Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin

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gp600/megalin, an endocytic receptor, belongs to the low-density lipoprotein receptor family. It is most abundant in the renal proximal tubular cells, where it is implicated in the reabsorption of a number of molecules filtered through the glomerulus. The cytoplasmic tail (CT) of gp600/megalin contains a number of sequence similarities, which indicate that gp600/megalin might be involved in signal transduction. To find intracellular proteins that would interact with the gp600/megalin CT, a human kidney cDNA library was screened by using the yeast two-hybrid system. The phosphotyrosine interaction domain (PID) of the Disabled protein 2 (Dab2), a mammalian structural analogue of *Drosophila* Disabled, was found to bind to the gp600/megalin CT in this system. The interaction between these two proteins was confirmed by a binding assay *in vitro* and by the co-immunoprecipitation of both proteins from renal cell lysates. The gp600/megalin CT contains three ΨXNPXY motifs (in which Ψ represents a hydrophobic residue) that are potentially able to interact with PID. Analysis of the CT deletion and point-mutation variants of gp600/megalin by the two-hybrid system revealed that the third ΨXNPXY motif is most probably involved in this interaction. Dab2 is a mitogen-responsive phosphoprotein thought to be an adaptor molecule involved in signal transduction, and a suggested negative regulator of cell growth. Dab2 is the first intracellular ligand identified for gp600/megalin; gp600/megalin is the first known transmembrane receptor that interacts with the cytosolic protein Dab2. We speculate that their interaction might involve gp600/megalin in signal transduction pathways or might mediate the intracellular trafficking of this receptor.

Key words: Disabled 2, NPXY motif, phosphotyrosine interaction domain.

INTRODUCTION

The giant endocytic receptor glycoprotein gp600/megalin was first discovered independently by two groups of investigators [1,2] as a target autoantigen involved in the active Heymann nephritis of rats. Active Heymann nephritis of rats, an autoimmune glomerular disease, is an accepted immunohistological and clinical model of human membranous glomerulonephritis [3,4]. The target autoantigen of active Heymann nephritis is a renal glycoprotein with a molecular mass of approx. 600 kDa, variously named gp600, gp330, LRP-2 and 'megalin' [1,2,5,6]. This single-chain protein contains a C-terminal cytoplasmic domain (213 amino acid residues), a single transmembrane domain (22 residues), and an extremely large ectodomain (4400 residues). The protein belongs to the low-density lipoprotein (LDL) receptor family and is most closely related in structure to LDL receptor-related protein/α2-macroglobulin receptor (LRP) [6,7]. However, gp600/megalin and LRP differ in their numbers and arrangements of the repeated sequences and in their distributions in various cell types. In addition, their C-terminal cytoplasmic domains have no similarity except for three short ΨXNPXY sequences (in which Ψ represents a hydrophobic residue) and a few residues surrounding these sequences. Although gp600/megalin is undoubtedly involved in receptor-mediated endocytosis, its overall function in various tissues remains largely unknown at present. This protein is highly conserved in evolution [8] and the interruption of its gene in mice (knock-out) is mostly lethal. The few surviving pups die soon after birth from respiratory failure and have a malformed forebrain [9].

A number of protein ligands have been identified previously for the ectodomain of gp600/megalin, including plasminogen [10], complexes of tissue-type or urokinase-type plasminogen activator (tPA or uPA) and plasminogen activator inhibitor type 1 (PAI-1) [11,12], lactoferrin, lipoprotein lipase [12], fibronectin, laminin, type I collagen [13] and the so-called receptor-associated protein (RAP) [14,15]. It has been shown that this last is a chaperone involved in the correct folding of LDL-receptor family members during their maturation in the reticulum [16,17]. The biological role of the other interactions is not clear at present. However, an important physiological role had been suggested recently for the absorption of several molecules: albumin [18], the transcobalamin–vitamin B_{12} complex [19], apolipoprotein J [20], apolipoprotein H [21], retinol [22], and the vitamin D–vitamin-D-binding protein complex [23].

It has been noted that the C-terminal cytoplasmic domain of gp600/megalin or cytoplasmic tail (CT), which is the longest of the members of the LDL-receptor family, contains intriguing and potentially functional motifs. They include several SH3 (Src homology 3) recognition motifs, one SH2 (Src homology 2) recognition motif for the p85 regulatory subunit of phosphoinositide 3-kinase, and additional sites for protein kinase C, casein kinase II and cAMP-/cGMP-dependent protein kinase [24]. It has therefore been suggested that this protein might have a role not only in endocytic processes but also in signal transduction. Identification of the relevant intracellular partners

Abbreviations used: CT, cytoplasmic tail; GST, glutathione S-transferase; IRPT cells, immortalized rat proximal tubular cells; LDL, low-density lipoprotein; LRP, LDL receptor-related protein/α2-macroglobulin receptor; PID, phosphotyrosine interaction domain; Ψ, hydrophobic amino acid residue; QDO medium, quadruple dropout medium; SV40, simian virus 40.

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of gp600/megalin should shed light on the possible role of this endocytic receptor in signal transduction.

In the present study we have used the yeast two-hybrid system to screen a kidney cDNA library to identify proteins that can interact with the gp600/megalin CT. We have found that Dab2 adaptor protein, a candidate for the negative regulation of cell growth and tumour suppression, can bind through its phosphotyrosine interaction domain (PID) to the gp600/megalin CT. This interaction might involve gp600/megalin in the Ras- and/or Jun-signalling pathways mediated by the interaction of Dab2 with another adaptor protein Grb2 [25], involved in the Ras-signalling pathway [26] and in the regulation of the Jun-signalling pathway [27].

