Receptor-Mediated Endocytosis as a Selection Force to Enrich Bacteria Expressing Rhodostomin on Their Surface

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Endocytosis, receptor-mediated • Bacterial display • TraT lipoprotein • Rhodostomin

Abstract
Previously, we developed a TraT display system to express snake venom rhodostomin (RHO), a disintegrin, on the external surface of Escherichia coli [J Biomed Sci 6:64-70;1999]. To show a new potential use of the TraT display system, we employed a biotin labeling technique coupled with SDS-PAGE and flow cytometry analyses to further demonstrate and confirm the expression of TraT-RHO on the E. coli surface. We also showed that the expression of TraT-RHO on the cell surface not only facilitated the bacteria adhesion to BHK-21 cells but also induced bacterial internalization into BHK-21 cells. This feature allowed us to enrich the TraT-RHO expression bacteria about 10,000-fold starting with a mixture of TraT-RHO bacteria with ß-galactosidase-positive bacteria in a ratio of 10²:10⁷ through four cycles of BHK-21 cell endocytosis and replating of engulfed bacteria on agar plates. We therefore suggest that the TraT display system can be applied to select out bacteria expressing a specific peptide sequence from a large population of display library through the process of receptor-mediated endocytosis and reamplification cycles.

Introduction

The horizons of peptide and protein display technologies have rapidly expanded in molecular and cell biology study in recent years [4]. Phage and bacterial display systems have become popular tools to study protein-protein and protein-ligand interactions in various biomedical fields [4, 28, 29]. They are particularly useful in searching for an unidentified counterpart which can bind to known proteins, enzymes, or receptors from a large pool of peptide library [3, 4, 12]. Based on the principle of affinity selection, a specific peptide expressed on the surface of phage or bacteria can be obtained through a serial panning procedure [3, 11, 21, 23]. By extending the selection principle, a phage displaying an Arg-Gly-Asp (RGD)-containing peptide has been demonstrated to be internalized by mammalian cells via integrin-mediated endocytosis [14]. Subsequently, a phage display has been employed to isolate novel ligands for endocytosed receptors via a mammalian cell uptake and reamplification process [18, 19]. Theoretically, for the therapeutic purpose, these ligands can then be attached to delivery vectors and be targeted to cells through the process of endocytosis [20, 25], which enlarges a possible application of phage display.

Receptor-mediated endocytosis is a well-known natural pathway of virus and bacteria entry into cells. In particular, integrins serve as receptors for the entry of aden-
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Materials and Methods

Chemicals and Materials

All enzymes for molecular cloning were from New England Biolabs (Beverly, Mass., USA). Chemical drugs, Triton X-100, and fluorescein-labeled goat anti-rabbit IgG were purchased from Sigma (St. Louis, Mo., USA). Isopropyl-β-D-thiogalactoside (IPTG) for induction of fusion protein production was from Biosynth (Staad, Switzerland). The protein biotinylation kit was from Pierce (Rockford, Ill., USA). Rabbit polyclonal antibody against the RHO peptide 45–59 (anti-RHO4559), which recognizes the RGD loop of RHO, was a generous gift from Dr. S.H. Chen (National Cheng-Kung University, Tainan, Taiwan, ROC) [8]. Rabbit anti-Trat antibody was obtained by immunizing rabbits with recombinant Trat protein purified by SDS-PAGE as described before [9].

Plasmid Constructions

To obtain a plasmid expressing the gene of the green fluorescence protein (GFP) in E. coli, the HindIII-Vhal fragment from pEGFP-N2 (Clontech, Palo Alto, Calif., USA) was subcloned into the pGEM-3zf(-) (Promega, Madison, Wisc., USA) and designated as pEGFP-BE. pEGFP-BE is under lac promoter control and is able to induce GFP expression in E. coli when IPTG is added. The plasmid of pTrat-RHO(Kan′) was obtained by inserting the fragment of the kanamycin gene from pKiss (Pharmacia, Brussels, Belgium) into the EcoRV site within the ampicillin gene of pTrat-RHO [5, 6], resulting in a gain in function of the kanamycin gene but a loss of function of the ampicillin gene.

Analyses of Bacterial Surface Proteins by Biotinylation

For labeling of surface proteins, E. coli (strain XLI-blue) bearing pTrat-RHO or pGEX-2KS were washed with ice-cold PBS (pH 8.0) 3 times. Bacteria were adjusted to about 2.5 × 10^7 cells/ml in PBS solution. Sulfo-NHS-Biotin was then added at a final concentration of 0.5 mg/ml to cells. The reaction was stopped after 30 min incubation at room temperature and washed with ice-cold PBS 3 times. Bacteria were then lysed by SDS buffer and the total cellular proteins were separated by SDS-PAGE [19]. Proteins on gels were transferred onto a nitrocellulose paper and reacted with streptavidin-peroxidase. The biotinylated proteins were then visualized by the development of color, an oxidized form of 4-chloro-1-naphthol.

Antibody Preparation

Antibodies from rabbit sera or mouse ascites were purified by protein A or thiophilic chromatography. Briefly, for preparation of the thiophilic affinity column, the epoxide groups were first introduced into acetone and diethyl ether washed resin (LiChrosphere Si300, or Kieselgel 60, from Merck, Darmstadt, Germany) and then cleaved with sodium hydrosulfide in Tris buffer (0.2 M, pH 8.5) for 1 h at room temperature. The silica was activated by stirring with a large amount of divinyl sulfone (1.5 ml/g silica) in Tris buffer (0.2 M, pH 8.5). Serum samples or ascites were adjusted to 0.75 M ammonium sulfate and applied to a column equilibrated in 0.75 M ammonium sulfate. Washing was continued until the baseline was reached. Immunoglobulins were then eluted with 0.1 M NaCl. The purity of the antibodies was analyzed by SDS-PAGE and quantified with an imaging system from Taigen Bioscience (Taipei, Taiwan, ROC) as described before [9].

Flow Cytometry

E. coli (strain XLI-blue) with or without expression plasmids were grown in 10 ml LB medium supplemented with 50 mg/ml ampicillin with vigorous shaking at 37°C until OD_595 of 0.5 was reached. Protein production was then induced by adding 0.5 mM IPTG to the bacterial culture for 1–3 h. To analyze the Trat-RHO expression on the bacterial surface, rabbit polyclonal lg against anti-RHO4559 or antiserum against recombinant Trat was incubated with the IPTG-treated cells at 4°C for 1 h. After washing with ice-cold PBS, samples

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