

Epidermal Growth Factor Modulates Tyrosine Phosphorylation of a Novel Tensin Family Member, Tensin3

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Abstract

Here, we report the identification of a new tensin family member, tensin3, and its role in epidermal growth factor (EGF) signaling pathway. Human tensin3 cDNA encodes a 1445 amino acid sequence that shares extensive homology with tensin1, tensin2, and COOH-terminal tensin-like protein. Tensin3 is expressed in various tissues and in different cell types such as endothelia, epithelia, and fibroblasts. The potential role of tensin3 in EGF-induced signaling pathway is explored. EGF induces tyrosine phosphorylation of tensin3 in MDA-MB-468 cells in a time- and dose-dependent manner, but it is independent of an intact actin cytoskeleton or phosphatidylinositol 3-kinase. Activation of EGF receptor is necessary but not sufficient for tyrosine phosphorylation of tensin3. It also requires Src family kinase activities. Furthermore, tensin3 forms a complex with focal adhesion kinase and p130Cas in MDA-MB-468 cells. Addition of EGF to the cells induces dephosphorylation of these two molecules, leads to disassociation of the tensin3-focal adhesion kinase-p130Cas complex, and enhances the interaction between tensin3 and EGF receptor. Our results demonstrate that tensin3 may function as a platform for the disassembly of EGF-related signaling complexes at focal adhesions. (Mol Cancer Res 2004;2(4):225–32)

Introduction

Epidermal growth factor (EGF) is implicated in many normal and abnormal processes including development, cell proliferation, and malignant transformation. Overexpression of EGF receptor (EGFR) has been found in many human tumors, including breast, prostate, lung, brain, ovarian, bladder, colon, gliomas, and renal carcinoma, and has been correlated with an advanced tumor stage (1, 2). Binding of EGF to its receptor results in receptor dimerization, activation of the tyrosine kinase activity, and phosphorylation of specific tyrosine residues on the EGFR cytoplasmic tail as well as its downstream substrates

(3). The phosphorylated residues on EGFR in turn serve as docking sites for Src homology 2 (SH2) domain-containing proteins forming signaling complexes. Some substrates do not appear to interact with the receptor itself, but they are activated in a receptor-dependent manner. Formation of the signaling complexes and induction of the substrate activities are both essential for programming various biological processes stimulated by growth factors. Therefore, identification of the signaling complexes and the downstream substrates is of importance in revealing EGF signaling pathways.

One group of EGFR substrates is localized to focal adhesions, the transmembrane junctions between the extracellular matrix and the actin cytoskeleton. These focal adhesion molecules include focal adhesion kinase (Fak), paxillin, and p130Cas (4–8). Increased tyrosine phosphorylation of Fak, paxillin, and p130Cas was observed in cells treated with low concentration of EGF (<10 ng/ml; Refs. 4, 6, 7). However, stimulation with a high concentration of EGF (>50 ng/ml) resulted in dephosphorylation of these molecules (4, 8), suggesting that the downstream events of EGFR are also regulated by the concentration of EGF.

Tensin1 is a focal adhesion molecule that plays roles in mediating signal transduction and actin cytoskeleton organization (9, 10). The NH₂ terminus of tensin1 binds to actin filaments, whereas the center region retards the G-actin polymerization (11). The COOH terminus contains the SH2 and the phosphotyrosine (pTyr) binding (PTB) domain (12). Furthermore, extracellular matrix, growth factors, or oncogenes are known to induce tyrosine phosphorylation of tensin1 (12–16). Recent studies have shown that tensin1 regulates cell migration (17). The data from analyzing tensin1 knockout mice have demonstrated a critical role of tensin1 in renal function and wound healing (18, 19).

Recently, we have identified the tensin-related molecules tensin2 and COOH-terminal tensin-like protein (cten; Refs. 17, 20), indicating that tensin represents a new gene family. In the present study, we report the identification of tensin3 and its tyrosine phosphorylation induced by EGF. EGF induces dephosphorylation of Fak and p130Cas, which leads to disassociation of these two molecules from tensin3 in MDA-MB-468 cells. Moreover, EGF promotes tyrosine phosphorylation of the EGFR to enhance the interaction between tensin3 and EGFR. Our results show that tensin3 is a previously unidentified signaling component in EGF-mediated signaling pathway.

