Ligand-induced receptor-mediated endocytosis plays a central role in regulating signaling conveyed by tyrosine kinase receptors. This process depends on the recruitment of the adaptor protein 2 (AP-2) complex, clathrin, dynamin, and other accessory proteins to the ligand-bound receptor. We show here that besides AP-2 and clathrin, two other proteins participate in the endocytic process of the insulin-like growth factor receptor (IGF-1R); they are EHD1, an Eps15 homology (EH) domain-containing protein 1, and SNAP29, a synaptosomal-associated protein. EHD1 and SNAP29 form complexes with α-adaptn of AP-2 and co-localize in endocytic vesicles, indicating a role for them in endocytosis. EHD1 and SNAP29 interact directly with each other and are present in complexes with IGF-1R. After IGF-1 induction, EHD1 and IGF-1R co-localize intracellularly. Overexpression of EHD1 in Chinese hamster ovary cells represses IGF-1-mediated signaling, as measured by mitogen-activated protein kinase phosphorylation and Akt phosphorylation, indicating that EHD1 plays a role as a down-regulator in IGF-1 signaling pathway.

Tyrosine kinase receptors convey signals that affect central cellular processes of proliferation, differentiation, metabolism, and apoptosis (1). Upon binding to their cognate ligand, the receptors undergo autophosphorylation within specific motifs that result in extensive recruitment of accessory proteins (2–4). This process promotes rapid signaling after sequestration of the activated receptor via ligand-induced receptor-mediated endocytosis (5). After internalization, tyrosine kinase receptor-containing vesicles are directed through vesicular structures into endosomal compartments that are further sorted on microtubular tracks to lysosomes or proteasomes for degradation of the receptors (6–8). Clathrin-mediated endocytosis is a well studied process whereby essential components of the basal endocytic machinery like the AP-2

endophilin, and dynamin are recruited to the plasma membrane to promote the formation of clathrin-coated vesicle (9–12). The dynamic assembly of these complexes is mediated through various protein recognition modules. One of the modules is the EH domain that was first identified as a 100-amino acid sequence repeated 3 times in the N terminus of the EGF receptor pathway substrate Eps15 (13–18). The central region of Eps15 shares the characteristic heptad repeats of coiled-coil proteins, and the C terminus contains a prolin-rich region and a repeated DPF motif (16, 17). Intracellular localization to plasma membrane pits and vesicles, interaction with prominent proteins of the basal endocytic machinery like α-adaptn of AP-2, and interactions through the EH domain with other proteins harboring the NPFXD sequence like epsin have implicated Eps15 in receptor-mediated endocytosis (18–22). Moreover, functional inhibition of Eps15 by antibodies or dominant negative mutants abrogated endocytosis of EGF and transferrin, therefore supporting the notion that Eps15 mediates endocytosis of the EGF receptor (21, 23). Recently a family of EH domain-containing proteins harboring four members, EHD1–EHD4, was identified (24, 25).

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The abbreviations used are: AP, adaptor protein complex; EH, Eps15 homology; EGF, epidermal growth factor; EGF-R, EGF-receptor; Eps15, EGF receptor pathway substrate 15; EHM, EH domain deleted mutant; IGF-1, insulin-like growth factor 1; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; MAP, mitogen-activated protein; MAPK, MAP kinase; NTM, N-terminus deleted mutant; r-, recombinant; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; SNAP, synaptosomal-associated protein.

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nase polyclonal antibodies (442704-S) were purchased from Sigma. Anti-phospho-Akt polyclonal antibodies (Ser-473, 9272) and anti-Akt polyclonal antibodies (9272) were purchased from Cell Signaling Technology. Rabbit anti-recombinant EHD1 (rEHD1) polyclonal antibodies were prepared as described (24). To prepare anti-recombinant SNAP29 antibodies, the SNAP29 cDNA fragment with the homology described above was cloned into the EcoRI and XhoI restriction sites of pLexA to produce pLexA/EHD1/SNAP29. pB42AD vector encoding the SNAP29 gene was rescued and tested for interaction with yeast strains expressing the pLexA fusion proteins described above. pB42AD vector expressing a mutant form of SNAP29 (SNAP29/NTM) missing the first 30 amino acids of the N terminus, as well as SNAP29/EFYbisSNAP29, was purchased from Dr. J. D. Watson (University of California, Los Angeles). Rabbit anti-recombinant EHD1 (rEHD1) polyclonal antibodies were provided by Dr. D. Watson (University of California, Los Angeles). Rabbit anti-recombinant SNAP29 polyclonal antibodies, the open reading frame of SNAP29 (rSNAP29) were purified on nickel beads according to the supplier’s recommendations (Novagen), and rabbits were immunized by 5 injections of 0.5 mg/injection of the purified protein at 2–3-week intervals. Animals were bled 10 days after the final booster. Serum was separated from the bleed and stored at −80 °C.

