VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 21 February 2008
Revised form accepted: 06 May 2008
Published online: 25 July 2008

Volume 13 (2008) pp 632-648
DOI: 10.2478/s11658-008-0027-4

Research article

EHDS ARE SERINE PHOSPHOPROTEINS: EHD1 PHOSPHORYLATION IS ENHANCED BY SERUM STIMULATION

BORIS FICHTMAN[1], LIAT RAVID[2], DEBORA RAPAPORT and MIA HOROWITZ*
Department of Cell Research and Immunology, Faculty of Life Sciences, Tel-Aviv University, Ramat Aviv 69978, Israel

Abstract: Endocytic processes are mediated by multiple protein-protein interacting modules and regulated by phosphorylation and dephosphorylation. The Eps15 homology domain containing protein 1 (EHD1) has been implicated in regulating recycling of proteins, internalized both in clathrin-dependent and clathrin-independent endocytic pathways, from the recycling compartment to the plasma membrane. EHD1 was found in a complex with clathrin, adaptor protein complex-2 (AP-2) and insulin-like growth factor-1 receptor (IGF-1R), and was shown to interact with Rabenosyn-5, SNAP29, EHBP1 (EH domain binding protein 1) and syndapin I and II. In this study, we show that EHD1, like the other human EHDs, undergoes serine-phosphorylation. Our results also indicate that EHD1 is a serum-inducible serine-phosphoprotein and that PKC (protein kinase C) is one of its kinases. In addition, we show that inhibitors of clathrin-mediated endocytosis decrease EHD1 phosphorylation, while inhibitors of caveolin-mediated endocytosis do not affect EHD1 phosphorylation. The results of experiments in which inhibitors of endocytosis were employed strongly suggest that EHD1 phosphorylation occurs between early endosomes and the endocytic recycling compartment.

* Author for correspondence: e-mail: horwitzm@post.tau.ac.il;
[1]Current address: University of California San Diego, CA 92093, USA,
[2]Current address: The Weizmann Institute of Science, Rehovot, 76100, Israel

Abbreviations used: AP-2 – adaptor protein complex-2; BSA – bovine serum albumin; CHO – Chinese hamster ovary; cpm – counts per minute; dFCS – dialyzed FCS; DMEM – Dulbecco modified eagle medium; EHBP1– EH domain binding protein 1; FCS – fetal calf serum; FYVE – phenylalanine-tyrosine-valine-glutamate; IGF-1R – insulin-like growth factor-1 receptor; mDHFR – mouse dihydrofolate reductase; MEM-Alpha – minimal essential medium-alpha; ORF – open reading frame; PBS – phosphate buffered saline; PKC – protein kinase C; PVDF – polyvinidilen difluoride; TLC – thin-layer chromatography; TLE – thin-layer electrophoresis

Key words: Endocytosis, EHD1, EH domain, Phosphorylation

INTRODUCTION

Endocytosis and vesicular trafficking are complex processes that depend on a large number of protein-protein interactions mediated by specific modules. These processes may be regulated by phosphorylation and dephosphorylation [1, 2]. One such module is the EH domain, first identified in Eps15 [3], which binds an NPF (aspargine-proline-phenylalanine) sequence in the target proteins [4]. Among the EH domain-containing proteins are members of the EHD subfamily [4], characterized by three structural modules: an N-terminal domain, harboring nucleotide binding motifs [5, 6]; a central coiled-coil domain, involved in oligomerization; and a single C-terminal EH domain. There are four vertebrate paralogs [7, 8], two plant orthologs and one gene in *C. elegans* [9] and *Drosophila* [10].

Vertebrate EHDs show a distinct tissue expression pattern [7, 8, 11-13] and intracellular localization. EHD1 was localized mainly to the endocytic recycling compartment (ERC) [7] with some localization on the plasma membrane and early endosomes [14, 15], while EHD2 was localized to the plasma membrane [16, 17]. EHD3 was located on early endosomes and the perinuclear tubular and vesicular structures of the recycling compartment [12, 18] and EHD4 was localized to the plasma membrane, to vesicular structures [19] and to early endosomes [20]. EHD1 has been implicated in the control of recycling to the plasma membrane of cargo internalized by clathrin-dependent and clathrin-independent endocytic pathways, including: the transferrin receptor [9, 21], CFTR (cyctic fibrosis transmembrane regulator) [22], GLUT4 (insulin-responsive glucose transporter) [23], MHC-I (major histocompatibility complex) [24], AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) type glutamate receptors [25] and IGF-1R [26]. Total or partial absence of EHD1 or expression of its dominant negative mutants led to attenuated recycling to the plasma membrane [9, 15, 21, 24].

It was recently shown that EHD1 has the ability to bind directly to an array of phospholipids, preferably phosphoinositols with a phosphate at position 3 [11, 27]. Several proteins interact with EHD1, including Rabenosyn-5 [28], syndapins I and II [29, 30], EHBP1 [23], Rab11-FIP2 (Rab11-family interacting protein 2) [18] and SNAP29 [26, 30]. Rabenosyn-5 is involved in endosomal fusion events on early endosomal membranes, and binds to phosphatidylinositol 3-phosphate via its FYVE domain [31]. Syndapins have been implicated in vesicle formation and the coordination of endocytosis with regulation of the actin cytoskeleton [32]. They bind the EH domain of the EHDs through their NPF motif [29]. The EHBP1 protein, which connects actin dynamics with endocytosis, has five NPF motifs through which it interacts, most probably with the EH domain of EHD1 [23]. Rab11-FIP2 is a member of the family of Rab11-binding proteins that mediates endocytic recycling [33]. The interaction between

EHD1 and Rab11-FIP2 is mediated through the NPF motif of Rab11-FIP2 and the EH domain of EHD1 [18]. SNAP29 is a member of the SNAP family, which includes SNAP23 and SNAP25. It is a target membrane SNARE protein (t-SNARE) that has a role in vesicle docking and membrane fusion [34].

The initiation and regulation of endocytosis depend on phosphorylation and dephosphorylation [2]. Phosphorylation of the different subunits of the AP-2 complex provides negative or positive regulation of internalization [35, 36]. The recruitment and assembly of clathrin depend on Src-mediated phosphorylation of the clathrin heavy chain [37]. Tyrosine phosphorylation of Eps15 (epithelial growth factor receptor pathway substrate) by EGFR (epithelial growth factor receptor) has been shown to be essential for ligand-induced EGFR internalization [38].