EXPERIMENTAL

Materials

All oligonucleotides were synthesized by Gibco-BRL Life Technology (Grand Island, NY, U.S.A.). gp600/megalin C-terminal peptide (the last 17 residues of gp600/megalin plus the N-terminal cysteine residue for conjugation to keyhole limpet haemocyanin) was synthesized by Chiron Mimotopes (San Diego, CA, U.S.A.).

Antibodies

Antibodies against Dab2 (goat polyclonal anti-peptide N19 and D19; human-, rat- and mouse-specific) and horseradish-peroxidase-conjugated anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish-peroxidase-conjugated anti-rabbit antibody was purchased from Dynatech Diagnostics (South Windham, ME, U.S.A.). Antibody against gp600/megalin was prepared previously [28]. Antibody against a synthetic gp600/megalin C-terminal peptide conjugated to keyhole-limpet haemocyanin was prepared in rabbit at the Animal Facilities of the University of California at Davis. It was affinity-purified on C-terminal peptide immobilized on Sulfolink resin (Pierce, Rockford, IL, U.S.A.) and tested for specificity against gp600/megalin by Western blotting. Antibody against the glutathione S-transferase (GST) fusion protein GST–Dab2 PID was prepared in rabbit at the Animal Facilities of the University of California at Davis. It reacted on the Western blots with the same protein band as commercial anti-Dab2 antibody N19 or D19.

Two-hybrid library screening

cDNA coding for the human gp600/megalin CT was synthesized by PCR with Marathon PCR-ready human kidney cDNA (Clontech, Palo Alto, CA, U.S.A.) and a pair of oligonucleotide primers that were designed on the basis of the human megalin cDNA sequence [24]: N-primer, 5′-CCGAATTCCACTATAG-AAGGACCGGCTC-3′ (nt 13440–13462); C-primer, 5′-TTTC-TGCAGCTGGTATAGCTATACTTCAGAGTC-3′ (complementary nt 14083–14058). This PCR fragment was treated with *Eco*RI and *Pst*I restriction endonucleases and cloned into the pAS2-1 vector (Clontech) to serve as a bait. This construct (pAS2-1:human CT) was transformed into the yeast PJ69-2A strain. Expression of the recombinant protein (with predicted molecular mass approx. 50 kDa) containing the Gal4 DNA-binding domain and the gp600/megalin CT was confirmed by Western blotting with antibody directed against the synthetic gp600/megalin C-terminal peptide (the last 17 residues) (results not shown). After transformation, this strain was mated with the Y187 strain pre-transformed with a human kidney cDNA library cloned into the pACT2 vector (Clontech). Screening was performed on plates without two nutritional markers for plasmids (Leu and Trp) and two nutritional markers for protein interaction (Ade and His) [so-called quadruple dropout (QDO) medium] in accordance with the manufacturer's protocol. Of the 2×10^6 mated yeast clones, 10 were selected on the QDO medium. All of these clones were tested for β-galactosidase expression, an additional marker of the protein interaction. All 10 clones were active in this assay. To test that the interaction occurred between the cloned library protein and CT but not between the library protein and Gal4 DNA-binding domain of the fusion protein, the library plasmids (pACT2:library cDNA) were rescued from the yeast clones selected on the QDO medium, purified and co-transformed into the PJ69-2A strain along with the (1) pAS2-1:human CT or (2) pAS2-1 constructs. Clones that grew only if co-transformed with construct (1) but not construct (2) were considered to be demonstrating true interaction. The cDNA inserts of the library plasmids from true positive clones were sequenced at the sequencing facility of the University of California at Davis and compared with the other sequences in the databases with a BLAST search at the NCBI World Wide Web site.

Cell cultures

L2 rat yolk sac carcinoma cell line [29], expressing gp600/megalin [30], was kindly provided by Dr Wewer (University Institute of Pathological Anatomy, Copenhagen, Denmark). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum and antibiotics. The immortalized rat proximal tubular (IRPT) cell line, which produces both membrane-bound and soluble forms of gp600/megalin but not LRP [31,32], was a gift from Dr Ingelfinger (Department of Pediatrics, Harvard Medical School, Boston, MA, U.S.A.). The IRPT cells were maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 25 mM Hepes, pH 7.0, 0.1 mM non-essential amino acids and 5% (v/v) fetal bovine serum. The IRPT cells were passaged every 3 days and passages 35–45 were used in the experiments. Both cell lines were grown to 90% confluence in 100 mm Petri dishes for the experiments.

Construction, expression and purification of the GST–Dab2 PID fusion protein

The cDNA insert from the pACT2:oz116 plasmid (rescued from one of the true positive clones) was excised by *Eco*RI plus *Xho*I and subcloned into pGEX4T-2. This construct was expressed in the JM109 *Escherichia coli* strain; the fusion protein GST–oz116 was purified on a glutathione–Sepharose column (Pharmacia Biotech, Piscataway, NJ, U.S.A.). The oz116 insert contains Dab2 PID; this fusion protein is therefore also designated GST–Dab2 PID. The fusion protein contained residues 1–176 of human Dab2 [33].

