Eps15R Is a Tyrosine Kinase Substrate with Characteristics of a Docking Protein Possibly Involved in Coated Pits-mediated Internalization*

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Eps15R was identified because of its relatedness to eps15, a gene encoding a tyrosine kinase substrate bearing a novel protein-protein interaction domain, called EH. In this paper, we report a biochemical characterization of the eps15R gene product(s). In NIH-3T3 cells, three proteins of 125, 108, and 76 kDa were specifically recognized by anti-eps15R sera. The 125-kDa species is a bona fide product of the eps15R gene, whereas p108 and p76 are most likely products of alternative splicing events. Eps15R protein(s) are tyrosine-phosphorylated following epidermal growth factor receptor activation in NIH-3T3 cells overexpressing the receptor, even at low levels of receptor occupancy, thus behaving as physiological substrates. A role for eps15R in clathrin-mediated endocytosis is suggested by its localization in plasma membrane-coated pits and in vivo association to the coated pits’ adapter protein AP-2. Finally, we demonstrate that a sizable fraction of eps15R exists in the cell as a complex with eps15 and that its EH domains exhibit binding specificities that are partially distinct from those of eps15. We propose that eps15 and eps15R are multifunctional binding proteins that serve pleiotropic functions within the cell.

Eps15R is a recently identified protein, which displays sequence and structural homologies with eps15, a substrate for the epidermal growth factor receptor (EGFR), and other receptor tyrosine kinases (1–3). The predicted amino acid sequences of eps15R and eps15 identify modular proteins (Refs. 1–4, and Fig. 1A). The most relevant feature of eps15R and eps15 is the presence, in their N-terminal regions, of three copies of a novel protein-protein interaction domain, the EH domain (for Eps15 Homology), which binds to the amino acid motif NPF (asparagine-proline-phenylalanine) of target proteins and is conserved throughout evolution (3, 4). The central regions of eps15R and eps15 present the characteristic heptad repeats of coiled-coil proteins (1, 3, 5) and may dictate a rod-like configuration. The C termini of eps15R and eps15 contain a proline-rich region and a repeated aspartic acid-proline-phenylalanine (DPF) motif. The proline-rich region has been shown to bind to the SH3 domain of Crk (6).

Several lines of evidence implicate eps15R and eps15 in receptor-mediated endocytosis. First, eps15 is localized in plasma membrane-coated pits and vesicles (7) and binds in vivo to the α-subunit of the clathrin adapter AP-2 (8–10). The region of eps15 responsible for AP-2 binding is located within its C terminus domain (9, 10); the analogous region of eps15R is also able to interact with AP-2 in vitro (10). Second, other EH-containing proteins appear to be involved in endocytosis. The yeast EH-containing End3p and Pan1p are necessary for endocytosis of the α-mating factor and lipophilic dye and for normal organization of the actin cytoskeleton (11–13). In addition mutagenesis studies in End3p and Pan1p suggested that the presence of EH domains is necessary for their function(s) (11–13). Finally, the sequence NPFXD, which bears striking similarity to the NPF motif identified as the binding target for the EHs of eps15 and eps15R, has been recently shown to represent a novel class of internalization signals in yeast (14).

The participation of EH-containing proteins in endocytosis might be a particular aspect of their more general involvement in the processes of intracellular routing. This hypothesis is based on the characteristics of known and putative EH-containing proteins and EH interactors. A screening of prokaryotic expression libraries with the EH domains of eps15 identified, among other proteins, NUMB as an EH-binding protein (4). NUMB is involved in cell fate determination through asymmetrical distribution at mitosis (15–17), a function that requires sorting of NUMB within dividing cells. In addition, a frequent characteristic of EH-binding proteins is the presence of multiple NPF motifs, which mirrors the frequent tandem arrangement of EH domains. Thus, screening of data bases for proteins containing multiple NPFs yielded a number of potential EH interactors. Among these are synaptojanin that participates in synaptic vesicle recycling (18); SCAMP 37 that is part of a family of molecules that are components of membranes that function in cell surface recycling (19); the Drosophila dorsal protein that establishes a nuclear ventral-to-dorsal gradient that is altered or absent in dorsalized or ventralized embryos, thus probably determining cell fate along the dorsal-ventral...
axis (20). We therefore have proposed that EH-containing proteins are involved in the regulation of intracellular sorting processes (4).

The elucidation of the function(s) of the EH domain and of EH-bearing proteins will require the biochemical and biological characterization of several of them. The present studies were undertaken to identify the binding abilities and biochemical features of the eps15R gene product.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—NIH-3T3 cells and NIH-3T3 cells overexpressing EGFR (NIH-EGFR, Ref. 21) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) plus 10% calf serum. When indicated cells were serum-starved for 24 h or for the indicated lengths of time, and EGFR (Upstate Biotechnology) treatment was performed for the indicated periods at 37 °C at a concentration of 0.17–17 nM (1–100 ng/ml). Human tumor cell lines of epithelial and mesodermal derivation were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Human tumor cell lines of hematopoietic derivation were propagated in RPMI plus 10% fetal calf serum.

