Available online at www.sciencedirect.com
SCIENCE DIRECT
Archives of Biochemistry and Biophysics 449 (2006) 130–142
ABB
www.elsevier.com/locate/yabbi

Role of Rab5 in insulin receptor-mediated endocytosis and signaling

C.M. Hunker, I. Kruk, J. Hall, H. Giambini, M.L. Veisaga, M.A. Barbieri *

Department of Biological Sciences, Florida International University, University Park, Miami, FL 33199, USA

Received 27 October 2005, and in revised form 25 January 2006
Available online 3 March 2006

Abstract

Activated insulin receptors recruit various intracellular proteins leading to signal generation and endocytic trafficking. Although activated receptors are rapidly internalized into the endocytic compartment and subsequently degraded in lysosomes, the linkage between insulin receptor signaling and endocytosis is not well understood. This study utilizes both overexpression and depletion of Rab5 proteins to show that they play a critical role in both insulin-stimulated fluid phase and receptor-mediated endocytosis. Specifically, Rab5:WT and Rab5:Q79L (a GTP-hydrolysis defective mutant) enhance both types of endocytosis in response to insulin, while Rab5:S34N (a GTP-binding defective mutant) has the opposite effect. Morphological analysis indicates that both Rab5 and insulin receptor are found on early endosomes, but not at the plasma membrane. In addition, expression of Rab5:WT and Rab5:Q79L enhance both Erk1/2 and Akt activation without affecting JN- and p38-kinase activities, while the expression of Rab5:S34N blocks both Erk1/2 and Akt activation. Consistent with these observations, DNA synthesis is also altered by the expression of Rab5:S34N. Taken together, these results demonstrate that Rab5 is required for insulin receptor membrane trafficking and signaling.

Keywords: Signal transduction; Rab5; Endocytosis; Insulin receptor

Receptor-mediated endocytosis is an essential mechanism for several important physiological processes. It is well established that the insulin receptor undergoes endocytosis upon insulin stimulation, which, in turn, produces both metabolic and mitogenic responses in cells expressing this receptor [11,19,20,28,30,39]. Metabolic responses include rapid increases in cellular uptake and storage of glucose, lipids, and amino acids.

In addition, the mitogenic responses to insulin appear to involve many of the pathways utilized by other growth factors whose receptors have intrinsic tyrosine kinase activity [2,22,32,39,41]. Like the epidermal growth factor (EGF)-receptor, insulin receptor is internalized into intracellular vesicular compartments following ligand binding, activation of intrinsic tyrosine kinase activity, and autophosphorylation [34]. This, in turn, will induce the binding of several factors, including the insulin receptor substrates (IRS-1 and IRS-2) [35,12].

Once phosphorylated and activated, IRS recruits and activates a variety of signaling molecules (for example, the p85 subunit of phosphatidylinositol (PI) 3-kinase and the Grb2/Sos complex) [10,9,14,40] in a similar fashion to the phosphorylated carboxy-terminal domain of the EGF-receptor [37,42]. After internalization, insulin and its receptor promptly dissociate in response to the acidic pH found in endosomes. Dissociation of insulin from its receptor allows receptor recycling and degradation. Only upon final delivery to the lysosome and/or some prelysosomal degradative compartment are the receptors completely inactivated [32,22,23,17,25,44].

Following receptor internalization, Rab5 (a member of the small GTP-binding protein family) plays a key role in regulating the trafficking of several receptors, including EGF-receptor and Transferrin-receptor [4,36]. Newly formed endocytic vesicles carrying the activated receptor and other cargo fuse with early endosomes and gain access to the endocytic pathway. Endosome fusion is a Rab5-dependent process. Using an in vitro assay that measures early endosome fusion, activation of Rab5 was found

* Corresponding author.
E-mail address: barbieri@fiu.edu (M.A. Barbieri).

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.01.020

to be rate limiting for this process [6,8,26]. Over-expression of Rab5a and/or an activated Rab5a mutant has also been shown to stimulate both EGF receptor uptake and fluid phase endocytosis. Interestingly, we have demonstrated that activation of EGF-receptor regulates the nucleotide status of Rab5, a process that is dependent on selective domains in the cytoplasmic tail of the EGF-receptor and on the activation of Ras [4]. Furthermore, the activation of the small GTPase Ras has been implicated in the regulation of both Raf/Erk1/2 and PI3k/Akt pathways, as well as on fluid phase endocytosis [10].

