計畫名稱　GP135之擇植及其在犬腎上皮細胞中表現之研究

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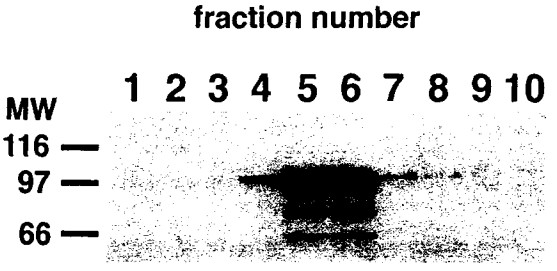
Epithelia comprise a widespread and important tissue type that forms boundaries and regulates vectorial transport of ions and solutes between different compartments of the body. The kidney nephron is such an example where different epithelial cell types form a tubular monolayer to separate the ultrafiltrate from the underlying interstitium and functions in urine concentration, ionic reabsorption, and volume regulation. The performance of these various physiological functions depends on a polarized distribution of membrane ion channels or transporters. For example, the interaction of Na^+/K^+-ATPase with cytoskeleton proteins fodrin and ankyrin helps anchor Na^+/K^+-ATPase at the basolateral domain of renel tubular cells to set up a sodium gradient, and this gradient provides the driving force for the transportation of many other solutes and ions. An aberrant localization of Na^+/K^+-ATPase at the apical domain of renal tubules may contribute to the clinical manifestations found in polycystic kidney disease.

GP135 is an apical transmembranous protein, and is found only in the thick ascending limb of loop of Henle in mouse nephron. This suggests GP135 is likely to be a channel, pump, or transporter protein involved in solute or ionic transportation in renal tubules. From previous studies, we found GP135 was abnormally trapped in an intracellular compartment, instead of being targeted to the apical plasma membrane, due to the expression of constitutively active Rac1 small GTPase. The implication of Rac1 as a signaling molecule controling actin cytoskeleton organization and the knowledge that quite a few ionic channel or pump proteins are anchored at specific subcellular regions via their interaction with actin cytoskeleton suggest Rac1 might regulate the function of GP135 through its effect on actin cytoskeleton. In order to test this hypothesis and study the function of GP135, I propose a project to clone GP135. The strategy I would adopt is based on an expression cloning technique using a monoclonal antibody (3F2) which recognizes nicely GP135 expressing cells. Madin-Darby canine kidney (MDCK) cDNA library would be constructed first in lambda phage vectors and used subsequently to transfect CHO and HEK293 cells which lack the expression of endogenous GP135. Cell trapping of magnetically labeled cells in conjunction with FAC sorting would allow easy and quick selection of positive GP135 expressing cells.

In previous year, we were making MDCK cDNA library capable of expressing membranous proteins. In order to construct a cDNA library enriched in genes encoding secretory proteins or proteins targeted to cell membranous, we followed the protocol designed by Mechler BM to recover ER membrane-accociated RNA (Methods in Enzymology 152: 241-248, 1987). In brief, exponentially growing cells were treated with cyclohexamide at 25 μM for 30 minutes to prevent translated peptides released from ribosomes. Then, the cells were scraped from the culture plates with rubber policeman and pelleted by low speed centrifugation. After cells were lysed in cold hypotonic solution, the cells were homogenized by Dounce Homogenizer. The cracked cell lysate was cleared off nuclei before loaded into a sucrose discontinuous gradient (2.5 M-1.98M-1.3M sucrose gradient). Then the lysate was spinned at 90,000 g for 15 hours before fractionated into 10-12 fractions. Free ribosomes and free mRNA will be present in the load zone while membrane-associated ribosomes and mRNA will be at the interface between the 1.98M and 1.3M sucrose steps. Membrane-bound mRNAs were confirmed by examining the fractions with antibodies to ER marker such as calnexin and HSP72.

As shown below in Figure 1, the anti-calnexin western blotting does show the ER marker protein calnexin is concentrated in fraction 5 and 6 (HSP72 also showed similar finding. Data not shown.)

Figure 1. Anti-calnexin (an ER marker) Fractions from the polysome collection were further separated in a 15-30% continuous sucrose gradient to enrich polysome (ribosomes stringed together along the mRNAs) containing parts. The result was confirmed by western blotting of the fractions.