Protein binding *in vitro*

L2 and IRPT cell lysates were prepared by solubilization of the cells, grown in 100 mm Petri dishes, in 1 ml of PBSC buffer [PBS containing 1% (v/v) Nonidet P40, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$] supplemented with 1 mM PMSF, 5 μg/ml aprotinin and 0.1 mM $NaVO_4$. GST–Dab2 PID (6 μg) or GST (6 μg) bound to 10 μl of glutathione–Sepharose were incubated for 2 h at 4 °C with 1 ml of cell lysates. The resin was collected by centrifugation

and washed three times with PBSC buffer. Proteins were eluted with SDS gel-loading buffer, separated by SDS/PAGE [6% (w/v) gel], transferred to Immobilon P membrane (Millipore Corp., Bedford, MA, U.S.A.) and subjected to Western blot analysis with rabbit anti-(gp600/megalin C-terminal peptide) antiserum diluted 1:200.

Co-immunoprecipitation of gp600/megalin and Dab2

L2 and IRPT cell lysates, prepared as described above, and kidney lysate, prepared as described below (Western Blotting), were incubated for 90 min at 4 °C with (1) 10 μl of rabbit anti-(gp600/megalin) antiserum [28], (2) 2 μg of rabbit anti-(gp600/megalin C-terminal peptide) antibody, (3) 2 μg of irrelevant rabbit anti-(transcription factor D) antibody (Santa Cruz Biotechnology), (4) 10 μl of rabbit anti-(Dab2 PID–GST fusion protein) antiserum or (5) 10 μl of normal rabbit serum; 15 μl of Protein A–Sepharose was then added and incubation was continued for a further 1 h at 4 °C. Beads were washed three times with PBS buffer plus 0.05% (v/v) Tween 20 and three times with PBS. Proteins were eluted with SDS gel loading buffer, separated by SDS/PAGE [4–15% (w/v) gradient gel], transferred to Immobilon P membrane (Millipore) and subjected to Western blot analysis with rabbit polyclonal anti-(GST–Dab2 PID) antiserum (diluted 1:200), anti-(gp600/megalin C-terminal peptide) antiserum (diluted 1:200) or anti-(gp600/megalin) antibody (diluted 1:100) [28].

Western blotting

Total rat kidney lysate was prepared by the following procedure. A rat kidney was homogenized in 5 ml of buffer A [25 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/50 mM KCl/1 mM dithiothreitol] supplemented with 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 0.1 mM $NaVO_4$, then centrifuged at 10000 ***g***. Supernatant was collected, mixed with Triton X-100 [1% (v/v) final concentration], frozen in liquid nitrogen and stored at −80 °C. L2 and IRPT cell lysates were prepared as described above.

Proteins from kidney and cell culture lysates were separated by SDS/PAGE and transferred to Immobilon P membrane. Membrane was blocked in 5% (v/v) milk/0.1% (v/v) Tween 20 in PBS, incubated with appropriate primary antibodies diluted in the same solution for 2 h at room temperature and then washed three times with PBS/0.05% (v/v) Tween 20. After incubation with appropriate secondary antibody conjugated with horseradish peroxidase (dilution 1:20000 for anti-rabbit IgG and 1:2000 for anti-goat IgG), the membrane was washed three times with PBS/0.05% (v/v) Tween 20, twice with PBS, developed with a LumiGLO Chemiluminescent substrate kit (KPL, Gaithersburg, MD, U.S.A.) and exposed to film.

Human gp600/megalin C-tail deletion variants

Plasmid DNA pAS2-1:human CT was used to prepare C-tail deletion variants by PCR. N-terminal oligonucleotide primer 5′-CCGAATTCCACTATAGAAGGACCGGCTC-3′ and six deletion C-terminal primers (CTD1, 5′-TTGTCGACTAGGTTGGAGTTGGGTCTCTTCTC-3′; CTD2, 5′-TTGTCGACTAACTTTCCTTTTGCTCGTTCTCCATC-3′; CTD3, 5′-TTGTCGACTCATTTTGTCACCTGAGTTCCATCAG-3′; CTD4, 5′-TTGTCGACTATACAGTCACCTGGATTGGCTGAAC-3′; CTD5, 5′-TTGTCGACTACACTCCAATATCCATGTTAAGATC-3′; CTD6, 5′-TTGTCGACTAGGGCTTGACGAGACTGCTTAAGC-3′) were used to amplify the corresponding deletion variants of the gp600/megalin C-tail. Each of the C-terminal primers contained a stop codon (underlined). Amplified DNA was digested with *Eco*RI/*Sal*I and cloned into the pAS2-1 vector digested with the same enzymes. Corresponding constructs were designated CTDel-1 to CTDel-6. Each of them was co-transformed with oz116 plasmid (pACT2:oz116) into PJ69-2A yeast strain and plated on medium without Leu and Trp to select co-transformants. The co-transformants obtained were plated on QDO medium to verify interacting pairs. For testing the interactions semiquantitatively through β-galactosidase activity, the mutated constructs and oz116 plasmid were co-transformed into the Y187 strain and selected on medium without Leu and Trp.

Human gp600/megalin C-tail point-mutation variants

Plasmid DNA pAS2-1:human CT was used to prepare C-tail point-mutation variants by using a QuikChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Appropriate pairs of oligonucleotides (sequences available from the authors) for each site-directed mutation were designed and used in this work. We prepared the following point-mutation constructs: 1NA, 2NA, 3NA and 2KP. The number in each construct indicates the number (first, second or third) of the ΨXNPXY motif in Figure 4; the letters indicate the native and substituting residues. All mutations were confirmed by sequencing of the constructs. Each of these point-mutation variants was co-transformed with pACT2:oz116 (i.e. Dab2 PID) prey plasmid into Y187 yeast strain. Co-transformants were selected on the plates without Leu and Trp, then tested for β-galactosidase activity to measure the interaction between bait and prey.

