# μ-Opioid Receptor Cell Surface Expression Is Regulated by Its Direct Interaction with Ribophorin I[S]

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## ABSTRACT

The trafficking of the μ-opioid receptor (MOR), a member of the rhodopsin G protein-coupled receptor (GPCR) family, can be regulated by interaction with multiple cellular proteins. To determine the proteins involved in receptor trafficking, using the targeted proteomic approach and mass spectrometry analysis, we have identified that Ribophorin I (RPNI), a component of the oligosaccharide transferase complex, could directly interact with MOR. RPNI can be shown to participate in MOR export by the intracellular retention of the receptor after small interfering RNA knockdown of endogenous RPNI. Overexpression of RPNI rescued the surface expression of the MOR 344KFCTR348 deletion mutant independent of calnexin. Furthermore, RPNI regulation of MOR trafficking is dependent on the glycosylation state of the receptor, as reflected by the inability of overexpression of RPNI to affect the trafficking of the N-glycosylation-deficient mutants, or GPCRs that have minimal glycosylation sites. Hence, this novel RPNI chaperone activity is a consequence of N-glycosylation-dependent direct interaction with MOR.

Being a member of the GPCR superfamily and rhodopsin subfamily, opioid receptors (μ, δ, κ) have the putative structures of seven-transmembrane domains and an extracellular N terminus with multiple glycosylation sites (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993). When synthesis occurs, the completed core oligosaccharide is transferred from the dolichylpyrophosphate carrier to a growing, newly synthesized polypeptide chain, which is coupled through an N-glycosidic bond to the side chain of an asparagine residue. The oligosaccharyltransferase responsible for this transfer is a complex enzyme with its active site in the lumen of endoplasmic reticulum (ER) (Silberstein and Gilmore, 1996). During the translocation into the ER lumen, polypeptides on membrane-bound polysomes may be cotranslationally modified by N-glycosylation (Kreibich et al., 1983). In this process, the oligosaccharide transferase (OST) catalyzes the transfer of high mannose oligosaccharides, which are preassembled on lipid-anchored dolicholpyrophosphate moieties to an asparagine residue within an Asn-X-Ser/Thr consensus motif of nascent polypeptide chains facing the lumen of the ER (Abeijon and Hirschberg, 1992). Immediately after coupling to the polypeptide chain, terminal glucose and mannose residues are removed by ER glucosidases and mannosidases (Kornfeld and Kornfeld, 1985). When the glycoprotein moves to the Golgi complex, the glycan chains undergo further trimming of mannoses. N-glycosylation has been found to be an important factor in the regulation of protein folding, stability, sorting and secretion (Helenius and Aebi, 2001). For opioid receptors, this N-glycosylation is a rate-limiting step in their translocation to the cell membrane (Petaja-Repo et al., 2000). However, the detailed mechanism of the opioid receptors biosynthesis is still elusive. A truncated form (38–117) of GEC1 was found to specifically interact with the C-tail of the human κ-opioid receptor (KOR) and to be important for trafficking human KOR in the biosynthesis pathway (Chen et al.,

**ABBREVIATIONS:** GPCR, G protein-coupled receptor; ER, endoplasmic reticulum; OST, oligosaccharide transferase; KOR, κ-opioid receptor; N2A, neuro2A neuroblastoma cell; RPNI, ribophorin I; MOR, μ-opioid receptor; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; siRNA, small interfering RNA; GFP, green fluorescent protein; AR, adrenergic receptor; HA, hemagglutinin; FACS, fluorescence activated cell sorting; LC MS/MS, liquid chromatography-tandem mass spectrometry; EndoH, endoglycosidase H; PNGase F, peptide-N⁻-(N-acetyl-β-glucosaminyl)asparagine amidase; IP, immunoprecipitation; co-IP, coimmunoprecipitation; AR, adrenergic receptor; MOR5ND, MOR with Asn9, Asn31, Asn38, Asn46, and Asn53 residues at the N terminus all mutated to Asp; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; DOR, δ-opioid receptor; BiP, ER luminal binding protein; C2, MOR with the 344KFCTR348 sequence deleted.

2006). Whether proteins similar to GEC1 participate in MOR maturation process remains unknown.

To identify the binding partners of MOR, we purified the MOR complexes from neuroblastoma neuro2A (N2A) cells stably expressing (His)6-tagged MOR using $Ni^{2+}$-resin affinity column chromatography. We found that ribophorin I (RPNI), a member of the oligosaccharide transferase family that was assumed to be responsible for *N*-glycosylation of newly synthesized proteins, could interact with MOR specifically. Furthermore, our results showed that RPNI was a critical mediator of the MOR transport from the ER to the cell membrane. Our data demonstrate that, in addition to *N*-glycosylation, RPNI acts as a novel key regulator in the transport of nascent receptors, thus affecting MOR function.

## Materials and Methods

**Expression of (His)6-MOR in the N2A Cells.** The rat MOR tagged with the (His)6-epitope at the N terminus was subcloned in pCDNAmp vector. N2A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 10% fetal calf serum (DMEM growth medium) in a 10% $CO_2$ incubator. These N2A cells were then transfected with 10 $\mu$g of the (His)6-MOR plasmids. The colonies surviving the antibiotic G418 (Geneticin) selection (1 mg/ml) were isolated. The cell clone that expressed MOR at the level of 0.8 pmol/mg protein was used in receptor complex purification.

**Cell Culture and Transient Transfection.** N2A cells were maintained in DMEM containing 10% fetal bovine serum and penicillin/streptomycin. Cells were plated on 100-mm dishes (for immunoprecipitation studies) or six-well plates (for flow cytometry studies) at a density of 250,000 cells/ml, and grown to 80% confluence. Transfections were performed using the Superfect transfection reagent (QIAGEN, Valencia CA). Transfection medium was replaced with medium containing fresh serum 12 to 18 h after transfection, and cells were harvested 24 to 48 h later.

**Purification of Receptor Complex and Mass Spectrometry.** Sixty 150-mm dishes of stably expressing (His)6-MOR N2A cells and 60 150-mm dishes of control N2A cells were lysed with 1% Triton X-100 at 4°C for 2 h. Lysate was collected and centrifuged at 10,000*g* for 15 min at 4°C. Supernatant was collected and purified by $Ni^{2+}$ resin columns (Invitrogen, Carlsbad, CA). Then the columns were washed 10 times with wash buffer and eluted with elution buffer provided by the kit (Invitrogen). Eluates were concentrated with Amicon concentration cells (Millipore, Billerica, MA). Protein was separated by SDS-PAGE and silver-stained, and the presence of MOR was identified by Western analysis.

**Proteolytic Digestion.** Silver-stained gel bands were excised, dried, and destained by incubating in 15 mM $K_3Fe(CN)_6$ and 50 mM $Na_2S_2O_3$ at 24°C for 15 min, and then washed with 100 mM $NH_4HCO_3$. Destained gels were dried and rehydrated in 50 mM $NH_4HCO_3$ and 5 mM $CaCl_2$ solution with 0.01 mg/ml sequence-grade modified porcine trypsin (Promega, Madison, WI) and incubated at 37°C overnight. Trypsinized fragments were collected by sonicating the gel pieces in 50 $\mu$l of 25 mM $NH_4HCO_3$ and again after adding 50 $\mu$l of 50% acetonitrile. The supernatant was collected and sonicated repeatedly in 50 $\mu$l of 5% formic acid and again after adding 50 $\mu$l of 50% acetonitrile. The supernatant was pooled. DTT was added to a final concentration of 1 mM, and the sample was dried and frozen at $-80$°C for matrix-assisted laser desorption ionization/time-of-flight spectroscopy or LC MS/MS.

**LC MS/MS Spectrometry.** Before LC MS/MS analysis, the sample was reconstituted with load buffer, and the entire sample was injected. LC MS/MS methods were as described in Kapphahn et al. (2003). LC MS/MS results were analyzed by ProteinPilot Software 2.0, software revision number 50861 (Applied Biosystems, Inc.) (Shilov et al., 2007). The search engine uses biological modification invoked through the search effort (i.e., semi- and nontrypsin peptides are included in the search) as the search parameters. Protein Database was NCBI's nr mouse subset database from musculus_NCBInr_CTM_20061212 and was appended to a contaminants database (107,806 proteins total).

