Differential Nucleocytoplasmic Trafficking between the Related Endocytic Proteins Eps15 and Eps15R*

Viviane Poupon‡§, Simona Polo¶, Manuela Vecchiţ, Gwendal Martin, Alice Dautry-Varsat¶, Nadine Cerf-Bensussan†, Pier Paolo Di Fiore¶¶, and Alexandre Benmerah$$$ From the ‡INSERM E9925, Faculté Necker-Enfants Malades, 156 rue de Vaugirard, 75730 Paris Cedex 15, France, §Unité de Biologie des Interactions Cellulaires, URA-CNRS 1960, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France, the ¶Department of Experimental Oncology, European Institute of Oncology, Via Ripamonti 435, 20141, Milan, Italy, the §§University of Milan, Medical School, 20122, Milan, Italy, and $$$The FIRC, Institute for Molecular Oncology, 20134 Milan, Italy

Eps15 and Eps15R are constitutive components of clathrin-coated pits that are required for clathrin-dependent endocytosis. The most striking difference between these two related proteins is that Eps15R is also found in the nucleus, whereas Eps15 is excluded from this compartment at steady state. To better understand the individual functions of these two proteins, the mechanisms responsible for their different localization were investigated. Interestingly, some mutants of Eps15 were found in the nucleus. This nuclear localization was correlated with the loss of the last ~100 amino acids of Eps15, suggesting the presence of a nuclear export signal (NES) within this region. As expected, the last 25 amino acids contain a leucine-rich sequence matching with classical NESs, show a leptomycin B-sensitive nuclear export activity, and bind to the exportin CRM1 in a leucine residue-dependent manner. In contrast, no NES could be found in Eps15R, a result in keeping with its constitutive nuclear localization that appears to be regulated by alternative splicing. Altogether, these results are the first characterization of nucleocytoplasmic shuttling signals for endocytic proteins. They also provide an explanation for the different nuclear localization of Eps15 and Eps15R and further evidence for a possible nuclear function for Eps15 protein family members.

Formation of clathrin-coated vesicles from plasma membrane clathrin-coated pits (CCPs) is the initial step of the major pathway for receptor-mediated endocytosis. The role of clathrin, AP-2 complexes, and dynamin in this process is now well delineated (1–5). Over the past few years, several new CCP-associated proteins were identified, including amphiphysins, epsins, and proteins containing EH (Eps15-Homology) domains, mainly Eps15 and intersectin/ese/dap160, all required for clathrin-dependent endocytosis (6–12).

Eps15 was the first identified member of the EH-domain-containing proteins found in CCPs (13, 14). Its function in clathrin-dependent endocytosis seems to be restricted to the early events leading to CCP formation. On the other hand, Eps15 is not found in clathrin-coated vesicles (15). On the other, Eps15 mutants inhibit CCP assembly (16, 17). Eps15 is composed of three structural domains (18, 19). The N-terminal (DI) domain contains three copies of the evolutionary conserved EH domain (20), a protein-protein binding module that recognizes NPF (asparagine-proline-phenylalanine) motifs (21) present in several proteins including the epsins (8, 9) and Hrb/RIP/RAB (21), a protein involved in the nuclear export of the HIV Rev protein (22–24). The central domain (DII) is involved in the oligomerization of Eps15 (25, 26). The C-terminal domain of Eps15 (DIII) is characterized by the presence of 15 repeats of a DPF (aspartate-proline-phenylalanine) motif. Most of these repeats are present in the AP-2 binding domain, which spans more than 100 amino acids (621–739) (11, 27) and contains at least three independent AP-2 binding sites (28).

Besides its well demonstrated involvement in clathrin-dependent endocytosis (10, 11, 16, 29), a possible nuclear function for Eps15 was suggested by recent studies showing that Eps15, like some other proteins from CCPs, is able to accumulate in the nucleus when nuclear export is inhibited (30, 31). Altogether with the results showing that Eps15 is tyrosine-phosphorylated in response to several growth factors (14, 18), these data have suggested its role in signal transduction and our most recent results indicate that Eps15 may be involved in transcription regulation (31). Interestingly, Eps15R, a protein closely related to Eps15 that shares the same structural organization (20) and the same protein binding pattern, i.e. NPF containing proteins and AP-2 complex, is constitutively found in the nucleus (32). This constitutive nuclear localization contrasts with what is observed for Eps15 that is not found in this compartment at steady state. In this study, the mechanisms responsible for such different nucleocytoplasmic trafficking were investigated.

