Interaction of lp-dlg/KIAA0583, a Membrane-associated Guanylate Kinase Family Protein, with Vinexin and β-Catenin at Sites of Cell-Cell Contact*  

Received for publication, October 28, 2002, and in revised form, March 17, 2003  
Published, JBC Papers in Press, March 25, 2003, DOI 10.1074/jbc.M211004200  

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Vinexin is a recently identified cytoskeletal protein and plays a key role in the regulation of cytoskeletal organization and signal transduction. Vinexin localizes at sites of cell-extracellular matrix adhesion in NIH3T3 fibroblasts and at sites of cell-cell contact in epithelial LLC-PK1 cells. Expression of vinexin promotes the formation of actin stress fiber, but the role of vinexin at sites of cell-cell contact is unclear. Here we identified lp-dlg/KIAA0583 as a novel binding partner for vinexin by using yeast two-hybrid screening. lp-dlg/KIAA0583 has a NH2-terminal coiled-coil-like domain, in addition to four PDZ domains, an Src homology (SH) 3 domain, and a guanylate kinase domain, which are conserved structures in membrane-associated guanylate kinase family proteins. The third SH3 domain of vinexin bound to the region between the second and third PDZ domain of lp-dlg, which contains a proline-rich sequence. lp-dlg colocalized with vinexin at sites of cell-cell contact in LLC-PK1 cells. Furthermore, lp-dlg colocalized with β-catenin, a major adherens junction protein, in LLC-PK1 cells. Co-immunoprecipitation experiments revealed that both endogenous and epitope-tagged deletion mutants of lp-dlg/KIAA0583 associated with β-catenin. We also showed that these three proteins could form a ternary complex. Together these findings suggest that lp-dlg/KIAA0583 is a novel scaffolding protein that can link the vinexin-vinculin complex and β-catenin at sites of cell-cell contact.

Cell-cell adhesion is important for cell polarity, tissue morphogenesis development, and homeostasis (1–3). To this end, epithelial cells exhibit specialized structures involved in cell-cell contacts such as tight junctions and adherens junctions. Adherens junctions contain the transmembrane cell adhesion molecules, cadherins and nectins, which mediate the calcium-dependent and -independent cell-cell adhesion (1, 3, 4), respectively. The cytoplasmic domain of cadherin binds to β-catenin, which then binds to α-catenin. α-Catenin binds to actin and actin-binding proteins such as vinculin, α-actinin, and ZO-1, resulting in the link of cadherin to the actin cytoskeleton (3, 5, 6). The cytoplasmic domain of nectin binds to l-afadin, which then binds to actin and a vinculin-binding protein ponsin (4, 7, 8). Multiple protein complexes of these cytoplasmic proteins play important roles in communicating between cell adhesion systems, regulating cell-cell adhesion, and transducing signals into cells.

Vinexin is a protein localizing at cell-cell and cell-extracellular matrix junctions (9). There are at least two types of vinexin, vinexin α and vinexin β, which share a common carboxyl-terminal sequence containing three SH (Src homology) 3 domains. The larger vinexin α has an additional amino-terminal sequence containing a sorbin homology domain. Vinexin is a member of a novel adaptor protein family, including ArgBP2 and ponsin, all of which have a sorbin homology domain in the NH2-terminal half and three SH3 domains in the COOH-terminal half (8–12). Vinexin binds to vinculin, which also localizes at cell-cell and cell-matrix junctions, through its first and second SH3 domains and enhances actin stress fiber formation and cell spreading (9). Furthermore, vinexin β regulates the anchorage dependence of extracellular signal-regulated kinase activation induced by epidermal growth factor (13, 14). Therefore, vinexin plays a crucial role in regulating cell-extracellular matrix communication, but little is known about the function of vinexin at cell-cell junctions.

