Enterophilin-1, a New Partner of Sorting Nexin 1, Decreases Cell Surface Epidermal Growth Factor Receptor*

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The intestinal epithelium undergoes continuous and rapid renewal, with proliferation of the multipotent stem cells limited to the crypts of Lieberkühn. At the top of the crypt, cells lose their proliferative ability and complete differentiation during a highly organized migration along the crypt-villus axis (1–3). Two events are important to intestinal epithelial cell differentiation: the transition from stem cells to committed proliferative cells and the mechanisms responsible for the irreversible loss of proliferative potential as the committed cells start to differentiate. However, little is known regarding how these two events take place.

The regulation of epithelial cell growth and functional differentiation is susceptible to various influences along the crypt-villus axis, including growth factor-derived signals. The overall importance of growth factors in intestinal epithelial cell renewal is underscored by the fact that the normal intestinal development is severely perturbed in EGFR1 knock-out mice (4). Interestingly, EGF binding in situ along the crypt-villus axis is higher in crypt than in villus enterocytes (5). These data correlated with studies performed with colon adenocarcinoma Caco-2 cells indicating that the expression of cell surface EGFR is dramatically decreased in well differentiated Caco-2 cells (6). Even if the cell growth arrest is well known to be associated with the differentiation process, the link between these two phenomena remains a confused point in the study of epithelial cell differentiation.

We have recently described enterophilins as a new family of intestinal proteins, with a carboxyl-terminal B30.2 domain and an extended leucine zipper in their amino-terminal part. Three members were identified: enterophilin-1 (Ent-1), enterophilin-2 (Ent-2), and a short form of Enterophilin-2 (Ent-2S). Ent-1 contains up to 45 regular heptad repeats and corresponds to a 65-kDa protein. Interestingly, Ent-1 was mostly expressed in the mid-crypt-villus axis, when cells stop their proliferation to start the differentiation process. In human intestinal epithelial carcinoma Caco-2 cells, Ent-1 ortholog expression pattern was positively correlated to growth arrest and terminal differentiation program. In addition, transfection of HT-29 cells with Ent-1 full-length cDNA inhibited cell growth and promoted an increase in alkaline phosphatase activity, an intestinal differentiation marker. Taken together, these results suggested a close relationship between Ent-1 expression and the enterocyte differentiation program (7).

To investigate the role of Ent-1 in growth arrest associated with enterocytic differentiation, we performed a yeast two-hybrid screen to identify proteins interacting with Ent-1. We report herein that Ent-1 interacts with sorting nexin 1 (SNX1). The sorting nexins are an emerging family of proteins (for a review see Ref. 8), characterized by the presence of a phox homology domain (9, 10). They are involved in the intracellular trafficking of several membrane receptors (11–13). SNX1, the best studied member of this family, recognizes the lysosomal targeting sequence code in EGFR. Moreover, SNX1 overexpres-

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The abbreviations used are: EGFR, EGF receptor; EGF, epidermal growth factor; ERA1, early endosome antigen 1; Ent-1, enterophilin-1; GFP, green fluorescent protein; SNXs, sorting nexins; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; RPE, R-phycocerythin.
sion decreases the amount of EGFR on the cell surface as a result of enhancing trafficking in the endosome-to-lysosome pathway (11). Furthermore, recent data demonstrate that mice lacking both SNX1 and SNX2 display alterations in proper cellular trafficking (14). We confirm the association of Ent-1 with SNX1 by biochemical and immunofluorescence experiments in mammalian cells. Furthermore, Ent-1 causes a significant diminution of cell surface EGFR. In this context, identification of SNX1 as an Ent-1 partner and their cooperative role in cell surface EGFR decrease provide more evidence about the involvement of Ent-1 in the inhibition of cell proliferation associated with the enterocytic differentiation process.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Full-length Ent-1 cDNA, the “leucine zipper” region, and the B30.2 domain were amplified by PCR using oligonucleotides containing Ncol and SalI restriction sites, and PCR products were inserted in pAS2-1 (Clontech Laboratories) at the corresponding sites. First, full-length Ent-1 containing vector pAS2-1 was used as a bait to screen an epitHELial HeLa cell cDNA library constructed in pGAD GH vector at EcoRI/HindIII restriction sites (Clontech Laboratories). The yeast host strain CG-1945 (Clontech Laboratories) was transformed with the bait and library plasmids. Positive transformants on medium lacking leucine, tryptophan, and histidine, and a colony lift filter assay was performed to test β-galactosidase activity. Harvesting positive library clones were retransformed into strain SFY526 (Clontech Laboratories) with full-length Ent-1 bait and tested for expression of LacZ reporter gene to confirm the specificity of the interaction. cDNA inserts of true positive clones were sequenced and submitted to the database. To pinpoint the interacting domain of Ent-1, the leucine zipper region or the B30.2 domain constructions were amplified by PCR using oligonucleotides containing XhoI restriction sites, and PCR products were inserted in pAS2-1 vector (Clontech Laboratories) to generate pDpRed2-N1-Ent-1 construct, encoding COOH-terminal domain, and the B30.2 domain constructions were used in a one to one interaction test with positive library plasmids in the yeast strain SFY526 and tested for positive interaction as described above.