In this study, we show that all human EHDs are serine phosphoproteins. EHD1 is a serum-inducible serine-phosphoprotein. We also show that EHD1 phosphorylation occurs between the early endosomes and the endocytic recycling compartment during clathrin-mediated endocytosis.

MATERIALS AND METHODS

Construction of plasmids

To construct HAEHD1, a 1.7-kb *EagI* fragment of EHD1 was cloned into the *NotI* site of pCruz HA^{TM} vector (Santa Cruz Biotechnology, Inc. CA, USA). To construct MycEHD2, MycEHD3 and MycEHD4, 1.7-kb cDNA fragments from the corresponding cDNAs were cloned into a CMVneoMYC vector (donated by Prof. Sima Lev of the Weizmann Institute of Science, Rehovot, Israel) between the *XhoI* and *EcoRV* sites (for EHD2), *NotI* and *NsiI* sites (for EHD3) and *NotI* and *XhoI* sites (for EHD4). Site-directed mutagenesis was employed to mutate all the serines in EHD1 to alanines. The reactions were performed in 25 μl containing 50 ηg of HAEHD1, 25 ρmoles of each dNTP, 20 ρmoles of a primer containing the mutation, and 1.25 units of PFU turbo DNA polymerase in PFU buffer (Stratagene, La Jolla, CA, USA) at 37ºC for 1 h. Mutations were validated by sequencing.

Cells and transfections

HEK293 cells were grown in DMEM supplemented with 10% FCS. CHO cells were grown in F-12 medium supplemented with 10% FCS at 37ºC. CHO cells stably expressing HAEHD1 (CHOHAEHD1) were generated by cotransfecting CHO cells with pBABE-puro plasmid (Addgene Inc. Cambridge, MA/USA) and HAEHD1, using FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. 48 h later, cells were split into dishes with a medium containing 7 μg/ml of puromycin (Sigma, Steinheim, Germany). Puromycin-resistant clones were tested for HA-EHD1 expression using anti-HA antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Stably expressing MycEHD2 (CHOMycEHD2) and MycEHD3

(CHOMycEHD3) were generated by transfecting CHO cells, deficient for the DHFR gene, with MycEHD2 or MycEHD3 plasmids and a vector expressing the mouse DHFR cDNA, as above. Selection was performed with MEM-Alpha (Biological Industries, Beit Haemek, Israel). In the case of CHOMycEHD3, MycEHD3 overexpression was amplified by 100 nM methotrexate (Sigma, Rehovot, Israel). The expression of Myc-tagged EHDs was followed using anti-myc antibody (Cell Signaling Technology, Boston, MA, USA).

Metabolic labeling with ^{32}P-orthophosphate

For EHD1, sub-confluent CHOHAEHD1 or HEK293 cells transiently transfected with HAEHD1 were incubated in phosphate-free DMEM for 30 min before labeling with 0.07-0.2 mCi/ml of ^{32}P-orthophosphate (Amersham Pharmacia Bioscience, Freiburg, Germany) for 4-6 h. When indicated, the cells were starved overnight for FCS in the appropriate media, after which they were incubated in serum-free/phosphate-free DMEM for 30 min and labeled with ^{32}P-orthophosphate for 4-6 h. Stimulation was performed with dFCS for the indicated times before lysis. ^{32}P-orthophosphate (0.05 mCi/ml) was added overnight to the CHOMycEHD2, CHOMycEHD3 and HEK293 cells transiently transfected with MycEHD4.

Lysis, immunoprecipitation, and immunoblotting

The cells were washed with ice-cold PBS and scraped into a lysis buffer (10 mM Hepes pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% glycerol, 40 mM NaF, 1 mM Na_3VO_4, 0.1 mM ammonium molybdate, 0.5% NP-40) supplemented with the protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin (Sigma, Rehovot, Israel), and 1 µg/ml leupeptin (Sigma, Rehovot, Israel). Following centrifugation (13,000 g for 10 min at 4ºC), the supernatants were subjected to immunoprecipitation by the addition of the required antibodies and protein-A-conjugated agarose beads (Roche Applied Science, Bazel, Switzerland). Following incubation for 12 h at 4ºC, the immunocomplexes were washed at least 3 times with lysis buffer, mixed with Laemmli sample buffer and boiled for 3 min. The proteins were resolved on 10% SDS-PAGE and transferred to a PVDF (Boehringer, Mannheim, Germany) or nitrocellulose (Schleicher&Schuell, Dassel, Germany) membrane, and subjected to further analysis or decorated with the relevant antibodies. Detection was carried out using horseradish peroxidase conjugated to a specific secondary antibody followed by an enhanced chemiluminescence reaction (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

***In-vitro* phosphorylation assay**

Twenty four hours after transfection of HEK293 cells with the appropriate plasmids, they were lysed in a lysis buffer (10 mM Hepes pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% glycerol, 40 mM NaF, 1 mM Na_3VO_4, 0.1 mM NH_4MoO_4, 0.5% NP-40) supplemented with the protease inhibitors: PMSF (1 mM), aprotinin (1 µg/ml) and leupeptin (1 µg/ml). The lysates were incubated

on ice for 20 min followed by centrifugation at 13,000g for 10 min at 4ºC, and the supernatants were subjected to immunoprecipitation with the appropriate antibodies. The immunoprecipitates were washed five times with lysis buffer and once with doubled distilled water. The kinase assay was performed at 30ºC for 10 min in 30 µl of reaction buffer (50 mM MES pH 6.0, 1.25 mM EGTA, 12.5 mM $MgCl_2$, 0.125 mM ATP, 5 µCi of γ-^{32}P-ATP and 20 ηg of PKC (Calbiochem, La Jolla, CA, USA) in a total volume of 30 µl. The proteins were resolved through 10% SDS-PAGE and transferred to a membrane which was autoradiographed.

Partial acid hydrolysis of proteins immobilized on PVDF membrane

Partial acid hydrolysis of the PVDF-immobilized proteins was carried out as described previously [39]. Following X-ray exposure, the PVDF membrane, containing the ^{32}P-labeled proteins, was rewetted with methanol, rinsed twice with PBS, and washed for 5 min with 50 mM Tris pH 7.5, 150 mM NaCl, and 0.2% Tween-20. Proteins were stained in 50 mM Tris pH 6.5, 150 mM NaCl and 0.2% Tween-20, containing 1 µl/ml indian ink (Royal Talens, Apeldoorn, the Netherlands). The membrane was rinsed in 50 mM Tris pH 6.5, 150 mM NaCl and 0.2% Tween-20 for 2 min. The stripes containing the proteins of interest were excised and placed in tubes containing 200 µl 6 N HCl, which were then filled with argon. Hydrolysis was performed at 110ºC for 1 h. The supernatants were dried, resuspended in 100 µl of water and dried again. 10 µl of first dimension buffer (2.5% formic acid, 7.8% glacial acetic acid, pH 1.9) containing 15 parts buffer to 1 part cold phosphoamino acid standards (1 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine in deionized water) was added to the mix.