Affinity Purification—One grain of rat tissues was homogenized in 4 ml of 20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM MgCl2, 0.2% Triton X-100 buffer supplemented with protease inhibitor mixture (Sigma, P8340) and phosphatase inhibitor mixture (Sigma, P5726). The homogenates were centrifuged for 15 min at 14,000 rpm in SS-34 rotor. The extract was affinity-purified on rEHD1 bound to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to manufacturer’s manual. Column-bound material was washed three times with PBS, and three elutions were collected with 0.1 M glycine, pH 2.5, which was titrated immediately by 1 M Tris, pH 8.8. Starting material, flow-through, washes, and elutions were resolved on SDS-PAGE and subjected to Western analysis. For affinity purification of rat proteins on a 1D gel, the gel was cut into columns, rat tissues were homogenized as described above. Half the extracts were incubated with 3 μg/ml purified rSNAP29 for 2 h at 4 °C. The all the extracts were further incubated for 2 h at 4 °C with 30 pl of PBS-washed (50% slurry) nickel beads (Novagen). The beads were washed three times with 0.2% Triton X-100 Hepes buffer and boiled in Laemmli sample buffer containing 5% β-mercaptoethanol (Sigma).

IGF-1 Induction of VEGF Promoter in EHD1-expressing Cells—NIH/IGF-1R cells (NIH3T3 cells overexpressing IGF-1R, a kind gift of Dr. D. LeRoith, NIH) were transiently transfected with either pCDNA1 (Invitrogen), pCDNA1/EHD1 (24), pVEGF/CD4 reporter plasmid (a kind gift of Dr. S. Luria, Sti Biotechnology, Israel), or both. 24 h later, the medium was changed to DCCM-1 medium (Biological Industries, 05-010-1) overnight. The cells were induced with/without 50 ng/ml IGF-1 for another 18 h. The cells were harvested, and cytoplasmic RNA was extracted. The expression of CD4 and EHD1 was monitored by reverse transcriptase-PCR reactions with the following primers: for CD4, 5′-GAGTTCTCTAAGAGACCT-3′, 5′-GAAGTTTGGCCGAGAATG-3′, and for EHD1, 5′-CATCGCCGCTATGACCATG-3′, 5′-GATGATGCGTATGTG-3′. The PCR reactions were transferred to nitrocellulose filters, and a Southern blot was performed with specific probes for the expressed genes.

In Vitro Complex Formation and Immunoprecipitation—For in vitro complex formation, 2 × 104 NIH/IGF-1R cells were serum-starved for 10 h in DCCM-1 medium without insulin or IGF-1 supplemented with 2 ml 1-glutamine solution and PSEN-STREP solution (Biological Industries). The cells were treated with 100 μg/ml human IGF-1 (PeproTech) for different time intervals. After the cells were harvested, and cytoplasmic RNA was extracted. The expression of CD4 and EHD1 was monitored by reverse transcriptase-PCR reactions with the following primers: for CD4, 5′-GAGTTCTCTAAGAGACCT-3′, 5′-GAAGTTTGGCCGAGAATG-3′, and for EHD1, 5′-CATCGCCGCTATGACCATG-3′, 5′-GATGATGCGTATGTG-3′. The PCR reactions were transferred to nitrocellulose filters, and a Southern blot was performed with specific probes for the expressed genes.

Western Blot Analysis—Proteins were resolved on SDS-PAGE and transferred to a nitrocellulose blot, and the immunoblot was decorated with the relevant antibodies for 2 h at room temperature or overnight at 4 °C. Detection was carried out using horseradish peroxidase conjugated secondary antibody followed by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech).