Vinulin is a part of the cadherin-catenin junctional complex and is involved in apical junctional organization (15–18), suggesting that its binding partner vinexin may have important roles at sites of cell-cell contact. In this study, we identified a membrane-associated guanylate kinase (MAGUK) family protein lp-dlg/KIAA0583 as a vinexin-binding protein. lp-dlg colocalized with vinexin and β-catenin at sites of cell-cell contact. lp-dlg was also co-immunoprecipitated with β-catenin. These findings suggest that lp-dlg/KIAA0583 is a scaffolding protein that can link the vinexin-vinculin complex and β-catenin at sites of cell-cell contact.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The plasmid pGBT9-vinexin β was constructed by subcloning the full-length vinexin β into the EcoRI/SalI site of pGBT9 (Clontech). Yeast two-hybrid screening was performed as described previously (9). Briefly, the yeast strain HP7c was transformed first with pGBT9-vinexin β and subsequently with a human placenta cDNA library (Clontech) fused to the GAL4 transcriptional activating domain. Transformants were screened for tryptophan, leucine, and histidine autotrophy. Histidine-positive colonies were further tested for β-galactosidase activity. Prey plasmids from dual-posi-
binding assays were performed as described previously (12). In the

region of lp-dlg was amplified by PCR and subcloned into p401F.

PCR-based Full-length lp-dlg cDNA Cloning—A 5'-rapid amplification of
cDNA ends experiment was performed using Human Placenta Maternal
Cot-1 (Clontech) cDNA as described previously (13). Primer sets 5’-GACTGATGCCAGCTGTTATCTTGTCAC and AP1 and 5’-
CCAGTCATTGACCCTTAAGCGGC and AP2 were used for the first
PCR and second PCR, respectively. The several PCR fragments gener-
ated by independent PCR were ligated into pCR2.1 (Invitrogen) and
sequenced to exclude the fragments containing nucleotide errors intro-
duced by the PCR reaction artificially. The cDNA fragment from one of
the longest clones was combined with KIAA0583 cDNA (provided by
Dr. Takahiro Nagase) using AfiIII to construct the full-length lp-dlg cDNA. The resulting full-length cDNA was subcloned into pGZ21 (9) for
expressing as GFP-tagged protein.

Antibodies—The cDNA insert of the clone (pMW6) isolated by the yeast
cell hybrid screening was subcloned into pEXEX7-1 (Amerham Biosciences) for expressing as GST-tagged protein and designed pGST-
MW6. Rabbit anti-lp-dlg antiserum was raised against GST-MW6. Polyclonal antibodies were affinity purified using GST-MW6 covalently
conjugated to Affi-Gel 10 (Bio-Rad) followed by the adsorption with
Affi-Gel 10 conjugated with GST. Anti-FLAG antibody M2, anti-GFP
antibody, and anti-β-catenin antibody were obtained from Sigma, Santa
Cruz Biotechnology, and Transduction Laboratories, respectively. An-
ti-HA antibodies were purchased from Roche Diagnostics and Santa
Cruz Biotechnology.

Northern Blotting—A cDNA fragment of 1557 base pairs of the
lp-dlg coding region was radiolabeled using a random Primer DNA labeling kit (2.0 (TAKARA) and then used to probe the Human
Multiple Northern blot (Clontech) containing 2 μg of poly(A)⁺ RNA, as described previously (9).

In Vitro Binding Assay Using Affinity Precipitation—The GST-fused proteins containing vinexin deletion mutants, GST-1stSH3, GST-
2ndSH3, GST-3rdSH3, GST-3rdSH3/WF, and GST-3rdSH3/YV were de-
scribed previously (14). Full-length vinexin β was subcloned into pEXEX7-1. The cDNA insert of pMW6 was subcloned into p401F (9) to
make FLAG-tagged MW6. To construct FLAG-tagged lp-dlg deletion
mutants (644–909, 842–879, and 880–909), the correspond-
ing region of lp-dlg was amplified by PCR and subcloned into p401F. In vitro binding assays were performed as described previously (12). In brief, COS-7 cells were transiently transfected with various GST-
tagged constructs and washed twice with phosphate-buffered saline and
lysed in Triton xylene buffer (1% Triton X-100, 100 μg/ml p-amidi-
nophenylmethanesulfonyl fluoride hydrochloride, 10 μg/ml aprotinin,
10 μg/ml leupeptin). The lysates were incubated with 3 μg of each GST
fusion protein and glutathione-Sepharose 4B (Amerham Biosciences)
at 4 °C for 3 h. After four washes with lysis buffer, co-precipitated
proteins were resolved by 8% SDS-PAGE and analyzed by Western blot
with anti-FLAG M2 antibody.

