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Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors

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The involvement of Ral and its downstream molecules in receptor-mediated endocytosis was examined. Expression of either Ral^{G23V} or Ral^{S28N}, which are known to be constitutively active and dominant-negative forms, respectively, in A431 cells blocked internalization of epidermal growth factor (EGF). Stable expression of Ral^{G23V} or Ral^{S28N} in CHO-IR cells also inhibited internalization of insulin. Internalization of EGF and insulin was not affected by full-length RalBP1 which is an effector protein of Ral, but was inhibited by its C-terminal region which binds directly to Ral and POB1. POB1 is a binding protein of RalBP1 and has the Eps15 homology (EH) domain. Deletion mutants of POB1 inhibited internalization of EGF and insulin. However, internalization of transferrin was unaffected by Ral, RalBP1, POB1 and their mutants. Epsin and Eps15 have been reported to be involved in the regulation of endocytosis of the receptors for EGF and transferrin. The EH domain of POB1 bound directly to Epsin and Eps15. Taken together with the observation that EGF and insulin activate Ral, these results suggest that Ral, RalBP1 and POB1 transmit the signal from the receptors to Epsin and Eps15, thereby regulating ligand-dependent receptor-mediated endocytosis.

Keywords: endocytosis/epsin/POB1/Ral/RalBP1

Introduction

Ral is a member of the small GTP-binding protein (G protein) superfamily and consists of RalA and RalB (Chardin and Tavitian, 1986; Feig *et al*., 1996; Bos, 1998). Like other small G proteins, Ral has a GDP-bound inactive and a GTP-bound active form. The GDP-bound form of Ral is converted to the GTP-bound form by Ral-GDP dissociation stimulator (RalGDS), while the GTP-bound form is changed to the GDP-bound form by Ral GTPase-activating protein (RalGAP) (Feig *et al*., 1996; Bos, 1998). We have found that RalGDS is a putative effector protein of Ras (Kikuchi *et al*., 1994). Indeed, RalGDS stimulates the GDP–GTP exchange of Ral in a Ras-dependent manner in COS cells, and a dominant-negative form of Ral blocks Ras-dependent transformation of NIH 3T3 cells (Urano *et al*., 1996; Kishida *et al*., 1997). Ral is required for Src- and Ras-dependent activation of phospholipase D and regulates the initiation of border cell migration induced by Ras in *Drosophila* oogenesis (Jiang *et al*., 1995; Lee *et al*., 1996). RalGDS and Raf synergistically stimulate cellular proliferation and gene expression (White *et al*., 1996; Okazaki *et al*., 1997). Furthermore, exposure of cells to epidermal growth factor (EGF) and insulin increases the GTP-bound active form of Ral through Ras and RalGDS (Kishida *et al*., 1997; Wolthuis *et al*., 1998), and the post-translational lipid modifications of Ras and Ral are important for this signaling pathway (Hinoi *et al*., 1996; Kishida *et al*., 1997; Matsubara *et al*., 1999). These results indicate that RalGDS and Ral act downstream of Ras and mediate Ras functions. However, the cellular functions of Ral are not yet fully understood.

One possible clue to clarify the mode of action of Ral is RalBP1 (Ral-binding protein 1) (Feig *et al*., 1996). RalBP1 has a Ral-binding domain in its C-terminal region and binds to the GTP-bound form of Ral but not to the GDP-bound form. A mutation in the effector loop of Ral impairs its interaction with RalBP1, and RalBP1 inhibits the activity of RalGAP toward Ral (Feig *et al*., 1996; Hinoi *et al*., 1996). These results suggest that RalBP1 is an effector protein of Ral. RalBP1 also has a RhoGAP homology domain in its central region and exhibits GAP activity toward Rac1 and CDC42 but not RhoA (Feig *et al*., 1996). Therefore, RalBP1 may link Ral to Rac or CDC42. However, no report has yet shown that RalBP1 regulates the activities of Rac or CDC42 in intact cells.

To clarify the functions of RalBP1, we have identified a novel protein named POB1 (for partner of RalBP1) as a binding protein of RalBP1 (Ikeda *et al*., 1998). POB1 has a single Eps15 homology (EH) domain in its N-terminal region, and two proline-rich motifs and a coiled-coil structure in its C-terminal region. The activated Ral forms a complex with POB1 through RalBP1. POB1 directly binds to Grb2 but not to Nck or Crk. Furthermore, EGF induces tyrosine phosphorylation of POB1 and leads to formation of a complex between EGF receptor and POB1. These results suggest that RalBP1 and POB1 are involved in EGF signaling.

POB1 belongs to the EH domain-containing protein family (Di Fiore *et al*., 1997). The EH domain originally was identified as a motif present in three copies in the N-terminal region of Eps15 (Fazioli *et al*., 1993). Eps15 is a substrate of EGF receptor kinase and is constitutively associated with the plasma membrane and clathrin adaptor protein complex AP-2 (Fazioli *et al*., 1993; Di Fiore *et al*., 1997). Membrane-bound Eps15 is associated mainly with

clathrin-coated pits and vesicles. Furthermore, expression of the AP-2-binding region of Eps15 in CV-1, COS and HeLa cells inhibits internalization of the EGF and transferrin receptors (Carbone *et al*., 1997). Thus, Eps15 is involved in receptor-mediated endocytosis. The EH domain has been found in several proteins of mammals, yeast, insects and nematodes, thus establishing its evolutionary conservation (Di Fiore *et al*., 1997). Proteins with the EH domain include Eps15R (for Eps15-related), Reps1 (for RalBP1-associated Eps-homology domain protein), Intersectin, Pan1 and End3. Eps15R has 47% amino acid identity with and exhibits similar characteristics to Eps15 (Wong *et al*., 1995; Coda *et al*., 1998). The POB1-related protein Reps1 has been identified as a RalBP1-binding protein (Yamaguchi *et al*., 1997). Intersectin has five Src homology 3 domains in addition to two EH domains and is involved in the regulation of internalization of the transferrin receptor (Yamabhai *et al*., 1998; Sengar *et al*., 1999). Pan1 and End3 are *Saccharomyces cerevisiae* dimeric partners which are necessary for endocytosis of the α-mating factor receptor and for normal organization of the actin cytoskeleton (Tang *et al*., 1997), and a mutagenesis study of End3 suggests that the presence of its EH domain is necessary for its function (Benedetti *et al*., 1994). Therefore, it has been inferred that the EH domain-containing proteins generally regulate endocytosis. However, whether POB1 is involved in the regulation of endocytosis is not known.

Ral is present not only in the plasma membrane along with Ras but also in intracellular vesicles (Feig *et al*., 1996; Bos, 1998). Therefore, it has been suggested that Ral may be involved in intracellular vesicle transport in addition to the regulation of gene expression and cellular proliferation (Feig *et al*., 1996; Bos, 1998). This prompted us to examine whether Ral and its downstream molecules, RalBP1 and POB1, regulate endocytosis. We demonstrate here that Ral, RalBP1 and POB1 are involved in the regulation of internalization of EGF and insulin in A431 and CHO-IR cells, whereas they do not affect internalization of transferrin. Furthermore, we have purified two proteins which bind to the EH domain of POB1 from bovine brain membrane extract: one is Eps15 and the other is epsin, which was isolated previously as a binding protein of Eps15 (Chen *et al*., 1998). We show that POB1, Eps15 and epsin interact with one another. Since it has been demonstrated that Eps15 and epsin regulate endocytosis of the receptors for EGF and transferrin, these results suggest that Ral, RalBP1 and POB1 transmit the signal from the receptors to epsin and Eps15, resulting in the regulation of ligand-dependent, receptor-mediated endocytosis.

Results

Effects of Ral on internalization of EGF in A431 cells

To clarify the possible role of Ral in endocytosis, we expressed HA-Ral (wild type) or its mutants in A431 cells and examined whether they affect internalization of tetramethylrhodamine-conjugated EGF (rhodamine–EGF), since we found previously that EGF activates Ral through Ras and RalGDS (Kishida *et al*., 1997). Ral^{G23V} is not sensitive to RalGAP, and the GTP-bound form exists dominantly in intact cells (Hinoi *et al*., 1996; Kishida *et al*., 1997). Ral^{S28N} is relatively insensitive to RalGDS and functions in a dominant-negative manner in Ras-dependent transformation (Hinoi *et al*., 1996; Urano *et al*., 1996). $Ral^{G23V/C203S}$ is not geranylgeranylated at the C-terminal region and is localized in the cytosol fraction (Matsubara *et al*., 1997). Expression of Ral^{G23V} in A431 cells did not affect the binding of EGF to the cells at 4°C (Figure 1A and B) but inhibited internalization of EGF at 37°C (Figure 1C and D). Expression of Ral^{S28N} blocked internalization of, but not the binding of, EGF (Figure 1E–H). In contrast, A431 cells expressing wild-type Ral or $Ral^{G23V/C203S}$ bound to and internalized EGF as well as untransfected cells did (Figure 1I–P). These results suggest that expression of either the GTP- or GDP-bound form of Ral inhibits internalization of EGF and that the membrane localization of Ral through its C-terminal geranylgeranylation is important for its function of regulating internalization of EGF.

Effects of Ral on internalization of insulin in CHO-IR cells

We also examined whether Ral affects internalization of insulin in CHO-IR cells, since insulin activates Ral (Wolthuis *et al*., 1998). To this end, we established CHO-IR cells that stably express HA-Ral (wild type) or its mutants (Figure 2A). When these cells were fractionated into the cytosol and the membrane fractions, Ral^{G23V}, Ral^{S28N} and wild-type Ral were detected in both the membrane and the cytosol fractions, whereas $Ral^{G23V/C203S}$ was detected only in the cytosol fraction (Figure 2B). Expression of Ral and its mutants in CHO-IR cells did not affect the insulin-binding activity (Figure 2C). Insulin stimulation of the stable transformants resulted in a similar extent of tyrosine autophosphorylation of the insulin receptor β-subunit to that in wild-type CHO-IR cells (Figure 2D). To determine the effects of Ral on internalization of insulin, wild-type CHO-IR cells and CHO-IR cells expressing Ral or its mutants were incubated with [^{125}I]insulin at 4°C and further incubated without insulin at 37°C (Figure 2E). In the wild-type cells, ~55% of the cell surface insulin underwent internalization by 15 min. Expression of HA-Ral (wild type) or HA-$Ral^{G23V/C203S}$ did not affect internalization of insulin, whereas expression of HA-Ral^{G23V} or HA-Ral^{S28N} resulted in ~30–50% reduction of the internalization (Figure 2E). The degree of inhibition of internalization of insulin by Ral mutants was higher at 7.5 min than at 15 min, suggesting that Ral may affect an initial event of internalization of the insulin receptor, such as clathrin coating, rather than the endosomal fusion and recycling. Taken together, these results strongly suggest that Ral regulates endocytosis of the EGF and insulin receptors. This is the first demonstration that Ral is involved in intracellular vesicle transport.