MAP Kinase and Akt Phosphorylation—CHO cells and CHO cells overexpressing EHD1 (CHO/EHD1) were serum-starved for 10 h and treated with 100 ng/ml human IGF-1 (PeproTech) for different time intervals. The cells were extracted in Laemmli sample buffer, and the proteins were resolved on SDS-PAGE and subjected to Western analysis using anti-phospho-MAP kinase and anti-MAP kinase antibodies or anti-phospho-Akt and anti-Akt antibodies. The autoradiographs were quantitated by phosphorimaging, and the values for the phosphorylated-MAPK or phosphorylated Akt were normalized to the MAPK or Akt values, respectively.

To explore the proteins that associate with IGF-1R a kind gift of Dr. D. Watson (University of California, Los Angeles). Rabbit anti-recombinant EHD1 (rEHD1) polyclonal antibodies were provided by Dr. D. Watson (University of California, Los Angeles). Rabbit anti-recombinant SNAP29 polyclonal antibodies, the open reading frame of SNAP29 (rSNAP29) were purified on nickel beads according to the supplier’s recommendations (Novagen), and rabbits were immunized by 5 injections of 0.5 mg/injection of the purified protein at 2–3-week intervals. Animals were bled 10 days after the final booster. Serum was separated from the bleed and stored at −80 °C.

Affinity Purification—One grain of rat tissues was homogenized in 4 ml of 20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM MgCl2, 0.2% Triton X-100 buffer supplemented with protease inhibitor mixture (Sigma, P8340) and phosphatase inhibitor mixture (Sigma, P5726). The homogenates were centrifuged for 15 min at 14,000 rpm in SS-34 rotor. The extract was affinity-purified on rEHD1 bound to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to manufacturer’s manual. Column-bound material was washed three times with PBS, and three elutions were collected with 0.1 M glycine, pH 2.5, which was titrated immediately by 1 M Tris, pH 8.8. Starting material, flow-through, washes, and elutions were resolved on SDS-PAGE and subjected to Western analysis. For affinity purification of rat proteins on a 1D gel, the gel was cut into columns, rat tissues were homogenized as described above. Half the extracts were incubated with 3 μg/ml purified rSNAP29 for 2 h at 4 °C. The extracts were further incubated for 2 h at 4 °C with 30 pl of PBS-washed (50% slurry) nickel beads (Novagen). The beads were washed three times with 0.2% Triton X-100 Hepes buffer and boiled in Laemmli sample buffer containing 5% β-mercaptoethanol (Sigma).

RESULTS

Interaction of EHD1 with Proteins of the Endocytic Machinery and IGF-1R—To explore the proteins that associate with EHD1, rat testis extract was affinity-purified on rEHD1 affinity column. Eluted proteins were subjected to Western analysis. As depicted in Fig. 1a, a-adenovirus AP-2 and the heavy chain of clathrin, proteins characteristic of the endocytic machinery, were found in complexes with rEHD1. REHD1 formed complexes with three proteins with molecular masses of 63, 68, and 80 kDa, which were detected by anti-reHED1 antibodies. The 63-kDa protein represents EHD1 and EHD3, whereas the 68-kDa represents EHD2 and EHD4 (25). Actually...
we have shown that EHD1 is able to undergo self-dimerization, as demonstrated for Eps15 (27) and to form hetero-oligomers with EHD3,\(^2\) EHD2, and EHD4.\(^3\) The proteins designated as 1 and 2, Fig. 1a, may represent other unknown proteins that share an antigenic determinant(s) with EHD1, are unable to bind EHD1, and are recognized by anti-EHD1 antibodies. The 80-kDa protein may represent another unknown EH domain-containing protein that shares antigenic determinant(s) with EHD1, is able to bind EHD1, and is recognized by anti-EHD1 antibodies. Interestingly, EHD1 formed complexes with the β-chain of the IGF-1 receptor. These results indicate association of EHD1 with proteins of the endocytic machinery and with the IGF-1 receptor.