**Immunoprecipitation and Western Blot Analysis.** Confluent cells were washed in phosphate-buffered saline (PBS) at 4°C and lysed for 30 min in solubilization buffer (1% Triton X-100, 150 mM NaCl, and 1 mM EGTA, pH 7.4) containing mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at 4°C. Lysate was centrifuged for 30 min at 10,000*g*, and the supernatant was collected and assayed for protein by the BCA method (Pierce, Rockford, IL). A total of 500 $\mu$g of protein was precleared with Protein G Sepharose beads (Sigma-Aldrich) and incubated with 1 $\mu$g of mouse anti-HA antibodies for 2 to 3 h followed by incubation with 30 $\mu$l of Protein G Sepharose for 3 h, all at 4°C. Sepharose beads were pelleted by brief centrifugation at 10,000*g*, at 4°C, and washed three times with solubilization buffer. Proteins were eluted by resuspending in two volumes of 2× SDS-sample buffer (10 mM Tris, 15 mM SDS, 20 mM dithiothreitol, 20% glycerol, and 0.02% bromphenol blue, pH 6.8) followed by incubation at 65°C for 30 min. Proteins were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corporation), and immunoblotted with anti-FLAG M2 (Sigma-Aldrich) primary antibodies and detected by anti-mouse alkaline phosphatase-linked secondary antibodies (Bio-Rad Laboratories, Hercules, CA) in Tris-buffered saline containing 5% powdered milk and 0.1% Tween 20, unless indicated otherwise.

**In Vitro Translation.** Each construct was translated in vitro using the TNT Coupled Transcription/Translation System (Promega, Madison, WI) following the company's protocol. This protocol simplifies in vitro translation by starting with cDNA. The addition of RNA polymerase to the translation mixture eliminated the separate synthesis of RNA from DNA. In brief, the TNT buffer, rabbit reticulocyte lysate, RNA polymerase, amino acid mixture, and RNase inhibitor were added to a 0.5-ml microcentrifuge tube placed on ice. One microgram of plasmid DNA was added to the tube and briefly spun to mix reaction components to the bottom of tube. The reaction was then incubated at 30°C for 90 min.

**Gel-Overlay Assay.** Protein samples were separated by SDS-PAGE gel and transferred to Immobilon-P membranes (Millipore Corporation), incubated with in vitro translation product of FLAG-RPNI for 1 h at room temperature. The membrane was washed for five min three times at room temperature with Tris-buffered saline/Tween 20 and immunoblotted with anti-FLAG primary antibodies (Sigma Aldrich). The association of the in vitro-translated product was detected with anti-mouse alkaline phosphatase-linked secondary antibodies (Bio-Rad Laboratories) diluted in Tris-buffered saline containing 5% powdered milk and 0.1% Tween 20.

**Construct siRNA of RPNI.** The GenScript's siRNA design center siRNA Target Finder and siRNA Construct Builder (http://www.genscript.com/rnai.html) was used to design siRNA sequence for RPNI with following nucleotide sequences: Sense1: GATCCCGTT-GTTCTCGTAATGTACTTTGTTGATATCCGCAAAGTACATTAC-GAGAACAATTTTTTTCCAAA; Antisense1, AGCTTTTGGAAAAAAT-TGTTCTCGTAATGTACTTTGCGGATATCAACAAAGTACATTAC-GAGAACAACGG; Sense2, GATCCCATCTTCAGTGCATACTGGT-CATTGATATCCGTGACCAGTATGCACTGAAGATTTTTTTCCAAA; Antisense2, AGCTTTTGGAAAAAAATCTTCAGTGCATACTGGTC-ACGGATATCAATGACCAGTATGCACTGAAGATGG; Sense3, GATCCCGTATTGACAGTCTCATCAAAGTTTGATATCCGACTTT-GATGAGACTGTCAATATTTTTTCCAAA; Antisense3, AGCTTTTG-GAAAAAATATTGACAGTCTCATCAAAGTCGGATATCAAACTTT-GATGAGACTGTCAATACGG.

**Confocal Microscopy.** Cells transfected with GFP-tagged vector, GFP-tagged RPNI, and GFP-tagged RPNI siRNA were grown in six-well culture plates on glass coverslips to 60 to 75% confluence and fixed in 3.7% paraformaldehyde for 30 min. Adherent cells were treated with lysis buffer (0.1% Triton X-100, 150 mM NaCl, and 1

mM EGTA, pH 7.4) for 20 min. Then cells were incubated with mouse anti-HA antibody (1:1000) for 1 h, followed by washing with PBS. Cells then were incubated with goat anti-mouse Alexa Fluor 594 (1:1000). All antibody incubations were performed in PBS with 10% bovine serum albumin. Cells were dried briefly and mounted onto glass slides using Vectashield (Vector Labs, Burlingame, CA). HA-tagged MOR was visualized by immunofluorescence, using a CARVII Confocal Imager (BD Biosciences, San Jose, CA) with a Leica DMIRE2 fluorescence microscope (Leica, Wetzlar, Germany).

**Fluorescence Flow Cytometry.** Cells were grown in 12-well culture plates to 80 to 85% confluence. Before the addition of antibodies, cells were rinsed twice with serum-free DMEM. Then the cells were incubated at 4°C for 60 min in serum-free DMEM with anti-HA antibody (1:500). Afterward, the cells were washed twice with serum-free DMEM and incubated with Alexa Fluor 633-labeled goat anti-mouse IgG secondary antibody (1:400) at 4°C for 1 additional hour. Then the cells were washed and fixed with 3.7% formaldehyde before quantifying the receptor immunoreactivity with fluorescence-activated cell sorting (FACScan; BD Biosciences). Fluorescence intensity of 10,000 cells was collected for each sample. CellQuest software (BD Biosciences) was used to calculate the mean fluorescence intensity of the cell population. In our study, two-color flow cytometry was used. A stained nontransfected sample (Mock) was processed with the cytometer to adjust the voltages on forward- and side-scatter detectors for viewing the populations of interest. The R1 region was adjusted around the population. The fluorescent detector voltages were adjusted to place the unstained events in the lower left quadrant. After that, each sample was installed and data were required. Only the cells that transfected with GFP were used to obtain the data on MOR fluorescence. All FACS analyses were conducted three times with triplicate samples in each experiment.

## Results

**Identification of RPNI Direct Binding to MOR.** To identify components of MOR signaling complexes, (His)6-MOR stably expressed in N2A cells was partially purified with a $Ni^{2+}$ resin column, and the proteins within the receptor complex were separated as described under *Materials and Methods*. Silver staining of the gels revealed multiple protein bands including (His)6-MOR identified subsequently with Western analysis, which migrated as a diffused band with molecular mass between 65 and 70 kDa (Supplemental Fig. S1A). Several protein bands were excised and analyzed using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry as described. One of the proteins with significant mascot probability score (>65) was tentatively identified to be RPNI. The protein band around 85 kDa was demonstrated conclusively as being RPNI with the LC MS/MS spectrometry analysis. Five distinct peptides were identified from MS/MS spectra with >90% confidence (representing 9% sequence coverage) and three peptides were identified with <90% confidence. Total coverage (all peptides) was 13.6% by amino acid sequence (Table 1). Equivalent protein candidates reported were gi|74207369, gi|55715894, gi|48474583, and gi|31543605. They all refer to the sequence of RPNI. The distribution of the five peptides within the RPNI amino acid sequence was summarized in Fig. 1A. To confirm whether MOR interacts with RPNI and whether this interaction occurs in intact cells, we carried out coimmunoprecipitation studies with lysate from N2A cells transiently transfected with MOR and RPNI. Expression of full-length RPNI tagged at the N terminus with the FLAG epitope was confirmed by direct Western analyses of the total lysates (Fig. 1B, c). Interaction of RPNI with MOR was de-

termined by coimmunoprecipitation of the complex with antibodies against the HA epitope, followed by immunoblotting with anti-FLAG antibody. RPNI did not coimmunoprecipitate with the receptor when cells were transfected with either HA-MOR or FLAG-RPNI alone (Fig. 1B, a, lanes 1 and 2). However, RPNI was detected in anti-HA immunoprecipitates from cells that were cotransfected with both HA-MOR and FLAG-RPNI, indicating that MOR interacts with FLAG-RPNI in mammalian cells (Fig. 1B, a, lanes 3 and 4). Likewise, coimmunoprecipitation of HA-MOR with FLAG-RPNI was observed only in cells cotransfected with both HA-MOR and FLAG-RPNI using anti-FLAG but not in cells transfected with either one of these two constructs (Fig. 1B, b). Same results were also obtained when digitonin was used to extract the complex to preserve the OST physiological activity (Supplemental Fig. S2B). Interestingly, although RPNI showed interaction with MOR in both Triton X-100 treated and digitonin treated immunoprecipitates, other components of OST complex only showed in digitonin treated immunoprecipitates (Supplemental Fig. S2B). Such observations could be due to the fact that the four components of OST complex link to each other when OST stability was maintained in digitonin (Fu et al., 1997) during co-IP of RPNI with MOR. RPNI interaction with MOR does not depend on the OST complex glycosylation activity. Only when the OST stability was maintained in digitonin, the individual components of OST complex were co-IP with MOR. Meanwhile, when the stability of OST complex was disrupted by Triton X-100, only RPNI but not RPNII, OST48, or Dad1 was coimmunoprecipitated with MOR (Supplemental Fig. S2).