Results

Identification of Human Tensin3 cDNA Sequence

In our effort to isolate genes related to tensin1, we have identified three homologues. Tensin2 and cten are reported

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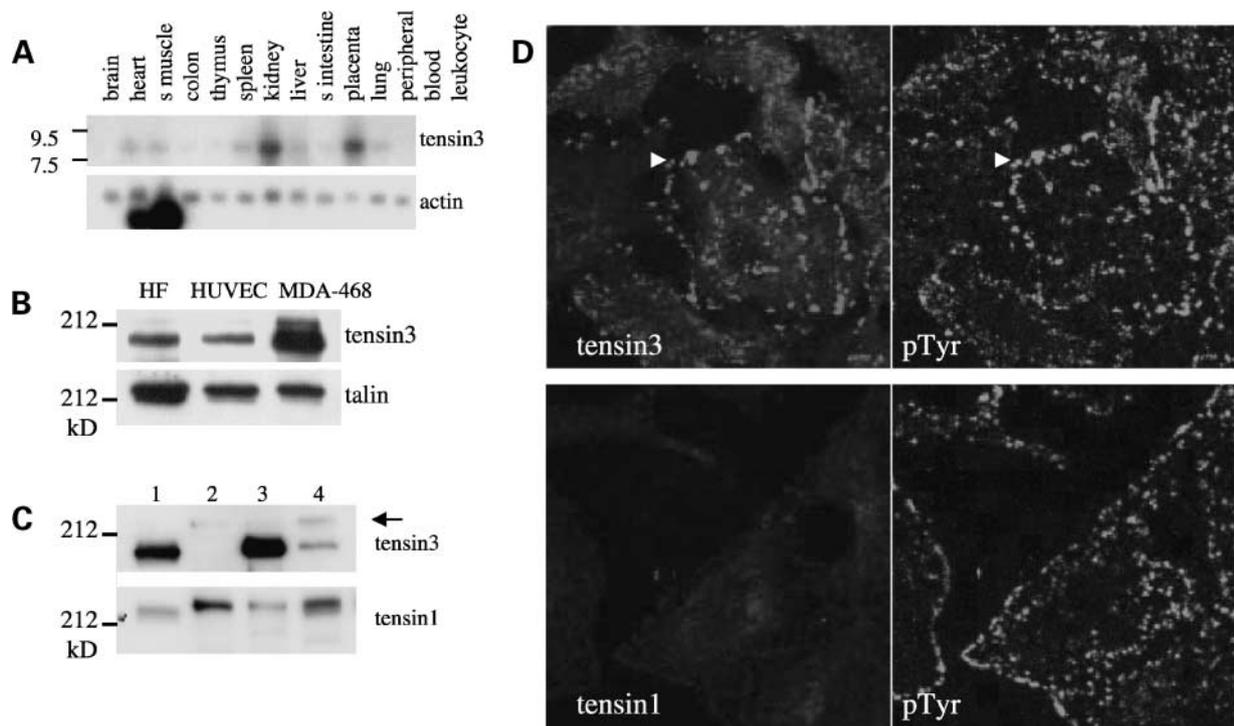


FIGURE 2. Expression of tensin3. **A.** Northern blot analysis of tensin3 (*upper*) in human tissues. The blots were stripped and probed with actin cDNA probe (*lower*). **B.** Equal amounts of cell lysates prepared from human fibroblasts, HUVEC, and MDA-MB-468 cells were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with tensin3 antibodies. The membrane was stripped and probed with antibodies to talin for comparison. **C.** MDA-MB-468 whole cell lysate (*lane 1*), MDA-MB-468 lysates immunoprecipitated with tensin1 (*lane 2*) or tensin3 (*lane 3*) antibodies, and hFOB whole cell lysate (*lane 4*) were immunoblotted with tensin3 antibodies (*upper*) and reprobbed with tensin1 antibodies (*lower*). Arrow, weak tensin1 signals detected by tensin3 antibodies. **D.** A549 cells grown on coverslips were fixed and labeled for tensin3, tensin1, and pTyr. Samples were viewed under LSM 510 laser scanning microscope. Arrowheads, focal adhesion staining. Note that tensin1 is not expressed in A549 cells.

domains. In addition, there are 32 tyrosine residues in tensin3 and 13 of them are potential phosphorylation sites predicted by the Prediction Servers on the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk>). These tyrosine residues are 30, 49, 59, 159, 291, 312, 333, 354, 549, 780, 802, 855, and 1256.

