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SHORT NOTE
Rapid Isolation of Endosomes from BHK Cells: Identification of DPP IV (CD26) in Endosomes

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In plasma membrane glycoproteins the peripheral monosaccharides of the N-glycan side chains are degraded faster than the core oligosaccharides and the protein backbone. This intramolecular heterogenous turnover is a typical characteristic of membrane glycoproteins and is termed remodeling or reprocessing. The mechanism of the reprocessing has been shown first for dipeptidyl peptidase IV (DPP IV, CD26). However, it is still a question in which subcellular compartment the enzyme machinery for the reprocessing is located. Since lysosomes could be excluded, it has been proposed that the responsible glycosidases are located at the plasma membrane, in endosomes, or in the trans-Golgi network. The present study is concerned with the possible role of endosomes in this process of reprocessing. We transfected nonpolarized BHK cells with rat DPP IV cDNA. By establishing a fast and efficient method to purify endosomes, we could identify for the first time significant amounts of DPP IV in endosomes and we suggest therefore that endosomes are closely related with the regulation of reprocessing of plasma membrane glycoproteins.

INTRODUCTION

Dipeptidyl peptidase IV (DPP IV, CD26, EC 3.4.14.5) is an ectopeptidase cleaving X-Pro from the NH_2-terminus and is expressed as a transmembrane glycoprotein in a variety of tissues in mammals [1]. Physiological substrates for DPP IV include substance P, β-casomorphin, growth hormone-releasing factor, and the neuropeptides Y and YY [2, 3]. In addition to its function as a peptidase, DPP IV is involved in other cellular processes that are apparently unrelated to its enzymatic activity. For example, DPP IV participates in signal transduction of T-helper lymphocytes [4] and is reported to be associated with adenosine desaminase and CD45 [5, 6]. Furthermore, DPP IV acts as a cell adhesion molecule by mediating cell binding to collagen [7, 8].

Rat DPP IV is a type II membrane glycoprotein consisting of 767 amino acids with a molecular weight of 110 kDa as determined by SDS–PAGE [9]. The molecule appears to be a homodimer in its native state [10]. The extracellular portion of DPP IV can be divided into three parts: a membrane-proximal domain rich in N-glycans, followed by a cysteine-rich region, and finally, near the C-terminus of the protein, the active site of the peptidase [11].

It has been shown previously in rat liver that the peripheral monosaccharides (*N*-acetylneuraminic acid, D-galactose, L-fucose) of the N-glycan chains of the DPP IV glycoprotein have a three times shorter half-life compared to the core glycans (D-mannose, *N*-acetylglucosamine) or the protein backbone. Furthermore, DPP IV becomes reglycosylated and is reinserted into the plasma membrane [12]. In contrast to rat liver, the rapid turnover of N-glycans in rat hepatoma tissue is extended to the core glycans (D-mannose) [13]. This mechanism, termed intramolecular heterogenous turnover or reprocessing [12, 14], is not restricted to DPP IV but is also found in other integral membrane glycoproteins [14] and does not occur in serum glycoproteins [15]. The molecular mechanism and the biological significance of the reprocessing is not understood. Since it is still an open question in which subcellular compartment, endosomes, the trans-Golgi network (TGN), or the plasma membrane, the reprocessing of DPP IV occurs, we transfected rat DPP IV into BHK cells and established a fast and efficient method to separate subcellular compartments. Using Western blot analysis we were able to detect significant amounts of rat DPP IV in endosomal compartments of BHK cells.

MATERIALS AND METHODS

Cell culture. Wild-type BHK cells (BHK) and BHK cells transfected with rat DPP IV (BHK 2/5) were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum. Experiments were always carried out when cells had just reached confluency after passage. Horseradish peroxidase (HRP) was internalized by fluid-phase endocytosis as described in [16].

Antibodies. Monoclonal anti-DPP IV antibody 13.4 [17] was used as serum-free cell culture supernatant and monoclonal anti-rab5 antibody was a gift from Dr. R. Jahn (New Haven) and used as ascites.

