行政院國家科學委員會專題研究計畫 期中進度報告

GP135 之極性及功能性研究(2/3)
期中進度報告(精簡版)

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研究計畫成果報告

I.簡介（Introduction）與背景說明

Multicellular organisms depend on polarized epithelia to carry out key physiological functions, such as vectorial flows of individual solutes across the renal tubular or gastric mucosa cells. In order to perform these vital functions, the epithelial cells maintain an asymmetrical distribution of lipid and protein components at the plasma membranes in the presence of a myriad of constant endocytotic and secretory activities. This protein and lipid polarity is achieved first in the trans-Golgi network complex where biomembrane components are sorted into different vesicles for apical or basolateral delivery (1, 2). Although various signals have been linked to the targeting codes for protein delivery, a great deal of the mechanistic details about the sorting of membranous proteins are still unraveled. The basolateral sorting signals described to date reside in the cytoplasmic domains, and on occasions the signals bear similarity to the acidic tyrosine or di-leucine motif found in the endocytic proteins. In contrast to the more conserved nature of basolateral sorting signals, apical signals appear to be more diverse. These include N-, and O-glycans found in many apical residental and secretory proteins, and the small glycolipid moiety found in glycophosphatidylinositol (GPI)-anchored proteins (GPI-APs). These signal codes, often found on the luminal side of the transported proteins, have been postulated to provide proteins a partition access to the sphingolipid-cholesterol rafts whose destiny is to apical domain.

Gp135/cPCLP1 (canine podocalyxin like protein 1) is the most used apical marker protein in polarized Madin-Darby canine kidney (MDCK) cells. It is recently found to be the canine homologue of podocalyxin (PC). It is demonstrated Gp135 and NHERF2 participate in a pre-apical complex and loss of Gp135 function results in defective cystogenesis of collagen-embedded MDCK cells. Furthermore, it is also linked to the HGF/SF induced tubulogenesis. Although Gp135 has been used for the apical hallmark for decades, the apical sorting signal and the mechanism by which it is specifically delivered to the apical domain in polarized MDCK cells have never been characterized. In this study, we identify Gp135 relies on a bipartite signal for faithful apical targeting, and there are apparently independent signals existing in its extracellular O-glycosylation rich region and its cytoplasmic PDZ domain binding motif.

II.關鍵材料及方法 (Subjects and Methods)

Immunofluorescence and Confocal Microscopy

MDCK cells, cultured on 12-mm-diam Transwell filters (Costar Corp., Cambridge, MA) for 4 or 5 d, would be fixed in 2% paraformaldehyde in Dulbecco's PBS solution containing 1.8 mM Ca++ and 0.5 mM Mg++ (CMPBS). Cells would be permeabilized with 0.075% saponin in CM-PBS containing 0.2% BSA, incubated with either mouse anti-myc or anti-CD7 antibodies, and diluted 1:1,000 in the same buffer. After washes with CM-PBS–saponin–BSA, filters would be incubated with fluorescein-conjugated goat anti–mouse IgG and RNase, each diluted 1:200 in CM-PBS–saponin–BSA. Nuclei would be subsequently labeled with propidium iodide. Immunofluorescent images would be collected using a confocal microscope (Leica).

Steady State Localization of full-length and Mutant GP135

Apical and basolateral plasma membrane proteins would be biotinylated as described previously (3). Briefly, confluent monolayers of MDCK cells on filters (Transwell; Costar Corp.) would be washed thrice in ice-cold CM-PBS. 500 g/ml sulfo-NHS-SS-biotin, diluted from a 200 mg/ml stock in DMSO, would be added to either apical (0.67 ml) or basolateral (1.33 ml) chambers. Surfaces not receiving biotin would be incubated in CM-PBS alone. Filters would be incubated 20 min on ice, and then fresh buffer and biotin would be applied and filters were incubated another 20 min. Biotinylation reactions would be quenched by washing cells in five changes of CM-PBS containing 50 mM NH4Cl and 0.2% BSA.

Filters would be excised from plastic collars and monolayers solubilized by incubation for 1 h at 4°C in lysis buffer (150 mM NaCl, 20 mM TrisHCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.2% BSA, and a protease inhibitor cocktail including 1 mM PMSF and 10 g/ml each of antipain, pepstatin A, and leupeptin). Extracts would be precleared by incubation for 1 h with fixed *Staphylococcus aureus* cells (Pansorbin), and cleared supernatants would be incubated with avidin-agarose for 2 h at 4°C with end-over-end rotation. Avidin-agarose precipitates would be washed as described previously for immunoprecipitates (4). Bound proteins would be released from beads by boiling in SDS-PAGE sample buffer containing 100 mM dithiothreitol, separated by SDS-PAGE, and transferred to membranes (Immobilon-P; Millipore Corp., Waters Chromatography, Milford, MA). Blots would be incubated with mouse anti-MYC (9E10) by ECL and the signals would be quantified using a phosphorimager (Molecular Dynamics, Inc.).