We also performed RT-PCR using E-cadherin specific primers to amplify E-cadherin signals from the fractions we retrieved after sucrose density gradient. Since E-cadherin is an membranous protein, we expect to find whether the fractions which are supposed contain the membranous proteins are exactly where E-cadherin signals reside. . As shown below in Figure 2, the E-cadherin RNA is more concentrated in fraction 5 and 6 while GAPDH signals are uniformly distributed over all the fractions.

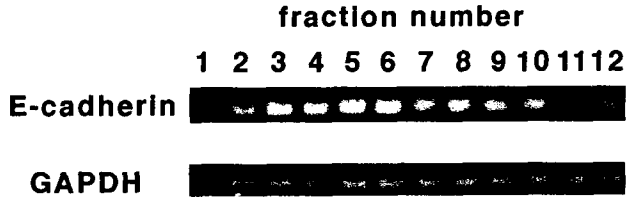


Figure 2. RT-PCR were performed using E-cadherin, a membranous protein, and GAPDH, a cytosolic protein, specific primers. The starting template was 1 μg RNA from each fraction. Equal fraction of the PCR product was loaded.

We then pooled the RNA from fraction 4 to fraction 6, and synthesized cDNA library Using SuperScript™ plasmid system kit from Gibco-BRL company. The protocol was summarized as follows:

After growing the transformants on thirty 50 square cm bacterial platest, there were totally 1,700,000 independent primary transformants isolated after initial screening, and we randomly picked up 15 colonies and performed miniprep of the plasmid DNAs. Then we restriction digested the plasmids to relieve the inserts using the cloning enzymes, Sal I and Not I. As shown below, all the clones contain inserts (there was no empty vector among the 15 clones we randomly picked), and the average insert size was about 3 kb in size.

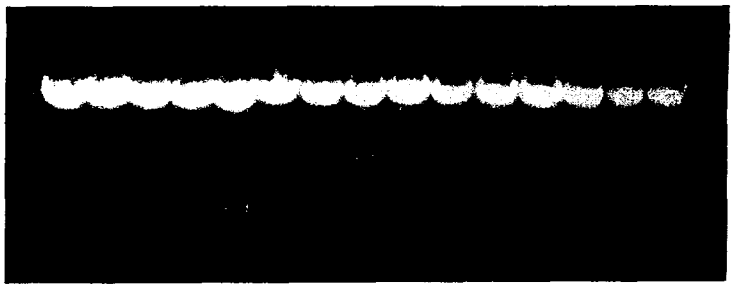


Figure 3. Sal I and Not I digestion pattern of 15 randomly picked clones of the MDCK cDNA library which is enriched in membranous proteins encoding genes.

Finally, we made a mega-preparation of the cDNA after we retrieved the plasmid DNA from the primary transformants. We got totally 25 microgram of cDNA. We then transfected the cDNA into the LLC-PK1, MDBK, HEK293, and OKP cells, which do not express any GP135 antibody stainable proteins on surface. In order to see whether we could identify any known membranous proteins in the transfected cells, we used a monoclonal antibody, rr-I, which recognizes extracellular domain of E-cadherin, to perform immunofluorescence in the c-DNA transfected cells. Below is an example of OKP cells transfected with the cDNA library and stained with the E-cadherin extracellular domain antibody, rr-I.

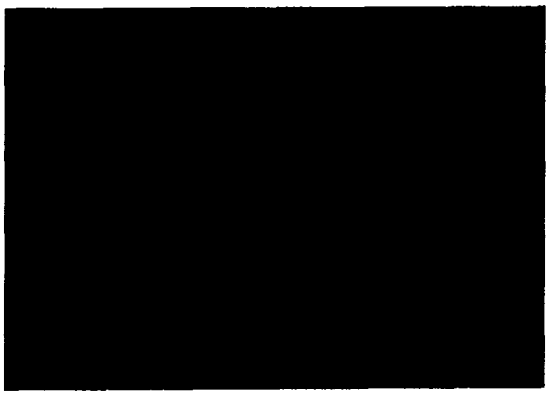


Figure 4. OKP cells were transfected with MDCK cDNA library using Lipofectamine 2000. The transfected cells were trypsinized and plated onto collagen coated cover slips right after transfection. The cells were then fixed, without any further premeabilization, and then stained with rr-I, a monoclonal antibody which recognizes extracellular domain of E-cadherin.