Two-dimensional (2D) thin-layer electrophoresis and phosphoamino acid identification

TLE was carried out using the Hunter thin-layer electrophoresis system (HTLE-7000, CBS Scientific, Inc. Del Mar, CA, USA) as described [40]. Briefly, following acid hydrolysis, samples containing 50-100 cpm were applied to a TLC cellulose plate (Merck, Darmstadt, Germany). Separation was carried out with 2.5% formic acid, 7.8% glacial acetic acid, pH 1.9 for 20 min at 1.5 kV for the first dimension, and with 5% glacial acetic acid, 0.5% pyridine, pH 3.5 for 16 min at 1.3 kV for the second dimension. Standard phosphoamino acids were visualized by ninhydrin staining. ^{32}P-labeled phosphoamino acids were identified by autoradiography.

Phosphopeptide mapping

^{32}P-labeled proteins were immunoprecipitated, separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and autoradiographed. Phosphopeptide mapping was carried out as described elsewhere [40]. Stripes containing the proteins of interest were excised from the membrane and blocked with 0.5% polyvinylpyrrolidone (Sigma, Steinheim, Germany) in 100 mM acetic acid for

30 min at 37ºC. Following washes with water (1 ml × 5) and with 50 mM NH_4HCO_3 (1 ml × 2), a tryptic digest was performed overnight at 37ºC in 200 µl of 50 mM NH_4HCO_3 containing 2 µg of modified trypsin (Proteomics Grade, Sigma, Rehovot, Israel), after which 2 µg of enzyme were added for an additional 4 h at 37ºC. Following the removal of the membrane and evaporation of the remaining liquid in a speed-vac, 20 µl of the first dimension buffer (2.2% formic acid, 7.8% glacial acetic acid, pH 1.9) was added and evaporated, and 5-10 µl of the same buffer was added. First dimension TLE separation was carried out using the HTLE-7000 system as above. Samples containing 200-1000 cpm were applied to a TLC cellulose plate and separated in first dimension buffer for 25 min at 1 kV, followed by TLC in 62.5% isobutyric acid, 1.9% butanol, 4.8% pyridine, 2.9% glacial acetic acid, pH 3.5 for the second dimension. The plates were dried and autoradiographed.

Quantitation and proteomic tools

Blots were scanned using an Image Scan scanner (Amersham Pharmacia Biotech, UK), and the intensity of each band was measured by the image master densitometer 1Dprime (Amersham Pharmacia Biotech, UK). The prediction of kinases was performed using the NetPhosK 1.0 program (http://www.cbs.dtu.dk/services/NetPhosK/).

RESULTS AND DISCUSSION

EHD1 is a serine phosphoprotein: its phophorylation is enhanced by serum stimulation

Increasing evidence suggests that phosphorylation plays a regulatory role in endocytosis. Therefore, we decided to examine whether EHD1 is a phosphoprotein. The results (Fig. 1A) show that EHD1 is a phosphoprotein. Its phosphorylation is enhanced by serum stimulation. The phosphorylation level of EHD1 starts to increase following 5 min of serum induction, reaches its peak after 15 min, and returns to its basal level after 30 min of serum induction. Phosphoamino acid analysis revealed that EHD1 is a serine phosphoprotein (Fig. 1B).

PKC is one of the kinases responsible for basal phosphorylation of EHD1

According to computer predictions, PKC may phosphorylate EHD1. To test this possibility, the phosphopeptide map of EHD1, phosphorylated *in vitro* by PKC, was compared to that of EHD1 immunoprecipitated from cells labeled with ^{32}P-orthophosphate. As shown (Fig. 2), EHD1 phosphorylated in cells shares four overlapping peptides (peptides 1-4, Fig. 2) with PKC-phosphorylated EHD1, providing evidence that EHD1 is a substrate for PKC phosphorylation in cells. However, the major serum-inducible phosphopeptides (peptides 1, 5 and 6, Fig. 2) are absent from the *in vitro* phosphorylation reaction, suggesting that serum-stimulated phosphorylation of EHD1 is not PKC-mediated. Thus, our results indicate that PKC is one of the kinases that phosphorylate EHD1.

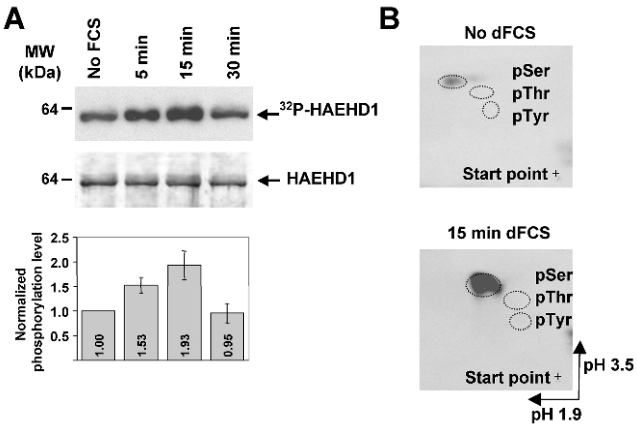

Fig. 1. EHD1 undergoes serum-stimulated phosphorylation on serine(s). A – Following overnight FCS starvation, CHOHAEHD1 cells were labeled with ^{32}P-orthophosphate for 4 h. In the last 30 min of labeling, the cells were stimulated with 10% dFCS for 0, 5, 15 and 30 min. HAEHD1 was immunoprecipitated with anti-HA antibody and separated through 10% SDS-PAGE, and the corresponding blot was subjected to autoradiography (upper panel) and interaction with anti-HA antibody (lower panel). The chart shows the mean ratio of the phosphorylation level normalized to the protein amount based on band-density measurements from four experiments (means ± SD). B – HAEHD1 was labeled and purified as described in A. Following autoradiography, the membrane was stained with indian ink, and bands corresponding to HAEHD1 were excised, quantified and subjected to phosphoamino acid analysis. The digested material was separated on two-dimensional TLE and autoradiographed. Standard phosphoamino acids were located by ninhydrin staining (dotted circles).

