Noc2, a Putative Zinc Finger Protein Involved in Exocytosis in Endocrine Cells*

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We have cloned a cDNA encoding a novel protein of 302 amino acids (designated Noc2, no C2 domain) that has 40.7% amino acid identity with and 77.9% similarity to the N-terminal region of rabphilin-3A, a target molecule of Rab3A. However, unlike rabphilin-3A, Noc2 lacks two C2 domains that are thought to interact with Ca²⁺ and phospholipids. Noc2 is expressed predominantly in endocrine tissues and hormone-secreting cell lines and at very low levels in brain. Immunoblot analysis of subcellular fractions of the insulin-secreting cell line MIN6 and immunocytochemistry reveal that Noc2 is a 38-kDa protein present in the cytoplasm. Overexpression of Noc2 in PC12 cells cotransfected with growth hormone enhances high K⁺-induced growth hormone secretion. Screening a mouse embryonic cDNA library with the yeast two-hybrid system shows that Noc2 interacts with the LIM domain-containing protein zyxin, a component of the cytoskeleton, and this interaction is further confirmed by the communoprecipitation experiment. Accordingly, Noc2 is probably involved in regulated exocytosis in endocrine cells by interacting with the cytoskeleton.

EXPERIMENTAL PROCEDURES
cDNA Cloning of Noc2—700,000 plaques of a rat islet cDNA library were hybridized under low stringency conditions (17) using a mouse rabphilin-3A cDNA (16) as a probe. Five clones encoded a partial rabphilin-3A-related protein (designated Noc2). A full-length Noc2 clone was isolated from a RINm5F cDNA library. Both strands of DNA were sequenced.

RNA Blot Analysis—RNA blot analysis was performed under standard stringent hybridization conditions with 32P-labeled 580-base pair encoding the Rab3A-binding domain of rabphilin-3A as a probe. We identified a novel protein that has a high similarity to the Rab3A-binding domain of rabphilin-3A but lacks the C2 domain. Our data also suggest that Noc2 is involved in regulated exocytosis in endocrine cells by interacting with the cytoskeleton.
cein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (15).

Transfection and Growth Hormone Secretion Assay—A full-length cDNA of Noc2 or β-galactosidase was inserted into the mammalian expression vector pBSTM116 bearing a mouse embryonic cDNA library. Approximately 1.96 × 10^6 transformants were screened for growth on Yc plate medium (2% glucose, 0.5% ammonium sulfate, 1% succinic acid, and 0.12% yeast nitrogen base) containing 0.5 mM 3-amino-1,2,4-triazole but lacking tryptophan, histidine, uracil, and leucine. His^- colonies were then picked and grown in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity as described (20). Four His^- and LacZ^- positive clones were obtained with this screening. The plasmids were recovered and transformed into Escherichia coli.

Immunoprecipitation—The protein coding region Noc2 cDNA was subcloned into frame in pGEX-2T (Pharmacia). The periplasmic fraction of Noc2 was transformed into JM109. The expressed protein was affinity-purified by glutathione-Sepharose 4B (Pharmacia). The N-terminal fragment of mouse zyxin (amino acids 343–564) cDNA was subcloned in frame into pFLAG-MAC (Kodak/IIBI, New Haven, CT). The expressed protein was affinity-purified by Anti-FLAG M2 Affinity Gel (Kodak/IIBI), according to the manufacturer’s instructions. For coimmunoprecipitation experiments, the purified GST-Noc2 (1 µg) was incubated at 4 °C for 1 hr with the purified FLAG-zyxin (1 µg) in the binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 0.5 mg/ml bovine serum albumin, and 5 mM β-mercaptoethanol), and glutathione-Sepharose 4B (Pharmacia) was then added. The mixture was incubated at 4 °C for 1 hr. After washing three times with the binding buffer, the mixture was eluted with the elution buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and 10 mM glutathione) and was then subjected to immunoblot analysis. Coimmunoprecipitation with Anti-FLAG M2 Affinity Gel (Kodak/IIBI) was performed similarly.