Immunoprecipitation—COS-7 cells were transiently transfected with
p401F-MW6 with or without expression plasmid for GFP-tagged vin-
exin β (9). COS-7 cells were lysed as described above and equal amounts of
lysates were incubated with 5 μg of anti-FLAG antibody M2 for 1 h at
4 °C. The immunocomplexes were incubated with protein G-Sepharose
for 1 h and washed four times with lysis buffer. The bound proteins were detected as described above with anti-FLAG antibody. To examine the interaction of FLAG-vinexins with endogenous lp-dlg, FLAG-tagged vinexin genes were subcloned into the pLRT-X, which was then transfected into HEK293T-On (Tet-On) expressing a tetracycline-On (Tet-On) expression system. After 24 h, the cells were then lysed and immunoprecipitated with anti-FLAG M2 antibody as described above. The bound proteins were detected using
anti-GFP antibody. To detect the interaction of endogenous lp-dlg with
β-catenin, endogenous lp-dlg from LLC-PK1 cells was immunoprecipi-
tated with anti-lp-dlg antibody. Co-precipitated β-catenin was detected as described above using anti-β-catenin antibody.

Immunostaining—For immunostaining of endogenous lp-dlg with GFP-vinexin β, LLC-PK1 cells were transfected with GFP-tagged vin-
exin β using LipofectAMINE (Invitrogen). The cells were fixed with
cold methanol at room temperature for 1 min. For co-immunostaining of
endogenous lp-dlg and β-catenin, LLC-PK1 cells were fixed with acet-
ene at room temperature for 30 s. Immunofluorescence staining was
performed as described previously (9). The fluorescence images were
obtained using an Axiovert microscope (Carl Zeiss) equipped with a
MicroRadiance confocal laser scanning microscope (Bio-Rad).

RESULTS

Identification of lp-dlg/KIAA0583 as a Novel Vinexin Binding Partner—Vinexin binds to vinculin and localizes at cell-cell and
matrix junctions, but little is known about the function of
vinexin at cell-cell junctions. To elucidate the function of
vinexin at sites of cell-cell contact, we first performed the yeast
two-hybrid screening using mouse vinexin β as a bait to isolate
vinexin-binding proteins localizing at sites of cell-cell contact.

The cDNA library constructed from human placenta poly(A)⁺ RNA was screened, and four independent clones were isolated
as positive clones for both histidine autotrophy and α-amin-
acids. Sequence analysis revealed that one of these
clones yielded the longest clone in this study. BLAST search analysis of
this clone isolated by two-hybrid screening. The region used as a probe
for Northern blotting in Fig. 2 is shown as a solid line. Amino acid residues
were numbered from the first methionine of lp-dlg. B, predicted amino acid sequence of lp-dlg (GenBank™/EMBL/BibJ accession numberAB918651) is shown. PDZ domains are underlined once. The SH3
domain is underlined twice. The GUK domain is boxed.

![Fig. 1. Schematic diagram of the domain structure of lp-dlg and its predicted amino acid sequences. A, lp-dlg contains the NH2-terminal coiled-coil region, four PDZ domains, an SH3 domain, and a GUK domain. Each domain is depicted as a black bar. The boundaries of the domains are indicated by vertical lines. The region that binds to vinexin is shaded. The predicted amino acid sequence of lp-dlg is shown below the diagram. The amino acid residues that are conserved in all PDZ domains are shown as solid line. The GUK domain is a cDNA fragment of 1–1557 base pairs of the lp-dlg coding region, which is labeled with a radioactively labeled random primer. The region of lp-dlg containing the GUK domain is shown by the solid line. The predicted amino acid sequence of lp-dlg (GenBank™/EMBL/BibJ accession numberAB918651) is shown. PDZ domains are underlined once. The SH3 domain is underlined twice. The GUK domain is boxed.](https://www.jbc.org/)

![A](https://www.jbc.org/)

![B](https://www.jbc.org/)
p-dlg was also part of KIAA0583 gene product (23) although there were no overlapping regions of p-dlg with pMW6 cDNA. Because KIAA0583 was predicted not to be a full-length cDNA, we first performed 5'-rapid amplification of cDNA ends (RA-PCR) to construct the full-length cDNA (Fig. 1A). This protein, named lp-dlg (large type of p-dlg), has four PDZ domains, an SH3 domain, and a GUK domain, which are conserved structures among most MAGUK family proteins. In addition to these conserved domains, lp-dlg has a coiled-coil region, which can work as a protein interacting region, at the NH2 terminus.