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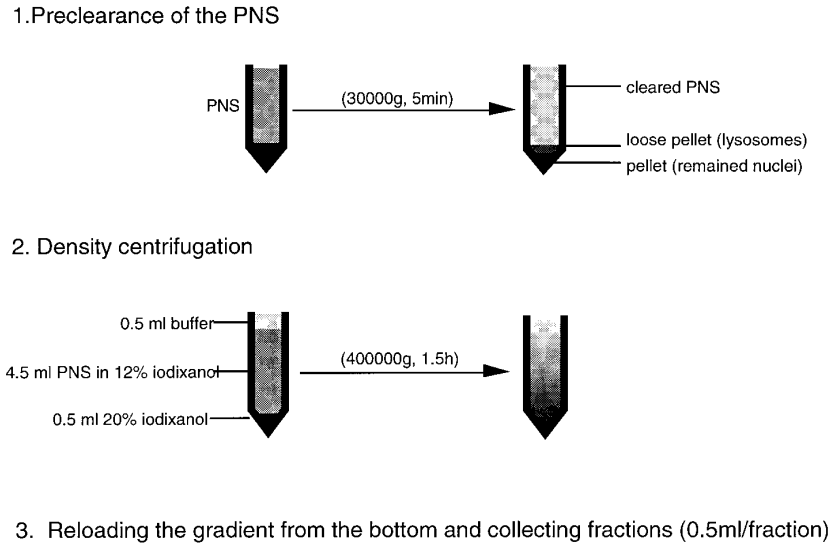

SCHEME 1

Polyclonal anti-rab6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to mouse or rabbit immunoglobulins conjugated to HRP or fluorescein isothiocyanate (FITC) were obtained from Dianova (Hamburg, Germany) and Sigma, respectively.

Cellular fractionation. Cells were homogenized in buffer containing 250 m*M* glucose, 3 m*M* imidazole, 0.5 m*M* EDTA at pH 7.4 by passing them 10 times through a syringe with a G22 × 1.25 needle. Cell homogenates were centrifugated at 3000*g* for 10 min to get a postnuclear supernatant (PNS). PNS was further centrifugated at 30,000*g* for 5 min to pellet remaining nuclei and dense lysosomes. Cleared PNS was adjusted to a 12% iodixanol (Nycomed, Oslo) solution using a 60% iodixanol stock solution and homogenization buffer, added on top of 0.8 ml 20% iodixanol cushion in a 6-ml Sorvall ultracentrifugation tube, and centrifuged in a vertical Sorvall rotor (TV-865; Sorvall) at 400,000*g* for 1.5 h without brake. Fractions of 0.5 ml were collected from the bottom of the tube and either further analyzed or diluted with homogenization buffer to 6 ml and membranes were then pelleted by further ultracentrifugation at 100,000*g* (for overview see Scheme 1).

Analytical procedures. Protein determination was performed in 96-well ELISA plates using 100 μl bicinchonic acid protein reagent (Pierce) and 25-μl samples. Plates were evaluated in a 96-well ELISA reader (Spectra) at 570 nm.

HRP activity was determined in 96-well ELISA plates and visualized by incubation with H_2O_2 and 2,2-azinodi-3-ethylbenzthiazoline sulfonate 6 (Sigma) as HRP substrates, according to the manufacturer's instructions. The color reaction was terminated by addition of 1% aqueous SDS and the optical density of the reaction products was determined at 405 nm.

Glycosidase activities were determined in 96-well ELISA plates using the respective *p*-nitrophenylpyranoside (Sigma) as glycosidase substrate. Twenty-five microliters of each sample was incubated with 100 μl *p*-nitrophenylpyranoside in acetate buffer, pH 4.2 (3 mg/ml), and incubated for 1 h at 37°C. Reaction was stopped by adding 200 μl 1 *M* Na_2CO_3 and the optical density of the reaction products was determined at 405 nm.