Plasmids—Ent-1 full-length cDNA was cloned in pcDNA3.1/myc-his vector (Invitrogen™) as previously described (7). We thus obtained the pcDNA3/myc-his-Ent-1 vector, encoding a myc-tagged protein in the COOH-terminal region. Full-length Ent-1 was cut off from pcDNA3/myc-his vector using KpnI/ApaI sites and was inserted in pEGFP-C2 plasmid (Clontech Laboratories) to encode green fluorescent protein (GFP)-tagged protein in the NH2-terminal region (pEGFP-C2-Ent-1). Ent-1 full-length cDNA was also cloned in pDsRed2-N1 vector (Clontech Laboratories) to generate pDpRed2-N1-Ent-1 construct, encoding COOH-terminal DsRed-tagged Ent-1 protein. The NH2-terminal GFP-tagged SNX1 (pEGFP-C1-SNX1) and the NH2-terminal FLAG-tagged SNX1 (pcDNA3/FLAG-SNX1) constructs were generously provided by Dr. Gordon N. Gill (University of California at San Diego, La Jolla). Ent-1 full-length cDNA was cloned in pcDNA3.1/myc-his vector (Invitrogen™) supplemented with 10% fetal bovine serum (Invitrogen™) and 100 μg/ml penicillin/streptomycin (Invitrogen™) in a humidified atmosphere containing 5% CO2. Nonessential amino acids (0.1 mM; Invitrogen™) were added to the culture medium of Caco-2 cells. All cells were transfected with the different plasmids using FuGENETM™ (Roche Applied Science), except Caco-2 cells, which were transfected with a cationic lipid, LipofectAMINE™ (Invitrogen™), according to the manufacturer’s protocols.

Immunoprecipitation Experiments—Caco-2 cells were co-transfected with both pcDNA3/myc-his-Ent-1 and pcDNA3/FLAG-SNX1. The experiments were performed 48 h after transfection, and all of the procedures were done at 4°C. The cells were washed three times with phosphate-buffered saline (PBS) and lysed for 15 min with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol) containing protease and phosphatase inhibitors (1 mM phenylmethanesulfon fluor fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 20 mM NaF, 2 mM Na3VO4). Insoluble debris were removed by centrifugation at 14,000 × g for 15 min, and the supernatant was subjected to a precolling with 20 μl of protein G-Sepharose for 30 min. Ent-1 was immunoprecipitated for 2 h with 5 μg of monoclonal anti-Myc antibody, followed by 50 μl of protein G-Sepharose for 1 h. After washing three times in buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol) containing protease and phosphatase inhibitors, the bound proteins were eluted by boiling the beads in Laemmli buffer (15). The precipitated proteins were then submitted to SDS-polyacrylamide gel electrophoresis and detected by immunoblotting.

Confluence-induced Differentiation of Caco-2 Cells—Caco-2 cells were seeded at 18,000 cells/cm2 and grown in 60-mm dishes in the complete medium. The medium was changed every 2 days. The cells were washed twice in PBS and scraped at 4°C in PBS containing protease and phosphatase inhibitors (1 mM phenylmethanesulfon fluor fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 20 mM NaF, 2 mM Na3VO4) at various times up to 23 days after plating. The samples were sonicated, and the protein concentration was determined according to the method of Bradford (16). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis. Then Ent-1 and EGFR expression were analyzed by immunoblotting.

