Epidermal Growth Factor-like Repeats of Thrombospondins Activate Phospholipase Cγ and Increase Epithelial Cell Migration through Indirect Epidermal Growth Factor Receptor Activation*

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Thrombospondin (TSP) 1 is a trimeric multidomain protein that contains motifs that recognize distinct host cell receptors coupled to multiple signaling pathways. Selected TSP1-induced cellular responses are tyrosine kinase-dependent, and TSP1 contains epidermal growth factor (EGF)-like repeats. Specific receptor interactions or functions for the EGF-like repeats have not been identified. We asked whether one or more biological responses to TSP1 might be explained through EGF receptor (EGFR) activation. In A431 cells, TSP1 increased autophosphorylation of Tyr-1068 of EGFR in a dose- and time-dependent manner. The ability of TSP1 to activate EGFR was replicated by the tandem EGF-like repeats as a recombinant protein. The three EGF-like repeats alone produced a high level of Tyr-1068 phosphorylation. EGF-like repeats from TSP2 and TSP4 also activated EGFR. Tyr-1068 phosphorylation was less when individual EGF-like repeats were tested or flanking sequences were added to the three EGF-like repeats. TSP1 and its EGF-like repeats also increased phosphorylation of EGFR Tyr-845, Tyr-992, Tyr-1045, Tyr-1086, and Tyr-1173, activated phospholipase Cγ, and increased cell migration. No evidence was found for binding of the EGF-like repeats to EGFR. Instead, EGFR activation in response to TSP1 or its EGF-like repeats required matrix metalloprotease activity, including activity of matrix metalloprotease 9. Access to the ligand-binding portion of the EGFR ectodomain was also required. These findings suggest release of an endogenous EGFR ligand in response to ligation of a second unknown receptor by the TSPs.

Thrombospondin (TSP)2 is an ~420-kDa trimeric glycoprotein composed of three identical 145-kDa polypeptide chains linked by disulfide bonds (1). TSP1 is one of five TSP family members (2). Each subunit of TSP1 and TSP2 contains the following structural elements: an NH2-terminal globular domain of the laminin G domain and concanavalin A-like lectin/glucanase superfamily; an α-helical region that presumably forms a parallel homotrimeric coiled coil as in matrilin-1; a von Willebrand factor C domain-like region of matrix metalloprotease 9. Access to the ligand-binding portion of the EGFR ectodomain was also required. These findings suggest release of an endogenous EGFR ligand in response to ligation of a second unknown receptor by the TSPs.

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‡ The abbreviations used are: TSP, thrombospondin; BS3, bis(sulfo)succinimidyl)suberate; E123, epidermal growth factor-like repeats 1–3; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; MMP, matrix metalloprotease; PLCγ, phospholipase Cγ; PTK, protein-tyrosine kinase; PVDF, polyvinylidene difluoride; siRNA, small interfering RNA; TSR, thrombospondin type 1 repeat; aa, amino acid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
protein (16). Therefore, the NH2-terminal globular domain, the
TSRs and calcium-binding repeats, and the COOH-terminal
domain of TSP1 all exhibit receptor binding activities that elicit
distinct host cell responses. Aside from minimal recognition by
integrins (14), no such receptor binding activity or biological
function has been ascribed to the type 2 EGF-like repeats of
TSP1. TSP1 is secreted by numerous cell types and is present in
the extracellular matrix (17). TSP1 was first demonstrated in the
release of thrombin-stimulated platelets (18). It is
expressed by endothelial cells, smooth muscle cells, fibroblasts,
keratinocytes, cells of monocytes/macrophage lineage, and
tumor cells (17). Much of our understanding of TSP1 biology
has been established in these cell systems. TSP1 is also
expressed in epithelia, is abundant in the basement membranes
underlying these cells (17), and participates in epithelial cell
responses, including re-epithelialization during wound healing
(8), bronchial epithelial cell morphogenesis and development
(19), and migration of epithelium-derived tumor cells (20). In
human epithelium-derived cancer cells, EGF increases TSP1
expression (21). TSP1 null mice display epithelial cell alter-
ations (22, 23).

In previous studies, we demonstrated that TSP1 increases
tyrosine phosphorylation of the zonula adherens proteins
γ-catenin and p120ctn (24), an event that can occur downstream
of the EGF receptor (EGFR) (25, 26), also referred to as HER1 or
ErbB1 (27). EGFR contains an NH2-terminal, ligand-binding
ectodomain that is coupled to an intracellular catalytic domain
and its tyrosine autoprophosphorylation sites (27). Ligand
binding to the EGFR ectodomain induces receptor homodimerization
and heterodimerization with other ErbB family members,
intrinsic kinase activity, and autophosphorylation of specific
lysine residues which, in turn, serve as docking sites within
the cytoplasmic domain for signaling molecules (27). High
affinity EGFR ligands share a 45–55-aa EGF motif with six spa-
tially conserved cysteine residues that form three intramole-
cular disulfide bonds that dictate their tertiary conformation (28).
These ligands are synthesized as transmembrane precursor
proteins that are cleaved by cell surface matrix metallopro-
teases (MMP) (28) and ADAMs (a disintegrin and metallopro-
teinases) (29–32) to release mature growth factors for auto-
crine/paracrine stimulation. EGFR ligands that specifically
activate EGFR include EGF, transforming growth factor α, amphiregulin,
and others that activate both EGFR and ErbB4, including heparin-binding EGF, betacellulin, and epiregulin
(28).