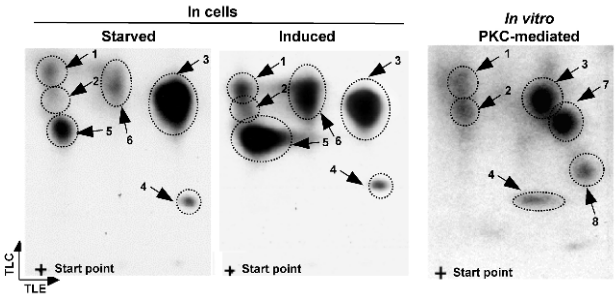

Fig. 2. Peptide maps of EHD1 phosphorylated in cells and *in vitro*. 24 h after transfection of HEK293 cells with HAEHD1-expressing plasmid, the cells were labeled with ^{32}P-orthophosphate for 4 h and stimulated with dFCS for 15 min. Following immunoprecipitation with anti-HA antibody, 10% SDS-PAGE and blotting, the membrane was autoradiographed. Protein bands were excised from the membrane and subjected to trypsin digestion. The digested material was separated on 2D TLE/TLC, followed by autoradiography. For *in vitro* phosphorylation, HAEHD1 was immunoprecipitated with anti-HA antibody and subjected to *in vitro* phosphorylation by PKC kinase as described in the Materials and Methods section. The phosphorylation products were separated on 10% SDS-PAGE and blotted, and the membrane was autoradiographed. The protein bands corresponding to HAEHD1 were subjected to trypsin digestion and TLE/TLC separation as described. The numbers denote the different peptides.

All mammalian EHDs are serine phosphoproteins

Since EHD2, EHD3, and EHD4 are highly homologous to EHD1, we decided to test whether they are also phosphoproteins. To do that, cells stably expressing either EHD2 (CHOMycEHD2) or EHD3 (CHOMycEHD3) and HEK293 cells transiently transfected with MycEHD4 were labeled with ^{32}P-orthophosphate and stimulated with serum for different periods. The cell lysates were immunoprecipitated with anti-myc antibody and resolved through a 10% SDS-PAGE, which was blotted and autoradiographed. The results (Fig. 3, A-C) demonstrate that EHD2, EHD3 and EHD4 are phosphoproteins. Their phosphorylation is not stimulated by serum. Phosphoamino acid analysis demonstrated that EHD2, EHD3 and EHD4 are serine phosphoproteins. EHD4 may also be phosphorylated on threonine (Fig. 3C, bottom panel). Since EHD2, EHD3 and EHD4 undergo phosphorylation which is not serum stimulated, it is plausible that their phosphorylation is important either for their correct conformation or localization, or their interaction with other proteins.

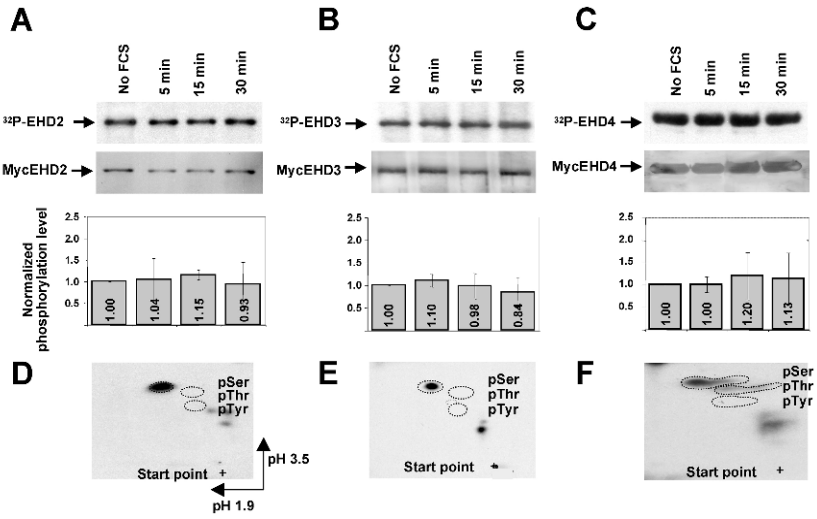

Fig. 3. EHD2, EHD3 and EHD4 are serine-phosphoproteins. CHOMycEHD2 (A), CHOMycEHD3 (B), or HEK293 cells transiently transfected with MycEHD4 (C) were starved in a medium devoid of FCS overnight and then labeled overnight with ^{32}P-orthophosphate. Prior to lysis, the cells were stimulated with 10% dFCS for the indicated times. Immunoprecipitation was carried out with anti-myc antibody and interacting proteins were separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane, which was autoradiographed (upper panels) or subjected to interaction with anti-myc antibody (lower panels). The blots were scanned as described in the Materials and Methods section. The outlined charts show the results of the quantitation of protein phosphorylation measured in three different experiments. The phosphorylation level at time 0 was determined as 1, and the intensity was normalized according to the protein level. D-F – Proteins immunoprecipitated as above were separated through 10% SDS-PAGE, transferred to a PVDF membrane and processed for phosphoamino acid analysis. Following HCl digestion, the mixtures were loaded onto TLC plates and separated by 2D TLE following autoradiography. Standard phosphoamino acids were visualized by ninhydrin staining (marked with circles).

EHD1 phosphorylation depends on clathrin-mediated endocytosis

Serum contains a large number of biologically active molecules that can affect different processes in cells by binding to their specific receptors, including receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs), which internalize as a result of binding to these ligand molecules. Since we have shown that EHD1 phosphorylation is stimulated by serum, we tested different RTK and GPCR ligands for their ability to induce EHD1 phosphorylation. Platelet-derived growth factor (PDGF), b-fibroblast growth factor (bFGF), insulin, lisophosphatidic acid (LPA) and the GPCR ligand LPA increased EHD1 phosphorylation in a concentration-dependent manner (Fig. 4A, upper panel). It is worth noting that both RTKs and GPCRs internalize through clathrin-mediated pathways [41].