MATERIALS AND METHODS

Cells and Antibodies—HeLa and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, penicillin, and streptomycin (Invitrogen). The mouse monoclonal anti-Eps15 and the rabbit polyclonal anti-Eps15R antibodies were described previously (32). Alexa-488 goat anti-
mouse and Alexa-594 goat anti-rabbit immunoglobulins were obtained from Molecular Probes (Eugene, OR).

Amplification and Cloning of Alternative Splice Forms—Total RNA from NIH3T3 cells were extracted using the Rneasy minikit from Qiagen according to manufacturer's instructions. The cDNA used as a template for polymerase chain reaction (PCR) was obtained by reverse transcription of mRNA from NIH3T3 cells using Superscript II reverse transcriptase and oligo(dT) from Invitrogen. DNA fragments corresponding to the C-terminal domain of Eps15R (nucleotides 1722–2748, amino acids 567–907) were amplified using High-Fidelity Taq polymerase from Invitrogen. Amplified DNA fragments were purified from agarose gel and cloned into PGEMT or PCR3.1 vectors using PCR products from Invitrogen. DNA fragments corresponding to the putative Eps15R NLS were generated as described for Eps15 (see above). Finally, to generate complete splice forms of Eps15R, the 5' part of full-length Eps15R was replaced by the corresponding BamHI/XhoI fragment from the three characterized splice forms (ERB, ERC, and ERD).

All the constructs were checked by nucleotide sequencing.

Generation of the GFP Constructs—The cDNA of human eps15 subcloned in pBluescript II KS (Stratagene, La Jolla, CA) was obtained in the laboratory (33) and used as a template to generate the different cDNA fragments used in this study. The Eps15 constructs encoding each structural domains (DI, DII, DIII), the C-terminal deleted mutant (EΔPC), and the mutant with an internal deletion of the central coiled-coil domain (EΔCC) were described previously (11, 17, 27). Briefly, the deleted mutants were obtained by PCR and introduced within Eps15 subcloned between the BamHI/XhoI sites of the PGEX5.1 vector (Amersham Biosciences, Inc.) using appropriate restriction sites. The point mutations of the putative Eps15R NES were generated as described for Eps15 (see above).

To generate the EΔ(5–30) construct, the region corresponding to the C-terminal part of the EΔCC construct was removed using ClaI (+1937) and XhoI sites and exchanged with a ClaI/XhoI fragment obtained from the EΔC construct lacking the 46 last amino acids (17). The point mutations of the putative monopartite nuclear localization signal KRK were generated by a two-step PCR. Alanine substitutions for threonine were performed using the calcium phosphate transfection kits from CLONTECH or Invitrogen. For fluorescence microscopy, transfected HeLa cells were grown on coverslips and used 1 or 2 days after transfection. When a treatment with leptomycin B (a kind gift of Dr B. Wolff, Novartis) was performed, the cells were incubated at 37°C for at least 30–60 min in Dulbecco's modified Eagle's medium-2% fetal calf serum containing 20 ng leptomycin B. The cells were then washed in cold PBS and fixed in 3.7% paraformaldehyde and 0.03% sucrose for 30 min at 4°C. The cells were then washed in PBS and mounted on microscope slides in 100 mg/ml Mowiol (Calbiochem), 25% glycerol (VV), 100 mM Tris-HCl, pH 8.5.

For immunofluorescence, HeLa cells grown on coverslips were washed in PBS and fixed as described above, then washed once in PBS, and after quenching for 10 min in 50 mM NH4Cl in PBS, washed again in PBS supplemented with 1 mg/ml bovine serum albumin. The cells were then incubated with the first antibodies in permeabilization buffer (PBS with 1 mg/ml bovine serum albumin and 0.1% Triton X-100) for 30–45 min at room temperature. After two washes in the permeabilization buffer, the first antibodies were replaced by the secondary antibodies. After two washes in permeabilization buffer and one in PBS, the cells were mounted on microscope slides as described above.

The samples were examined under an epifluorescence microscope (Axiophan II, Zeiss) attached to a cooled CCD camera (Spot-2, Diagnostic Instruments) or under a confocal microscope (LSM 510, Zeiss).