RESULTS

Structure of Noc2—The sequence of the longest insert (1934 base pairs) of λ clones contains an open reading frame encoding a 302-amino acid protein (Mr = 33431.2) having 40.7% identity with and 77.9% similarity to the amino acid sequence of the N-terminal region (amino acids 1–304) of rat rabphilin-3A (Fig. 1). However, unlike rabphilin-3A, it lacks the C2 domains and is therefore designated Noc2 (no C2 domain). Interestingly, Noc2 has a putative metal ion-binding domain containing a cluster of cysteine residues that is also conserved in rabphilin-3A. There are a potential protein kinase A-dependent phosphorylation site and three potential protein kinase C-dependent phosphorylation sites in Noc2.

Tissue Expression of Noc2—RNA blot analysis revealed that a single 2.2- or 2.6-kb transcript of Noc2 is expressed at very high levels in pancreatic islets; at moderate to high levels in ovarian, adrenal, pituitary, and PC12 cells and the insulin-secreting cell lines RINm5F (rat), MIN6 (mouse), and HIT-T15 (hamster); and at low levels in testis, the rat GH-secreting cell line GH3, and the mouse adrenocorticotropic hormone-secreting cell line AtT20 (Fig. 2). Noc2 mRNA is detected at only very low levels in brain after a longer exposure (data not shown). Noc2 protein is detected as a 38-kDa protein in total proteins prepared from MIN6 cells and PC12 cells (data not shown).

Subcellular Localization—Fig. 3A shows the immunoblot analysis of subcellular fractions from mouse MIN6 cells probed with anti-Noc2 and anti-synaptotagmin III antibodies. Noc2 was detected as a 38-kDa protein in fractions 2–8, peaking at fraction 3. In contrast, synaptotagmin III, which is present in large dense core vesicle in MIN6 cells (15), was detected in fraction 9 and 10 (Fig. 3A). Fig. 3B shows the representative of double-immunostaining of MIN6 cell for Noc2 and insulin. Noc2 was detected diffusely and partially dotted in the cytoplasm of MIN6 cells.

Enhancement of GH Secretion from PC12 Cells Overexpressing Noc2—Total amounts of GH produced in PC12 cells transfected with human GH ranged from 3 to 10 ng/35-mm dish. The basal secretion of GH (expressed as a percentage of GH amounts released into the medium containing 4.7 mM K^+ stimulation, GH secretion (60 mM) stimulation, GH secretion

![Fig. 1. Comparison of amino acid sequences of rat Noc2 and rat rabphilin-3A.](image)

![Fig. 3A.](image)

![Fig. 3B.](image)

DISCUSSION

In the present study we have identified a novel protein designated Noc2. Noc2 has structural features characterized by 1) a high degree of similarity to the N-terminal region of rabphilin-3A, 2) no C2 domain, and 3) no Putative transmembrane region. Interestingly, Noc2 has a putative zinc finger motif composed of a cysteine-rich sequence that is conserved among rabphilin-3A and a recently identified putative Rab3 effector, RIM (21). Because rabphilin-3A has been shown to bind Rab3A via this domain in a zinc-dependent manner (22), we assumed that Noc2 might also interact with Rab3 members. However,
Noc2 did not bind Rab3A, Rab3B, or Rab3C under the conditions in which rabphilin-3A binds Rab3A, although we cannot exclude the possibility that Noc2 might bind other members of the Rab3 subfamily or their related proteins.

To search for a molecule that interacts with Noc2, we screened a mouse embryonic cDNA library using the yeast two-hybrid system (20) and identified zyxin as a candidate. Coimmunoprecipitation experiments confirmed that Noc2 interacts with zyxin. Zyxin is a cytoskeletal element that exhibits protein interaction in a zinc-dependent manner (24). It is possible, therefore, that the zinc finger domain of Noc2 interacts with one of the LIM domains in zyxin. In fact, we have found by the yeast two-hybrid system that the zinc finger containing N-terminal fragment (amino acids 1–200) of Noc2 interacts with the fragment between the first and part of the third LIM terminal region of zyxin has been shown to interact with zyxin. Zyxin is a cytoskeletal element that exhibits protein interaction in a zinc-dependent manner (24). This domain participates in protein-protein interaction in a zinc-dependent manner (24). It is possible, therefore, that the zinc finger domain of Noc2 interacts with one of the LIM domains in zyxin. In fact, we have found by the yeast two-hybrid system that the zinc finger containing N-terminal fragment (amino acids 1–200) of Noc2 interacts with the fragment between the first and part of the third LIM domain of zyxin (data not shown).

