THE PX-RICS/14-3-3ζ/θ COMPLEX COUPLES N-CADHERIN/β-CATENIN WITH DYNEIN/DYNACTIN TO MEDIATE ITS EXPORT FROM THE ENDOPLASMIC RETICULUM*

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We have recently shown that β-catenin-facilitated export of cadherins from the endoplasmic reticulum (ER) requires PX-RICS, a β-catenin-interacting GTPase-activating protein for Cdc42. Here we show that PX-RICS interacts with isoforms of 14-3-3 and couples the N-cadherin/β-catenin complex to the microtubule-based molecular motor dynein/dynactin. Similar to knockdown of PX-RICS, knockdown of either 14-3-3ζ or θ resulted in the disappearance of N-cadherin and β-catenin from the cell-cell boundaries. Furthermore, we found that PX-RICS and 14-3-3ζ/θ are present in a large multiprotein complex that contains dynein/dynactin components as well as N-cadherin and β-catenin. Both RNAi- and dynamitin-mediated inhibition of dynein/dynactin function also led to the absence of N-cadherin and β-catenin at the cell-cell contact sites. Our results suggest that the PX-RICS/14-3-3ζ/θ complex links the N-cadherin/β-catenin cargo with the dynein/dynactin motor, and thereby mediates its ER export.

In general, cargo proteins to be exported from the endoplasmic reticulum (ER) have characteristic amino acid sequences called “ER export motifs” that facilitate their ER exit (1-4). Classic cadherins (simply referred to cadherins hereafter) (5-9), however, are known to have no functional ER export motifs, and their efficient ER exit requires complex formation with β-catenin at the ER immediately after cadherin synthesis (10-12). We have recently shown that this β-catenin-facilitated ER export of cadherins...
requires PX-RICS, a β-catenin-interacting GTPase-activating protein (GAP) for Cdc42 (13).

PX-RICS is an alternatively spliced isoform of RICS [RhoGAP involved in the β-catenin-N-cadherin and N-methyl-D-aspartate (NMDA) receptor signaling] and contains phox homology (PX) and src homology 3 (SH3) domains in its N-terminal region (14,15). RICS is expressed predominantly in neurons of the brain and localized to the growth cone and postsynaptic density (PSD), where it regulates neurite extension and presumably NMDA signaling (14). In contrast, PX-RICS is expressed in a wide variety of tissues and cell lines and localized to the ER and cis-Golgi (13,15). Our recent study has revealed that PX-RICS facilitates ER-to-Golgi transport of the N-cadherin/β-catenin complex through its direct interaction with β-catenin, Cdc42, γ-aminobutyric acid type A receptor-associated protein (GABARAP) and phosphatidylinositol-4-phosphate (PI4P) (13). This finding suggests that PX-RICS is a key molecule in a novel intracellular transport system that is independent of known ER export motifs, and provides a molecular basis that explains why the assembly of cadherins with β-catenin is essential for efficient ER exit of cadherins. However, the precise molecular mechanisms by which PX-RICS triggers ER exit of the N-cadherin/β-catenin complex remain to be elucidated. To address this issue, we attempted to identify PX-RICS-interacting scaffold proteins involved in the PX-RICS-dependent forward transport mechanism. Here we report that PX-RICS and its novel binding partner 14-3-3 proteins link the N-cadherin/β-catenin cargo with the dynein/dynactin motor, thereby regulates the amount of surface N-cadherin available for cell-cell adhesion.

**EXPERIMENTAL PROCEDURES**

_Antibodies-_ Rabbit polyclonal antibody to PX-RICS was generated as described previously (15). The commercially available antibodies used in this study were as follows: mouse anti-14-3-3ε, mouse anti-β-catenin and mouse anti-p150Glued antibodies were purchased from BD Biosciences; mouse anti-GFP antibody [Living colors A.v. monoclonal antibody (JL-8)] from Invitrogen; rabbit anti-Myc antibody from MBL; mouse anti-α-tubulin antibody (DM1A) from Merck; mouse anti-N-cadherin (13A9) and mouse anti-DYNC1I (74.1) antibodies from Millipore; mouse anti-Myc (9E10), mouse anti-pan-14-3-3 (H-8), rabbit anti-14-3-3ζ (C-16), goat anti-14-3-3β (A-15), goat anti-14-3-3γ (A-12), goat anti-14-3-3η (E-12) and goat anti-Sec23 (E-19) antibodies from Santa Cruz Biotechnology; mouse anti-FLAG (M2) and mouse anti-14-3-3θ (3B9) antibodies from Sigma; rabbit anti-calnexin antibody from Stressgen; mouse anti-transferrin receptor antibody (H68.4) from Zymed. Rabbit and mouse IgGs were from Millipore.

**PX-RICS-derived mutants-** Details of the PX-RICS-derived mutants used in this study are
as follows: 1433BR, a fragment of PX-RICS (amino acids 1763-1828) that contains a 14-3-3-binding motif (amino acids 1793-1798); ΔRSKSDP and 1433BR-ΔRSKSDP, mutants of PX-RICS and 1433BR lacking the 14-3-3-binding motif, respectively; S1796A and 1433BR-SA, mutants of PX-RICS and 1433BR in which Ser\(^{1796}\), the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) phosphorylation site in the 14-3-3 binding motif, is replaced with Ala. All mutant forms were generated by PCR-based mutagenesis.