In addition to these ‘‘authentic’’ ErbB ligands, EGF-like
sequences are present in many other proteins, including TSP1 (33–35). EGF-like repeats in the γc chain of laminin-5 (34)
and in the counter-adhesive protein, tenasin-C (35), have been
demonstrated to activate EGFR. EGFR not only responds to
direct binding of EGF motif-containing ligands, but it can be
transactivated by heterologous receptors, including G protein-
coupled receptors (36) and integrins (37). Whether TSP1 elicits
biological responses through EGFR and/or other ErbB recep-
tors, either through direct binding or transactivation, is not
known. Here, we provide evidence that the EGF-like repeats of
TSP1 and other TSP family members, likely through an MMP-
mediated indirect process, activate EGFR and that this activa-
tion is coupled to downstream signaling events and cellular
responses that can explain aspects of TSP1 bioactivity.

MATERIALS AND METHODS

Human Intact TSP1 Preparation—Human platelet TSP1 was
purified as described (24). Briefly, fresh human platelets (Birm-
ingham American Red Cross, Birmingham, AL) were throm-
bin-stimulated, and the platelet releasate was applied to a hepa-
arin-Sepharose CL-6B (Pharmacia, Piscataway, NJ) affinity
column preequilibrated with Tris-buffered saline (TBS-C: 0.01
M Tris-HCl, 0.15 M NaCl, 0.1 mM CaCl2, pH 7.4). The bound
TSP1 was eluted and applied to an A0.5 M gel filtration column
(Bio-Rad) pre-equilibrated with TBS-C, pH 7.4.

Preparation of Recombinant TSP Proteins—Baculovirus-ex-
pressed recombinant human TSP1 domains were purified after
secretion as described (38–40). These recombinant proteins
(numbered from the initiating methionine of the full-length
subunit) include the following: 1) the NH2-terminal heparin-
binding domain + oligomerization domain + von Willebrand
factor-C domain (aa 19–374) (NoC); 2) the von Willebrand
factor-C domain + TSRs 1–3 (aa 312–548) (CP123); 3) TSR
repeat 3 + EGF-like repeats 1–3 (aa 491–691) (P3E123); 4)
EGF-like repeats 1–3 (aa 549–691) (E123); 5) EGF-like repeats
1 and 2 (aa 549–647) (E12); 6) EGF-like repeat 2 (aa 590–647)
(E2); 7) EGF-like repeat 3 (aa 648–691) (E3); 8) EGF-like
repeats 1–3 to the COOH terminus (aa 549–1170) (E123CaG); 9)
the third EGF repeat to the COOH terminus (aa 648–1170)
(E3CaG); and 10) the wire and COOH-terminal lectin-like
domain (aa 692–1170) (CaG). In addition, two baculovirus-ex-
pressed TSP2 constructs, P3E123 (aa 493–693) and E123CaG
(aa 551–1172), and two TSP-4 constructs, E1234 (aa 286–462)
and E1234CaG, were prepared (40). Protein concentration and
purity were determined by absorbance at 280 nm using an
extinction coefficient based on the amino acid composition and
by PAGE in SDS with and without prior reduction.

Cell Culture—Human epidermoid carcinoma A431 (Amer-
ican Type Culture Collection, Manassas, VA) were cultured in
Dulbecco’s modified Eagle’s medium (ATCC) enriched with
10% fetal bovine serum (FBS; Hyclone Laboratories, Logan,
UT), 5 mM l-glutamine, nonessential amino acids, and vitamins
in the presence of penicillin (50 units/ml) and streptomycin (50
μg/ml) (Sigma).

Knockdown of EGFR and MMP9 through RNA Interference—
Small interfering RNA (siRNA) duplex products designed to
target EGFR and MMP9, as well as an irrelevant control duplex
siRNA not corresponding to any known sequence in the human
genome, were introduced into A431 cells (Dharmacon, Lafay-
ette, CO) (41). First, 5 × 105 A431 cells were centrifuged (200 ×
g, 10 min), after which the pellet was resuspended in 100 μl of A431 Nucleofector solution (Amaxa Biosystems) and incu-
bated with 4.0 μg of siRNA duplexes. The A431 cell/siRNA mixture was transferred to an Amaxa-certified cuvette and sub-
jected to programmed electroporation (program X-001)
(Amaxa Biosystems). The MMP9 siRNA-transfected cells were
cultured for increasing times, and the supernatants were con-
centrated and assayed for MMP9 in an MMP9 ELISA kit (Cal-
biochem). At these same time points, the EGFR siRNA-trans-
fected cells were lysed and processed for immunoblotting with

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anti-EGFR antibody (BD Biosciences). To confirm equivalent protein loading and transfer, blots were stripped with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mmol/liter Tris-HCl, pH 6.7, and reprobed with 0.5 ng/ml murine anti-physarum β-tubulin IgG2b (Roche Applied Science) followed by HRP-conjugated anti-mouse IgG (BD Transduction Laboratories) and again developed with enhanced chemiluminescence (ECL).

**EGFR Activation**—To determine whether TSP1 activates EGFR, A431 cells were serum-starved for 6 h, after which they were exposed for 0.5 h to increasing concentrations of recombinant human EGFR (R & D Systems, Inc), TSP1, or media alone, or they were exposed for increasing times to a fixed concentration of EGF (100 ng/ml or 16.7 nM), TSP1 (30 μg/ml or 214 nM), or media alone. In selected experiments, cells were pretreated for 2 h with the EGFR-selective tyrphostin, AG1478 (5 μM) (Calbiochem) (42), the MMP2/MMP9 inhibitor IV, SB-3CT (1 μM) (Calbiochem) (43), the EGFR ectodomain-blocking antibody, GR13L (Calbiochem) (44), a murine monoclonal anti-human MMP9 neutralizing antibody (Calbiochem) (45), or a species- and isotype-matched antibody control, B7-1/CD80 (R & D Systems, Inc.).

