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Recovery of functional peptide transporter PepT1 in budded baculovirus fraction

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Abstract

Transporters play a critical role in many physiological and pathological states and expression of the functional transporter protein is essential in exploring its kinetics and developing effective drugs. We describe here the recovery of functional transporter protein in the baculovirus fraction. We introduced a gene encoding human peptide transporter PepT1, important for the absorption of protein hydrolytic products or peptide-mimetic drugs, into a baculovirus vector. After infection, a large amount of PepT1 appeared in the budded virus fraction compared with Sf9 cells. Uptake of [^{14}C]glycylsarcosine was markedly increased in an acidic condition and showed a clear overshoot in PepT1-expressing virus fraction. The apparent Michaelis constant for [^{14}C]glycylsarcosine was 0.55 ± 0.06 mM. [^{14}C]Glycylsarcosine uptake was inhibited by di- and tripeptides and orally active β-lactam antibiotics. These results suggest that functional PepT1 recovers efficiently in a budded virus fraction, and, thus, this expression system will be a useful tool for characterization and screening of peptide-mimetic drugs in drug discovery.

Keywords: Transporter; PepT1; Sf9; Baculovirus; Budded virus; Uptake

Transporters play a significant role in the maintenance of vital activities in the cells by transporting the essential substances in and the dispensable substances out. In addition, transporters expressed in various tissues have been considered to be one of the determinant factors of absorption, distribution, and excretion of drugs [1–3]. Recently, the relationship between abnormal functions of transporters and the pathogenesis of several diseases has gained much interest [4–6].

Peptide transporter PepT1 consists of 12 membrane spanning domains and is one of the well-characterized transporters in the SLC family [7]. PepT1 is under intense investigation in many laboratories because of its nutritional importance in absorption of protein hydrolytic products including di- or tripeptides from the small intestine, as well as in reabsorption of filtered di- or tripeptides from the renal proximal tubule. Recent studies have shown that many peptide-mimetic drugs including the numerous amino β-lactam antibiotics of the cephalosporin and penicillin classes [8], angiotensin-converting enzyme inhibitors such as captopril and the ester prodrugs of enalapril [9], and valyl prodrugs of ganciclovir and acyclovir [10,11] gain entry into the systemic circulation via PepT1 and show good oral availability. On the other hand, the result that PepT1 is highly expressed in the human pancreatic cancer cell lines may have impact on future therapeutic strategies in the treatment of

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pancreatic cancer or pancreatic cancer-derived metastasis [12]. These reports suggest that PepT1 is an important transporter in drug discovery.

The baculovirus expression system offers the advantage of high level recovery of heterologous transporter proteins in insect cells [13–17]. The system has been utilized for determining whether or not a drug is a substrate or inhibitor of a transporter [18]. PepT1 expressed by this system is an attractive method of screening or assessing pharmacologically interesting peptide-mimetic drugs in drug discovery. The major drawback of this high yield system, however, is the relatively large amount of contamination of non-functional expression products [13]. The display of intact membrane protein on the extracellular baculovirus particle was first demonstrated by Loisel et al. [19]. They showed that the virally displayed β-adrenergic receptor was in a glycosylated mature form and functional compared to the receptor protein recovered from Sf9 cell membrane [19]. This observation led to the effective reconstitution of leukotriene B4 receptors and trimeric G-proteins on the baculovirus envelope [20]. In addition to membrane receptors, endoplasmic reticulum (ER)[1] proteins were also displayed in budded viruses but in a less aggregated and less degraded form compared with the proteins expressed in the Sf9 cell membrane [21].

We describe here the recovery of functional PepT1 in budded baculovirus fraction. This provides a useful tool for characterization and screening of inhibitors and activators of PepT1 in drug discovery.

Materials and methods

Recombinant baculovirus construction and Sf9 cell culture

Expression vectors for human PepT1 were constructed as described below. The cDNA fragments encoding the ORF of human PepT1 derived from human kidney cDNA (Marathon-ready cDNA, Clontech, Palo Alto, CA) were amplified by PCR using gene-specific primer set. After their nucleotide sequences were confirmed, the PCR products were then cloned into *Bam*HI–*Xho*I site of vector pBlueBacHis2 (Invitrogen, Carlsbad, CA), and the baculovirus vectors expressing human PepT1 (pBlueBacHis2-PepT1) were constructed. The recombinant viruses were generated from Sf9 cells co-transfected with the vector for human PepT1 and Bac-N-Blue viral DNA (Invitrogen) by homologous recombination and sequential plaque purification and amplification. Sf9 cells were cultured in Grace's media (Invitrogen) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL), and 0.1% Pluronic F-68 (Gibco-BRL) in 1000 ml spinner flasks at 27 °C.

Sf9 cells and budded baculovirus preparation

Budded baculoviruses were isolated basically according to the method of Loisel et al. [19]. The culture medium of Sf9 cells infected with recombinant viruses for 48, 72, 96, or 120 h was centrifuged at 1000*g* for 15 min at 4 °C to precipitate cells; the supernatants were further centrifuged at 45,000*g* for 30 min at 4 °C. Pellets were then resuspended in ice-cold phosphate-buffered saline (PBS) and centrifuged at 1000*g* for 15 min at 4 °C to eliminate any remaining cells or debris. This supernatant was finally centrifuged at 45,000*g* for 30 min at 4 °C and the precipitate, used as the isolated baculovirus fraction, was resuspended in ice-cold PBS and stored at 4 °C until use. For preparation of the Sf9 cell lysate, the cells were resuspended in isotonic buffer (PBS containing 1% Triton X-100 and complete protease inhibitor mixture [Roche Applied Science]). After mixing at 4 °C for 30 min, the supernatant was prepared by centrifugation at 1000*g* for 10 min and used for Western blotting. Protein concentration was determined by DC Protein Assay Reagent (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Western blotting

SDS–PAGE was carried out as described by Laemmli [22], however, samples were not heat-treated in order to minimize aggregation. For Western blotting, proteins were resolved by SDS–PAGE and transferred to a PVDF membrane (Hybond-P, Amersham). After blocking with Block Ace (Snow Brand Milk Products, Japan) for 1 h at room temperature, the membrane was immunoblotted with monoclonal anti-polyhistidine antibody (His-1, Sigma, St. Louis, MO). Immunoreactive proteins were detected by an ECL Plus kit (Amersham Biosciences, Piscataway, NJ), and the amount of PepT1 was quantified by NIHimage using 75 kDa protein in a 6× His Protein Ladder (Qiagen, Germany) as a standard.

Sucrose density gradient centrifugation and electron microscopy

The budded virus was overlaid onto a 25–56% linear sucrose density gradient and centrifuged at 100,000*g* for 90 min in a SW28 rotor. Fractions (1.5 ml) were collected from the top of the gradient, and the distribution of PepT1 was examined by Western blotting with the monoclonal anti-polyhistidine antibody and SDS–PAGE with Coomassie brilliant blue staining. After confirming the budded virus fraction by SDS–PAGE and Western blotting, 2 μl of the budded virus fraction was applied to a carbon-coated 400-mesh copper grid that had been pre-coated with colloidion film. The grid was stained with 2% (wt/vol) phosphotungstic acid (pH 6.7). The material was immediately observed under an AKASHI EM-002A electron microscope (Topcon, Tokyo, Japan).

[1] *Abbreviations used:* ER, endoplasmic reticulum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution.