BLASTp search of NGP-1 sequence showed that similar sequences are found in genomes of diverse eukaryotes with the G-domain displaying a high degree of sequence homology. This region in NGP-1 shows features characteristic of the G-domain present in many regulatory GTPases, which act as molecular switches in different cellular processes such as control

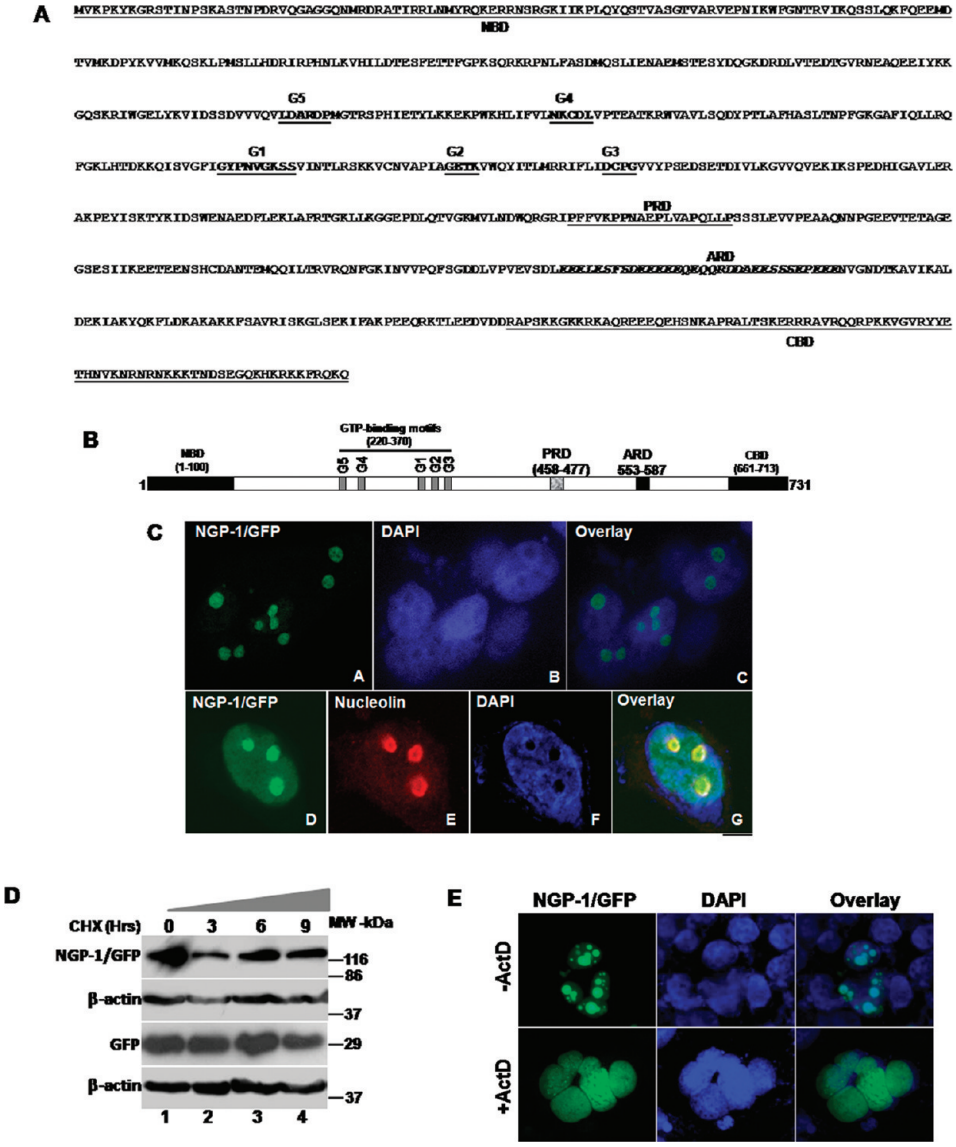

Figure 1. NGP-1 is a nucleolar protein. (A) Depiction of different functional motifs of NGP-1. The amino- and carboxyl-terminal basic amino acid-rich domains (NBD and CBD, respectively) are underlined. The G-motifs from G1 to G5 are highlighted in bold and underlined; the proline-rich domain (PRD) is underlined, and the acidic amino acid-rich domain (ARD) is highlighted in italics. (B) Diagrammatic representation of different functional domains of NGP-1. (C) Colocalization of NGP-1 with nucleolin in the nucleolus. The mammalian expression vector carrying NGP-1−GFP protein was transfected in Cos-7 cells in chamber slides. After 16−24 h, the cells were fixed with a 3% paraformaldehyde solution in 1× PBS and permeabilized with 1% Triton X-100. Fixed cells were probed with the anti-nucleolin antibody followed by staining with the secondary antibody conjugated with Alexa Fluor 594. The nucleus was stained with DAPI, and the images were captured via Carl Zeiss laser scanning confocal microscopy using Zen2009. Green fluorescence indicates NGP-1−GFP protein localization and the red nucleolin. The yellow fluorescence in the merged image suggests that NGP-1 is colocalized with nucleolin in the nucleolar compartment. The bar is 10 μm. (D) The GFP fusion with NGP-1 did not alter the stability of NGP-1−GFP fusion protein. NGP-1−GFP and GFP expression vectors were transfected in 293T cells as described above and were treated with cycloheximide (CHX) to block the translation of protein 24 h after transfection. Cells were collected at different time periods after CHX treatment, and Western blot analysis was performed using anti-GFP antibodies to determine the steady state levels of fusion proteins. (E) Cos-7 cells were transfected with the expression vector encoding NGP-1−GFP protein. After 16 h, the medium was replaced with either fresh medium (control) or medium containing 5 μg/mL actinomycin D (Act-D) and incubation was continued for an additional 3 h. The subcellular distribution of NGP-1−GFP protein was determined directly with a fluorescence microscope.

of cell growth and differentiation, protein trafficking, and signal transduction.[18,21,40] Domain search analysis identified five conserved G-domains that define NGP-1 as a GTP-binding protein and basic amino acid- rich regions both in the amino- and carboxyl-terminus (Fig. 1A,B). In addition, we noticed a proline-rich domain (PRD) and an acidic amino acid-rich domain (ARD) toward the carboxyl terminus of NGP-1 (Figure 1A,B). G-domains of nearly all regulatory GTPases have five conserved polypeptide loops designated G1−G5, which form contact sites for the guanine nucleotide. Rather than the usual G1-G2-G3-G4-G5 sequence found in the superfamily of regulatory GTPases, the G1 motif (Gx4GK(S/T) in NGP-1 is in between the G4 (NKxD) and G2 (GxT) as G5-G4-G1-G2-G3 sequence, which has been described as a circularly permuted

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G-motif. The spacing between individual G-motifs in NGP-1 is similar to that of known G-proteins. The domain structure of NGP-1, like other known nucleolar GTPases, Nug2p, Ngp1p, nucleostemin, and GNL3L, is similar to that of HSR1/MMR1 GTP-binding protein GNL-1 and members of the Ylqf/YawG family of GTPases.

NGP-1 Localizes to the Nucleolus. The existence of several closely related large MMR1-HSR1 GTPase family proteins suggests the possibility that they may have shared or independent roles in regulating different cellular events from the nucleolus. To improve our understanding of the nucleolar transport mechanism(s) of one of the members of the MMR1-HSR1 family of GTPases, full-length NGP-1 was amplified from the HeLa cDNA library and cloned as a carboxyl-terminal fusion with enhanced Green Fluorescent Protein (eGFP) as described in Materials and Methods. The subcellular localization of NGP-1−GFP protein was assessed by fluorescence microscopy upon transient expression in Cos-7 cells. We selected GFP as a partner for generating fusion protein because it allows the visualization of tagged proteins without antibody staining and is known to localize to the nucleus when attached to a functional NLS. The predicted size of the NGP-1−GFP fusion protein is around 106 kDa, which exceeds the passive diffusion limit of the nuclear pore complex (NPC). Immunofluorescence analysis showed that NGP-1−GFP protein is mainly localized in subnuclear foci with some nucleoplasmic staining in Cos-7 cells (Figure 1C). GFP, in comparison, was diffusely distributed both in the nucleus and in the cytoplasm (data not shown). The subnuclear foci were labeled with antibody against the known nucleolar protein, nucleolin, to confirm the localization of NGP-1. Results in Figure 1C suggest that NGP-1 colocalizes with nucleolin in the nucleolar compartment. Western blot analysis of whole-cell lysates confirmed that the fusion protein had a molecular mass of 120 kDa. Further, HeLa cells were transfected with NGP-1−GFP expression plasmid, and the stability of the fusion protein was tested in the presence of translational inhibitor cycloheximide (CHX). Transfected cells were treated with CHX, and the cell lysates were subjected to Western blot analysis using anti-GFP antibodies. Results suggest that the fusion of GFP with NGP-1 does not alter the stability of the fusion protein (Figure 1D). Treatment of NGP-1−GFP protein-expressing Cos-7 cells with RNA polymerase inhibitor actinomycin D (Act-D) (Act-D blocks transcription by intercalating between nucleotide base pairs and induces nucleolar reorganization) resulted in the redistribution of NGP-1 from the nucleolus to the nucleoplasm, with weak cytoplasmic staining in contrast to that of the untreated cells (Figure 1E). These results suggest that ongoing transcription is critical for efficient retention of NGP-1 in the nucleolus. In conclusion, NGP-1 appears to possess specific signals for its nuclear transport as well as for its nucleolar retention.

Both Amino- and Carboxyl-Terminal Domains Independently Regulate Nuclear/Nucleolar Targeting of NGP-1. To understand the mechanism(s) underlying the nucleolar transport of NGP-1, we analyzed the amino acid sequence of NGP-1. Such an analysis indicated the presence of positively charged residues in both amino- and carboxyl-terminal regions of the protein. Because the positively charged amino acid-containing regions act as nuclear/nucleolar targeting signals for many cellular and viral proteins,[21,30−33] we generated variants of NGP-1−GFP protein harboring truncations in the amino and carboxyl termini (Figure 2A). Wild type NGP-1 or its truncation mutants were expressed in Cos-7 cells, and their subcellular localization was assessed by immunofluorescence microscopy. Mutant proteins containing amino acids 1−100 or 661−731 of NGP-1 retained their ability to localize to the nucleolus and nucleoplasm like wild-type protein (Figure 2A,B). In contrast, NGP-1 mutant proteins lacking amino (amino acids 1−100) and carboxyl termini (amino acids 661−731) localized only in the cytoplasm (Figure 2B). A mutant with a deletion of a predicted loop sequence (Δ554−587) retained the wild-type nucleolar localization, suggesting that the loop region may not be critical for the NGP-1 subcellular distribution (Figure 2B). To further define whether the nucleolar transport mediated by the amino- and carboxyl-terminal fragments of NGP-1 is a signal-mediated process, we used NGP-1^{1-100} as a representative and generated chimeric protein containing a DsRed monomer at the amino terminus and GFP at the carboxyl terminus of NGP-1^{1-100} (Figure 2C). The DsRed monomer is a mutant form of red fluorescent protein from *Discosoma* sp. reef coral and is an ideal choice for use as a red fluorescent fusion tag.[41,42] It has been expressed as a fusion tag with a large panel of proteins with diverse functions and subcellular locations.[41,42] Chimeric NGP-1^{1-100} containing DsRed and GFP (RNG) was transfected in HeLa cells, and the expression was assessed by Western blot analysis using anti-GFP antibodies. Results in Figure 2D indicate that the fusion proteins were expressed with the appropriate molecular mass as expected. Both DsRed−GFP (RG) and DsRed−NGP-1^{1-100} −GFP (RNG) expression plasmids were transfected in HeLa cells, and we tested whether NGP-1^{1-100} is sufficient to transport the fusion protein to nucleolus like the full-length protein. The chimeric protein RNG containing NGP-1^{1-100} was efficiently targeted to the nuclear/nucleolar compartment (Figure 2E) in contrast to the diffused pattern observed with DsRed−GFP protein (Figure 2E). These data suggest that NGP-1^{1-100} is sufficient to transport the heterologus proteins to the nuclear/nucleolar compartment by a signal-mediated process. Results in Figure 2F suggest that both amino- (amino acids 1−100) and carboxyl-terminal (amino acids 661−731) nucleolar localization signals (NoLS) independently translocate NGP-1 as well as the heterologous protein, GFP, to the nucleolar compartment. A careful examination of localization patterns reveals that NGP-1^{1-100} retains wild-type nucleolar localization except for a few nucleoli lacking fluorescence in the fibrillar center (FC) (Figure 2F). In contrast, NGP-$1^{661-731}$ was distributed mainly in the granular component (GC) and the dense fibrillar component (DFC) without any fluorescence in the FC region of the nucleolus (Figure 2F,G), suggesting that both amino- and carboxyl-terminal NoLSs are essential for the efficient localization of NGP-1 in the nucleolar compartment. Western blot analysis of the transfected cell lysates confirms that all the NGP-1 deletion mutants containing GFP fusion protein expressed the correct size polypeptides (data not shown). These results suggest that both the amino- (amino acids 1−100) and carboxyl-terminal (amino acids 661−731) NoLSs contained necessary and sufficient information to translocate and retain NGP-1 in the nucleolus independently. Our data provide evidence that NGP-1 encodes two independent nucleolar localization signals (NoLS) and is able to translocate NGP-1 as well as the heterologous cytoplasmic proteins to the nucleolus by a signal-mediated process.

Detailed analysis of amino acid sequences within the amino- and carboxyl-terminal NoLS showed clusters of positively charged lysines and arginines (Figure 3A,D). To identify the