Because we could not use the expression cloning strategy to isolate cDNA encoding GP135, we are trying to use protein chemistry methodology to purify GP135 and use mass spectrometry or N-terminal sequencing to identify the protein:

Below is the current protocol we use to purify GP135 from MDCK cells:

(1) Fractionation to enrich membranous portion from the homogenized lysates
(2) Use Wheat Germ Beads to purify glycoproteins after fractionation
(3) Affinity purify the preparation after eluting the previous fraction using Anti-GP135 antibody beads

STEP (1) Fractionation to enrich membranous portion from the homogenized lysates

Standard membrane enrichment procedure : (all on ice or 4°C)

Scrape and homogenize cells (using dounce homogenizer)

⬇

Low speed centrifugation (3000 rpm, 5 to discard intact cells and nuclei

⬇

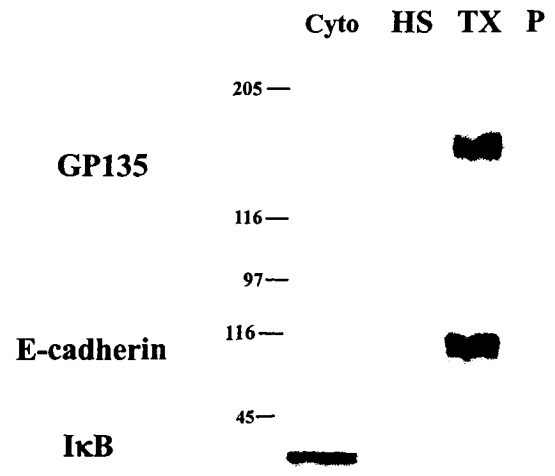
High speed centrifugation (14000 rpm, 1hr) to separate plasma membrane sheet (pellet) and cytosolic fraction (supernatant)

⬇

Add buffer w/ detergent to extract protein from pellet

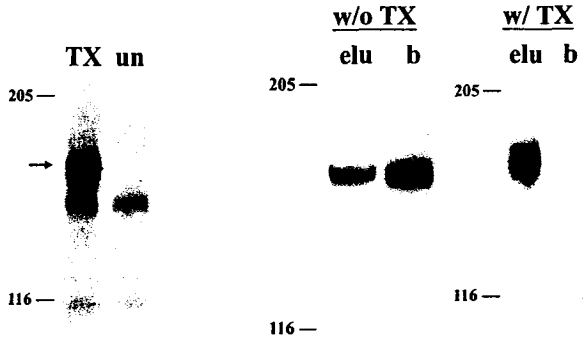
Results:

Cellular fractionation result —



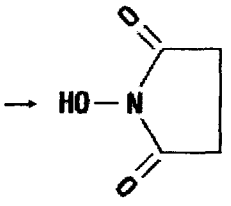
Affinity binding — **Wheat Germ Lectin** beads

- **Specificity : N-acetyl-β-D-glucosaminyl residue and sialic acid residue**
- **Sample binding: ~ 4.5hr @ 4°C**
- **Sample elution: 0.8M GlcNAc w/ 2% Triton X-100 O/N @ 4°C**



Affinity binding —
anti-GP135 antibody NHS coupled beads

- **NHS (N-hydroxysuccinimide):**

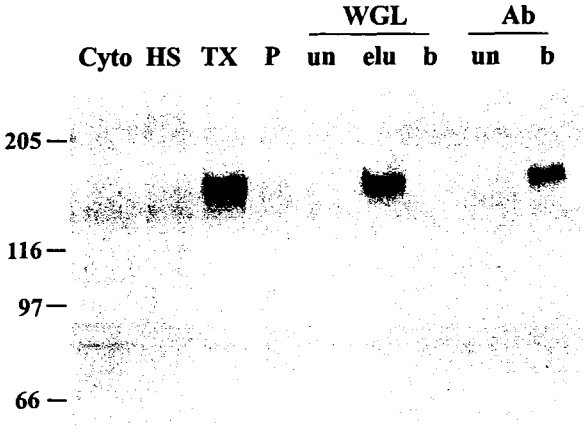


NHS
M.W. 115.10

- **Sample binding:** O/N @ 4°C or 3hr @ RT
- **Sample elution:** SDS sample buffer boiling