Expression of Tensin3 in Various Tissues and Cell Types

To examine the expression of tensin3 in various tissues, we have performed Northern blot analysis using a cDNA fragment unique to tensin3 as a probe. A single band ~ 8 kb was detected in some but not all tissues (Fig. 2A). In particular, the expression was strong in mRNA isolated from the kidney and the placenta. A low expression level was observed in heart, skeletal muscle, spleen, liver, and lung. This expression pattern is similar with tensin1 and tensin2 (17), whereas *cten* is restricted to the prostate and the placenta (20).

After we had completed the cloning of tensin3, we found that the amino acid sequence derived from tumor endothelial marker-6 (Genbank accession no. AF378756) was identical to our tensin3 sequence, although tumor endothelial marker-6 lacked the first 240 residues of tensin3. Tumor endothelial marker-6 was originally identified by serial analysis of gene expression as a tumor endothelia marker (21, 22), but no further characterization of this molecule has been reported to date. We raised antibodies against tensin3 to study its expression in human umbilical vein endothelial cells (HUVEC), epithelia cells (MDA-MB-468), and fibroblasts by immunoblotting

(Fig. 2B). Our results demonstrate that tensin3 is expressed as a 180-kDa protein in all three cell types with higher expression in MDA-MB-468, indicating that tensin3 is not endothelia specific. The blot was stripped and reprobbed with talin antibodies and similar amount of talin was detected in HUVEC and MDA-MB-468, whereas fibroblasts contained slightly more talin (Fig. 2B). To examine the specificity of anti-tensin3 antibodies, tensin1 and tensin3 were immunoprecipitated from MDA-MB-468 (tensin2 was not detected in MDA-MB-468) and immunoblotted with either anti-tensin3 or tensin1 antibodies. As shown in Fig. 2C, anti-tensin1 antibodies did not cross-react with tensin3. However, anti-tensin3 antibodies were able to recognize tensin1, but the signals were very weak and inconsistent in samples from MDA-MB-468 presumably due to very low expression of tensin1 in these cells (Fig. 2C, *lane 1*). Because there was a significant difference in the molecular masses between tensin1 (220 kDa) and tensin3 (180 kDa), we were able to distinguish tensin1 from tensin3 on immunoblotting (Fig. 2C, *lane 4*). The subcellular localization of tensin3 proteins was examined by immunofluorescence. Because anti-tensin3 antibodies weakly cross-reacted with other tensins, we used A549 cells for this study, which did not express detectable tensin1 (Fig. 2D) or tensin2 (data not shown), to be unequivocally certain that the labeling was on tensin3. As expected, tensin3 is localized to focal adhesions (Fig. 2D), demonstrating that focal adhesion localization is a common feature of all tensin family proteins characterized to date.

EGF Induces Tyrosine Phosphorylation of Tensin3 in MDA-MB-468 Cells

Because tensin3 is highly expressed in MDA-MB-468 cells, a human breast carcinoma overexpressing EGFR, we examined whether tensin3 participated in EGF-mediated signal transduction pathways. Initially, we found that increased tyrosine phosphorylation of tensin3 was detected in EGF-treated cells. A dose-response study was performed to determine what concentration of EGF was required (Fig. 3A). In parallel, we also examined the activation of the EGFR as judged by its tyrosine phosphorylation. Stimulation of the cells with 10 ng/ml EGF was able to induce tyrosine phosphorylation of tensin3 and EGFR. EGF concentrations > 100 ng/ml did not further increase the phosphorylation of either tensin3 or EGFR. The time course for tensin3 tyrosine phosphorylation exposed to 100 ng/ml EGF was also analyzed (Fig. 3B). Our results showed the tyrosine phosphorylation level of tensin3 peaked at 30 min after exposure to EGF. These results demonstrate that EGF induces tyrosine phosphorylation of tensin3 in a time- and dose-dependent manner.

EGF-Induced Tyrosine Phosphorylation of Tensin3 Is Independent of the Integrity of the Actin Cytoskeleton or Phosphatidylinositol 3-Kinase Activity but Requires EGFR and Src Family Kinase Activities

Because it has been shown by other researchers that tyrosine phosphorylation of focal adhesion molecules including Fak, paxillin, and p130Cas by EGF stimulation required an intact actin cytoskeleton (4, 8), we examined the potential role for an intact actin cytoskeleton in the tyrosine phosphorylation of tensin3 in response to EGF. The results showed that although the actin cytoskeleton in MDA-MB-468 cells was completely disrupted by 1 μ M of the F-actin disrupting agent cytochalasin D (Fig. 4A), the EGF-induced tyrosine phosphorylation of tensin3 and EGFR was not inhibited by cytochalasin D (Fig. 4B).