Immunoblotting—The protein samples were submitted to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and subjected to immunoblotting according to standard protocols (17). Rabbit polyclonal anti-Ent-1 peptide antibody was obtained from Eurogentec as previously described (7). Monoclonal antibody against c-Myc epitope (9E10) and polyclonal antibody against epidermal growth factor receptor (10D5) were from Santa Cruz Biotechnology. Monoclonal antibody against flag (M2) was purchased from Sigma, and monoclonal antibody against GFP was from Roche Applied Bioscience. Revelation was done with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Promega) and ECL (Amersham Biosciences) detection system.

Immunofluorescence Microscopy—COS-7 cells were grown on sterile glass coverslips and transfected with pEGFP-C2-Ent-1 or pEGFP-C1-SNX1 or co-transfected with both pcDNA3/myc-his-Ent-1 and pEGFP-C1-SNX1. For EGF stimulation, the cells were serum-starved for 24 h. The cells were incubated with 100 ng/ml EGF for 1 h at 4°C, rinsed twice in PBS, and finally incubated with warm medium at 37°C for 10, 15, or 30 min. The cells were washed three times with ice-cold PBS, fixed for 15 min with 3% paraformaldehyde, permeabilized for 2 min with 0.2% Triton X-100, and saturated for 30 min with 0.2% gelatin. The cells were then incubated 60 min with the primary antibody (anti-EEA1 from Transduction Laboratories or anti-c-Myc (9E10) from Santa Cruz Biotechnology), and then immunostaining was performed with TRIT-conjugated anti-mouse IgG secondary antibody (Southern Bio-technology Associates). The coverslips were examined with a Zeiss Axioskop microscope or with a confocal microscope (Zeiss, LSM 510, Axivert 100).

Size Exclusion Chromatography—COS-7 cells were co-transfected with pEGFP-C2-Ent-1 and pcDNA3/FLAG-SNX1, grown for 24 h, and then serum-starved for an additional 24 h. The cells were incubated with 100 ng/ml EGF for 1 h at 4°C, rinsed twice in PBS, and finally incubated with warm medium at 37°C for 10 min to synchronize EGFR endocytosis. As previously described by Chin et al. (18), COS-7 cells were lysed for 30 min in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 1% Nonidet P-40, 50 μM phenylmethanesulfon fluoride, 10% glycerol, using a flow rate of 0.5 ml/min, and 0.25-ml fractions were collected after a delay of 10 min. The column was calibrated with protein standards (Bio-Rad), including thyroglobulin (670 kDa), y globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B-12 (1.35 kDa). Equal volumes of fractions were subjected to immunoblotting analysis.

Flow Cytometry—COS-7 cells were transfected with pEGFP-C2-Ent-1, pEGFP-C1-SNX1, or empty vector and grown for 72 h. Then cells were collected by treatment with 2 mM EDTA in PBS and fixed in 1% formaldehyde for 30 min, and nonspecific sites were saturated with 1% bovine serum albumin for 30 min. Cell surface EGFR was detected by incubation with the primary antibody (clone LA1) for 15 min with 0.05% N-vinylpyrrolidone and with a R-phycocerythrin (RPE)-conjugated goat anti-mouse IgG secondary antibody (Dako) for 30 min. The cells were then analyzed by flow cytometry (Beckman Coulter XL 4C). Expression of the GFP-fused proteins was monitored by fluorescence measurement, and the surface binding of the anti-EGFR antibody was measured specifically on the transfected cell population. To evaluate the effect of Ent-1 and SNX1 on plasma membrane EGFR, we co-transfected COS-7 cells with pDsRed2-N1-Ent-1 and pEGFP-C1-SNX1 or both empty vectors. Cell surface EGFR was similarly immunolabeled, except for the secondary antibody: RPE-Cy5-conjugated rabbit anti-mouse IgG antibody.
Enterophilin-1 Interacts with Sorting Nexins 1 and 2—To elucidate the role of Ent-1 in endocytotic differentiation, we performed a two-hybrid screening of epithelial HeLa cell library using full-length Ent-1 as a bait. Twenty-four positive clones were rescued and confirmed by retransformation into a second yeast host strain SFY526. The positive clones from the yeast two-hybrid screen were sequenced, and their predicted amino acid sequences were aligned below the domain structure of SNX1 (A) and SNX2 (B) analyzed with the SMART program.