**In Vitro Complex Formation between EHD1 and IGF-1R—**An *in vitro* assay was developed to confirm the interaction of EHD1 with IGF-1R. NIH3T3 cells over-expressing the human IGF-1 receptor (NIH3T3/IGF-1R) (28) were treated with IGF-1 for different time intervals. Extracts prepared from these cells were incubated with rEHD1 and further subjected to immunoprecipitation using monoclonal anti-IGF-1R α-chain antibodies, and the Western blot was decorated with either anti-IGF-1R β-chain antibodies or anti-EHD1 antibodies. As depicted in Fig. 1b, the level of the IGF-1 receptors recognized by both anti-α-chain and anti-β-chain antibodies was elevated at up to 5 min of IGF-1 treatment, whereas within 10 min of treatment, their level declined. Minor changes were observed in the level of IGF-1R precursor, which migrated as a 180-kDa protein, indicating that only the mature receptors responded to IGF-1 induction, presumably by conformational changes that prevented their antibody recognition. rEHD1 was shown to associate with IGF-1R. The association increased within 5 min of IGF-1 induction and decreased to basal level after 15 min of treatment. These results demonstrate an association between rEHD1 and IGF-1R that depends on IGF-1 stimuli.

**Intracellular Colocalization of IGF-1R and EHD1—**To confirm the interaction between IGF-1R and EHD1, we tested their intracellular localization. CHO cells overexpressing EHD1 were serum-starved overnight, treated with IGF-1 for 1 h, fixed, and reacted with anti-EHD1 rabbit polyclonal antibodies or anti-IGF-1R monoclonal antibodies as detailed under “Experimental Procedures.” The cells were visualized using confocal microscopy. As indicated in Fig. 1c, there is an intracellular colocalization of IGF-1R and EHD1, confirming their interaction.

**MAP Kinase and Akt Phosphorylation in EHD1 Overexpressing Cells—**IGF-1 mediates the MAPK and Akt-signaling cascades (29). To verify whether EHD1 influences the signal transduction by IGF-1 receptor, phosphorylation of MAP kinase (MAPK) or Akt was followed in CHO cells and in CHO cells overexpressing the EHD1 gene (CHO/EHD1)\(^4\) in response to IGF-1. As depicted in Fig. 2a, CHO cells with IGF-1 leads to phosphorylation of MAPK within 2.5 min, which reaches high levels at 15 min of treatment. In contrast, in CHO/EHD1 cells, the phosphorylation of MAPK is retarded, and at 15 min, the phosphorylation is 2.3-fold reduced compared with the parental cells. As indicated in Fig. 2b, IGF-1 treatment leads to Akt phosphorylation within 2.5 min, which reaches high levels at 5 min of treatment. In contrast, in CHO/EHD1 cells, the phosphorylation of Akt is retarded, and at 5 min, the phosphorylation is 2.2-fold reduced compared with the parental cells. Therefore, overexpression of EHD1 reduces IGF-1-induced signaling as measured by MAPK and Akt phosphorylation, suggesting that EHD1 is a down-modu-

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\(^2\) Rotem-Yehudar and M. Horowitz, unpublished results.

\(^3\) R. Galperin and M. Horowitz, manuscript in preparation.