Protein interaction determined by β-galactosidase activity

The appropriate Y187 transformants were grown overnight in the liquid medium without Leu and Trp, diluted 1:5 into rich YPD medium and grown for a further 4–6 h until D_{600} reached 0.4–0.8. Yeast cells (1.5 ml) were pelleted, washed in Z-buffer [60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$ (pH 7)] and finally resuspended in 300 μl of Z-buffer. Cell suspension (100 μl) was used to measure the cell concentration by D_{600}. The rest of the cells were subjected to three cycles of freezing in liquid nitrogen and thawing at 37 °C; they were then centrifuged, and duplicate samples of 20 μl of supernatant for each clone were mixed with Galacton-Star reaction mixture (Clontech). The β-galactosidase activity was measured by counting chemiluminescent light in the β-counter containing two photomultipliers (Wallac Model 1409). The resulting counts were normalized for each sample by using the corresponding D_{600} value to express activity in arbitrary units. The scale was chosen so that the average value for the positive control was 100 arbitrary units. Yeast co-transformed with pTD1-1 and pVA3-1 plasmids (Clontech) served as a positive control [two proteins encoded by these plasmids p53 and simian virus 40 (SV40) large T-antigen are known for their strong interaction]. Yeast co-transformed with pVA3-1 and pAS2-1:human CT served as a negative control. Results from three independent experiments were averaged. Yeast lysate prepared from equal quantities of cells containing C-tail variants were tested for the expression of the Gal4 DNA-binding domain:C-tail variant fusion proteins by Western blotting with anti-(C-terminal peptide) antiserum (diluted 1:200).

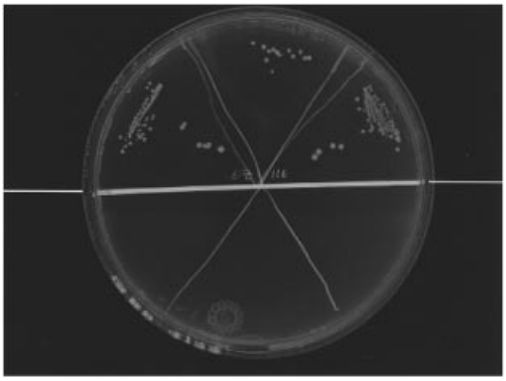

Figure 1 True interaction between the gp600/megalin CT and oz116 protein (i.e. Dab2 PID)

PJ69-2A yeast strain was co-transformed with the pACT2:oz116 and pAS2-1:human CT or pAS2-1 plasmids. Co-transformants were selected on the plates without Trp and Leu. Three colonies from each co-transformed strain were plated on QDO medium to verify the true interactions. Co-transformants containing pACT2:oz116 and pAS2-1:human CT (upper half) but not pACT2:oz116 and pAS2-1 (lower half) grew on this selective medium. The photograph was taken after incubation at 30 °C for 5 days. Similar results have been obtained for oz117 and oz103 plasmids (results not shown).

RESULTS

PID of Dab2 interacts with the human gp600/megalin CT in the two-hybrid system

Ten clones survived on selective QDO medium during the screening of a human kidney cDNA library in the two-hybrid system. Three of these ten clones (oz116, oz117 and oz103) were found to be true positives; seven were false positives. Figure 1 demonstrates the true-positive interaction between hybrid proteins encoded by pACT2:oz116 and pAS2-1:human C-tail plasmids. Library cDNA species from these three true-positive clones were sequenced. Two cDNA species (oz116 and oz117) revealed identical sequences. By comparison with sequence databases this sequence was identified as a partial sequence of the human Dab2 cDNA [33] (GenBank accession no. U39050). The cloned oz116 cDNA fragment had a length of approx. 580 bp and coded for the first 176 residues of Dab2. The cDNA fragment also included 6 bp of the 5′ untranslated region of Dab2. This added two residues to the N-terminus of Dab2, which was fused to the Gal4-activation domain. The cloned oz116 cDNA included the entire PID of Dab2 [33]. The only difference from the published structure was at nt 343–344 (numbering according to the human Dab2 sequence; GenBank accession no. U39050). In our sequence these nucleotides were GC, as opposed to CG in the published sequence. This difference was present in both oz116 and oz117 clones and led to the amino acid replacement of Arg-84 in the published sequence by Ala-84 in our sequence. Alanine is present at this position in both rat [34] (GenBank accession no. U95177) and mouse [35] (GenBank accession no. U18869) Dab2 sequences. Although the pre-transformed human kidney cDNA library was prepared with oligo(dT) primer, these clones (oz116 and 117) did not contain the 3′-end of cDNA. Instead, cDNA synthesis was apparently initiated in the mRNA region coding for the $K^{173}KKEEEKKK^{181}$ sequence of Dab2, which is rich in oligo(A) sequences [33].

