EAST, an Epidermal Growth Factor Receptor- and Eps15-associated Protein with Src Homology 3 and Tyrosine-based Activation Motif Domains*

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1 The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; EGF, epidermal growth factor; EGFR, EGF receptor; TAM, tyrosine-based activation motif; α-, amino acid(s); CEFH, chicken embryo heart fibroblast; PCR, polymerase chain reaction; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; Pipes, 1,4-piperazinediethanesulfonic acid; STAM, signal transducing adaptor molecule.

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We describe the cloning and characterization of a new cytoplasmic protein designated epidermal growth factor receptor-associated protein with SH3- and TAM domains (EAST). It contains an Src homology 3 domain in its midregion and a tyrosine-based activation motif in its COOH terminus. Antibodies to EAST recognize a 68-kDa protein that is present in most chicken tissues. An epidermal growth factor (EGF)-dependent association between the EGF receptor (EGFR) and EAST was shown by reciprocal immunoprecipitation/immunoblotting studies with specific antibodies. Activated EGFR catalyzed the tyrosine phosphorylation of EAST, as judged by an in vitro kinase assay with both immunoprecipitated and purified EGFR. Immunoprecipitation/immunoblotting experiments also demonstrated an association between EAST and eps15, an EGFR substrate associated with clathrin-coated pits and vesicles, which is essential in the endocytotic pathway. The association between EAST and eps15 was not affected by EGF treatment. In immunofluorescence microscopy, EAST was shown to partially colocalize with clathrin. The sequence of the NH2-terminal portion of EAST shows a high degree of similarity with a group of proteins involved in endocytosis or vesicle trafficking. Thus, EAST is a novel signal transduction component probably involved in EGF signaling and in the endocytotic machinery.

Signal transduction proteins are characterized by their capacity to specifically associate with other proteins to form multimolecular assemblies (1). In the case of receptor tyrosine kinases and receptor tyrosine kinase-induced intracellular signaling, such protein interactions are mediated by distinct protein domains. Among these, the Src homology 2 (SH2) domain, which binds Tyr(P) residues in a specific context, and the Src homology 3 (SH3) domain, which binds sequences characterized by polyproline tracts, are the best known (2). In the epidermal growth factor receptor (EGFR), for instance, binding of the epidermal growth factor (EGF) leads to the phosphorylation of multiple tyrosine residues by the kinase activity of the receptor. These Tyr(P) residues serve as docking sites for various downstream, SH2-containing, signaling elements (3). These, in turn, can associate with other signaling proteins or substrates via other binding modules, such as SH3 domains.

As new proteins and interactions are being discovered, new motifs are also disclosed. Thus, the phosphotyrosine-binding domain present in Shc and IRS-1, for example, recognizes Tyr(P) in a manner different from SH2 domains (4). Similarly, a distinct new type of tyrosine-containing, SH2-binding domain, termed tyrosine-based activation motif (TAM), has been found in antigen receptor molecules (5). TAM-containing receptors lack an intrinsic kinase activity and use TAM motifs to recruit and activate nonreceptor protein tyrosine kinases, such as members of the Src and Syk families (6), as their effectors.

Many of the known signaling pathways are still only partially characterized, and their regulation is poorly understood, rendering the search for new interacting proteins a topic of high priority. For instance, elimination of critical SH2-binding sites of the platelet-derived growth factor and fibroblast growth factor receptors does not abrogate mitogenic signaling, suggesting that as yet unidentified proteins interact with these receptors (7, 8). The identification of novel receptor-associated components is also required to fill the gaps in our knowledge of the internalization of receptor tyrosine kinases (9). The internalization of these receptors contributes to their down-regulation and thus is an important part of signal transduction (10, 11).

Fazioli et al. (12) recently found a new EGFR substrate that they designated eps15, for EGFR pathway substrate clone 15. Eps15 is phosphorylated by EGFR in response to the activation of the latter by EGF. The unique domain structure of eps15 makes this protein a novel type of EGFR substrate. However, no direct interaction between the EGFR and eps15 could be shown. More recently, a specific interaction between the SH3 domain of Crk and the conserved proline-rich motif of eps15 was demonstrated, suggesting that Crk could mediate the binding of eps15 to the EGFR (13). Intriguingly, eps15 binds to proteins operating in endocytosis, such as α-adaptin (14, 15) and clathrin (16), and associates with clathrin-coated pits (17). More recently, eps15 was shown to be essential in receptor-mediated endocytosis (18).