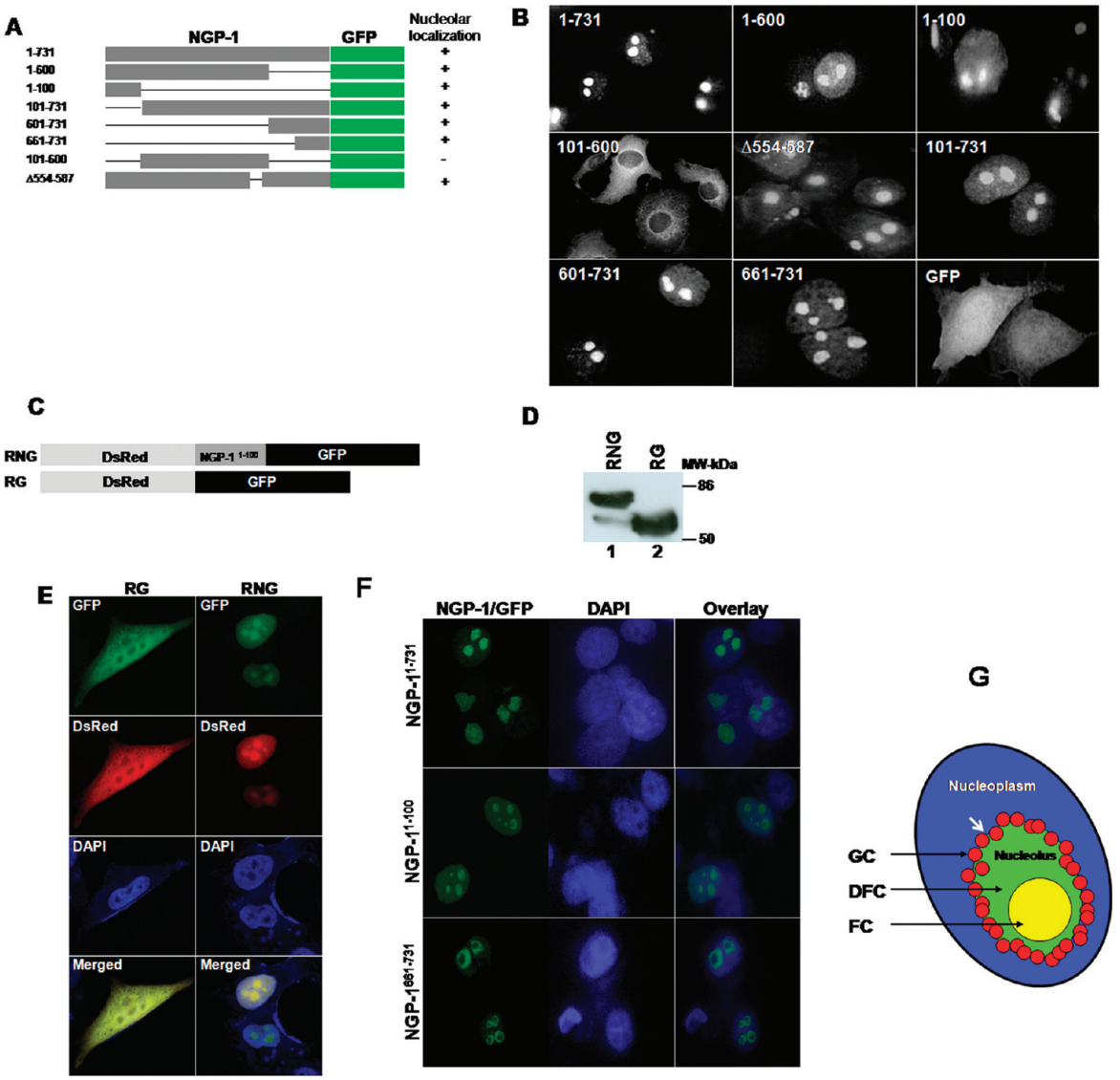

Figure 2. Identification of nucleolar localization signals in NGP-1. Cos-7 cells were transiently transfected with the expression vectors encoding GFP-tagged wild-type and various amino- and carboxyl-terminal deletion mutants of NGP-1. The numbers on the side of each panel correspond to the amino acids within NGP-1 that were expressed (A). Sixteen hours after transfection, the cells were fixed, and the subcellular distribution of NGP-1 was assessed directly with a fluorescence microscope (B). (C) Schematic representation of DsRed−NGP-1^{1-100}−GFP and DsRed−GFP proteins. (D) Expression of DsRed−NGP-1^{1-100}−GFP and DsRed−GFP fusion proteins. Indicated fusion constructs were transfected in 293T cells, and expression was assessed by Western blot analysis using anti-GFP antibodies as described in Materials and Methods. (E) Subcellular localization of DsRed−NGP-1^{1-100}−GFP and DsRed−GFP fusion proteins. HeLa cells were transfected with the indicated expression vectors, and the subcellular distribution was detected by Carl Zeiss laser scanning confocal microscopy. The results suggest that NGP-1^{1-100} is sufficient to confer the nuclear/nucleolar localization of DsRed and GFP fusion protein. (F) NGP-1 encodes two independent NoLSs. Cos-7 cells were transiently transfected with GFP fusion clones containing minimal NGP-1 NoLS. After being transfected for 16 h, the cells were fixed, and the localization of NGP-1−GFP fusion proteins was assessed by confocal microscopy after the nucleus had been stained with DAPI. The bar is 10 μm. (G) Cartoon showing a single nucleus with morphological subcompartments of the nucleolus. Abbreviations: FC, fibrillar center; DFC, dense fibrillar component; GC, granular component. The white arrow indicates the accumulation of GFP signal at the granular component on the nucleolus. A summary of the intracellular localization of the NGP-1 protein fragments is shown in panel A.

critical residues that are important for nucleolar retention, we used site-directed mutagenesis to generate variants of NGP-1 in which all the basic amino acid residues were exchanged with alanines individually or in combination within both NoLSs (Figure 3A,D). Western blot analysis of the transfected cell lysates indicated that all the variants of the NGP-1−GFP fusion protein were expressed with correct size polypeptides (Figure 3B), suggesting that mutations did not alter the expression profile of mutant proteins. GFP-tagged forms of these NGP-1 variants were transiently expressed in Cos-7 cells as described in Materials and Methods, and their subcellular localization was assessed by fluorescence microscopy. Results in Figure 3C indicate that replacement of R39 and R40 within NGP-1^{1-100} as well as R682−R684, K711−A713, and R724, K725, and A726 within NGP-$1^{661-731}$ (Figure 3E) did not alter the wild-type nucleolar localization pattern. We observed a diffused nuclear localization for NGP-1^{1-100}

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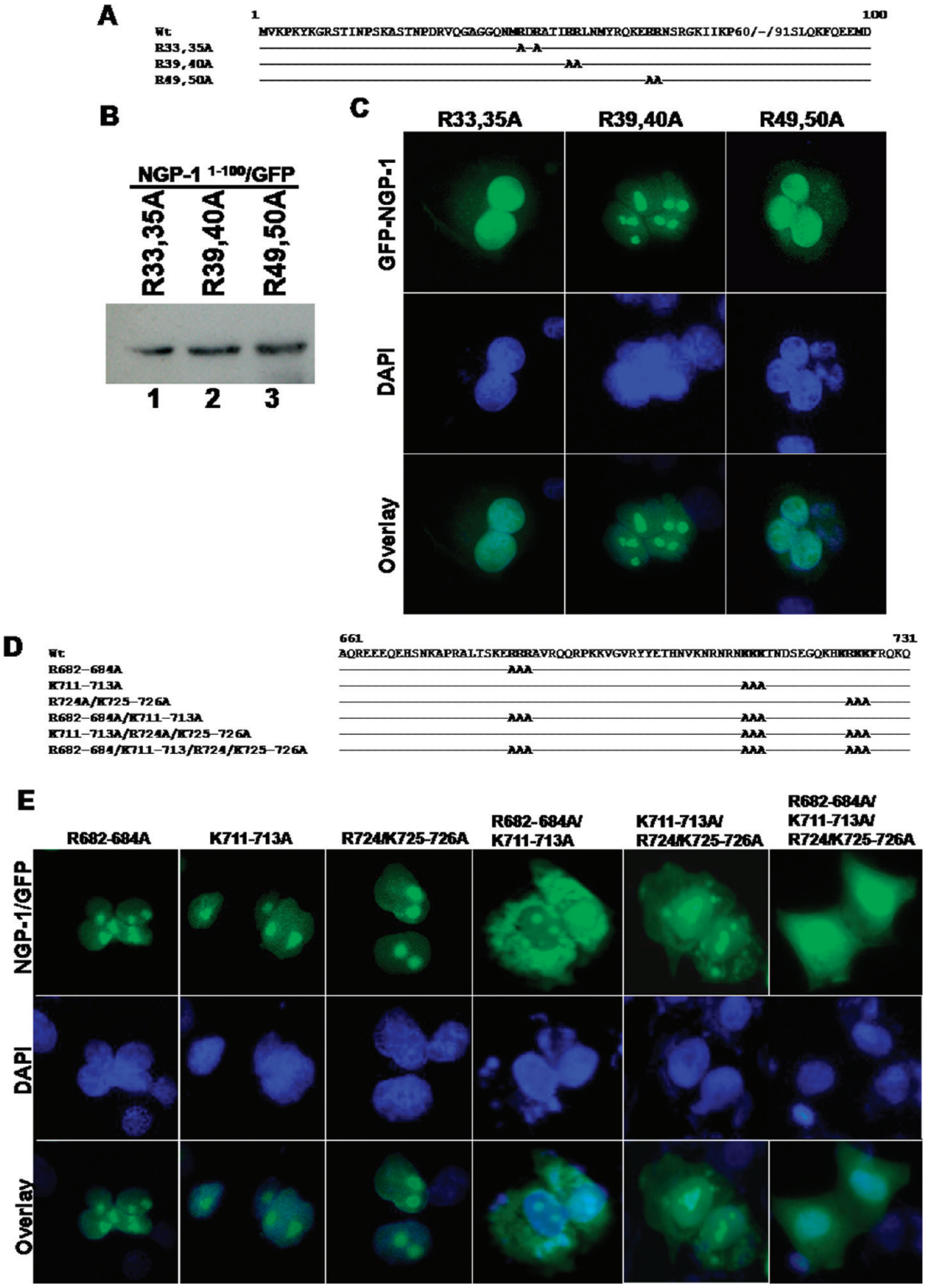

