EAST, a novel EGF receptor substrate, associates with focal adhesions and actin fibers

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Abstract EAST is a novel epidermal growth factor receptor (EGFR) substrate. It interacts with Eps15, another EGFR substrate which is involved in receptor endocytosis. In this study we show that EAST associates with focal adhesions and actin filaments. First, in immunofluorescence and electron microscopy analysis, an extensive codistribution of EAST with vinculin, paxillin and actin filaments was seen. Second, overexpression of the NH_2 terminus of EAST led to a formation of actin-rich microspikes and membrane protrusions. Third, in cosedimentation assay EAST showed a direct association with actin. These results suggest that EAST is involved in the EGFR-regulated reorganization of the actin cytoskeleton and may be part of a link between cytoskeleton and endocytic machinery.

Key words: Actin; EAST; EGFR; Focal adhesion

1. Introduction

Epidermal growth factor receptor (EGFR) is a prototype of the receptor protein-tyrosine kinases (RPTK) and serves as a thoroughly studied model of RPTK-mediated signal transduction. Its signaling is initiated by ligand-induced dimerization and subsequent auto-/transphosphorylation of the receptor [1]. Specific phosphorylated tyrosines then serve as binding sites for the downstream SH2 domain-containing effector molecules.

Besides initiating mitogenic signaling, binding of EGF to the EGFR also results in dramatic changes in cell morphology [2]. These include formation of membrane ruffles, cell rounding and increased actin polymerization which is dependent on the tyrosine kinase activity of the receptor [2,3]. A number of studies have shown that the EGFR is associated with the actin cytoskeleton, and cosedimentation of the EGFR with purified actin has been demonstrated [4]. Based on such observations, cytoskeleton has been suggested to play an important role in EGF-induced signal transduction [5].

We have recently cloned a novel EGFR and Eps15-associated protein EAST (EGFR-associated protein with SH3 and TAM domains), which serves as a substrate for the EGFR and Src kinase [6,7]. It also associates with Eps15, a well-established component of the machinery driving endocytic uptake of EGFR [8]. Based on its properties, we have suggested that EAST is involved in the endocytosis of the EGFR [6]. In this study, we show that EAST associates with focal adhesions and actin filaments, and, thus, suggest that EAST is also involved in the EGFR-initiated cytoskeletal reorganization.

2. Materials and methods

2.1. Cell culture

Human embryonal skin fibroblasts (HES) and HeLa cells were maintained in EMEM (Gibco BRL) containing 10% heat-inactivated fetal bovine serum (HyClone), 2 mM glutamine, 1% nonessential amino acids and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B). HER-14 and MDCK cells were grown in DMEM (Gibco BRL) and EMEM, respectively, containing 10% heat-inactivated fetal bovine serum and antibiotics.

To disrupt the actin cytoskeleton, HES cells were treated with 1 μM cytochalasin D (Sigma) for 1 h at 37°C before fixation.

Transient transfections and the cDNA constructs were as described previously [6].

2.2. Immunofluorescence and electron microscopy

For immunofluorescence microscopy, cells were grown on glass coverslips and fixed with 4% paraformaldehyde in PEM buffer (100 mM PIPES, pH 6.8, 5 mM EGTA, 2 mM $MgCl_2$) with 0.2% Triton X-100 for 10 min, followed by post-fixation in −20°C ethanol for 2 min. The rest of the staining procedure was as described previously [6,9]. Cells were viewed under an Olympus BH2 fluorescence microscope equipped with appropriate filters.

For immunoelectron microscopy, HES cells were grown on plastic to near confluence, fixed and stained (see above). The samples were post-fixed with 2% glutaraldehyde for 30 min at room temperature, embedded in epon-polymer and prepared for viewing in transmission electron microscopy mode in a Philips 410 LS microscope.