**Immunoprecipitations, Immunoblotting, and In Vitro Protein Studies**—Immunoprecipitation, immunoblotting, and in vitro immunoprecipitation experiments were performed as described previously (1, 22). Typically, we employed 50–100 μg of total cellular proteins for direct immunoblot analysis and 3–5 μg of total cellular proteins for immunoprecipitation/immunoblotting experiments. Immunoblots were decorated with the appropriate primary antibody (see below), and detection was with horseradish peroxidase conjugated with specific secondary antisera followed by enhanced chemiluminescence reagents.

For co-immunoprecipitation studies cells were lysed with a buffer containing 1% Triton X-100 (Pierce), 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 5 mM EGTA, protease inhibitors (4 μM phenylmethylsulfonyl fluoride and 100 μg/ml aprotinin), and phosphatase inhibitors (10 mM sodium orthovanadate and 20 mM sodium pyrophosphate); cell lysates were used immediately, without freeze/thaw/thawing. Immunoprecipitations and co-immunoprecipitations were performed for 1 h, and immune complexes were recovered by adsorption to Gamma Bind G-agarose (for monoclonal antibodies, Pharmacia Biotech Inc.) or to Protein A-agarose (for polyclonal antibodies, Pharmacia).

In _in vitro_ binding experiments, 10–20 μg of GST fusions proteins or wild type GST were incubated with 1–3 mg of total cellular proteins for 1 h at 4 °C and subsequently with agarose-bound glutathione, followed by immunoprecipitation with the monoclonal antibody, as described (1, 22). In the experiment shown in Fig. 7C, _in vitro_ bindings were performed on [35S]methionine-labeled proteins (30 × 10⁶ cpm/lane).

Antibodies used were as follows: (i) two polyclonal sera (numbers 1 and 2), specific for the eps15R gene product and generated against the region amino acids 216–266, Fig. 1A, expressed as a GST fusion protein. The recombinant fusion protein was used to immunize two different rabbits. Sera were purified by affinity chromatography onto the GST-eps15R immunogen co-valently conjugated to amino- linker (Fig. 1A). Except where expressly indicated, affinity purified anti-eps15R sera were employed as follows: (ii) an anti-eps15 polyclonal serum generated against the full-length eps15 protein expressed in bacteria in a GST fusion background (1, 22); (iii) a commercial anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology); (iv) four anti-adaptins antibodies: the α-31 anti-α-adaptin polyclonal serum generated against the ear of rat α-adaptin residues 635–938 and specific for α-adaptin (25), a monoclonal anti-α-adaptin recognizing both αA and αC adaptins (Sigma), a monoclonal AP-6 (provided by ATCC) specific for both αA and αC adaptins, and a monoclonal anti-γ-adaptin recognizing only human γ-adaptin (Sigma); (v) commercial anti-phospholipase C-γ, anti-SP1, and anti-H-RAS antibodies (Santa Cruz Biotechnology); and (vi) the E7 anti-EGFR peptide serum (26).

**Transfections and in Vitro Transcription/Translation Studies**—The eps15R cDNA, under the transcriptional control of a Moloney murine leukemia virus-long terminal repeat (pCEV-eps15R, Ref. 3), was transfected into NIH-3T3 cells by the calcium phosphate precipitation method (27).

[35S]Methionine-labeled eps15R protein was synthesized by _in vitro_ transcription/translation, using a commercial kit (Promega) and the linearized pCEV-eps15R.

**Subcellular Fractionation**—NIH-EGFR cells, in logarithmic phase of growth, were washed three times with cold phosphate-buffered saline containing 0.5 μM sodium orthovanadate and scraped into a hypotonic lysis buffer, HBL (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, 10 mM NaF). Cells were incubated on ice for 10 min and Dounce-homogenized (40 strokes) with a tight-fitting pestle. The homogenate was then pelleted at the total pellet and then subjected to high speed centrifugation (375 × g) for 5 min. The pellet (nuclear fraction) was further washed for five times with HBL containing 0.1% Nonidet P-40 to remove membrane and/or cytosolic contamination. The soluble, post-nuclear fraction was centrifuged again at low speed for 10 min to remove any nuclear contamination and then subjected to high speed centrifugation (150,000 × g) for 30 min. The pellet (membrane fraction) was washed with HBL and centrifuged again to recover the final membrane fraction. The supernatant (cytosolic fraction) was centrifuged again at high speed to remove membrane contamination, and the supernatant was defined as the cytosolic fraction. All fractions were adjusted to 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 3 mM MgCl₂, 0.5% deoxycholate, 0.1% SDS, 0.2% Nonidet P-40, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 50 mM NaF and then centrifuged at 12,000 × g for 15 min to remove insoluble material. All procedures were performed on ice. Each fraction was assayed for the presence of eps15 and phospholipase Cγ as cytosolic markers, SP1 as nuclear marker, Hα-Ras and EGFR as membrane markers.