In addition to the actin cytoskeleton, it has also been demonstrated by other laboratories that the activation of phosphatidylinositol 3-kinase (PI3-kinase) is required for the tyrosine phosphorylation of Fak, paxillin, and p130Cas (4, 5, 23–25). Therefore, we examined the effect of PI3-kinase inhibitor, LY294002, on the EGF-induced tensin3 tyrosine phosphorylation. As shown in Fig. 4C, incubation with LY294002 had no effect on the EGF-induced tyrosine phosphorylation of tensin3.

To further confirm that the EGF-induced tyrosine phosphorylation of tensin3 was dependent on the autophosphorylation of EGFR, we used the EGFR specific inhibitor, AG1478, to block the phosphorylation of EGFR. As shown in Fig. 4D, the tyrosine phosphorylation of EGFR was inhibited by AG1478. Concomitantly, the tyrosine phosphorylation of tensin3 was suppressed. In contrast, the kinase inhibitor specific for platelet-derived growth factor receptor (AG1296) had no effect on EGFR or tensin3 phosphorylation (data not shown). These data demonstrated that the observed tyrosine phosphorylation change of tensin3 induced by EGF was indeed dependent on the EGFR pathway. Because activation of EGFR was shown to activate Src tyrosine kinase (26, 27) and Src was able to phosphorylate tensin1 (12), we examined whether EGF-induced

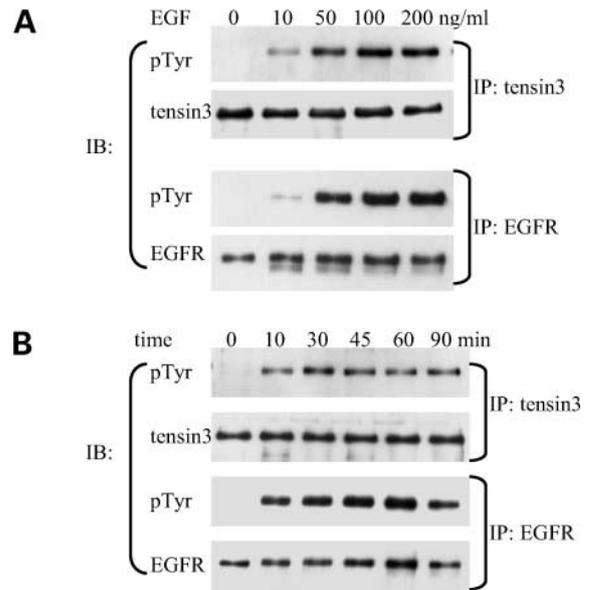


FIGURE 3. EGF stimulates tensin3 tyrosine phosphorylation in MDA-MB-468 cells. Serum-starved MDA-MB-468 cells were treated with 0–200 ng/ml EGF for 30 min (**A**) or treated with 100 ng/ml EGF for 0–90 min (**B**). The cells were lysed and immunoprecipitated with antibodies against tensin3 or EGFR and analyzed by immunoblotting with tensin3 or EGFR antibodies. The membrane was stripped and probed with anti-pTyr antibodies.

tensin3 tyrosine phosphorylation required the activation of Src family kinase by using the Src family specific inhibitor PP2. As shown in Fig. 4D, 5 μ M PP2 was able to inhibit the tyrosine phosphorylation of tensin3 but only had minimal effect on the tyrosine phosphorylation of EGFR. These results suggest that both EGFR and Src family kinase activations are required for EGF-induced tyrosine phosphorylation of tensin3. To test whether Src could phosphorylate tensin3, green fluorescent protein (GFP)-tensin3 constructs were cotransfected with kinase inactive (K295L) or active (Y529F) form of c-Src mutants into HEK293 cells. As shown in Fig. 4E, cotransfection with Src (Y529F), but not Src (K295L), was able to induce tyrosine phosphorylation of GFP-tensin3, indicating that tensin3 could be a direct substrate of Src kinase.