**Cell culture and transfection**- HEK293T, COS-7 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone). Cells were transfected with plasmids using FuGENE 6 (Roche). For CaMKII inhibition, COS-7 cells were treated with 10 µM KN-93 (Merck) for 24 h.

**Identification of PX-RICS-interacting proteins**- HEK293T cells were transfected with pcDNA3.1(+)−FLAG-PX-RICS and binding proteins were analysed by direct nano-flow liquid chromatography/electrospray tandem mass spectrometry, as described earlier (16).

**Protein expression and purification**- For preparation of GST-fusion proteins, cDNA fragments were subcloned into pGEX-5X-3 (GE Healthcare). GST-fusion proteins were synthesized in Escherichia coli and isolated by adsorption to glutathione-Sepharose (GE Healthcare).\(^{35}\)S-labeled proteins were synthesized by in vitro transcription-translation in the presence of \[^{35}\text{S}\]methionine using the TNT\(^{TM}\) coupled reticulocyte lysate system (Promega).

**In vitro pull-down assays**- Lysates from COS-7 cells (500 µg of protein) or in vitro-translated proteins were incubated with 2 µg of GST or GST-fusion proteins in binding buffer [10 mM Tris-HCl (pH 6.8), 140 mM NaCl, 1 mM EDTA, 0.1 % Triton X-100, protease inhibitor cocktail] for 1 h at 4 °C and then with glutathione-Sepharose for 1 h at 4 °C. Beads were washed extensively with binding buffer and bound proteins were fractionated by SDS-PAGE followed by immunoblotting or autoradiography. For dephosphorylation experiments, lysates from COS-7 cells (500 µg of protein) were treated with 10 U of BAP (Takara Bio Inc.) for 1 h at 30 °C prior to pull-down assays. For phosphorylation experiments, in vitro translated PX-RICS was treated with 50 ng of rat brain CaMKII (Merck) for 2 h at 30 °C in the presence of 1 mM Ca\(^{2+}\), 2 mM ATP, 2.5 µg of bovine brain calmodulin (Millipore) in 50 µl reaction mixture prior to pull-down assays.

**Immunoprecipitation and immunoblotting**- Cells were lysed in lysis buffer T [10 mM Tris-HCl (pH 6.8), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor cocktail, phosphatase inhibitor cocktail]. The lysates were precleared with protein A-Sepharose (GE Healthcare) for 1 h at 4 °C. Precleared lysates (500 µg of protein) were incubated with 5 µg of antibody for 1 h at 4 °C, and then the immunocomplexes were adsorbed to protein A-Sepharose for 1 h at 4 °C. After washing extensively with lysis buffer T,
immunoprecipitates were resolved by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The blots probed with primary antibodies were visualized with alkaline phosphatase-conjugated secondary antibodies (Promega).

**Immunofluorescence** - HeLa cells were plated on coverslips in 6-well tissue culture plates (1 × 10^5 cells per well). After 48 h of incubation at 37 °C, cells were fixed with cold methanol for 20 min at -20 °C (for PX-RICS, 14-3-3 or N-cadherin staining) or 2% paraformaldehyde for 30 min at 4 °C (for β-catenin staining), and permeabilized with 0.2% Triton X-100 in Tris-buffered saline (TBS) for 5 min. The cells were double-stained with the appropriate combination of primary antibodies for 60 min at room temperature. Staining patterns were visualized by incubating with Alexa Fluor 488- or Alexa Fluor 594-labelled donkey secondary antibody (Invitrogen) for 60 min at room temperature. The cell images were obtained with a LSM510META laser scanning confocal microscope (Zeiss).

**RNAi experiments** - Sequences of human CaMKII isoforms targeted by shRNAs are as follows:
- shRNA-CAMK2A (for the α isoform), 5'-GGGACACCACTACCTGATCTT-3';
- shRNA-CAMK2B (for the β isoform), 5'-CCGCCCTCTGAAAGCATTCCAA-3';
- shRNA-CAMK2D (for the δ isoform), 5'-ATGCCCGCTCTGACCATATATA-3';
- shRNA-CAMK2G (for the γ isoform), 5'-TGCCGCTAGCCATCGAAGTACA-3';
- shRNA-control, 5'-TGAGAGTATGACATCCGG-3'.

DNA oligonucleotides encoding shRNAs were subcloned into the H1 promoter-driven vector pSUPERretro.puro (OligoEngine). Sequences of Stealth RNAi (Invitrogen) that target the human 14-3-3ζ, θ, DYNC1I2 or p150^Glued gene are as follows:
- siRNA-ζ-1, 5'-CAGGUGUAGUAUUGUGGGGUACUUU-3';
- siRNA-ζ-2, 5'-CAGUUAACAUUUAGGGAGUUAUCUG-3';
- siRNA-θ-1, 5'-CCAAACACUUAUGUAGAGCAGGACUAAA-3';
- siRNA-θ-2, 5'-CAGUUAACAUUUAGGGAGUUAUCUG-3';
- siRNA-θ-1, 5'-CCAAACACUUAUGUAGAGCAGGACUAAA-3';
- siRNA-θ-2, 5'-CAGUUAACAUUUAGGGAGUUAUCUG-3';
- siRNA-DYNC1I2-1, 5'-GGAGAUUGGAUUUAUGGAAUCUCAA-3';
- siRNA-DYNC1I2-2, 5'-GGGCGAGCGAAUAGCAAGCAGGCUUUGUA-3';
- siRNA-p150-1, 5'-GGGCAGAAGACAAAGCAAAGCUAAA-3';
- siRNA-p150-2, 5'-ACCAUGACUGCGUUCUGGUGCGUUG-3'.