DPP IV activity was determined in 96-well ELISA plates. A 150-μl sample in 100 m*M* Tris–HCl, pH 8.0, was incubated with 10 μl of a 10 mg/ml stock solution of glycylpropyl-*p*-nitroanilidtosylate (Bachem, Switzerland) as substrate for DPP IV. Plates were incubated for 1 h at 37°C. Reaction was terminated by addition of 100 μl CH_3COO-Na (1 *M*, pH 4.5), and the optical density of the reaction products was determined at 405 nm.

Immunoblotting. Samples were electrophoresed on SDS–polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose filters. The blots were blocked with 4% fat-free dry milk powder in PBS, incubated with the respective primary antibodies, washed with PBS, and incubated with the appropriate secondary antibodies (Sigma, Dianova). After washing, labeled proteins were detected by enhanced chemiluminescence detection (Amersham Buchler) according to the manufacturer's instructions and visualized by exposing the blots to Kodak XR-5 films for periods between 10 and 120 s. When necessary, blots were stripped in buffer containing 100 m*M* 2-mercaptoethanol, 2% SDS, 50 m*M* Tris–HCl, pH 6.7, for 30 min at 57°C. The blots were then washed and equilibrated in PBS before reusing.

Establishment of a BHK cell clone expressing rat DPP IV. Plasmid cDNA encoding rat DPP IV and Geneticin resistance [18] was introduced into BHK cells by electroporation (Bio Rad) using the following conditions: 20 μg DNA was added to 10^7 BHK cells in 500 μl medium. Cells were incubated on ice for 10 min and electroporation was performed using the following parameters: 0.3 V, 100 Ω, 960 μFD. Cells were incubated again on ice for 10 min. Selection was carried out for 2 months using medium containing 700 mg/liter Geneticin (Gibco). Geneticin-resistant cells were cloned by limiting dilution, and expression of rat DPP IV of these cell clones was tested by immunofluorescence using monoclonal anti-DPP IV antibodies.

Immunofluorescence. BHK cells transfected with rat DPP IV were grown in plastic dishes with 3.5-cm diameter and fixed with 3% formaldehyde in PBS for 10 min at room temperature (RT). Cells were blocked in PBS containing 0.5% BSA and 0.1 *M* glycine for 20 min at RT, stained with primary antibodies for 30 min at RT, and incubated with FITC-conjugated secondary antibodies (Sigma) for 20 min at RT. Labeled cells were washed with PBS and covered with a glass coverslip in elvanol. Dishes were viewed on a Zeiss Axiolab microscope.

RESULTS AND DISCUSSION

DPP IV Expression of BHK Cells

Indirect immunofluorescence analysis of the transfected BHK cell clone revealed that all cells ex-