EGF Promotes the Interaction Between Tensin3 and EGFR and Disrupts the Tensin3-Fak-p130Cas Complex in MDA-MB-468 Cells

Because tensin3 contains the SH2 and PTB domains and EGF treatment alters the pTyr level of several proteins, we tested the effect of EGF treatment on the association of tensin3 and pTyr proteins. Interestingly, we detected more pTyr proteins associated with tensin3 in non-EGF-treated cells than in EGF-treated cells. As shown in Fig. 5A, pTyr proteins >200 kDa, ~200 kDa, and 120–130 kDa were coimmunoprecipitated with tensin3 in non-EGF-treated cells and only 170–180 kDa proteins were associated with tensin3 in EGF-treated cells. Immunoblot analysis using tensin3 antibodies confirmed that the 170–180 kDa band was tensin3. Because both pTyr levels

of tensin3 and EGFR were increased on EGF stimulation and both molecular masses were ~ 170 – 180 kDa, we speculated that EGFR might associate with tensin3 after EGF treatment. Indeed, EGFR was presented in tensin3 immunocomplexes (Fig. 5A). To rule out the association was due to the weak cross-reactivity of tensin3 antibodies to tensin1, we used EGFR antibodies for coimmunoprecipitation and found that tensin3 was also detected in EGFR immunocomplexes (Fig. 5B). In addition, tensin3 was coimmunoprecipitated with transfected hemagglutinin (HA)-tagged EGFR after EGF stimulation in Rat1 fibroblasts (Fig. 5C). These results demonstrated that EGF not only induced the tyrosine phosphorylation of tensin3 and EGFR but also enhanced the interaction between EGFR and tensin3.

Because Fak and p130Cas migrate ~ 125 – 130 kDa, we examined whether Fak and p130Cas interacted with tensin3. Figure 5A showed that both Fak and p130Cas were coimmunoprecipitated with tensin3 prior to EGF treatment. In addition, we examined whether the lack of interactions among tensin3, Fak, and p130Cas were due to dephosphorylation of Fak and p130Cas and found that pTyr levels of Fak and p130Cas were significantly decreased on EGF stimulation (Fig. 5B), which is also consistent with other report (8). These results demonstrated that tyrosine phosphorylation of Fak and p130Cas might be critical for their interactions with tensin3. Finally, we tested whether the COOH-terminal region of tensin3, which

contains the SH2 and PTB domains, was responsible for the interaction with these pTyr proteins. GFP-tensin3 (1149–1445) was transiently transfected into MDA-MB-468 cells, and the recombinant proteins were immunoprecipitated with anti-GFP antibodies and immunoblotted by anti-pTyr antibodies. As shown in Fig. 5D, GFP-tensin3 (1149–1445) was able to interact with similar pTyr proteins, which were confirmed to be p130Cas, Fak, and EGFR by immunoblotting (Fig. 5D). These data indicate that the SH2 and/or PTB domains of tensin3 are responsible for the interaction of tensin3 with pTyr proteins.

Discussion

In this study, we have identified a new tensin family member, tensin3. By use of *in vitro* binding assays, we have shown previously that the NH₂-terminal region of tensin1 binds to actin filaments (11), whereas the SH2 domain, but not the PTB, at the COOH-terminal end interacts with pTyr-containing proteins (12, 28). In fact, the PTB domain of tensin3 binds to the NPXY motifs in integrin β cytoplasmic tails in a non-tyrosine-phosphorylated fashion (29). Given the high sequence similarities of both regions among tensins, the actin binding, PTB, and integrin binding activities likely are conserved in tensin3. Despite the high sequence similarities at both ends of tensins, the middle regions are highly divergent. Previously, others and we have demonstrated that a fragment within the middle region