Stealth RNAi negative control low GC duplex (Invitrogen) was used as a control. HeLa cells were transfected with the shRNA expression
constructs or cotransfected with Stealth RNAi
and pSUPERretro.puro using Lipofectamine 2000
(Invitrogen), cultured for 24 h and then treated
with puromycin (4 µg/ml) for 48 h to remove
untransfected cells. Surviving cells were
subjected to immunoblotting,
immunofluorescence, cell dissociation assays, or
cell surface biotinylation assays. For rescue
experiments, HeLa cells were cotransfected using
Lipofectamine 2000 with Stealth RNAi and the
expression plasmid carrying only the coding
region of 14-3-3ζ or θ.

Surface biotinylation assays- Biotinylation assays
of cell surface proteins were performed as
described (17). siRNA-transfected cells were
washed three times with ice-cold phosphate-buffered saline (PBS) containing 1
mM CaCl₂ and 0.5 mM MgCl₂ [PBS(+)] and
incubated with 0.25 mg/ml EZ-Link
Sulfo-NHS-LC-Biotin (Pierce) in PBS(+) for 20
min at 4 °C with gentle agitation. After an
immediate rinse with ice-cold quenching buffer
[50 mM glycine in PBS(+)], cells were further
washed three times in ice-cold quenching buffer
for 5 min each. The cells were lysed in lysis
buffer T and cleared lysates (0.5 mg of protein)
were incubated with 60 µl of 50 % slurry of
streptavidin-agarose beads (Pierce) for 2 h at 4 °C.
Beads were washed five times in lysis buffer T
and then once with PBS. Bound proteins were
analyzed by immunoblotting.

Cell dissociation assays- Cells were scraped in
PBS(+) and suspended by 10 times repeated
pipetting. The extent of cell dissociation was
quantified by counting the number of cell clumps
(Np) and the total number of cells (Nc) and was
represented by the ratio Nc/Np.

Density gradient ultracentrifugation- The density
gradient ultracentrifugation was performed as
described elsewhere (18). Subconfluent HeLa
cells (1 × 10⁵) were washed twice with ice-cold
PBS(+) and lysed in 1 ml of lysis buffer D [25
mM Hapes-KOH (pH 6.8), 150 mM NaCl, 2 mM
EDTA, 1 % digitonin, protease inhibitor cocktail,
phosphatase inhibitor cocktail] at 4 °C for 30 min.
After centrifugation at 17,000 × g for 30 min, the
supernatant (2 mg of protein/0.5 ml) was layered
over an 11.5 ml 10-40 % (w/v) linear sucrose
density gradient containing 25 mM Hapes-KOH
(pH 6.8), 150 mM NaCl, and 0.4 % digitonin.
After centrifugation for 15 h at 35,000 rpm in a
Beckman SW40 rotor, 12 fractions containing 1
ml each were collected from the top of the tube
and were subjected to immunoblotting or
immunoprecipitation. Protein mobility markers
[High molecular weight native marker kit (GE
Healthcare)] were applied to a parallel gradient
and their fraction positions were determined by
2-15% native PAGE followed by Coomassie
staining.

Live cell imaging of N-cadherin and PX-RICS-
N-cadherin-GFP and mCherry-PX-RICS
expression constructs were created by inserting
full-length human N-cadherin and PX-RICS
cDNAs into pEGFP-N1 and pmCherry-C1
(Takara Bio Inc.), respectively. Live cell imaging
was performed using a Revolution XD system
(Andor Technology).
RESULTS

PX-RICS interacts with 14-3-3ζ and θ at the ER

We attempted to identify PX-RICS-interacting proteins involved in the PX-RICS-dependent forward transport mechanism using liquid chromatography-based electrospray tandem mass spectrometry (16). We found that PX-RICS could interact with the β, γ, ε, ζ and η isoforms of 14-3-3 proteins, which have been reported to be involved in diverse biological processes, including intracellular protein transport (19-21). In vitro pull-down assays confirmed that PX-RICS interacts with all seven human isoforms of 14-3-3 proteins (Fig. 1A). 14-3-3σ showed a relatively low binding activity for PX-RICS, whereas the other isoforms exhibited similar high activities.

We next examined whether PX-RICS is associated with 14-3-3s in vivo. When lysates from HeLa cells were subjected to coimmunoprecipitation experiments, 14-3-3s were found to coprecipitate with PX-RICS, and vice versa (Fig. 1B). We also investigated whether 14-3-3s are colocalized with PX-RICS, which is present at the ER (13). We found that both PX-RICS and 14-3-3s are stained as reticular or punctate patterns and a significant fraction of these proteins colocalize especially in the perinuclear region (Fig. 1B). These results suggest that PX-RICS is associated with 14-3-3s at the ER in living cells.