Effects of RalBP1 on internalization of EGF and insulin

There are several proteins that may be involved in the Ral signaling pathway. The structures of RalBP1 and POB1 are shown schematically in Figure 3. RalBP1 is a putative effector protein of Ral, but its function is not clear (Feig *et al*., 1996). To examine the involvement of RalBP1 in internalization of EGF, we expressed

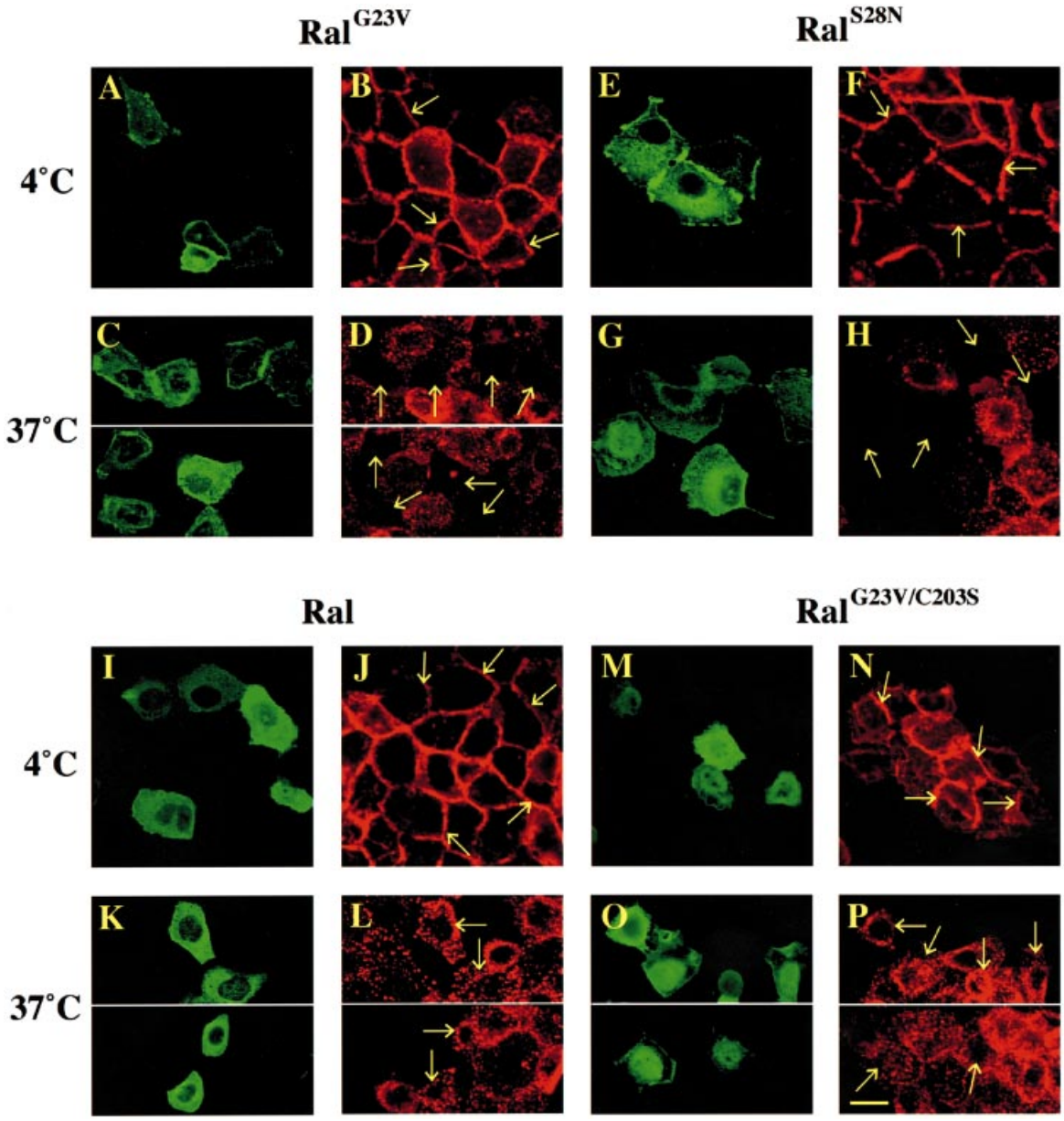

Fig. 1. Effects of Ral on internalization of EGF. A431 cells transiently expressing HA-Ral^{G23V} (**A**, **B**, **C** and **D**), HA-Ral^{S28N} (**E**, **F**, **G** and **H**), HA-Ral (wild type) (**I**, **J**, **K** and **L**) or HA-Ral$^{G23V/C203S}$ (**M**, **N**, **O** and **P**) were incubated with rhodamine–EGF for 1 h at 4°C (A, B, E, F, I, J, M and N) or allowed to internalize rhodamine–EGF for 15 min at 37°C (C, D, G, H, K, L, O and P). Transfected cells were visualized with the anti-HA antibody in green (A, C, E, G, I, K, M and O). Internalized rhodamine–EGF was visible as a red punctate/vesicular pattern of staining (D, H, L and P). Cells expressing Ral are indicated by arrows. Bar, 20 μm. The results shown are representative of five independent experiments.

Myc-RalBP1 (full length), Myc-RalBP1-(1–415) or Myc-RalBP1-(364–647) in A431 cells. A431 cells expressing RalBP1 or its mutants bound EGF as well as untransfected cells (Figure 4A, B, E and F; data not shown). A431 cells expressing Myc-RalBP1 (full length) or Myc-RalBP1-(1–415) internalized EGF as well as untransfected cells (Figure 4C and D; data not shown). In contrast, the cells expressing Myc-RalBP1-(364–647) failed to internalize EGF (Figure 4G and H), indicating that the C-terminal region of RalBP1 containing the Ral- and POB1-binding regions inhibits internalization of EGF. To examine whether RalBP1 is involved in internalization of insulin, we established CHO-IR cells stably expressing Myc-RalBP1 (full length) or its deletion mutants. The expression levels of Myc-RalBP1 (full length), Myc-RalBP1-(1–415) and Myc-RalBP1-(364–647) were similar as assessed by immunoblot analysis (data not shown). Expression of these proteins did not change the binding of [^{125}I]insulin to the cells or tyrosine autophosphorylation of the β-subunit of the insulin receptor in response to insulin compared with wild-type CHO-IR cells (data not shown). Although expression of Myc-RalBP1 (full length) did not affect internalization of insulin, Myc-RalBP1-(1–415) inhibited it by 60% in contrast to its lack of effect on internalization of EGF (Figure 5A). Myc-RalBP1-(364–647) also inhibited internalization of insulin (Figure 5A). Since RalBP1-(1–415) shows GAP activity toward Rac and CDC42 (Feig *et al*., 1996), these results suggest that the signal from Ral to Rac/CDC42 may be involved in internalization of insulin. Although we do not know the