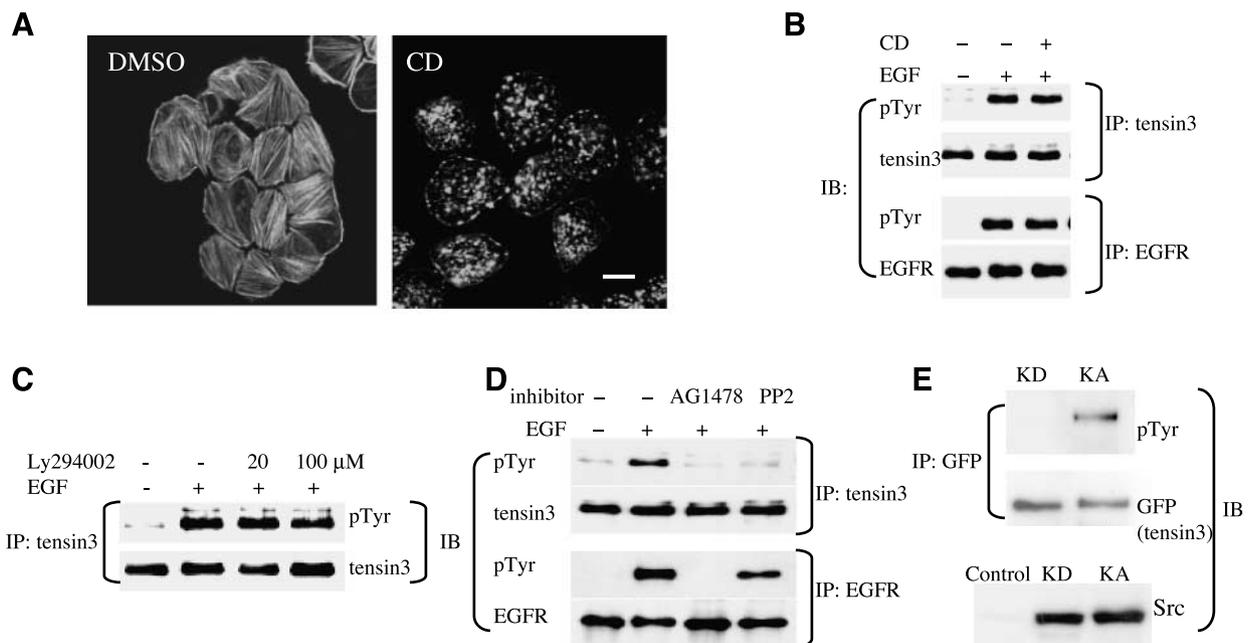


FIGURE 4. Tyrosine phosphorylation of tensin3 induced by EGF is independent of actin cytoskeleton network or PI3-kinase activity but requires EGFR and Src family kinase activities. Serum-starved MDA-MB-468 cells treated with DMSO or $1 \mu\text{M}$ cytochalasin D for 1 h were either fixed for detection of actin filaments by using fluorescently conjugated phalloidin (**A**) or stimulated with 100 ng/ml EGF for 30 min, immunoprecipitated with antibodies for tensin3 or EGFR, and immunoblotted with indicated antibodies (**B**). **C.** Cells were treated with or without LY294002 followed by incubation with 100 ng/ml EGF for 30 min. Cell lysates were immunoprecipitated with tensin3 antibodies and immunoblotted with indicated antibodies. **D.** Cells were treated with or without $2.5 \mu\text{M}$ AG1478 or $5 \mu\text{M}$ PP2 for 15 min followed by incubation with 100 ng/ml EGF for 30 min. Cell lysates were immunoprecipitated with tensin3 or EGFR antibodies and immunoblotted with the indicated antibodies. **E.** Cell lysates were prepared from HEK293 cells transiently cotransfected with GFP-tensin3 and kinase-dead (KD) c-Src (K295L) or kinase-active (KA) c-Src (Y529F) and immunoprecipitated with goat anti-GFP antibodies followed by immunoblotting with antibodies against pTyr or GFP. Whole cell lysates prepared from HEK293 cells (control) and above transfectants were immunoblotted with anti-Src antibodies to show the expression levels of Src proteins. Bar, $10 \mu\text{m}$.