Despite the high sequence homology among 14-3-3 isoforms, not all ligands show equal affinities for different 14-3-3 isoforms (19,22). We therefore examined whether PX-RICS preferentially interacts with particular isoform(s) of 14-3-3s using isoform-specific antibodies. We found that the β, γ and η isoforms were expressed at the extremely low levels, but the ε, ζ and θ isoforms were abundantly expressed in HeLa cells (data not shown). In vivo pull-down assays with HeLa cell lysates revealed that the ζ and θ isoforms, but not the ε isoform, coprecipitated with PX-RICS (Fig. 1C and data not shown). Immunofluorescent staining also revealed that PX-RICS is colocalized with the ζ and θ isoforms, but not the ε isoform, in the perinuclear region (Fig. 1C and data not shown). These results suggest that PX-RICS interacts specifically with the ζ and θ isoforms of 14-3-3 proteins.

CaMKII-mediated phosphorylation of PX-RICS regulates its binding to 14-3-3ζ

PX-RICS contains a putative 14-3-3-binding motif RSKSDP (R, Arg; S, Ser; K, Lys; D, Asp; P, Pro) near its C-terminus, which is consistent with the well-characterized consensus sequence for 14-3-3 binding, RSXŜP/TPXP (X, any amino acid; ŜP, phospho-Ser; T̂P, phospho-Thr) (19,22). To determine whether this motif acts as a 14-3-3-binding site, we constructed a deletion mutant of PX-RICS (ΔRSKSDP) in which the entire RSKSDP sequence has been removed. In vitro pull-down assays revealed that GST-14-3-3ζ was able to precipitate FLAG-tagged wild-type PX-RICS, but not ΔRSKSDP, from COS-7 cell lysates (Fig. 1D). Thus, 14-3-3 proteins may bind
Phosphorylation of a serine/threonine residue in the 14-3-3-binding motif is known to be required for interaction with 14-3-3s (19, 22). Hence, we investigated whether binding of PX-RICS to 14-3-3s is dependent on serine phosphorylation of the RSKSDP sequence. To this end, we utilized a point mutant of PX-RICS (S1796A) in which Ser1796, the second serine residue in the 14-3-3-binding motif, was replaced with Ala. We found that S1796A lacks the ability to interact with GST-14-3-3ζ (Fig. 1D). To confirm this, we performed pull-down assays using lysates from PX-RICS-expressing COS-7 cells that had been pretreated with bacterial alkaline phosphatase (BAP). We found that GST-14-3-3ζ failed to precipitate PX-RICS from the BAP-treated lysates (Fig. 1E). These results suggest that phosphorylation of Ser1796 in the 14-3-3-binding motif of PX-RICS is required for its interaction with 14-3-3s.

Inspection of the amino acid sequence of PX-RICS revealed that its 14-3-3-binding motif overlaps with a motif required for phosphorylation by CaMKII, RXXS/T (23). We therefore asked whether CaMKII phosphorylates Ser1796 of PX-RICS and, if so, whether this phosphorylation promotes the interaction between PX-RICS and 14-3-3s. In vitro translated wild-type PX-RICS and S1796A were treated or not with CaMKII, and then subjected to pull-down assays using GST-14-3-3ζ. We found that the amount of wild-type PX-RICS associated with 14-3-3ζ was increased more than three-fold by CaMKII treatment (Fig. 1F). In contrast, the amount of S1796A associated with 14-3-3ζ was not changed by CaMKII treatment. Furthermore, when we coexpressed FLAG-tagged PX-RICS and Myc-tagged 14-3-3ζ in COS-7 cells and treated the cells with the cell-permeable CaMKII inhibitor KN-93, coprecipitation of PX-RICS with 14-3-3ζ was markedly inhibited (Fig. 1G). Similarly, shRNA-mediated knockdown of CaMKII isoforms reduced the interaction between endogenous PX-RICS and 14-3-3 proteins (Fig. 1H). Taken together, these results suggest that CaMKII-mediated phosphorylation of PX-RICS at the 14-3-3-binding motif enhances its ability to interact with 14-3-3 proteins.

14-3-3ζ/θ is required for PX-RICS-mediated N-cadherin/β-catenin transport: 14-3-3 proteins are known to be involved in intracellular protein transport (19-21). Thus, we examined whether 14-3-3ζ and θ are involved in PX-RICS-mediated ER-to-Golgi transport of the N-cadherin/β-catenin complex. We generated siRNAs that target the 3’-untranslated region (UTR) of human 14-3-3ζ (siRNA-ζ-1 and -2) and θ (siRNA-θ-1 and -2), respectively (supplemental Fig. S1A) and examined their effects on the intracellular localization of N-cadherin and β-catenin. When HeLa cells were transfected with siRNA for 14-3-3ζ or θ, N-cadherin and β-catenin disappeared from the cell-cell contact sites (Fig. 2A). Double immunofluorescent staining with antibodies to N-cadherin and calnexin, an ER-resident protein, revealed that a significant fraction of N-cadherin was retained in the ER.
Double knockdown of 14-3-3ζ and θ resulted in no apparent synergistic effect on the intracellular localization of N-cadherin and β-catenin (Fig. 2A). Cell surface biotinylation assays also revealed that the amount of cell surface N-cadherin is markedly reduced in siRNA-expressing cells (Fig. 2B). We further examined by cell dissociation assays whether knockdown of 14-3-3ζ or θ affects the cell adhesion ability of HeLa cells. We found that, after mechanical dispersal in the presence of Ca²⁺, control cells remained as large aggregates but cells transfected with siRNA against 14-3-3ζ or θ dissociated efficiently to nearly single cells (Fig. 2C). These results suggest that knockdown of 14-3-3ζ or θ leads to the reduction in the amount of N-cadherin at the cell surface and consequent reduction in the cell-cell adhesion ability. These phenotypes are identical to those of PX-RICS- and GABARAP-knockdown cells (13), suggesting that 14-3-3ζ and θ are involved in PX-RICS-mediated transport of the N-cadherin/β-catenin complex.