In Vitro Binding Assays—GST recombinant proteins were produced and purified as described previously (27, 31). Human CRM1 and RanQ69L mutant containing plasmids were kind gifts of M. Yoshida and Dr. J. Mattaj, respectively. In vitro binding experiments were performed as described previously (31). Briefly, human CRM1 was translated in vitro using the TnT Coupled Reticulocyte Lysate System from Promega in the presence of [35S]methionine (Amersham Biosciences, Inc.). Then, 15 μg of each GST construct were incubated with

**Fig. 1. Intracellular localization of endogenous Eps15 and Eps15R.** HeLa cells grown on coverslips were fixed, permeabilized, and processed for fluorescence microscopy using the 3T mouse monoclonal antibody against Eps15 (a) and the anti-Eps15R rabbit polyclonal antibody (b), revealed by an Alexa 488 and an Alexa 594-labeled secondary antibodies, respectively. The cells were then observed under an epifluorescence microscope attached to a cooled CCD camera. Insets show higher magnifications of representative areas. Eps15 and Eps15R colocalized in clathrin-coated pits (arrows). Eps15R, but not Eps15, shows a bright nuclear staining (arrowheads).

**Fig. 2. Nuclear localization of an Eps15 mutant.** HeLa cells transiently transfected with Eps15 (a and b) or EΔPC (c and d) GFP constructs were fixed and processed for fluorescence microscopy as described under “Material and Methods.” a and c, the focus was made on the planar plasma membrane adherent to the coverslip to show the CCP localization of the constructs. b and d, the focus was made on the central region of the cell to better observe the nucleus that was identified using phase contrast (not shown). The EΔPC construct lacks the last 131 COOH-terminal amino acids (see Fig. 8).
20 µl of in vitro translated [35S]Met-labeled hCRM1 in binding buffer (50 mM Hepes/KOH, pH 7.5, 200 mM NaCl, 5 mM (CH₃COO)₂Mg, 1% boiled fetal bovine serum, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) in the presence or absence of 2 mM GTP-loaded RanQ69L. The incubation was performed at 4 °C for 2 h. The beads were subsequently washed with binding buffer, and proteins were resolved by SDS-PAGE followed by autoradiography.

RESULTS AND DISCUSSION

Eps15 and Eps15R are constitutive components of CCPs (13, 14, 32) in which they colocalize (Fig. 1, insets). The most striking difference between the distribution of the two proteins is that Eps15R shows a bright nuclear staining, whereas Eps15 is not found in this compartment at steady state (Fig. 1, arrowheads).

Transport of large proteins (>50 kDa) through nuclear pores is mediated by import and export machineries, which, respectively, recognize NLSs and NESs. Both classical NLS and NES are short peptidic sequences, lysine-arginine-rich and leucine-rich, respectively, which are sufficient to drive nuclear import and export of carrier proteins (reviewed in Refs. 35 and 36). We recently showed that Eps15 is translocated in the nucleus in the presence of leptomycin B (LMB) (31), a drug that specifically inhibits the CRM1-dependent nuclear export pathway (37–41). This result and the fact that Eps15 is excluded from the nucleus at steady state (Figs. 1a and 6a) suggested the presence of a classical leucine-rich NES within Eps15.

To precisely map this signal, GFP-tagged Eps15 mutants were tested for their nuclear localization at steady state. The expected results was that mutants lacking the NES containing region should be allowed to accumulate in the nucleus. Addition of the GFP to the NH₂ terminus of Eps15 did not modify its intracellular localization (11, 17), the resulting construct showing a punctate staining at the plasma membrane (Fig. 2a). As shown previously, the mutant EΔP/C, deprived of the 131 last COOH-terminal amino acids (Fig. 8), is efficiently targeted to CCPs (Fig. 2c). However, in contrast to what was observed for Eps15 (Fig. 2b), this mutant was found in the nucleus with a clear pattern of nucleolar exclusion (Fig. 2d). This was in keeping with the presence of a NES in the last 131 amino acids (766–896) of Eps15 that are lacking in the EΔP/C construct. This hypothesis was supported by the fact that a leucine-rich region was found between amino acids 870 and 896, which matches well with previously characterized NESs (Fig. 3A). To assess the presence of a functional NES within this region, its nuclear