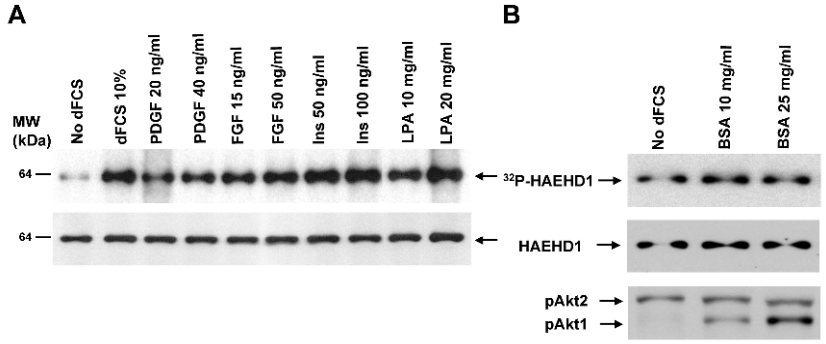

Fig. 4. EHD1 phosphorylation is stimulated by RTK and GPCR ligands, but not by albumin. Preconfluent CHOHAEHD1 cells were serum starved overnight and labeled with ^{32}P-orthophosphate for 4 h. Prior to lysis, the cells were stimulated as follows: A – with growth factors at the marked concentrations for 15 min, or B – with bovine serum albumin (BSA) at the marked concentrations for 15 min. HAEHD1 was immuoprecipitated, separated through 10% SDS-PAGE and transferred to a nitrocellulose membrane, which was autoradiographed (upper panels) and then subjected to interaction with anti-recombinant mouse EHD1 antibodies [7] (A – lower panel, B – middle panel) or with anti-phosphoAkt1/2 antibodies (Cell Signaling Technology, Boston, MA, USA), (B – lower panel). PDGF – platelet-derived growth factor; FGF – fibroblast growth factor; Ins – insulin; LPA – lysophosphatidic acid; pAkt1 – phosphorylated Akt1; pAkt2 – phosphorylated Akt2.

Albumin is an abundant protein in the plasma that undergoes caveolin-mediated endocytosis [42]. Since EHD1 was shown to play a role in the recycling of MHC class I molecules internalized through a caveolin-mediated pathway [24], we tested whether albumin induction affects EHD1 phosphorylation. The results (Fig. 4B, upper panel) show that treatment with BSA had no detectable effect on EHD1 phosphorylation, although it increased the phosphorylation of Akt1 kinase in CHOHAEHD1 cells (Fig. 4B, lower panel), which was used as a positive control for BSA-mediated signaling [43].

To summarize, our results strongly suggest that the activation of clathrin-mediated endocytosis by RTK and GPCR ligands increases EHD1

phosphorylation. Stimulation of caveolin-mediated endocytosis by BSA has no effect on EHD1 phosphorylation. These results imply that phosphorylation of EHD1 is mediated by clathrin-dependent endocytosis.

Inhibitors of clathrin-dependent endocytosis reduce EHD1 phosphorylation
To confirm the results suggesting that EHD1 phosphorylation occurs during clathrin-mediated endocytosis, pharmacological treatments that specifically inhibit clathrin- or caveolin-mediated endocytosis were employed. Treatment of cells with 0.45 M sucrose specifically inhibits clathrin-mediated endocytosis, arresting coated pit formation [44], while monodansylcadaverine (MDC)

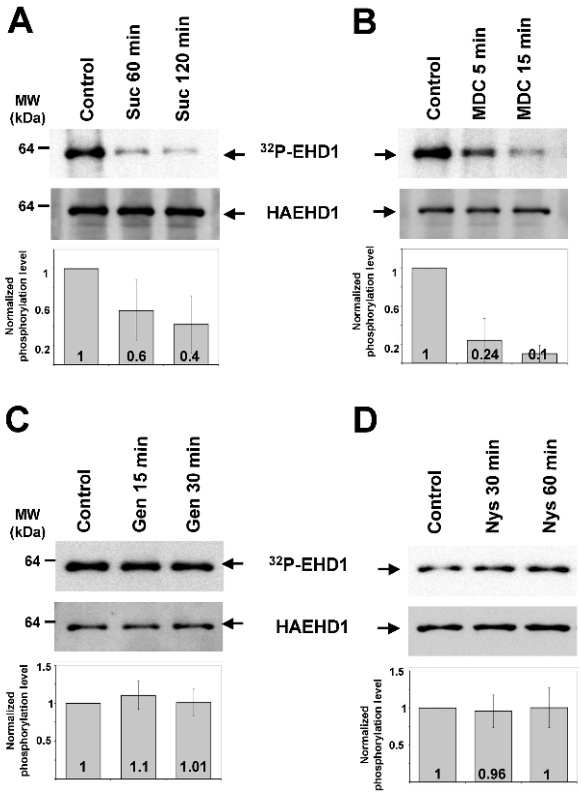

Fig. 5. EHD1 phosphorylation is sensitive to treatment with sucrose and MDC, but not to genistein and nystatin. CHOHAEHD1 cells were grown and labeled with ^{32}P-orthophosphate in phosphate-free DMEM, supplemented with 1% dFCS for 4 h. Prior to lysis, the cells were treated with: A – hypertonic medium (0.45 M sucrose), B – MDC (400 μM), C – genistein (200 μM) or D – nystatin (25 μg/ml) for the indicated times. Following immunoprecipitation and separation through 10% SDS-PAGE, the proteins were transferred to a membrane, autoradiographed, blotted and subjected to interaction with anti-EHD1 antibodies. The outlined charts show the results of the quantitation of protein phosphorylation, measured in three different experiments. The phosphorylation level at time 0 was determined as 1. Suc – sucrose, MDC – monodansylcadaverine, Gen – genistein, Nys – nystatin.

specifically inhibits clathrin-mediated internalization by stabilizing clathrin cage assembly [45]. We decided to test whether these treatments affect EHD1 phosphorylation. The results indicated that treatment with sucrose (Fig. 5A) or MDC (Fig. 5B) effectively inhibited EHD1 phosphorylation, suggesting that EHD1 phosphorylation depends on intact clathrin-mediated endocytosis and that phosphorylation occurs downstream of clathrin-coated vesicle formation.

Inhibitors of caveolin-dependent endocytosis do not affect EHD1 phosphorylation

Genistein specifically inhibits caveolin-mediated endocytosis without affecting clathrin-dependent internalization [46]. Nystatin is a cholesterol-binding drug that removes cholesterol from membranes and thereby selectively disrupts caveolae while not affecting clathrin-coated pits, actin fibers, or other submembranous structures [46]. The effect of these drugs on EHD1 phosphorylation was tested. As evident from the results neither genistein (Fig. 5C) nor nystatin (Fig. 5D) had any effect on EHD1 phosphorylation. The presented data supports our earlier suggestion that EHD1 phosphorylation depends on intact clathrin-mediated endocytosis, but not on caveolin-mediated endocytosis.