**Calculation of Stoichiometry of In Vivo Interaction between eps15 and eps15R**—One hundred and 550 μg of total cellular proteins were immunoprecipitated with the anti-eps15 and the anti-eps15R number 2 sera, respectively (see Fig. 5A), and detected in Immunoblot with the anti-eps15 antibody. On the same gel, increasing amounts (10, 30, 45, and 53 μg) of total cellular proteins were also loaded, to serve as a reference. In a parallel experiment, 850 and 400 μg of total cellular proteins were immunoprecipitated with the anti-eps15 and the anti-eps15R sera, respectively (see Fig. 5B), and detected in immunoblot with the anti-eps15R serum. On the same gel, increasing amounts (10, 15, and 20 μg) of total cellular proteins were also loaded, to serve as a reference. Autoradiographic signals were quantitated in a PhosphorImager screen connected by harvest laser and a Chemi-Imager machine background determined on an equal scale of the autoradiogram displaying no specific signals. To estimate the fraction of eps15 co-immunoprecipitating with eps15, we compared densitometric readings of the total pool of eps15 in cells (assessed by direct immunoblot with anti-eps15 antibody, see Fig. 5A), the percentage of the eps15 pool that co-immunoprecipitated with eps15R, and the efficiency of immunoprecipitation shown. The reciprocal experiment was performed to estimate the amounts of eps15R co-immunoprecipitating with eps15. Details of the densitometric readings are given in the legend to Fig. 5.

**Immunofluorescence Staining**—Cells grown on coverslips were fixed with freshly prepared 3.7% formaldehyde for 12 min at room temperature or 30 min on ice and permeabilized with Ca²⁺/Mg²⁺-free phosphate buffered saline containing 0.1% Triton X-100, 1% bovine serum albumin for 5 min at room temperature. Coverslips were then incubated for 1 h with primary antibody (mouse monoclonal X-22 or AP.6.8 and affinity purified anti-eps15R number 1 preclared by centrifugation at 100,000 × g for 10 min), washed extensively, and then incubated with secondary anti-mouse IgG and anti-rabbit IgG labeled with Texas Red or fluorescein (Jackson Technology) in the same buffer at room temperature. Coverslips were mounted in Fluoromount (Fisher) containing 1 mg/ml para-phenylenediamine. A Nikon Diaphot 300 microscope equipped with 100 × objective lens and the single fluorochrome filter sets for either Texas Red or fluorescein were used for visualization and recording the images.

**RESULTS**

Identification of the Product(s) of the eps15R Gene—To characterize the eps15R protein(s), we initially generated polyclonal sera specific for the predicted product of the eps15R gene. As shown in Fig. 1A, the predicted sequences of murine eps15 and eps15R bear significant homology and co-linearity (1, 3, 4). However, a search of 51 amino acids, between the second and the third EH domain of eps15R (amino acid positions 216 to 266, Fig. 1A), is unique to this protein and not present in eps15. We engineered a GST fusion protein containing this region of eps15R and used it as an antigen to generate polyclonal sera, which were then affinity purified on the
immobilized antigen. As shown in Fig. 1B, two independently raised sera (indicated as #1 and #2) recognized in NIH-3T3 cells at least three major bands of 125, 108, and 76 kDa, respectively (lane LYS). In a panel of human cell lines (Fig. 1C), the anti-eps15R serum ubiquitously recognized bands in the same molecular weight range, although putative eps15R products frequently appeared as doublets or triplets, a phenomenon possibly due to cell cycle-specific post-translational modifications (see below).

The specificity of recognition of the 125-, 108-, and 76-kDa bands was confirmed in experiments in which total cell lysates from NIH-3T3 cells were first immunoprecipitated with anti-eps15R sera (from two different rabbits, indicated as #1 and #2) and then blotted with the same sera. As shown in Fig. 1B, the three major bands of 125, 108, and 76 kDa were readily detectable in anti-eps15R immunoprecipitates but not in immunoprecipitates obtained with preimmune sera from the same animals (Pre #1 and Pre #2). To exclude cross-reactivity with the related eps15 protein, we compared in direct immunoblot the bands recognized by the anti-eps15R serum with those recognized by a specific anti-eps15 serum. The anti-eps15 serum recognized a major band of ~150 kDa and a weaker doublet of ~125 kDa, as described previously (1). However, in the side-by-side comparison, none of the bands recognized by the anti-eps15 serum co-migrated with those recognized by anti-eps15R (Fig. 1D). In particular, the 125-kDa eps15R-specific band migrated slightly faster than the ~125-kDa band recognized by anti-eps15R (Fig. 1D).

The 125-kDa Species Is a Bona Fide Product of the eps15R Gene—The predicted molecular mass of eps15R is 99 kDa; none of the anti-eps15R-reactive bands matched this size. To investigate the nature of the discrepancy between the electrophoretic mobility of the eps15R products and the predicted molecular weight, we performed in vitro transcription/translation (TnT) of the eps15R cDNA, in parallel to the eps15 cDNA.