---

2 The nuclear staining obtained with the polyclonal anti-Eps15R antibody is specific, since it is lost when incubations are done in the presence of the Eps15R-derived fusion protein used as immunogen (data not shown).
Fig. 5. The activity of Eps15 NES depends on leucine and isoleucine residues. HeLa cells transiently transfected with E766-896 and the E766–896 construct, corresponding to the COOH-terminal 131 amino acids region of Eps15 fused to GFP (Fig. 8), displayed a distribution clearly different from that of GFP alone, with a cytoplasmic staining surrounding a dark area corresponding to the nucleus (Fig. 3B, panel a). Therefore, addition of the COOH-terminal domain of Eps15R and to a mutant lacking the last 50 amino acids of Eps15 and Eps15R constructs. HeLa cells transiently transfected with D3R (c) or ER747 (d) GFP constructs, corresponding to the COOH-terminal domain of Eps15R and to a mutant lacking the last 160 amino acids of Eps15R, respectively (see Fig. 8), were fixed and processed for fluorescence microscopy. e, immobilized GST-E766–896 (lanes 2 and 3), GST-E766–896 (lanes 4 and 5), GST-E766–896 (lanes 6 and 7), and GST-E766–896 (lanes 8 and 9) were incubated with in vitro translated CRM1, in the presence (lanes 2, 4, 6, and 8) or absence (lanes 3, 5, 7, and 9) of a GTP-loaded constitutively active mutant of the Ran GTPase (RanQ69L). Lane 1 represents one-fourth of the amount of CRM1 used in the assay.

export activity was further tested using GFP. GFP is a 26-kDa protein that passively diffuses through the nuclear pore and can be detected both in the cytoplasm and nucleus (Fig. 3B, panel a). The E766–896 construct, corresponding to the COOH-terminal 131 amino acids region of Eps15 fused to GFP (Fig. 8), displayed a distribution clearly different from that of GFP alone, with a cytoplasmic staining surrounding a dark area corresponding to the nucleus (Fig. 3B, panel b). Therefore, addition of the COOH-terminal domain of Eps15 encompassing amino acids 766–896 leads to nuclear exclusion of GFP. This nuclear exclusion was not simply due to an increased molecular weight of the resulting fusion protein compared with GFP alone but rather to constitutive export from the nucleus. First, in contrast with the E766–896 construct, the E766–890 construct, lacking the 6 COOH-terminal amino acids,3 was found both in the cytoplasm and nucleus, with a staining pattern similar to GFP alone (data not shown). A 6-amino acid difference was unlikely to cause differences in passive diffusion through nuclear pores sufficient to explain the difference between the two constructs. These data rather suggest that the last 6 amino acids are required for nuclear export activity (see below). Second, the presence of a functional NES was further confirmed using LMB. As shown in Fig. 3B, the E766–896 construct, normally excluded from the nucleus (b), was found both in the cytoplasm and nucleus after a 30-min incubation with LMB (c). The effect of LMB was specific in that no nuclear accumulation of GFP-DII construct, corresponding to the central domain of Eps15 (Fig. 8), was observed upon LMB treatment (d and e). Altogether, these results show the presence of a functional classical NES in the COOH-terminal part of Eps15.

The shuttling between the cytoplasm and nucleus of proteins with predominant plasma membrane localization has already been described. This is the case for β-catenin, which colocalizes with E-cadherin in adherent junctions (43), and for the α-actinin-binding protein Zyxin normally found in focal adhesion structures (44). More recently, it was shown that epsin, an Eps15-interacting protein also found in CCPs (8), is also able to undergo nucleocytoplasmic shuttling (30, 46). As observed for epsin, endogenous or GFP-tagged Eps15 was not found in the nucleus at steady state (Figs. 1a, 2a, and 6a). Treatment with LMB for up to 4 h did not lead to appreciable nuclear localization of Eps15 (data not shown). However, longer treatments (12–16 h) led to nuclear accumulation of a fraction of the total

3 The last 6 amino acids of Eps15 were chosen to be deleted, because they are not conserved in Eps15R (see Fig. 6).
pool of Eps15 (31), suggesting that nuclear entry of Eps15 is a slow and/or regulated process.