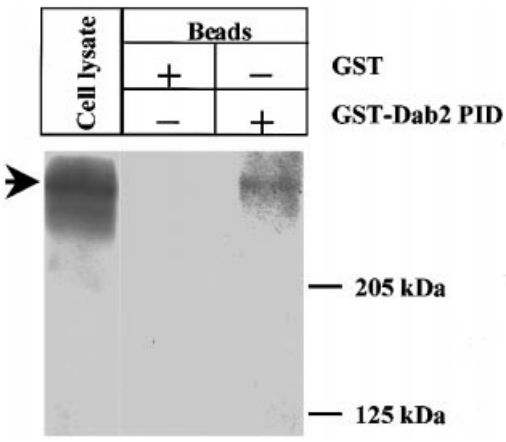

Figure 2 Binding of gp600/megalin and Dab2 PID *in vitro*

GST–Dab2 PID fusion protein or GST alone was bound to glutathione–Sepharose beads and used for the binding of gp600/megalin from the L2 cell lysate. Immunoblotting with anti-(gp600/megalin C-terminal peptide) antiserum revealed that GST–Dab2 PID is able to precipitate megalin (arrowed) while GST alone is not. The positions of molecular mass markers are indicated at the right.

Binding of gp600/megalin and Dab2 PID *in vitro*

GST–Dab2 PID fusion protein was prepared as described in the Experimental section and used to confirm the ability of the gp600/megalin CT and Dab2 to interact with each other. Figure 2 shows that GST–Dab2 PID, but not GST, bound to the glutathione–Sepharose resin, can bind gp600/megalin from the L2 cell lysate. The L2 yolk-sac carcinoma cells are known to express gp600/megalin abundantly [30]. Similar results (results not shown) were obtained with IRPT cells, which are also known to express gp600/megalin [31,32]. These experiments indicate clearly that gp600/megalin and Dab2 PID can interact *in vitro*.

Expression of Dab2 in renal cells and co-immunoprecipitation of gp600/megalin and Dab2 from cell lysate

The fact that we isolated Dab2 cDNA from a human kidney cDNA library indicates that mRNA for this protein is expressed in kidney tissue. We used Western blotting to demonstrate the expression of Dab2 protein in the rat L2 and IRPT cells (Figure 3) and in rat kidney. All these cells express gp600/megalin abundantly. Dab2 can be detected in all of them as a dominant p96 isoform.

To confirm the interaction between gp600/megalin and Dab2 *in vivo* we tested the kidney, L2 and IRPT cell lysates for the co-immunoprecipitation of these two proteins. Figure 3 shows the results of the experiment with IRPT cells. It is clear that Dab2 was co-precipitated by anti-(C-terminal peptide) antibodies but not by irrelevant antibody against transcription factor D. Moreover, gp600/megalin can be co-precipitated with anti-(Dab2 PID) antibody but not with normal rabbit serum. The co-precipitation of gp600/megalin by anti-(Dab2 PID) antibody was always more efficient than the co-precipitation of Dab2 by any anti-(gp600/megalin) antibodies. This is related to the inefficient precipitation of gp600/megalin by anti-(gp600/megalin) antibodies during the short incubation necessitated by the high level of degradation of the gp600/megalin C-tail noted in the cell lysate even in the presence of protease inhibitors (A. V. Oleinikov and S. P. Makker, unpublished work). Similar co-

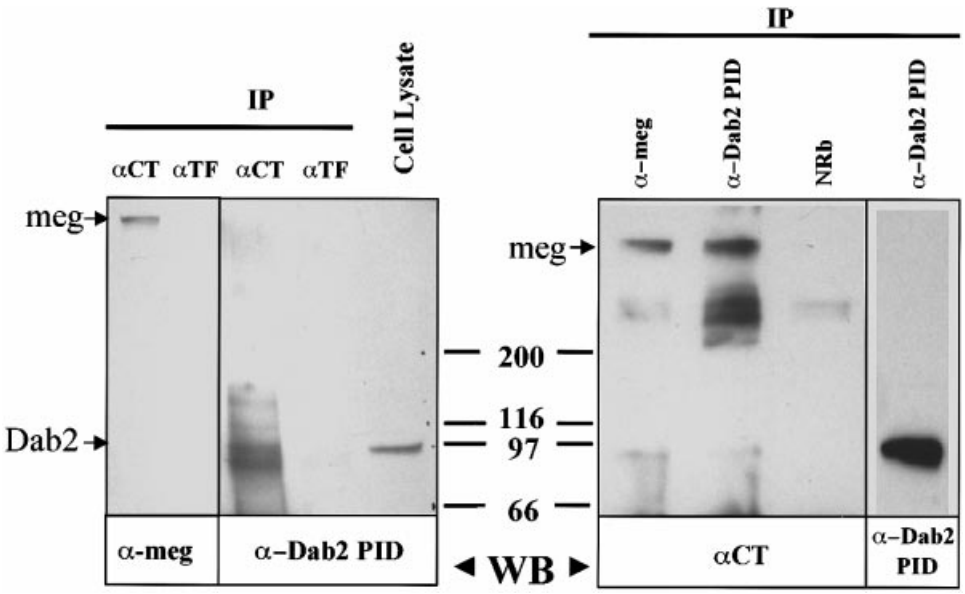

Figure 3 Co-immunoprecipitation of gp600/megalin and Dab2 from cell lysate

IRPT cell lysate was immunoprecipitated (IP) with 2 μg of rabbit anti-(gp600/megalin C-terminal peptide) (αCT) or with 2 μg of irrelevant rabbit antibody against transcription factor D (αTF), and with 10 μl of rabbit anti-(Dab2 PID) antiserum (α-Dab2 PID), 10 μl of anti-(gp600/megalin) antibody (α-meg) or 10 μl of normal rabbit serum (NRb). Precipitated proteins along with total IRPT cell lysate protein were separated by SDS/PAGE [4–15% (w/v) gradient gel] and subjected to Western blotting (WB) with anti-(C-terminal peptide) (αCT), anti-(gp600/megalin) antibody (α-meg) or rabbit polyclonal anti-(Dab2 PID) antibody (α-Dab2 PID). The positions of molecular mass markers are indicated (in kDa) in the centre.

precipitation of the two proteins was obtained with kidney and L2 cell lysates (results not shown).