Figure 3. Identification of critical amino acids in the amino- and carboxyl-terminal NoLSs for NGP-1 nucleolar translocation. Schematic representation of expression vectors containing mutations within NGP-1^{1-100} (A) and NGP-$1^{661-731}$ (D). All the site-specific mutants were generated by Quick-change site-directed mutagenesis as described in Materials and Methods. All the indicated mutants were transfected into Cos-7 cells. After 16−24 h, the cells were fixed, and the subcellular distribution of NGP-1^{1-100} (C) and NGP-$1^{661-731}$ (E) was determined by fluorescence microscopy. Expression of NGP-1 mutant proteins from the total transfected cell lysates was assessed by Western blot analysis with anti-GFP monoclonal antibodies (1:1000 dilutions). Respective fusion constructs are indicated at the top (B). Results from the subcellular localization analysis suggest that clusters of positively charged amino acids with both amino- and carboxyl-terminal domains are critical for efficient localization of NGP-1 into the nucleolar compartment.

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when R33, R35, R49, and R50 were replaced with alanines (Figure 3C), suggesting that amino acid residues R33, R35, R49, and R50 are critical for efficient targeting of NGP-1^{1-100} to the nucleolar compartment. The exchange of R682, R683, and R684 together with K711, K712, and K713 with alanine residues resulted in cytoplasmic localization with some nucleolar staining of NGP-$1^{661-731}$ (Figure 3E). However, the replacement of K711, K712, and K713 combined with R723, K724, and K725 showed intense nucleolar localization with some cytoplasmic signals (Figure 3E). On the other hand, exchange of all basic residues within NGP-$1^{661-731}$ resulted in diffused localization in both cytoplasm and nucleus similar to GFP localization pattern (Figure 3E). These results provide evidence that multiple repeats of positively charged residues like arginine and lysine are critical for the formation of a functional NLS/NoLS for NGP-1.

NGP-1 Interacts with Receptors in the Classical Nuclear Transport Pathways. To improve our understanding of the mechanism of NGP-1 nuclear/nucleolar transport, we sought transport receptors that specifically interact with NGP-1. Sequence comparison of both N- and C-terminal NoLSs of NGP-1 with other known nucleolar transport signals suggests that the N-terminal NoLS of NGP-1 is rich in arginine residues in contrast to the C-terminal NoLS, which is rich in lysine residues (Figure 4A). Recent reports suggested that proteins that are localized into the nucleolus preferentially interact with importin-β directly through their ariginine-rich NoLS without the involvement of importin-α.[35,36] The C-terminal NoLS of NGP-1 is rich in lysine residues but translocates the cargo to the nucleolus, leading to the hypothesis that this NoLS may possess different receptor binding (direct or piggyback) specificities to transport NGP-1. To identify the transport receptor for NGP-1, we first tested whether NGP-1 interacts with importin-α or importin-β by a series of GST pull-down assays. HeLa cell lysates containing equal amount of NGP-1/GFP and GFP were mixed with glutathione Sepharose beads that had been prebound to either GST or GST fused to importin-α or importin-β (Figure 4D). Four hours after incubation, the beads were washed and the bound proteins as well as a fraction of the input proteins were examined by SDS−10% PAGE followed by Western blot analysis using anti-GFP antibodies (Figure 4B). An interaction between NGP-1 and importin-β was readily detected (Figure 4B, lane 2), and we observed a weak interaction of NGP-1 with importin-α (Figure 4B, lane 1). In addition, the lack of interaction between GFP and importin-β or importin-α as well as NGP-1 and GST (data not shown) served to illustrate the specificity of the interaction of NGP-1 with transport receptors. These data suggest that NGP-1 interacts with both receptors in the classical nuclear transport pathway for efficient translocation into the nucleolar compartment.

To investigate the involvement of the interaction of importin-α and importin-β in NGP-1 nuclear/nucleolar transport, we further determined the binding specificity of NGP-1 NoLS with both importin-α and importin-β. Toward this end, we first checked whether both the amino- and carboxyl-terminal NoLSs of NGP-1 interact with importin-α and importin-β independently by the above-mentioned GST pull-down assay. HeLa cell lysates containing an equal amount of NGP-1^{1-100}, NGP-$1^{661-731}$, or GFP were mixed with glutathione Sepharose beads that were prebound to either GST or GST fused to importin-α or importin-β. Four hours after incubation, the beads were washed and Western blot analysis was performed using anti-GFP antibodies as described above. Results from Figure 4C indicate that NGP-1^{1-100} interacts very strongly with importin-β (top panel, lane 1) compared with importin-α (middle panel, lane 1). In contrast, NGP-$1^{661-731}$ interacts strongly with importin-α (middle panel, lane 2) compared with importin-β (top panel, lane 2). Further, a lack of interaction between GST and NGP-1^{1-100} (Figure 4C, lane 1) as well as NGP-$1^{661-731}$ (Figure 4C, lane 2) served to illustrate the specificity of the interaction of NoLSs with importin-β and importin-α. HIV-1 Rev-NLS (Figure 4C, lane 3) and SV40-NLS (Figure 4C, lane 4) were used as positive controls for importin-β and importin-α interactions, respectively. GFP was used as a negative control, and no interaction was observed with both transport receptors (Figure 4C, lane 5). An equal amount of GST, GST−importin-α protein, or GST−importin-β protein bound with glutathione Sepharose beads was used in each in vitro pull-down assay (Figure 4D). These data provide evidence that NGP-1 interacts with both importin-β and importin-α through amino- and carboxyl-terminal NoLSs, respectively, for its efficient translocation into the nuclear/nucleolar compartments.

To improve our understanding of whether the amino-terminal NoLS, NGP-1^{1-100}, interacts with importin-β directly without importin-α, importin-α was immuno-depleted from HeLa cell lysates containing equal amount of NGP-1^{1-100}/GFP or GFP (Figure 4E) by incubating the lysates with anti-importin-α antibody. Four hours after incubation, immune complex containing importin-α was removed by addition of Protein A agarose beads to the lysates. Equal amounts of lysates (with and without importin-α) were resolved via SDS−10% PAGE followed by Western blot analysis with anti-importin-α antibodies as described above. Results in Figure 4F confirmed that importin-α was completely depleted from lysates containing NGP-1^{1-100}−GFP protein (top panel, lane 3) and GFP (top panel, lane 4). Equal amounts of lysates (with and without importin-α) containing NGP-1^{1-100}−GFP protein or GFP were mixed with glutathione Sepharose beads that were prebound to either GST−importin-β or GST. Four hours after incubation, the beads were washed extensively and Western blot analysis was conducted using anti-GFP antibodies as described above. Results from Figure 4G indicate that NGP-1^{1-100} interacts directly with importin-β (lane 1) and no interaction was observed with GST (lane 3). These results suggest that the amino-terminal NoLS of NGP-1 directly interacts with importin-β without importin-α and efficiently targets NGP-1 as well as heterologous protein to the nucleolar compartments.