The RPNI interaction with MOR can be demonstrated to occur within the cellular context, which is not a consequence of detergent extraction by the immunoprecipitation studies with the mixed lysates. When N2A cells separately transfected with MOR or RPNI were mixed together immediately before detergent addition for lysate preparations and co-IP, anti-HA did not coimmunoprecipitate FLAG-RPNI from mixed cells transfected with either MOR or RPNI while anti-HA could coimmunoprecipitate the FLAG-RPNI from N2A cotransfected with both MOR and RPNI (Fig. 1C). The direct interaction between RPNI and MOR can be demonstrated further with gel-overlay experiments as described under *Materials and Methods*. As shown in Fig. 1D a, in vitro translation resulted in the production of the full-length FLAG-RPNI. After incubating the Immobilon-P membrane containing the SDS-PAGE separated total lysate from N2A cells with the in vitro translated products, positive interac-

TABLE 1
Peptides identified using tandem mass spectrometry corresponding to mouse RPNI
Confidence (%) as reported from Protein Pilot Results Summary. The percentage of total coverage is 13.6%. High confidence coverage (>90%) is 9%.

| Peptide | Confidence |
|---|---|
| | % |
| AVTSEIAVLQSR | 99 |
| LPVALDPGSK | 99 |
| NIQVDSPYDISR | 99 |
| SEDVLDYGPFK | 97 |
| DIPAYSQDTFK | 91 |
| DISTLNSGK | 23 |
| QPDSGISSIR | 6 |
| DTYLENEK | 3 |

tion in the lane containing lysate from MOR-expressed N2A cells, but not in the lane from wild type N2A cells, was observed after excess in vitro translated product was removed by repeated washings (Fig. 1D, b). The location of the in vitro translated FLAG-RPNI product coincided with the location of HA-MOR, as determined by immunoblotting with anti-HA antibody (Fig. 1D, c). These data indicate that RPNI directly interacts with MOR, not via a protein scaffold or complex.

To demonstrate whether RPNI interacts with MOR endogenously expressed, MOR from mouse hippocampus tissue was immunoprecipitated by the anti-MOR C-tail polyclonal antibodies. Mouse cerebellum was used as the negative control to determine the specificity of the antibodies used. It showed that MOR C-tail antibodies could IP the endogenous RPNI from hippocampus but not from cerebellum extracts (Supplemental Fig. S3). To examine whether RPNI could associate with other glycosylated GPCRs, N2A cells were cotransfected with $\alpha_{1A}$-adrenergic receptor ($\alpha$1a-AR) and RPNI transiently. Co-IP studies indicated that RPNI also interacted with $\alpha$1a-AR similar to the RPNI interaction with MOR (i.e., FLAG-RPNI) was pulled down by anti-HA only in N2A cells expressing both FLAG-RPNI and HA-$\alpha$1a-AR (Supplemental Fig. S4A). Because RPNI is within the glycosyltransferase complex, the interaction between RPNI and MOR and between RPNI and $\alpha$1a-AR can occur within the ER, before being transported to Golgi during the maturation

of these GPCRs. The presence of MOR in ER or Golgi of N2A transiently transfected with MOR can be observed with confocal microscopy studies using specific organelle markers. The colocalization of MOR and RPNI within the ER was observed in such confocal microscopy studies (Supplemental Fig. S5A).

**Ability of RPNI to Affect Cellular Location of MOR.** To examine the effect of RPNI interaction on MOR cellular trafficking, three RPNI siRNA (1, 2, 3) and one scramble siRNA were constructed as described under *Materials and Methods*. siRNA constructs were transfected into N2A cells and the GFP fluorescence was used to screen for similar transfection efficiency before lyses. The efficiency of RPNI siRNA knockdown was demonstrated as Fig. 2A. Only the siRNA3 construct exhibited significant knockdown of the RPNI content in N2A cells. Thus, siRNA3 and scramble siRNA as control were used in subsequent studies. N2A cells stably expressing HA-MOR were transfected with GFP-pRNAT H1.1/hygro vector (0.1 $\mu$g), GFP-scramble RPNI siRNA (0.1 $\mu$g), or GFP-RPNI siRNA3 (0.1 $\mu$g). MOR distribution was detected with mouse anti-HA antibody. We observed no difference in MOR distribution among cells transfected with scramble RPNI siRNA and the control vector. Comparing cells with no GFP fluorescence and those with, MOR was detected only on the cell surface in cells transfected with the scramble siRNA (Fig. 2B). In N2A cells expressing the RPNI siRNA as indicated by the GFP fluorescence, the
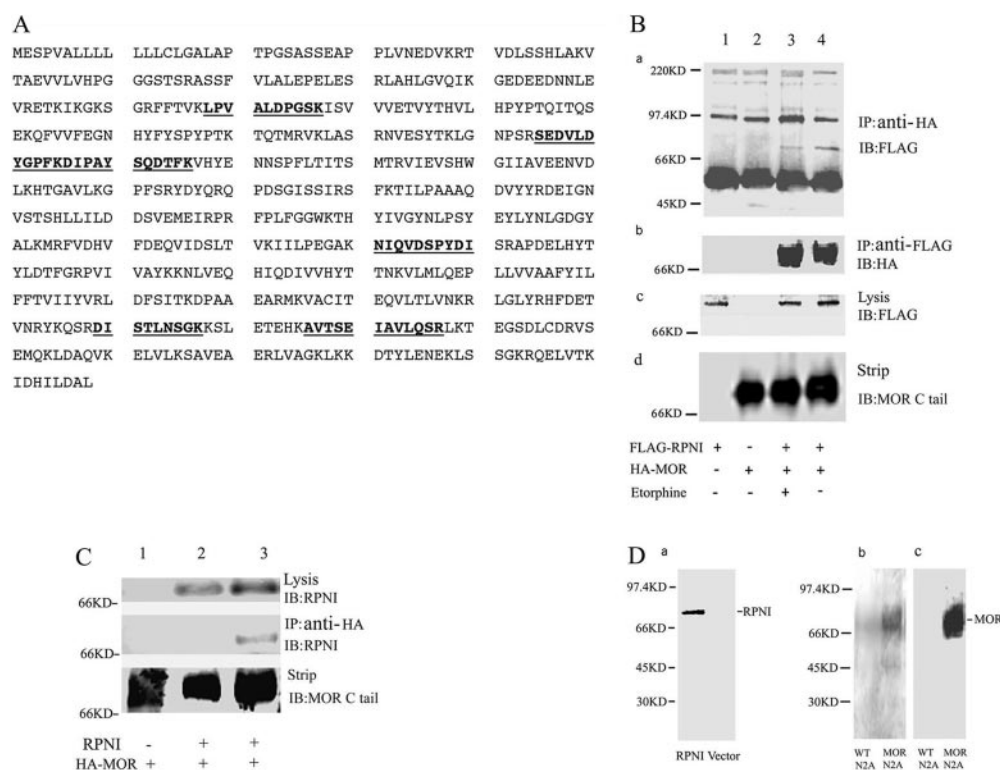


**Fig. 1.** Identification of RPNI as a MOR-associated protein. A, LC MS/MS analysis of the protein band corresponding to RPNI. The bold and underlined sequences represent the RPNI amino acid sequences identified using tandem mass spectrometry after in-gel digestion of the protein-staining band. The protein sequence refers to gi|31543605. B, coimmunoprecipitation of FLAG-RPNI and HA-MOR. a, FLAG-RPNI was detected after HA-MOR was immunoprecipitated (IP) from the cell lysate; b, HA-MOR was detected after FLAG-RPNI was immunoprecipitated from the cell lysates; c, the expression level of FLAG-RPNI was determined in 1/20 of the total lysate used in IP experiments; d, MOR was detected with rabbit polyclonal antibodies directed against the C-tail of MOR after HA-MOR was immunoprecipitated from the cell lysates. C, co-IP of FLAG-RPNI and HA-MOR in mixed cells that individually express FLAG-RPNI and HA-MOR. Lane 1, cells only express HA-MOR as negative control; lane 2, mixed cells that individually express FLAG-RPNI and HA-MOR; lane 3, cells that express both FLAG-RPNI and HA-MOR as positive control. D, gel overlay of RPNI with MOR. a, in vitro translation products of FLAG-RPNI, as indicated by Western analysis using anti-FLAG antibody; b, gel overlay of FLAG-RPNI on membranes containing SDS-PAGE separated N2A extract from cells expressing or not expressing HA-MOR; c, Western analysis of MOR expression.