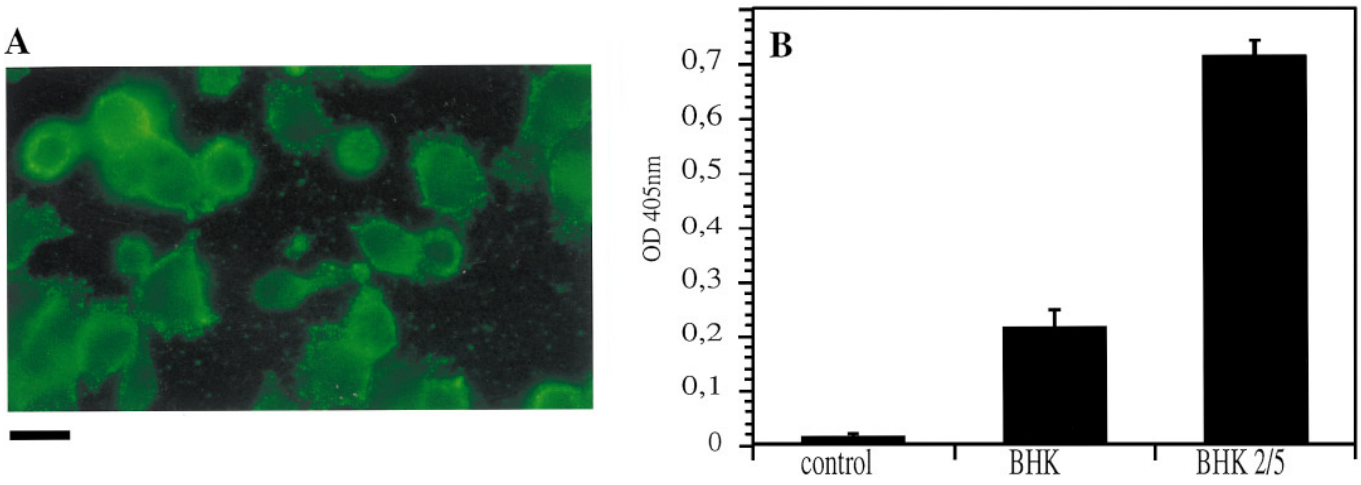

FIG. 1. (A) Immunofluorescence staining of rat DPP IV-transfected BHK cells. Adherent growing BHK cells transfected with rat DPP IV were fixed and stained with monoclonal anti-DPP IV antibody. Bar represents 10 μm. (B) Determination of DPP IV activity in transfected and untransfected BHK cells. DPP IV enzyme activity was determined in solubilysates of wild-type BHK cells (BHK) and in BHK cells transfected with rat DPP IV (BHK 2/5). The optical density of the reaction product was measured at 405 nm. Background of the color reaction products was determined in the absence of cell solubilysates (control). Mean values +/− standard deviations from two independent experiments carried out in duplicates are shown.

pressed rat DPP IV at their surface. All plasma membrane regions of the adherent growing cells were strongly stained (Fig. 1A). No difference in growth rate or adhesive properties were observed between the rat DPP IV-transfected and wild-type BHK cells (data not shown). In agreement with these data, high levels of enzyme activity of DPP IV could only be determined in transfected BHK cells and not in wild-type BHK cells (Fig. 1B).

Distribution of Subcellular Compartments in the Density Gradient

We then established conditions to prepare endosomal fractions from BHK cells using several centrifugation

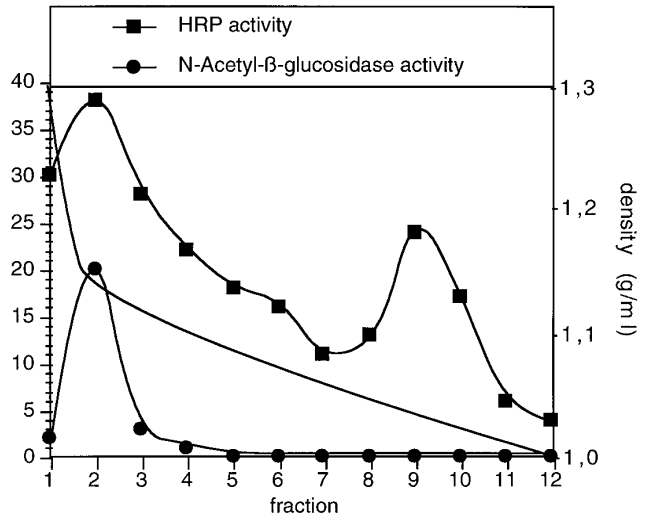

FIG. 2. Density fractionation of a 30,000*g* supernatant. Gradients were collected (0.5 ml) dense end first (fraction 1) and relative activity of endocytosed HRP as marker for endosomes and of *N*-acetyl-β-glucosidase activity as marker for lysosomes was determined for each fraction. Density of each fraction of the gradient is indicated on the right in g/ml.