Since 14-3-3 proteins are known to function as homo- or heterodimers that can interact with a wide variety of substrate proteins (19,22), we examined the functional interaction between 14-3-3ζ and θ. When 14-3-3ζ lacking its 3'-UTR was overexpressed in cells transfected with siRNA targeting the 3'-UTR of 14-3-3ζ, N-cadherin and β-catenin were found to be localized at the cell-cell boundaries (supplemental Fig. S1B and S2). However, overexpression of 14-3-3ζ did not restore the localization of N-cadherin and β-catenin in cells transfected with siRNA against 14-3-3θ. Similar results were obtained when 14-3-3θ lacking its 3'-UTR was overexpressed. In addition, the aberrant localization of N-cadherin and β-catenin in cells transfected with siRNAs against 14-3-3ζ and θ was rescued only by cotransfection of 14-3-3ζ and θ, but not 14-3-3ζ or θ alone (supplemental Fig. S1B and S2). These results suggest that heterodimeric 14-3-3ζθ is involved in transport of the N-cadherin/β-catenin complex.

Complex formation between PX-RICS and 14-3-3ζ/θ is essential for N-cadherin/β-catenin transport- We next assessed the significance of the interaction between PX-RICS and 14-3-3ζ/θ in transport of the N-cadherin/β-catenin complex. For this purpose, we expressed in HeLa cells a fragment of PX-RICS fused to EGFP (EGFP-1433BR) that interferes with the interaction between PX-RICS and 14-3-3ζ/θ (Fig. 3A and supplemental Fig. S1C) and examined its effects on the subcellular localization of N-cadherins and β-catenin. We found that the amounts of N-cadherin and β-catenin at the cell-cell contact sites were markedly reduced in EGFP-1433BR-expressing cells compared to the surrounding untransfected cells (Fig. 3B). In contrast, such changes were not induced by expression of mutant forms of EGFP-1433BR, EGFP-1433BR-ΔRSKSDP or EGFP-1433BR-SA (Fig. 3B), which are unable to interfere with the interaction between PX-RICS and 14-3-3ζ/θ (supplemental Fig. S1C). These results suggest that the interaction between PX-RICS and
14-3-3ζ/θ is essential for PX-RICS-mediated transport of the N-cadherin/β-catenin complex. PX-RICS/14-3-3-associated dynein/dynactin is responsible for N-cadherin/β-catenin transport.

Recent proteomic analyses of putative 14-3-3-binding partners have identified many proteins that are involved in intracellular vesicular transport, including the dynein/dynactin motor (20,21,24-28). Thus, we explored whether PX-RICS and 14-3-3ζ/θ are associated with components of forward transport machineries. In vivo pull-down assays showed that PX-RICS and 14-3-3ζ/θ coimmunoprecipitated with the intermediate chain of cytoplasmic dynein (DYNClI) and the p150 subunit of dynactin (p150Glued) (Fig. 4A). Consistent with this result, immunofluorescent staining revealed that the intracellular localization of these proteins overlapped significantly in the perinuclear region (Fig. 4A). In contrast, Sec23, a core component of the COPII coat protein complex, did not coprecipitate with PX-RICS or 14-3-3ζ/θ (data not shown). To further confirm the presence of a large multimeric complex assembled from the above-mentioned proteins, we fractionated digitonin-solubilized proteins on a linear sucrose density gradient. Pull-down assays revealed that N-cadherin, β-catenin, PX-RICS, 14-3-3ζ/θ, DYNClI and p150Glued coimmunoprecipitated with PX-RICS from fractions corresponding to a molecular mass larger, but not smaller, than 669-kD (Fig. 4B and data not shown). Taken together, these results suggest that N-cadherin, β-catenin, PX-RICS, 14-3-3ζ/θ, DYNClI and p150Glued are components of the multiprotein complex having a molecular mass of > 669-kD.