RESULTS

Enterophilin-1 Interacts with Sorting Nexins 1 and 2—To pinpoint the binding domains of Ent-1 with its identified partners, we used the two-hybrid one to one interaction assay with the leucine zipper part or the B30.2 domain as a bait. Twenty-four positive clones were rescued and confirmed by retransformation into a second yeast host strain SFY526. The positive clones from the second screening were sequenced; nine clones encoded sorting nexin proteins, a family of proteins playing an important role in protein trafficking among various organelles. Sequence analysis indicated that these clones encoded SNX1 and six encoded SNX2 as depicted by protein alignments presented in Fig. 1 (A and B, respectively). These data also showed that the clones contained only a part of the coiled coil regions and the EGFR binding domain of SNX1, indicating that these minimal regions are sufficient for binding to Ent-1.

To pinpoint the binding domains of Ent-1 with its identified partners, we used the two-hybrid one to one interaction assay with the leucine zipper part or the B30.2 domain as a bait. The results showed that the B30.2 domain was unable to bind SNX1 and SNX2. Unfortunately, the leucine zipper part of Ent-1 was toxic for the two yeast strains. Accordingly, we investigated whether Ent-1-associated vesicles could be identified with an anti-Ent-1 antibody (12). Immunoprecipitates were then submitted to SDS-polyacrylamide gel electrophoresis, and SNX1 and Ent-1 proteins were detected by immunoblotting with an anti-FLAG antibody and an anti-Myc antibody, respectively.

FLAG-tagged SNX1 were co-expressed in Caco-2 cells, and Ent-1 was subjected to immunoprecipitation with an anti-Myc antibody. Immunoprecipitated complexes were analyzed by immunoblotting, and data revealed the presence of both Ent-1 and SNX1, confirming the interaction between these two proteins in intestinal cells (Fig. 2).

Enterophilin-1 Co-localizes with SNX1 on Vesicular and Tubulovesicular Structures—Identification of SNXs as Ent-1 partners led us to focus on the cytoplasmic localization of Ent-1 by fluorescence microscopy. COS-7 cells were co-transfected with COOH-terminally tagged Ent-1 and NH2-terminally tagged SNX1 displayed a vesicular and tubulovesicular signal. We observed that part of both staining patterns perfectly overlapped (Fig. 3, bottom panel, inset), indicating that within the cell, Ent-1 and SNX1 co-localized in similar structures. However, we noticed that Ent-1 was also located in cellular areas lacking SNX1 staining. Similarly, SNX1 was found in cytoplasmic pools that did not contain Ent-1. Thus, Ent-1 and SNX1 showed substantial but not complete co-localization.

Furthermore, we confirmed that NH2-terminally tagged Ent-1 displayed a cytoplasmic distribution similar to COOH-terminally tagged Ent-1 in different epithelial cell lines. Indeed, we also observed vesicular and tubulovesicular structures with pEGFP-C2-Ent-1 in COS-7 cells, as well as in Madin-Darby canine kidney, HeLa, and Caco-2 cells (data not shown).

Ent-1-containing Structures Are Different from EEA1-positive Early Endosomes—Because SNX1 has been reported to be substantially associated to early endosomal membranes (13, 20), we investigated whether Ent-1-containing vesicles corresponded to early endosomes. COS-7 cells were transfected with pEGFP-C2-Ent-1 and the early endosomal specific marker EEA1 was labeled with a specific antibody after various times of EGF stimulation (100 ng/ml) as indicated under “Experimental Procedures”. Confocal microscopy analysis revealed that Ent-1 was still present on vesicular and tubulovesicular structures in cells stimulated for 10, 15, or 30 min with EGF (Fig. 4). However, no merge was observed with EEA1 staining at any time of stimulation, suggesting that Ent-1-containing vesicles were different from the previously defined EEA1-positive early endosomes (Fig. 4). By contrast, SNX1 partially overlapped with EEA1 signal under the same conditions of EGF stimula-
Enterophilin-1 and SNX1 Coexist in Macromolecular Complexes Containing EGFR in the Cell—Sorting nexins are supposed to act as a multimeric protein complex named the retroplex by analogy with their yeast orthologs (22). Thus, we checked for the presence of Ent-1 in such macromolecular complexes by gel filtration chromatography. COS-7 cells were co-transfected with pEGFP-C2-Ent-1 and pDNA3/FLAG-SNX1. After stimulation with 100 ng/ml EGF for 10 min to stimulate EGFR endocytosis, the cytosolic fractions were prepared and loaded onto the column. The different fractions were analyzed for the presence of GFP-Ent-1, FLAG-SNX1, and endogenous EGFR by Western blotting. An important signal for Ent-1 was obtained from fractions 26–30, corresponding to large complexes of ~670–720 kDa that perfectly matched the Ent-1-enriched complexes. As with Ent-1 and SNX1, EGFR presented an elution profile in fractions 22–28, overlapping the Ent-1 and SNX1 peaks in fraction 26 corresponding to ~670-kDa heteromeric protein complexes. With Ent-1 and SNX1, EGFR was not found as monomeric form (~60 kDa for the FLAG-tagged SNX1). Endogenous EGFR presented an elution profile in fractions 22–28, overlapping the Ent-1 and SNX1 peaks in fraction 26 corresponding to ~670-kDa heteromeric protein complexes. As with Ent-1 and SNX1, EGFR was not found as monomeric form. We can notice that a part of the three proteins was observed in the void volume (fractions 1–15) that corresponded to large molecular mass complexes excluded from the column. Alternatively, this could be the result of nonspecific aggregation. Nevertheless, gel filtration data demonstrated a convincing overlap between Ent-1 and SNX1 elution profiles and strongly suggested the existence of Ent-1/SNX1/EGFR multimeric complexes involved in EGF-induced EGFR endocytosis.