For double-labeling experiments, monoclonal antibodies to vinculin (a gift from Prof. Ismo Virtanen) and to paxillin (Zymed) were used alongside with the EAST antibody [6]. For fluorescence microscopy, labeling of actin was done by using bodipy-conjugated phallacidin (Molecular Probes). For electron microscopy, actin was visualized by using monoclonal actin antibody C4 (Boehringer Mannheim). The polyclonal hemagglutinin (HA) antibody (Y-11) was from Santa Cruz Biotechnology. The binding of the antibodies was visualized by using Texas Red (Jackson ImmunoResearch Laboratories)- or fluorescein isothiocyanate (Caltag Laboratories)-conjugated antibodies to rabbit or mouse IgG.

2.3. Actin cosedimentation assay

The assay was performed essentially as described by Wu et al. [10]. Briefly, chicken gizzard actin (Sigma) was allowed to polymerize in F buffer (2 mM Tris-HCl, pH 8.0, 1 mM ATP, 0.2 mM DTT, 0.2 mM $CaCl_2$, 50 mM KCl and 2 mM $MgCl_2$) at 37°C for 1 h. Then the polymerized actin was incubated with the bacterially produced GST-EAST fusion protein on ice for an additional hour. After high-speed centrifugation (130 000 × *g*, 1 h, at 4°C), the supernatant and the pellet were separated on 10% SDS-PAGE and analyzed by Coomassie Brilliant Blue staining.

The production and the purification of the NH_2-terminal GST-EAST fusion protein (aa 4–188) has been described previously [6].

3. Results

In order to study the subcellular localization of EAST, we carried out immunofluorescence microscopy on cultured cells

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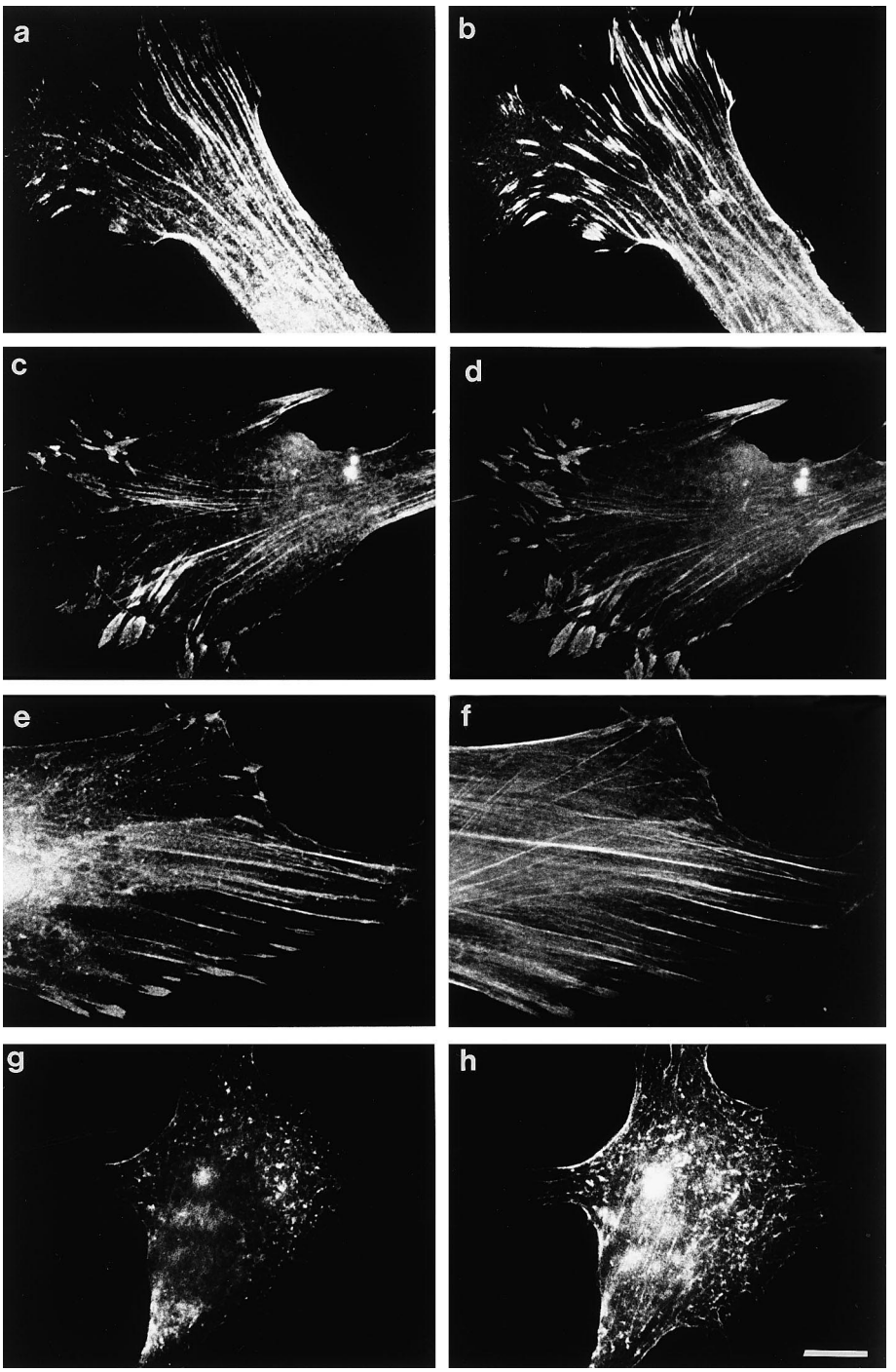