Recently, it has been shown that proteins of the Armadillo-like family, such as β-catenin or p120 catenin, are prevented from entering in the nucleus due to their binding to N- and E-cadherin at the plasma membrane (47, 48). Eps15 is constitutively complexed with AP-2 in plasma membrane CCPs (13, 14, 28, 33). To define whether this association could prevent its nuclear translocation, the nucleocytoplasmic shuttle activity of ΔAP-2, an Eps15 construct deprived of all AP-2 binding sites (Fig. 8), was analyzed. This mutant, localized in the cytoplasm at equilibrium (17), and was not detected in the nucleus even after a 30 min LMB treatments (data not shown), a condition under which no appreciable nuclear accumulation of wild type Eps15 occurs (data not shown and Ref. 31). This short LMB treatment was then chosen to maximize the effects ascribable to altered dynamics in nucleocytoplasmic trafficking of the mutants under scrutiny. Altogether, these results suggest that nuclear entry of Eps15 is not regulated through its association with AP-2 at the plasma membrane. Homo- and heterooligomerization of proteins might also regulate nucleocytoplasmic shuttling, by masking either NES or NLS (49–51). This possibility was tested using the EΔCC mutant of Eps15 that lacks the coiled-coil domain required for oligomerization (25, 26). As shown in Fig. 4, EΔCC is found in the cytoplasm but not in the nucleus at steady state (Fig. 4c), but it efficiently translocates into the nucleus upon short LMB treatment (Fig. 4b), showing its constitutive nucleocytoplasmic shuttling. The same results was found using a Myc-tagged version of the EΔCC construct (data not shown), showing that nucleocytoplasmic shuttling of ΔECC was not tag-dependent. Altogether, these data suggest that fast access of Eps15 to the nucleus is negatively regulated by its oligomerization state. This could explain why Eps15 is slowly imported into the nucleus. At steady state the great majority of Eps15 is engaged in supramolecular structures (dimers and tetramers (25)). Thus, nuclear import of Eps15 might only concern a small fraction of the Eps15 pool or even just the neosynthesized protein in a dynamic competitive process between oligomerization and nuclear traffic.

The fact that the EΔCC construct, a 100-kDa protein, is efficiently translocated in the nucleus suggests the presence of a NES or of equivalent molecular devices within Eps15. The primary sequence of Eps15 was first analyzed using the PSORT II program (52) to identify putative classical NLSs. A short basic sequence similar to monopartite NLS was found between the second and the third EH domains (amino acids 214–216). This potential NLS KRK was mutated to KRA, KAA, or AAA, and the mutations were introduced in the EΔCC context (Fig. 8). All these mutants were translocated into the nucleus upon LMB treatment, in a way indistinguishable from the EΔCC construct (data not shown), showing that nuclear accumulation of Eps15 constructs is not mediated by a classical lysine-arginine-rich NLS. Further sequence analysis using a more recent program specifically raised to identify classical lysine-rich and M9 type NLSs (53) did not reveal any of these signals in Eps15. Altogether, these results suggest that Eps15 does not contain any canonical NLS. However, some of our data indicate that the nuclear localization of Eps15 constructs depends on the NH2-terminal domain of the protein. A GFP construct encompassing the NH2-terminal domain of Eps15, DI (Fig. 8), had mainly a nuclear localization (Fig. 4c). The DI construct, which encompasses the central domain of Eps15, was only found in cytoplasm (Fig. 4d). The ΔΔA/P/C construct, which encompasses DI and DII domains (Fig. 8), showed an intracellular localization similar to the DI construct (Fig. 4e). Altogether, these data strongly suggest that the NH2-terminal EH domains are responsible for nuclear localization. This was further confirmed by the fact that the EΔEH/A/P/C construct that corresponds to the EΔA/P/C construct lacking EH domains is excluded from the nucleus (Fig. 4f). Then, nuclear import of Eps15 seems to be NLS-independent and EH domains appears to be required. An NLS-independent import pathway has already been described for β-catenin that is imported in the nucleus through its interaction with the LEF/TCF transcription factor (54, 45). An EH domain-binding protein may thus be involved in the nuclear import of Eps15, and epsin, the major partner of Eps15 EH domains (8), is an interesting candidate as it also shows constitutive nucleocytoplasmic shuttling (30, 46).