As shown in Fig. 2A (right panel), the 35S-labeled product of the eps15R TnT contained a major species of ~125 kDa, in addition to several other smaller products. We interpreted the 125-kDa band as the primary translational product of the eps15R cDNA, and the smaller bands as degradation products or as the results of the translation of incompletely elongated mRNAs. The 125-kDa band was specifically and quantitatively immunoprecipitated by the anti-eps15R serum, but not by anti-eps15 (Fig. 2A, right panel) and co-migrated with authentic p125eps15R present in cell lysates (not shown). Of note, eps15 obtained by TnT of the eps15 cDNA was not immunoprecipitated by the anti-eps15R serum (Fig. 2A, left panel), thus further confirming the specificity of this reagent.

The eps15R cDNA, under the transcriptional control of a Moloney murine leukemia virus-long terminal repeat (pCEV-eps15R, Ref. 3) was then transfected into NIH-3T3 cells. Under these conditions, only the 125-kDa species was overexpressed when proteins from the transfectant were compared with those of mock-transfected NIH-3T3 cells (Fig. 2B). Thus the sum of our results indicates that p125 is a bona fide product of the eps15R gene, and it is encoded by the full-length cDNA previously isolated and described (3). The aberrant migration of p125eps15R is also reminiscent of that of p150eps15 that has a calculated mass of 98 kDa (1). In both cases, altered migration appears to be due to the C-terminal portions of the...
proteins (data not shown), possibly because of their high proline content.

We do not have formal proof that p108 and p76 are products of the eps15R gene. However, as will become subsequently clear, they share all tested biochemical properties with p125\(^{\text{eps15R}}\) and p76\(^{\text{eps15R}}\), thus the possibility that they also originate from the eps15R locus is very likely.

The p125\(^{\text{eps15R}}\) tyrosine-phosphorylated following EGF stimulation and is physically associated with other Tyr(P)-containing proteins—Eps15R was originally identified as a substrate for the EGFR. We thus tested whether eps15R shares the same property. To this end we treated NIH-EGFR cells, overexpression of the EGFR. We have previously demonstrated that in NIH-3T3 cells at a concentration of 1 \(\mu\)g, as indicated under “Experimental Procedures.” Mass populations of cells transfected with pCEV (NO) or pCEV-eps15R (eps15R) were then obtained by selection in 22, 16, and 9 a.u. for p125\(^{\text{eps15R}}\), p108\(^{\text{eps15R}}\), and p76\(^{\text{eps15R}}\), respectively; eps15R-transfected cells (eps15R lane), 22, 16, and 18 a.u. for p125\(^{\text{eps15R}}\), p108\(^{\text{eps15R}}\), and p76\(^{\text{eps15R}}\), respectively.

Since no Tyr(P) content could be demonstrated in p108\(^{\text{eps15R}}\) and p76\(^{\text{eps15R}}\) (Fig. 3A), we concluded that these two eps15R isoforms associate in vivo with other Tyr(P)-containing proteins. The nature of these Tyr(P)-containing proteins remains to be established.

We next analyzed whether p125\(^{\text{eps15R}}\) is a physiological substrate in the EGFR-activated pathway. To do this, we employed conditions of EGF stimulation able to elicit a sizable mitogenic signal but under which only a few thousand EGFRs were stimulated in NIH-EGFR cells. We have previously demonstrated that in NIH-EGFR cells half-maximal mitogenic stimulation is obtained at an EGF concentration of 0.1–0.2 nM (1 ng/ml EGF). At this concentration around 50% of the high affinity EGF-binding sites would be occupied at equilibrium (22). We estimated that in NIH-EGFR cells, this would correspond to less than 10,000 receptors (22). NIH-EGFR cells stimulated with 0.17 nM EGF displayed readily detectable p125\(^{\text{eps15R}}\) phosphorylation (Fig. 3D). Thus, p125\(^{\text{eps15R}}\) is phosphorylated following activation of the EGFR kinase in vivo, at physiological levels of receptor activation.

In Vivo Association between eps15R and eps15—We have previously shown that eps15 is present as a dimer/oligomer in vivo and that this property is determined by the coiled-coil region of eps15 that serves as a dimerization interface (28). It previously shown that eps15 is present as a dimer/oligomer in vivo and that this property is determined by the coiled-coil region of eps15 that serves as a dimerization interface (28). It was of interest, therefore, to establish whether eps15R products can participate to the formation of oligomers with eps15. We elected to approach this problem by showing co-immunoprecipitation between eps15 and eps15R. To this end, lysates of NIH-3T3 cells, obtained in mild lysis conditions to preserve protein-protein interactions were subjected to immunoprecipitation with anti-eps15R followed by immunoblot with eps15R and vice versa. The results are shown in Fig. 4, A and B, respectively. We could demonstrate that eps15 was immunoprecipitated by anti-eps15R sera, and vice versa, but not by the respective preimmune sera. Since the antibodies employed are specific and do not show cross-reaction with the related species (Figs. 1D and 2A), our results strongly suggest complexing between eps15 and eps15R in vivo. Of note (Fig. 4B), all three eps15R isoforms were co-immunoprecipitated by the anti-eps15R serum.