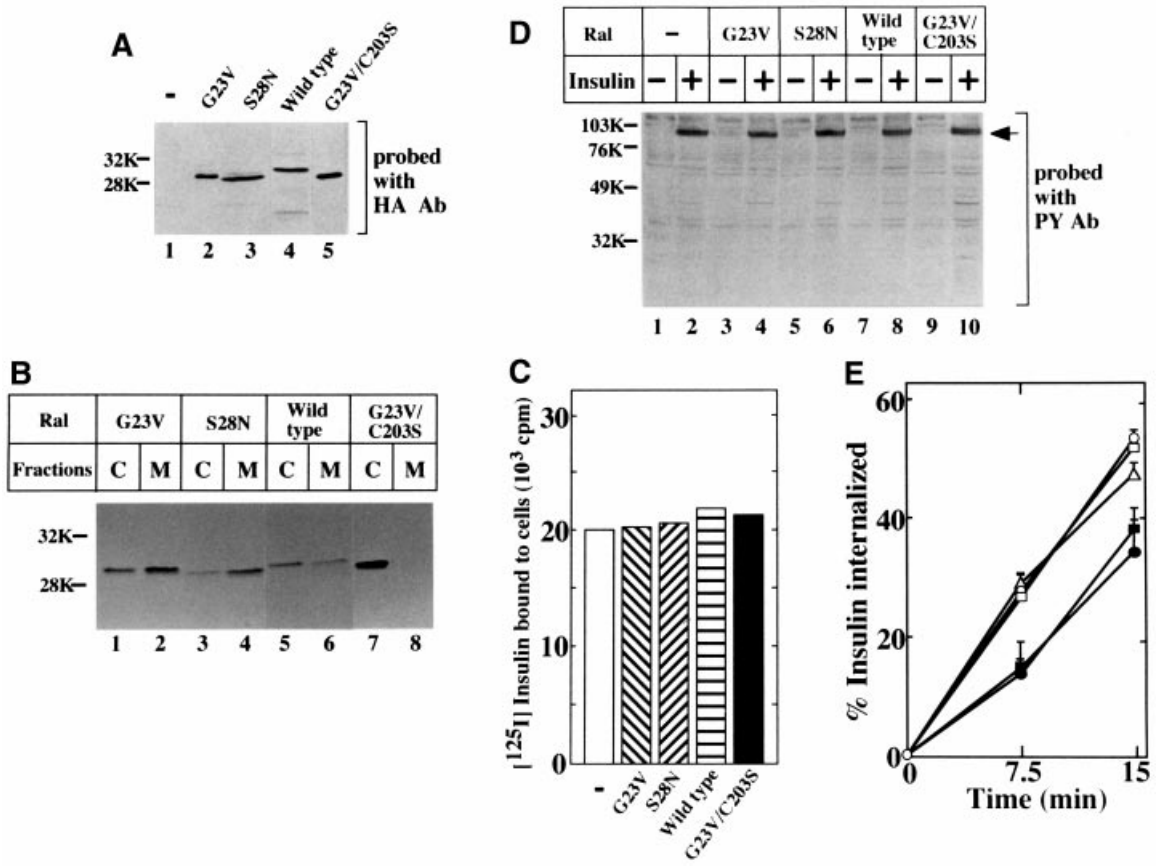

Fig. 2. Effects of Ral on internalization of insulin. (**A**) Expression of Ral^{G23V}, Ral^{S28N}, Ral (wild type) or Ral$^{G23V/C203S}$ in CHO-IR cells. The lysates (20 μg of each protein) of CHO-IR cells stably expressing HA-Ral^{G23V} (lane 2), HA-Ral^{S28N} (lane 3), HA-Ral (wild type) (lane 4) or HA-Ral$^{G23V/C203S}$ (lane 5) were probed with the anti-HA antibody. Wild-type CHO-IR cells were used as a control (lane 1). '–', No transfection; G23V, Ral^{G23V}; S28N, Ral^{S28N}; Wild type, Ral (wild type); G23V/C203S, Ral$^{G23V/C203S}$. (**B**) Subcellular localization of Ral in CHO-IR cells. Aliquots of the membrane and cytosol fractions of CHO-IR cells expressing HA-Ral^{G23V} (lanes 1 and 2), HA-Ral^{S28N} (lanes 3 and 4), HA-Ral (wild type) (lanes 5 and 6) or HA-Ral$^{G23V/C203S}$ (lanes 7 and 8) were probed with the anti-HA antibody. C, cytosol fraction; M, membrane fraction. (**C**) Insulin-binding activity. Wild-type CHO-IR cells (□) and CHO-IR cells expressing HA-Ral^{G23V} (▧), HA-Ral^{S28N} (▨), HA-Ral (wild type) (▤) or HA-Ral$^{G23V/C203S}$ (■) were incubated with 10 nM [^{125}I]insulin for 5 h at 4°C, and then the cells were washed and lysed. The bound radioactivity was measured with an autogamma counter. (**D**) Autophosphorylation activity of the insulin receptor. Wild-type CHO-IR cells (lanes 1 and 2) and CHO-IR cells expressing HA-Ral^{G23V} (lanes 3 and 4), HA-Ral^{S28N} (lanes 5 and 6), HA-Ral (wild type) (lanes 7 and 8) or HA-Ral$^{G23V/C203S}$ (lanes 9 and 10) were incubated with (+) or without (–) 1 μM insulin for 10 min at 37°C. Aliquots of the lysates (20 μg of each protein) of these cells were probed with the anti-phosphotyrosine antibody. The arrow indicates the position of the insulin receptor β-subunit. PY, phosphotyrosine. (**E**) Internalization of insulin. Wild-type CHO-IR cells (○) and CHO-IR cells expressing HA-Ral^{G23V} (●), HA-Ral^{S28N} (■), HA-Ral (wild type) (□) or HA-Ral$^{G23V/C203S}$ (△) were incubated with 10 nM [^{125}I] insulin for 5 h at 4°C, and then further incubated without insulin for the indicated periods at 37°C. The results shown are means ±SE of five independent experiments.

reasons for the different effects of RalBP1-(1–415) on the endocytosis of the EGF and insulin receptors, these findings are the first demonstrations of the physiological functions of RalBP1.

Effects of POB1 on internalization of EGF and insulin

POB1 has been identified as a binding protein of RalBP1 and is suggested to be involved in EGF signaling (Ikeda *et al.*, 1998). Since POB1 has the EH domain, it is speculated that POB1 is involved in internalization of EGF, like Eps15. Enhanced green fluorescent protein (EGFP)–POB1 (full length) or EGFP–POB1-(126–227) (EH domain) was expressed in A431 cells. Expression of these proteins did not affect the binding of EGF to the cells (Figure 4I, J, M and N), whereas the EH domain but not full-length POB1 inhibited internalization of EGF (Figure 4K, L, O and P). These results suggest that POB1 is not involved in the binding of EGF but regulates its internalization and that the EH domain is important for the function of POB1. To examine the effects of POB1 on internalization of insulin, we established CHO-IR cells that stably express HA-POB1 (full length) and HA-POB1-(322–521), but failed to establish the cells stably expressing HA-POB1-(1–374). POB1-(322–521) contains a binding site for RalBP1 (Ikeda *et al.*, 1998). The expression levels of HA-POB1 (full length) and HA-POB1-(322–521) were similar as assessed by immunoblot analysis (data not shown). Expression of these proteins did not affect the insulin-binding activity of the cells (data not shown). Insulin stimulation of these stable transformants resulted in a similar extent of tyrosine autophosphorylation of the insulin receptor β-subunit as in wild-type CHO-IR cells (data not shown). Expression of HA-POB1 (full length) did not affect internalization of insulin, whereas expression of HA-POB1-(322–521) resulted in ~30–40% reduction of the internalization (Figure 5B). Taken together, these results indicate that

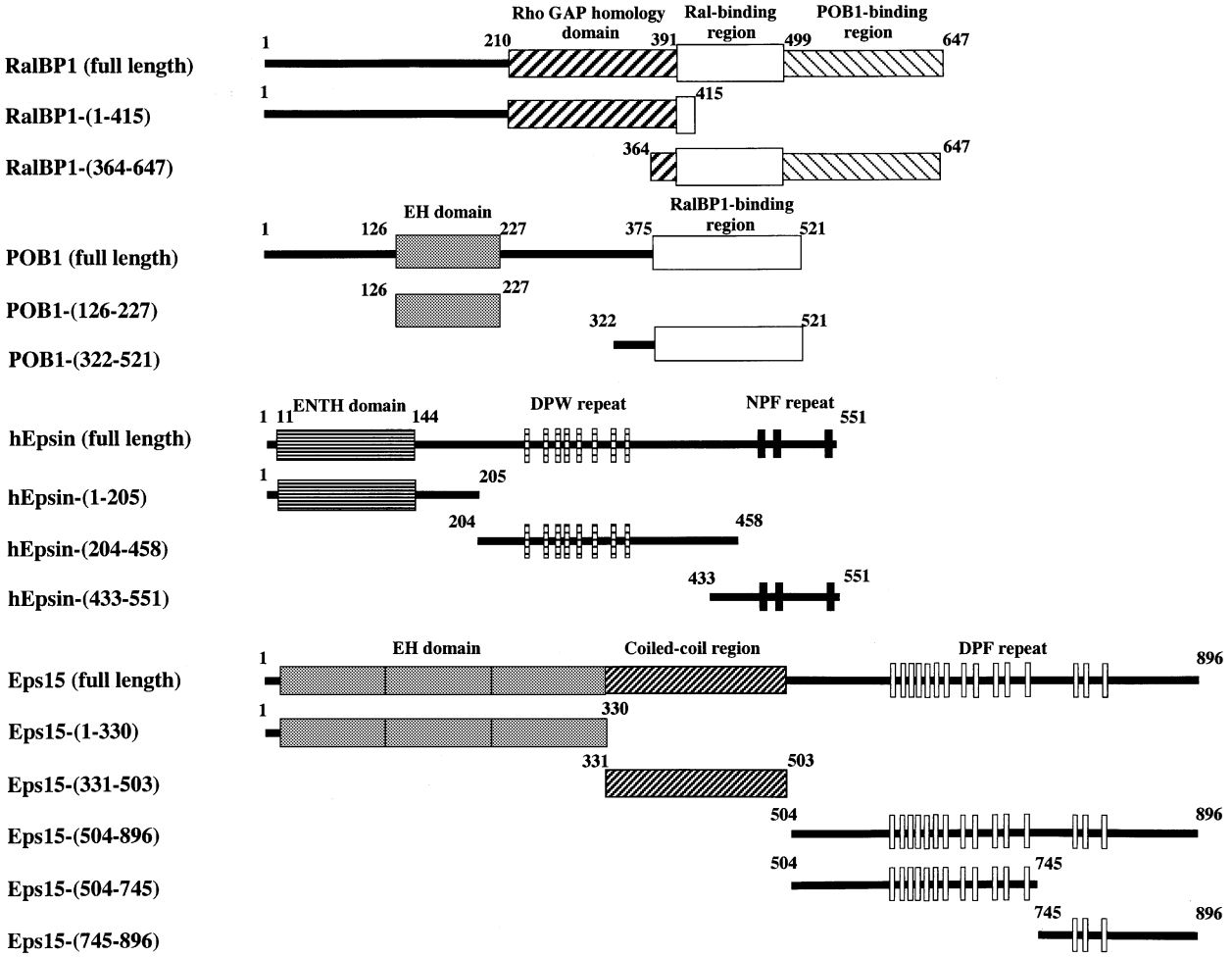

Fig. 3. Schematic representations of RalBP1, POB1, epsin, Eps15 and their deletion mutant constructs used in this study.

POB1 is also involved in the regulation of endocytosis of the receptors for EGF and insulin.

Effects of Ral, RalBP1 and POB1 on internalization of transferrin

Receptor-mediated endocytosis is generally separated into a ligand-dependent process and a ligand-independent process. Endocytosis of the EGF and insulin receptors involves both processes. We examined whether Ral, RalBP1 and POB1 affect the ligand-independent process by the use of [^{125}I]transferrin, because the transferrin receptor is a constitutively recycled membrane protein whose ligand, transferrin, mediates cellular iron uptake (Qian and Tang, 1995). The endocytosis of the transferrin receptor in CHO cells has been well characterized (McGraw and Maxfield, 1990). CHO-IR and CHO cells exhibited the same internalization rate constant for transferrin, which is the fraction of surface transferrin receptors internalized per minute (data not shown). Therefore, we analyzed CHO-IR cells expressing Ral, RalBP1, POB1 or their mutants by this transferrin internalization assay. In contrast to EGF and insulin, neither wild-type Ral nor the Ral mutants affected internalization of transferrin (Figure 6A), and nor did RalBP1, POB1 or their deletion mutants (Figure 6B and C). These results demonstrate that Ral, RalBP1 and POB1 are not involved in the regulation of endocytosis of the transferrin receptor, suggesting that these proteins do not regulate the ligand-independent process.