Targeting of Metabolically Labeled Proteins

To examine initial delivery of proteins to apical and basolateral membrane domains, a biotin targeting assay would be used (4). Briefly, 4 or 5-d-old filter-grown cultures would be pulse labeled for 20 min in [35S]cysteine and chased for various times in medium containing excess unlabeled cysteine. At each chase time, filters would be placed on an ice bath, biotinylated from the apical or basolateral surface, lysed, and precleared as described above. Lysates would be incubated overnight with mouse anti-MYC (9B7.3) and immune complexes would be collected by incubation for 2 h with
protein A–Sepharose (5 mg/ml). Immunoprecipitates would be eluted by boiling in 5% SDS, diluted 50-fold with lysis buffer, and then biotinylated proteins would be recovered by reprecipitation with avidin-agarose. Proteins would be separated by SDS-PAGE and analyzed with a phosphorimager (Molecular Dynamics Inc.).

III.重要之結果 (Results)

Fig. 1. Domain specific steady state distribution of Gp135/CD7 chimeric proteins. *Left*. The diagrams show schematically the structural features of the domain swapping constructs. **m**: Myc epitope tag; **Ex**: extracellular domain; **TMC**: transmembrane and cytosolic domains. **Solid box**: Myc epitope tag; **strippled box**: Gp135; **empty ellipse**: CD7. *Middle*. Steady state surface biotinylation, immunoprecipitation, SDS-PAGE separation, nitrocellulose filter transferring, and HRP-avidin hybridization were performed as described in the Materials and Methods. A representative fluorography was shown. **AP**: apical. **BL**: basolateral. The fluorographic signals were quantified by ImageGauge software program. The percentage of each chimeric distributed at the apical domain was analyzed by quantifying the fluorographic

signals and shown as the mean of a triplicated experiment. *Right*. The representative confocal X-Z section images of the Myc-tagged Gp135/CD7 chimeric proteins were shown. Green channel: anti-Myc staining; red: staining for tight junction marker ZO1 and propidium iodide staining for nuclei. Bar:10 μm

Figure 1

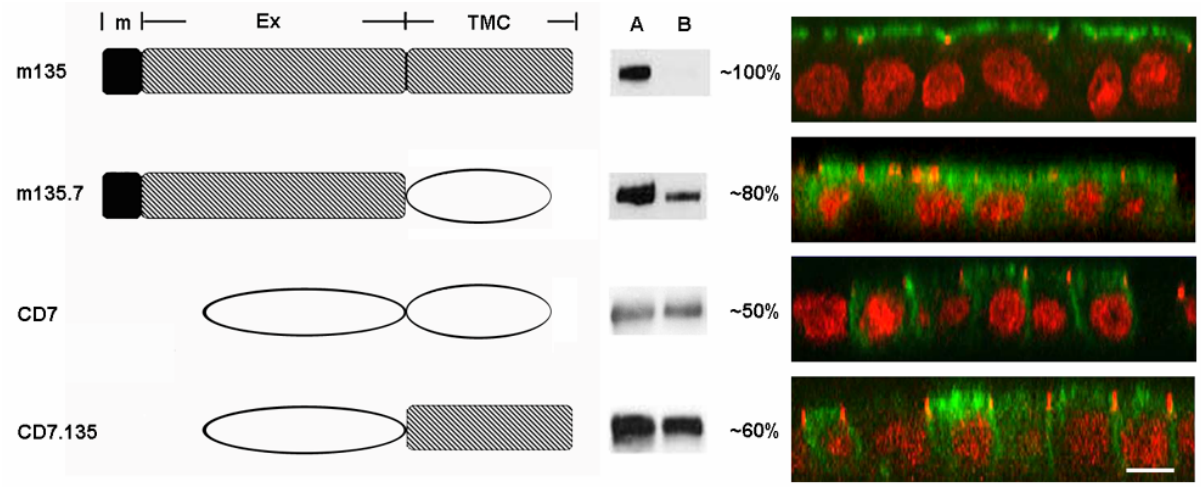
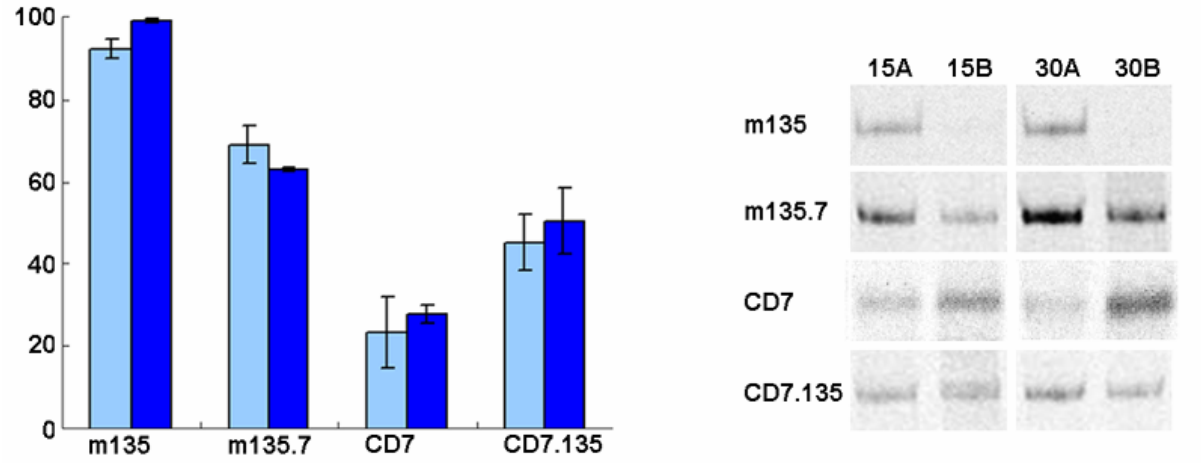