majority of MOR was detected to be located intracellularly (Fig. 2C). In the same field, in cells not expressing the RPNI siRNA as indicated by the absence of GFP fluorescence, MOR was observed on the cell surface only. After siRNA transfection, total MOR expression was not altered as indicated by Western analyses of the total lysate (Fig. 2D). The cell surface MOR level with siRNA transfection was quantified with FACS using N2A cells transfected with GFP-Vector as the basal fluorescence level. The cell surface receptor fluorescence decreased from $100 \pm 5.4$ to $41.96 \pm 2.9\%$ of the basal level after RPNI siRNA transfection (Fig. 2D). These data demonstrate that RPNI regulates MOR cell surface expression. To eliminate the possibility that overexpression and knockdown of RPNI could affect the overall MOR glycosylation state leading to the removal of misfolded protein, the glycosylation states of MOR after overexpressing or knocking down of RPNI were detected by Western blot analyses after the treatment with endoglycosidase H (EndoH) and peptide-$N^-$-($N$-acetyl-$\beta$-glucosaminyl)asparagine aminase (PNGase F). As summarized in Fig. 3A a, majority of MOR migrated in SDS-PAGE with a molecular mass of ~65 to 70 kDa. There was a minor protein band with molecular mass of ~45–46 kDa detected by the HA antibodies, probably the immature form of MOR. Pretreatment of the lysate with EndoH re-

duced the molecular mass of MOR slightly, whereas pretreatment with PNGase F reduced the molecular mass of MOR to ~46 kDa, similar to that of the nascent polypeptide. Overexpression of RPNI eliminated the nonmatured MOR protein band but did not alter the receptor sensitivity toward EndoH and PNGase F (Fig. 3A, b). siRNA knockdown of RPNI resulted in an increase in the amount of immature MOR form but did not alter the receptor sensitivity to these two enzymes (Fig. 3A, c). Therefore, the observed cellular location of MOR under the conditions of overexpression of RPNI or knockdown of RPNI with siRNA does not reflect an alteration in the glycosylation states of the receptor.

It is noteworthy that interaction of RPNI with MOR seems to involve the glycosylation states of the receptor. As shown in Fig. 3B, gel overlay studies with the in vitro-translated RPNI product indicated that only MOR not treated with the enzymes could bind with RPNI. Pretreatment of MOR with either EndoH or PNGase F eliminated the RPNI interaction with the receptor (Fig. 3B, b). Because Western analyses indicated similar levels of receptor among various enzyme treatments (Fig. 3B, a), the absence of interaction in such gel
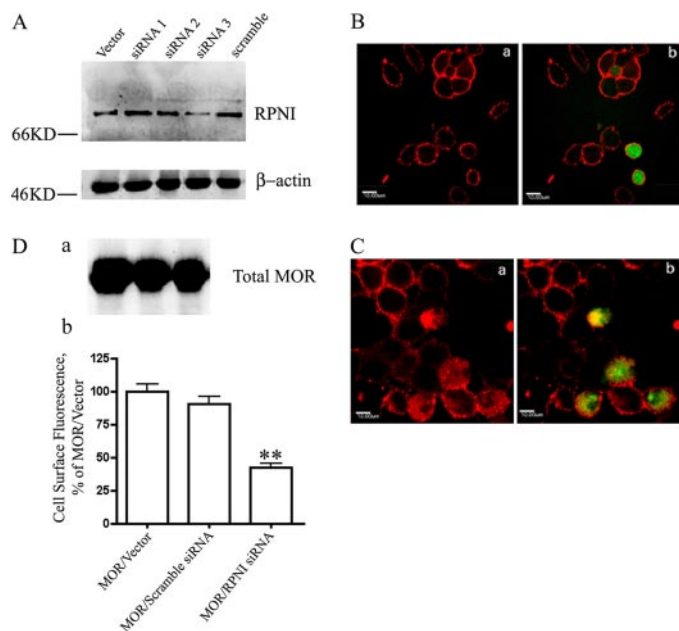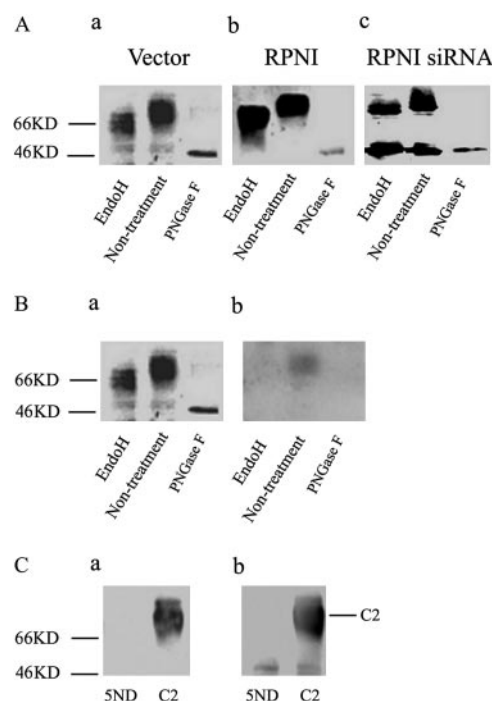


**Fig. 2.** siRNA-mediated knockdown of RPNI decreased cell surface expression of MOR in N2A cells. N2A cells were transfected with GFP-tagged vector, GFP-tagged scramble RPNI siRNA or GFP-tagged RPNI siRNA for 48 h. A, efficiency of siRNA used on knocking down endogenous RPNI. siRNA3 significantly reduced endogenous RPNI expression compared with others. $\beta$-Actin was used as control and transfection efficiency was controlled as described under *Results*. B, transfection of cells with 0.1 $\mu$g of scramble GFP-RPNI siRNA did not affect the expression of MOR on cell surface. C, transfection of 0.1 $\mu$g of GFP-RPNI siRNA decreased the expression of MOR on cell surface. In both B and C: a, HA-MOR was stained by the mouse anti-HA monoclonal antibody and detected with the goat anti-mouse antibodies conjugated with Alexa Fluor 594; b, merge MOR with GFP-tagged RPNI scramble siRNA or GFP-tagged siRNA. Scale bar equals 10 $\mu$m. D, a, expression level of MOR in 1/20 fraction of each sample, which showed total MOR of each sample are almost the same; b, FACS analyses of cell surface MOR expression in N2A cells transfected with RPNI siRNA as described under *Materials and Methods*. The bars represent the average $\pm$ S.E.M. from three separated experiments carried out in triplicate. $**$, $p < 0.01$ compared with cells transfected with vector.

**Fig. 3.** Glycosylation state of MOR when overexpressing or knocking down RPNI. A, Western blot to detect MOR, MOR after EndoH digestion, and MOR after PNGase F digestion. a, MOR in extract from N2A-MOR transfected with vector, after EndoH digestion, and after PNGase F digestion; b, extract from N2A-MOR overexpressing RPNI, after EndoH digestion, and after PNGase F digestion; c, MOR in extract from N2A-MOR transfected with RPNI siRNA, MOR after EndoH digestion, and MOR after PNGase F digestion. B, gel overlay of RPNI with MOR, EndoH-digested MOR, and PNGase F-digested MOR. a, Western blot to detect N2A extract from cells expressing MOR, EndoH-digested MOR, and PNGase F-digested MOR. b, gel overlay of in vitro translated FLAG-RPNI on membrane containing SDS-PAGE separated N2A extract from cells expressing MOR, EndoH-digested MOR, and PNGase F-digested MOR were carried out as described under *Materials and Methods*. C, gel overlay of in vitro-translated FLAG-RPNI with MOR C2 mutant or MOR5ND mutant. a, gel overlay of FLAG-RPNI on membranes containing SDS-PAGE separated N2A extract from cells expressing MOR C2 mutant or MOR5ND mutant; b, Western analysis was used to detect different glycosylated forms of MOR C2 and MOR5ND mutants using anti-MOR C tail polyclonal antibody.

overlay studies suggested the high mannose or some hybrid types of *N*-linked carbohydrates on MOR were essential for RPNI interaction. Combined with the data of RPNI IP from mixed cell lysates (Fig. 1C), it indicates that carbohydrate moiety is indispensable for RPNI binding. To further demonstrate whether RPNI interacts directly with the carbohydrate moiety or not, the MOR partially purified with wheat germ agglutinin was digested with PNGase F and gel overlay studies were carried out with the in vitro translation product of RPNI. No interaction was observed between RPNI and MOR in the PNGase F-treated sample (Fig. 3B, b).

The inability of RPNI to interact with the PNGase F-treated MOR could be due to the changes in the receptor's conformation after enzyme digestion thus affecting the recognition between these proteins, the role of CHO in RPNI binding was investigated with other GPCRs. Because our data showed RPNI could bind to glycosylated adrenergic receptor ($\alpha$1a-AR) (Supplemental Fig. S4A), whether RPNI could have distinct interaction among GPCRs that exhibit high sequence homology but different glycosylation states was investigated, such as the nonglycosylated $\alpha$2B-AR and the highly glycosylated $\alpha$2C-AR. As shown in Supplemental Fig. S4, B and C, RPNI was not co-IP with $\alpha$2B-AR, whereas RPNI did co-IP with $\alpha$2C-AR. Despite the highly conserved motifs of them (Dong and Wu, 2006), our data suggested that there might not be specific binding motif for RPNI. *N*-glycosylation plays an important role in RPNI interaction with GPCR.