In this paper, we describe the cloning, the primary and functional properties of a new signal transduction protein that associates with the EGFR and eps15. Because of its binding properties and domain structure, we have named it EGF...
receptor-associated protein with SH3- and TAM domains (EAST). We suggest that EAST, together with eps15, is involved in EGFR-mediated signaling and in the regulation of the endocytic machinery.

**EXPERIMENTAL PROCEDURES**

**General Procedures**—Standard solutions, buffers, and procedures for the purification and precipitation of DNA, restriction enzyme digestion, and ligation reactions were as described in Sambrook et al. (19). Sequencing was done by the dideoxynucleotide chain termination method of Sanger (22), using the Th17 Sequencing kit (Pharmacia Biotech Inc.) or an automated ABI PRISM 377XL DNA Sequencer (Perkin-Elmer). Synthetic oligonucleotides were obtained from the Oligonucleotide Core Facility of Biocenter Oulu or from Pharmacia. For sequence analysis and alignments, the GCG, CLUSTAL W, and COILS programs were used.

**cDNA Cloning of EAST**—Fragments of mRNAs encoding for SH3 domain-containing proteins expressed in chicken brain were amplified by reverse transcription and polymerase chain reaction (PCR) using degenerate oligonucleotide primers (20). The original clone of EAST was extended by PCR cloning from different chicken libraries and RNA and by library screening with CDNA probes. The libraries used included a chicken brain cdNA library (5'cloned in Agt10 and ligated to pGEM-T-Easy (Promega) and a chicken genomic library in the EMBL SPh7/T vector (CLONTECH). RNA was prepared as described earlier (20). 5' rapid amplification of cDNA ends was done using the 5'AmpliFINDER rapid amplification of cdNA ends kit (CLONTECH). Library screening was performed as follows. Filters containing 1 × 10^4 plaques/filter were incubated overnight with an EAST probe in hybridization buffer (50% formamide, 5 mM NaCl, 50 mM phosphate). They were then resuspended in 40 μl of kinase buffer containing unlabeled ATP at a final concentration of 50 μM. Incubation was carried out for 20 min at 4 °C. The complexes were then washed twice with kinase buffer to remove the excess ATP. 5 μg of normal or mutated recombinant EAST protein and 6 μCi of [γ-32P]ATP were added, and the mixtures were incubated for 15 min at 37 °C. EAST was then collected by incubating with either glutathione-Sepharose 4B beads or anti-EAST antibody-conjugated IgG-agarose. The beads were then washed twice with kinase buffer, boiled in 2× Laemmli's sample buffer, and subjected to SDS-PAGE (as above) and autoradiography. The direct phosphorylation assay using a purified EGFR kinase domain (Stratagene) was done according to the manufacturer's instructions.

**Northern Blot Analysis**—The cdNA corresponding to the nucleotides 60–231 of the coding region of EAST was amplified by PCR and used as a probe. Preparation of total RNAs and mRNAs were as described previously (20), and the hybridization was performed as above.

**Cell Culture**—Cells of chicken embryo heart fibroblasts (CEHF) were established, and the cells were grown as described previously (20). HER-14 cells (a gift from Dr. J. Schlessinger, New York University Medical Center) were maintained in 10% fetal bovine serum in RPMI-1640 medium (Gibco-BRL) supplemented with 1% nonessential amino acids, 2 mM glutamine, 1% bovine serum, and then stimulated with EGF (100 ng/ml). HeLa cells, grown to 70–90% confluency, were washed with ice-cold PBS and subjected to immunofluorescence microscopy. The cells were grown on glass coverslips. Cells were washed in Hanks' salts solution and then fixed for 10 min with 4% paraformaldehyde in PEM buffer (100 mM Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl2, and 0.2% Triton X-100). After washing in PBS, the cells were incubated with 10% fetal bovine serum in PBS-glycine for 30 min. They were then washed in PBS, sonicated, and incubated on x-ray film (Kodak). Positive plaques were then collected, plated, and rescreened.