Enterophilin-1 Enhances the Decrease of Cell Surface EGF Receptors—Gel filtration data led us to check for the effects of Ent-1 on EGFR cell surface pool. COS-7 cells were co-transfected with pEGFP-C2-Ent-1, pEGFP-C1-SNX1, or empty vector for 72 h, and cell surface EGFR was immunolabeled. By flow cytometry, cell surface EGFR expression was specifically monitored in the GFP- or GFP-Ent-1-transfected cells as indicated under “Experimental Procedures.” Our results showed that Ent-1 induced a significant decrease of 35% of plasma mem-

**Fig. 3.** Ent-1 co-localizes with SNX1 on vesicles and on tubulovesicular structures. COS-7 cells were co-transfected with pcDNA3/Myc-His-Ent-1 and pEGFP-C2-SNX1, grown for 48 h, and processed for fluorescence microscopy. The cells were labeled with primary monoclonal anti-Myc antibody, followed by detection with a secondary antibody conjugated to Texas Red (upper panel). SNX1 was identified by the green fluorescence emitted by GFP (middle panel). The arrows indicate vesicular and tubulovesicular structures clearly visible in both panels. The merge image shows that Ent-1 and SNX1 co-localized on vesicles and tubulovesicular structures (lower panel). The inset in each panel corresponds to higher magnification, showing the best overlapping structures.

**Fig. 4.** Ent-1 containing structures are distinct from the EEA1-positive early endosomes. COS-7 cells were transfected with pEGFP-C2-Ent-1 (A, D, and G), serum-starved for 24 h, stimulated with 100 ng/ml EGF for 10 min (A–C), 15 min (D–F), or 30 min (G–I), and processed for immunofluorescence with primary anti-EEA1 antibody, followed by detection with secondary antibody conjugated to TRITC (B, E, and H). The merge of the two signals is represented in C, F, and I. Ent-1 vesicles are largely distinct from EEA1-positive early endosomes.
brane EGFR, compared with the GFP-transfected cell population used as control (Fig. 7A). A similar decrease was obtained with cells overexpressing SNX1 in our experimental conditions (Fig. 7A), in agreement with previously published data (11). These results indicated that overexpressed Ent-1 was able to promote the down-regulation of cell surface EGFR.

To evaluate the cooperation between Ent-1 and SNX1 in the regulation of EGFR endocytosis, we co-transfected COS-7 cells with pDsRed2-N1-Ent-1 and pEGFP-C1-SNX1. Cell surface EGFR was immunolabeled and identically analyzed on double-transfected cell population. We demonstrated that Ent-1 and SNX1 displayed a synergetic effect on cell surface EGFR removal because a 65% decrease of plasma membrane EGFR was quantified in double-transfected cells (Fig. 7B). Taken together, these data suggested a cooperative effect of Ent-1 and SNX1 on cell surface EGFR removal during endocytosis.