The effect of inhibitors of vesicular trafficking on EHD1 phosphorylation

The results presented imply that EHD1 phosphorylation depends on clathrin-mediated endocytosis. To further explore at what stage of the endocytic pathway it occurs, the effect of several inhibitors was studied. The fungal metabolite wortmannin is an irreversible inhibitor that blocks the activity of the PI3-kinase of mammalian cells [47]. It reduces the delivery of internalized transferrin from early endosomes to the recycling compartment by preventing the binding of EEA1 (early endosomal antigen 1) to endosomes [42, 48, 49]. As presented in Fig. 6A, wortmannin significantly inhibited EHD1 phosphorylation.

Since nocodazole treatment results in microtubule disassembly and the inhibition of microtubule-based vesicle transport, its effect on EHD1 phosphorylation was tested in CHOHAEHD1. Interestingly, nocodazole treatment increased EHD1 phosphorylation (Fig. 6B), implying that dephosphorylation of EHD1 is microtubule-dependent.

Taking all the results together, we can assume that the phosphorylation of EHD1 occurs during the microtubule-dependent vesicular translocation from early endosomes to the endocytic recycling compartment. This assumption fits well with the results showing maximum phosphorylation after 15 min of serum induction and the results of a previous study in a similar cell system (CHO cells) which showed that following 15 min of exposure to transferrin, most of it was found in the endocytic recycling compartment [50]. Based on this assumption and since EHD1 has an established role in recycling from the endocytic recycling compartment to the plasma membrane [24], we hypothesize that phosphorylation precedes recycling from the endocytic recycling compartment.

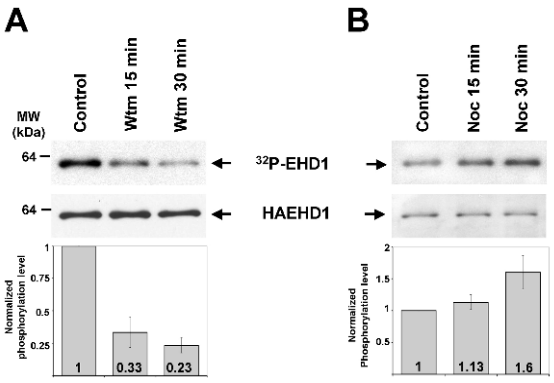

Fig. 6. EHD1 phosphorylation is wortmannin and nocodazole sensitive. CHOHAEHD1 cells were grown and labeled as described in the legend to Fig. 4. Prior to lysis, the cells were treated with A –100 nM wortmannin or B – 10 μM nocodazole for the indicated times. Following immunoprecipitation and separation through 10% SDS-PAGE, proteins were transferred to a membrane, autoradiographed, blotted and subjected to interaction with anti-EHD1 antibodies. The outlined charts show the results of the quantitation of protein phosphorylation measured in three different experiments. The phosphorylation level at time 0 was determined as 1. Wtm – wortmannin, Noc – nocodazole.

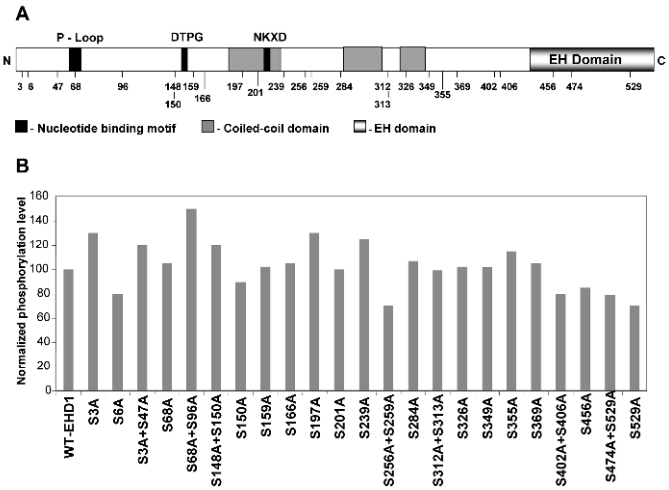

Fig. 7. EHD1 serine mutants do not show a significant decrease in the level of phosphorylation. A – A map of EHD1 and its domains showing all 26 serine sites. P-loop, DTPG and NKXD are indicated. The numbers denote serine amino acids. B – A summary of the phosphorylation assays performed in HEK293 cells expressing different serine point mutations in the EHD1 protein. Transfection and labeling with ^{32}P-orthophosphate (4 h labeling with 0.07 mCi/ml) was performed as described in the Materials and Methods section. The levels of phosphorylation were normalized to the protein level as detected by western blotting, and WT-EHD1 was considered as 100. Each column is based on 1-3 experiments. Note that some mutants show a higher phosphorylation level, while others present a decreased phosphorylation level. All these changes were regarded as experimental variations.

The biological significance of EHD1 phosphorylation during clathrin-mediated endocytosis is still unknown. As a first stage in studying the potential impact of EHD1 phosphorylation on endocytosis, we undertook a systematic mutagenesis approach. All 26 serines in EHD1 (Fig. 7A) were mutated to alanines and the phosphorylation of all the mutated proteins was tested by transfecting plasmids expressing them into HEK293 cells. None of the mutants showed any substantial decrease in phosphorylation level compared to WT-EHD1, implying that more than one serine is phosphorylated in EHD1 (Fig. 7B). This is consistent with the results of the phosphopeptide analysis (Fig. 2) and with the results of a study in which the proteome analysis of adipogenesis was carried out [51]. The authors used several techniques to follow changes in the protein expression profile that occur during differentiation of 3T3-L1 fibroblasts into adipocytes. They found that EHD1 appeared on 2D gels in four different forms, which change in abundance during induced adipogenesis without any change in the level of EHD1 expression, suggesting that these changes are due to post-translational modifications.

We also tested the ability of several truncation mutants to undergo phosphorylation in cells in tissue culture. The smallest truncation, which showed the typical EHD1 localization but was not phosphorylated, contained the N-terminal 336 amino acids (out of 535) of EHD1 (see Fig. 7A). There are 8 serines in the missing region (S349, S355, S369, S402, S406, S456, S474 and S529) out of which only 4 (S349, S456, S474 and S529) are conserved among vertebrates with 4 EHDs, including human, mouse, dog, fugu and xenopus. While the truncation mutant was not phosphorylated, the mutant with four serine to alanine mutations in the conserved serines (S349, S456, S474 and S529) did not present any decreased phosphorylation, suggesting that the truncation was unable to perform a function necessary for phosphorylation (data not shown).