As the ΔECC construct showed fast nuclear translocation upon leptomycin B treatment, we decided to use this construct to definitively characterize Eps15 NES. We first verified that the identified NES was also functional in the EΔCC context.
Indeed, the EΔ(CC+C) construct, corresponding to EΔCC lacking the 47 last amino acids (Fig. 8), is found in the nucleus (data not shown). Leucine or isoleucine residues were shown to be required for CRM1-dependent NES activity (37–40). Three different point mutants were designed to test whether this was also the case for Eps15 NES. In these mutants, characteristic leucine and isoleucine residues were replaced by alanine in the E/H9004CC context (Fig. 5). All the resulting GFP constructs showed a clear nuclear localization compared with the wild-type construct (Fig. 5, b–d), showing that nuclear export activity of the NES was lost in all the mutants. In addition, the same mutants were tested for their ability to bind to the exportin CRM1 in the presence of an activated mutant of Ran (RanQ69L), a small GTPase that regulates the binding of CRM1 to NES-containing proteins (38). In this in vitro binding assay, Eps15 as well as EΔCC were able to precipitate CRM1 in a RanQ69L-dependent manner (Fig. 6, lane 4, and Fig. 5, lane 8, respectively). As expected, the ability to precipitate CRM1 was lost for all the NES mutants (compare Fig. 5e, lanes 2, 4, and 6–8). Altogether with the data showing that the nuclear export activity of the COOH-terminal region of Eps15 is inhibited by LMB treatment (Figs. 3 and 4), these results further demonstrate that the COOH-terminal leucine-rich region of Eps15 shows all the characteristics of a classical NES.

The presence of a NES within Eps15 is likely to explain its exclusion from the nucleus at steady state. A simple explanation for the constitutive nuclear localization of Ep15R is that it lacks a functional NES and therefore is allowed to
accumulate in this compartment. The COOH-terminal domain is the less conserved part between Eps15 and Eps15R (20) except for the last ~50 amino acids that contain the NES in Eps15 (Fig. 6). However, isoleucine 890 that is required for Eps15 NES (see above) is not conserved in Eps15R. Three different experiments were designed to test our hypothesis. First, the intracellular localization of a GFP-Eps15R full-length construct was compared with that of GFP-Eps15. As shown in Fig. 6, only GFP-Eps15R could be found in the nucleus. The nuclear staining was faint but clearly detected compared with GFP-Eps15 (Fig. 6, a and b). The fact that the GFP-Eps15R construct could be found in the nucleus is in keeping with a lack of NES(s) in the protein. The lack of NES in Eps15R was further suggested by the fact that the two overlapping constructs, D3R and ER747, that correspond to the 660–907 and 1–747 regions, respectively, showed clear nuclear staining (Fig. 6, c and d), suggesting that they were not exported from this compartment. Finally, the lack of NES in Eps15R was confirmed using the in vitro CRM1 binding assay. As expected, GST-Eps15 (Fig. 6, lane 4), but not GST-Eps15R (Fig. 6c, lane 2), was able to precipitate CRM1.

Altogether the data presented above show that Eps15 contains a functional classical leucine-rich NES and that such a signal is absent in Eps15R. These data help to explain the difference of nuclear localization observed between the two proteins. On the one hand, Eps15 is actively exported from the nucleus and then excluded from this compartment at steady state; on the other, Eps15R is allowed to accumulate in this compartment. However, the in vivo situation is likely to be more complicated, since the staining pattern of full-length GFP-Eps15R (Fig. 6b) is quite different to what observed for the endogenous protein that shows a bright nuclear staining (Fig. 1b). Western blotting experiments showed that several Eps15R immunoreactive bands could be found in all tested cell lines, suggesting that Eps15R undergoes extensive alternative splicing (32). As the additional bands show lower molecular weights than the one corresponding to full-length Eps15R, we wondered whether they could be responsible for the strong nuclear staining observed when cells were stained with anti-Eps15R antibody. To characterize putative Eps15R alternative splice forms, DNA fragments were amplified by RT-PCR from NIH3T3 cells mRNA as described under “Material and Methods.” Interestingly, in addition to amplification products of the predicted size, several additional shorter bands were also found (data not shown), corresponding to three different alternative splice forms bearing deletions in the 747–861 region (Fig. 7, top panel). Their intracellular localization was investigated using GFP fusion proteins. As shown in Fig. 7, they all showed increased nuclear staining compared with that of full-length Eps15R (compare d, f, and h to b). The stronger nuclear staining of the splice forms was not due to a loss of CCP localization, since they all showed a clear punctate staining at the plasma membrane similar to full-length Eps15R (compare c, e, and g to a). The 747–861 region deleted in the splice forms is therefore likely to be involved in the regulation of nuclear access of Eps15R. The same kind of study was performed for Eps15 (data not shown). Only one alternative splice event could be found corresponding to the insertion of a 37-amino acid sequence between the second and the third EH domain (Fig. 8). This splice event was already described by Bernard et al. (42) and is conserved from human to mice, since it could be found in mouse expressed sequence tag data banks (IMAGE clone 586592). The intracellular localization of the corresponding GFP construct was very similar to that of Eps15 and was also excluded from the nucleus even after LMB treatment (data not shown).