We then estimated the stoichiometry of interaction between eps15 and eps15R. To this end, lysates of NIH-3T3 cells, ob-
tained under mild conditions of lysis to preserve protein-protein interactions, were immunoprecipitated with the anti-eps15R serum and analyzed in immunoblot with anti-Tyr(P). The lane Lysate shows the position of the eps15R products and was obtained by loading 50 μg of total cellular proteins on the same gel and staining it separately with the anti-eps15R serum. Molecular mass markers are indicated in kDa. The position of p125 is indicated by an arrowhead and lettering.

**Fig. 3. Tyrosine phosphorylation of eps15R product(s).** A, kinetic of tyrosine phosphorylation following EGFR activation. NIH-EGFR cells were either mock-treated (○) or treated with EGF at either 10 or 100 ng/ml (1.7 and 17 nM, respectively), for the indicated lengths of time at 37 °C. Total cellular proteins (2 mg) were immunoprecipitated with the anti-eps15R serum and analyzed in immunoblot with anti-Tyr(P). The lane Lysate shows the position of the eps15R products and was obtained by loading 50 μg of total cellular proteins on the same gel and staining it separately with the anti-eps15R serum.

B, quantitative analysis. Densitometric readings of the blot in A were obtained and converted into arbitrary units. Data are plotted as % of the maximum tyrosine phosphorylation of eps15R that was achieved for a treatment with 100 ng/ml EGF for 5 min. ●, tyrosine phosphorylation of p125 at 17 nM EGF; ○, tyrosine phosphorylation of p125 at 1.7 nM EGF. C, co-immunoprecipitation of eps15R gene products with other Tyr(P)-containing proteins. NIH-EGFR cells were either mock-treated (●) or treated with EGF at 100 ng/ml for 5 min at 37 °C (+). Total cellular proteins (2 mg) were immunoprecipitated with the either the anti-eps15R serum (eps15R lanes) or with an anti-Tyr(P) monoclonal (Tyr(P)) or with an irrelevant monoclonal antibody (control) and analyzed in immunoblot with the anti-eps15R serum. Molecular mass markers are indicated in kDa. The positions of p125, p108, and p76 are indicated by arrowheads and lettering.

D, tyrosine phosphorylation of eps15R under physiological conditions of EGFR activation. NIH-EGFR cells were either mock-treated (○) or treated with EGF at either 1 or 10 ng/ml (0.17 and 1.7 nM, respectively), for 5 min at 37 °C. Total cellular proteins (2 mg) were immunoprecipitated with the anti-eps15R serum and analyzed in immunoblot with anti-Tyr(P). The lane Lysate shows the position of the eps15R products and was obtained as in A. The position of p125 is indicated by an arrowhead and lettering.

The reciprocal experiment, i.e. immunoprecipitation with anti-eps15 followed by immunoblot with anti-eps15R, yielded similar results, indicating an upper value of 22% of association of the eps15R pool with eps15 (Fig. 5, A and B).
The rodent protein C-terminal region of eps15R was able to bind to AP-2 interaction between AP-1 and eps15R, since the available anti-eps15R antibodies allowed us to test the interaction and the Golgi-specific adapter AP-1 (8–10). The availability of eps15R and the clathrin adapter complexes AP-1 (8–10) were performed, we recovered an additional C- adaptin-ear fusion protein (Fig. 6C). It should be noted, however, that recovery of p76eps15R appeared lower than that of the other isoforms (Fig. 6C, compare the signal in direct immunoblot versus that obtained in the in vitro binding). Evidence supports the notion that p108eps15R and p76eps15R are the products of alternative splicing occurring in the region encoding the C terminus of the proteins. In addition we have shown that binding of the C terminus of eps15 to α-adaptin depends on the additive influence of three short amino acid determinants (10). Thus the weaker interaction of p76eps15R with the α-adaptin ear might be due to lack of one or more binding determinants in this putatively alternatively spliced isoform.

Binding Properties of eps15R Products Mediated by Their EH Domains—One of the most interesting structural features of eps15R is the presence of three EH domains in its N terminus (Refs. 1, 3, and 4, and Fig. 1A). We have previously demonstrated that the EH domains of eps15 and eps15R bind to phage displayed peptides containing the amino acid motif NPF (asparagine-proline-phenylalanine) (4). In addition we identified, by direct screening of prokaryotic expression libraries, a number of NPF-containing proteins that bind to the EH domains of eps15. These proteins include the human homologue of the Drosophila NUMB protein (h-NUMB, Refs. 15–17), a numb related protein (h-NUMB-R, Ref. 4), the cellular co-factor of the HIV REV protein, RAB (h-RAB, Refs. 29 and 30), and a RAB-related gene (h-RAB-R, Ref. 4). Furthermore, three partial clones encoding for anonymous proteins, designated ehb3, ehb10, and ehb21 (for eh-binding), were also identified (4). It was therefore of interest to test whether the binding properties of eps15R overlap those of eps15 or are distinct.