**Migration Assay**—A431 cells were cultured to confluence in the wells of 24-well plates (Corning Glass, Corning, NY). Using a sterile 200-μl pipette tip, a single wound was made across the diameter of each monolayer, after which cell debris was removed by washing with HEPES buffer and 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaCl, 1 mM EGTA, 1 mM phosphatidylserine, 500 μM para-aminobenzamidine, and 1 mM phosphatidylcholine (all purchased from Sigma). The EC lysates were resolved by electrophoresis on a 4–12% SDS-polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The blots were probed with murine monoclonal anti-phospho-EGFR (Tyr-1068) followed by HRP-conjugated goat anti-mouse IgG (Pierce) and developed with ECL (Amer-sham Biosciences). To confirm equivalent protein loading, blots were stripped and reprobed for β-tubulin. For each immunoblot, densitometric quantification of phospho-EGFR Tyr-1068 signal was normalized to the β-tubulin signal for the same lane on the same stripped and reprobed blot.

**Cross-competition Binding Studies**—To determine whether E123 binds to the same receptor and/or same portion of the EGFR ectodomain as do high affinity EGFR ligands, binding of fluoroprobe-labeled EGF and E123 to suspended A431 cells was studied with flow cytometry. Purified recombinant E123 was dialyzed versus borate buffer (Slide-A-Lyzer Mini Dialysis Unit, Pierce), reacted with fluorescein isothiocyanate (FITC) reconstituted in dimethylformamide, and again dialyzed to remove excess unconjugated FITC dye (EZ-Label FITC protein labeling kit, Pierce). A431 cells in fluorescence-activated cell sorter tubes (0.5–1.0 × 10⁶ cells/tube) were incubated for 10 min at 4 °C with increasing final concentrations of either FITC-EGF (Invitrogen) or FITC-E123. To define binding over time, A431 cells were incubated at 4 °C with either FITC-EGF (50 ng/ml) or FITC-E123 equimolar to native TSP1 at 30 μM (Calbiochem) (43), the EGFR ectodomain-blocking antibody, GR13L (Calbiochem) (44), or a species- and isotype-matched antibody control, B7-1/CD80 (R & D Systems, Inc.) followed by goat anti-rabbit HRP-conjugated IgG (Pierce) and developed with ECL (48). To confirm equivalent protein loading and transfer, blots were stripped and reprobed for total PLCγ with rabbit anti-human PLCγ antibody (Cell Signaling Technology).

**Migration Assay**—A431 cells were cultured to confluence in the wells of 24-well plates (Corning Glass, Corning, NY). Using a sterile 200-μl pipette tip, a single wound was made across the diameter of each monolayer, after which cell debris was removed by washing with HEPES buffer and 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaCl, 1 mM EGTA, 1 mM phosphatidylserine, 500 μM para-aminobenzamidine, and 1 mM phosphatidylcholine (all purchased from Sigma). The EC lysates were resolved by electrophoresis on a 4–12% SDS-polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The blots were probed with murine monoclonal anti-phospho-EGFR (Tyr-1068) followed by HRP-conjugated goat anti-mouse IgG (Pierce) and developed with ECL (Amer-sham Biosciences). To confirm equivalent protein loading, blots were stripped and reprobed for β-tubulin. For each immunoblot, densitometric quantification of phospho-EGFR Tyr-1068 signal was normalized to the β-tubulin signal for the same lane on the same stripped and reprobed blot.

Patterns of EGF Tyrosine Phosphorylation in Response to EGF, TSP1, and E123—A431 cells were serum-starved for 6 h after which they were exposed to media alone, EGF (10 ng/ml or 1.67 nM, 10 min), native TSP1 (30 μg/ml or 214 nM, 0.5 h), or an equimolar concentration (3.8 μg/ml or 214 nM, 1 h) of TSP1 E123 (aa 549–691). The cells were lysed and the lysates processed for immunoblotting with a series of defined, epitope-mapped, rabbit polyclonal anti-phospho-EGFR antibodies that recognize Tyr-845, Tyr-992, Tyr-1045, and Tyr-1173 (Cell Signaling Technology, Inc., Beverly, MA) and Tyr-1086 (Zymed Laboratories Inc.) all contained within the cytoplasmic domain of EGFR (27). As stated above, a murine monoclonal antibody was use to probe for phospho-EGFR Tyr-1068. The blots were stripped and reprobed with anti-β-tubulin antibodies.
FITC-E123 (2 μg/ml) for increasing times. To establish binding specificity, FITC-EGF (3 μg/ml) was incubated for 10 min at 4°C with increasing concentrations of unlabeled EGF (up to 500-fold relative to labeled ligand), and FITC-E123 (5 μg/ml) was incubated for 10 min at 4°C with increasing concentrations of unlabeled E123 (up to 500-fold relative to labeled ligand). Finally, cross-competition binding studies were performed using FITC-EGF with increasing concentrations of unlabeled E123 and FITC-E123 with increasing concentrations of unlabeled E123 and FITC-E123 with increasing concentrations of unlabeled EGF. The cells were washed and resuspended in PBS and analyzed by FACSscan (BD Biosciences).