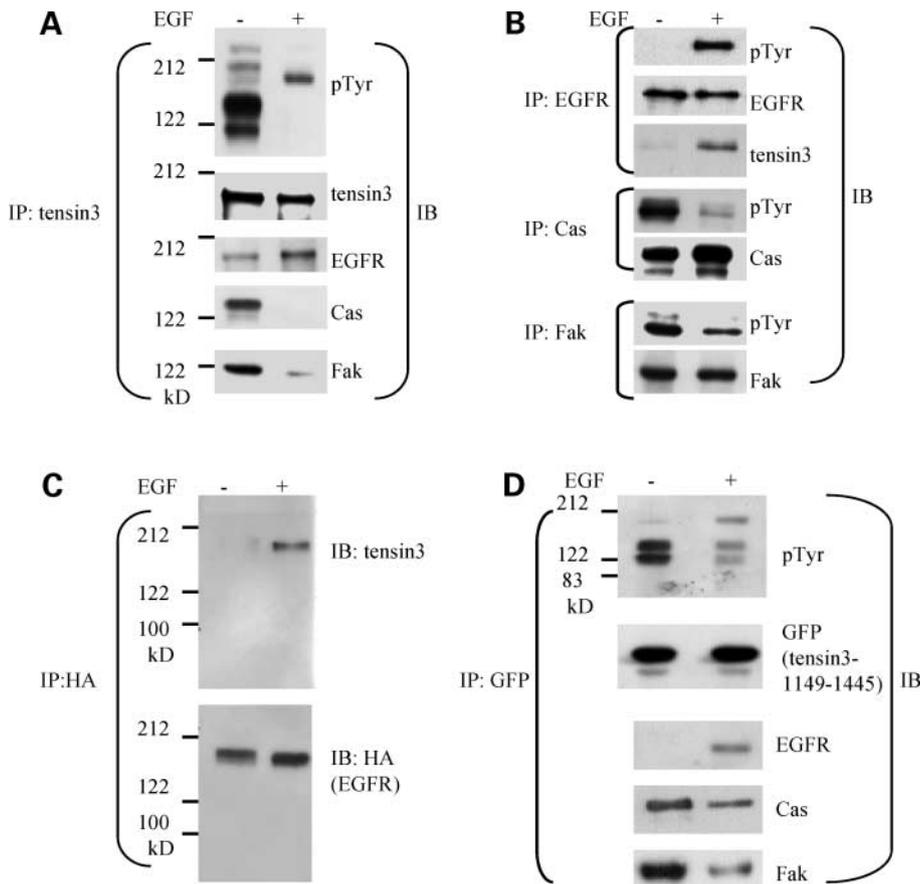


FIGURE 5. EGF promotes the interaction between tensin3 and EGFR, reduces tyrosine phosphorylation of Fak and p130Cas, and disrupts the tensin3-Fak-p130Cas complexes. Serum-starved MDA-MB-468 cells stimulated with or without 100 ng/ml EGF for 30 min were lysed and immunoprecipitated with antibodies against tensin3 (**A**), EGFR, p130Cas, or Fak (**B**) followed by immunoblotting with indicated antibodies. **C.** Rat1 fibroblasts transiently transfected with HA-EGFR were treated with or without EGF for 30 min. Cells were lysed and immunoprecipitated with anti-HA antibodies followed by immunoblotting with anti-tensin3 or HA antibodies. **D.** MDA-MB-468 cells transiently transfected with GFP-tensin3 (1149–1445) containing the SH2 and PTB domains were serum starved and treated with or without 100 ng/ml EGF for 30 min. Cell lysates were immunoprecipitated with anti-GFP antibodies followed by immunoblotting with antibodies against indicated molecules.

of tensin1 is able to retard the actin polymerization by binding to the barbed end of actin (11, 30). The corresponding region of tensin2 is very rich in proline residues, which may provide potential binding sites for interacting modules, such as SH3 and WW domains (31). Because the middle region of tensin3 does not share sequence homology with any proteins or domains in the databases, it is difficult to predict its function at present.

One common feature of tensin family members is the focal adhesion localization. Analysis of the focal adhesion localization of tensin1 has identified two focal adhesion binding (FAB) sites within the molecule (28). Interestingly, one FAB site is in the conserved NH₂-terminal region, while the other is in the conserved COOH terminus. Because the targeting sites are also found at the same regions in tensin2, the FAB sites of tensin3 likely are also localized to these regions.

Our studies have demonstrated that EGF-induced tyrosine phosphorylation of tensin3 appears to occur in a Src family kinase-dependent manner because incubation of cells with the Src specific inhibitor PP2 blocked phosphorylation of tensin3 but not of the EGFR. Although we cannot rule out the possibility that another PP2-sensitive kinase other than a Src family kinase could phosphorylate tensin3, this possibility is remote, because analysis of the crystal structure of a Src family member Hck bound to PP2 indicates that the specificity of PP2 lies in the binding of the chlorine group to a hydrophobic pocket that is only found in Src family kinases (32). Furthermore, we detected increased tyrosine phosphorylation of tensin3 in cells

transfected with constitutive active form of c-Src mutant but not with kinase inactive mutant. These results suggest that Src, but not EGFR, is the major kinase that directly phosphorylates tensin3 in EGF-stimulated cells.

The role of tyrosine phosphorylation of tensin3 is currently unknown. Apparently, tyrosine phosphorylation of tensin3 provides docking sites for other SH2/PTB domain-containing signaling molecules. Alternatively, tyrosine phosphorylation may induce conformation change of tensin3 and in turn alter the binding activities of its actin binding, SH2, PTB, or FAB domains. Among 13 potential tyrosine phosphorylation sites, 8 sites localize to the actin binding and FAB domains, while one site is within the SH2 domain. It will require the identification of the specific phosphorylation sites to further elucidate these possibilities.