Identification of POB1-EH domain-binding proteins

It has been shown that the EH domain of Eps15 is a protein-binding module and that several proteins interact with Eps15 through their Asn–Pro–Phe (NPF) motifs (Di Fiore *et al*., 1997). Therefore, we tried to identify the proteins that bind to the EH domain of POB1. Bovine brain membrane extract was applied to glutathione *S*-transferase (GST) and GST–POB1-EH columns. After the columns had been washed, elution was performed stepwise with serial addition of 250 mM NaCl, 500 mM NaCl, 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS), 1% CHAPS and 10 mM glutathione. Several proteins bound to the GST–POB1-EH column in preference to the GST column. However, the assay gave reproducible results only with a 130 kDa protein (p130) and an 84 kDa protein (p84). These proteins were eluted from the column with 0.5% CHAPS (Figure 7A, lanes 1 and 2). To clarify the primary structures of p130 and p84, the purified proteins were subjected to amino acid sequencing. Two peptide sequences derived from p130 were determined: one was KTDLDLD-GYVSGQEVK and the other was KTNEVQELQNDLD-RETSSLQELEA. These were identical to the deduced amino acid sequence of Eps15R. One peptide sequence

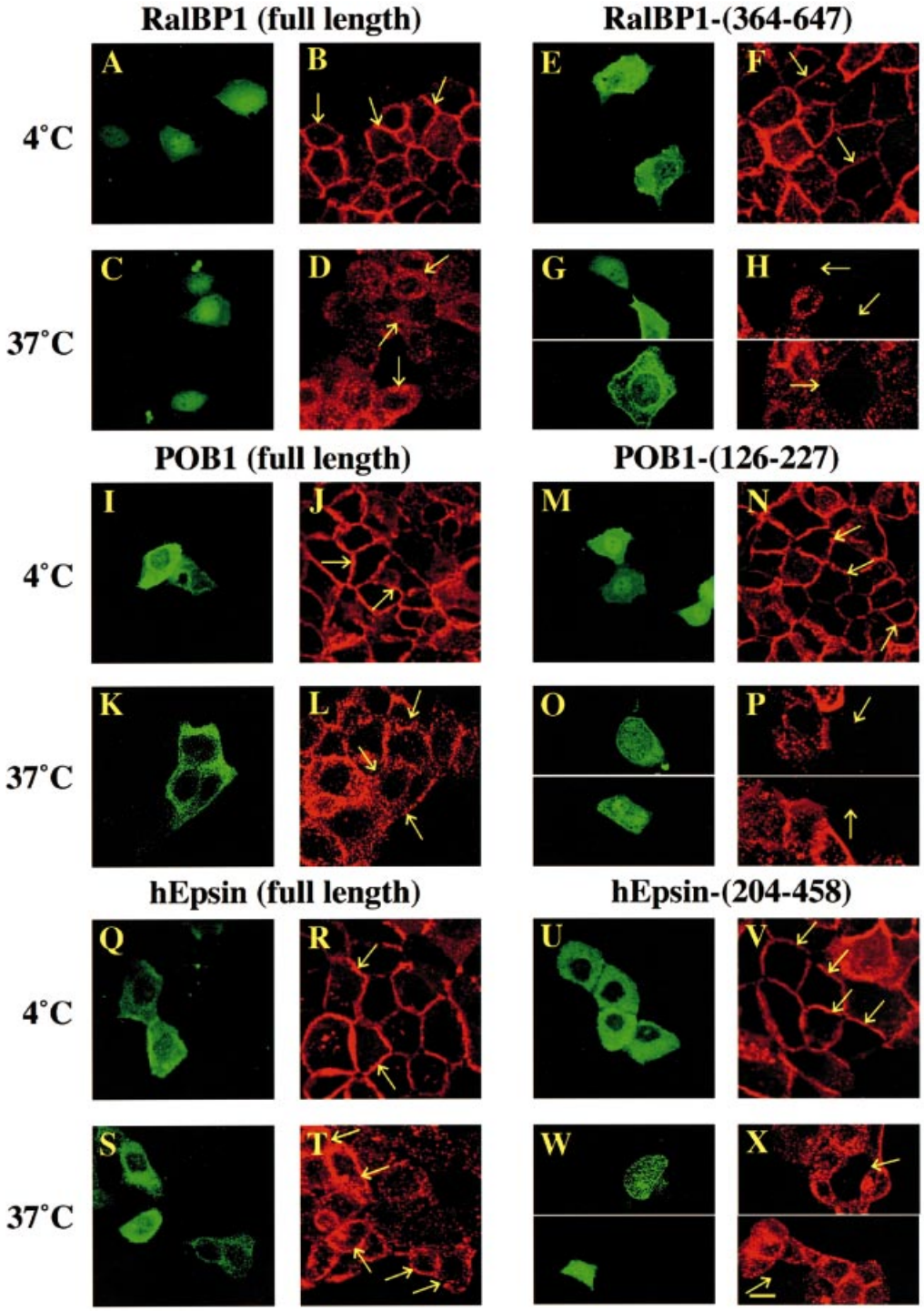

Fig. 4. Effects of RalBP1, POB1 and epsin on internalization of EGF. A431 cells transiently expressing Myc-RalBP1 (full length) (**A**, **B**, **C** and **D**), Myc-RalBP1-(364–647) (**E**, **F**, **G** and **H**), EGFP–POB1 (full length) (**I**, **J**, **K** and **L**), EGFP–POB1-(126–227) (EH domain) (**M**, **N**, **O** and **P**), EGFP–hEpsin (full length) (**Q**, **R**, **S** and **T**) or EGFP–hEpsin-(204–458) (**U**, **V**, **W** and **X**) were incubated with rhodamine–EGF for 1 h at 4°C (B, F, J, N, R and V), and then allowed to internalize rhodamine–EGF for 15 min at 37°C (D, H, L, P, T and X). Transfected cells were visualized either with the anti-Myc antibody in green (A, C, E and G) or spontaneous fluorescence of EGFP (I, K, M, O, Q, S, U and W). Internalized rhodamine–EGF was visible as a red punctate/vesicular pattern of staining (D, H, L, P, T and X). Cells expressing RalBP1, POB1 or hEpsin are indicated by arrows. Bar, 20 μm. The results shown are representative of three independent experiments.

derived from p84 was KNIVHNYSEAEI. This sequence was identical to the sequence of epsin which had been identified as a binding protein of Eps15 and shown to be involved in clathrin-mediated endocytosis (Chen *et al.*, 1998). We have already isolated human epsin (hEpsin) as a binding protein of POB1 (Morinaka *et al.*, 1999). hEpsin is 90% identical with rat epsin (Figure 7B). The N-terminal region of epsin has the evolutionarily conserved domain called ENTH (for epsin N-terminal homology) domain, the central region is characterized by the presence of eight repeats of the Asp–Pro–Trp (DPW) motif and the C-terminal region contains three NPF motifs, which are

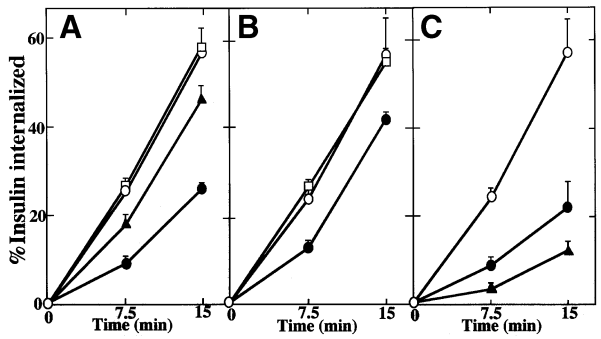

Fig. 5. Effects of RalBP1, POB1 and epsin on internalization of insulin. (**A**) RalBP1. The insulin internalization activities of wild-type CHO-IR cells (○) and CHO-IR cells expressing Myc-RalBP1 (full length) (□), Myc-RalBP1-(1–415) (●) or Myc-RalBP1-(364–647) (▲) were measured. (**B**) POB1. The insulin internalization activities of wild-type CHO-IR cells (○) and CHO-IR cells expressing HA-POB1 (full length) (□) or HA-POB1-(322–521) (●) were measured. (**C**) Epsin. The insulin internalization activities of wild-type CHO-IR cells (○) and CHO-IR cells expressing Myc-hEpsin-(1–205) (▲) or Myc-hEpsin-(204–551) (●) were measured. The results shown are means ± SE of five independent experiments.

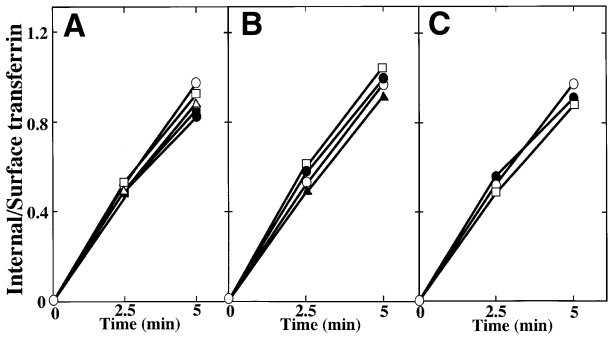

Fig. 6. Effects of Ral, RalBP1 and POB1 on internalization of transferrin. (**A**) Ral. The transferrin internalization activities of wild-type CHO-IR cells (○) and CHO-IR cells expressing HA-Ral^{G23V} (●), HA-Ral^{S28N} (■), HA-Ral (wild type) (□) or HA-Ral$^{G23V/C203S}$ (△) were measured. (**B**) RalBP1. The transferrin internalization activities of wild-type CHO-IR cells (○) and CHO-IR cells expressing Myc-RalBP1 (full length) (□), Myc-RalBP1-(1–415) (●) or Myc-RalBP1-(364–647) (▲) were measured. (**C**) POB1. The transferrin internalization activities of wild-type CHO-IR cells (○) and CHO-IR cells expressing HA-POB1 (full length) (□) or HA-POB1-(322–521) (●) were measured. The results shown are means of three independent experiments.

known to constitute the binding sequence of the EH domain (Figures 3 and 7B). Epsin binds to AP-2 and is involved in the clathrin-dependent internalization of the receptors for EGF and transferrin (Chen *et al*., 1998).

An anti-epsin rabbit polyclonal antibody was generated by immunizing rabbits with hEpsin-(1–205). When the homogenate of rat brain was probed with this antibody, a single band was observed at the molecular mass of ~84 kDa (Figure 7C). The band corresponding to epsin was detected in various tissues including liver, spleen and testis, and weakly in lung and thymus, but not in heart or kidney (Figure 7C). The molecular mass of Myc-hEpsin expressed in COS cells was the same as that of p84 in rat tissues (Figure 7C). Furthermore, p84 bound to the GST–POB1-EH column was recognized by the anti-epsin antibody, whereas no protein bound to the GST column reacted with this antibody (Figure 7A, lanes 3 and 4), indicating that epsin cDNA encodes p84. The structures of epsin and Eps15 are shown in Figure 3.