**Immunoblotting and Immunoprecipitation**—Cells, grown to 70–90% confluency, were washed with ice-cold PBS and then scraped in a lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 25 mM NaF, 10 μM ZnCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were incubated on ice for 10 min and cleared by centrifugation for 10 min at 14,000 g, and the supernatants were collected and used in the assays. For immunoprecipitations, 1–4 mg of proteins were used. Antibodies were added to the cleared lysates and incubated for 2 h at 4 °C. Anti-rabbit or anti-mouse IgG-agarose beads were then added, and the incubation was continued for another 2 h at 4 °C. In some cases, cells were pretreated by incubating them with a secondary conjugate for 2 h at 4 °C. Beads were washed 3–5 times with the lysis buffer and then boiled in 2× Laemmli sample buffer for 5 min. The solubilized immunoprecipitates were separated by 10% SDS-PAGE, transferred onto nitrocellulose filters (Schleicher & Schuell) using a Semi-Dry Blottter (Kontes), and subjected to immunoblot analysis. The blots were developed by the ECL method. For repetitive probing, the filters were stripped for 40 min at 62 °C in a stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol), washed extensively, and reprobed.

**Kinase Assays**—The intracellular immunocomplex kinase assay was performed as follows. HER-14 cells were lysed (as above), and the lysates were used for immunoprecipulation using a monoconal anti-EGFR antibody and anti-mouse IgG-agarose beads. The beads were washed once, and one with 0.5% Nonidet P-40, 140 mM NaCl, 25 mM Hepes, pH 7.2, 1 mM MgCl2, 1 mM MnCl2, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM β-glycerophosphate. They were then resuspended in 40 μl of kinase buffer containing unlabeled ATP at a final concentration of 50 μM. Incubation was carried out for 20 min at 4 °C. The complexes were then washed twice with kinase buffer to remove the excess ATP. 5 μg of normal or mutated recombinant EAST protein and 6 μCi of [γ-32P]ATP were added, and the mixtures were incubated for 15 min at 37 °C. EAST was then collected by incubating with either glutathione-Sepharose 4B beads or anti-EAST antibody-conjugated IgG-agarose. The beads were then washed twice with kinase buffer, boiled in 2× Laemmli's sample buffer, and subjected to SDS-PAGE (as above) and autoradiography. The direct phosphorylation assay using a purified EGFR kinase domain (Stratagene) was done according to the manufacturer's instructions.

**Immunofluorescence Microscopy**—For immunofluorescence microscopy, the cells were grown on glass coverslips. Cells were washed in Hanks' salt solution and then fixed for 10 min with 4% paraformaldehyde in PEM buffer (100 mM Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl2, and 0.2% Triton X-100). After washing in PBS, the cells were incubated with 10% fetal bovine serum in PBS-glycine for 30 min. They were then overlaid with primary antibody for 30 min, washed, and incubated with tetramethylrhodamine isothiocyanate-conjugated secondary antibody (Caltag Laboratories) for another 30 min. Double-staining experiments were performed as described previously (20). The cells were viewed under an Olympus BH2 fluorescence microscope equipped with appropriate filters.

**GFP Fusion Proteins**—The full-length, NH2-terminal (aa 1–205) and COOH-terminal (aa 260–469) constructs were subcloned into the EcoRI/HindIII cloning site of the pR5K transfection vector (a gift from Dr. J. Schlessinger). Transient transfections were done using FUGENE 6-reagent (Boehringer Mannheim) according to the manufacturer's instructions. Mutations were produced using the QuickChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing.

**Immunofluorescence Microscopy**—For immunofluorescence microscopy, the cells were grown on glass coverslips. Cells were washed in Hanks' salt solution and then fixed for 10 min with 4% paraformaldehyde in PEM buffer (100 mM Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl2, and 0.2% Triton X-100). After washing in PBS, the cells were incubated with 10% fetal bovine serum in PBS-glycine for 30 min. They were then overlaid with primary antibody for 30 min, washed, and incubated with tetramethylrhodamine isothiocyanate-conjugated secondary antibody (Caltag Laboratories) for another 30 min. Double-staining experiments were performed as described previously (20). The cells were viewed under an Olympus BH2 fluorescence microscope equipped with appropriate filters.
A