Enterophilin-1 Expression Correlates with the Decrease of EGFR during the Differentiation of Caco-2 Cells—To investigate the physiological relevance of the effects of Ent-1 overexpression on cell surface EGFR, we analyzed the expression patterns of both proteins in relation to enterocyte differentiation. Human colon carcinoma Caco-2 cell line is a well characterized model for the study of intestinal differentiation. We thus performed the confluence-induced differentiation of...
Intestinal differentiation marker (data not shown). These re-
appearance of alkaline phosphatase activity, typically used as
plating (7, 23). The functional differentiation was followed by
Caco-2 cells start to proliferate after a lag time of 2 days after
weak expression of EGFR that could be expected knowing that
expression beginning at day 14, when the human ortholog of
Western blot analysis showed a marked decrease of EGFR
ning at day 9, sustained up to 23 days in culture. Interestingly,
as a 65-kDa protein, displayed an increased expression begin-
culture. As previously shown (7), the cells reached conflu-
A
B
Fig. 7. Ent-1 enhances the EGFR removal from the cell surface. A, 
COS-7 cells were transfected with pEGFP-C2-Ent-1 (shaded bar) or pEGFP-
C1-SNX1 (white bar) or empty vector (control, black bar) and grown for 72 h.
The cells were then labeled with monoclonal anti-EGFR (LA1) primary antibody
and RPE-conjugated secondary antibody (red fluorescence). The relative density
of cell surface EGFR was specifically evaluated in the
transfected cell population by flow cytometry. B, COS-7 cells were co-trans-
fected with pEGFP-C1 and pDsRed2-N1 (control, stippled black bar) or
pEGFP-C1-SNX1 and pDsRed2-N1-Ent-1 (stippled shaded bar) and grown
for 72 h. The cells were then similarly labeled with monoclonal anti-EGFR
(LA1) primary antibody and (RPE-Cy5)-conjugated secondary antibody
for 72 h. The cells were then labeled with monoclonal anti-EGFR
expression during intestinal epithelial differentiation.

**Fig. 8.** Endogenous Ent-1 expression correlates with the decrease of EGFR expression during Caco-2 cell spontaneous differen-
tiation. Caco-2 cells were grown in 60-mm dishes and harvested at various times up to 23 days after plating. Equal amounts of proteins
were subjected to SDS-polyacrylamide gel electrophoresis. Ent-1 was
detected with rabbit polyclonal anti-Ent-1 peptide antibody, and EGFR
was detected with polyclonal anti-EGFR antibody.

RESULTS demonstrated a correlation between the Ent-1 expression
pattern and the decrease of EGFR expression during intestinal
epithelial differentiation.

**DISCUSSION**

In this study, we sought partners of Ent-1 by a two-hybrid
screening of an epithelial HeLa cell cDNA library. We dem-
strated that Ent-1 interacted with sorting nexins 1 and 2. We
first focused our investigations on the interaction of Ent-1 with
SNX1, because SNX1 is the best studied mammalian member
of the sorting nexin family. The association between Ent-1 and
SNX1 was confirmed by immunoprecipitation experiments in
epithelial mammalian cells. We also co-localized Ent-1 and SNX1
in vesicular and tubulovesicular structures. These results
were consistent with recent studies that described sorting
nexins in such vesicular and tubulovesicular compartments
(19, 24). As suggested by Kurten et al. (24), these tubulovesi-
cular structures are not always detectable and may therefore
represent transient entities. Ent-1-containing structures were
clearly different from early endosomal marker EEA1 staining
after EGF-induced EGFR endocytosis, whereas a certain pro-
portion of SNX1 vesicles overlapped with EEA1-positive early
endosomes. Such partial co-localization is consistent with re-
cent data showing that SNX1-containing vesicles were part of
the early endocytic compartment but were partially distinct
from previously defined EEA1-containing endosomes and recy-
cling transferrin receptor-containing endosomes (19, 20, 24).
Further studies will attempt to more precisely define such
EEA1-negative vesicles containing both Ent-1 and SNX1.

