FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 46, No. 4, 2008
pp. 419-421

Co-transfection of EYFP-GH and ECFP-rab3B in an experimental pituitary GH3 cell: a role of rab3B in secretion of GH through porosome

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Abstract: Recently, in order to elucidate the role of rab3B in porosome, we have observed the incorporation of rab3B in the secretion of GH through porosome under confocal laser scanning microscopy (CLSM). Transfected cells with GH-EYFP fusion protein and rab3B-ECFP fusion protein were observed under CLSM, which showed the colocalization of EYFP-GH and ECFP-rab3B in the budding configuration of secretory process. These structural and functional images of rab3B imply the incorporation of rab3B in the secretion of GH through porosome.

Key words: GH, rab3B, porosome, EYFP, ECFP

Introduction

Low molecular weight GTP-binding proteins of the rab family are known to act as the central regulators of vesicular traffic, and they are associated with membrane vesicles or granules that are undergoing exocytotic fusion with the plasma membrane [1,2]. Rab3B is the major form found in the anterior pituitary [1]. Lledo *et al.* reported that antisense oligonucleotides against rab3B specifically and reversibly block the expression of rab3B, and inhibit Ca^{2+}-dependent exocytosis in pituitary cells [1]. A number of proteins are involved in vesicular budding and fusion in cells [3,4]. Among these proteins, the *N*-ethylmaleimide-sensitive factor (NSF)-attachment protein receptors (SNAREs) were implicated as the minimal fusion machinery capable of fusing opposing bilayers [4]. Employing a variety of approaches, including atomic force microscopy and electrophysiology, Jena and his research group were able to resolve the molecular mechanism of SNARE-induced membrane fusion in cells [5-7]. Their studies demonstrate that in the presence of Ca^{2+}, target- (t-) SNAREs (syntaxin and SNAP-25/23) and vesicle- (v-) SNARE (vesicle associated membrane protein (VAMP)) in opposing bilayers interact in a circular array to form conducting channels, porosome [5]. T-SNAREs are located at the cell plasma membrane (present at the base of porosomes), and v-SNAREs are present at the secretory vesicle membrane. Previously, we investigated the modulations of the intracellular dynamics of growth hormone (GH), rab3B, SNARE proteins such as SNAP-25 and syntaxin, in rat pituitary cells, caused by growth hormone-releasing hormone (GHRH) and somatostatin (SRIF) [8,9]. These proteins display a close relation-

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Folia Histochem Cytobiol. 2008:46(4): 419 (419-421)
doi: 10.2478/v10042-008-0069-5

ship in GH secretion [8,9]. In order to examine more specific relationship among molecules that play important roles in transportation and secretion of pituitary hormone, we developed an experimental pituitary cell line that has secretory granules of GH linked to enhanced yellow fluorescein protein (EYFP), which enabled us to visualize the real-time movement of the molecules in a living cell [10]. Recently, in order to elucidate the role of rab3B in porosome, we have observed the incorporation of rab3B in the secretion of GH through porosome under confocal laser scanning microscopy (CLSM). In this paper, we describe the synergistic dynamics of rab3B and GH in porosome with illustrative fluorescein imaging of rab3B and GH in an experimental cell line.

Materials and methods

Construction of rat EYFP- GH-1 plasmid for transfection of GH3 cell. The rat GH cDNA clone pRGH-1 and the EYFP-expression construct pEYFP-N1 were obtained from the American Type Culture Collection (Manassas, VA, USA) and Clontech Laboratories, Inc., (Palo Alto, CA, USA), respectively. The GH-EYFP fusion construct pCMV-Sig- EYFP-GH-1 was derived from pEYFP-N1 and contained a sequence encoding the rat GH signal peptide (1 to 26 in the rat GH amino-acid sequence) and the EYFP-coding segment, followed by another rat GH coding sequence (27 to 217 in the rat GH amino-acid sequence).