1  MELSASNPYE GVSEKATNKH NSNDGMLIM DICKQGVP STGNGCRAF
51  MRRVNHVPM VAGCALTLLG ACVSQGRIF KHVCSQFVPA TEARGWNNKA
101  KQVVSPLKNT IMVYVREEQF KQPQCLLSIA TIKSLKKEGV TTPAASGQT
151  TNAARKSSIL EKMKEDIDIR KAEISLQEQI KQKMETQSKL YPSARIQQTN
201  QQNLARKVAL YPEAVENDEL YFEQSETF YLVQDTUWKK GNNRHGSVLP
251  EQSNTSIVDI VEGFPTVQYQ SEVQDANTTE EKKAAREPAV IDERDMQRT
301  VQLOSIDPOY LNLDTHLDOLK EVTQCOEQPM DSEEKLDIDIR KHEISLQDNV
351  KVLKAELEK QMSETPMYS AYXCLHESQA QYPTSESGSV QSYVQFQSG
401  KNYMCQGQIVQ TVSGQYGLGP DQMQLASLP QNNISSXCLN YTKSBRQMC
451  HNKAITQWR FKSKTHER*

Fig. 1. Structure and predicted amino acid sequence of EAST. A, amino acid sequence of EAST. The SH3 domain is underlined, and the TAM is in bold type. B, schematic representation of the structure of EAST.

RESULTS

Sequence and Domain Structure of EAST—The amino acid sequence of EAST, deduced from the full-length cDNA isolated and sequenced as described under “Experimental Procedures,” is shown in Fig. 1A. The protein consists of 468 amino acids with a calculated molecular mass of 52,406 Da. The methionine start codon is in partial agreement with the Kozak consensus sequence. The open reading frame ends with two sequential start codon is in partial agreement with the Kozak consensus with a calculated molecular mass of 52,406 Da. The methionine

Association of EAST with EGFR—Several tyrosine-phosphorylated proteins, representing putative EAST-interacting proteins, co-precipitated with EAST (data not shown). The most prominent of these had a molecular mass of about 180 kDa, suggestive of EGFR. This was confirmed by immunoblotting with anti-EGFR antibody (Fig. 5A). The co-immunoprecipitation of EGFR with EAST, seen even in serum-starved, non-stimulated cells, was clearly enhanced after EGFR treatment (Fig. 5A). Immunoprecipitation with anti-EGFR antibodies and blotting with anti-EAST antibodies again demonstrated a distinct co-immunoprecipitation of the two proteins, the extent of which was significantly increased upon EGFR treatment (Fig. 5B). The results demonstrate an EGFR-dependent association between EGFR and EAST.

To determine whether the EGFR-induced phosphorylation of EAST is because of a direct action of EGFR or is mediated by some other proteins, we performed an in vitro immunocomplex kinase assay utilizing specific anti-EGFR antibodies to immunoprecipitate EGFR and bacterially produced EAST as a substrate; EAST was phosphorylated under these conditions (Fig. 6A). Although suggestive of a direct effect of EGFR on EAST, it does not rule out the possibility that the phosphorylation is because of some other kinase(s) present in the immunocomplex. Therefore, the experiment was repeated by using purified, recombinant EGFR kinase domain and bacterially produced...
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**Fig. 2. Sequence alignments.** A, sequence alignment of the TAM of EAST with a representative set of TAMs. B, alignment of the amino acid sequence of EAST with STAM, VPS27, Hrs, and Tom-1b. A single fully conserved residue is indicated by an *asterisk*, and a strong (>60%) conservation is indicated by a *dot*. The residues are *shaded* according to the following scheme: white letter on a black background and black letter on a gray background when an identical residue or similarity, respectively, has an occurrence of more than 60% in a position. The accession numbers of the sequences are as follows: EAST, AJ224514; STAM, U43900; Tom-1b, Y08741; Hrs, D84064; VPS27, P40344.

EAST. Fig. 6B shows that EAST was also phosphorylated under these conditions. Then we repeated the experiment by digesting the fusion protein with thrombin, which leads to a cleavage of EAST from its fusion partner GST. A distinct phosphorylation of EAST was seen, verifying that EAST is the target of the kinase activity (Fig. 6B). We also made a preliminary attempt to characterize the sites of phosphorylation in EAST. For that purpose, NH2- and COOH-terminal parts of EAST were expressed as fusion proteins and subjected to phosphorylation assay as above. Phosphorylation of both fusion proteins was seen with a slightly stronger intensity in the NH2-terminal half (Fig. 6B). These results strongly suggest that EAST is a direct substrate of EGFR. However, there are no typical motifs, such as SH2 and phosphotyrosine-binding domains, that could serve as EGFR-binding sites in EAST. On the other hand, there are two tyrosine residues, Tyr359 and Tyr372, in the TAM domain. By analogy with other TAM-containing proteins, these residues are potential phosphorylation sites. To investigate the possibility that they are the targets of the EGF-induced phosphorylation, both residues were mutated to phenylalanines, and the phosphorylation assay was repeated using the mutated EAST as a substrate. The results indicate that the wild-type and mutated EAST are phosphorylated to similar extents (Fig. 6B), suggesting that tyrosines other than Tyr359 and Tyr372 of the TAM are the phosphorylation targets of the EGFR.