Chemical Cross-linking Experiments—To establish a direct receptor-ligand interaction between E123 and cell surface-expressed EGFR ectodomain, E123 (3.8 μg/ml or 214 nM) and cells were co-incubated in the presence of the H2O-soluble, cell-permeable, chemical cross-linking reagent, bis(sulfosuccinimidyl) suberate (BS3) (Mr = 572.43) (Pierce) as described (50). The two ends of this homobifunctional reagent cross-link amine groups and are separated by a flexible 11.4D spacer arm. The two ends of this homobifunctional reagent cross-link amine groups and are separated by a flexible 11.4D spacer arm. The reaction mixture was incubated for another 0.5 h at room temperature after which the cross-linking reaction was terminated by the addition of glycine (250 mM). The cells were washed and solubilized in the presence of glycine (250 mM). The lysates were precleared by incubation for 1 h at 4°C with protein G cross-linked to agarose (Sigma) and pre-loaded with a species- and isotype-matched irrelevant antibody (AFAP IgG1, BD Transduction Laboratories). The lysates were then incubated overnight at 4°C with anti-EGFR antibody (2.5 μg of antibody/500 μg of lysate) (Pharmingen) or an equivalent concentration of the irrelevant antibody control. The resultant immune complexes were immobilized by incubation with protein G cross-linked to agarose for 2 h at 4°C. After centrifugation, the pellet was washed and boiled in sample buffer, and the eluted EGFR-binding proteins were resolved by 6% SDS-PAGE and transferred to PVDF. The blots for the EGFR immunoprecipitates were probed for either E123 with murine monoclonal C6.7 anti-human TSP1 antibody that recognizes the second EGF-like repeat (47) (Fig. 9, lanes 2–5), or EGF with rabbit polyclonal anti-human EGF antibody (Santa Cruz Biotechnology, Inc.) (lanes 6–9). Purified recombinant E123 were used as a positive control (Fig. 9, lane 1).

Detection of MMPs—A431 cells were incubated for increasing times with TSP1 (30 μg/ml or 214 nM), E123 (3.8 μg/ml or 214 nM), EGF (100 ng/ml or 16.7 nM), or media alone. Supernatants were collected into Tris-glycine SDS buffer, loaded onto precast 10% zymogram gels containing 1 mg/ml gelatin polymerized within the gel (Invitrogen), and the proteins electrophoretically resolved. The gels were incubated with renaturing buffer (0.5 h, room temperature), followed by developing buffer 2× (0.5 h at room temperature and 4 h and 37°C), stained (Simply Blue 0.5%, 0.5 h), and destained (methanol/acetic acid/H2O, 50:10:40) according to the manufacturer’s protocol. Clear bands of lysis against a dark blue background were noted, which corresponded to gel mobility of active MMPs. In other experiments, the same concentrated supernatants described above were assayed for MMP9 protein using an MMP9 ELISA kit (Calbiochem) or assayed for MMP9 catalytic activity for a fluorogenic substrate linked to a quencher molecule (Fluorokine E, R & D Systems, Minneapolis, MN). To enhance the sensitivity of the fluorogenic assay, the incubation time of samples with the fluorogenic substrate was prolonged from the prescribed 2–24 h, after which fluorescence (excitation = 355 nm, emission = 425 nm) was assayed (Fluoroskan Ascent; Thermo Scientific, Fremont, CA).

Statistical Methods—Analysis of variance was used to compare the mean responses among experimental and control conditions.
FIGURE 2. Structure-function analysis of TSP1-induced EGFR activation. A, schematic of baculovirus-derived recombinant TSP1 domains. B, A431 cells were exposed for 0.5 h to equimolar concentrations (214 nM) of baculovirus-derived recombinant TSP1 domains, including the following: lane 1, NH2-terminal heparin-binding domain / oligomerization / procollagen domain (NoC); lane 2, procollagen domain / properidin repeats 1–3 (CP123); lane 3, properidin repeat 3 + EGF-like repeats 1–3 (P3E123); lane 4, EGF-like repeats 1–3 (E123); lane 5, EGF-like repeats 1–3 + Ca2+ -binding repeats + COOH terminus (E123-CaG); lane 6, EGF-like repeat 3 to COOH terminus (E3CaG); and lane 7, COOH terminus (CaG). C, TSP1 (214 nM) was preincubated with either of two antibodies, C6.7 and HB8432, each targeting its EGF-like repeats, or a species- and isotype-matched antibody control, B7-1, after which the TSP1 was incubated with A431 cells. D, A431 cells were exposed for 1 h to equimolar concentrations (214 nM) of E123, E12, E2, or E3 or media alone. A431 cells were exposed for 0.5 h to increasing concentrations of recombinant TSP1 EGF-like repeats (E123) or media alone (E) or exposed for increasing exposure times to a fixed concentration of recombinant TSP1 EGF-like repeats (3.8 μg/ml or 214 nM), a concentration equimolar to 30 μg/ml TSP1, or media alone (F). G, A431 cells transfected with EGFR targeting (lane 2) or control (lane 1) siRNAs were cultured for 48 h, after which they were exposed for 1 h to E123 (214 nM) or media alone. Cells were lysed and processed for immunoblotting with anti-phospho-EGFR (Tyr-1068) antibodies. The blots were stripped and reprobed with anti-β-tubulin antibody to indicate protein loading and transfer. IB, immunoblot; IB*, immunoblot after stripping; each of these blots are representative of ≥ 2 independent experiments. H, for each phospho-EGFR Tyr-1068 immunoblot (see Fig. 1A and Fig. 2E), densitometric quantification of each Tyr(P)-1068 signal was normalized to the β-tubulin signal for the same lane on the same stripped and reprobed blot. Vertical bars represent mean (± S.E.) fold increase of arbitrary densitometry units of Tyr(P)-1068 signal normalized to arbitrary densitometry units of β-tubulin signal, each relative to the simultaneous control. n = 3, * significantly increased compared with the equimolar concentration of E123 at p < 0.05.
groups for all experiments. The Dunnett and Scheffé F tests were used to determine significant differences between groups. A p value of <0.05 was considered significant.