**RPNI Enhances Cell Surface Expression of the C2 Mutant but Not the Five *N*-Glycosylation Mutants.** Previous studies indicated that the export of MOR could be facilitated by a chemical chaperone such as naloxone (Chaipatikul et al., 2003). In particular, naloxone could rescue an export-deficient MOR mutant lacking a motif at the proximal carboxyl tail 344KFCTR348 (C2). Colocalization of C2 and MOR in ER has been reported (Chaipatikul et al., 2003). The C2 mutant receptor had similar glycosylation state as the wild-type receptor (Supplemental Fig. S6A); gel overlay studies indicated it could interact with RPNI directly (Fig. 3C, a). Whether RPNI could increase the export of the C2 mutant was examined with confocal microscopy using anti-HA conjugated with Alexa Fluor 488 to detect the receptor location and goat anti-mouse antibody conjugated with Alexa Fluor 594 to detect the FLAG-RPNI. As shown in Fig. 4A, cell-surface expression of C2 mutant was observed only in N2A cells expressing both C2 mutant and RPNI. Meanwhile, in N2A cells without RPNI expression (i.e., cells exhibiting only the Alexa488 fluorescence), a majority of the C2 mutant was detected intracellularly (Fig. 4A, b). RPNI and naloxone seem to have similar mechanisms in regulating C2 mutant expression. Using FACS analyses to determine the cell surface C2 level, we could demonstrate that the cell surface expression of C2 was dependent on the amount of RPNI plasmid used to transfect the cells. The addition of 1 $\mu$M naloxone in cells expressing RPNI increased the amount of C2 on cell surface, but only at the lower RPNI plasmid
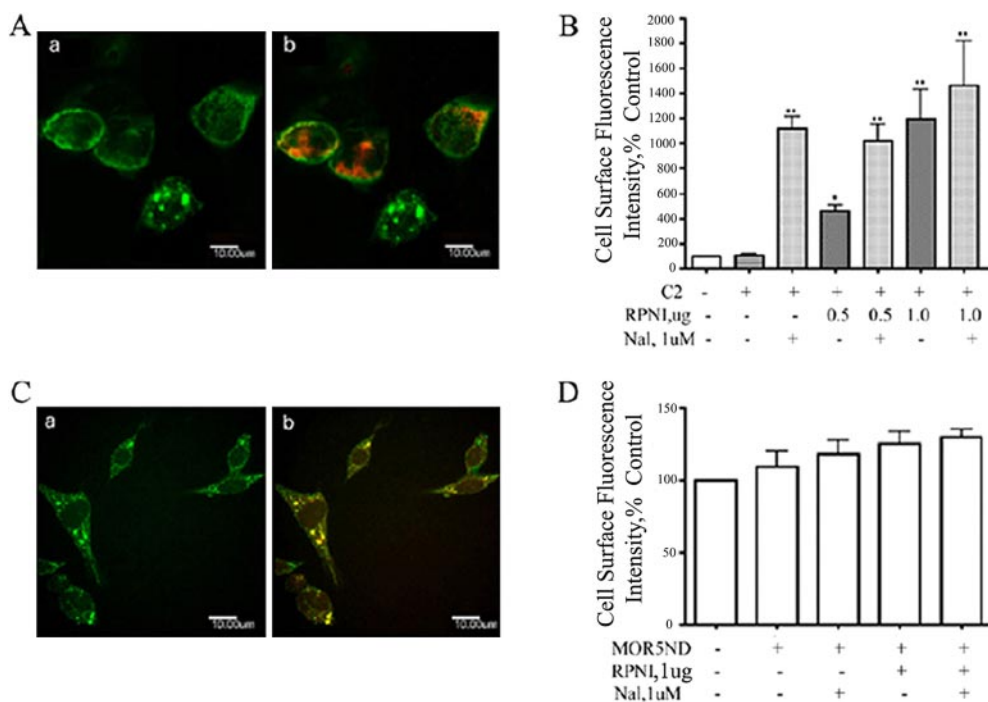


**Fig. 4.** RPNI rescues C2 cell surface expression but not five *N*-glycosylation-site mutants of MOR. A, RPNI up-regulate the cell surface expression of export deficient MOR C2 mutant. a, wild-type (WT) N2A cells were cotransfected with HA-MOR C2 mutant and FLAG-RPNI. C2 mutant expression was detected by rabbit anti-HA antibody conjugated with Alexa Fluor 488; b, merge of MOR C2 mutant and FLAG-RPNI that was determined by staining with mouse anti-FLAG monoclonal antibody and detected with goat anti-mouse antibodies conjugated with Alexa Fluor 594. Scale bar equals 10 $\mu$m. B, FACS analyses of C2 cell surface expression in the presence of naloxone and overexpression of RPNI. N2A cells were transiently transfected with vector or 0.5 $\mu$g of RPNI or 1 $\mu$g of RPNI and MOR C2 mutant. The bars represent averages $\pm$ S.E.M. of immunofluorescence in cells treated and not treated with 1 $\mu$M naloxone after transfection in $n = 3$ experiments. C, RPNI failed to regulate the export of five *N*-glycosylation-site mutants of MOR (MOR5ND). a, WT N2A cells were cotransfected with (His)6-MOR5ND mutant and FLAG-RPNI. MOR5ND mutant expression was detected by rabbit anti-(His)6 antibody conjugated with Alexa Fluor 488; b, merge of MOR5ND mutant and FLAG-RPNI that was determined by staining with mouse anti-FLAG monoclonal antibody and detected with goat anti-mouse antibodies conjugated with Alexa Fluor 594. Scale bar, 10 $\mu$m. D, inability of RPNI or naloxone to rescue cell surface expression of MOR5ND as determined by FACS analyses. The bars represent the averages $\pm$ S.E.M. of three separate experiments carried out in triplicate.

concentration (Fig. 4B). The level of C2 expressed on cell surface in the presence of naloxone and RPNI did not significantly exceed the level observed when naloxone alone was used to rescue the C2 export. The inability of naloxone to increase the cell surface expression of C2 in N2A cells overexpressed with RPNI could be the exhaustion of the intracellularly located receptor export pools at the high level of RPNI. However, this did not seem to be the case, because intracellularly located C2 could be observed in N2A cells overexpressing RPNI (Fig. 4A). These data suggest that RPNI may have chaperone activities similar to that observed with the chemical chaperone naloxone.

Because RPNI was known to have oligosaccharyltransferase activity (Kelleher et al., 1992) and RPNI could not interact with MOR pretreated with EndoH or PNGase F (Fig. 3B), we investigated whether the putative glycosylation residues (Asn at the N-terminal domain of MOR) had roles in RPNI export function. The putative glycosylation sites in MOR (Asn9, Asn31, Asn38, Asn46, and Asn53) were all mutated to Asp (MOR5ND). MOR5ND transiently transfected into N2A cells was observed to be retained at the ER (Supplemental Fig. S6B). Similar to the mobility of the PNGase F-pretreated MOR, MOR5ND in SDS-PAGE exhibited an apparent molecular mass of ~46 kDa (Supplemental Fig. S6A). In addition, geloverlay studies indicated that MOR5ND, similar to MOR pretreated with EndoH or PNGase F, was unable to interact with RPNI (Fig. 3C, a). When the cellular location of the receptor was monitored by confocal microscopy, MOR5ND was observed as intracellular location despite overexpression of RPNI (Fig. 4C). A majority of the receptor immunofluorescence was detected intracellularly regardless of RPNI level (Fig. 4C, b). Likewise, FACS analyses of the MOR5ND receptor mutant demonstrated minimal cell surface expression. Overexpression of RPNI resulted in a slight but not significant change in cell-surface MOR5ND mutant receptor immunofluorescence. It is noteworthy that addition of naloxone did not result in an increase in cell-surface expression of MOR5ND receptor mutant either, regardless of the presence or absence of RPNI overexpression (Fig. 4D).

To determine whether chaperone function of RPNI is dependent on N-glycosylation, single mutation mutants in each of the five putative glycosylation Asn sites of MOR were generated. As shown in Supplemental Fig. S7B, all five single glycosylation site mutants exhibited a slightly faster mobility in SDS-PAGE, indicative of a smaller molecular mass protein species than wild-type MOR. In some regards, their molecular masses are similar to that of MOR pretreated with EndoH (Fig. 3A). It is noteworthy that, in contrast to the wild type MOR, all the single glycosylation site mutants could not pull down the FLAG-RPNI (Supplemental Fig. S7A). Hence, RPNI should have minimal consequence on these mutants' intracellular trafficking. When the cell surface expression of these mutants was determined by FACS analyses, it was shown that RPNI could not regulate the plasma membrane insertion of these mutants (Fig. 5B). Although variable degrees of cell surface expression of the mutant receptors were observed (Fig. 5A), coexpression of RPNI with any one of the five mutants did not increase these mutant receptors export to the cell surface. Such data suggest that RPNI's interaction with MOR and subsequent chaperone function requires full N-glycosylation of the receptor.