We next investigated whether dynein/dynactin is involved in N-cadherin/β-catenin trafficking by siRNA-mediated depletion of DYNClI and p150Glued. There are two distinct genes for DYNClI, DYNClII and DYNClI2. The former is expressed exclusively in neurons, whereas the latter is expressed ubiquitously (29). Thus, we targeted DYNClII for siRNA-based silencing of DYNClI in HeLa cells (supplemental Fig. S1D). In DYNClI2-knockdown cells, N-cadherin and β-catenin disappeared from the cell-cell boundaries as in PX-RICS-, 14-3-3ζ- or θ-knockdown cells (Fig. 5A). A significant fraction of N-cadherin was retained in the ER, as shown by coimmunostaining with calnexin (Fig. 5A). Silencing of p150Glued also led to a similar phenotype (supplemental Fig. S1D and Fig. 5A). We also disrupted dynactin motor function by overexpression of dynamitin, which disassembles dynactin, an activator of cytoplasmic dynein (30) (supplemental Fig. S1E). In dynamitin-expressing cells, the amounts of N-cadherin and β-catenin at the cell-cell boundaries were markedly reduced compared to the surrounding untransfected cells (Fig. 5B). These results suggest that PX-RICS/14-3-3ζ/θ-dependent transport of the N-cadherin/β-catenin complex is mediated by dynein/dynactin.

PX-RICS moves together with N-cadherin. To obtain further evidence for the involvement of PX-RICS in N-cadherin transport, we attempted...
to visualize the intracellular movement of N-cadherin and PX-RICS using time-lapse microscopic imaging of HeLa cells expressing N-cadherin-GFP and mCherry-PX-RICS. We found that N-cadherin-GFP and mCherry-PX-RICS are colocalized on the same vesicles and move together around the ER/Golgi-like field (Fig. 6A and supplemental Video S1). Some vesicles carrying both N-cadherin-GFP and mCherry-PX-RICS showed straight, long-ranged and centrifugal movements (Fig. 6B). These results are consistent with our notion that PX-RICS plays an important role in N-cadherin transport.

**DISCUSSION**

Despite the growing list of membrane proteins requiring 14-3-3 for efficient cell surface expression, the molecular mechanism by which 14-3-3 proteins exert this effect remains poorly understood (20,21). In this study, we have shown that PX-RICS interacts with 14-3-3ζ/θ to couple the N-cadherin/β-catenin cargo with the dynein/dynactin motor, and thereby mediates its ER-to-Golgi transport. Our finding provides novel insights into how proteins of the 14-3-3 family promote the cell surface expression of membrane proteins.

In our knockdown/rescue experiments, we have found that exogenous expression of 14-3-3ζ or θ can restore the intracellular localization of N-cadherin and β-catenin in 14-3-3ζ- or θ-knockdown cells, but not in 14-3-3ζ- or θ-knockdown cells (supplemental Fig. S1B and S2). In addition, the localization of N-cadherin and β-catenin in 14-3-3ζ/θ-doubly knockdown cells was rescued only by cointraduction of both ζ and θ isoforms, but not by either ζ or θ isoform (supplemental Fig. S1B and S2). These results suggest that heterodimeric 14-3-3ζ/θ is involved in transport of the N-cadherin/β-catenin complex. Thus, it is reasonable that knockdown of either ζ or θ isoform yields similar phenotypic outputs. N-cadherin was found to be stuck in the ER as clearly demonstrated by double immunofluorescence (Fig. 2A), suggesting that these knockdown phenotypes could be attributed to blocked ER-to-Golgi transport, but not to aberrant post-Golgi trafficking, including aberrant surface expression, membrane anchoring, endocytosis or recycling.

Putting our previous and present findings together, we propose a possible model for the action of PX-RICS and its interacting molecules during ER exit of the N-cadherin/β-catenin complex (supplemental Fig. S3). Our previous results suggest that PX-RICS-mediated ER-to-Golgi transport of the N-cadherin/β-catenin complex is dependent on multiple protein-protein (PX-RICS-GABARAP, -Cdc42 and -β-catenin) and protein-lipid (PX-RICS-PI4P) interactions (13). The PX domain of PX-RICS has the highest binding affinity for PI4P (15), which is a predominant phosphoinositide in the ER and Golgi membranes and plays important roles in vesicular budding and/or fusion (31-34). GABARAP is known to be
associated with the ER membrane presumably through its hydrophobic phosphatidylethanolamine tail (35,36). Thus, PX-RICS may be selectively recruited to the ER membrane through its coincident interaction with PI4P and GABARAP (32) and link the N-cadherin/β-catenin cargo with GABARAP. GABARAP may act as a binding cue to stabilize the interaction between PI4P and PX-RICS (32). 14-3-3ζ/θ heterodimer may serve as a linker to mediate the interaction between ER-anchored PX-RICS and the dynein/dynactin motor complex (19-21). Importantly, GABARAP is also known to have the ability to bind microtubules (37). The concerted action of these molecules may provide the driving force for ER exit and/or transport of the N-cadherin/β-catenin cargo along GABARAP-bound microtubules. The substrate diversity of 14-3-3 proteins may enable them to utilize other adaptor proteins in place of PX-RICS, thus allowing the dynein/dynactin motor to carry different cargo proteins without canonical ER export motifs. Similar cargo-adaptor-motor systems have also been proposed for the kinesin superfamily of proteins, another family of cytoplasmic molecular motors (38-40). Thus, the diversity of adaptors may be a common feature by which limited types of molecular motors can recognize a wide array of cargos.