Fig. 1. Immunofluorescence localization of EAST. Double-immunofluorescence staining for EAST (a, c, e, g) and for vinculin (b), paxillin (d) and actin (f, h) in HES cells. In figures g and h, the cells were pretreated with cytochalasin D before fixation. Bar = 10 μm.

by using the EAST antibody [6]. In human embryonal skin fibroblasts (HES), EAST displayed three distribution patterns: (i) as patches at the ventral surface of the cells, resembling focal adhesions, (ii) along filamentous structures contiguous with the ventral patches and resembling actin fibers, and (iii) less conspicuous 'spot'-like structures, possibly representing vesicular structures. The patch-like structures were seen especially along the cell perimeter in the protruding areas of the cell, compatible with the notion that they represent focal adhesions (Fig. 1a, c, e).

In order to positively identify the subcellular structures associated with EAST, double-staining experiments were performed by using as 'markers' antibodies to two well-known focal adhesion proteins, vinculin and paxillin. A virtually complete colocalization of EAST with both vinculin (Fig. 1a, b) and paxillin (Fig. 1c, d) was seen, validating the idea that the 'patches' represent focal adhesions. An extensive co-distribution was also seen with filamentous actin in double-staining experiments employing the EAST antibody and the bodipy-conjugated phallacidin; EAST staining overlapped with the phallacidin-decorated actin fibers (Fig. 1e, f). Intriguingly, EAST was not seen homogenously along the entire