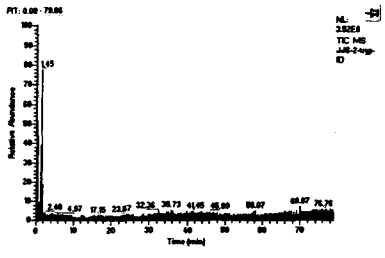
Enriched membranes ➡ WG lectin beads binding

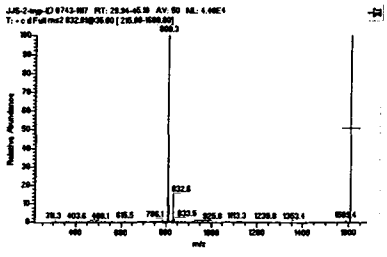
➡ Antibody coupled beads binding



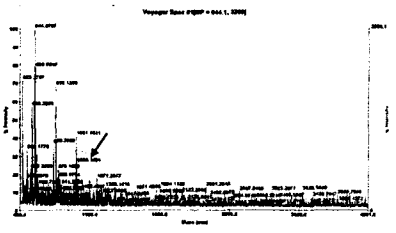
Mass Spectroscopy Analysis

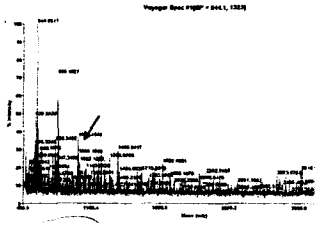
- **LC-Mass: no matches in databank**



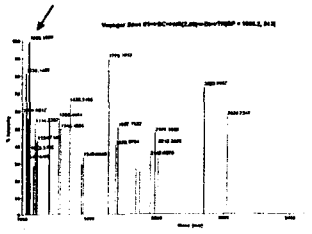


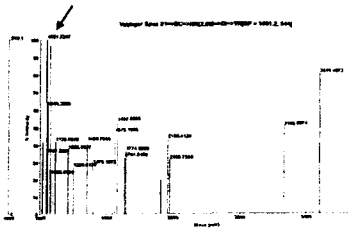
- **MALDI-TOF: no enough samples for analysis**





Higher intensity **Lower intensity**





More GP135 is needed for mass spectroscopy or other analysis —

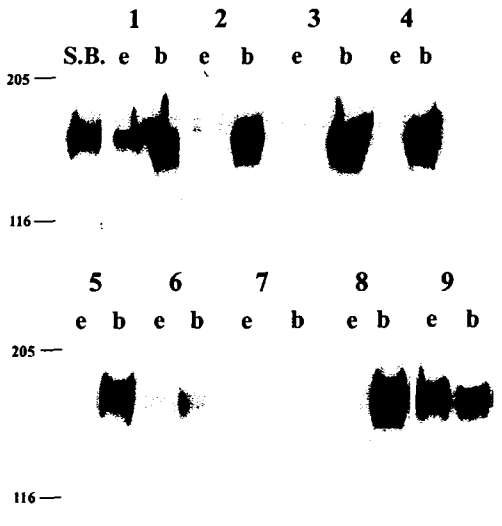
I. Larger scale purification

II. Concentration of final volume

➡ **a. Concentrate antibody to decrease the volume of antibody coupled beads**

b. Find ways to efficiently elute GP135 from antibody coupled beads

Try to elute GP135 from antibody crosslinked beads

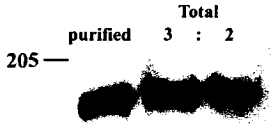


1. Phosphate acid pH12.5
2. 3.5M MgCl2
3. 5M LiCl
4. H2O
5. 50% ethylene glycol pH6.5
6. 50% ethylene glycol pH11.5
7. 2M Gn·HCl
8. 2M Urea
9. 0.5% SDS