RESULTS

TSP1 Activates EGFR—We previously found that broad spectrum PTK inhibition protects against selected TSP1-induced cell responses (24). TSP1 contains EGF-like repeats (1) and increases ZA protein tyrosine phosphorylation (24), an activity that can be ascribed to EGFR signaling (25–27). We therefore asked whether TSP1 activates EGFR. After serum starvation, increasing concentrations of TSP1 were presented to high EGFR-expressing A431 cells (Fig. 1A). TSP1 at concentrations ≥5 µg/ml (36 nM) increased phosphorylation of EGFR on Tyr-1068. On a molar basis, the minimal TSP1 concentration required for EGFR activation (5 µg/ml, Fig. 1A) was >40-fold greater than the minimal concentration of EGF (5 ng/ml; 0.83 nM; Fig. 1C). TSP1 (214 nM) stimulated EGFR phosphorylation by 3 min with further time-dependent increases up to 60 min (Fig. 1B). These kinetics were similar to those observed with EGF (16.7 nM) treatment (Fig. 1D). Prior PTK inhibition with the EGF-receptor-selective tyrphostin, AG1478, blocked EGFR activation in response to either EGF or TSP1 (Fig. 1E). Transfection of A431 cells with EGFR targeting siRNAs knocked down EGFR protein >90% relative to cells transfected with control siRNA (Fig. 1F, lanes 1 and 2). Prior knockdown of EGFR blocked EGFR Tyr-1068 phosphorylation in response to TSP1 (Fig. 1F, lane 4). These data indicate that TSP1 increases phosphorylation of EGFR at Tyr-1068 in a concentration- and time-dependent manner that is blocked by either an inhibitor of EGFR autophosphorylation or knockdown of EGFR through siRNA technology.

Structure-Function Studies of TSP1-induced EGFR Activation—To define which sequences within TSP1 activate EGFR, the high EGFR-expressing A431 cells were exposed to baculovirus-encoded recombinant TSP1 constructs that correspond to overlapping sequences from the full-length protein (Fig. 2A) (38–40). Each domain, at a concentration equimolar to the monomeric subunit of TSP1 at 30 µg/ml, i.e. 214 nM, was tested for EGFR activation as measured by Tyr-1068 phosphorylation (Fig. 2B). Only the three recombinant constructs containing EGF-like repeats 1–3 were active (Fig. 2B, lanes 3–5). The E123 alone (Fig. 2B, lane 4) produced the highest level of Tyr-1068 phosphorylation. The third EGF-like repeat fused to the Ca2+-binding type 3 repeats, E3CaG, did not increase EGFR Tyr-1068 autophosphorylation (Fig. 2B, lane 6). Preincubation of TSP1 with either of two antibodies that bind to E123, C6.7, and HB8432 (46, 47) each diminished EGFR Tyr-1068 phosphorylation (Fig. 2C, lanes 4 and 5). These data indicate that the EGF-like repeats are necessary and sufficient for TSP1-induced EGFR activation.

To establish which of the three EGF-like repeats is responsible for EGFR activation, equimolar concentrations of E123, E12, E2, and E3 were compared for their abilities to stimulate EGFR Tyr-1068 phosphorylation (Fig. 2D). E123 (Fig. 2D, lane 2) stimulated the highest level of Tyr-1068 phosphorylation; E12 stimulated EGFR phosphorylation but to a lesser extent than E123 (Fig. 2D, lane 3); phosphorylation in response to E2 was still less (Fig. 2D, lane 4); and phosphorylation in response to E3 was comparable with that seen in response to E12 (Fig. 2D, lane 5). Again, E3 flanked by the COOH terminus did not increase Tyr-1068 phosphorylation (Fig. 2B, lane 6). Thus, the ability to activate EGFR appears to reside in each of the three EGF-like repeats and is enhanced when all three are assembled in tandem. Interestingly, addition of either the third TSR to form P3E123 (Fig. 2B, lane 3) or the COOH-terminal wire and lectin-like modules to form E123CaG (Fig. 2B, lane 5) attenuated the activity of E123.

When A431 cells were exposed for 1 h to increasing concentrations of purified E123, EGFR Tyr-1068 phosphorylation was increased at concentrations ≥2 µg/ml (112.6 nM) (Fig. 2E). When A431 cells were exposed for increasing times to a fixed concentration of E123 (3.8 µg/ml or 214 nM) (Fig. 2F). EGFR Tyr-1068 phosphorylation was increased after 3–10 min (lanes 2 and 3) with maximum phosphorylation at 30–60 min (lanes 4 and 5). Prior EGFR knockdown completely blocked EGFR Tyr-1068 phosphorylation in response to E123 (Fig. 2G, lane 2). To directly compare the relative potencies of native TSP1 and E123 to activate EGFR, quantitative densitometry of each phospho-EGFR Tyr-1068 signal was normalized to the β-tubulin signal for the same lane on the same stripped and reprobed blot. The mean normalized Tyr(P)-1068 value for the simultaneous media controls was assigned a value 1.0. The normalized Tyr(P)-1068 values for cells challenged with either TSP1 or E123 were expressed as fold-increase relative to the simultaneous control (Fig. 2H). When normalized Tyr(P)-1068 values in response to the TSP1 stimulus were compared with normalized values in response to equimolar concentrations of E123, at each of four tested concentrations, TSP1 was 2.1–2.8-fold more potent than was E123. Thus, the molar potency of TSP1 monomer was at least 2-fold greater than that for E123, and the stimulus-to-response lag time preceding EGFR activation for E123 (10 min) was only slightly prolonged compared with that seen for native TSP1 (3 min) (Fig. 1B).