In summary, we have shown that all human EHDs are serine-phosphoproteins. EHD1 phosphorylation is stimulated by serum during clathrin-mediated endocytosis, most probably during the transport of vesicles between the early endosomes and the ERC.

Acknowledgements. This work was partially supported by the Jacqueline Seroussi Memorial Foundation for Cancer Research, the Kodesh Institute for Research on Cancer Development and Prevention and the German-Israeli BioDisc program (to Mia Horowitz).

REFERENCES

1. Wong, W.T., Kraus, M.H., Carlomagno, F., Zelano, A., Druck, T., Croce, C. M., Huebner, K. and Di Fiore, P.P. The human eps15 gene, encoding a tyrosine kinase substrate, is conserved in evolution and maps to 1p31-p32. **Oncogene** 9 (1994) 1591-1597.

2. Slepnev, V.I., Ochoa, G.C., Butler, M.H., Grabs, D. and De Camilli, P. Role of phosphorylation in regulation of the assembly of endocytic coat complexes. **Science** 281 (1998) 821-824.
3. Di Fiore, P.P., Pelicci, P.G. and Sorkin, A. EH: a novel protein-protein interaction domain potentially involved in intracellular sorting. **Trends Biochem. Sci.** 22 (1997) 411-413.
4. Santolini, E., Salcini, A.E., Kay, B.K., Yamabhai, M. and Di Fiore, P.P. The EH network. **Exp. Cell Res.** 253 (1999) 186-209.
5. Lee, D.W., Zhao, X., Scarselletta, S., Schweinsberg, P.J., Eisenberg, E., Grant, B.D. and Greene, L. E. ATP binding regulates oligomerization and endosome association of RME-1 family proteins. **J. Biol. Chem.** 280 (2005) 17213-17220.
6. Vetter, I.R. and Wittinghofer, A. The guanine nucleotide-binding switch in three dimensions. **Science** 294 (2001) 1299-1304.
7. Mintz, L., Galperin, E., Pasmanik-Chor, M., Tulzinsky, S., Bromberg, Y., Kozak, C.A., Joyner, A., Fein, A. and Horowitz, M. EHD1-an EH-domain-containing protein with a specific expression pattern. **Genomics** 59 (1999) 66-76.
8. Pohl, U., Smith, J.S., Tachibana, I., Ueki, K., Lee, H.K., Ramaswamy, S., Wu, Q., Mohrenweiser, H.W., Jenkins, R.B. and Louis, D.N. EHD2, EHD3, and EHD4 encode novel members of a highly conserved family of EH domain-containing proteins. **Genomics** 63 (2000) 255-262.
9. Grant, B., Zhang, Y., Paupard, M.C., Lin, S.X., Hall, D.H. and Hirsh, D. Evidence that RME-1, a conserved C. elegans EH-domain protein, functions in endocytic recycling. **Nat. Cell Biol.** 3 (2001) 573-579.
10. Smith, C.A., Dho, S.E., Donaldson, J., Tepass, U. and McGlade, C.J. The Cell Fate Determinant Numb Interacts with EHD/Rme-1 Family Proteins and Has a Role in Endocytic Recycling. **Mol. Biol. Cell** 15 (2004) 2698-3708.
11. Blume, J.J., Halbach, A., Behrendt, D., Paulsson, M. and Plomann, M. EHD proteins are associated with tubular and vesicular compartments and interact with specific phospholipids. **Exp. Cell Res.** 313 (2007) 219-231.
12. Galperin, E., Benjamin, S., Rapaport, D., Rotem-Yehudar, R., Tolchinsky, S. and Horowitz, M. EHD3: a protein that resides in recycling tubular and vesicular membrane structures and interacts with EHD1. **Traffic** 3 (2002) 575-589.
13. George, M., Ying, G., Rainey, M.A., Solomon, A., Parikh, P.T., Gao, Q., Band, V. and Band, H. Shared as well as distinct roles of EHD proteins revealed by biochemical and functional comparisons in mammalian cells and C. elegans. **BMC Cell Biol.** 8 (2007) 3.
14. Naslavsky, N. and Caplan, S. C-terminal EH-domain-containing proteins: consensus for a role in endocytic trafficking, EH? **J. Cell Sci.** 118 (2005) 4093-4101.

15. Rapaport, D., Auerbach, W., Naslavsky, N., Pasmanik-Chor, M., Galperin, E., Fein, A., Caplan, S., Joyner, A.L. and Horowitz, M. Recycling to the plasma membrane is delayed in EHD1 knockout mice. **Traffic** 7 (2006) 52-60.
16. Guilherme, A., Soriano, N.A., Bose, S., Holik, J., Bose, A., Pomerleau, D.P., Furcinitti, P., Leszyk, J., Corvera, S. and Czech, M.P. EHD2 and the novel EH domain binding protein EHBP1 couple endocytosis to the actin cytoskeleton. **J. Biol. Chem.** 279 (2004) 10593-10605.
17. Daumke, O., Lundmark, R., Vallis, Y., Martens, S., Butler, P.J. and McMahon, H.T. Architectural and mechanistic insights into an EHD ATPase involved in membrane remodelling. **Nature** 449 (2007) 923-927.
18. Naslavsky, N., Rahajeng, J., Sharma, M., Jovic, M. and Caplan, S. Interactions between EHD Proteins and Rab11-FIP2: A Role for EHD3 in Early Endosomal Transport. **Mol. Biol. Cell** 17 (2006) 163-177.
19. Shao, Y., Akmentin, W., Toledo-Aral, J.J., Rosenbaum, J., Valdez, G., Cabot, J.B., Hilbush, B.S. and Halegoua, S. Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. **J. Cell Biol.** 157 (2002) 679-691.
20. Sharma, M., Naslavsky, N. and Caplan, S. A role for EHD4 in the regulation of early endosomal transport. **Traffic** 9 (2008) 995-1018.
21. Lin, S.X., Grant, B., Hirsh, D. and Maxfield, F.R. Rme-1 regulates the distribution and function of the endocytic recycling compartment in mammalian cells. **Nat. Cell Biol.** 3 (2001) 567-572.
22. Picciano, J.A., Ameen, N., Grant, B. and Bradbury, N.A. Rme-1 regulates the recycling of the cystic fibrosis transmembrane conductance regulator. **Am. J. Physiol. Cell Physiol.** 285 (2003) 1009-1018.
23. Guilherme, A., Soriano, N.A., Furcinitti, P.S. and Czech, M.P. Role of EHD1 and EHBP1 in perinuclear sorting and insulin-regulated GLUT4 recycling in 3T3-L1 adipocytes. **J. Biol. Chem.** 279 (2004) 40062-40075.
24. Caplan, S., Naslavsky, N., Hartnell, L.M., Lodge, R., Polishchuk, R.S., Donaldson, J.G. and Bonifacino, J.S. A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. **Embo J.** 21 (2002) 2557-2567.
25. Park, M., Penick, E.C., Edwards, J.G., Kauer, J.A. and Ehlers, M.D. Recycling endosomes supply AMPA receptors for LTP. **Science** 305 (2004) 1972-1975.
26. Rotem-Yehudar, R., Galperin, E. and Horowitz, M. Association of insulin-like growth factor 1 receptor with EHD1 and SNAP29. **J. Biol. Chem.** 276 (2001) 33054-33060.
27. Naslavsky, N., Rahajeng, J., Chenavas, S., Sorgen, P.L. and Caplan, S. EHD1 and Eps15 interact with phosphatidylinositols via their EH-domains. **J. Biol. Chem.** 282 (2007) 16612-16622.
28. Naslavsky, N., Boehm, M., Backlund, P.S., Jr. and Caplan, S. Rabenosyn-5 and EHD1 interact and sequentially regulate protein recycling to the plasma membrane. **Mol. Biol. Cell** 15 (2004) 2410-2422.