We employed GST fusion proteins encoding the mentioned EH-binding proteins to perform in vitro binding experiments. As shown in Fig. 7A, GST-RAB and GST-RAB-R, but not GST-NUMB, GST-NUMB-R, GST-ehb3, GST-ehb10, and GST-ehb21, were able to bind eps15R products. All of the above GST fusions were able to interact with eps15 (Ref. 4, see also Fig. 7A for selected positive controls). We also employed GST fusion proteins containing the three EH domains of eps15 and eps15R (GST-eps15EH and GST-eps15REH, respectively) to perform in vitro binding experiment on [35S]methionine-labeled extracts from NIH-3T3 cells. As shown in Fig. 7B, the binding patterns of the two fusion proteins were partially distinct,

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![Fig. 4. In vivo interaction between eps15 and eps15R. A, lysates from NIH-3T3 cells were either immunoprecipitated (1 μg) with an anti-eps15R serum or with the corresponding preimmune serum (PRE), or loaded directly (100 μg) onto the gel (lane IP −). Detection in immunoblot was with the anti-eps15 antibody. B, lysates from NIH-3T3 cells were either immunoprecipitated (1 μg) with an anti-eps15 serum of with the corresponding preimmune serum (PRE), or loaded directly (100 μg) onto the gel (lane IP −). Detection in immunoblot was with the anti-eps15R antibody. Molecular mass markers are indicated in kDa.](http://www.jbc.org/content/308/10/3088/F2.large.jpg)
Subcellular Localization of eps15R Product(s)—We next determined the subcellular localization of the eps15R products. To this end, NIH-EGFR lysates were fractionated into cytosolic (C), nuclear (N), and membrane (M) fractions, as described under “Experimental Procedures.” Aliquots of each fraction, along with an aliquot of the total cellular proteins (T), representative of the same number of cells, were then analyzed in immunoblot with the anti-eps15R serum. As shown in Fig. 8, eps15R product(s) were localized mainly to the soluble (C) fraction. However, a significant amount of eps15R products was also present in the nuclear fraction, representing ~5–10% of the total eps15R pool. Detection of eps15R in the nuclear fraction was not due to contamination by other fractions. This was demonstrated by staining identical immunoblots with specific soluble (eps15 and phospholipase C-γ) membrane (EGFR and Ras) or nuclear (SP1) fraction markers, which showed near purity of the subcellular fractions (Fig. 8).

The subcellular localization of eps15R was further analyzed by immunofluorescence experiments. It has been previously demonstrated that eps15 is located in clathrin-coated pits (7). To study whether eps15R is also located in coated pits, double immunofluorescence staining of NIH-3T3 cells with Eps15 and AP-2 and clathrin antibodies was performed. Fig. 9 shows that Eps15R staining produced a punctuated pattern that is typical of plasma membrane clathrin-coated pits. Most dots corre-
In this paper we report the biochemical characterization of eps15R, an intracellular signal transducer previously identified by virtue of its homology to eps15, an EGF receptor kinase substrate (4–6). We identified at least three proteins, in murine fibroblasts, which showed specific cross-reactivity with two independently raised anti-eps15R sera: p125, p108, and p76. By Tnt experiments and ectopic expression in mouse fibroblasts, we demonstrated that p125 is a bona fide product of the eps15R gene. The nature of the other bands remains to be clarified; they can represent degradation fragments, products of alternatively spliced mRNAs, or specifically cross-reacting proteins. It is, however, unlikely that p108 and p76 are degradation products originating from p125, since their levels do not increase under conditions in which p125 levels are artificially augmented, as obtained by transfection of the eps15R cDNA.

Of the remaining possibilities, we favor origin from alternatively spliced mRNAs. This is based on the fact that p108 and p76 share several biochemical properties with p125 including the production of EH-containing proteins, which specifically recognized by GST-fusion proteins containing either the three EH domains of eps15R or of eps15 (GST-eps15REH and GST-eps15EH, respectively) or with control GST. Bands specifically recognized by GST-eps15REH are indicated by arrowheads. Molecular mass markers are indicated in kDa.