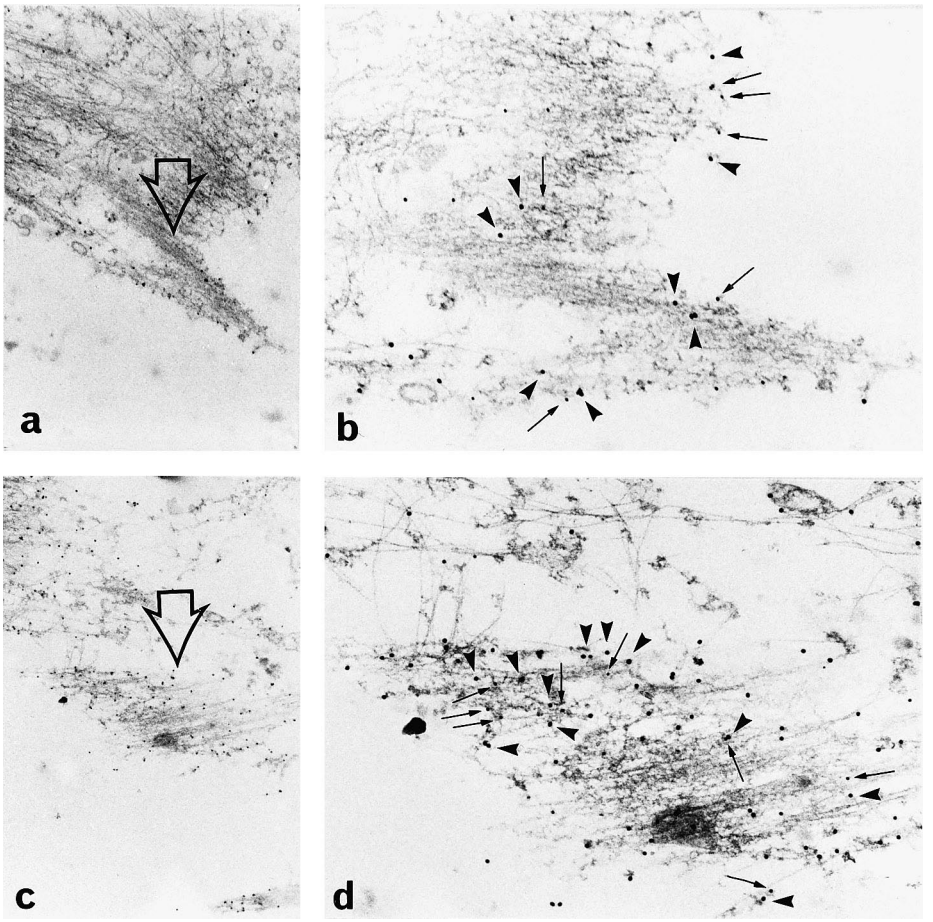

Fig. 2. Immunoelectron microscopical localization of EAST. Low (a, 14000×; c, 12000×) and high (b, d) magnification transmission electron migrographs of HES cells double-stained for EAST and vinculin (a, b), and for EAST and actin (c, d). Binding of the EAST antibody was detected by using anti-rabbit Ig conjugated with small (10 -nm) gold particles (arrows). Binding of vinculin and actin antibodies was visualized by using anti-mouse Ig conjugated with larger (15 nm) gold particles (arrowheads).

lengths of the individual fibers, but, instead, was more conspicuous in the focal adhesion-associated ends of the fibers.

A similar distribution of EAST as in HES cells was also seen in MDCK and HeLa cells, indicating that the location is not species or cell-type specific. Furthermore, in MDCK cells, EAST could also be seen in cell-cell contact areas (not shown). Controls of the immunostaining experiments included staining with the EAST antibody preincubated with the bacterially produced EAST; no staining was seen, attesting to the specificity of the staining patterns (not shown).

It is important to note that the staining pattern described above was only seen when the cells were fixed by using a cross-linking fixative paraformaldehyde followed by Triton X-100 treatment, a widely used procedure which is considered to preserve most of the cytoskeletal elements. If methanol, another widely used fixative which precipitates but does not cross-link proteins, was applied, only the vesicular staining was seen while the filamentous and focal adhesion-associated staining was abolished or greatly attenuated (not shown). This altered staining pattern could not be due to disruption of these structures upon methanol treatment, since both actin fibers and focal adhesions could be seen in methanol-fixed cells (not shown). Neither do we believe that it is due to masking effect by the fixative. The most likely explanation is that actin fiber- and focal adhesion-associated EAST is solubilized and extracted by methanol. This would suggest that there are two types of EAST in cells; one associated with actin which is methanol extractable and the other associated with the vesicles which resists methanol fixation.