We performed Northern blot analysis to determine the tissue distribution of lp-dlg mRNA. A human multiple tissue Northern blot was hybridized with the cDNA of the NH2-terminal region of lp-dlg (Fig. 1A). A major band of 8.5-kb transcripts was detected (Fig. 2A). A faint band of 4.5 kb was detected in some tissues (Fig. 2A). The expression level of lp-dlg was the highest in the placenta and modest in the brain, heart, skeletal muscle, and kidney. Interestingly, the short form of the alternative splicing variant, p-dlg, was reported to be expressed at a high level in placenta but not in the brain or heart (23), suggesting that splicing is regulated in a tissue-specific manner.

To confirm the expression of lp-dlg in cultured cells, Western blot analysis was performed using anti-lp-dlg polyclonal antibody. Two major protein bands of 250 and 200 kDa were detected at high levels in LLC-PK1 cells and moderately in other cell lines (Fig. 2B). Both proteins were also detected by another anti-lp-dlg antibody purified from a different rabbit serum (data not shown), suggesting that they are isoforms. To determine which proteins are translated from lp-dlg mRNA, full-length cDNA of lp-dlg was transfected into COS-7 cells. Proteins of 250 kDa were detected in addition to endogenous proteins (data not shown), suggesting that the 250-kDa protein is translated from lp-dlg mRNA and that the 200-kDa protein is from another splicing variant of lp-dlg.

Interaction of Vinexin with lp-dlg—To determine the region of the lp-dlg binding site in vinexin, various deletion mutants of vinexin were fused with the GAL4 DNA-binding domain (Fig. 3A). The two-hybrid system using these deletion mutants as bait plasmids and pMW6 as a prey plasmid were performed. Transformants containing vinexin β, 2nd+3rdSH3, and 3rdSH3 showed histidine autotrophy, suggesting that vinexin β binds to lp-dlg through its third SH3 domain (Fig. 3B). To further confirm the interaction of vinexin β with lp-dlg in vitro, various SH3 domains of vinexin β were expressed and purified as GST fusion proteins. These GST fusion proteins were immobilized on a glutathione-Sepharose 4B and incubated with cell lysates from COS-7 cells expressing FLAG-MW6. The bound proteins were analyzed by immunoblotting using an anti-FLAG antibody. Consistent with the results of the yeast two-hybrid system, GST-vinexin β and GST-3rdSH3 were able to interact with FLAG-MW6 (Fig. 4A). In contrast, neither GST-1stSH3 nor GST-2ndSH3 bound to FLAG-MW6 (Fig. 4B), suggesting that the interaction of the third SH3 domain of vinexin and lp-dlg is specific. Furthermore, the 3rdWF mutant (tryptophan residue at position 306 to phenylalanine) and the 3rdYV mutant (tyrosine residue at position 324 to valine), which lost the binding ability to the target protein Sos (14), could not interact with FLAG-MW6 (Fig. 4B).

To map the vinexin binding site in lp-dlg, various deletion mutants of lp-dlg (Fig. 5A) tagged with the FLAG epitope were transfected into COS-7 cells. These mutants were expressed comparably in COS-7 cells (Fig. 5B). Cell lysates were then incubated with GST-vinexin β. Immunoblotting using anti-FLAG antibody against the bound proteins showed that vinexin β interacted with FLAG-MW6, 644–909, 842–909, and 842–879 strongly but not 880–909 (Fig. 5B). Similar results were also obtained using GST-3rdSH3 in place of GST-vinexin β (data not shown). These results suggest that the third SH3 domain of vinexin β binds to the

![Fig. 2. Northern blotting and Western blotting of lp-dlg. A. Northern blotting using lp-dlg. The cDNA fragment, isolated by 5'-rapid amplification of cDNA ends shown in Fig. 1A, was labeled with 32P and hybridized with poly(A)-RNA resolved by electrophoresis and transferred to a nylon membrane. Autoradiography of the blot is shown with the mobilities and sizes of molecular weight markers. B. Protein samples extracted from COS-7, LLC-PK1, HeLa, and Madin-Darby canine kidney cells were resolved by SDS-PAGE, followed by Western blot analysis using the affinity purified anti-lp-dlg antibody.](image-url)
region of 842–879 of lp-dlg, which contains proline-rich putative SH3 binding sequences.