REFERENCES

FOOTNOTES

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The abbreviations used are: ER, endoplasmic reticulum; GAP, GTPase-activating protein; RICS, RhoGAP involved in the β-catenin-N-cadherin and N-methyl-D-aspartate receptor signaling; NMDA, N-methyl-D-aspartate; PX, phox homology; SH3, src homology 3; PSD, postsynaptic density; GABARAP, γ-aminobutyric acid type A receptor-associated protein; PI4P, phosphatidylinositol-4-phosphate; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; BAP, bacterial alkaline phosphatase; UTR, untranslated region

FIGURE LEGENDS

FIGURE 1. Phosphorylation of PX-RICS by CaMKII enhances its interaction with 14-3-3 proteins. A, Binding of PX-RICS with 14-3-3s in vitro. Lysates from COS-7 cells expressing FLAG-tagged PX-RICS were incubated with GST or GST-fusion proteins as indicated and bound proteins were analyzed by immunoblotting with anti-FLAG antibody. The lower panel shows Coomassie staining of GST and GST-fusion proteins used in pull-down assays. GST-GABARAP was used as a positive control.
Binding of PX-RICS with 14-3-3s in vivo. HeLa cell lysates were subjected to immunoprecipitation with the antibodies indicated above each lane, followed by immunoblotting with the antibodies indicated on the right. Solid arrowheads denote 14-3-3s. The open arrowhead denotes the light chain of the antibodies used in immunoprecipitation. Immunofluorescent staining of HeLa cells with anti-PX-RICS and anti-pan-14-3-3 antibody are shown in the lower panel. C, Specific interaction of PX-RICS with the ζ and θ isoforms of 14-3-3s. HeLa cell lysates were subjected to immunoprecipitation with anti-PX-RICS antibody followed by immunoblotting with antibodies specific for 14-3-3ζ or θ. Open arrowheads indicate the light chain of the antibodies used in immunoprecipitation. The lower panel shows immunofluorescent staining of HeLa cells with anti-PX-RICS antibody plus 14-3-3ζ- or θ-specific antibody. D, The internal RSKS^{1796}DP sequence of PX-RICS is responsible for its binding to 14-3-3s. Lysates from COS-7 cells expressing FLAG-tagged wild-type PX-RICS or its mutant forms (∆RSKSDP or S1796A) were incubated with GST-14-3-3ζ, and bound proteins were analyzed by immunoblotting with anti-FLAG antibody. E, Phosphorylation of PX-RICS is required for its binding to 14-3-3s. Lysates from COS-7 cells expressing FLAG-PX-RICS were treated with buffer (-) or BAP (+) and incubated with GST-14-3-3ζ. Bound proteins were analyzed by immunoblotting with anti-FLAG antibody. F, CaMKII-mediated phosphorylation of Ser^{1796} in the 14-3-3-binding motif of PX-RICS enhances its binding to 14-3-3s. In vitro-translated wild-type and mutant PX-RICS (PX-RICS-WT and -S1796A) were treated with buffer (-) or CaMKII (+), and then subjected to pull-down assays with GST-14-3-3ζ. The representative results of densitometric quantification of precipitated PX-RICS are shown in the lower panel. G, Binding of PX-RICS to 14-3-3 is inhibited by a CaMKII inhibitor. COS-7 cells expressing FLAG-PX-RICS and/or Myc-tagged 14-3-3ζ were treated with KN-93 as indicated. The amount of PX-RICS bound to 14-3-3ζ was analyzed. H, Decreased interaction of endogeneous PX-RICS with 14-3-3 proteins due to CaMKII knockdown. Lysates from HeLa cells transfected with an empty shRNA vector (EV), mixture of four shRNAs for CaMKII isoforms (CaMKII), or control shRNA were analyzed by immunoprecipitation followed by immunoblotting.

FIGURE 2. 14-3-3ζ/θ is involved in ER-to-Golgi transport of the N-cadherin/β-catenin complex. A, Knockdown of 14-3-3ζ or θ results in the disappearance of N-cadherin and β-catenin at the cell-cell boundaries and the ER accumulation of N-cadherin. HeLa cells transfected with the indicated siRNAs were subjected to immunofluorescent staining with anti-N-cadherin plus anti-calnexin or anti-β-catenin antibody. Arrows indicate N-cadherin or β-catenin enriched at the sites of cell-cell contact. Arrowheads indicate the absence of N-cadherin and β-catenin at the borders between two neighboring cells. Scale bars, 10 µm. B, Upper panel, Knockdown of 14-3-3ζ or θ inhibits the surface expression of N-cadherin. The amounts of total and surface N-cadherin in siRNA-transfected HeLa cells were evaluated by surface
biotinylation assays. Transferrin receptor (TfR) and α-tubulin were used for positive and negative controls, respectively. Lower panel, Surface expression of N-cadherin was quantified by measuring the band intensity of the biotinylated fraction (SA-pull-down in the upper panel) compared with that of total input. Representative results of the three independent experiments are shown. C, Ca\(^{2+}\)-dependent cell adhesion is abrogated by knockdown of 14-3-3ζ or θ. HeLa cells expressing the indicated siRNAs were dissociated by pipetting in the presence of Ca\(^{2+}\). Cell adhesion activity was quantified by counting the total number of cells (N\(_c\)) and the number of particles (cell clumps) (N\(_p\)). Error bars represent the mean ± s.d. (n = 6).