Rabphilin-3A has been shown to interact with α-actinin, a component of the cytoskeleton, and possibly to be involved in the predocking and docking process of synaptic vesicles by linking Rab3A to the cytoskeleton (25). This Rab3A-rabphilin-3A system is suggested to regulate reorganization of actin filaments (25). Interestingly, a proline-rich domain in the N-terminal region of zyxin has been shown to interact with α-actinin (26). Accordingly, Noc2 may participate in the reorganization of actin filaments by interacting with zyxin.

Although rabphilin-3A is expressed predominantly in brain, rabphilin-3A contains an unusual proline-rich N terminus followed by three tandemly arrayed LIM domains (23). The LIM domain contains the cysteine-rich consensus sequence (CX_{2}CX_{16–23}HX_{2}CX_{2}CX_{16–21}CX_{2}f(C/H/D) (24). This domain participates in protein-protein interaction in a zinc-dependent manner (24). It is possible, therefore, that the zinc finger domain of Noc2 interacts with one of the LIM domains in zyxin. In fact, we have found by the yeast two-hybrid system that the zinc finger containing N-terminal fragment (amino acids 1–200) of Noc2 interacts with the fragment between the first and part of the third LIM domain of zyxin (data not shown).

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Although rabphilin-3A is expressed predominantly in brain, Noc2 was expressed predominantly in endocrine cells and is poorly expressed in brain. Overexpression of rabphilin-3A in PC12 cells enhances high K\(^{+}\)-induced GH secretion from the cells transfected with GH (27). We have found that overexpression of Noc2 in PC12 cells also enhances high K\(^{+}\)-induced secretion, further suggesting involvement of Noc2 in Ca\(^{2+}\)-triggered secretion. Because Noc2 lacks the C2 domain and is not present in large dense core vesicle in MIN6 cells, it remains to be determined whether Noc2 interacts directly or indirectly with vesicle-associated proteins having the C2 domain. Considering these findings together, Noc2 participates in regulated exocytosis in endocrine cells probably by interacting with the cytoskeleton.

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FIG. 2. Northern blot analysis of Noc2. The sizes of the hybridized transcripts of Noc2 are indicated. The size of the transcripts of Noc2 is 2.2 kb in rat tissues and rat-derived endocrine cell lines and 2.6 kb in mouse-derived cell lines. 20 μg of total RNA, except for thyroid and pituitary (10 μg), were loaded.

FIG. 3. Subcellular localization of Noc2. A, immunoblot analysis of postnuclear fractions. Upper panel, Noc2; lower panel, synaptotagmin III (Syt.III). Noc2 is detected as 38 kDa in fractions 2–8, and synaptotagmin III is detected as 63 kDa in fractions 9 and 10. B, identification of Noc2 protein in MIN6 cells by confocal laser microscopy. MIN6 cells were immunostained with anti-Noc2 and anti-insulin antibodies and visualized by fluorescence confocal microscopy. Left panel, Noc2; right panel, insulin.

FIG. 4. Effect of Noc2 overexpression on GH secretion from PC12 cells. [a], effect of Noc2 on high K\(^{+}\) (60 mM)-induced GH secretion; [b], effect of Noc2 in basal GH secretion; [c], control in high K\(^{+}\)-induced GH secretion; [d], control in basal GH secretion. Each point was assayed in duplicate or triplicate. The values are the average of three independent experiments. Filled squares and filled circles are offset 1 min for clarity.

FIG. 5. Coimmunoprecipitation of Noc2 and zyxin. Lanes 1 and 4, GST-Noc2 alone (50 ng). Lane 2, FLAG-zyxin coimmunoprecipitated with GST-Noc2 by glutathione-Sepharose 4B. Lanes 3 and 6, FLAG-zyxin alone (30 ng). Lane 5, GST-Noc2 coimmunoprecipitated with FLAG-zyxin by Anti-FLAG Affinity Gel. Upper panel, immunoblot analysis with anti-Noc2 antibody; lower panel, immunoblot analysis with anti-FLAG M2 antibody.
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