Association of EAST with Eps15—Eps15 is an EGFR substrate (12) with a molecular mass of about 150 kDa. A band of that size was present on the Tyr(P) immunoblot of the anti-EAST immunoprecipitate (data not shown). Reciprocal immunoprecipitation/immunoblotting experiments using anti-eps15 and anti-EAST antibodies revealed that eps15 and EAST co-immunoprecipitated. This association was independent of EGF treatment, because equal amounts of coprecipitated proteins were present in the immunocomplexes before and after treatment of HER-14 cells with EGF (Fig. 7).

In *vitro* studies with synthetic peptides have shown that eps15 homology domains of eps15 recognize a short core motif, NPF, in interacting proteins (26, 27) and that the interaction can be abolished by a point mutation of any of these three amino acids. An NPF motif is present in the NH2 terminus of EAST (aa 7–9). To test whether this motif mediates a direct interaction between EAST and eps15, we overexpressed an HA-tagged, mutated NH2-terminal EAST (aa 1–205) in HER-14 cells and performed immunoprecipitations with anti-HA antibody. The substitution of Asn by Asp could not abolish the association between EAST and eps15 (data not shown).

**Immunolocalization of EAST**—A single band of 68 kDa was recognized by affinity-purified anti-EAST antibodies in immunoblot analyses of CEHF lysates (Fig. 3B), whereas no bands were seen with the preimmune serum (data not shown). A closer examination of the staining revealed a dotted pattern.
or vesicular membrane-associated staining. Often, it was also seen in cell protuberances, which probably represent membrane ruffles. In some cells, the staining pattern partially coincided with actin fibers and focal adhesions (data not shown).

In view of the association between EAST and the endocytosis-associated eps15, we investigated the subcellular distribution of EAST in relation to proteins involved in receptor-mediated endocytosis. This was studied in HeLa cells using antibodies to clathrin, a marker for clathrin-coated pits and vesicles formed during the early stages of endocytosis; EAST displayed a membrane-associated distribution, with spot-like condensations (Fig. 9A), and a partial colocalization with clathrin (Fig. 9B). This strongly suggests that EAST is localized in structures associated with endocytosis.

**Fig. 5.** Association of EAST with native EGFR. HER-14 cells were serum-starved overnight and then treated with EGF (100 ng/ml) for 10 min. A, anti-EAST immunoprecipitates (IP) were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using anti-EGFR antibody (upper panel). The filters were stripped and reprobed with anti-EAST antibody (lower panel). B, anti-EGFR immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting using anti-EAST antibody (upper panel). The filters were stripped and reprobed with anti-EGFR antibody (lower panel).

**Fig. 6.** Tyrosine phosphorylation of wild-type and mutated EAST by EGFR. A, in vitro immunocomplex kinase assay; EGFR was immunoprecipitated from HER-14 cell lysates using anti-EGFR antibody. The immunocomplexes were then incubated with EAST in the presence of [γ-32P]dATP. B, kinase assay with purified EGFR kinase domain in the presence of [γ-32P]dATP. The proteins were separated by SDS-PAGE and visualized by autoradiography. WT, wild-type full-length EAST; FF-TAM, full-length EAST with Tyr359 and Tyr372 of the TAM mutated to phenylalanines; NT, NH2 terminus of EAST; CT, COOH terminus of EAST; t.d., thrombin digestion.

**Fig. 3.** Expression of EAST in chicken tissues and cell lines. A, Northern hybridization analysis. kb, kilobases. B, immunoblot analysis.

**Fig. 4.** Tyrosine phosphorylation of EAST. HER-14 cells were serum-starved overnight and then treated with EGF (100 ng/ml). The anti-EAST immunoprecipitates (IP) were resolved by SDS-PAGE and analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody.