**RPNI Rescues C2 without Increasing the Mutant Receptor Interaction with Calnexin.** The transit of proteins from ER is determined by its interaction with calnexin. Pharmacological chaperones were reported to rescue some GPCR mutants by altering their interaction with calnexin (Robert et al., 2005). It is probable that RPNI-enhanced cell surface expression of MOR is a reflection of the receptor interaction with calnexin. Hence, the export deficient mutant C2 was used to examine whether RPNI could increase C2 glycosylation level and subsequent calnexin binding. MOR and the C2 mutant were immunoprecipitated from N2A cells transfected either with HA-MOR and vector (Fig. 6A, lane 1), with HA-C2 and RPNI (Fig. 6A, lane 2), with HA-C2 and vector treated with naloxone (Fig. 6A, lane 3), with HA-C2 and vector treated with the proteosome inhibitor MG132 (Fig. 6A, lane 4), or with HA-C2 and vector treated with saline (Fig. 6A, lane 5). The relative C2 receptor level was determined using HA-MOR as the reference. The increase in the total cellular level of C2 in N2A cells overexpressed RPNI was similar to those observed in cells treated with naloxone or proteosome inhibitor MG132 (Fig. 6B). However, FACS analysis showed that overexpression of RPNI, such as addition of naloxone (Chaipatikul et al., 2003), increased the total C2 cell surface level, whereas MG132 did not (Fig. 6C). In addition, in contrast to wild-type MOR, in which strong interaction with calnexin was detected in the immunoprecipitate (Fig. 6, A, lane 1, and D), C2 did not exhibit strong interac-
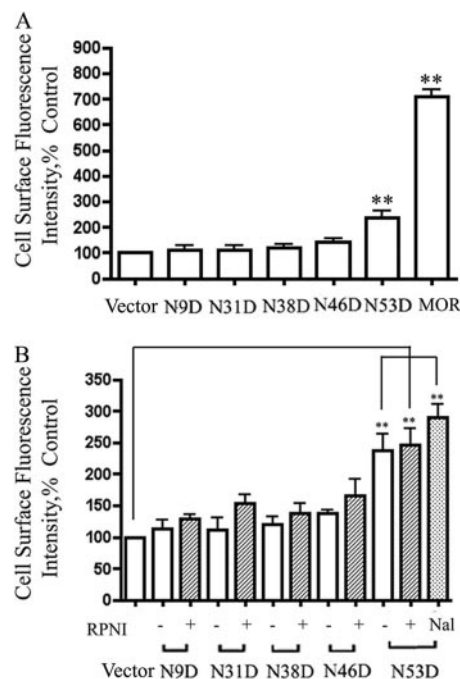


Fig. 5. Inability of RPNI to affect the export of five single N-glycosylation-site mutants. A, N2A cells were transiently transfected either with MOR mutant or MOR for 48 h before cell surface receptor levels were determined by FACS analyses, as described under *Materials and Methods*. The bars represent the averages ± S.E.M. of three separate experiments carried out in triplicate. B, N2A cells were transiently transfected either with MOR mutant and vector or with MOR mutant and 1 μg of RPNI for 48 h before cell surface receptor levels were determined by FACS analyses, as described under *Materials and Methods*. The □, ▨, and ▦ bars represent the averages ± S.E.M. in three separated experiments carried out in triplicate in the absence and presence of RPNI overexpression or treatment with 1 μM naloxone, respectively; **, $p < 0.01$ compared with cells transfected with vector.

tion with calnexin (Fig. 6, A, lanes 2–5, and D). Overexpression of RPNI or pretreatment with naloxone did not increase the receptor-calnexin interaction. These data suggest that the RPNI chaperone function, similar to that of naloxone, is not due to an increase in calnexin binding to MOR.

**RPNI Interacts with DOR and KOR and Enhances Their Export.** Because RPNI was shown to interact with other highly glycosylated GPCR such as $\alpha$1a-AR and $\alpha$2C- but not $\alpha$2B-AR (Supplemental Fig. S4), whether RPNI could interact with δ-opioid receptor (DOR) or κ-opioid receptor (KOR) and had similar effect in these receptors' export was investigated. Coimmunoprecipitation studies were performed in lysate from N2A cells transiently transfected with HA-DOR or HA-KOR, together with either FLAG-RPNI or vector control. Similar to our observations with MOR, RPNI was observed to co-IP with DOR and KOR (Fig. 7, A and C). RPNI was not coimmunoprecipitated with anti-HA when N2A cells were not transfected with the HA-DOR or HA-KOR. FACS analyses indicated that RPNI also could affect DOR or KOR export. Overexpression of RPNI significantly increased the DOR level on the cell surface by $33.2 \pm 7.8\%$ (Fig. 7B) but did not significantly increase KOR cell surface expression (Fig. 7D). In comparison, when N2A cells were transfected with RPNI siRNA, both DOR and KOR cell surface expression were significantly reduced by $38.1 \pm 3.9$ and $22 \pm 4.4\%$, respectively.
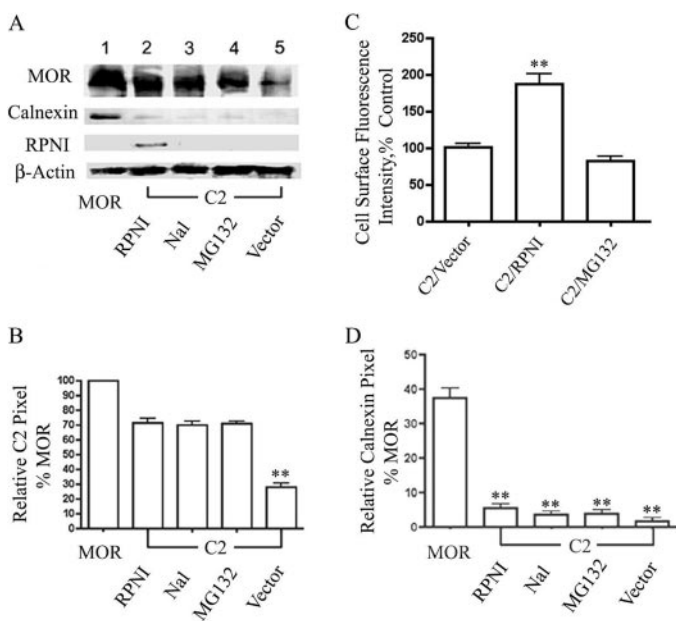


**Fig. 6.** RPNI did not increase C2 and calnexin interaction. N2A cells were transfected either with HA-MOR and vector (A, lane 1), HA-C2 mutant and FLAG-RPNI (A, lane 2), HA-C2 mutant and vector treated with naloxone (A, lane 3), HA-C2 mutant and vector treated with 10 μM MG132 16 h before harvesting (A, lane 4), or HA-C2 mutant and vector (A, lane 5). Immunoprecipitation and Western analyses were carried out as described under *Materials and Methods*. B, relative intensities of C2 mutant levels using HA-MOR as a reference, and the amount of β-actin immunoactivity in each lane was used for loading control. The bars represent the average ± S.E.M. of three separate experiments. C, FACS analyses of cell surface MOR C2 mutant after overexpression of RPNI, or the addition of MG132. D, relative intensities of calnexin co-IP with HA-MOR using HA-MOR immunoreactivity as a reference, and β-actin immunoactivity in each lane was used for loading control. The bars represent the average ± S.E.M. of three separate experiments.

## Discussion

RPNI was originally identified as an integral component of rough microsomal membranes and seemed to be related to the bound ribosomes (Kreibich et al., 1978). Currently, RPNI is accepted as a member of the OST family. The mammalian OST is an oligomeric complex composed of three membrane proteins located within the ER: RPNI, ribophorin II, and OST48 (Kelleher et al., 1992). Later, DAD1, an antiapoptotic protein, also was found to be a subunit of the mammalian OST (Kelleher and Gilmore, 1997). The biosynthesis of membrane proteins at the ER involves the integration of the polypeptide at the Sec61 translocon, together with a number of maturation events that can occur both during and after synthesis, such as N-glycosylation and signal sequence cleavage (Wilson et al., 2005). The transmembrane domain of RPNI has been suggested to function as a dolichol binding site (Kelleher et al., 1992). It is also reported that RPNI may function to retain potential substrates in close proximity to the catalytic subunit of the OST (sttp3), thereby stochastically improving the efficiency of the N-glycosylation reaction in vivo (Wilson et al., 2005). Other evidence has shown that RPNI may be multifunctional and facilitate additional processes [for example, ER quality control (Wilson et al., 2005)].