Gp600/megalin CT interacts with Dab2 PID, most probably through its third ΨXNPXY motif

Six C-terminally truncated deletion variants of the gp600/megalin CT were prepared and cloned into the pAS2-1 vector to test their interaction *in vivo* with Dab2 PID by using the yeast two-hybrid system. These variants, along with the gp600/megalin CT sequence and some of the consensus motifs, are shown in Figure 4. Each of these variants was co-transformed with pACT2:oz116 (i.e. Dab2 PID) prey plasmid into the yeast strain PJ69-2A. Co-transformants were selected on the plates without Leu and Trp and then plated on QDO medium to verify the bait and prey interaction. Figure 5 (top panel) shows the results of this experiment after 10 days of incubation. It is clear that, whereas deletion variants CTDel-1 to CTDel-3 can interact with Dab2 PID, variants CTDel-4 to CTDel-6 cannot. However, the growth rate of the CTDel-3 variant was markedly lower than that of the other two deletion mutants. This might indicate that the third ΨXNPXY motif is responsible for the Dab2 binding but, on its removal (CTDel-3), the second ΨXNPXY-like motif (it contains a Lys residue instead of Pro) of the megalin CT might interact with lower affinity and help a colony to survive. This hypothesis was tested in the two-hybrid system by measuring β-galactosidase expression, a marker whose expression depends on the strength of interaction between gp600/megalin CT and Dab2 PID (Figure 5, middle panel) and can be quantified. We also prepared and tested for β-galactosidase activity several point-mutation variants of the gp600/megalin CT in the ΨXNPXY motifs to demonstrate directly the motif's involvement in the interaction (Figure 5, middle panel). This experiment revealed that (1) the interaction between the gp600/megalin C-tail and Dab2 PID is as strong as between p53 and SV40 large T-antigen, two proteins known for their strong interaction; (2) the removal of the third ΨXNPXY motif has a major and marked effect on the binding of Dab2 PID. The point mutation N → A in this motif completely prevented the interaction with Dab2 PID and consequently the expression of β-galactosidase. This is also an internal control on the specificity of interaction, because the same bait protein with a single point mutation does not interact with prey protein. The mutation in the first motif did not affect the interaction significantly. Mutation N → A in the second motif decreased the interaction but did not eliminate it. The control experiment (Figure 5, bottom panel) indicated that the level of expression of the C-tail point-mutation variants was similar to that of the intact CT and could not be accounted for by the absence of or decrease in the β-galactosidase activity.

DISCUSSION

Using three different approaches, a two-hybrid system for the screening of a human kidney cDNA library, a binding assay *in vitro* and co-immunoprecipitation of proteins expressed *in vivo*, we have identified Dab2 as an intracellular ligand of endocytic receptor gp600/megalin. This receptor, which is present in the proximal renal tubules, is implicated in the reabsorption of various molecules filtered through the glomerulus. In addition, this protein is important in the development of the mammalian organism because disruption of its gene is mostly lethal to embryos [9]. On the basis of the presence of several structural motifs in gp600/megalin CT that are common to the proteins involved in signal transduction pathways it has been suggested