FIG. 6. Interaction of eps15R with the AP-2 complex. A, co-immunoprecipitation of eps15R with α-adaptin. Total cellular lysates from NIH-3T3 cells were obtained in mild lysis conditions and either loaded directly on the gel (100 µg, lane −) or immunoprecipitated (IP) (2 µg) with anti-eps15 (eps15), anti-eps15R (eps15R), preimmune (PRE), or anti-α-adaptin (AP-2) sera. Detection in immunoblot was with an anti-α-adaptin serum. The bloting antibody was monoclonal anti-α-adaptin recognizing both αA and αC adaptins (Sigma). The anti-α-adaptin used in immunoprecipitation experiments was the α-31 serum specific for αC-adaptin. The positions of αA and αC are indicated by arrowheads and lettering. B, co-immunoprecipitation of eps15R with γ-adaptin. Total cellular lysates from NIH-3T3 cells were either loaded directly on the gel (100 µg, lane −) or immunoprecipitated (2 µg) with anti-eps15R (eps15R), preimmune (PRE), or anti-γ-adaptin (AP-1) sera. Detection in immunoblot was with an anti-γ-adaptin serum. C, in vitro binding. A GST fusion protein containing the ear of α-adaptin (α-ear), or GST, were challenged with 1 mg of total cellular lysates from NIH-3T3 cells, followed by immunoblot detection with the anti-eps15R serum. The lane Lysate was loaded with 50 µg of total cellular proteins. Molecular mass markers are indicated in kDa.

FIG. 7. In vitro binding of NPF-containing proteins to eps15R. A, in vitro binding of eps15R to GST fusions of EH-binding proteins. Total cellular lysates from NIH-3T3 cells (1 mg/lane) were incubated with the indicated GST fusion proteins (10 µg) for 1 h at 4 °C. Specifically bound eps15R was detected by immunoblot with either an anti-eps15R or an anti-eps15-serum. Lanes marked − were loaded with 50 µg of total cellular lysate to serve as reference for the positions of eps15R and eps15 products (also indicated by arrowheads). B, in vitro binding. Thirty million trichloracetic acid-precipitable cpm of [3H]methionine-labeled total protein from NIH-3T3 cells were used in each lane. Labeled proteins were incubated with GST fusion proteins containing either the three EH domains of eps15R or of eps15 (GST-eps15REH and GST-eps15EH, respectively) or with control GST. Bands specifically recognized by GST-eps15REH are indicated by arrowheads. Molecular mass markers are indicated in kDa.
whether the eps15 and eps15R complexes are heterodimers (i.e. exists with eps15 or eps15R or whether a hierarchy of interactions not know whether all binders can simultaneously associate homodimers (28) and eps15R exist in the cell as a mixture of eps15/eps15 and eps15R, and their overlapping biochemical properties, the question arises as to the putative function of an eps15-eps15R complex. A possibility is that it serves a role in bringing together different proteins specifically recruited by the EH domains of the two components of the complex. Indeed, in this study, we provide evidence for a certain degree of specificity in the binding properties of the EH domains of eps15 and eps15R. Thus eps15R-eps15 complexes could juxtapose EH-binding proteins that could not be brought together by eps15/eps15 or eps15R/eps15R homodimers.

This possibility is further suggested by our finding that eps15 and eps15R exist in the cell as a mixture of eps15/eps15 homodimers (28) and eps15R-eps15 complexes. We do not know whether the eps15-eps15R complexes are heterodimers (i.e. whether they interact directly) or whether they both interact with a common binding partner. In addition, although the presence of eps15R/eps15R homodimers has not been ascertained yet, one can speculate, by analogy, that they also exist. Given the extensive co-linearity and homology between eps15 and eps15R, and their overlapping biochemical properties, the question arises as to the putative function of an eps15-eps15R complex. A possibility is that it serves a role in bringing together different proteins specifically recruited by their EH domains (Refs. 3 and 4 and this paper), by their heptad-containing coiled-coil regions (28), and by their C-terminal domains, which have been shown to bind the SH3 domain of crk in vitro (6), in addition to a-adaptin. All these interactions cannot immediately be reconciled with an exclusive role in endocytosis. We do not know whether all binders can simultaneously associate with eps15 or eps15R or whether a hierarchy of interactions exists in vivo. If this were the case, eps15 and eps15R may function as docking proteins capable of facilitating macromolecular assembly of intracellular proteins.

FIG. 8. Subcellular localization of the eps15R gene product(s). NIH-EGFR cells in logarithmic phase of growth were harvested, and subcellular fractions were obtained as described under "Experimental Procedures" and are identified as C (cytosolic fraction), N (nuclear fraction), and M (membrane fraction). Aliquots of each fraction or of the total cellular lysate (T), representative of the same number of cells (5 x 10⁶), were then analyzed by immunoblot for the presence of eps15R, eps15, phospholipase C-γ (PLC-γ), SP1, H-ras, and EGFR.

abilities which include those mediated by their EH domains (Refs. 3 and 4 and this paper), by their heptad-containing coiled-coil regions (28), and by their C-terminal domains, which have been shown to bind the SH3 domain of crk in vitro (6), in addition to a-adaptin. All these interactions cannot immediately be reconciled with an exclusive role in endocytosis. We do not know whether all binders can simultaneously associate with eps15 or eps15R or whether a hierarchy of interactions exists in vivo. If this were the case, eps15 and eps15R may function as docking proteins capable of facilitating macromolecular assembly of intracellular proteins.