Cytochalasin D prevents actin polymerization and causes disruption of the actin-based cytoskeleton [11]. Due to this property, it is widely used as a probe to explore the actin dependency of the distribution of cell organelles and proteins. We therefore asked whether it would affect the localization of EAST in cultured cells. Upon treatment of HES cells with cytochalasin D, disruption of the actin fibers was seen; instead of fibers, F-actin was seen as distinct spots (Fig. 1h). In double-staining, EAST was seen in the same spots (Fig. 1g). This indicates that distribution of EAST is dependent on the integrity of the actin cytoskeleton and strongly suggests that EAST is closely associated with the actin cytoskeleton.

We also used immunoelectron microscopy to study the subcellular localization of EAST. As is shown in Fig. 2, EAST colocalizes in immunoelectron microscopical analysis with both vinculin (Fig. 2a, b) and actin (Fig. 2c, d). These results further strengthen the notion that EAST is a component of focal adhesions and actin fiber assemblies.

We next wanted to know whether overexpression of EAST affects the organization of the actin cytoskeleton. For that purpose, we transfected cells with cDNAs encoding hemag-

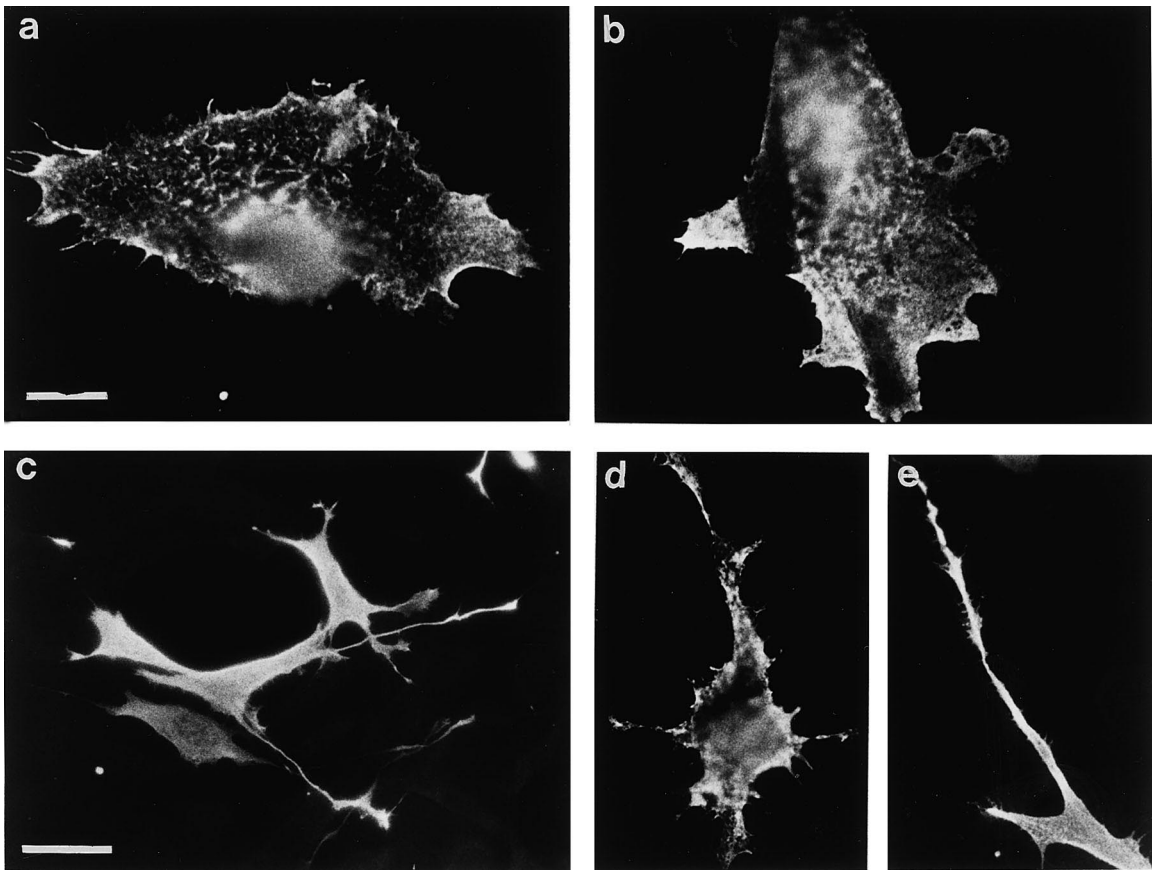

Fig. 3. Overexpression of the NH_2 terminus of EAST. HeLa (a), MDCK (b) and HER-14 (c–e) cells were transfected with the HA-tagged NH_2-terminal part of EAST, fixed and stained by using anti-HA antibody. Bars=10 μm (a, b, d, e) and 20 μm (c).

glutinin epitope (HA)-tagged NH_2- (aa 1–205) and COOH-terminal (aa 260–469) fragments of EAST and detected the expressed polypeptides by using anti-HA antibodies. The NH_2-terminal portion of EAST encompasses the VHS domain, a distinct domain found in a group of proteins involved in vesicular trafficking ([12], Lohi and Lehto, submitted). The COOH-terminal portion of EAST contains the TAM motif, known to serve as a binding site for SH2 domain-containing proteins, e.g. in immunoreceptors [13]. We were unable to overexpress the full-length EAST and the SH3 domain of EAST, due to their rapid degradation and/or toxicity to the cells.