**Fig. 9.** Distribution of EAST in relation to clathrin (A) and clathrin-coated pits and vesicles (B).
COOH terminus, although quite distinct, was still associated with vesicular structures. This may indicate that the NH2- and COOH-terminal regions of EAST associate with different endocytotic vesicles at different stages of endocytosis. We were unable to study the localization of overexpressed, full-length, EAST protein in cultured cells because of its rapid degradation.

DISCUSSION

In this study we report the cloning and characterization of a new 68-kDa protein, which we have named EAST. It contains an SH3 domain, a TAM domain, and an NH2-terminal region similar to the NH2 termini of proteins involved in endocytosis. EAST associates with eps15 and EGFR. In immunofluorescence microscopy, a membrane-associated and vesicular staining, coinciding with that of clathrin, was seen.

The present results link EAST to both EGFR and eps15, suggesting that EAST could serve as a linker between these two molecules. By virtue of its TAM and SH3 domains, EAST appears to be a bona fide signaling protein that could play a role in pathways involving tyrosine kinases. This was demonstrated by showing that EAST co-immunoprecipitates with the EGFR from lysates of EGF-treated HER-14 cells and that it becomes phosphorylated on tyrosine in response to EGF. A distinct phosphorylation of EAST was seen already within 1 min after stimulation, a time scale that corresponds well to the autophosphorylation of EGFR. The association between EAST and the EGFR was confirmed in reciprocal co-immunoprecipitation experiments using anti-EGFR and anti-EAST antibodies. Significantly more EGFR and EAST were seen complexed after EGF treatment, indicating that the phosphorylation of either one or both of the components enhances the interaction.

Co-immunoprecipitation experiments to determine whether eps15 interacts with EAST were prompted by the presence of a band of about 150 kDa in anti-EAST immunoprecipitates, and an interaction between eps15 and EAST could be confirmed by reciprocal co-immunoprecipitation using anti-eps15 and anti-EAST antibodies. Contrasting with the case of EGFR, there was no difference between naïve and EGF-treated cells in the amount of EAST co-immunoprecipitating with eps15, indicating that the eps15-EAST interaction is not dependent on EGF-induced phosphorylation. Whether the interaction is direct or not remains yet to be elucidated, because the association between EAST and eps15 could not be abolished by mutating the NPF motif of EAST.

Originally, eps15 was isolated in an effort to uncover as yet uncharacterized effector pathways emanating from the EGFR and erbB-2/neu receptors (12). Eps15 has a unique domain structure. There are three eps15 homology domains in the amino terminus, and DPF (aspartic acid-proline-phenylalanine) repeats in the carboxyl terminus. In the central region, there is a domain with an α-helical conformation and a probable coiled-coil supramolecular organization. From the immunoprecipitation experiments using lysates of unstimulated cells and cells stimulated either with EGF or with other growth factors, it was concluded that the phosphorylation of eps15 is relatively specific to the EGF and the EGFR (12, 16). However, attempts to demonstrate a direct interaction between the EGFR and eps15 failed, leaving unanswered the question of whether eps15 is a direct substrate of the EGFR or whether it binds to and is phosphorylated by a kinase(s) downstream of the EGFR (12). More recently, Schumacher et al. (13) isolated eps15 in an expression library search for proteins that bind to the SH3 domain of Crk. They suggested that Crk might be a crucial link between the EGFR and eps15 in the mitogenic signaling emanating from the EGFR. However, the interaction between eps15 and Crk could not be shown by co-immunoprecipitation from cell lysates.

FIG. 7. Association of EAST with native eps15. HER-14 cells were serum-starved overnight and treated with EGF (100 ng/ml) for 10 min. A, anti-EAST immunoprecipitates (IP) were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using anti-eps15 antibody (upper panel). The filters were stripped and reprobed with anti-EAST antibody (lower panel). B, anti-eps15 immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting using anti-EAST antibody (upper panel). The filters were stripped and reprobed with anti-eps15 antibody (lower panel).