Fig. 2. Plasma membrane delivery of newly synthesized Gp135/CD7 chimeric proteins. Polarized MDCK cells stably expressing various chimeric proteins were metabolically labeled with ^{35}S-Met/Cys for 30mins, chased 15mins or 30mins, then cell surface biotinylated on either apical or basolateral membrane, and processed for immunoprecipitation using the anti-Myc mAb (9E10). The chimeric proteins were eluted from the cross linked antibody beads by acidic glycine buffer, recaptured with avdin agarose, and separated by SDS-PAGE. The percentage of each newly synthesized chimeric targeted to the apical domain was shown as a bar histogram (light blue:15mins chasing ;Deep blue:30mins chasing). The values were the means and the S.E.s of three identical samples. A representative result was shown below the histograms. 15(A/B)/30(A/B), 15mins/30mins chasing apically or basolaterally

Figure 2



IV.討論 (Discussion)與成果之貢獻

To identify the apical sorting determinants of Gp135/cPCLP1, we started with constructing various domain swapping mutants of Gp135 and CD7. Because CD7 was previously reported to display a non-polarized membranous expression pattern in MDCK cells, we fathom that the steady state distribution of these chimerics in polarized MDCK cells would be revealing regarding the apical sorting signals of Gp135/cPCLP1. Indeed, steady state biotinylation and confocal section imaging study confirmed CD7 was evenly distributed at the apical and the basolateral domains (Figure 1). Further analysis revealed substitution of the extracellular or the intracellular domain of Gp135 with the homologous domain of CD7 resulted in a chimeric still predominantly residing at the apical domain (the m135.7 or the CD7.135 chimeric in Figure 1). Because we could not express any amino-terminally tagged CD7 construct at plasma membrane which failed to exit endoplasmic reticulum (data not shown), we complemented our myc-tagged constructs study with a series of FLAG tagged constructs (data not shown). To our surprise, when the FLAG epitope was tagged to CD7, it conferred CD7 with a basolateral polarization characteristic (80% of steady steady CD7-FLAG protein was at the basolateral surface as demonstrated by the biotinylation study (data not shown). We reasoned the FLAG epitope (MDYKDDDDK) bears certain similarity to the previously identified acidic tyrosine signals found in some basolateral targeted proteins such as LDL receptor and directs the basolateral targeting of the CD7-FLAG proteins. Although CD7-FLAG protein was mainly basolateral, the 135.7F and its complementary construct 7.135F showed 75% and 50% distribution at the apical domain respectively (data not shown). These findings together with the myc-tagged chimerics study indicate both extracellular and intracellular domains of Gp135 contain apical targeting signals.

V. 參考文獻 (References)

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