Until this study, no specific functions could be ascribed to the two related proteins, Eps15 and Eps15R, that both bind AP-2- and NPF-containing proteins, localize in CCPs, are required for clathrin-dependent endocytosis, and are phosphorylated in response to epidermal growth factor (10, 11, 14, 18, 28, 32, 33). The results obtained here show that they are clearly involved in different nucleocytoplasmic trafficking events. The function of these two related proteins in the nucleus is not clear, but their opposite nuclear localization suggests that they are likely to be different or differently regulated. The fact that Eps15 undergoes nucleocytoplasmic shuttling suggests that it could be involved in signal transduction from CCPs to the nucleus, and our recent study suggests that this function could be related to transcription regulation (31). In the case of Eps15R, its nuclear localization at steady state rather suggests a constitutive nuclear function. The mutants and the splice forms described here should enable us to generate the tools that would help to better understand what could be the function of these “endocytic” proteins in the nucleus.

Acknowledgments—We thank J. Mattaj, M. Yoshida, and B. Wolff for their kind gifts of reagents. We also thank C. Dargemont for a very useful introduction to the nucleocytoplasmic field and S. Benichou for his helpful comments.

REFERENCES
1. Mellman, I. (1996) Ann. Rev. Cell Dev. Biol. 12, 575–625
2. Hirst, J., and Robinson, M. S. (1998) Biochim. Biophys. Acta 1404, 173–193
3. Schmid, S. L., McNiven, M. A., and De Camilli, P. (1998) Curr. Opin. Cell Biol. 10, 504–512
4. McNiven, M. A. (1998) Cell 94, 151–154
5. Kirchhausen, T. (1999) Ann. Rev. Cell Dev. Biol. 15, 705–732
6. Shupnik, O., Low, P., Grahe, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P., and Brodin, L. (1997) J. Cell. Biol. 138, 1053–1062
7. Wigg, P., Kohler, K., Vallis, Y., Doyle, C. A., Owen, D., Hunt, S. P., and McMahon, H. T. (1997) Mol. Cell. Biol. 7, 2003–2015
8. Chen, H., Fre, S., Slepnev, V. I., Capua, M. B., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998) Nature 394, 793–797
9. Rosenthal, J. A., Chen, H., Slepnev, V. I., Pellegrini, L., Salcini, A. E., Di Fiore, P. P., and De Camilli, P. (1999) J. Cell Biol. 140, 3959–3965
10. Carbone, R., Fre, S., Iannolo, G., Battelli, F., Mancini, P., Pelicci, P. G., Torrisi, M. R., and Difore, P. P. (1999) J. Cell Biol. 145, 1189–1197
11. Damm, D., Schumacher, C., Hage, W., Verkleij, A. J., Henegouwen, P. J. (1997) J. Cell. Biol. 136, 811–821
12. Cupper, P., Judavh, A. P., and Kirchhausen, T. (1998) J. Biol. Chem. 273, 1847–1850
13. Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Durart-Varsat, A. (1999) J. Cell Sci. 112, 1385–1391
14. Benmerah, A., Poupin, V., Cerf-Bensussan, N., and Dautry-Varsat, A. (2000) J. Biol. Chem. 275, 3299–3305
15. Poxoli, F., Minichelli, L., Motokura, B., Wong, W. T., and Di Fiore, P. P. (1993) Mol. Cell. Biol. 13, 5814–5821
16. Wong, W. T., Kraus, M. H., Carminogho, F., Zelano, A., Druck, T., Croce, C. M., Huebner, K., and Di Fiore, P. P. (1994) Oncogene 9, 1501–1507
17. Shupnik, O., Schumacher, C. A. E., Romano, A., Castagnino, P., Pelicci, P. G., and Di Fiore, P. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 92, 9520–9524
18. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkovna, O., Cesaroni, G., Pelicci, P. G., and Difore, P. P. (1997) Genes Dev. 11, 2239–2249