Copy number of GP135

I. Western: c.f. purified sample and total lysate

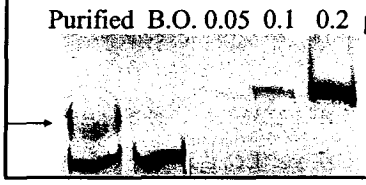
➡ To determine **cell number** of certain purified sample



➡ **X = 2 * 10^5 cells**

II. Silver stain: c.f. purified sample and BSA std

➡ To determine the **amount** of the purified sample



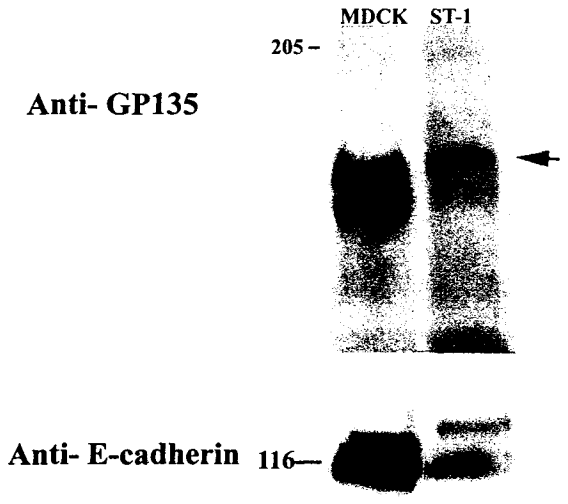
➡ **5X = 0.1 μg**

Mole of X : 0.02 * 10^{-6} (g) **/ 165 * 10^3** (M.W.) = **1.2 * 10^{-13}** (moles)

Copy number of GP135 : 1.2 * 10^{-13} (moles) **x 6 * 10^{23} / 2 * 10^5** (cell number) = **3.6* 10^5**

1μg : 10^7 cells

10 pmole : 1.7 * 10^7 cells



No strong enough evidences to tell if there's GP135 homolog in ST-1 cells

Future work — to get sequence information of GP135

- **Solve problems of "amount" to get better MALDI-TOF spectrum.**
- **Any practicable protein identification strategies other than mass spectroscopy analysis?**
- **Perform deglycosylation to enhance protein identification via MS analysis.**
- **Study other biochemical properties of GP135, e.g. 2D electrophoresis, immunoprecipitation, its' membrane linkage means.., to know better about it.**

(1) A mammalian cDNA expression library could be constructed in pCMV-SPORT 6 vector™ (from Invitrogen). MDCK cells have been used as a model system to study cell-cell adhesion, cell-substratum adhesion, cell motility, and cell polarity. However, a well characterized and useful expression library has been elusive. By generating a MDCK cDNA expression library, the research on the field of epithelial physiology could be greatly benefited.

(2) GP135 has been found for more than 15 years now, but it has not been cloned. GP135, unlike the other apical marker proteins, most of which are GPI anchored protein, is an apical transmembrane protein. This makes it an intriguing marker protein to study the signaling motif involved in apical membrane targeting since those GPI anchored proteins lack intracellular domains. By comparing the sequence motif of GP135 and $p75^{NTR}$ involved in apical targeting, we could learn about the mechanism in setting up cellular polarity.

(3) As I explained in details previously in the introduction section, GP135 is likely a protein involved in renal tubular function. Furthermore, the modulation of its subcellular localization by Rac1 small GTPase activity implies a signaling pathway by which Rac1 could regulate important physiological function. If indeed there is the functional relationship between GP135 and Rac1, I would next explore the mechanism involved in this regulation. Since I have made Rac1 mutant (both constitutively active and dominant negative) expressing MDCK cells and MDCK cells do have endogenous GP135. It is appropriate to say that this project could be integrated into other projects carried in my lab now regarding the study of Rac1 biology.

(4) Once GP135 is cloned and functional analysis indicates it is a protein with significant physiology function. More extensive and ambitious studies, such as yeast two hybrid analysis and immunoprecipitation to isolate interacting proteins, or transgenic mice study, should be followed to extend the horizon of understanding this protein.

(5) If GP135 is indeed proved to be a channel or transporter protein involved in solute or electrolyte transportation, pharmacological study could be followed to develop potential agonist or antagonist which should be quite useful in medical practice.