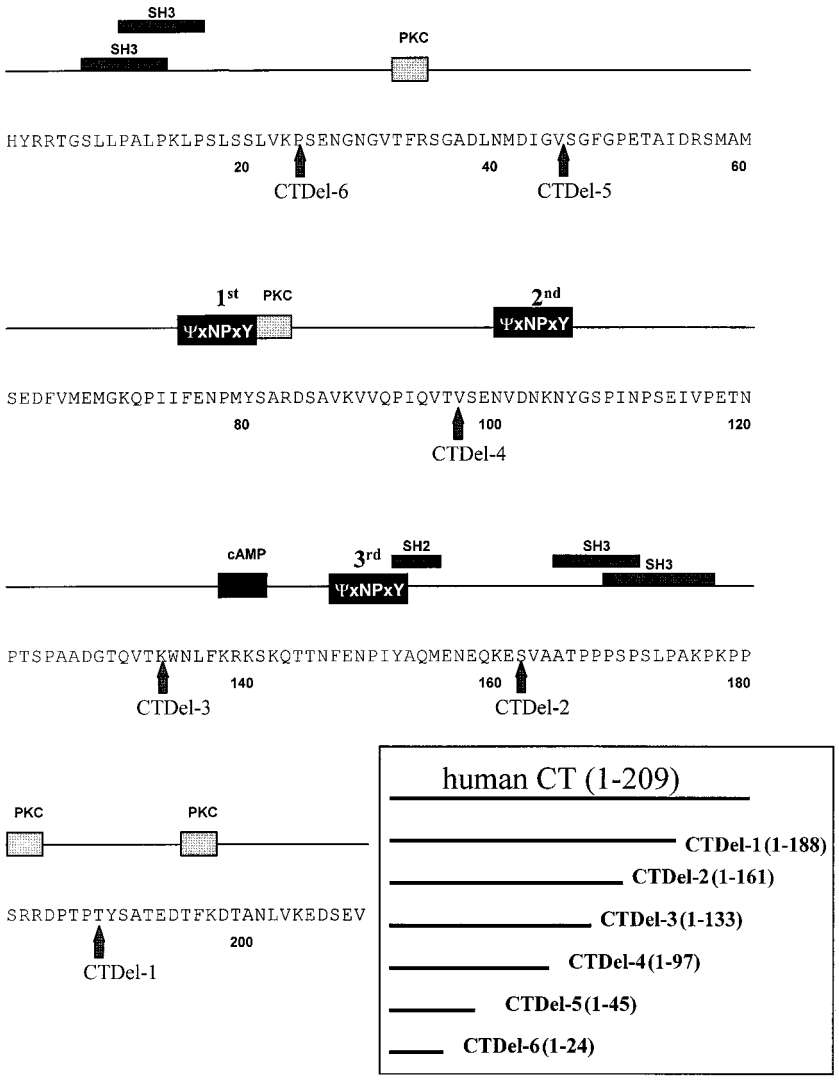

Figure 4 Diagram of the human gp600/megalin CT deletion variants

Residue number 1 corresponds to the first residue of the gp600/megalin CT. Arrows indicate C-terminal ends of the corresponding deletion variants. Abbreviations: PKC, protein kinase C phosphorylation motif; cAMP, cAMP-dependent protein kinase phosphorylation motif. SH2 and SH3 recognition motifs have been indicated previously in [24]. Other motifs were identified with the program OMIGA (Oxford Molecular Ltd). Three ΨXNPXY motifs are numbered starting from the most N-terminal.

that gp600/megalin might have a role in signal transduction [24]. It would therefore be of great interest to identify the intracellular proteins that can interact with gp600/megalin.

Our results show clearly that the cytosolic adaptor protein Dab2 is expressed in kidney and L2 and IRPT cell lines, and that the gp600/megalin CT interacts with Dab2 PID both *in vitro* and *in vivo*. We also found by immunofluorescence microscopy (results not shown) that Dab2 is localized to the renal proximal tubule brush border, a place of gp600/megalin expression [36]. It has recently been demonstrated that another protein from the Dab family, Dab1, can bind through its PID to the CT of LRP [37,38], a protein homologous with gp600/megalin. Both gp600/megalin and LRP belong to the LDL-receptor family. Dab1, however, is expressed predominantly in the brain [39,40], whereas the expression of Dab2 seems to be tissue-independent [33]. It has been shown that Dab1–LRP interaction most probably occurs through the second ΨXNPXY motif in the CT of LRP [37,38]. The ΨXNPXY motif, together with a few surrounding residues, organizes a tight-turn structure in which Asn is the most critical residue for the interaction [41–43]. LRP contains two ΨXNPXY motifs; gp600/megalin contains three (the second is a ΨXNPXY-like motif because it contains K instead of P). The necessity of interacting with several different PID-containing proteins might determine the presence of several ΨXNPXY motifs in the LRP and gp600/megalin CTs.

The second ΨXNPXY motif of LRP together with a few surrounding residues is most closely similar to the third gp600/megalin ΨXNPXY motif area [37]. Our results from the experiments with nested deletions of the C-tail and mutations of the most critical residue Asn in all ΨXNPXY motifs demonstrated that this third motif was the one most likely to be involved in the interaction with Dab2 PID (Figure 5). This is interesting in that the two homologous receptors interact with the two homologous adaptor proteins at the homologous motifs. However, we could

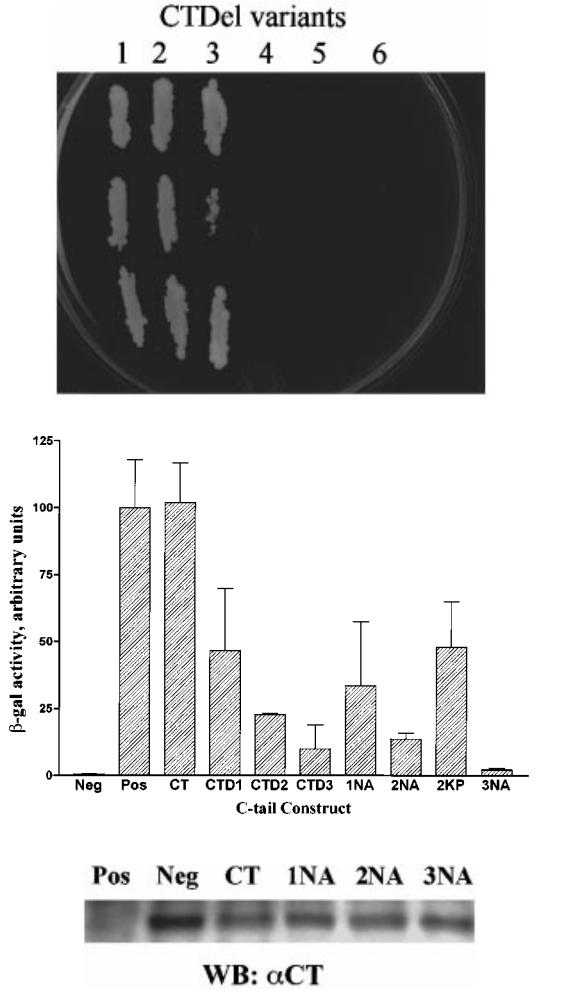

Figure 5 Interaction of gp600/megalin CT variants with Dab2 PID in the two-hybrid system