Using an affinity column purification paradigm, we have identified RPNI as a MOR-interacting protein. The interaction between RPNI and MOR was confirmed by coimmunoprecipitation studies in transiently transfected N2A cells and direct gel overlay studies using the in vitro translation products of a RPNI cDNA construct. By means of both confocal immunofluorescence microscopy and FACS analysis, our data demonstrated that the expression level of MOR could be regulated by RPNI level. RPNI seems to mediate MOR exocytotic activity, which is clearly related to its OST activity. For example, RPNI can stimulate the transport of the MOR C2 mutant from the ER to the cell surface, but not the export of the glycosylation-deficient MOR mutant-MOR5ND. This phenomenon suggests that RPNI not only is involved in biosynthesis of nascent polypeptides as previously reported, but also plays a pivotal role in their maturation and plasma membrane expression.

How RPNI can regulate MOR plasma membrane expression requires further investigation. However, our studies reveal several possible scenarios in which RPNI may affect MOR export.

First, our data showed that RPNI's rescuing of MOR C2 mutant was not due to endoplasmic reticulum-associated degradation, because its substrate MOR could not rescue C2 (Law et al., 2005), and MG132 only enhanced intracellular accumulation of C2 mutant but not on cell surface, contrary to RPNI overexpression (Fig. 6C). Furthermore, overexpression of RPNI has differential effects on the export of various opioid receptors and MOR mutants (Figs. 4 and 7).

Second, our data suggested that RPNI could serve as chaperone for MOR. RPNI has been suggested to be involved in ER quality control (de Virgilio et al., 1998; Wilson et al., 2005). Antibodies produced against the cytoplasmic domain of RPNI interfere with protein translocation across the rough ER by preventing ribosome targeting to the Sec61 complex (Yu et al., 1990). OST was demonstrated to be adjacent to the protein translocation channel (Shibatani et al., 2005), allowing the cotranslational modification of the nascent polypep-

tide as it enters the lumen of the rough ER (Chen et al., 1995). Most newly synthesized membrane and secretory proteins are delivered to the Sec61 translocon that mediates their integration into the lipid bilayer (Lecomte et al., 2003). The Sec61 translocon forms an aqueous pore that is gated by the luminal Hsp70-BiP (Alder and Johnson, 2004; Alder et al., 2005). Accumulating evidence has revealed that the ER molecular chaperone BiP is a master regulator of ER function. BiP is responsible for maintaining the permeability barrier of the ER during protein translocation, directing protein folding and assembly, and targeting misfolded proteins for retrograde translocation so they can be degraded by the proteasomes (Hendershot, 2004; Alder et al., 2005). Hsp70 (BiP), together with RPNI, already has been found to interact with the angiotensin-converting enzyme receptor (Santhamma and Sen, 2000). It is noteworthy that we identified one of the proteins copurified with (His)6-MOR to be Bip. Thus, similar to the chemical chaperone of MOR-naloxone (Chaipatikul et al., 2003), RPNI/Hsp70 could function as chaperone to rescue MOR C2 mutant transport to cell surface.

As the prelude of chaperone-assisted folding and oligomerization, formation of disulfide bonds already has started when the growing nascent peptide chains enter the luminal compartment (Bergman and Kuehl, 1979a,b). Proteins that fail to fold or oligomerize properly are prevented from export and are degraded (Hurtley and Helenius, 1989; Doms, 1990; Parodi, 2000). During folding, polypeptides with $N$-linked oligosaccharides interact transiently and specifically with two ER-unique chaperones, calnexin and calreticulin. Calnexin transiently interacts with different glycoproteins during folding and maturation (Hebert et al., 1996). The disulfide bonds in the opioid receptor may link to calnexin and affect the plasma membrane expression of the opioid receptor. It is noteworthy that C2 mutant is different from some other calnexin-retained GPCR mutants (i.e., V2 vasopressin receptor and V1b/V3 receptor mutants) (Morello et al., 2001; Robert et al., 2005), which may account for different mutants having different effects on the interaction of calnexin. From Fig. 6, calnexin was detected when MOR was immunoprecipitated, contrary to C2 coexpressed with RPNI. Coexpression of C2 and RPNI did not affect the total amount of cellular glycosylated receptor, compared with C2 with naloxone and C2 with proteosome inhibitor MG132. Under all these conditions, interaction between C2 and calnexin was not detected (Fig. 6D). However, the coexpression of RPNI with C2 helps to stabilize C2 and prevents its degradation compared with C2 cotransfected with vector control (Fig. 6, A and B). The data suggest that overexpression of RPNI could
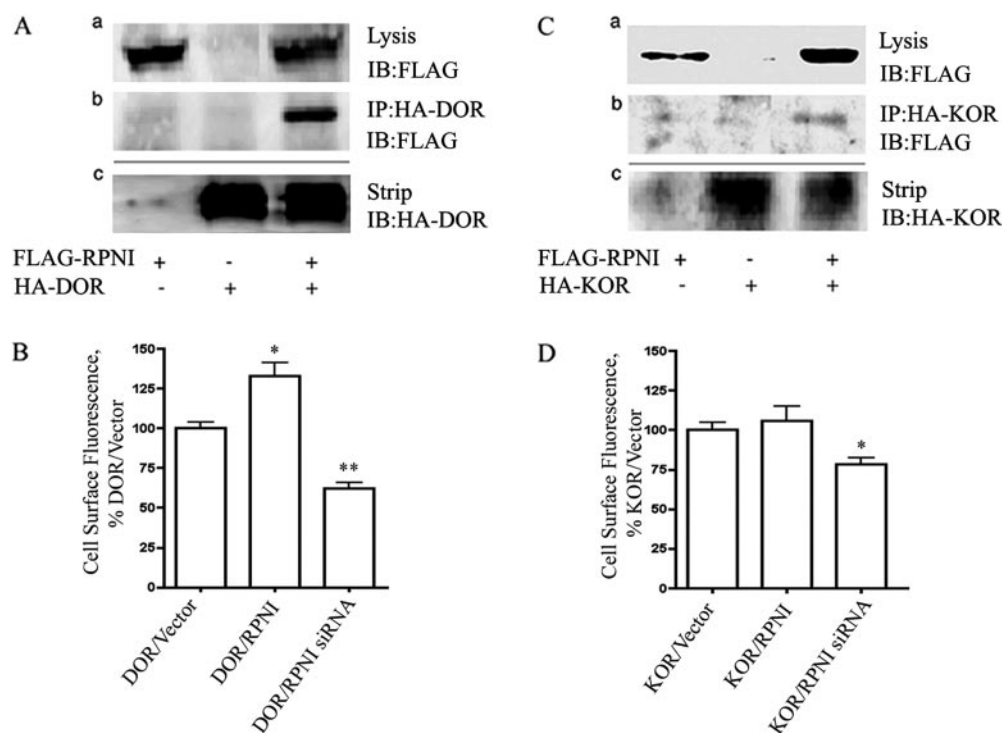


Fig. 7. Interaction between RPNI and DOR and KOR affects receptor export. A, co-IP of RPNI with DOR. N2A cells were transiently transfected either with vector and HA-DOR, vector and FLAG-RPNI, or FLAG-RPNI and HA-DOR, as described under *Materials and Methods*. a, RPNI expression was detected with mouse anti-FLAG antibody in 1/20 total cell lysates; b, RPNI was detected with rabbit anti-FLAG after HA-DOR was immunoprecipitated from the cell lysates using mouse anti-HA antibodies; c, the amount of HA-DOR immunoprecipitated and loaded on each lane was demonstrated after stripping the blots used to detect RPNI. B, increase of DOR export by RPNI. N2A cells were transiently transfected either with HA-DOR and vector, HA-DOR and RPNI, or HA-DOR and RPNI siRNA for 48 h before cell-surface receptor levels were determined by FACS analyses, as described under *Materials and Methods*. The bars represent the averages ± S.E.M. of $n = 3$ experiments carried out in triplicate. *, $p < 0.05$; **, $p < 0.01$ compared with cells transfected with vector. C, co-IP of RPNI with KOR. N2A cells were transfected either with vector and HA-KOR, vector and FLAG-RPNI, or FLAG-RPNI and HA-KOR, as described under *Materials and Methods*. a, RPNI expression was detected with mouse anti-FLAG antibody in 1/20 total cell lysates; b, RPNI was detected with rabbit anti-FLAG after HA-KOR was immunoprecipitated from the cell lysates using mouse anti-HA antibodies; c, the amount of HA-KOR immunoprecipitated and loaded on each lane was demonstrated after stripping the blots used to detect RPNI. D, decrease in KOR export after RPNI knockdown. N2A cells were transiently transfected either with HA-KOR and vector, HA-KOR and FLAG- RPNI, or HA-KOR and RPNI siRNA for 48 h before cell surface receptor levels were determined by FACS analyses, as described under *Materials and Methods*. The bars represent the averages ± S.E.M. of $n = 3$ experiments carried out in triplicate. *, $p < 0.05$ compared with cells transfected with vector.

enhance cell surface expression of C2, which might reflect RPNI-associated ER quality control processes to facilitate C2 mutant's export out of ER other than the calnexin pathway.