SNX1 and SNX2 are the mammalian orthologs of the yeast
vacuolar protein sorting Vps5p (25). Vps5p is a subunit of a
large multimeric complex, termed the retromer complex,
involved in retrograde transport of proteins from endosomes to
the trans-Golgi network (26). In mammalian cells, homodimer-
ization and heterodimerization of SNXs have been reported
(22, 24), and it has been proposed that complex formation
between SNXs may be necessary for organizing functional
units for receptor sorting and degradation. We showed a perfect
co-elution of Ent-1 and SNX1 in 310–670-kDa macromolecular
complexes. Part of EGFR was also detected in the same com-
plexes. We also demonstrated that neither Ent-1 and SNX1 nor
EGFR eluted in their respective monomeric form. These data
suggested that the three proteins interacted with each other or
with other proteins in ~435–670-kDa macromolecular com-
plexes. This was consistent with the work of Chin et al. (18),
which demonstrated the elution of SNX1 in similar size com-
plexes and the lack of monomeric-SNX1 form. Additionally,
SNX1, but not Ent-1, was detected in higher molecular mass
complexes (above 1700 kDa). Because a certain proportion of
SNX1 and Ent-1 co-localized by immunofluorescence, these results emphasized that both proteins could interact with each other in the same multimeric complexes. Ent-1 could be a component of macromolecular complexes involved in endocytosis of EGFR. However, EGFR endocytosis is a very dynamic process, involving continuously remodeled complexes and structures. Thus, the presence of EGFR in Ent-1/SNX1-enriched complexes could be transient, strictly depending on well-defined macromolecular complex formation along the EGFR endosome-to-lysosome pathway.

SNXs have been shown to modulate endocytosis of a variety of receptors (for a review, see Ref. 8), including EGFR (11), protease-activated receptor-1 (13), or low density lipoprotein receptors (27). Overexpressed SNX1 was clearly involved in the decrease of EGFR on the cell surface as a result of enhancing the rate of constitutive and ligand-induced degradation (11). We then investigated the role of Ent-1 on EGFR degradation. Our results showed that overexpression of Ent-1 significantly decreased the cell surface EGFR. Interestingly, EGFR degradation was strongly decreased when Ent-1 and SNX1 were co-expressed. These results highlighted the synergistic effect of both proteins and were in favor of a role of Ent-1 in cell surface EGFR removal by endocytosis through its interaction with SNX1. Ent-1 could thus be considered as a new partner of SNX1, such as ACK2 (activated Cdc42-associated kinase 2), which promotes EGFR degradation through its interaction with SNX9 (28) or Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) that inhibits EGFR endocytosis through its binding to SNX1 (18). It will be interesting now to define whether Ent-1 could regulate other types of cell surface receptors as described for SNXs.

EGFR is an important mediator of intestinal epithelial cell proliferation. In fact, undifferentiated crypt cells display a higher EGFR binding activity than differentiated villus enterocytes (5), which is correlated with the decrease of EGFR surface expression along the crypt-villus axis (6). Our data indicate that Ent-1 expression during spontaneous differentiation of Caco-2 cells correlates with the decrease of EGFR expression. Among several intestinal epithelial cell lines, Caco-2 cells have proven to be the most useful in vitro models. Indeed, Caco-2 cells are unique in their ability to initiate spontaneous differentiation on reaching confluence under normal culture conditions and undergo a maturation process closely resembling the differentiation of normal intestine (23). Furthermore, we recently published that the onset of Ent-1 expression corresponded to cell growth arrest, preceding functional differentiation. Additionally, we reported a decrease of proliferation in Ent-1-transfected HT-29 cells (7). In this context, Ent-1-mediated cell surface EGFR removal adds to our understanding of the regulation of growth arrest in intestinal epithelium. This is directly related with the reported decrease of EGFR at the mid-villus axis (5).

To summarize, we demonstrated that Ent-1 was a new partner of SNX1. According to our two-hybrid results, we hypothesized an interaction mediated by the coiled-coil structures present in both the COOH-terminal region of SNXs and the NH2-terminal leucine zipper part of Ent-1. Works in progress in our laboratory presently aim at defining the exact SNX1-binding regions on Ent-1 by generating Ent-1 mutants defective in binding SNX1. Additionally, Ent-1 was localized with SNX1 on vesicular and tubulovesicular structures, which were different from EEA1-positive early endosomes. Interestingly, Ent-1 co-eluted with SNX1 in macromolecular complexes containing part of EGFR and induced cell surface EGFR removal. Moreover, Ent-1 and SNX1 displayed a cooperative effect on EGFR degradation, strongly increasing plasma membrane EGFR removal. Further studies will bring new insights to precise exact molecular mechanisms by which Ent-1 and SNX1 regulate receptor vesicular trafficking. These data provide new evidence about Ent-1 involvement in down-regulation of mitogenic signal leading to cell growth arrest and enterocyte differentiation.

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