Construction of rat rab3B-enhanced cian fluorescein protein (ECFP) plasmid for transfection of GH3 cell. prab3B-ECFP was constructed as follows. A cDNA fragment encoding rat rab3B was amplified by RT-PCR and cloned in-frame into the EcoRI/BamHI sites of pECFP-N1 (Clontech Laboratories, Inc.), which expresses rab3B-ECFP fusion proteins in the mammalian cell. Plasmids were purified using Qiagen midi-prep kit (Qiagen GmbH, Hilden, Germany), and were verified by nucleotide sequencing.

GH3 cell culture. GH3 cells were maintained at 37°C in a 5% CO_2 in DMEM/Ham's F-12 medium supplemented with 2.5% heat-inactivated fetal bovine serum (FBS), 10% horse serum (HS), 100U/ml of penicillin and 100 μg/ml of streptomycin at 70% confluency on poly-L-lysin coated 35-mm glass base dishes.

Transfection. Transfection of GH3 cells was performed using lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA). Briefly, GH3 cells at 70% confluency on poly-L-lysin-coated dishes were co-transfected with pGH-EYFP and prab3B-ECFP using lipofectamine 2000 in low serum Opti-MEM using lipofectamine 2000 for 4-5 h. The transfected cells were washed and incubated in DMEM/Ham's F-12 medium for 24-36h before microscopic observation, harvest for immunoblot analyses, or selection of stable transformants in DMEM/Ham's F-12 medium supplemented with f.c. 250 μg/ml of Geneticin.

Western blotting for pCMV- sig- EYFP-GH-1 and pCMV-ECFP-rab3B. To prepare whole cell extracts for immunoblot analyses, transfected cells were heated at 100°C in Laemmli sample buffer for 10 min, sonicated and cleared by centrifugation. The extracts were fractionated on a 12.5% SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane. For the detection of GH-EYFP fusion protein and rab3B-ECFP fusion protein, anti GFP antibody (anti-green fluorescent protein, rabbit IgG, Molecular Probe, Invitrogen Corp., Carlsbad, CA, USA) was used, and signals were detected with ECL-Plus chemiluminescence reagents.

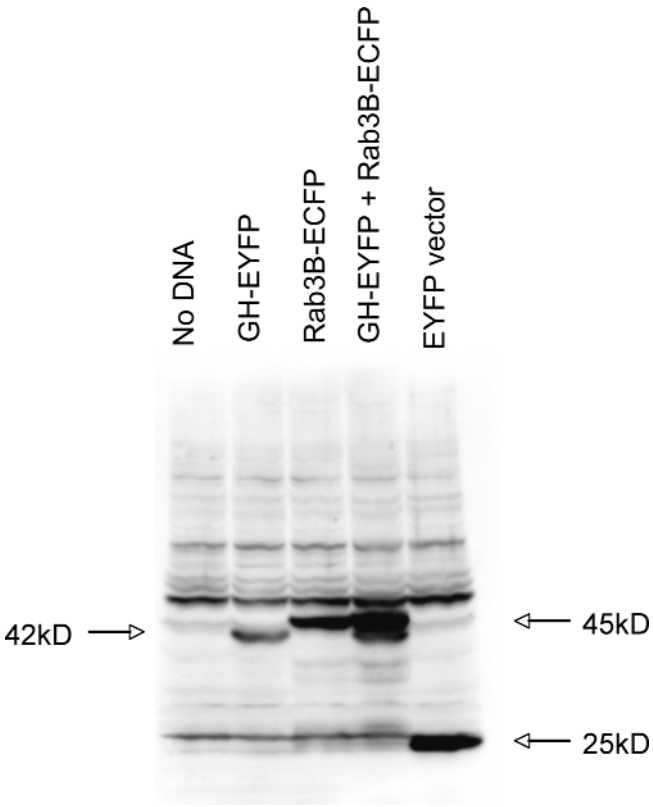

Fig. 1. In the whole cell extracts of transfected cells with GH-EYFP fusion protein and rab3B-ECFP fusion protein, the Western blotting showed 42kD band of GH-EYFP fusion protein and 45kD band of rab3B-ECFP fusion protein.

Visualization of the dynamics of EYFP-GH and ECFP-rab3B. Cells were observed under CLSM for the inspection of the dynamics of EYFP-GH and ECFP-rab3B.