In HeLa (Fig. 3a) and MDCK cells (Fig. 3b), transient overexpression of the NH_2 terminus of EAST led to a conspicuous increase in a number and length of microspikes, and to a formation of large cell protrusions. In HER-14 cells, the formation of microspikes and long foot-like projections was also seen (Fig. 3c–e). They all appeared strongly positive in staining with the anti-HA antibody demonstrating the localization of the expressed polypeptides in these cell compartments. The cells also exhibited membrane ruffles along the dorsal surface of the cell. The abundance of actin in microspikes and pseudopod-like cell protrusions and the mechanistic role of actin in their emergence [14] suggest that the NH_2 terminus of EAST induces the reorganization of actin.

The COOH terminus of EAST was localized in transfection studies to perinuclear vesicles [6]. No formation of membrane protrusions or microspikes was seen. Interestingly, the COOH-terminal EAST-containing vesicles frequently formed linearly arranged 'chains', as if they were positioned along cytoplasmic filaments (not shown).

In order to study whether there is a direct association between EAST and actin, we performed an actin cosedimentation assay. Bacterially produced NH_2-terminal GST-EAST fusion protein was incubated with the polymerized actin. The reaction mixture was then subjected to high-speed centri-

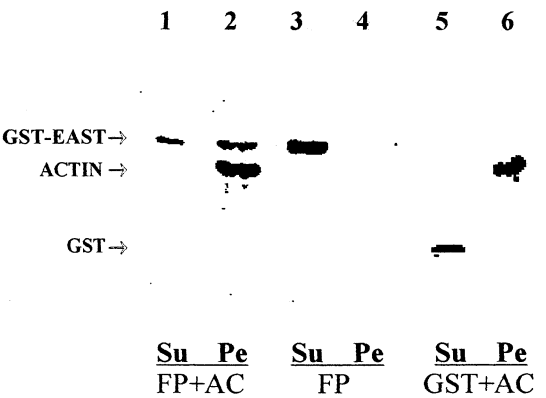

Fig. 4. Actin cosedimentation assay. Polymerized actin (AC) was incubated with the NH_2-terminal GST-EAST fusion protein (lanes 1 and 2) or GST (lanes 5 and 6), or the NH_2-terminal GST-EAST fusion protein (FP) was incubated alone (lanes 3 and 4). The supernatant (Su) and pellet (Pe) fractions were then fractionated in SDS-PAGE and stained with Coomassie Brilliant Blue.