It has been shown that EGF promotes cell migration that is mediated in a large part by dephosphorylation of Fak (8). Our studies have shown that Fak not only is dephosphorylated but also dissociated with tensin3 on EGF stimulation. Interestingly, we have recently shown that both tensin1 and tensin2 are able to promote fibronectin-stimulated cell migration (17). Furthermore, we have demonstrated that tensin1 mutants that do not localize to focal adhesions retard cell migration, whereas the mutant containing the SH2 domain deletion has no effect on cell migration, suggesting that the subcellular localization and binding to pTyr proteins are two important factors (28). Although we have not yet shown that tensin3 shares the capacity, it is

reasonable to speculate that tensin3 may also regulate cell motility in a similar mechanism based on common sequence motifs. If this is the case, tensin3 may regulate EGF-stimulated cell migration by releasing Fak and p130Cas from the tensin3 signaling complex and recruiting the EGFR to the complex.

Materials and Methods

Cell Culture and Plasmids

HUVEC and human fibroblasts purchased from Clonetics (Palo Alto, CA) were cultured in media recommended by the company. MDA-MB-468, A549, HEK293, and hFOB cells purchased from American Type Culture Collection (Manassas, VA) were cultured in DMEM supplemented with 10% FCS. Before treating with EGF, 70–80% confluent MDA-MB-468 cells in 100 mm dishes were washed thrice with PBS and starved in serum-free medium overnight. The pLNCX vectors containing kinase inactive (K295L) or active (Y529F) form of c-Src mutants were kindly provided by Dr. Sheila Thomas (Harvard Medical School, Boston, MA). pLXSN containing human EGFR cDNA with HA-tag was a kind gift from Dr. Albert Wong (Thomas Jefferson University, Philadelphia, PA).

Generation of Anti-Tensin3 Polyclonal Antibody

Purified glutathione *S*-transferase-tensin3 polypeptide (amino acids 418–577) was used to immunize rabbits. The same polypeptide, after removed from glutathione *S*-transferase by factor Xa, was used to make affinity column for antibody purification. Serum was loaded onto the preequilibrated column and anti-tensin3 polyclonal antibody was eluted with 100 mM glycine (pH 2.5) after washing with equilibration buffer [10 mM Tris-HCl (pH 7.4)] followed by 0.5 N NaCl in the equilibration buffer. Eluted antibody was immediately neutralized with 1 M Tris-HCl (pH 8.0) and concentrated by using Centricon (Millipore, Billerica, MA). Other antibodies used in this report were mouse anti-pTyr antibodies (P-Tyr-100, Cell Signaling Technology, Beverly, MA), mouse anti-EGFR (SC528, Santa Cruz Biotechnology, Santa Cruz, CA, for immunoprecipitation), rabbit anti-EGFR (SC1005, Santa Cruz Biotechnology, for immunoblotting), mouse anti-HA (SC7392, Santa Cruz Biotechnology), rabbit anti-GFP (SC8334, Santa Cruz Biotechnology, for immunoblotting), goat anti-GFP (600-101-215, Rockland, Gilbertsville, PA, for immunoprecipitation), mouse anti-Fak (BD610087, BD Biosciences, San Diego, CA), mouse anti-paxillin (BD610619, BD Biosciences), mouse anti-talin (T3287, Sigma Chemical Co., St. Louis, MO), mouse anti-v-Src (Ab-1, Calbiochem, San Diego, CA), and rabbit anti-tensin1 (S. H. Lo).

EGF Induction, Preparation of Cell Lysates, and Immunoprecipitation

Starved cells were incubated in 3 ml fetal bovine serum-free medium containing 0–200 ng/ml EGF for indicated period of time. Then, the cells were washed with ice-cold PBS and solubilized in lysis buffer [0.1% Triton X-100, 50 mM Tris-HCl (pH 8), 0.5 M NaCl, 0.2 mM EDTA, 1 µg/ml aprotinin, 0.75 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM DTT, and 1 µM phenylmethylsulfonyl fluoride]. For coimmunoprecipitation, cells were lysed in immunoprecipitation buffer [0.02% Triton

X-100, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.2 mM EDTA, and protease inhibitor cocktail]. Cell lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4°C. Total protein concentration was determined by Bio-Rad assay kit (Hercules, CA) using BSA as a standard. Five hundred micrograms of cell lysates were immunoprecipitated with 3 µg anti-tensin3 polyclonal antibody by rotating at 4°C for 2 h (3 mg of cell lysate proteins were used for coimmunoprecipitation assays) followed by the addition of 20 µl of 50% protein A-Sepharose slurry and rotating for 1 h. Protein A beads were collected and washed with lysis buffer six times and boiled in 20 µl of protein loading buffer for 5 min. Similarly, cell lysates were immunoprecipitated with anti-EGFR monoclonal antibody. Samples were subjected to 10% SDS-PAGE and transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Piscataway, NJ) for immunoblotting.

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