FIGURE 3. Complex formation of PX-RICS with 14-3-3ζ/θ is essential for ER-to-Golgi transport of the N-cadherin/β-catenin complex. A, Schematic representation of PX-RICS-derived mutants. PX, Phox homology domain; SH3, Src homology 3 domain; GAP, GTPase-activating protein domain; GBR, GABARAP-binding region; Granin, granin motif; Pro-rich, polyproline stretch; CBR, β-catenin-binding region. Green rectangles indicate the EGFP tag. The 14-3-3-binding motif is intact in EGFP-1433BR but deleted or mutated in the other two mutants. B, Inhibition of the interaction between PX-RICS and 14-3-3ζ/θ results in the disappearance of N-cadherin and β-catenin from the cell-cell boundaries. HeLa cells were transfected with the indicated constructs and processed for immunostaining with anti-N-cadherin or anti-β-catenin antibody. Arrows indicate N-cadherin and β-catenin enriched in the cell-cell boundaries. Arrowheads indicate the N-cadherin- or β-catenin-negative boundaries of HeLa cells expressing PX-RICS-derived mutants. Scale bars, 10 µm.

FIGURE 4. The PX-RICS-14-3-3ζ/θ complex links the N-cadherin/β-catenin complex with the cytoplasmic dynein/dynactin motor. A, Upper panels, PX-RICS and 14-3-3ζ/θ are associated with the cytoplasmic dynem/dynactin complex. HeLa cell lysates were subjected to immunoprecipitation with anti-PX-RICS or anti-14-3-3ζ/θ antibody followed by immunoblotting with antibodies specific for DYNC1I or p150Glued. Open arrowheads indicate DYNC1I or p150Glued coimmunoprecipitated with PX-RICS or 14-3-3ζ/θ. Asterisks indicate proteins immunoprecipitated nonspecifically. Lower panels, PX-RICS, 14-3-3ζ and 14-3-3θ were colocalized with DYNC1I and p150Glued at the perinuclear region in HeLa cells. Scale bars, 10 µm. B, PX-RICS assembles with N-cadherin, β-catenin, 14-3-3ζ/θ, DYNC1I and p150Glued to form a large multiprotein complex. Left panel, Lysates from HeLa cells solubilized with 1 % digitonin were fractionated by sucrose density gradient (10-40 %) ultracentrifugation and each fraction was subjected to immunoblotting with the indicated antibodies. Arrows at the top indicate the mobilities of protein molecular weight markers. Right panel, Fractions 7 and 8, the highest molecular weight fractions that contain all of the above-mentioned proteins, were pooled and immunoprecipitated
with anti-PX-RICS antibody followed by immunoblotting with the indicated antibodies.

FIGURE 5. Inhibition of dynein/dynactin function leads to the absence of N-cadherin and β-catenin at the cell-cell contact sites. A, Knockdown of DYNC1I or p150<sup>Glued</sup> results in the disappearance of N-cadherin and β-catenin from the cell-cell boundaries. HeLa cells transfected with the indicated siRNAs were subjected to immunofluorescent staining with anti-N-cadherin plus anti-calnexin or anti-β-catenin antibody. Arrows indicate N-cadherin or β-catenin enriched at the sites of cell-cell contact. Arrowheads indicate the absence of N-cadherin and β-catenin at the borders between two neighboring cells. Scale bars, 10 µm. B, The disappearance of N-cadherin and β-catenin from the cell-cell boundaries due to dynamitin-mediated abrogation of dynein motor function. Arrows indicate N-cadherin and β-catenin enriched in the cell-cell boundaries. Arrowheads indicate the N-cadherin- or β-catenin-negative boundaries of HeLa cells expressing Myc-tagged dynamitin. Scale bars, 10 µm.

FIGURE 6. Time-lapse images of N-cadherin-GFP and mCherry-PX-RICS in HeLa cells. Corresponding video images are available in supplemental Video S1. A, Stills from Video S1 (at 27.2 sec) of a HeLa cell expressing N-cadherin-GFP (green) and mCherry-PX-RICS (red). EG, ER/Golgi-like field; N, nucleus. A scale bar, 5 µm. B, Magnified time-lapse images of the region boxed in A. Arrowheads indicate a typical moving vesicle that harbors both N-cadherin-GFP and mCherry-PX-RICS. A scale bar, 3 µm.
A

PX-RICS  DYNC1I  Merge  PX-RICS  p150\textsuperscript{Quad}  Merge

14-3-3\textsubscript{ζ}  DYNC1I  Merge  14-3-3\textsubscript{ζ}  p150\textsuperscript{Quad}  Merge

14-3-3\textsubscript{θ}  DYNC1I  Merge  14-3-3\textsubscript{θ}  p150\textsuperscript{Quad}  Merge

B

KDa

[Image of protein gel analysis]

Fraction No. 1 2 3 4 5 6 7 8 9 10 11 12

IP

Lysate  Rabbit IgG  PX-RICS

N-cadherin  \beta\text{-}catenin  PX-RICS  14-3-3\textsubscript{ζ}+\theta  DYNC1I  p150\textsuperscript{Quad}
A

N-cadherin-GFP  |  mCherry-PX-RICS  |  Merge

B

Elapsed time

27.2 s  28.8 s  30.4 s  32.1 s

N-cadherin-GFP  |  mCherry-PX-RICS  |  Merge

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