To confirm the in vivo association of vinexin β with lp-dlg, co-immunoprecipitation experiments were performed. GFP-tagged vinexin β was transfected with or without FLAG-MW6 into COS-7 cells. Equal amounts of total protein lysates were immunoprecipitated using anti-FLAG antibody. As shown in Fig. 6A, GFP-vinexin β was co-immunoprecipitated with FLAG-MW6. To verify the interaction of vinexin with full-length lp-dlg, FLAG-tagged vinexin α and β were expressed in LLC-PK1 cells. Total cell lysates were immunoprecipitated using anti-FLAG antibody, and co-immunoprecipitated endogenous lp-dlg was examined by immunoblotting using anti-lp-dlg antibody. Precipitated lp-dlg was detected in cells expressing both FLAG-tagged vinexin α and β, but not in LLC-PK1 (Fig. 6B). Together these observations suggest that vinexin β interacts with lp-dlg both in vitro and in vivo.

Subcellular Localization of lp-dlg—To determine whether lp-dlg and vinexin are colocalized at sites of cell-cell contact, GFP-tagged vinexin β was transfected into LLC-PK1 cells where lp-dlg was expressed at a high level. The subcellular localization of vinexin β was observed by GFP fluorescence, and the same cells were also stained with anti-lp-dlg polyclonal antibody and Alexa 568-labeled secondary antibody. GFP-tagged vinexin β showed the localization at both sites of cell-cell and cell-extracellular matrix junctions, in addition to a diffuse pattern (Fig. 7A). Endogenous lp-dlg was also concentrated at sites of cell-cell contact and colocalized with vinexin β (Fig. 7A). Interestingly, lp-dlg was not concentrated at sites of cell-extracellular matrix junction (Fig. 7A, inset). Furthermore, lp-dlg was partially colocalized with β-catenin, a major component of adherens junctions (Fig. 7B). We also examined the localization of lp-dlg protein in frozen tissue sections of mouse placenta, where lp-dlg mRNA was expressed at high levels (Fig. 2A), and found that lp-dlg and β-catenin were also colocalized in tissues (data not shown).

Interaction of lp-dlg with β-Catenin—Co-localization of lp-dlg and β-catenin both in cultured cells and in tissues raises the possibility of the interaction between these two molecules. To examine this possibility, FLAG-tagged lp-dlg mutants were transiently expressed in COS-7 cells by transfection with GFP-tagged β-catenin. Equal amounts of total protein lysates were immunoprecipitated using anti-FLAG antibody. As shown in Fig. 8A, GFP-β-catenin was co-immunoprecipitated with FLAG-MW6 but not with vector alone, suggesting that lp-dlg can associate with β-catenin. FLAG-MW6 also contained the binding site for β-catenin both in cultured cells and in tissues (see Fig. 2F). We also examined the localization of lp-dlg protein in frozen tissue sections of mouse placenta, where lp-dlg mRNA was expressed at high levels (Fig. 2A), and found that lp-dlg and β-catenin were also colocalized in tissues (data not shown).
that endogenous β-catenin was co-immunoprecipitated with endogenous lp-dlg. β-Catenin was not detected in the precipitate using preimmune serum. E-cadherin and α-catenin showed very weak association with lp-dlg compared with that of β-catenin (data not shown). These results suggest that lp-dlg associates with β-catenin as well as vinexin at sites of cell-cell contact.

To determine whether lp-dlg forms a ternary complex with vinexin β and β-catenin, GFP-vinexin β and HA-β-catenin were transiently expressed in COS-7 cells with or without FLAG-MW6, and then immunoprecipitated with anti-HA antibody. GFP-vinexin β was barely co-precipitated with HA-β-catenin without FLAG-MW6 (Fig. 8C). However, GFP-vinexin β was co-precipitated significantly with HA-β-catenin when FLAG-MW6, which includes the binding sites for both vinexin β and β-catenin, was coexpressed (Fig. 8C). This observation suggests that lp-dlg can form a ternary complex with vinexin β and β-catenin and function as a scaffolding protein.