fugation and separated on SDS-PAGE. As shown in Fig. 4, the NH_2-terminal part of EAST pelleted with the polymerized actin (Fig. 4, lanes 1 and 2). When, as a control, the fusion protein was centrifuged alone, it remained in the supernatant (Fig. 4, lanes 3 and 4). GST, the fusion partner of bacterially produced EAST, did not pellet with the polymerized actin (Fig. 4, lanes 5 and 6). These results show that there is a direct association between F-actin and EAST in vitro, and that the interaction is mediated by the NH_2 terminus of EAST.

4. Discussion

The present results show that EAST binds and colocalizes with actin and colocalizes with actin-associated focal adhesion components. Moreover, overexpression of the NH_2-terminal part of EAST elicits morphological changes that are compatible with the reorganization of the actin cytoskeleton. In view of the direct association between EGFR and EAST [6], this suggests that EAST may participate in EGF-induced changes in the cellular cytoskeleton.

There are numerous studies which indicate that altered morphology, cytoskeletal changes and actin reorganization are integral parts of EGF-induced phenotypic changes in cultured cells [15,16]. In regard to actin, EGF treatment stimulates actin polymerization and formation of actin-containing membrane ruffles [2]. Also, a formation of a signaling complex has been proposed which includes EGFR, PLC-γl and filamentous actin [17]. In accordance with this, direct association between the EGFR and actin has been shown and the actin binding site in EGFR has been identified [4]. However, the molecular mechanisms that mediate the cytoskeletal effects of EGF are mostly unknown.

The role of the actin cytoskeleton in receptor function is unclear. Experimental evidence for two mechanisms have been presented. One is that the association with the cytoskeleton facilitates the function of receptor [18]. The other postulates that binding of the receptor to the actin cytoskeleton would allow a precise localization of the receptor and its associated signaling compartments in the cell surface-cytoplasm interface [5]. It is also known that actin cytoskeleton is critically involved in endocytosis, a pivotal mechanism of growth factor receptor downregulation [19]. This is indicated, e.g. by the results which show that actin cytoskeleton is essential in receptor-mediated endocytosis [20] and that Rac and Rho, the well-known regulators of actin reorganization [21,22], are involved in endocytosis [23]. On the other hand, profilins form complexes with proteins involved in both the regulation of actin assembly and the vesicular trafficking, and are suggested to link the actin cytoskeleton to endosomal structures [24]. Recently, a novel member of the Rho family, RhoD, was cloned and shown to regulate both the endosome dynamics and the arrangement of cell surface and actin cytoskeleton [25].

We have suggested that EAST is involved in the endocytosis of EGFR [6]. This is based on the following observations: First, EAST associates with Eps15, another EGFR substrate which is indispensable for EGF-induced receptor endocytosis [6,8]. Second, EAST colocalizes partially with clathrin, a requisite element of receptor endocytosis. Moreover, EAST, in its domain structure, is similar to some endocytosis-associated proteins, such as VPS27 and Hrs ([6]; Lohi and Lehto, submitted). Thus, the present results which demonstrate that EAST associates with actin, strongly suggest that EAST could be involved in the mechanism coordinating or linking endocytic machinery with the EGF-induced cytoskeletal reorganization. In this sense it is intriguing that Pan1p, the yeast homologue of Eps15, plays a dual role in being involved in endocytosis and in the organization of the cortical actin cytoskeleton [26]. Moreover, Eps15 stimulates actin polymerization in vitro [19].

Accordingly, we suggest that EAST is involved in the regulation of the actin cytoskeleton. Given its features as an EGFR substrate and a putative endocytosis-associated regulator, it can be hypothesized that EAST functions at the interface linking the actin cytoskeleton to the EGFR endocytosis.

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