Interaction of Eps15 with hEpsin

The results in Figure 7A suggest three possibilities: (i) that the EH domain of POB1 binds to epsin complexed with Eps15R, (ii) that it binds to Eps15R complexed with epsin, or (iii) that it binds to Eps15R and epsin individually. We have shown that the EH domain of POB1 directly binds to the NPF motifs in the C-terminal region of hEpsin (Morinaka *et al*., 1999). Since Eps15R exhibits the same characteristics as Eps15 (Coda *et al*., 1998), we confirmed that the hEpsin which we isolated indeed associates with the EH domain of Eps15. Lysates of COS cells expressing Myc-hEpsin (full length) or its deletion mutants were incubated with GST–Eps15-EH or GST. GST–Eps15-(1–330) (EH domain) precipitated Myc-hEpsin (full length) and Myc-hEpsin-(433–551), but not Myc-hEpsin-(1–205) or Myc-hEpsin-(204–458) (Figure 8A). Furthermore, the overlay assay showed that the EH domain of Eps15 binds to hEpsin-(433–551) directly (data not shown). Since hEpsin-(433–551) contains three NPF motifs, these results indicate that Eps15 and hEpsin interact with each other through the EH domain and the NPF motifs, and are consistent with previous observations (Chen *et al*., 1998).

Interaction of POB1 with Eps15

To examine whether POB1 binds to Eps15, the lysates of COS cells expressing HA-Eps15 or HA-Eps15R were incubated with GST–POB1-EH or GST. GST–POB1-EH precipitated both HA-Eps15 and HA-Eps15R but not GST (Figure 8B). Since these results could not rule out the possibility that POB1 associates with Eps15 or Eps15R through epsin, we asked whether the EH domain of POB1 binds to Eps15 directly. To this end, maltose-binding protein (MBP)-fused deletion mutants of Eps15 were purified, and overlay analyses were performed using GST–POB1-EH and GST–Eps15-EH as probes. The molecular masses of MBP–Eps15-(1–330), MBP–Eps15-(331–503), MBP–Eps15-(504–896), MBP–Eps15-(504–745) and MBP–Eps15-(745–896) on SDS–PAGE were 82, 73, 115, 80 and 65 kDa, respectively (data not shown). The EH domain of POB1 bound to MBP–Eps15-(504–896) but not to MBP–Eps15-(1–330) or MBP–Eps15-(331–503) (Figure 8C). Neither GST nor GST–Eps15-EH bound to any mutant of MBP–Eps15 (Figure 8C). Furthermore, in residues 504–896 of Eps15, the EH domain of POB1 bound to MBP–Eps15-(504–745) but not to MBP–Eps15-(745–896) (Figure 8D). It is notable that Eps15-(504–745) does not have the NPF motifs. This clearly shows that the EH domain of POB1 binds to a protein which lacks the NPF motifs.

These experiments were performed with overexpressed proteins. We therefore examined next whether the POB1 EH domain actually binds to endogenous epsin and Eps15. Eps15 and epsin were detected using their specific antibodies in COS cells and bovine brain (Figure 8E, lanes 1 and 2). When the overlay assay was performed with GST–POB1-EH, GST–POB1-EH directly interacted with endogenous epsin and Eps15 (Figure 8E, lanes 3 and 4), but GST alone did not (data not shown). Taken together, these results indicate that POB1, epsin and Eps15 interact

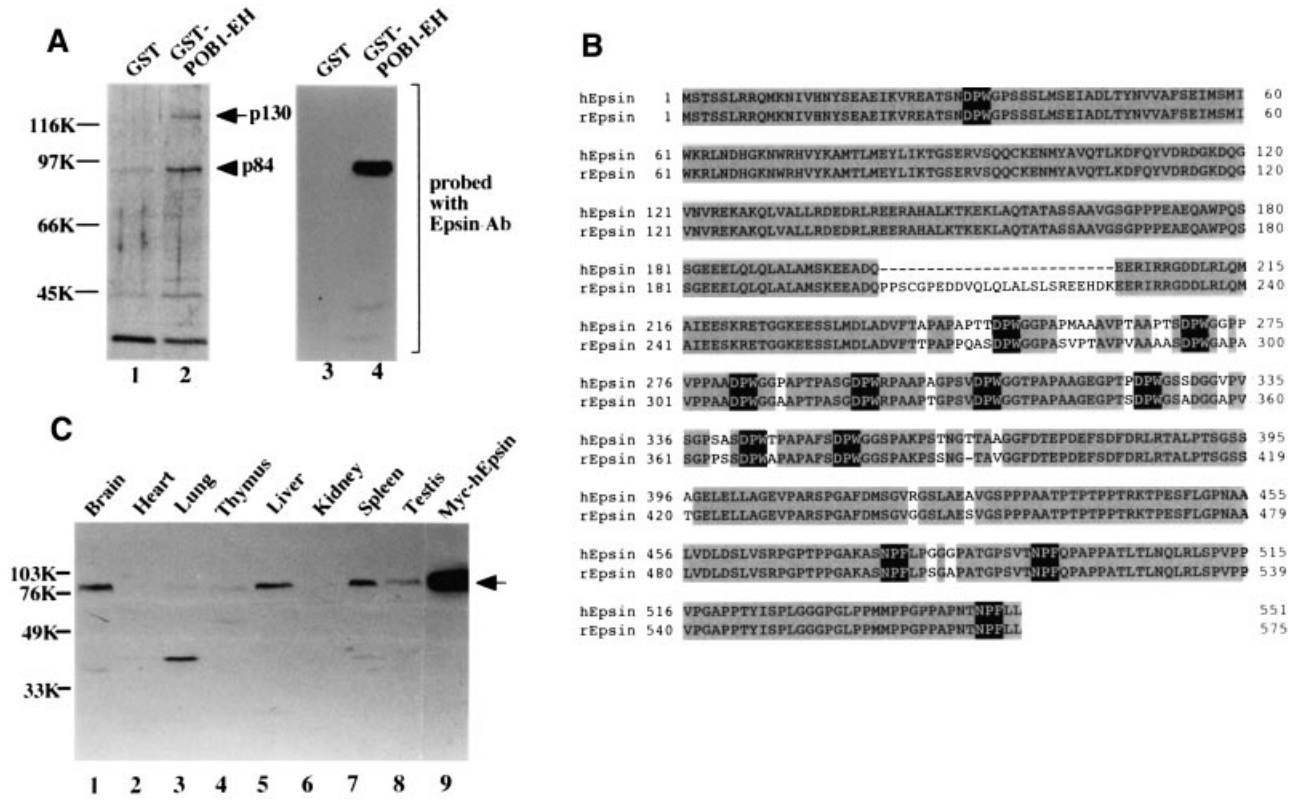

Fig. 7. Identification of POB1-EH domain-binding proteins. (**A**) p84 and p130. The bovine brain membrane extract was applied separately to the GST and GST–POB1-EH columns. After the columns had been washed, bound proteins were eluted sequentially with 250 mM NaCl, 500 mM NaCl, 0.5% CHAPS, 1% CHAPS and 10 mM glutathione. Aliquots were subjected to SDS–PAGE and visualized with silver staining. Proteins eluted with 0.5% CHAPS from the GST (lane 1) and GST–POB1-EH (lane 2) columns are shown. The arrow and arrowhead indicate the positions of p130 and p84, respectively. Proteins eluted with 0.5% CHAPS from the GST (lane 3) and GST–POB1-EH (lane 4) columns were probed with the anti-epsin antibody. (**B**) Alignment of amino acid sequences of human epsin (hEpsin) and rat epsin (rEpsin). Residues indicated with dark shading are identical amino acids. The DPW and NPF motifs are highlighted by black boxes. (**C**) Tissue distribution of epsin. Homogenates of various rat tissues (100 μg of each protein) (lanes 1–8) and COS cells overexpressing Myc-hEpsin (20 μg of protein) (lane 9) were probed with the anti-epsin antibody. The relationship between epsin and the protein with a molecular mass of 40 kDa in lung is not known. The arrow indicates the position of epsin.

with one another and suggest that they form a ternary complex.

Effects of epsin on internalization of EGF and insulin

It has been demonstrated that epsin regulates endocytosis of the receptors for EGF and transferrin (Chen *et al*., 1998). Consistent with those observations, neither EGFP–hEpsin (full length) nor EGFP–hEpsin-(204–458) affected the binding of EGF to A431 cells (Figure 4Q, R, U and V). Internalization of EGF in A431 cells was inhibited by expression of EGFP–hEpsin-(204–458) but not by expression of EGFP–hEpsin (full length) (Figure 4S, T, W and X). Furthermore, we established CHO-IR cells stably expressing Myc-hEpsin-(1–205) or Myc-hEpsin-(204–551) to examine whether epsin is involved in internalization of insulin. The expression levels of Myc-hEpsin-(1–205) and Myc-hEpsin-(204–551) were similar as assessed by immunoblot analysis (data not shown). Expression of these proteins did not affect the ability of the insulin receptor to bind to insulin or to auto-phosphorylate its tyrosine residues (data not shown). Expression of Myc-hEpsin-(1–205) inhibited internalization of insulin and resulted in ~80% reduction of the internalization (Figure 5C). Myc-hEpsin-(204–551) also inhibited internalization of insulin, but to a lesser extent than Myc-hEpsin-(1–205) (Figure 5C). Taken together, these findings show that epsin regulates internalization of the receptors for EGF, insulin and transferrin, and can act on the ligand-independent process of receptor-mediated endocytosis.