Top panel: six gp600/megalin CT deletion variant plasmids (CTDel-1 to CTDel-6) were co-transformed with the pACT2:oz116 (i.e. Dab2 PID) plasmids into the yeast strain PJ69-2A and selected on the medium without Trp and Leu. Three clones of each co-transformant were plated on the QDO medium to test the interaction between Dab2 PID and the truncated CT variants. The CTDel-1 and CTDel-2 variants grew well after 5 days of incubation. The CTDel-3 variant, missing the third ΨXNPXY motif, was able to interact with Dab2 but grew more slowly than CTDel-1 and CTDel-2. The photograph was taken after incubation at 30 °C for 10 days. Middle panel: interaction of the CT and three deletion and four point-mutation CT variants with Dab2 PID, measured by β-galactosidase activity. The positive control shows interaction between p53 and SV40 large T-antigen; the negative control shows interaction between gp600/megalin C-tail and SV40 large T-antigen. Three independent experiments with different clones for each construct in each experiment were performed. The bars show means $\pm$ S.D. Bottom panel: Western blot (WB) with anti-(gp600/megalin C-terminal peptide) (αCT) of the yeast lysates prepared from the indicated variants demonstrates a similar expression of the CT and point-mutation CT variant fusion proteins. Note that the positive control did not contain CT fusion protein but the negative control did.

not completely exclude a role for the second ΨXNPXY-like motif in this interaction. It is possible that the deletion in CTDel-3 construct provides better three-dimensional access to the second ΨXNPXY motif than it does in the full-length variant 3NA, in which the intact second motif did not contribute to the interaction. It is unlikely that significant overall structural changes in the other part of the C-tail might explain the absence of activity in the 3NA mutant because, as far as the peptide containing the ΨXNPXY motif is accessible for the interaction, the rest of the protein molecule is not generally important [38,42,43].

Although the structural domain interacting with the ΨXNPXY motif was named PID, the phosphorylation of the Tyr residue might have different effects on the interaction. In LRP the Tyr phosphorylation actually inhibits its binding to Dab1 [38]. It is not clear at present how the phosphorylation of Tyr might affect the interaction between gp600/megalin and Dab2. This issue will be resolved in future experiments.

Dab2 phosphoprotein has been described with different names in different species. It has been shown to have several isoforms resulting from alternative splicing. These isoforms and their names include p96, p93 and p67 in mouse [35], the p105 isoform of DOC2 ('differentially expressed in ovarian carcinoma') in human [33] and the p82 and p59 isoforms of C9 in rat [34]. In our experiments we have found that the molecular mass of the Dab2 present in rat L2 and IRPT cells and in rat kidney is approx. 96 kDa. We believe that this 96 kDa form is the same as that described as p82 by Tseng et al. [34], and that the difference in the molecular mass in the two studies resulted from the difference in polyacrylamide gel composition and from the use of different molecular mass markers.

The function of Dab2 is not clear at present, though several experiments argue for its role in signal transduction. In addition, no membrane receptors that can interact with Dab2 in the cytoplasm have yet been described. It has been shown that this protein is mitogen-responsive: it was phosphorylated at a Ser residue on treatment with colony-stimulating factor and was therefore suggested to be involved in the early steps of colony-stimulating factor signalling [35]. Dab2 is also up-regulated in rat ventral prostate after castration, suggesting that it can be regulated directly or indirectly by the androgen receptor [34], and it is down-regulated in, or absent from, ovarian carcinoma cell lines [44] and in gestational trophoblastic disease tissues [45]. When Dab2 was expressed in carcinoma [46] or choriocarcinoma [45] cell lines it significantly decreased the growth rate of the cells in culture. It also can suppress the tumour growth *in vivo* [46]. It has recently been shown that Ser phosphorylation in Dab2 inhibited the PMA-induced activity of the transcription factor activator protein 1. This phosphorylation might therefore be a molecular key for Dab2 tumour-suppressive function [47]. It has also been shown that Dab2 binds via its C-terminal proline-rich sequences to the SH3 domains of Grb2 [25]. Grb2, SH3-SH2-SH3 adaptor protein, is involved in most growth-factor-receptor signalling pathways, including the Ras and Jun signalling pathways [26,27,48,49]. It is interesting that both Grb2 [48] and gp600/megalin [9] are important in embryonic development. We speculate that the interaction of the gp600/megalin C-tail with signalling adaptor molecule Dab2 might involve gp600/megalin in signalling pathways.

In contrast, the ΨXNPXY motifs are also important for internalization of the receptors through the clathrin-coated pits [50]. The interactions of some adaptor protein(s) with this motif might mediate this process. Dab2 is expressed in a tissue-independent manner and is able to interact with the ΨXNPXY motif of endocytic receptor. If this interaction provides further steps of the endocytotic events then Dab2 might be a candidate as an adaptor molecule involved in the endocytosis of megalin at least.

In conclusion, we have identified the cytosolic adaptor protein Dab2 as an intracellular partner of endocytic receptor gp600/megalin. This finding suggests that the interaction between Dab2 and gp600/megalin might involve this endocytic receptor in signal transduction pathways or it might be important for the

intracellular trafficking of gp600/megalin. In addition, both possibilities might co-exist. Future experiments will clarify these hypotheses.

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