Positively Charged Residues within Both NoLSs Are Critical for the Interaction of NGP-1 with Transport Receptors. Immunofluorescence studies showed that the exchange of positively charged residues within both amino- and carboxyl-terminal NoLSs resulted in abrogation of NGP-1 nucleolar localization. Because we observed a strong affinity of NGP-1^{1-100} for importin-β and of NGP-$1^{661-731}$ for importin-α (Figure 4C), variants of NGP-1^{1-100} and NGP-$1^{661-731}$ were used to define the amino acid residues that are critical for their interaction with importin-β and importin-α, respectively. All the basic amino acids within NGP-1^{1-100} and NGP-$1^{661-731}$ were exchanged by Quick-change site-directed mutagenesis as described in Materials and Methods. HeLa cell lysates containing equal amounts of the indicated variant of NGP-1^{1-100} and NGP-$1^{661-731}$ were mixed with glutathione Sepharose beads that were prebound with GST fused with importin-β and importin-α, respectively. Four hours after incubation, beads were washed extensively and the eluted protein complexes were resolved via SDS−12% PAGE followed

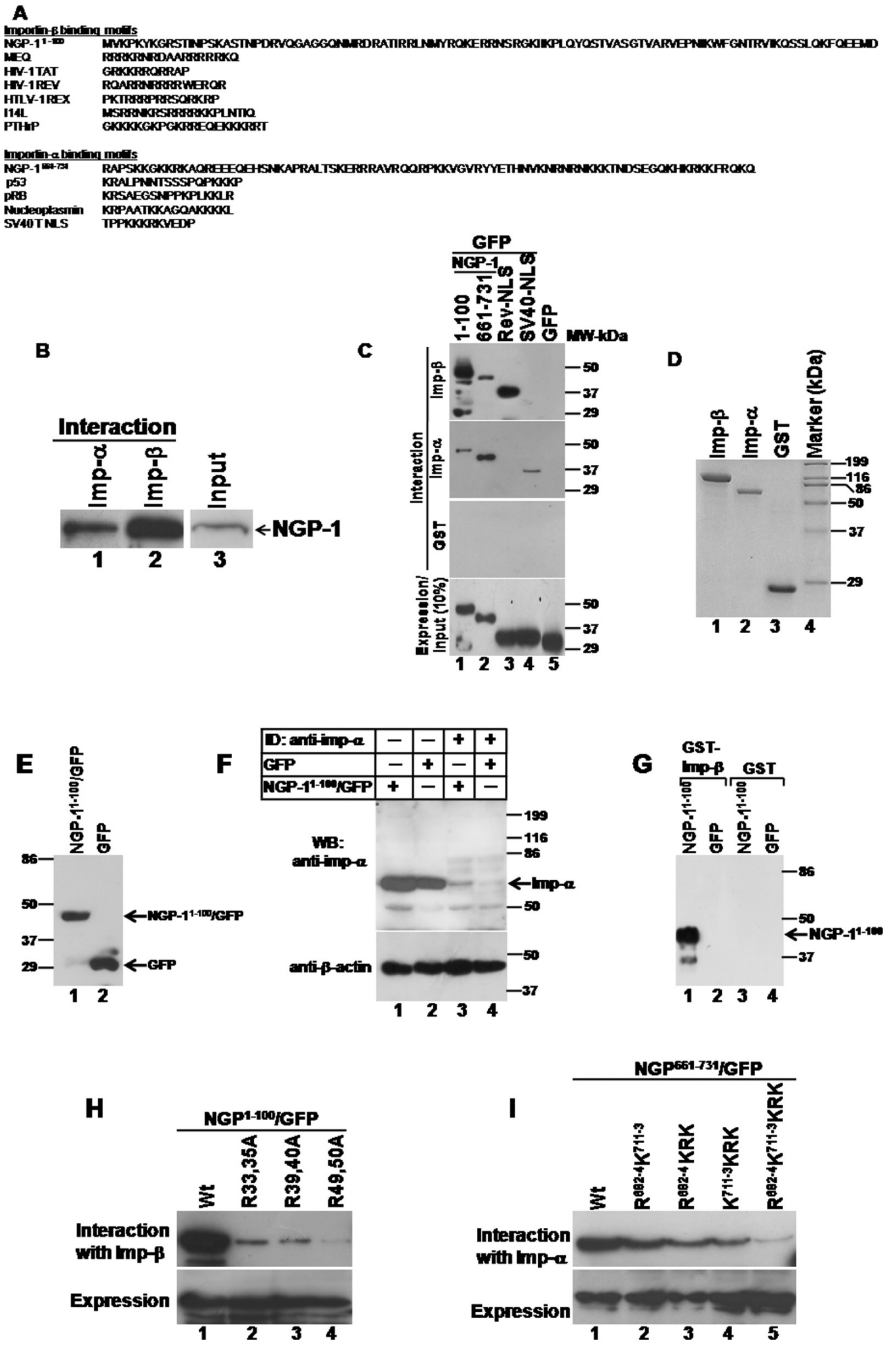

Figure 4. NGP-1 interacts with receptors in the classical nuclear transport pathway. (A) Comparison of the NGP-1 nuclear/nucleolar targeting signal with other known transport signals from proteins of cellular and viral origin. (B) Cell lysates containing NGP-1−GFP fusion protein were mixed with Sepharose beads prebound with importin-α or importin-β, and the bound proteins were aassessed by Western blot analysis using anti-GFP antibodies. (C) GST−importin-β or GST−importin-α protein was immobilized on glutathione Sepharose beads and incubated with cell lysates containing NGP-1^{1-100}, NGP-$1^{661-731}$, HIV-1 Rev-NLS, SV40-NLS, and GFP. Bound proteins were resolved via SDS−12% PAGE followed by Western blot analysis using anti-GFP antibodies as described in Materials and Methods. (D) Importin-α and importin-β were expressed and purified as GST fusions. Purified fusion proteins were dialyzed and rebound with glutathione Sepharose beads, and equal amounts of GST−importin-α and GST−importin-β proteins were used in all the in vitro pull-down assays. (E) Importin-α was depleted from HeLa cell lysates containing equal amounts of NGP-1^{1-100}/GFP or GFP by immunoprecipitation with anti-importin-α antibodies. (F) Depletion of importin-α was confirmed by Western blot analysis using anti-importin-α antibodies as described in Materials and Methods. (G) Glutathione Sepharose beads containing an equal amount of GST−importin-β protein or GST were incubated with cell lysates (with or without importin-α) containing NGP-1^{1-100}−GFP protein or GFP. Bound proteins were resolved via SDS−10% PAGE followed by Western blot analysis using anti-GFP antibodies as described in Materials and Methods. Results suggest that NGP-1^{1-100} may be able to interact with importin-β directly without importin-α. HeLa cell lysates containing equal amounts of indicated variants of NGP-1^{1-100} (H) and NGP-$1^{661-731}$ (I) were mixed with glutathione Sepharose beads containing GST−importin-β or GST−importin-α protein, and the bound proteins were resolved via SDS−12% PAGE followed by Western blot analysis using anti-GFP antibodies.

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by Western blot analysis using anti-GFP antibodies. Results suggest that the replacement of arginines within NGP-1^{1-100} altered its interaction with importin-β (Figure 4H, lanes 2−4). The exchange of R49 and R50 completely impaired the binding of NGP-1^{1-100} to importin-β (Figure 4H, lane 4), and this is in accordance with the subcellular localization patterns observed for mutant proteins (Figure 3C). These data suggest that the arginine residues at positions 49 and 50 play a vital role in binding of NGP-1 to importin-β for its efficient nucleolar targeting. Similarly, variants of NGP-$1^{661-731}$ such as R682-4A, R711-3A, and K724-5A showed weak interaction with importin-α in the in vitro GST pull-down assay (Figure 4I, lanes 2−4) compared with that of wild-type NGP-$1^{661-731}$ (Figure 4I, lane 1), though they retained their nucleolar localization (Figure 3E). In contrast, the exchange of all positively charged residues (R682-4A, R711-3A, and K724-5A) completely impaired the interaction of NGP-$1^{661-731}$ with importin-α (Figure 4I, lane 5), and this is consistent with the diffused cytoplasmic localization of the mutant protein (Figure 3E). These results indicate that the clusters of positively charged residues within NoLSs are important for the interaction of NGP-1 with both importin-α and importin-β for efficient translocation as well as retention in the nucleolar compartment.