To determine whether the EGF-like modules of other members of the TSP gene family also activate EGFR, A431 cells were
incubated with equimolar concentrations (214 nM) of two recombinant TSP2 constructs, P3E123 and E123CaG, and two recombinant TSP4 constructs, E1234 and E1234 CaG (Fig. 3). Each of these four EGF motif-containing proteins increased EGFR Tyr-1068 phosphorylation. As was seen with the TSP1 constructs, addition of the wire and lectin-like modules to the EGF-like repeats of TSP4 attenuated activity (Fig. 3, lanes 6 versus 7).

Pattern of EGFR Tyrosine Autophosphorylation in Response to Full-length TSP1 Versus TSP1 EGF-like Repeats—There are ≳10 tyrosine residues within the cytoplasmic domain of EGFR that have been identified as autophosphorylation sites (27). Upon phosphorylation, a number of these residues serve as docking sites for specific signaling and adaptor molecules. We asked whether TSP1 or E123 would stimulate phosphorylation of the same tyrosine residues as would be stimulated by EGF. Concentrations and incubation times for each agonist were chosen based on results presented in Fig. 1, A—D, and Fig. 2, E and F. TSP1 (214 nM, 0.5 h), E123 (214 nM, 1 h), and EGF (1.67 nM, 10 min) each increased phosphorylation of Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, Tyr-1086, and Tyr-1173 (lanes 1–4). In other experiments, A431 cells were exposed to a fixed concentration of TSP1 (30 μg/ml or 214 nM) for increasing times, after which the cells were lysed and the lysates processed for EGFR Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, Tyr-1086, and Tyr-1173 (lanes 1–4). Therefore, EGFR phosphorylation in response to TSP1 and E123 was comparable with that seen in response to EGF.

TSP1 Activates PLCγ—TSP1 and E123 activate EGFR (Figs. 1–4), stimulating autophosphorylation of EGFR Tyr-992 and Tyr-1173 (Fig. 4A). Upon EGFR activation, these two phosphotyrosines serve as docking sites for PLCγ (51). To relate these phosphorylation events to downstream signaling, phosphorylation of Tyr-992 and Tyr-1173 in response to the TSP1 stimulus was studied over time (Fig. 4, B and C). Phosphorylation of Tyr-992 was evident at ≳5 min with maximal phosphorylation at 30 min, whereas Tyr-1173 phosphorylation was not seen until ≳10 min with further increases at 30 and 60 min. We then asked whether TSP1 or E123 activates PLCγ through EGFR activation. In A431 cells, TSP1 at ≳5 μg/ml (36 nM) increased PLCγ activation; this activation plateaued at TSP1 ≳20 μg/ml (144 nM) (Fig. 5A). When A431 cells were exposed to TSP1 (214 nM) for increasing exposure times, PLCγ was activated at ≳5 min with further time-dependent increments through 60 min (Fig. 5B). The EGFR-selective PTK inhibitor, AG1478, completely blocked TSP1-induced PLCγ activation (Fig. 5C). Similarly, prior knockdown of EGFR also blocked PLCγ activation in response to TSP1 (Fig. 5D, lane 4). E123 (214 nM) also activated PLCγ (Fig. 5E, lanes 1 and 2), and
again, prior EGFR knockdown prevented this activation (Fig. 5E, lanes 3 and 4). These findings indicate that the E123 and EGF-like repeats of TSP1 each increased cell migration 5.3-, 4.6-, and 2.1-fold, respectively (Fig. 6, A and B). During this same period, no increases in cell proliferation as measured by [3H]thymidine incorporation could be detected (data not shown). The increased cell migration in response to EGF, TSP1, and E123 each was diminished by 88, 88, and ~100%, respectively, by EGFR-selective PTK inhibition with AG1478 (Fig. 6, A and B). When AG1478-exposed (5 μM, 48 h, n = 12) and media control (n = 12) A431 cells were analyzed for cytotoxicity in an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, no difference was found (1.20 ± 0.03 versus 1.22 ± 0.03; p = 0.56). Transfection of A431 cells with EGFR-targeting siRNAs knocked down EGFR protein >90% relative to cells transfected with control siRNA at 48, 72, and 96 h (Fig. 7A, lanes 4, 6, and 8). At 24 h, EGFR protein was only knocked down ~75% (Fig. 7A, lane 2). Prior knockdown of EGFR diminished the migratory response to EGF, TSP1, and E123 by ~60, ~93, and ~100%, respectively (Fig. 7, B and C). These findings indicate that the EGF-like repeats of TSP1 stimulate epithelial cell migration through EGFR activation. Because TSP1 increased migration ~2.2-fold more than did equimolar concentrations of E123 (Figs. 6 and 7), each normalized to its simultaneous control, it is conceivable that an additional domain(s) outside the EGF-like repeats also contributes to the migratory response.