FIG. 8. Immunolocalization of EAST in CEHFs. A, CEHFs stained with anti-EAST antibody. B, CEHFs stained with anti-EAST antibody pretreated with the antigen. Bar, 10 μm.
The present results clearly show an association between EAST and EGFR on the one hand and between EAST and eps15 on the other hand. Moreover, immunoprecipitation with anti-EAST antibodies also brings down both the EGFR and eps15, strongly suggesting that EAST serves as a “bridge” between the EGFR and eps15. The notion of a direct interaction between EAST and the EGFR has to be reconciled with the fact that in EAST there are no domains, such as SH2 or phosphotyrosine binding, that are usually found in proteins binding to activated receptor tyrosine kinases. The lack of an obvious binding motif is not unique to EAST, however, because c-Cbl, a proto-oncogene protein that becomes strongly phosphorylated and associates with activated EGFR, is also devoid of a distinct receptor-binding motif (28). The possibility of nonphosphotyrosine-mediated binding of the cytoplasmic tail of EGFR should also be considered. In fact, phosphorylation of eps15 occurs even in cells carrying a mutant EGFR in which all five auto-phosphorylation sites are abolished (29), suggesting that the association of eps15 with the EGFR, whether direct or indirect, is not based on “conventional” phosphotyrosine recognition.

Recent studies have convincingly shown that eps15 is closely associated with the endocytic machinery. First, it associates with several proteins known to be components of clathrin-coated pits and vesicles, including clathrin and adaptor protein 2 (14–16). Second, it localizes to the coated pits (17). Third, microinjection of anti-eps15 antibodies to cells inhibits the internalization of the EGFR and of the transferrin receptor (18). In view of the distinct role(s) of eps15 in endocytosis and receptor internalization, it is reasonable to propose that EAST also plays a role in these processes.

In immunofluorescence microscopy, EAST exhibited a general membrane-associated staining and spot-like condensations, which, by their staining with anti-clathrin antibodies, were identified as clathrin-coated pits and vesicles. Thus, both the physical interaction between eps15 and EAST and the codistribution of both proteins with clathrin-associated vesicles are indicative of a role for EAST in endocytosis. Its exact role in coated pit formation or regulation remains, however, to be elucidated. It is tempting to speculate that EAST could serve as a linker between the EGFR and eps15 and thus contribute to the internalization of the EGFR.

Protein data bank sequence comparisons revealed a number of proteins exhibiting sequence similarity with EAST. The highest degree of homology was seen with STAM, a protein originally discovered on the basis of its tyrosine phosphorylation in response to interleukin-2 treatment (22). EAST and STAM share the same SH3/TAM domain organization, which would warrant the claim that they are parent members of a new class of signal transduction proteins with this signature. To our knowledge, EAST and STAM are the first examples of nonreceptor proteins possessing a TAM. Given the role of the TAM in immunoreceptors as a docking site for various nonreceptor kinases, we hypothesize that EAST also has a bridge role, by relaying the linkage between EGFR and the endocytic machinery. The putative TAM-binding partner remains to be identified.

A local homology was seen between the NH2 termini of EAST, VPS27, Hrs, and Tom-1. Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) is a phosphotyrosine protein induced by hepatocyte growth factor, EGF, and platelet-derived growth factor (24), and it was recently shown to be associated with early endosomes (30). Hrs-2, a homologue of Hrs, was shown to be associated with SNAP-25 and is thought to modulate vesicular transport (31). VPS27 is also implicated in vesicular trafficking in yeast, in which it controls vacuolar and endocytic traffic through a prevacuolar compartment (23). An association with vesicular/endosomal trafficking appears to be a common denominator between EAST, STAM, Hrs, and VPS27, suggesting a role for the homologous NH2-terminal region in the regulation of these processes. More direct evidence supporting this notion was obtained in transfection experiments, in which the NH2-terminal portion of EAST colocal-
ized distinctly with clathrin. The fourth member of the group, Tom-1 (25), is only partially characterized, and its function is still unknown.

In conclusion, the present data strongly suggest that EAST is involved in the early stages of endocytosis and, more specifically, in the formation of clathrin-coated pits and vesicles. Hence, EAST may play an important role in the down-regulation of receptor tyrosine kinases, especially the EGFR, and may thus regulate the signaling potency of the EGFR. We propose that EAST serves as a scaffolding device in receptor-mediated endocytosis, bringing together the EGFR, eps15, eps15-associated proteins, and other uncharacterized components of the complex machinery of the early stages of endocytosis.