29. Braun, A., Pinyol, R., Dahlhaus, R., Koch, D., Fonarev, P., Grant, B.D., Kessels, M.M. and Qualmann, B. EHD proteins associate with syndapin I and II and such interactions play a crucial role in endosomal recycling. **Mol. Biol. Cell** 16 (2005) 3642-3658.
30. Xu, Y., Shi, H., Wei, S., Heng Wong, S. and Hong, W. Mutually exclusive interactions of EHD1 with GS32 and Syndapin II. **Mol. Membr. Biol.** 21 (2004) 269-277.
31. Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B. and Zerial, M. Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. **J. Cell Biol.** 151 (2000) 601-612.
32. Kessels, M.M. and Qualmann, B. The syndapin protein family: linking membrane trafficking with the cytoskeleton. **J. Cell Sci.** 117 (2004) 3077-3086.
33. Cullis, D.N., Philip, B., Baleja, J.D. and Feig, L.A. Rab11-FIP2, an adaptor protein connecting cellular components involved in internalization and recycling of epidermal growth factor receptors. **J. Biol. Chem.** 277 (2002) 49158-49166.
34. Steegmaier, M., Yang, B., Yoo, J.S., Huang, B., Shen, M., Yu, S., Luo, Y. and Scheller, R.H. Three novel proteins of the syntaxin/SNAP-25 family. **J. Biol. Chem.** 273 (1998) 34171-34179.
35. Ricotta, D., Conner, S.D., Schmid, S.L., von Figura, K. and Honing, S. Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. **J. Cell Biol.** 156 (2002) 791-795.
36. Wilde, A. and Brodsky, F.M. In vivo phosphorylation of adaptors regulates their interaction with clathrin. **J. Cell Biol.** 135 (1996) 635-645.
37. Ihara, Y., Yasuoka, C., Kageyama, K., Wada, Y. and Kondo, T. Tyrosine phosphorylation of clathrin heavy chain under oxidative stress. **Biochem. Biophys. Res. Commun.** 297 (2002) 353-360.
38. Confalonieri, S., Salcini, A.E., Puri, C., Tacchetti, C. and Di Fiore, P.P. Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. **J. Cell Biol.** 150 (2000) 905-912.
39. Kamps, M.P. and Sefton, B.M. Acid and base hydrolysis of phosphoproteins bound to immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. **Anal. Biochem.** 176 (1989) 22-27.
40. Boyle, W.J., van der Geer, P. and Hunter, T. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. **Methods Enzymol.** 201 (1991) 110-149.
41. Lin, F.T., Krueger, K.M., Kendall, H.E., Daaka, Y., Fredericks, Z.L., Pitcher, J.A. and Lefkowitz, R.J. Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1. **J. Biol. Chem.** 272 (1997) 31051-31057.
42. Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M. and Stenmark, H. EEA1

links PI(3)K function to Rab5 regulation of endosome fusion. **Nature** 394 (1998) 494-498.
43. Jones, D.T., Ganeshaguru, K., Anderson, R.J., Jackson, T.R., Bruckdorfer, K.R., Low, S.Y., Palmqvist, L., Prentice, H.G., Hoffbrand, A.V., Mehta, A.B. and Wickremasinghe, R.G. Albumin activates the AKT signaling pathway and protects B-chronic lymphocytic leukemia cells from chlorambucil- and radiation-induced apoptosis. **Blood** 101 (2003) 3174-3180.
44. Heuser, J.E. and Anderson, R.G. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. **J. Cell Biol.** 108 (1989) 389-400.
45. Phonphok, Y. and Rosenthal, K.S. Stabilization of clathrin coated vesicles by amantadine, tromantadine and other hydrophobic amines. **FEBS Lett.** 281 (1991) 188-190.
46. Puri, V., Watanabe, R., Singh, R.D., Dominguez, M., Brown, J.C., Wheatley, C.L., Marks, D.L. and Pagano, R.E. Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. **J. Cell Biol.** 154 (2001) 535-547.
47. Arcaro, A. and Wymann, M.P. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. **Biochem. J.** 296 (Pt 2) (1993) 297-301.
48. Jones, A.T. and Clague, M.J. Phosphatidylinositol 3-kinase activity is required for early endosome fusion. **Biochem. J.** 311 (1995) 31-34.
49. Li, G., D'Souza-Schorey, C., Barbieri, M.A., Roberts, R.L., Klippel, A., Williams, L.T. and Stahl, P.D. Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. **Proc. Natl. Acad. Sci. USA** 92 (1995) 10207-10211.
50. Sheff, D.R., Daro, E.A., Hull, M. and Mellman, I. The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. **J. Cell Biol.** 145 (1999) 123-139.
51. Welsh, G.I., Griffiths, M.R., Webster, K.J., Page, M.J. and Tavare, J.M. Proteome analysis of adipogenesis. **Proteomics** 4 (2004) 1042-1051.