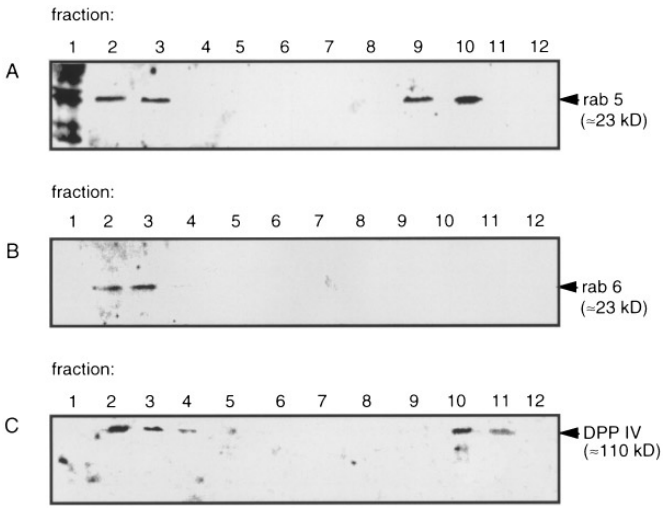

FIG. 3. Immunoblot analysis of each fraction after density fractionation. Aliquots of subcellular compartments of BHK cells transfected with rat DPP IV separated by density centrifugation were electrophoresed, transferred onto nitrocellulose filters, and probed with (A) monoclonal anti-rab5 antibody, (B) polyclonal anti-rab6 antibodies, and (C) monoclonal anti-DPP IV antibody.

steps followed by a density centrifugation. As a general marker of endosomal content, we used HRP endocytosed from the fluid phase. HRP enters early endosomal compartments 5 min after internalization and reaches late endosomes within 30 min at 37°C (data not shown but published in [19]). *N*-Acetyl-β-glucosidase activity was used as a marker for lysosomes. Peak levels of endocytosed HRP could be detected in fractions 1 to 4 and 8 to 11 (Fig. 2), which represent fractions containing endosomal structures (see next section). *N*-Acetyl-β-glucosidase activity was only detected in fractions 1 to 4 and not in fractions 8 to 11 (Fig. 2).

Detection of Rat DPP IV in Endosomes

Subcellular compartments enriched after density centrifugation were further analyzed by Western blot analysis. Small GTP-binding proteins (for review, see [20]) were used as intracellular compartment markers (rab5 for endosomes and rab6 for the Golgi). Rab5 could be detected in dense fractions 1 to 3, representing Golgi and plasma membrane, and also in endosomal fractions 9 to 10 (Fig. 3A). Golgi membranes were densely banded in fractions 2 and 3 (Fig. 3B). Transfected rat DPP IV was not only found in fractions 1 to 3, but also in fraction 10, which represent endosomal compartments, and in the even less dense fraction 11 (Fig. 3C).

In our experiments we could detect significant amounts of DPP IV in endosomal compartments of transfected BHK cells by Western blot analysis. Endosomes were identified by the endocytosed fluid-phase marker HRP and the GTPase rab5 after subcellular fractionation. Based on this finding we suggest that the mechanism of reprocessing of DPP IV is regulated via the endosomal pathway. Our most favored hypothesis to explain the reprocessing of DPP IV involves endosomes; deglycosylation of internalized glycoproteins could occur in endosomal compartments, deglycosylated glycoproteins are then further transported to the Golgi system, reglycosylated, and transported back to the plasma membrane.

During endocytosis, endosomes are generated by several vesicle fusion events. Early endosomes can be converted into late endosomes and both, early and late endosomes, are thought to communicate with the TGN via transport vesicles (for review, see [21]). Recent quantitative studies on the fluid-phase uptake of HRP revealed that it becomes distributed in early endosomal compartments within 2 min [22]. Since we have used 5-min HRP-labeling time and rab5 as endosomal marker, our data clearly support the hypothesis that the mechanism of the reprocessing of DPP IV is regulated via early endosomes. However, further work is necessary to identify the specific glycosidases involved in this process.

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