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**Fig. 1.** EHD1 interacts with AP-2, clathrin, and IGF-1R. *a,* extract from rat testes was affinity-purified on rEHD1 bound to CNBr-activated Sepharose 4B beads. Starting material (L), flow-through (FT), wash (W), and elutions (E1–E3) were resolved on SDS-PAGE and transferred into nitrocellulose, and a Western blot was employed using anti-IGF-1R β-chain antibodies, antibodies directed against α-adaptin/AP-2, and anti-clathrin heavy chain antibodies. The proteins were resolved on SDS-PAGE, and immunoblot was performed using anti-IGF-1R β-chain antibodies and anti-EHD1 antibodies. *b,* CHO cells overexpressing EHD1 were induced with IGF-1. The cells were stained with anti-EHD1 antibodies and anti-IGF-1R antibodies followed by relevant secondary-labeled antibodies. EHD1 (red), IGF-1R (green), and co-localization (yellow) were visualized by confocal analysis.
scriptase-PCR was performed. Monitoring EHD1 and CD4 expression pCDNA1/EHD1 (EHD1), and pVEGF/CD4 (CD4) were induced with phosphorylated-Akt were normalized to the MAPK and Akt values, phosphorymaging scanner, and the values for the phosphorylated-MAPK or anti-Akt antibodies (). The autoradiographs were quantitated by phospho- or anti-phospho-Akt antibodies (anti-MAPK antibodies ()) antibodies and Western analysis using anti-phospho-MAPK (lysed, and the proteins were resolved on SDS-PAGE and subjected to cell lysis, and the cells were treated with IGF-1 for different time intervals. The cells were found an of EHD1 down-regulates IGF-1-induced signaling. NIH3T3/IGF-1R cells were co-transfected with an EHD1 expression vector (pCDNA1/EHD1) and a reporter vector harboring the IGF-1R cells were co-transfected with an EHD1 expression vector (pCDNA1/EHD1) and a reporter vector harboring the EHD1 missing the N terminus (pLexA/EHD1/NTM) or the EH domain (pLexA/EHD1/EHM) as marked to the left margins. These strains were further transformed with constructs expressing pB42AD/SNAP29 or pB42AD/SNAP29/NTM, which encodes a truncated SNAP29 as marked at the top. Four isolated colonies from each transformation were seeded on selection plates containing either galactose/raffinose/uracyl-histidine–tryptophan–X-gal (X-gal), galactose/raffinose/urate–histidine–tryptophan–trehalose–(Leu–), or glucose/uracil–histidine–tryptophan–trehalose–(Ura–/His–/Trp–), as marked at the top. Growth on Leu– plates or blue colonies on the X-gal plates indicates protein-protein interaction. b, extracts from rat tissues were supplemented with (+) or without (−) rSNAP29 for 2 h and further incubated with nickel beads. The beads were washed, and bound material was separated on SDS-PAGE and subjected to Western analysis using anti-EHD1 antibodies. c, extracts from rat tissues were immunoprecipitated using anti-IGF-1R a-chain antibodies. Immuno-complexes were resolved on SDS-PAGE and transferred into nitrocellulose, and a Western blot was employed using either anti-rEHD1 antibodies or anti-rSNAP29 antibodies. d, extract from NIH3T3/IGF-1R cells was subjected to immunoprecipitation using anti-IGF-1R a-chain antibodies. Immuno-complexes were resolved on SDS-PAGE and transferred into nitrocellulose, and a Western blot was employed using either anti-rIGF-1R b-chain antibodies or anti-rSNAP29 antibodies. e, EGY48/p8op-lacZ Yeast strain was transformed with the plasmid pLexA/SNAP29 as marked in the left margins. This strain was further transformed with constructs expressing either pB42AD/IGF-1R b-chain cytoplasmic tail, pB42AD/SNAP29, or pB42AD/SNAP29/NTM, as marked in the right margins. Four isolated colonies from each transformation were seeded on selection plates containing either galactose/raffinose/urate–histidine–tryptophan–X-gal (X-gal), galactose/raffinose/urate–histidine–tryptophan–trehalose–(Leu–) or glucose/uracil–histidine–tryptophan–trehalose–(Ura–/His–/Trp–). Growth on Leu– plates or blue colonies on the X-gal plates indicates protein-protein interactions.

Isolation of SNAP29 as an EHD1-interacting Protein—We searched for potential binding partners of EHD1 using the yeast two-hybrid system. A bait construct comprising the human EHD1 fused to the LexA protein was used to screen a human brain cDNA library. One isolated clone encoded the human SNAP29. SNAP29, a 29-kDa synaptosomal-associated protein, shares structural homology with SNAP25 and SNAP3, proteins of the SNARE complex, participating in vesicular membrane fusion events (30). Unlike the latter, SNAP29 contains at its N terminus two motifs, NPFXXD sequence (amino acids 8–12) and PXXPXXP (amino acids 32–38), which are characteristic of proteins participating in trafficking, especially in endocytosis. As shown in Fig. 3a, SNAP29 was able to interact with EHD1, EHD3, and truncated EHD1 missing either its N
terminus or its C terminus, indicating it interacts with the central coiled-coil region of EHD1. On the other hand, a mutated version of SNAP29 lacking amino acids 1–38 or other non-relevant genes like Rubisco of tomato or human clathrin light chain (data not shown) were unable to interact with any of the EHD1-derived proteins (Fig. 3a). Nonetheless, the truncated SNAP29 was able to interact with SNAP29, although to a lesser extent than the wild type SNAP29 protein, as shown in Fig. 3d by growth on the LEU– plate. These results indicate that the interaction between SNAP29 and EHD1 is directed through modules within those 38 amino acids of SNAP29.

To further demonstrate the interaction between SNAP29 and EHD1, rat tissue extracts were affinity-purified on a rSNAP29 column. The eluates were subjected to SDS-PAGE, and the immunoblot was decorated with anti-EHD1 antibodies. As shown in Fig. 3b, EHD proteins from rat brain, heart, lung, and kidney bound to rSNAP29 and not to the control beads. This experiment indicates that SNAP29 binds to EHD proteins from various rat tissues, supporting the findings in yeast.