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One more line of evidence suggests additional biological roles for eps15 family members. During mouse embryogenesis, eps15 shows a restricted pattern of expression, at variance with its ubiquitous expression in adults (2), being expressed at E10 only in the liver primordium and in ganglia derived from the hindbrain neural crest, and at E12.5 also all along the central nervous system, in the ventricular zone where neuroblasts are located (31). The lack of expression of eps15 in other actively metabolizing and dividing tissues argues against an exclusive role in receptor-mediated endocytosis, since formation of coated pits is sustained in all developing tissues, regardless of the pattern of expression of eps15. Indeed at E12.5 the expression of eps15 seems to correlate better with that of NUMB (32), which is a target protein for its EH domain (4), being both present mainly in the periventricular undifferentiated neuroblasts but not in the differentiated neurons that migrate radially to reach their final cortical location (32, 33). Thus, in this particular setting eps15 might be involved in the asymmetric partitioning of NUMB at mitosis, through EH-mediated interactions (4). Indeed, the presence of EH domains in eps15 family members might have a prominent role in determining biological functions. The characteristics of EH-containing and EH-binding proteins have, in fact, prompted the hypothesis that the EH network is implicated in the regulation of protein transport, protein sorting, and organization of subcellular structures (4).

Comparative analysis of cellular localization uncovered an important difference between eps15 and eps15R. Both proteins co-localized with clathrin and AP-2 in coated pits (Ref. 7 and

FIG. 9. Subcellular co-localization of eps15R with AP-2 and clathrin. Formaldehyde-fixed NIH-3T3 cells were processed for double label immunofluorescence microscopy using the affinity purified anti-eps15R serum eps15R and the mouse monoclonal antibodies AP.6 (directed against the α-subunit of AP-2, α-adaptin) (a and b) or X-22 (directed against the clathrin heavy chain) (c and d). Rabbit and mouse primary antibodies were detected with corresponding secondary IgGs labeled with fluorescein (eps15R) or Texas Red (AP-2 or clathrin). a, anti-eps15R staining (fluorescein) in double labeling experiments for eps15R and α-adaptin, b, anti-α-adaptin staining (Texas Red) in double labeling experiments for eps15R and α-adaptin, c, anti-eps15R staining (fluorescein) in double labeling experiments for eps15R and clathrin, d, anti-clathrin staining (Texas Red) in double labeling experiments for eps15R and clathrin. Arrowheads point to examples of co-localization of the punctuated patterns of eps15R (a and c) and AP-2 (b) or clathrin (d) immunoreactivity.
Eps15R Protein

This association is not very strong, since both eps15 and eps15R partition in the cytosolic subcellular fraction, due to their release from the plasma membrane during the homogenization procedures (Ref. 1 and this paper). However, a significant fraction of eps15R can be found in the nucleus, both by immunofluorescence and biochemical subcellular fractionation. The nuclear localization of eps15R strengthens the hypothesis of multiple biological functions for eps15 family members. In particular, we predict that RAB, the cellular co-factor of the HIV-1 Rev protein, is an EH interactor that binds to eps15R and exhibits structural and topographical features compatible with those of a nucleoporin (29, 30). Thus it will be of interest to check whether eps15R, eps15, or other EH-containing proteins can mediate the function of RAB.

Whatever the pleiotropic functions of eps15 family members might be, they appear to be regulated in vivo, by different post-translational modifications. Both eps15 and eps15R are tyrosine-phosphorylated in vivo following activation of the EGFR kinase and appear to be physiological substrates in the EGFR-activated pathway (Ref. 1 and this paper). Moreover, eps15 appears to be mono-ubiquitinated in cells stimulated with EGF (34). Eps15R also seems to be subjected to post-translational modifications, in addition to tyrosine phosphorylation, as witnessed by the fact that all isoforms of eps15R show retarded gel mobility following EGFR activation (Fig. 3C). In the case of p125eps15R this could be possibly due to tyrosine phosphorylation. However, in the cases of p108eps15R and p76eps15R, other post-translational modifications, whose nature is not yet known, must account for it. Finally, both eps15 and eps15R are serine/threonine phosphorylated in vivo. The relevance of these modifications is still obscure. None of the binding functions of eps15 and eps15R are modulated by tyrosine phosphorylation, since they occur constitutively in vivo and can be reproduced with recombinantly expressed proteins in vitro. We are currently mapping tyrosine and serine/threonine phosphorylation sites on eps15 and eps15R. This knowledge, coupled to the development of bioassays for these proteins, as for instance modulation of NUMB or RAB activity, or influence on receptor-mediated endocytosis, should help in shedding light on the function(s) and biological role(s) of eps15 family members.

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Eps15R Is a Tyrosine Kinase Substrate with Characteristics of a Docking Protein Possibly Involved in Coated Pits-mediated Internalization
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