Discussion

Receptor-mediated endocytosis is a process in which eukaryotic cells continuously internalize receptor-bound ligands together with other plasma membrane proteins and lipids as well as extracellular solutes (Steinman *et al*., 1983; Trowbridge *et al*., 1993). The basic features of the pathway involve the internalization of receptor–ligand complexes via clathrin-coated pits to yield coated vesicles that rapidly lose their coats and fuse with a heterogeneous tubulovesicular network collectively referred to as early endosomes. Many molecules have been identified that function at different steps of vesicle transport (Rothman, 1994). Among these, the small G proteins of the Rab family serve critical regulatory roles in vesicular traffic (Novick and Zerial, 1997). Several Rab proteins are associated with the endocytotic pathway and play different roles in endocytosis and recycling. For example, Rab4 and Rab5 co-localize in early endosomes. Rab7 and Rab9 are associated with late endosomes. Rab5 regulates the

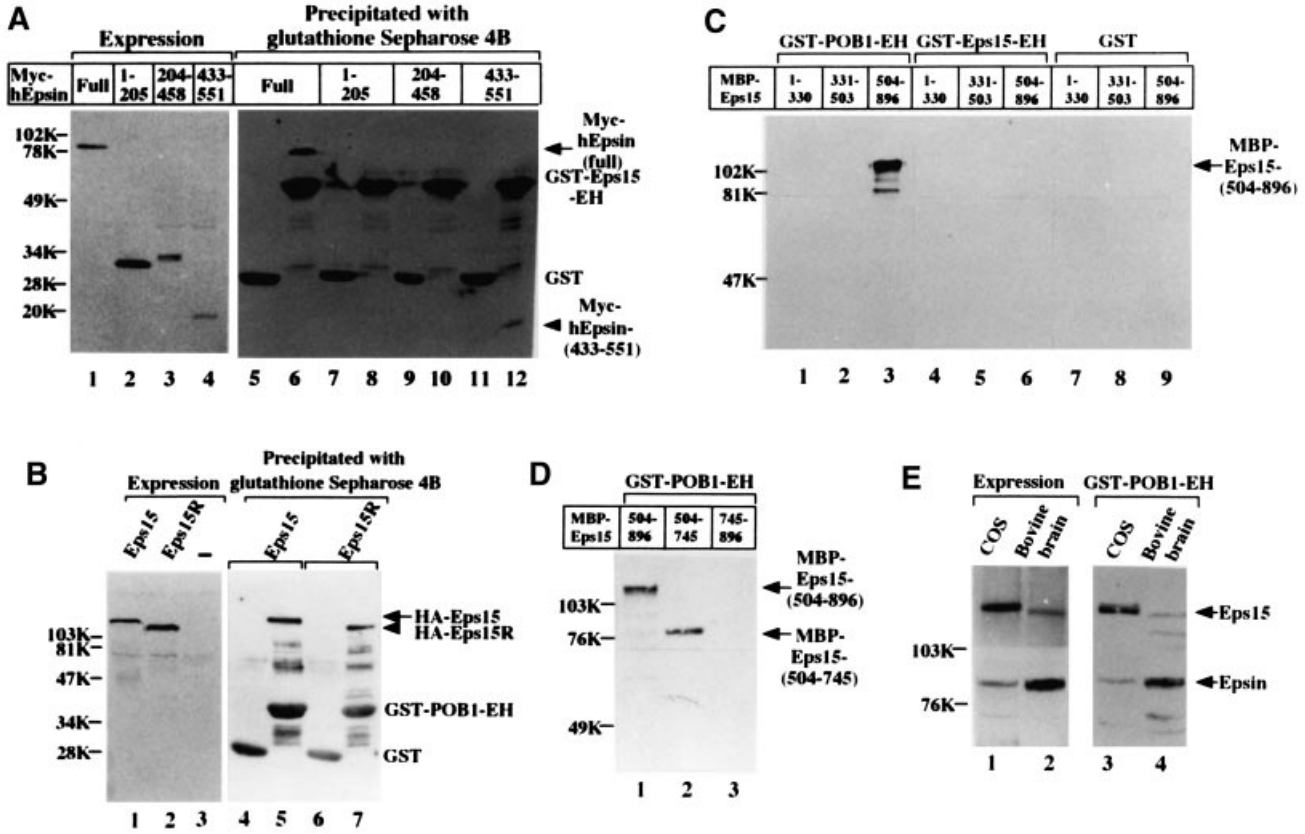

Fig. 8. Complex formation of POB1, epsin and Eps15. (**A**) Interaction of Eps15 with hEpsin. Lysates of COS cells expressing Myc-hEpsin (full length) (lanes 1, 5 and 6), Myc-hEpsin-(1–205) (lanes 2, 7 and 8), Myc-hEpsin-(204–458) (lanes 3, 9 and 10) or Myc-hEpsin-(433–551) (lanes 4, 11 and 12) were probed directly with the anti-Myc antibody (lanes 1–4) or precipitated with GST (lanes 5, 7, 9 and 11) or GST–Eps15-EH (lanes 6, 8, 10 and 12). The precipitates were probed with the anti-Myc antibody. The arrow and arrowhead indicate the positions of Myc-hEpsin (full length) and Myc-hEpsin-(433–551), respectively. GST–Eps15-EH and GST were non-specifically detected with the anti-Myc antibody. (**B**) Interaction of the EH domain of POB1 with Eps15 or Eps15R. The lysates of COS cells expressing HA-Eps15 (full length) (lane 1, 4 and 5) or HA-Eps15R (full length) (lanes 2, 6 and 7) were probed directly with the anti-HA antibody (lanes 1 and 2) or precipitated with GST (lanes 4 and 6) or GST–POB1-EH (lanes 5 and 7). The precipitates were probed with the anti-HA antibody (lanes 4–7). The lysates of COS cells transfected with an empty vector were used as a control (lane 3). The arrow and arrowhead indicate the positions of HA-Eps15 and HA-Eps15R, respectively. GST–POB1-EH and GST were non-specifically detected with the anti-HA antibody. (**C**) Direct interaction of POB1 with Eps15. Purified proteins (1.5 pmol each) of MBP–Eps15-(1–330) (lanes 1, 4 and 7), MBP–Eps15-(331–503) (lanes 2, 5 and 8) and MBP–Eps15-(504–896) (lanes 3, 6 and 9) were subjected to SDS–PAGE and transferred to nitrocellulose membranes. The membranes were overlaid with 6 nM GST–POB1-EH (lanes 1–3), GST–Eps15-EH (lanes 4–6) or GST (lanes 7–9), and the bound proteins were detected using the anti-GST antibody. (**D**) Direct interaction of POB1 with residues 504–745 of Eps15. Purified proteins (1.5 pmol each) of MBP–Eps15-(504–896) (lane 1), MBP–Eps15-(504–745) (lane 2) and MBP–Eps15-(745–896) (lane 3) were overlaid with 6 nM GST–POB1-EH and the bound proteins were detected by the anti-GST antibody. (**E**) Interaction of POB1 with endogenous Eps15 and epsin. COS cell lysates (lanes 1 and 3) and bovine brain homogenate (lanes 2 and 4) were probed directly with the anti-Eps15 and anti-epsin antibodies (lanes 1 and 2) or overlaid with 200 nM GST–POB1-EH (lanes 3 and 4). The results shown are representative of three independent experiments.

clathrin-coated pathway of the receptor internalization and transport into the early endosomes. In contrast, Rab4 is implicated in the regulation of membrane recycling from the early endosomes to the recycling endosomes or directly to the plasma membrane. It has been shown that Ral associates with synaptic vesicles and platelet-dense granules (Feig *et al*., 1996; Bos, 1998), suggesting that Ral may regulate the process of endocytosis or exocytosis. In this report, we have for the first time demonstrated that Ral and its downstream molecules, including RalBP1 and POB1, are involved in EGF- and insulin-dependent endocytosis. Our results show that mutants but not wild types of these proteins inhibit endocytosis. One explanation might be that endogenous levels of these proteins are not limited for endocytosis in intact cells and that mutants disrupt the complex formation among Ral, RalBP1, POB1 and their binding proteins, thereby inhibiting the functions of endogenous proteins. Since internalization of EGF, insulin and transferrin in A431, CHO-IR and CHO cells, respectively, has been characterized (Hari and Roth, 1987; Gamou *et al*., 1988; McGraw and Maxfield, 1990), it is possible that the roles of Ral and its downstream molecules in receptor-mediated endocytosis in these cells are representative of those that occur in other mammalian cells.

We have shown here that expression of Ral^{G23V} (the GTP-bound form) and Ral^{S28N} (the GDP-bound form) in A431 and CHO-IR cells inhibits EGF- and insulin-dependent endocytosis. These are similar to the actions of Rab2 in that both the GTP- and GDP-bound forms of Rab2 inhibit vesicle transport from the endoplasmic reticulum to the Golgi complex (Tisdale *et al*., 1992). These results indicate that the GDP–GTP exchange is critical for the regulation of receptor-mediated endocytosis by Ral. Expression of a constitutively active form of Ras (Ras^{G12V}) in A431 cells also inhibits internalization of

EGF (unpublished data). This result supports the observation that activated Ral inhibits endocytosis. However, because Ras has effector proteins other than RalGDS (Marshall, 1995), the roles of RalGDS in endocytosis remain to be clarified. It has been shown that protein kinase B (PKB) and Rab5 mediate Ras-dependent fluid phase endocytosis (Barbieri *et al*., 1998). Therefore, it is tempting to speculate that Ras regulates endocytosis through the RalGDS/Ral and PKB/Rab5 pathways additively or synergistically.

The post-translational modifications of small G proteins are critical for their activation and actions (Glomset *et al*., 1990; Takai *et al*., 1992). The C-terminal region of Ral has a CAAL motif (where C is cysteine, A is an aliphatic amino acid and L is leucine) and Ral has been found to be geranylgeranylated at its C-terminus. In the Ras/RalGDS/Ral signaling pathway, we have shown that the post-translational modification of Ras is required for determination of the subcellular localization of RalGDS, and that the modifications of both Ras and Ral are necessary for the signal from Ras to Ral through RalGDS in intact cells (Hinoi *et al*., 1996; Kishida *et al*., 1997; Matsubara *et al.*, 1999). We have also demonstrated in the present study that the C-terminal geranylgeranylation is necessary for the inhibitory action of Ral^{G23V} on EGF- and insulin-dependent endocytosis. These results are similar to the results showing that the post-translational modifications of Rab5 and Rab9 are required for regulation of vesicle transport (Gorvel *et al*., 1991; Lombardi *et al*., 1993). These results suggest that localization to the membrane fractions through the lipid modification is important for the function of Ral.

Although it has been suggested that RalBP1 links Ral to Rac and CDC42 because RalBP1 exhibits GAP activity toward Rac and CDC42, so far no report has verified this possibility. Indeed, expression of RalBP1 affects neither Rac- nor CDC42-dependent Jun N-terminal kinase activity (unpublished data). Since Rho subfamily members are involved in receptor-mediated endocytosis (Lamaze *et al*., 1996; Ridley, 1996), the effect of RalBP1 on endocytosis regulated by Rac and CDC42 remains to be elucidated. Our results show that the C-terminal region of RalBP1 which binds to both Ral and POB1 inhibits EGF- and insulin-dependent endocytosis and that the N-terminal region containing the RhoGAP homology domain inhibits insulin-dependent endocytosis. Although we do not know the mechanism of the different effects of RalBP1-(1–415) on internalization of EGF and insulin, complex formation of Ral, RalBP1 and POB1 could play a role in receptor-mediated endocytosis.