In Vivo Association of IGF-1R, SNAP29, and EHD Proteins—Immunoprecipitation of rat tissue extracts was performed using monoclonal antibodies directed against the α-chain of the IGF-1 receptor. The precipitates were resolved on SDS-PAGE and subjected to Western analysis using anti-EHD1 and anti-SNAP29 antibodies. As indicated in Fig. 3c, IGF-1 receptor formed complexes with EHD proteins derived from testes, brain, lung, and kidney. SNAP29 was found in complexes with IGF-1R in the same tissues as the EHD proteins. To confirm the interaction between SNAP29 and IGF-1R, extracts of NIH3T3/IGF-1R cells were subjected to immunoprecipitation with IGF-1 receptor. The precipitates were resolved on SDS-PAGE and transferred into nitrocellulose, and a Western blot was employed using anti-α-adaptin antibodies.

Association of SNAP29 with α-Adaptin of AP-2 Complex—To test the possible association of SNAP29 with proteins of the endocytic machinery, immunoprecipitation of rat tissue extracts was performed using anti-SNAP29 antibodies. The precipitates were resolved on SDS-PAGE and subjected to Western analysis using anti-α-adaptin of AP-2 antibodies. The results (Fig. 4) indicate that SNAP29 forms complexes with AP-2.

Intracellular Localization of EHD1 and SNAP29—The two-hybrid analysis and the co-immunoprecipitation results indicated interaction between EHD1 and SNAP29. Moreover, both proteins interacted with members of the endocytic machinery.
this interaction is mediated via the coiled-coil region of the proteins. These authors proposed that oligomerization of EH domain-containing proteins may be a general feature important for the functional activity of this family of proteins.

Results of experiments presented here implicate EHD1 in the endocytic process of the IGF-1R. IGF-1R bound to the rEHD1 column. Only a small fraction of IGF-1 receptors expressed in the rat testis was bound to rEHD1, presumably due to prerequisites like phosphorylation for such binding (Fig. 1a). It has already been shown for the EGF receptor that phosphorylation within specific motifs in the cytoplasmic tail is required for extensive recruitment of accessory proteins, among them Eps15 (4). IGF-1R and EHD1 were found in in vitro formed complexes that were immunoprecipitated with anti-α-chain of IGF-1R antibodies (Fig. 1b).

Complexes containing IGF-1R and EHD proteins were immunoprecipitated with anti-IGF-1R antibodies (Fig. 1c). Results of immunofluorescence analyses indicated colocalization of EHD1 in IGF-1R-containing vesicles (Fig. 1c). We also have results indicating that overexpression of EHD1 abrogates IGF-1-induced signaling. It has been shown that the IGF-1-signaling cascade induces the MAP kinase (extracellular signal-regulated kinase) and Akt (29, 31). Overexpression of EHD1 reduced 2.2 and 2.3 times the magnitude of IGF-1-induced signaling through the Ras-Raf pathway and the phosphatidylinositol 3-kinase pathway, respectively (Fig. 2), demonstrating that EHD1 is a down-modulator of these pathways. It is worth mentioning that recent publications indicated that endocytosis up-regulates signaling of activated receptors like the EGF receptor and the β2-adrenergic receptor (32). We could also show that overexpression of EHD1 down-regulates IGF-1-induced signaling by co-transfection of an EHD1 expression vector with a reporter vector harboring the CD4 cDNA under a VEGF promoter into NIH3T3/IGF-1R cells. We found a 10-fold reduction in the amount of CD4 RNA in cells overexpressing EHD1 versus those that do not over IGF-1 induction (Fig. 2c).

Using the two-hybrid analysis, we identified SNAP29 as an interacting partner of EHD1 (Fig. 3, a–c). SNAP29 was initially discovered as an interacting partner of syntaxin 3 in a two-hybrid screen and was shown to be ubiquitously expressed in mammalian tissues (30). SNAP29 displays 39% similarity to neuronal SNAP25 and 35% similarity to the ubiquitously expressed SNAP23 (30). The SNAP proteins were described as participating in the core SNARE complex that underlines membrane fusion processes (33–35). In the nerve terminal, the vesicle and plasma membrane are brought into close apposition through the formation of a four-helices-bundle structure. Vesicle-associated membrane proteins synaptobrevin/VAMP (vesicle-associated membrane protein)-vesicle SNAP receptor (v-SNARE) contribute one helix, whereas the target membrane proteins (t-SNARE) SNAP25 and syntaxin contribute three helices to form a parallel bundle referred to as the SNARE complex (33–35). Unlike SNAP25 and SNAP23, SNAP29 does not have palmitoylation sites for anchoring a distinct membrane compartment, but rather, it is able to interact with different syntaxins and VAMPs that are specifically localized to distinct compartments; therefore, it was suggested as involved in multiple transport steps (30).