Three-Dimensional Structural Model of NGP-1. The Ylqf GTPase from *Bacillus subtilis* was used to model the three-dimensional structure of NGP-1 using the 3D-Jigsaw server as described previously.[40] The final model (Figure 5A) was derived using amino acid residues 202−464 of NGP-1 that are 25% identical to those of Ylqf GTPase. The validity of the model could be readily checked with the conservation of residues in the hydrophobic core of the molecule and the exposure of hydrophilic residues to the solvent. The G-domain has the characteristic fold of P-loop-containing nucleoside triphosphate hydrolases that belong to the G-protein family.[26] The active site, as identified using the crystal structure of Ylqf GTPase in complex with GNP, a nonhydrolyzable GTP analogue, and a magnesium ion (Protein Data Bank entry 1PUJ), is located in the G-domain with several highly conserved residues surrounding GNP and a single magnesium ion. This further confirms the feature of the model, because the active site residues are coming from different parts of the primary structure of the protein. For example, Asp 258 interacts with N1 and N2 of the guanine moiety of GNP, Lys 323 is involved in binding to both β- and γ-phosphate groups of GNP, Ser 324 binds magnesium, and Ser 325 forms a hydrogen bond with the α-phosphate of GNP. The conserved residues within all G-domains were mutated to understand the role of these motifs in NGP-1 functions.

Conserved G-Domains Play an Important Role in the Nucleolar Retention of NGP-1. To understand the contribution of GTP-binding motifs to NGP-1 nuclear/nucleolar retention, we created mutant forms of NGP-1 [G5m (R227A/D228A/P229A), G4m (D258A), G1m (K323A), G1m (S324A), G1m (S325A), G2m (G343A/T345A), and G3m (P363A/G364A)] in which the conserved residues within G-domains were replaced with alanines (Figure 5B). Amino acids selected for mutagenesis of NGP-1 are conserved in different putative nuclear GTPases from diverse eukaryotic organisms as well as in the Ras family proteins. Western blot analysis of the transfected cell lysates indicates that exchange of conserved residues within G-domains did not alter the expression of NGP-1 mutant polypeptides (Figure 5D, bottom panel). Immunofluorescence analysis showed that mutant proteins G1m, G2m, and G3m continued to be localized in the nucleolus, in addition to intense cytoplasmic staining (Figure 5E). Exchange of conserved residues within domain G4 (G4m, D258A) resulted in a punctate localization pattern for the mutant protein throughout the nucleus, including the nucleolus (Figure 5E). NGP-1 variants containing mutations at conserved residues in G5 (G5m, RDP-A) showed efficient nuclear localization but were excluded from the nucleoli (Figure 5E). These results suggest that the GTP-binding motifs, G5 and G4, play a critical role in the efficient nucleolar retention of NGP-1. The replacement of conserved proline residues between residues 463 and 475 did not alter the wild-type nucleolar localization of mutant NGP-1 protein (Figure 5E), suggesting that the conserved proline motif is not required for nucleolar retention. The mutations within G5 completely blocked the nucleolar localization of NGP-1 even in the presence of both amino- and carboxyl-terminal functional NoLSs in NGP-1. These results demonstrate that both amino- and carboxyl-terminal NoLSs as well as the wild-type GTP-binding domains are required for the efficient nucleolar retention of NGP-1. This further suggests that NGP-1 uses its GTP binding property as a molecular switch to control the transition between the nucleolus- and nucleoplasm-localized states, involving the interaction between the NoLS and GTP-binding domains.

To improve our understanding of how GTP binding status influences the nucleolar targeting of NGP-1, we characterized the GTP binding ability of various G-domain NGP-1 mutants. Cos-7 cell lysates containing various G-domain mutants of NGP-1 were mixed with GTP-conjugated agarose, and the bound proteins were analyzed by Western blot analysis using anti-GFP antibodies. Results demonstrate that a specific retention of NGP-1 by the GTP-conjugated agarose (Figure 5C, top panel, lane 2, and Figure 5D, top panel, lane 1). The GTP binding ability of NGP-1 depends on motifs G4 and G5, as amino acid substitutions within these G-domains (Figure 5B) severely altered the GTP binding ability of NGP-1 mutant proteins (Figure 5D, top panel, lanes 5 and 6), though an equal amount of expression was observed for all G-domain mutant proteins (Figure 5D, bottom panel, lanes 1−6). In fact, mutation in G4 and G5 completely blocked the GTP binding ability of NGP-1 (Figure 5D, top panel, lanes 5 and 6) and failed to keep NGP-1 in the nucleolus (Figure 5E). This result and the molecular modeling of the GNL3L structure (Figure 5A) suggest that motifs G4 and G5 form a platform for binding of GTP to NGP-1. The known nucleolar GTP-binding protein nucleostemin was used as a positive control (Figure 5C, lane 1), and GFP was used as a negative control (Figure 5C, lane 3) to ensure the integrity of the assay. These findings demonstrate that motifs G4 and G5 play a critical role in the GTP binding activity of NGP-1 and further provide evidence that GTP binding directly influences the retention of NGP-1 in the nucleolar compartment.

NGP-1 Expression Is Upregulated in Actively Proliferating Cells. Recent reports suggest that expression of nucleolar GTPases is upregulated in cancer cells and stem cells. We have recently demonstrated that GNL3L, a putative nucleolar GTPase that is closely related to NGP-1, plays an important role in rRNA processing in the nucleolus.[18] The inhibition of nucleolar GTPase expression resulted in a reduced level of cell growth and induced apoptosis, suggesting an important role of GNL3L in cell proliferation.[18] To understand whether NGP-1 expression is upregulated in actively proliferating cells, we first analyzed the expression pattern of NGP-1 in mitogen-activated human primary peripheral blood mononuclear cells (hPBMCs) as well as in human neural precursor cells (hNPCs). Human PBMCs were