POB1 has characteristics similar to those of Eps15 in that both proteins have an EH domain, proline-rich motifs and a coiled-coil structure, and that both are tyrosine phosphorylated in response to EGF (Fazioli *et al*., 1993; Ikeda *et al*., 1998). Eps15 is implicated in internalization of the EGF and transferrin receptors (Carbone *et al*., 1997; Di Fiore *et al*., 1997). The EH domain appears to function as a protein–protein interaction module and it has been demonstrated that the amino acids Asn–Pro–Phe (NPF) form the core of an EH domain-binding motif (Salcini *et al*., 1997). Synaptojanin 1 possesses the NPF motifs, and its interaction with the EH domain of Eps15 has been demonstrated (Haffner *et al*., 1997). Synaptojanin 1 is a Grb2-binding protein with inositol 5-phosphatase activity, and participates in synaptic vesicle endocytosis (McPherson *et al*., 1994). Epsin binds to Eps15 through its NPF motifs (Chen *et al*., 1998). It has been demonstrated that the EH domain of Intersectin also binds to epsin (Yamabhai *et al*., 1998; Sengar *et al*., 1999). In yeast, the EH domain of Pan1 interacts with yAP180A and yAP180B, which have the NPF motifs (Wendland and Emr, 1998). yAP180s are homologous to mammalian AP180, which binds to clathrin and promotes its assembly into cages, suggesting that AP180 participates in membrane trafficking events (Morris *et al*., 1993). Thus, the interaction of EH domain-containing proteins with their binding partners could play a role in endocytosis. We have identified epsin as a binding protein of the EH domain of POB1. The EH domain and the C-terminal region of POB1 block the internalization of EGF and insulin, respectively. It is possible that the EH domain of POB1 competes with endogenous POB1 for binding to epsin, and that the C-terminal region of POB1 competes with endogenous POB1 for binding to RalBP1. Therefore, these results suggest that the complex formation of RalBP1, POB1 and epsin is also important for EGF- and insulin-induced endocytosis.

Furthermore, we have found that the EH domain of POB1 directly binds to the C-terminal region of Eps15. Since this region does not contain the NPF motif, the result demonstrates that the EH domain interacts with some motif other than the NPF motif. In yeast, the region containing the EH domain of Pan1 binds to the C-terminal region of End3, which does not have the NPF motif (Tang *et al*., 1997). These results suggest that the EH domain does not always require the NPF motif for its interaction with binding partners. Consistent with these observations, it has been reported that the first EH domain of End3 binds to a His–Thr/Ser–Phe (HT/SF) motif (Paoluzi *et al*., 1998). Since Eps15-(504–745) does not contain the HT/SF motif, the EH domain of POB1 could interact with Eps15 through a new motif. During the revision of this manuscript, Ese (Intersectin) has been reported to bind to Eps15 through the coiled-coil regions of the two proteins (Sengar *et al*., 1999). Taken together, these results suggest that EH domain-containing proteins associate with each other, and that the POB1–Eps15 and Intersectin–Eps15 complexes regulate receptor-mediated endocytosis.

It is likely that epsin and Eps15 are involved in the constitutive process of the endocytosis of the transferrin receptor through interaction with AP-2 and clathrin (Di Fiore *et al*., 1997; Chen *et al*., 1998). In contrast to epsin and Eps15, Ral, RalBP1 or POB1 do not affect internalization of transferrin. These results suggest that Ral, RalBP1 and POB1 are involved in the ligand-dependent process of endocytosis, that epsin and Eps15 are involved in the ligand-independent process, and that Ral, RalBP1 and POB1 transmit the signal from the receptors to epsin and Eps15, thereby regulating EGF- or insulin-dependent receptor-mediated endocytosis. Our current model for this is as follows (Figure 9). Once Ras is activated by extracellular signals such as EGF or insulin, Ras anchoring to the plasma membrane through its lipid modification recruits RalGDS to the plasma membrane where RalGDS then stimulates the GDP–GTP exchange

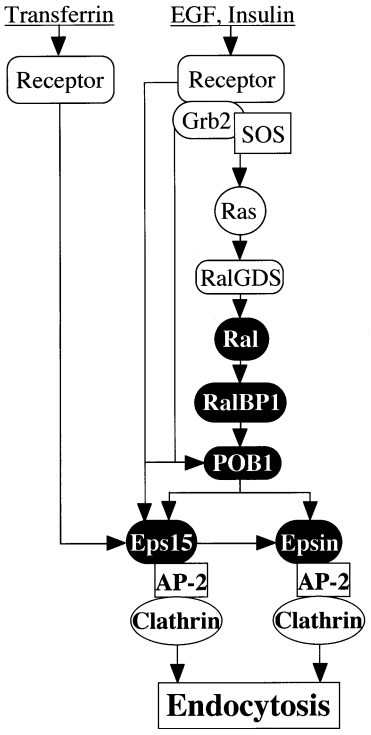

Fig. 9. Ral signaling pathway in the regulation of endocytosis of the EGF and insulin receptors. When Ras is activated by EGF or insulin through Grb2 and SOS, it recruits RalGDS to the plasma membrane, resulting in the activation of Ral. The activated Ral interacts with RalBP1, which forms a complex with POB1. POB1 associates with epsin and Eps15, which bind to the AP-2 and clathrin complex. Grb2 directly binds to POB1. EGF induces tyrosine phosphorylation of Eps15 and POB1. For further explanation, see text.

of Ral, which is also present in the plasma membrane. Activated Ral induces the translocation of RalBP1 complexed with POB1 to the plasma membrane. Finally POB1 associates with epsin and Eps15, which bind to the AP-2–clathrin complex, resulting in the formation of clathrin-coated vesicles with transmembrane receptors. Studies to clarify how assembly and disassembly of this complex are regulated temporally and spacially are under way. It has been demonstrated that the interactions of epsin and Eps15 with AP-2 are inhibited by phosphorylation and enhanced by dephosphorylation (Chen *et al*., 1999). It is intriguing to speculate that complex formations of Ral and its downstream molecules are regulated in a phosphorylation-dependent manner.

Materials and methods

Materials and chemicals

A431 cells (EGF receptor-overexpressing cells), CHO-IR cells (insulin receptor-overexpressing CHO cells) and the anti-GST and anti-MBP antibodies were kindly supplied by Drs E.Tahara (Hiroshima University, Hiroshima, Japan), Y.Ebina (Tokushima University, Tokushima, Japan) and M.Nakata (Sumitomo Electric Industries, Yokohama, Japan), respectively. Hygromycin-resistant CHO-IR cells which stably express Ral, RalBP1, POB1, hEpsin or their mutants were propagated as described (Okazaki *et al*., 1996). Human Eps15, Eps15R and epsin cDNAs were isolated by PCR using a Marathon ready cDNA library (Clontech Laboratories, Inc., Palo Alto, CA). GST and MBP fusion proteins were purified from *Escherichia coli* according to the manufacturer's instructions. Tetramethylrhodamine-conjugated EGF (rhodamine–EGF), [^{125}I]insulin and [^{125}I]transferrin were purchased from Molecular Probes, Inc. (Eugene, OR), Amersham Pharmacia Biotech, Inc. (Buckinghamshire, UK) and NEN Life Science Products, Inc. (Boston, MA), respectively. The anti-Eps15 antibody was from Transduction Laboratories, Inc. (Lexington, KY). Other materials and chemicals were from commercial sources.

Plasmid construction

pCGN/RalB, pCGN/RalB^{G23V}, pCGN/RalB^{S28N}, pCGN/RalB$^{G23V/C203S}$, pBJ-Myc/RalBP1, pBJ-Myc/RalBP1-(1–415), pBJ-Myc/RalBP1-(364–647), pGEX-2T/POB1-(126–227) (EH domain), pEF-BOS-Myc/hEpsin (full length), pEF-BOS-Myc/hEpsin-(1–205), pEF-BOS-Myc/hEpsin-(204–458), pEF-BOS-Myc/hEpsin-(433–551), pMAL-c2/hEpsin-(1–205), pMAL-c2/hEpsin-(204–458) and pMAL-c2/hEpsin-(204–551) were constructed as described (Matsubara *et al*., 1997; Okazaki *et al*., 1997; Ikeda *et al*., 1998; Morinaka *et al*., 1999). To construct pEGFP-c1/POB1 (full length), pUC19/POB1 (full length) was digested with *Xba*I, and then inserted into the *Xba*I-cut pEGFP-c1. To construct pUC19/POB1-(126–227) (EH domain), the 0.3 kb fragment encoding POB1-(126–227) with *Eco*RI and *Bgl*II sites was synthesized by PCR, digested with *Eco*RI and *Bgl*II, and inserted into the *Eco*RI- and *Bam*HI-cut pUC19. To construct pEGFP-c2/POB1-(126–227) (EH domain), pUC19/ POB1-(126–227) was digested with *Eco*RI and *Sal*I, and then inserted into the *Eco*RI- and *Sal*I-cut pEGFP-c2. To construct pEGFP-c1/hEpsin (full length), pBSKS/hEpsin (full length) was digested with *Xba*I, and then inserted into the *Xba*I-cut pEGFP-c1. To construct pEGFP-c2/hEpsin-(204–458), pBSKS/hEpsin (full length) was digested with *Sal*I, blunted with Klenow fragment and digested with *Bam*HI, and then inserted into pEGFP-c2 which had been digested with *Xba*I, blunted with Klenow fragment and digested with *Bam*HI. To construct pGEX-2T/Eps15-(1–330) (EH domain) and pMAL-c2/Eps15-(1–330) (EH domain), the 1.0 kb fragment encoding Eps15-(1–330) with *Bam*HI sites was synthesized by PCR. This fragment was digested with *Bam*HI and inserted into the *Bam*HI-cut pGEX-2T or pMAL-c2. To construct pMAL-c2/Eps15-(331–503), the 0.5 kb fragment encoding Eps15-(331–503) with *Xba*I sites was synthesized by PCR. This fragment was digested with *Xba*I and inserted into the *Xba*I-cut pMAL-c2. To construct pMAL-c2/Eps15-(504–896), the 1.2 kb fragment encoding Eps15-(504–896) with *Xba*I sites was synthesized by PCR. This fragment was digested with *Xba*I and inserted into the *Xba*I-cut pMAL-c2. To construct pMALc-2/Eps15-(504–745), pMALc-2/Eps15-(504–896) was digested with *Sac*I, blunted with T4 DNA polymerase and digested with *Xba*I, and the fragment encoding Eps15-(504–745) was inserted into pMALc-2 which had been digested with *Pst*I, blunted with T4 DNA polymerase and digested with *Xba*I. To construct pMALc-2/Eps15-(745–896), pMALc-2/Eps15-(504–896) was digested with *Sac*I and *Xba*I, and the fragment encoding Eps15-(745–896) was inserted into the *Sac*I- and *Xba*I-cut pMALc-2.