SNAP29 forms complexes with IGF-1R in vivo (Fig. 3, c and d). Moreover, SNAP29 binds directly to EHD1 (Fig. 3, a and b). This interaction is strong and is carried out through the central coiled-coil region of EHD1 (Fig. 3a). It seems that the N terminus of SNAP29 participates in the binding to EHD1 (Fig. 3a). However, we cannot exclude the possibility that the truncated form of SNAP29 missing this region has lost the conformation required for the binding. This mutant was able to bind SNAP29 (Fig. 3e), although the binding was inefficient; therefore, we cannot exclude the possibility that this region is involved in binding to both EHD1 and SNAP29. The mechanism whereby EHD1 and SNAP29 are recruited to the endocytic complex of the activated IGF-1R is not yet understood. However, according to the yeast two-hybrid assay, SNAP29 does not interact directly with the cytoplasmic tail of IGF-1R β-chain, as demonstrated in Fig. 3e, suggesting that the interaction between SNAP29 and IGF-1R depends on accessory proteins. Indeed, SNAP29 also formed complexes with α-adaptin of AP-2 (Fig. 4). EHD1 and SNAP29 co-localized to endocytic vesicles (Fig. 5). All these findings indicate participation of EHD1 and SNAP29 in endocytosis of the IGF-1R. Future studies will provide detailed characterization of the involvement of SNAP29 and EHD1 along specific intracellular traffic stations of the endocytic process.

Another EH domain-containing protein termed intersectin was found in a yeast two-hybrid screen as an interacting partner of SNAP25, a hallmark of the vesicle SNAP receptor (SNARE) complex, playing a role in exocytosis at nerve terminals (36). Intersectin interacts with dynamin, a prominent accessory protein of endocytosis that participates in the pinching process of the coated pit and binds to Ras exchange factor mSos1, thus providing indication for linkage between endocytosis and signaling processes (37). In another work, Adams et al. (38) demonstrated that intersectin overexpression activated Elk-1 transcription factor in a MAPK-independent manner, and this ability resided within the EH domain. Moreover, intersectin expression was enough to induce oncogenic transformation of rodent fibroblasts. These data suggest that intersectin may link endocytosis with regulation pathways important for cell growth and differentiation. Different from EHD1, intersectsin harbor several EH domains and three SH3 modules, enabling them, most probably, to participate in trafficking and signaling processes (36).

We propose that SNAP29 functions with EHD1, an EH domain-containing protein, to mediate IGF-1R endocytosis. Both these proteins were co-localized to endocytic vesicles, and both formed complexes with α-adaptin of AP-2 and IGF-1R. EHD1, which shares structural homology to Eps15, was found to associate with IGF-1R in response to IGF-1 stimuli and was shown to down-regulate IGF-1 signaling, suggesting it is involved in the sequestration of IGF-1R. SNAP29 and EHD1 were demonstrated to bind IGF-1R both in vitro and in vivo. The accumulated data indicate a role for these proteins in IGF-1R endocytosis.