**Effect of EGFR Ectodomain Blocking Antibodies on TSP1 Activation of**

**TSP1 EGF-like Repeats Activate PLCγ and EGFR**

![Figure 6](image_url)

**FIGURE 6. TSP1 increases cell migration through EGFR activation.** A431 cells were cultured to confluence in the wells of 24-well plates, after which they were wounded with a pipette tip, washed to remove cellular debris, and incubated for 48 h with EGF (10 ng/ml or 1.67 nM), TSP1 (30 μg/ml or 214 nM), E123 (3.8 μg/ml or 214 nM), or media alone in the presence or absence of the EGFR selective tyrphostin, AG1478 (5 μM) (n = 6). At 48 h, cellular migration into the wound was photographed in triplicate and quantified. A, representative photographs of wounded monolayers after 48 h of incubation with EGF, TSP1, E123, or media alone in the presence or absence of AG1478. Arrows in panels 2, 6, and 10 indicate increased cell migration into the wound. Magnification, ×40. B, vertical bars represent mean (± S.E.) migration into the wound at 48 h after incubation with EGF, TSP1, E123, or media alone in the presence or absence of AG1478. For each condition, n = 6. * significantly increased compared with the media control at p < 0.05. ** significantly decreased compared with the stimulus alone at p < 0.05.
in response to EGF (Fig. 8, lane 4), TSP1 (lane 7), or E123 (lane 10), whereas inclusion of the antibody control did not (lanes 3, 6, and 9). These data indicate that TSP1 and its EGF-like repeats activate EGFR through the ligand-binding portion of its ectodomain. Thus, activation appears to be due to either direct engagement of EGFR and/or through MMP-mediated release of a tethered EGFR ligand(s).

**Direct Interaction of E123 with EGFR Ectodomain Is Not Detectable**—To demonstrate a direct interaction between the EGF-like repeats of TSP1 and the EGFR ectodomain, purified recombinant E123 was studied vis-à-vis EGF in both cross-

**FIGURE 8. Immunoblockade of EGFR ectodomain inhibits EGFR activation.** A431 cells were exposed to EGF (10 ng/ml or 1.67 nM, 10 min), TSP1 (30 μg/ml or 214 nM, 0.5 h), E123 (3.8 μg/ml or 214 nM, 1 h), or media alone, each in the presence or absence of the EGFR ectodomain-blocking antibody GR13L or a species- and isotype-matched antibody control B7-1. The cells were lysed, and the lysates were processed for phospho-EGFR (Tyr-1068) immunoblotting. The blots were stripped and reprobed for β-tubulin to indicate protein loading and transfer. IB, immunoblot; IB*, immunoblot after stripping. This blot is representative of >2 independent experiments.
phorylation in response to either TSP1 or E123 was studied in the presence of the MMP2/MMP9 inhibitor, SB-3CT (43). Prior MMP inhibition decreased TSP1- and E123-induced EGFR Tyr-1068 phosphorylation by \( \approx 60\% \) (Fig. 10, lanes 4 and 6, and E). In contrast, EGF-induced EGFR Tyr-1068 phosphorylation was not influenced by prior MMP inhibition (Fig. 10, D, lane 9, and E). Because TSP1 increased both MMP9 protein (Fig. 10, A and B) and activity (Fig. 10C) and prior MMP inhibition partially blocked TSP1-induced EGFR activation (Fig. 10, D and E), we asked whether MMP9 was operative. Preincubation of cells with MMP9 neutralizing antibody for immunoblockade of surface expressed MMP9 diminished

**FIGURE 9.** Direct E123 interaction with EGFR ectodomain is not detectable. A431 cells were incubated for 10 min at 4 °C with a fixed subsaturation concentration of FITC-EGF (3 μg/ml) in the presence of increasing concentrations of either unlabeled EGF (A) or unlabeled E123 (B). In similar experiments, the cells were incubated for 10 min at 4 °C with a fixed subsaturation concentration of FITC-E123 (5 μg/ml) in the presence of increasing concentrations of either unlabeled E123 (C) or unlabeled EGF (D). A–D are representative of \( \approx 2 \) independent experiments. The cells were washed, resuspended in PBS, and analyzed by FACScan. E, in other experiments, A431 cells were serum-starved, washed to remove amine-containing media, and incubated for 0.5 h at 4 °C with E123 (214 nM), EGF (16.7 nM), or media alone in the presence of the chemical cross-linking reagent BS3 (final concentration 3 mM). The cross-linking reaction was terminated by the addition of glycine (250 mM), and the cells were lysed. Lysates were immunoprecipitated with anti-EGFR antibody, and the EGFR immunoprecipitate was resolved by 6% SDS-PAGE and transferred to PVDF. The blots were probed for either E123 with murine monoclonal (C6.7) anti-human TSP1 antibody that recognizes the second EGF-like repeat (lanes 1–5) or EGF with rabbit anti-human EGF antibody (lanes 6–9). Purified recombinant E123 was used as a positive control (lane 1). The blots were stripped and reprobed for β-tubulin. IP, immunoprecipitate; IB, immunoblot; IB*, immunoblot after stripping. This blot is representative of three experiments.
TSP1-induced EGFR Tyr-1068 phosphorylation by >65% (Fig. 11A, lane 3). Transfection of A431 cells with MMP9-targeting siRNAs knocked down MMP9 protein ~60% relative to cells transfected with control siRNA in both TSP1-treated and E123-treated cells (Fig. 11A). MMP9 levels in EGF-stimulated and media control cells were almost undetectable. Prior knockdown of MMP9 diminished EGFR Tyr-1068 phosphorylation by ~80% relative to the control siRNA-transfected cells in response to either TSP1 or E123 (Fig. 11C, lanes 4 and 6). Similarly, prior MMP9 knockdown decreased TSP1/E123-induced PLCγ activation by >80% compared with the simultaneous controls (Fig. 11D, lanes 4 and 6). In contrast, prior knockdown of MMP9 did not alter either EGF Tyr-1068 phosphorylation (Fig. 11C, lanes 7 and 8) or PLCγ activation (Fig. 11D, lanes 7 and 8) in response to EGF, excluding any nonspecific downstream signaling effects. These data suggest that EGFR and PLCγ activation in response to TSP1 is mediated, at least in part, through MMP activation, involving MMP9 and possibly one or more other undetected MMPs.