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REFERENCES

1. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080
2. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Science 259, 1157–1161
3. Pawson, T., and Schlessinger, J. (1993) Curr. Biol. 3, 434–442
4. Zhou, M. M, Ravichandran, K. S., Olejniczak, E. F., Petros, A. M., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W. S., Burakoff, S. J., and Fesik, S. W. (1995) Nature 378, 584–592
5. Keegan, A. D., and Paul, W. E. (1992) J. Biol. Chem. 267, 16271–16276
6. Songyan, Z., Sheslon, E. S., Mcglade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Rafnfsky, S., Feldman, R. A., and Canzler, L. C. (1994) Mol. Cell. Biol. 14, 2777–2785
7. Yu, J. C., Heidaran, M. A., Pierce, J. H., Gutkind, J. S., Lombardi, D., Ruggiero, M., and Aaronsen, S. A. (1991) Mol. Cell. Biol. 11, 3780–3785
8. Mohammadi, M., Tik, I., Sorokin, A., Burgess, W. H., Jaye, M., and Schlessinger, J. (1996) Mol. Cell. Biol. 16, 977–989
9. Riezman, H., Woodman, P. G., van Meer, G., and Marsh, M. (1997) Cell 91, 731–738
10. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
11. Seaman, M. N. J., Burd, C. G., and Emr, S. D. (1996) Curr. Opin. Cell Biol. 8, 549–556
12. Fazioli, F., Minichiello, L., Matoskova, B., Wong, W. T., and Di Fiore, P. P. (1993) Mol. Cell. Biol. 13, 5814–5828
13. Schumacher, C., Knudsen, B. S., Obuchi, T., Di Fiore, P. P., Glassman, R. H., and Hanafusa, H. (1995) J. Biol. Chem. 270, 15341–15347
14. Benmerah, A., Gagnon, J., Begue, B., Megerbance, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1995) J. Cell Biol. 131, 1831–1838
15. Benmerah, A., Begue, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1996) J. Biol. Chem. 271, 12111–12116
16. van Delﬁ, S., Schumacher, C., Hage, W., Verkleij, A. J., and Henegouwen, P. M. P. (1997) J. Cell Biol. 136, 811–821
17. Tebar, F., Sorkina, T., Sorokin, A., Ericsson, M., and Kirchhausen, T. (1996) J. Biol. Chem. 271, 28727–28730
18. Carbone, R., Fre, S., Iannolo, G., Belleudi, F., Mancini, P., Pelicci, P. G., Torrisi, M., and Di Fiore, P. P. (1997) Cancer Res. 57, 5498–5504
19. Sambrook, J., Prichard, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Meriäinen, J., Lehto, V.-P., and Wasenius, V.-M. (1997) J. Biol. Chem. 272, 22978–22984
21. Rotin, D., Margolis, B., Mohammadi, M., Daly, R. J., Daum, G., Li, N., Fischer, E. H., Burgess, W. H. M., Ulrich, A., and Schlessinger, J. (1992) EMBO J. 11, 559–567
22. Takeshita, T., Arita, T., Asan, H., Tanaka, N., Higuchi, M., Kuroda, H., Kaneko, K., Munakata, H., Endo, Y., Fujita, T., and Sugamara, K. (1996) Biochem. Biophys. Res. Commun. 235, 1035–1039
23. Piper, R. C., Cooper, A. A., Yang, H., and Stevens, T. H. (1995) J. Cell Biol. 131, 603–617
24. Komada, M., and Kitamura, N. (1995) Mol. Cell. Biol. 15, 6213–6223
25. Burk, O., Worpenberg, S., Haenig, B., and Klempnauer, K. H. (1997) EMBO J. 16, 1371–1380
26. Wong, W. T., Schumacher, C., Salcini, A. E., Romano, A., Castagnino, P., Pelicci, P. G., and Di Fiore, P. P. (1995) Proc. Natl Acad. Sci U S A. 92, 9530–9534
27. Salcini, A. E., Confoleni, S., Deria, M., Santolini, E., Tassi, E., Menenkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997) Genes Dev. 11, 2239–2249
28. Galisteo, M. L., Dikic, I., Batzer, A. G., Langdon, W. Y., and Schlessinger, J. (1995) J. Biol. Chem. 270, 20242–20245
29. Alvarez, C. V., Shon, K.-J., Miloso, M., and Bequinot, L. (1995) EMBO J. 14, 2701–2706
30. Kaneko, K., Munakata, H., Endo, Y., Fujita, T., and Sugamara, K. (1996) Biochem. Biophys. Res. Commun. 235, 1035–1039
31. Sanger, F., Nicklen, S., and Coulsen, A. R. (1977) Proc. Natl Acad. Sci U S A. 84, 5463–5467