EGF binding and internalization assay

The EGF internalization assay of A431 cells expressing HA-Ral, Myc-RalBP1, EGFP–POB1, EGFP–hEpsin or their mutants was performed as described previously (Carbone *et al*., 1997; Chen *et al*., 1998). Each plasmid was transfected into A431 cells which had been plated on glass coverslips coated with 2% gelatin using TransFast Reagent (Promega Corp., Madison, WI). When cells grew to 70–80% confluency at 24–48 h after the transfection, they were analyzed. The cells were placed in ice-cold binding medium [20 mM HEPES–NaOH pH 7.5, 130 mM NaCl, 0.1% bovine serum albumin (BSA)] for 30 min and treated with 1 μg/ml rhodamine–EGF in RPMI for 1 h at 4°C. To examine the binding of EGF to the cells, the cells were washed with phosphate-buffered saline (PBS) three times. To examine the internalization of EGF, the EGF-containing medium was replaced with warm RPMI, in which the cells were incubated further for 15 min at 37°C, followed by incubation in acidic washing buffer (0.2 M acetic acid pH 2.8, 0.5 M NaCl) for 15 min at 4°C in order to remove cell surface EGF. Then, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells expressing Myc- or HA-tagged proteins were permeabilized with 0.2% Triton X-100 and 0.2% BSA in PBS for 30 min at room temperature. The cells were stained with the anti-Myc or anti-HA antibody for 1 h at room temperature followed by Cy2-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech, Inc.) for 1 h at room temperature, and viewed with a confocal laser-scanning microscope (TCS-NT®, Leica-laser-technik GmbH, Heidelberg, Germany).

Insulin binding and internalization assay

The activities of the binding and the internalization of insulin in CHO-IR cells were determined as described previously (Hari and Roth, 1987; Biener *et al*., 1996). Confluent wild-type CHO-IR cells and CHO-IR

cells expressing Ral, RalBP1, POB1, hEpsin or their mutants (35 mm diameter dishes) were incubated with 10 nM [^{125}I]insulin (4–5×10^3 c.p.m./pmol) for 5 h at 4°C. To examine the insulin-binding activity of the cells, unbound ligands were removed by washing three times with cold PBS, the cells were lysed with PBS containing 1% Triton X-100 and 0.1% SDS, and the radioactivity was measured with an autogamma counter. Non-specific binding activity was determined in the presence of a large excess of unlabeled insulin, and was found to be <1.5% of the total binding. Internalization was initiated by adding warm binding medium (Ham's F12 containing 1 mg/ml BSA, 50 mM HEPES–NaOH pH 7.4) at 37°C after the cells had been washed with cold PBS three times. At various times, the medium was removed and the cells were put on ice, washed twice with the acidic washing buffer for 3 min, and then three times with cold PBS. The acid-extractable radioactivity represents surface-bound insulin. The acid-stripped cells were lysed with PBS containing 1% Triton X-100 and 0.1% SDS. The non-acid-extractable radioactivity represents internalized insulin. The rate of internalization of insulin was expressed as a percentage of internalized [^{125}I]insulin relative to the sum of surface-bound [^{125}I]insulin and internalized [^{125}I]insulin. To examine autophosphorylation of the insulin receptor, after 16 h serum starvation the cells were incubated with 1 μM insulin for 10 min at 37°C. The cells were lysed and the lysates were probed with the anti-phosphotyrosine antibody.

Transferrin internalization assay

The transferrin internalization assay was performed as described previously (McGraw and Maxfield, 1990). Wild-type CHO-IR cells and CHO-IR cells overexpressing Ral, RalBP1, POB1 or their mutants grown in 24-well plates were washed with and incubated in med1 (McCoy's 5′A medium salts containing 26 mM $NaHCO_3$, 1 mg/ml BSA and 20 mM HEPES–NaOH pH 7.4) for 1 h at 37°C. The cells were washed with med1 and incubated in med1 supplemented with 25 nM [^{125}I]transferrin for the indicated times. After the incubation, the plates were placed on ice and washed with PBS containing Ca^{2+} and Mg^{2+} (PBS+) and then with the acidic washing buffer for 5 min at 4°C. The cells were then washed three times with cold PBS+ and solubilized in 1 M NaOH, and the radioactivities of the lysates were counted in a gamma counter. Cell-associated radioactivity after the acid wash represents the internalized transferrin during the incubation at 37°C. The amount of the surface transferrin receptor was determined by incubating cells with 25 nM [^{125}I]transferrin on ice for 2 h followed by seven washes with cold PBS+ (surface transferrin binding). Internalization of transferrin was determined as the ratio of internalized transferrin/surface transferrin binding. To measure the non-specific binding, a large excess of non-radioactive diferric transferrin was added. The non-specific binding was <5% of the total binding. The surface transferrin binding was constant over the course of the experiment. The concentration of transferrin used was sufficient to saturate the surface transferrin receptors rapidly.

Identification and purification from bovine brain membrane extract of the proteins which bind to GST–POB1-EH

Bovine brains were obtained from the heads of freshly slaughtered cattle and frozen at –80°C until use. All procedures were carried out at 4°C. Cerebral tissue (~150 g, wet weight) was homogenized in a Potter–Elvehjem Teflon–glass homogenizer with 300 ml of buffer A [25 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA and 1 mM dithiothreitol (DTT)] containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 20 000 *g* for 30 min. The precipitates were suspended in 720 ml of buffer B (20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) containing 2 M NaCl and stirred for 1 h. The suspension was centrifuged again at 20 000 *g* for 30 min. Proteins in the supernatant were precipitated with 70% ammonium sulfate. The precipitate was dialyzed against buffer B and the dialysate was used as bovine brain membrane extract. GST–POB1-EH was incubated with glutathione–Sepharose 4B for 2 h, and the immobilized GST–POB1-EH was packed in a column (1 cm diameter). Approximately 3 mg of GST–POB1-EH bound to 1 ml of glutathione–Sepharose 4B. To identify proteins which bind to the EH domain of POB1, the bovine brain membrane extract (30 mg of protein) was applied separately to a glutathione–Sepharose 4B column containing GST (0.3 ml) and a glutathione–Sepharose 4B column containing GST–POB1-EH (0.3 ml) equilibrated with buffer B. After the columns had been washed with 3 ml of buffer C (10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT), stepwise elution was performed with 3 ml each of buffer C containing 250 mM NaCl, 500 mM NaCl, 0.5% CHAPS or 1% CHAPS followed by 3 ml of 10 mM Tris–HCl (pH 8.0) containing 10 mM glutathione, 5 mM $MgCl_2$ and 1 mM DTT. When each fraction was subjected to SDS–PAGE and visualized with silver staining, a 130 kDa protein (p130) and an 84 kDa protein (p84) were found to bind to the GST–POB1-EH column but not to the GST column, the bulk of these two proteins was eluted from the column by 0.5% CHAPS. To purify a large amount of p130 and p84, bovine brain membrane extract (100 mg of protein) was applied to a GST–POB1-EH column (1 ml) and the proteins were eluted by 0.5% CHAPS. The same procedures were repeated 10 times. The fractions containing p130 and p84 were concentrated to 80 μl using Centricon-10 (Amicon, Inc., Beverly, MA).

Amino acid sequencing

Purified p130 and p84 were resolved by SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The bands corresponding to p130 and p84 were digested by the lysyl endopeptidase, *Acromobacter* protease I (Iwamatsu, 1992). The resulting peptides were fractionated by C18 column chromatography and subjected to amino acid sequencing. Two sequences from p130 and one sequence from p84 were obtained.

Binding assay of POB1, epsin and Eps15

To show the interaction of Eps15 with epsin, COS cells expressing Myc-hEpsin (full length) or its deletion mutants were suspended in lysis buffer (50 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 5 mM EGTA, 10 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The suspension was sonicated and centrifuged at 100 000 *g* for 30 min, and the supernatant (6 mg/ml) was used as the lysate. The lysates (1 mg of protein) were incubated with 0.8 μM GST–Eps15-EH or GST for 3 h at 4°C. GST–Eps15-EH and GST were precipitated with glutathione–Sepharose 4B. The precipitates were washed twice with washing buffer (50 mM HEPES–NaOH pH 7.5, 1.5 mM $MgCl_2$, 5 mM EGTA), twice with the washing buffer containing 150 mM NaCl and twice with washing buffer, and then probed with the anti-Myc antibody. To show the interaction of POB1 with Eps15, the lysates of COS cells expressing HA-Eps15 or HA-Eps15R were incubated with 1 μM GST–POB1-EH or GST for 3 h at 4°C. The precipitates obtained with glutathione–Sepharose 4B were probed with the anti-HA antibody.

Overlay assay

The overlay assay was performed as described (McPherson *et al*., 1998). Various Eps15 deletion mutants were purified as MBP fusion proteins from *E.coli*. MBP–Eps15 deletion mutants (1.5 pmol each), COS cell lysates (100 μg of protein) and bovine brain homogenate (100 μg of protein) were subjected to SDS–PAGE and transferred to nitrocellulose membranes. GST–POB1-EH, GST–Eps15-EH or GST was incubated with the nitrocellulose membranes in overlay buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 3% BSA, 1 mM DTT, 0.1% Tween-20) at a final concentration of 6 or 200 nM for 12 h at 4°C. The membranes were then washed and probed with the anti-GST antibody.

Others

The anti-epsin rabbit polyclonal antibody was generated by immunizing rabbits with hEpsin-(1–205). For the experiments of tissue distribution of epsin, 7-week-old male rats of the Wistar strain were sedated with CO_2 gas and sacrificed by cervical dislocation. Brain, heart, lung, thymus, liver, kidney, spleen and testis were excised immediately and homogenized in 9 ml/g of tissue (wet weight) of homogenizing buffer (20 mM Tris–HCl pH 7.5, 0.25 M sucrose). The subcellular fractionation of CHO-IR cells was carried out as described previously (Hinoi *et al*., 1996).

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