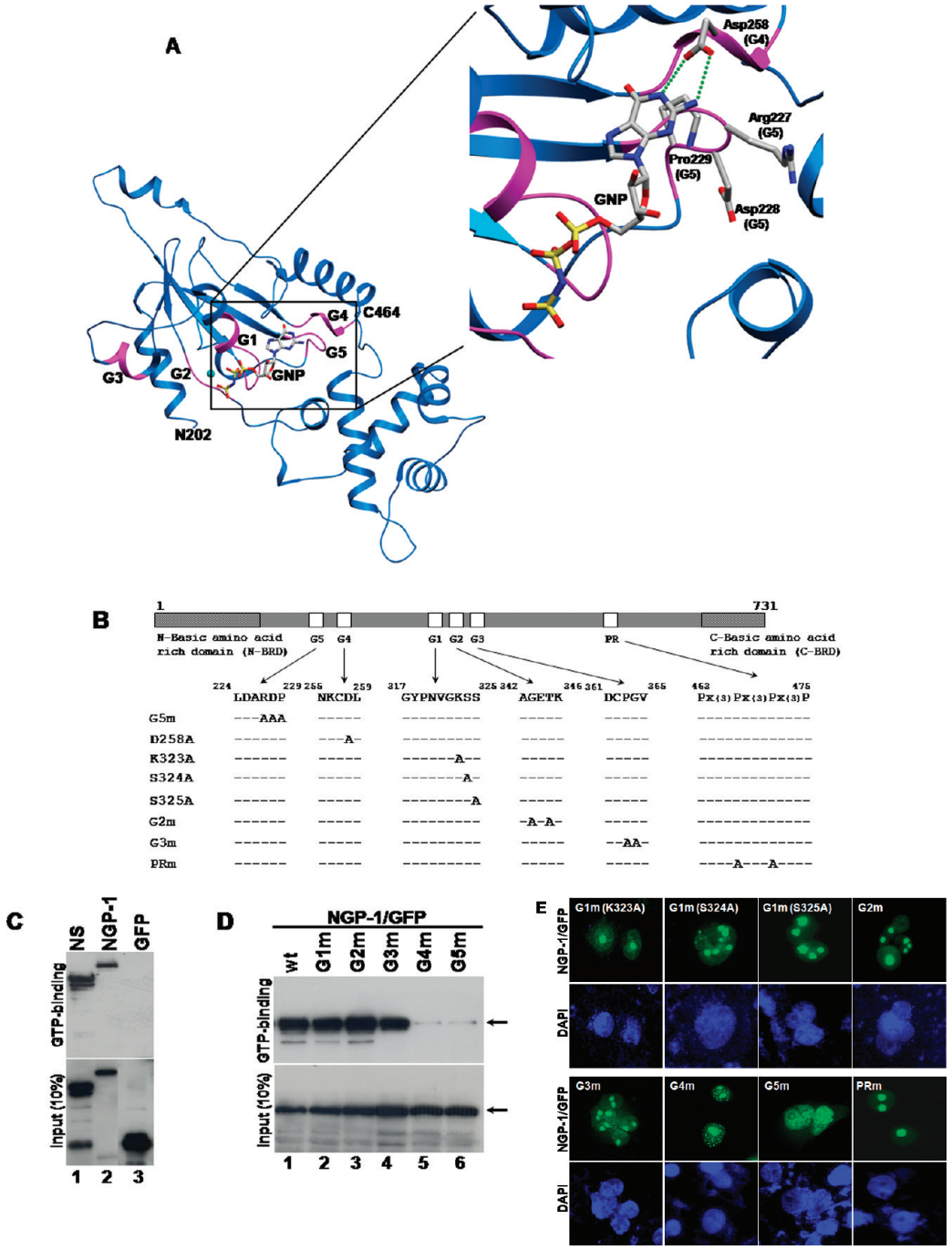

Figure 5. G-Domains modulate the nucleolar localization of NGP-1. (A) Ribbon diagram of the modeled structure of NGP-1 residues 202−464. G-Domains are colored differently; motifs G1 and G3−G5 are colored pink, and motif G2 is colored blue color along with GNP and a magnesium ion (green sphere). This panel was prepared using SETOR. (B) Conserved amino acids within different G-motifs of NGP-1 are exchanged by Quick-change mutagenesis and sequenced to verify their integrity. (C) NGP-1 binds to guanine nucleotide triphosphate (GTP). Cell lysates containing wild-type NGP-1 were incubated with GTP agarose resins. Nucleostemin (NS) and GFP were used as positive and negative controls, respectively. Bound proteins were resolved via SDS−10% PAGE followed by Western Blot analysis using anti-GFP monoclonal antibodies as described in Materials and Methods. (D) Expression vectors containing wild-type NGP-1 and its various indicated G-domain mutants were transfected in Cos-7 cells, and the lysates containing equal amounts of proteins were incubated with GTP agarose resins. Bound proteins were resolved via SDS−10% PAGE followed by Western blot analysis as described above. The results suggest that conserved residues within motifs G4 and G5 are critical for the GTP binding activity of NGP-1. (E) Expression vectors containing wild-type NGP-1 and its various indicated G-domain mutants were transfected into Cos-7 cells. After 16 h, the cells were fixed and the subcellular distribution of NGP-1−GFP fusion proteins was determined with a fluorescence microscope. Respective fusion constructs are indicated at the top.

isolated as described in Materials and Methods and induced with varying concentrations of Concanavalin-A (Con-A) for 7−12 h. Con-A is a lectin known to act as a lymphocyte mitogen and induces proliferation. After 12 h, the total RNA was isolated as

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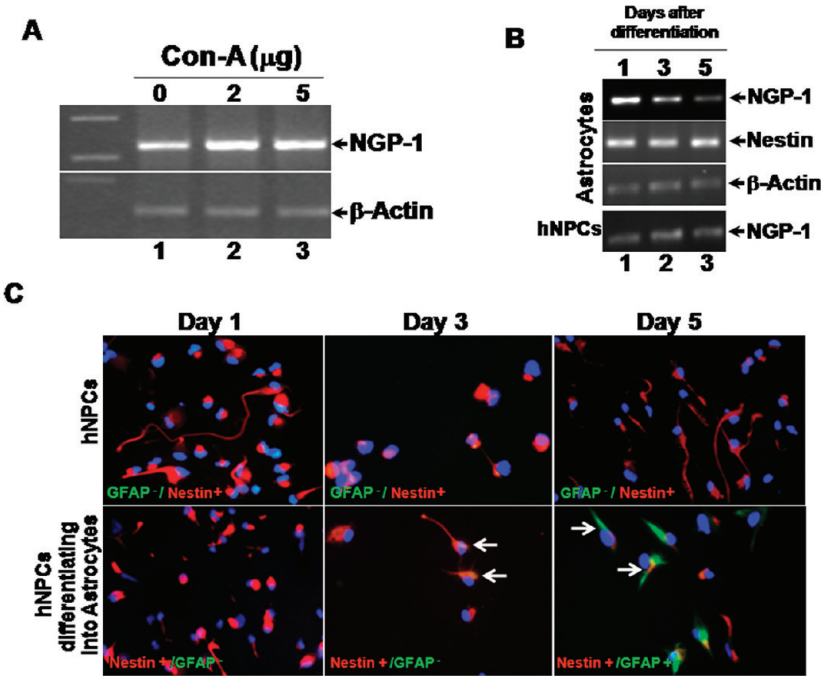

Figure 6. Expression of NGP-1 in actively proliferating cells. (A) Human primary PBMCs and neural precursor cells (B) were isolated as described in Materials and Methods. PBMCs were treated with different concentrations of mitogen Con-A. The human neural precursor cells (hNPC) were induced to differentiate into astrocytes as described in Materials and Methods. Total RNA was isolated from Con-A-treated PBMCs and from different stages of neural precursor cell differentiation. RNA was subjected to reverse transcription before being analyzed by semiquantitative PCR using specific primers for the indicated genes. β-Actin was used as an internal control and nestin as a neural precursor cell marker. (C) Expression of neural precursor cell marker, nestin (red) and the astrocytic marker GFAP (green) in hNPCs undergoing differentiation into astrocytic lineage on days 1, 3, and 5. As the hNPCs differentiate into astrocytes, increased levels of GFAP expression were observed. There was no GFAP expression noted in undifferentiated hNPCs (top panel).

described in Materials and Methods, and reverse transcription (RT) was performed followed by PCR analysis with NGP-1-specific primers to determine the levels of NGP-1 expression. Results in Figure 6A indicate that NGP-1 expression was induced in Con-A-treated PBMCs in a dose-dependent manner (Figure 6A, top panel, lanes 2 and 3) compared with an untreated culture (Figure 6A, top panel, lane 1). β-Actin was used as an internal control to show that an equal amount of cDNA was used for the experiments (Figure 6A, bottom panel, lanes 1−3). These data suggest that NGP-1 expression was activated upon cell proliferation. To improve our understanding of the relevance of expression of NGP-1 to cell proliferation, we isolated the human neural precursor cells (hNPCs) and differentiated them as described in Materials and Methods. The total RNA was isolated from the undifferentiated and differentiating hNPCs at various time intervals and subjected to RT-PCR to determine the levels of expression of NGP-1. Results in Figure 6B demonstrate that the level of expression of NGP-1 was gradually reduced when cells were progressing toward astrocytic differentiation (Figure 6B, top panel, lanes 2 and 3) compared with that of the undifferentiated hNPCs (Figure 6B, bottom panel, lanes 2 and 3). Nestin was used as a neural precursor cell marker and β-actin as an internal control to show that an equal amount of cDNA was used for PCR amplification (Figure 6A, lanes 1−3). As hNPCs differentiate into astrocytes, the level of expression of astrocyte cell-specific marker GFAP was elevated, especially at day 5 (Figure 6C, bottom panel). GFAP expression is specific to differentiating cells as the same is not present in undifferentiated hNPCs. The level of nestin remains constant in undifferentiated hNPCs (Figure 6C, top panel) at all the time points and is also seen in differentiating hNPCs. Colocalization of nestin with GFAP was observed on days 3 and 5 as well; this is usually not seen until astrocytic differentiation is complete (Figure 6C, bottom panel). These results suggest that the activation of NGP-1 expression is not simply a reflection of the proliferative state but is characteristic of an early multipotential state of the cells and further indicate that high-level NGP-1 expression may be critical for maintaining the active proliferation status of the cells.