Recently, it has been demonstrated that Ras-activated endocytosis is facilitated by the ability of Ras to directly regulate the Rab5a nucleotide exchange activity of Rin1 [38]. Rin1 contains an SH_2 (Src homology2) domain, a proline-rich domain, a Vps9 domain and a region involved in the binding of activated Ras (RBD) [1]. The SH_2 domain has been shown to interact specifically with tyrosine phosphorylated residues in the EGF-receptor tails and the Vps9 domain of Rin1 has been shown to serve as a Rab5-specific guanine nucleotide exchange factor (GEF)[1] [5,38]. Rin1's GEF activity is potentiated by the binding of activated H-Ras and also increases EGF-receptor endocytosis when co-expressed with Rin1 [38].

In this study, we demonstrate that Rab5 regulates fluid phase insulin-dependent endocytosis as well as insulin receptor-mediated endocytosis, and that both GTP binding and GTP hydrolysis are required for optimal function of Rab5. We also find that expression of the Rab5:S34N dominant negative mutant inhibits insulin-stimulated growth as well as activation of Akt and Erk1/2 kinases. However, the expression of Rab5:WT or the Rab5:Q79L constitutively activated mutant stimulates both insulin-dependent growth as well as activation of Akt and Erk1/2 kinases. These results suggest that Rab5 plays an important role in both insulin receptor-membrane trafficking and signaling.

Materials and methods

Materials

HepG2 and CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). INS-1, Adipocytes, and CHO-IR cells were generous gifts from Dr. Mike Mueckler (Washington University, MO) and Richard Roth (Stanford University, CA), respectively. PlateE and PhoA cells were generous gift from Dr. P.D. Stahl (Washington University, MO). The anti-human Rab7 was purchased from Calbiochem. A polyclonal antibody against GFP was obtained from Upstate, the anti-*myc* monoclonal antibody is from Invitrogen (Carlsbad, CA), and the anti-HA antibody is from Upstate. Transferrin, Goat anti-mouse, and anti-rabbit IgG conjugated to Alexa-546 were obtained from Molecular Probes (Eugene, OR). Mouse monoclonal and polyclonal anti-Rab5 antibodies were obtained from BD Biosciences Pharmingen and Santa Cruz, Biotechnology, Inc., respectively. The anti-IR-beta chain polyclonal antibodies for immunofluorescence were purchased from Upstate. The phospho-p42/44 (Erk1/2), phospho-Raf, and phospho-AKT antibodies were purchased from Cell Signaling Technology. The phospho-JNK and phospho-p38k antibodies as well as total anti-Erk1/2, AKT, p38k, Raf, and JNK were purchased from Sigma-Aldrich. Paraformaldehyde was obtained from Electron Microscopy Sciences (Ft. Washington, PA). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Fugene6 and Lipofectamine2000 were purchased from Roche and Invitrogen, respectively. Recombinant human insulin and IGF-I were purchased from Calbiochem and Upstate Biotechnology, respectively. All other reagents were from Sigma unless otherwise noted.

Construction of recombinant pMX-retroviruses

cDNAs of GFP-Rab5 were subcloned into the pMX-puromycin (pMX-puro) retrovirus system as described earlier [4]. The cDNA were used to transfect confluent PhoA and PlateE cell monolayers using a Fugene6-mediated procedure. Cells were maintained at 37 °C, and the media containing released viruses were harvested 48 h after transfection. Virus titers were generally between 10^8 and 10^9 plaque-forming units per ml. Virus stocks were aliquoted and kept frozen at −80 °C before use. HepG2-Rab5:WT and mutants cell lines were generated by expressing Rab5 constructs using pMX-puro as described earlier [4].

HRP endocytosis

Cell monolayers in 35-mm dishes were washed three times with serum-free α-MEM, and HRP uptake was initiated by addition of 1 ml of α-MEM containing 2 mg/ml HRP (Sigma) and 0.2% (wt/vol) bovine serum albumin (BSA) in the absence or in the presence of insulin as indicated in each figure. The uptake was conducted at 37 °C for the indicated time in each figure. After uptake, the cells were washed three times with phosphate-buffered saline (PBS) containing 0.2% BSA and then scraped into 1 ml of PBS. The dishes were rinsed once with 1 ml of PBS and the cell suspensions were pooled. Cells were centrifuged at 1200*g* for 3 min in a Beckman GPR centrifuge. Cell pellets were washed once by resuspension in 2 ml of PBS. Each cell pellet was lysed in 500 μl of PBS containing 0.1% (vol/vol) Triton X-100. Cell lysates were assayed for HRP activity as previously described [43].

Insulin internalization assay

The binding and the internalization activities of insulin in HepG2 cells were determined as described previously [20]. Confluent HepG2 cells were incubated with 100 pM

[1] *Abbreviations used:* IR, insulin receptor; Erk1/2, extracellular signal-regulated kinase, GEF, guanine nucleotide exchange factor; GFP, green fluorescence protein; JNK, jun N-terminal kinase; p38K, p38 protein kinase; siRNA, small interfering RNA; RNAi, RNA interference.