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REFERENCES
1. Stewart, C. E., and Rotwein, P. (1996) Physiol. Rev. 76, 1005–1026
2. Sorkin, A., and Waters, C. M. (1993) Bioessays 15, 375–382
3. Owen, D. J., and Evans, P. R. (1998) Science 282, 1237–1332
4. Marsh, M., and McMahon, H. T. (1999) Science 285, 215–220
5. Schmid, S. L. (1997) Annu. Rev. Biochem. 66, 511–548
6. Kirchhausen, T.; Bonifacino, J. S., and Riezman, H. (1997) Curr. Opin. Cell Biol. 9, 488–495
7. Levin, D. A., and Mellman, I. (1998) Biochim. Biophys. Acta 1401, 129–145
8. Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshumura, A., and Baron, R. (1999) J. Biol. Chem. 274, 31707–31712
9. Mellman, I. (1996) Annu. Rev. Cell Dev. Biol. 13, 575–625
10. Clague, M. J. (1998) Biochem. J. 336, 271–282
11. Schmid, S. L., McNiven, M. A., and De Camilli, P. (1998) Curr. Opin. Cell Biol. 10, 504–512
12. Simpson, F., Hussain, N. K., Qualmann, B., Kelly, R. B., Kay, B. K., McPherson, P. S., and Schmid, S. L. (1999) Nat. Cell Biol. 1, 119–124
13. Di Fiore, P. P., Pelicci, P. G., and Sorkin, A. (1997) Trends Biochem. Sci. 22, 411–413
14. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Meninkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997) Genes Dev. 11, 2239–2249
15. Coda, L., Salcini, A. E., Confalonieri, S., Pelici, G., Sorkina, T., Sorkin, A., Pelici, P. G., and Di Fiore, P. P. (1998) J. Biol. Chem. 273, 3003–3012
16. de Beer, T., Carter, B. E., Lobe-Rice, K. E., Sorkin, A., and Overduin, M. (1998) Science 281, 1357–1360
17. Mayer, B. J. (1999) Curr. Biol. 9, 70–73
18. Salcini, A. E., Chen, H., Iannolo, G., De Camilli, P., and Di Fiore, P. P. (1999) Int. J. Biochem. Cell Biol. 31, 805–809
19. Benmerah, A., Begue, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1996) J. Biol. Chem. 271, 12111–12116
20. Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A., and Cerf, B. N. (1998) J. Cell Biol. 140, 1055–1062
21. Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999) J. Cell Sci. 112, 1303–1311
22. Torrisi, M. R., Lotti, L. V., Belleudi, F., Gradini, R., Salcini, A. E., Confalonieri, S., Pelici, P. G., and Di Fiore, P. P. (1999) Mol. Biol. Cell 10, 417–434
23. Carbene, R., Fre, S., Iannolo, G., Belleudi, F., Mancini, P., Pelici, P. G., Torrisi, M. R., and Di Fiore, P. P. (1997) Cancer Res. 57, 5498–5504
24. Mintz, L., Galperin, E., Tultzinski, S., Bromberg, Y., Fein, A., Kozak, C., Joyner, A., and Horowitz, M. (1999) Genomics 59, 66–76
25. 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. (2000) Genomics 63, 255–262
26. Robinson, M. S. (1987) J. Cell Biol. 104, 887–895
27. Tebar, F., Confalonieri, S., Carter, R. E., Di Fiore, P. P., and Sorkin, A. (1997) J. Biol. Chem. 272, 15413–15418
28. LeRoith, D., and Roberts, C. T., Jr. (1993) Ann. N. Y. Acad. Sci. 692, 1–9
29. Werner, H., and Le Roith, D. (1997) Crit. Rev. Oncog. 8, 71–92
30. Steegmaier, M., Yang, B., You, J. S., Huang, B., Shen, M., Yu, S., Luo, Y., and Scheller, R. H. (1998) J. Biol. Chem. 273, 34171–34179
31. Miele, C., Rochford, J. J., Filippa, N., Giorgetti-Peraldi, S., and Van Obberghen, E. (2000) J. Biol. Chem. 275, 21695–21702
32. Ceresa, B. P., and Schmid, S. L. (2000) Curr. Opin. Cell Biol. 12, 204–210
33. Rothman, J. E. (1994) Adv. Second Messenger Phosphoprotein Res. 29, 81–96
34. Weiss, W. I., and Scheller, R. H. (1998) Nature 395, 328–329
35. Vogel, K., Cahaniols, J. P., and Roche, P. A. (2000) J. Biol. Chem. 275, 2959–2965
36. Okamoto, M., Schoch, S., and Sudhof, T. C. (1999) J. Biol. Chem. 274, 18446–18454
37. Tong, X. K., Hussain, N. K., de Heuvel, E., Kurakin, A., Abi-Jaoude, E., Quinn, C. C., Olson, M. F., Baranes, D., Kay, B. K., and McPherson, P. S. (2000) EMBO J. 19, 1263–1271
38. Adams, A., Thorn, J. M., Yamabhai, M., Kay, B. K., and O’Bryan, J. P. (2000) J. Biol. Chem. 275, 27414–27420