DISCUSSION

Human guanine nucleotide-binding protein NGP-1(GNL-2) is a nucleolar G-domain-containing protein that is highly conserved in the genomes of diverse eukaryotes. In this investigation, using a combination of site-specific mutagenesis, protein−protein interaction, and immunofluorescence analyses, we have demonstrated that NGP-1 harbors two nucleolar localization signals (NoLSs) that are sufficient to independently translocate a heterologous cytoplasmic protein to the nucleolus. Our data show that the N-terminal NoLS preferentially associates with the nuclear transport receptor, importin-β, while the C-terminal NoLS interacts specifically with importin-α to achieve nucleolar targeting of NGP-1. In addition, our results indicate that guanine nucleotide triphosphate (GTP) binding with NGP-1 acts as a molecular switch to control its transition between the nucleolus and the nucleoplasm, suggesting cross-talk between NoLSs and GTP-binding domains. The high-level expression of NGP-1 in mitogen-induced primary human PBMCs and in neuronal

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precursor cells suggests that it may play a critical role in maintaining the active proliferation status of the cells.

The interaction of NGP-1^{1-100} (N-terminus of NoLS) with importin-β supports the notion that NoLSs bound to importin-β are directly transported to the nucleus/nucleolus by a mechanism that is independent of importin-α. Our results regarding the NGP-1^{1-100} import pathway are similar to those for snRNP nuclear import reported previously by others.[30−37] In addition, NLSs that are rich in arginine residues, including Rev and Tat of HIV-1 and human rpl23a, bind directly to importin-β and are transported to the nucleolus.[32,33,43] On the other hand, the newly identified C-terminus of NoLS, NGP-$1^{661-731}$, that is rich in lysine residues transports the heterologous protein to the nucleolar compartment by an importin-α-mediated pathway. Surprisingly, data available from the literature demonstrate that all the known lysine-rich nuclear transport signals (NLSs of SV40, nucleophosmin, and p53) bind to importin-α and translocate the cargo to the nucleus but not to the nucleolus.[30,31] Though NGP-$1^{661-731}$ interacts with importin-α, the sequences are quite distinct from known importin-α interacting NLS sequences (Figure 4). These data suggest the possibility that amino acid residues other than the basic lysine and arginine residues within NGP-$1^{661-731}$ may be responsible for the specificity of interaction with transport receptors despite the fact that the basic residues may be critical for the interaction of NGP-1 with transport receptors. Furthermore, the observed nucleolar localization of NGP-$1^{661-731}$ suggests that lysine-rich transport signals may be able to target the cargo to different cellular compartments by any of the following mechanisms. (i) Interaction with importin-α may be sufficient to transport the cargo to the nucleolus in a manner independent of importin-β, similar to the nuclear transport of Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV),[44] and (ii) importin-α may be interacting with a novel receptor other than importin-β and translocates the cargo to the nucleolar compartment. It is likely that, upon entry into the nucleolus, NGP-1 may interact with other nucleolar proteins or RNA to be retained in the nucleolus. Such a view is supported by the altered subcellular localization of NGP-1 in cells treated with actinomycin D (Act-D) (see Figure 1E). This observation has led us to conclude that ongoing transcription may be required for efficient retention of NGP-1 in the nucleolus. Confocal microscopy demonstrated that each NoLSs individually translocates the heterologous protein only to the granular and dense fibrillar center of the nucleolus. In contrast, full-length NGP-1 (see Figure 2F) localized to the nucleolus, including the fibrillar center (site of rRNA transcription), revealing that both NoLSs are critical for efficient nucleolar targeting of NGP-1. The predicted amino acid sequence showed the presence of clusters of arginine residues within NGP-1^{1-100}. It is well-established that arginine-rich domains are known to interact with RNA in addition to its role in nucleolar transport.[32,33] This has prompted us to hypothesize that NGP-1 may play a role in rRNA transcription and/or processing. Further studies are warranted to determine whether NGP-1 interacts with RNA or other nucleolar components and to define its role in nucleolar functions.

Data from this investigation suggest that both the amino- and carboxyl-terminal NoLSs as well as the GTP-binding motifs regulate the nucleolar accumulation of NGP-1. Interestingly, NGP-1 mutants that failed to bind to GTP localize mainly in the nucleoplasm. Both NGP-1^{1-100} and NGP-$1^{661-731}$ are sufficient to independently target the GFP to the nucleolus, despite the additional requirement of the GTP-binding motifs for efficient nucleolar localization of full-length NGP-1. Recent reports suggest that the GTP-bound state regulates the nucleolar retention of putative GTPases, nucleostemin,[20] and GNL3L.[40] These findings suggest that an inhibitory mechanism gates the nucleolus targeting activity of full-length NGP-1. Small GTP-binding proteins use the conserved GTP binding structure as a molecular switch to regulate fundamental cellular functions. Drawing an analogy using other small GTPases, one can infer that the dynamic nucleolar retention may be regulated by the GTP-binding state of NGP-1. Although it is at present unknown whether the GTP-bound or non-GTP-bound NGP-1 represents the active form, our data demonstrate that disruption of this regulatory mechanism results in mislocalization of NGP-1. Further, nucleolar accumulation of NGP-1 appears to be dependent on its GTP-bound conformation.

The nucleolus functions as a factory for the assembly of ribosomal subunits. Discovery of a surprising variety of macromolecules in the nucleolus with no apparent ribosomal function indicates that it may play a role in nuclear export, sequestering of regulatory molecules, modification of small RNAs, assembly of ribonucleoproteins, and the control of aging. In these novel events, the nucleolus may serve as a privileged site for both recruitment and exclusion of regulatory complexes. Does the nucleolus serve as a "sequestration center" for the newly identified nucleolar putative GTPase, NGP-1? To the best of our knowledge, NGP-1 is one of the large G-domain-containing proteins reported to be localized in nucleoli. One of the current hypotheses is that certain nucleolar proteins upregulate the cell proliferation during tumorigenesis by modulating ribosome biogenesis. A growing body of evidence suggests that alteration of ribosome biogenesis may lead to uncontrolled cell proliferation and tumorigenesis. Nucleolar proteins have also been shown to regulate cell proliferation and growth by controlling ribosomal and nonribosomal functions of the nucleolus.[18,19] The upregulation of NGP-1 expression in the mitogen-activated human primary peripheral blood mononuclear cells as well as in the human fetal brain-derived neural precursor cells, in contrast to its downregulation in differentiating astrocytes, suggests that it may be critical for cell proliferation as well as the maintenance of the cells in a primitive state without senescence. This study provides evidence that NGP-1 is translocated to the nucleolus and regulates cell proliferation along with other molecules within the nucleolus. Further studies are required to understand whether NGP-1 can participate in the regulation of nucleolar sequestration of proteins with nonribosomal functions to control cell proliferation.

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■ ABBREVIATIONS

NGP-1, nucleolar GTP-binding protein 1; Act-D, actinomycin D; CHX, cycloheximide; Con-A, Concanavalin-A; DAPI, 4′, 6-diamidino-2-phenylindole; DFC, dense fibrillar component; FC, fibrillar center; G-protein, GTP-binding protein; GC, granular component; GFAP, glial fibrillary acidic protein; GNL-2, guanine nucleotide-binding protein-like 2; GNL3L, guanine nucleotide-binding protein-like 3-like; GTP, guanosine 5′-triphosphate; GTPase, guanosine 5′-triphosphatase; HIV-1, human immunodeficiency virus 1; hNPC, human neuronal progenitor cells; hPBMC, human peripheral blood mononuclear cells; NLS, nuclear localization signal; NoLS, nucleolar localization signal; NPC, nuclear pore complex; RPL23a, ribosomal protein L23a; vTF7-3, *Vaccinia* virus expressing T7 RNA polymerase.

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