19. Bogerd, H. P., Fridell, R., Madore, S., and Cullen, B. R. (1995) Cell 82, 485–494
20. Fritz, C. C., Zapf, M. L., and Green, M. R. (1995) Nature 376, 530–533
21. Doria, M., Salcini, A. E., Colombo, E., Parslow, T. G., Pelicci, P. G., and Di Fiore, P. P. (1999) J. Cell Biol. 147, 1379–1384
22. Cupper, P., Terhaar, E., Boll, W., and Kirchhausen, T. (1997) J. Biol. Chem. 272, 15413–15418
23. Benmerah, A., Bega, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1996) J. Biol. Chem. 271, 12111–12116
24. Daniell, L., De Camilli, P., Pelicci, P. G., and Bazzicalupi, P. (1997) Science 276, 259–263
25. Salcini, A. E., Hilliard, M. A., Croce, A., Arbucce, S., Luzzi, P., Tachetti, C., Daniell, L., De Camilli, P., Pelicci, P. G., Di Fiore, P. P., and Bazzicalupi, P.
30. Hyman, J., Chen, H., Di Fiore, P. P., De Camilli, P., and Brunger, A. T. (2000) J. Cell Biol. 149, 537–546.
31. Vecchi, M., Polo, S., Poupan, V., van de Loo, J. W., Benmerah, A., and Di Fiore, P. P. (2001) J. Cell Biol. 153, 1511–1517.
32. Coda, L., Scaleti, A. E., Confalonieri, S., Pelicci, P. G., and Di Fiore, P. P. (1996) J. Biol. Chem. 271, 3063–3072.
33. Benmerah, A., Gagnon, J., Bagwell, B., Megarbane, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1995) J. Cell Biol. 131, 1831–1838.
34. Coda, L., Salcini, A. E., Confalonieri, S., Pelicci, G., Sorkina, T., Sorkin, A., Pelicci, P. G., and Difiore, P. P. (1998) J. Biol. Chem. 273, 3003–3012.
35. Benmerah, A., Gagnon, J., Bagwell, B., Megarbane, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1995) J. Cell Biol. 131, 1831–1838.
36. Mattaj, I. W., and Englmeier, L. (1998) Annu. Rev. Biochem. 67, 265–306.
37. Gorlich, D., and Kutay, U. (1999) Annu. Rev. Cell Dev. Biol. 15, 607–660.
38. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997) Science 276, 141–144.
39. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) Cell 90, 1051–1060.
40. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) Nature 390, 308–311.
41. Stade, K., Ford, C. S., Gathrie, C., and Weis, R. K. (1997) Cell 90, 1041–1050.
42. Bernard, O. A., Mauchauffe, M., Mecucci, C., Van den Berghe, H., and Berger, R. (1994) Oncogene 9, 1039–1045.
43. Behrens, J., von Kries, J., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birrane, W. (1996) Nature 382, 638–642.
44. Nix, D. A., and Beckerle, M. C. (1997) J. Cell Biol. 138, 1139–1147.
45. Fagotto, F., Gluck, U., and Gumbiner, B. M. (1998) Curr. Biol. 8, 181–190.
46. Spradling, K. D., McDaniel, A. E., Lohi, J., and Pilcher, B. K. (2001) J. Biol. Chem. 276, 29257–29267.
47. Sadot, E., Simcha, I., Schutzman, M., Ben-Ze’ev, A., and Geiger, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15339–15344.
48. Van Hengel, J., Vanhoenacker, P., Staes, K., and Van Roy, F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7980–7985.
49. Latimer, M., Ernst, M. K., Dunn, L. L., Drutskaya, M., and Rice, N. R. (1998) Mol. Cell. Biol. 18, 2640–2649.
50. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999) EMBO J. 18, 1660–1672.
51. Adachi, M., Fukuda, M., and Nishida, E. (1999) EMBO J. 18, 5347–5358.
52. Horton, P., and Nakai, K. (1997) Intellig. Syst. Mol. Biol. 5, 147–152.
53. Cokol, M., Nair, R., and Rost, B. (2000) EMBO Rep. 1, 411–415.
54. Yokoya, F.,HashMap, N., Tachibana, T., and Yoneda, Y. (1999) Mol. Biol. Cell 10, 1119–1131.