Results and discussion

In the whole cell extracts of transfected cells with GH-EYFP fusion protein and rab3B-ECFP fusion protein, the Western blotting showed 42kD band of GH-EYFP fusion protein and 45kD band of rab3B-ECFP fusion protein (Fig. 1). Transfected cells with GH-EYFP fusion protein and rab3B-ECFP fusion protein were observed under CLSM, which showed the colocalization of EYFP-GH and ECFP-rab3B in the budding configuration of secretory process (Fig. 2). These structural and functional imagings of rab3B imply the incorporation of rab3B in the secretion of GH through porosome.

Rab3B is reported to be a key intracellular signaling molecule that can control exocytosis in anterior pituitary cells [1]. To elucidate the role of rab3B with regard to SNARE mechanisms, we undertook experiments to elucidate the role of rab3B in GH secretion and the mutual relationships with SNARE proteins

Folia Histochem Cytobiol. 2008:46(4): 420 (419-421)
doi: 10.2478/v10042-008-0069-5

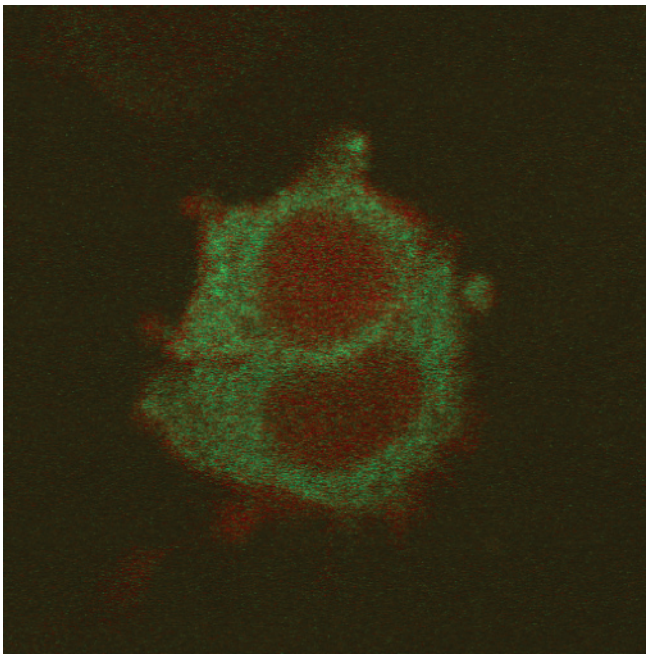

Fig. 2. Transfected cells with GH-EYFP fusion protein and rab3B-ECFP fusion protein were observed under CLSM, which showed the colocalization of EYFP-GH (green) and ECFP-rab3B (red) in the budding configuration of secretory process. These structural and functional images of rab3B imply the incorporation of rab3B in the secretion of GH through porosome.

such as SNAP-25 and syntaxin [8,9]. CLSM of immunohistochemical double stainings for SNAP-25, syntaxin and rab3B demonstrated co-localization of rab3B and these SNARE proteins in GHRH-treated rats, and their dissociation in SRIF-treated rats. These results suggest that rab3B plays a principal role in GH secretion from the anterior pituitary and that SNAP-25 and syntaxin act in association with rab3B in functional regulation of GH secretion. Moreover, in order to investigate, in real time, the transport and secretion of pituitary hormone, we have developed a stable experimental pituitary cell line, GH3 cell, which has secretory granules of GH linked to EYFP [10]. This GH3 cell has secretory granules of GH linked to EYFP, and secretes this molecule upon stimulated by Ca^{2+} influx or Ca^{2+} release from storage. This GH3 cell will be useful for the real-time visualization of the intracellular transport and secretion of GH. In the present experiment, using the experimental cell line expressing EYFP-GH and ECFP-rab3B, we have demonstrated the role of rab3B in the vesicular budding and GH secretion through porosome. These bioimages of rab3B support the role of rab3B in cooperation of GH secretion with SNARE system and porosome.

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Submitted: 9 March, 2008
Accepted after reviews: 12 May, 2008

Folia Histochem Cytobiol. 2008:46(4): 421 (419-421)
doi: 10.2478/v10042-008-0069-5