**DISCUSSION**

Vinexin is a vinculin-binding protein localized at cell-cell and cell-extracellular matrix junctions. We previously showed that vinexin enhances cell spreading and cytoskeletal organization and regulates the anchorage dependence of extracellular signal-regulated kinase activation, but the roles of vinexin at cell-cell junctions is not known. In this study, we identified a novel MAGUK protein, lp-dlg, as a binding partner of vinexin. We showed that lp-dlg binds to vinexin both using the two-hybrid system and by in vitro binding assay. Immunoprecipitation assay showed that they can also make a complex in vitro. In addition, lp-dlg and vinexin were colocalized at sites of cell-cell contact. lp-dlg was also colocalized with and bound to β-catenin, a major adherens junction protein. These observations suggest that lp-dlg is a novel binding partner of vinexin at sites of cell-cell contact.

β-Catenin connects the cytoplasmic domain of cadherin to α-catenin, which then binds to the actin cytoskeleton directly or indirectly through vinculin. In this study, we showed that both the endogenous and the deletion mutants of lp-dlg can bind to β-catenin. The region (472–644) containing the first and second PDZ domains of lp-dlg were required for this association. β-Catenin has been shown to have a PDZ-target like sequence (-TTL) at its COOH-terminal end and to bind to proteins containing PDZ domains (24, 25). Thus, the first or second PDZ domain of lp-dlg may mediate the interaction of lp-dlg with β-catenin. We also showed that lp-dlg can form a ternary complex with β-catenin and vinexin β. The function of this complex is unclear so far. However, it is possible that the β-catenin/lp-dlg/vinexin complex can link cadherin to actin cytoskeleton through vinexin binding to vinculin and contribute to the formation of cell-cell contacts. Alternatively, β-catenin/lp-dlg complex might compete with the vinexin binding to Sos, a guanine nucleotide exchange factor for Ras and Rac, and modulate the signaling, because both Sos and lp-dlg bind to the third SH3 domain of vinexin β (14). Further studies are necessary to examine these possibilities.

lp-dlg contains an NH2-terminal coiled-coil region, four PDZ domains, an SH3 domain, and a GUK domain, and belongs to the MAGUK protein family. This domain structure is slightly
different from typical MAGUK proteins, which have one or three PDZ domains and no coiled-coil regions. In addition, none of the four PDZ domains of lp-dlg have the GLGF motif, which is conserved in most PDZ domains and is necessary for binding to S/T-X-Φ (Φ is a hydrophobic residue). A recently proposed classification (26) also classified the four PDZ domains of lp-dlg into different groups from the PDZ domains of typical MAGUK proteins. Thus, lp-dlg may have different target proteins and different functions from other MAGUK proteins.

We showed that the third SH3 domain of vinexin β bound to the region of 842–879 of lp-dlg. This binding was specific, because neither the first nor the second SH3 domain of vinexin β bound to lp-dlg. In addition, the result that point mutations in the third SH3 domains disrupted the binding ability to lp-dlg suggests the specific interaction, although other proteins containing SH3 domain may also bind to lp-dlg. The region of 842–879 of lp-dlg included the proline-rich sequences, RagPlt-PPkPPRR. Two sequence motifs, RXXPXP and PXXPXR, were reported to be a consensus for binding to SH3 domains (27). Interestingly, the proline-rich sequences in 842–879 of lp-dlg contain both consensus motifs, suggesting that they mediate the interaction with the third SH3 domain of vinexin β.

During preparation of the present manuscript, another splicing variant of lp-dlg, DLG5, was reported (28). DLG5 was isolated from the heart as one of the genes located on chromosome 10q22, where familial atrial fibrillation was mapped, and excluded as a possible cause of familial atrial fibrillation (28). DLG5 contains an additional 45 amino acids at the NH2-terminal compared with lp-dlg. Both DLG5 and lp-dlg cDNA contain stop codons upstream of their first ATG in-frame, suggesting that both products include whole coding regions of each product. lp-dlg was isolated from placenta, where lp-dlg/DLG5 expression is high, and DLG5 from the heart, where lp-dlg/DLG5 expression is lower (Fig. 2). Thus, lp-dlg and DLG5 may represent the splicing variant expressed in the placenta and heart, respectively.

Acknowledgments—We thank Dr. T. Nagase, Dr. M. Ozawa, and Dr. M. Hagiwara for the generous gifts of KIAA0583 cDNA, β-catenin cDNA, and pLRT-X, respectively.

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J. Biol. Chem. 2003, 278:21709-21714.
doi: 10.1074/jbc.M211004200 originally published online March 25, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211004200

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