The chaperone activity of RPNI seems to be connected to its OST activities. Contrary to the MOR C2 mutant, another MOR plasma membrane expression-deficient mutant, MOR5ND, in which all five Asn residues at the N terminus were mutated to Asp, was unable to be expressed in plasma membrane despite RPNI overexpression. Furthermore, addition of naloxone did not result in cell-surface expression of MOR5ND. On the other hand, one of the single Asn mutants, N53D, was shown to express on the cell surface (Fig. 5) and interact with calnexin (Supplemental Fig. S8). Overexpression of RPNI or naloxone treatment did not increase the expression of the N53D mutant (Fig. 5). These results suggest that $N$-glycosylation is critical for naloxone or RPNI to function as a chaperone. A new model for OST recently was proposed in which RPNI might act as a chaperone or as an escort to promote the $N$-glycosylation of selected substrates by the catalytic STT3 subunits (Wilson and High, 2007). The importance of $N$-glycosylation in opioid receptor plasma membrane expression was demonstrated further with other opioid receptors—DOR and KOR, and the endoglycosidase studies. Although RPNI is known to form a complex with these two receptors, RPNI, as a chaperone, exhibits differential effects in their plasma membrane expressions. KOR has fewer $N$-glycosylation sites, and overexpression of RPNI has minimal effect on KOR plasma membrane expression. However, by knocking down RPNI level with siRNA, KOR plasma membrane expression decreased, illustrating that RPNI was important in KOR final destination. All these data suggest that RPNI's function depends not only on the glycosylation state of the receptor but also on the number of glycosylation sites.

A major finding of this study is the interaction of MOR with RPNI in neuroblastoma cells and the potential function of this association in regulating opioid receptor plasma membrane expression. Our data indicate that RPNI has functions other than being an oligosaccharyltransferase. The interaction of RPNI with MOR suggests that RPNI serves as a chaperone or controller of MOR transport from the ER to the cell surface. It remains to be determined whether RPNI and its interaction with MOR can regulate or be regulated by receptor signaling.

### Acknowledgments

### References

Abeijon C and Hirschberg CB (1992) Topography of glycosylation reactions in the endoplasmic reticulum. *Trends Biochem Sci* **17:**32–36.

Alder NN and Johnson AE (2004) Cotranslational membrane protein biogenesis at the endoplasmic reticulum. *J Biol Chem* **279:**22787–22790.

Alder NN, Shen Y, Brodsky JL, Hendershot LM, and Johnson AE (2005) The molecular mechanisms underlying BiP-mediated gating of the Sec61 translocon of the endoplasmic reticulum. *J Cell Biol* **168:**389–399.

Bergman LW and Kuehl WM (1979a) Co-translational modification of nascent immunoglobulin heavy and light chains. *J Supramol Struct* **11:**9–24.

Bergman LW and Kuehl WM (1979b) Formation of intermolecular disulfide bonds on nascent immunoglobulin polypeptides. *J Biol Chem* **254:**5690–5694.

Chaipatikul V, Erickson-Herbrandson LJ, Loh HH, and Law PY (2003) Rescuing the traffic-deficient mutants of rat $\mu$-opioid receptors with hydrophobic ligands. *Mol Pharmacol* **64:**32–41.

Chen C, Li JG, Chen Y, Huang P, Wang Y, and Liu-Chen LY (2006) GEC1 interacts with the $\kappa$ opioid receptor and enhances expression of the receptor. *J Biol Chem* **281:**7983–7993.

Chen W, Helenius J, Braakman I, and Helenius A (1995) Cotranslational folding and calnexin binding during glycoprotein synthesis. *Proc Natl Acad Sci U S A* **92:**6229–6233.

Chen Y, Mestek A, Liu J, Hurley JA, and Yu L (1993) Molecular cloning and functional expression of a $\mu$-opioid receptor from rat brain. *Mol Pharmacol* **44:**8–12.

de Virgilio M, Weninger H, and Ivessa NE (1998) Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J Biol Chem* **273:**9734–9743.

Doms RW (1990) Oligomerization and protein transport. *Methods Enzymol* **191:**841–854.

Dong C and Wu G (2006) Regulation of anterograde transport of $\alpha$2-adrenergic receptors by the N termini at multiple intracellular compartments. *J Biol Chem* **281:**38543–38554.

Evans CJ, Keith DE Jr, Morrison H, Magendzo K, and Edwards RH (1992) Cloning of a delta opioid receptor by expression cloning. *Science* **258:**1952–1955.

Fu J, Ren M, and Kreibich G (1997) Interactions among subunits of the oligosaccharyltransferase complex. *J Biol Chem* **272:**29687–29692.

Hebert DN, Foellmer B, and Helenius A (1996) Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. *EMBO J* **15:**2961–2968.

Helenius A and Aebi M (2001) Intracellular functions of N-linked glycans. *Science* **291:**2364–2369.

Hendershot LM (2004) The ER function BiP is a master regulator of ER function. *Mt Sinai J Med* **71:**289–297.

Hurtley SM and Helenius A (1989) Protein oligomerization in the endoplasmic reticulum. *Annu Rev Cell Biol* **5:**277–307.

Kapphahn RJ, Ethen CM, Peters EA, Higgins L, and Ferrington DA (2003) Modified alpha A crystallin in the retina: altered expression and truncation with aging. *Biochemistry* **42:**15310–15325.

Kelleher DJ and Gilmore R (1997) DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase. *Proc Natl Acad Sci U S A* **94:**4994–4999.

Kelleher DJ, Kreibich G, and Gilmore R (1992) Oligosaccharyltransferase activity is associated with a protein complex composed of ribophorins I and II and a 48 kd protein. *Cell* **69:**55–65.

Kieffer BL, Befort K, Gaveriaux-Ruff C, and Hirth CG (1992) The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U S A* **89:**12048–12052.

Kornfeld R and Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54:**631–664.

Kreibich G, Freienstein CM, Pereyra BN, Ulrich BL, and Sabatini DD (1978) Proteins of rough microsomal membranes related to ribosome binding. II. Crosslinking of bound ribosomes to specific membrane proteins exposed at the binding sites. *J Cell Biol* **77:**488–506.

Kreibich G, Sabatini DD, and Adesnik M (1983) Biosynthesis of hepatocyte endoplasmic reticulum proteins. *Methods Enzymol* **96:**530–542.

Law PY, Erickson-Herbrandson LJ, Zha QQ, Solberg J, Chu J, Sarre A, and Loh HH (2005) Heterodimerization of $\mu$- and 32 $\delta$-opioid receptors occurs at the cell surface only and requires receptor-G protein interactions. *J Biol Chem* **280:**11152–11164.

Lecomte FJ, Ismail N, and High S (2003) Making membrane proteins at the mammalian endoplasmic reticulum. *Biochem Soc Trans* **31:**1248–1252.

Morello JP, Salahpour A, Petäjä-Repo UE, Laperrière A, Lonergan M, Arthus MF, Nabi IR, Bichet DG, and Bouvier M (2001) Association of calnexin with wild type and mutant AVPR2 that causes nephrogenic diabetes insipidus. *Biochemistry* **40:**6766–6775.

Parodi AJ (2000) Protein glucosylation and its role in protein folding. *Annu Rev Biochem* **69:**69–93.

Petaja-Repo UE, Hogue M, Laperriere A, Walker P, and Bouvier M (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human $\delta$ opioid receptor. *J Biol Chem* **275:**13727–13736.

Robert J, Auzan C, Ventura MA, and Clauser E (2005) Mechanisms of cell-surface rerouting of an endoplasmic reticulum-retained mutant of the vasopressin V1b/V3 receptor by a pharmacological chaperone. *J Biol Chem* **280:**42198–42206.

Santhamma KR and Sen I (2000) Specific cellular proteins associate with angiotensin-converting enzyme and regulate its intracellular transport and cleavage-secretion. *J Biol Chem* **275:**23253–23258.

Shibatani T, David LL, McCormack AL, Frueh K, and Skach WR (2005) Proteomic analysis of mammalian oligosaccharyltransferase reveals multiple subcomplexes that contain Sec61, TRAP, and two potential new subunits. *Biochemistry* **44:**5982–5992.

Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, Hunter CL, Nuwaysir LM, and Schaeffer DA (2007) The Paragon Algorithm: a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol Cell Proteomics* **6:**1638–1655.

Silberstein S and Gilmore R (1996) Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *FASEB J* **10:**849–858.

Wilson CM and High S (2007) Ribophorin I acts as a substrate-specific facilitator of $N$-glycosylation. *J Cell Sci* **120:**648–657.

Wilson CM, Kraft C, Duggan C, Ismail N, Crawshaw SG, and High S (2005) Ribophorin I associates with a subset of membrane proteins after their integration at the sec61 translocon. *J Biol Chem* **280:**4195–4206.

Yu YH, Sabatini DD, and Kreibich G (1990) Antiribophorin antibodies inhibit the targeting to the ER membrane of ribosomes containing nascent secretory polypeptides. *J Cell Biol* **111:**1335–1342.

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