**DISCUSSION**

In these studies, we identified a novel function for the EGF-like repeats of TSP1. Exposure of high EGFR-expressing A431 cells to human platelet-derived trimeric TSP1 or baculovirus-encoded recombinant TSP1 EGF-like repeats increased EGFR tyrosine phosphorylation. The EGF-like repeats alone were sufficient to activate EGFR, whereas no other TSP1 domains could do so. EGF-like repeats from TSP2 and TSP4 also activated EGFR. Native TSP1 and E123 each increased phosphorylation of six distinct tyrosine residues within the cytoplasmic domain of EGFR (Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, Tyr-1086, and Tyr-1173). Immunoblockade of the ligand-binding portion of the EGFR ectodomain blocked EGFR activation in response to TSP1 or its EGF-like repeats. However, a direct binding interaction between E123 and EGFR could not be demonstrated. TSP1 and E123 each increased MMP9 expression and activity, and EGFR activation...
tion in response to either agonist was blocked, in part, by MMP2/MMP9-selective inhibition, immunoblockade of surface expressed MMP9, or knockdown of MMP9. These data support a scenario in which the EGF-like repeats of TSP1 stimulate MMP-mediated release of cell surface bound ligand(s), which then binds to the EGFR ectodomain. Finally, EGFR activation in response to TSP1 and its EGF-like repeats, including autophosphorylation of Tyr-992 and Tyr-1173, the docking sites for PLC-γ, was coupled to downstream PLC-γ activation and increased cell migration.

EGFR responds to direct binding of EGF motif-containing ligands (28) but can also be transactivated through a number of heterologous receptors (36, 37, 52, 54, 55). We first asked whether the EGF-like repeats of TSP1 directly engaged the EGFR ectodomain. Using two distinct experimental approaches, direct E123 binding to EGFR could not be detected (Fig. 9). However, preincubation of A431 cells with antibody that targets the ligand-binding portion of the EGFR ectodomain completely blocked EGFR activation in response to EGF, TSP1, and E123 (Fig. 8). The requirement for an unimpeded EGFR ectodomain (Fig. 8) in the absence of a detectable E123-EGFR interaction (Fig. 9) raised the possibility that TSP1 indirectly activates EGFR through MMP-mediated release of an EGFR ligand(s) (28, 54). Of interest, TSP1 reportedly up-regulates MMP-2 and -9 expression (56, 57), and in A431 cells, we found that TSP1 and its EGF-like repeats each increases MMP9 expression (Fig. 10B) and catalytic activity (Fig. 10C). That TSP1 increased MMP9 protein expression ≥2-fold more than did an equimolar concentration of E123 (Fig. 10B) implicates participation of TSP1 domains outside of the EGF-like repeats (58). Although TSP1 is known to increase MMP9 expression (56), it also directly inhibits enzymatic conversion of pro-MMP9 to MMP9 (58, 59), up-regulates expression of TIMP1 (21) and TIMP2 (56), and protects specific substrates from proteolysis (60). These bioactivities ascribed to TSP1 may make it more difficult to detect changes in MMP9 activity in response to the same molecule. Finally, pharmacologic inhibition of MMP activity (Fig. 10D), immunoblotting of surface expressed MMP9 (Fig. 11A), or siRNA-induced knockdown of MMP9 (Fig. 11B), each substantially but incompletely reduced EGFR activation in response to either TSP1 or E123. These combined data are compatible with a mechanism in which TSP1/E123 up-regulates MMP activity, including MMP9, to proteolytically release an EGFR ligand(s), which then binds to the EGFR ectodomain and activates EGFR signaling. TSP1 reportedly regulates MMP activity but through its NH2-terminal and TSR domains (58). How E123 might up-regulate MMP expression or activity is unclear. Beyond the classical MMPs, participation of either the closely related ADAMs and/or ADAMTs (a disintegrin and metalloproteinase with TSP motifs) families has not been excluded (61, 62). ADAM-9, -10, -12, and -17 each reportedly cleave and release one or more high affinity EGFR ligands (29–32). A431 cells express mRNAs for six high affinity EGFR ligands, including EGF, transforming growth...
factor α, amphiregulin, heparin-binding EGF, epiregulin, and betacellulin. Identification of the specific EGFR ligand(s) regulated by the EGF-like repeats of TSP1 is currently under study.

Although EGFR activation in response to the EGF-like repeats of TSP1 is, in part, MMP9-mediated (Figs. 10D and 11C), other mechanisms cannot be absolutely excluded. It is conceivable that TSP1 binds to EGFR with a binding affinity so low as to preclude detection by either cross-competition binding or chemical cross-linking studies (Fig. 9, C–E). Although each of the TSP1 EGF-like repeats contains the framework of six conserved cysteine residues that form the three intramolecular 1–3, 2–4, and 5–6 disulfide bonds required for EGFR engagement, the intervening residues dramatically diverge from corresponding sequences in high affinity EGFR ligands (28). Furthermore, unlike most EGF-like repeats that contain one residue between the fourth and fifth cysteines, the EGF-like repeats of the TSPs have two, potentially altering their structural flexibility (2). Two other proteins containing divergent EGF-like repeats, tenascin-C and laminin-5, have been shown to display low affinity binding to EGFR (34, 35). Multidomain TSP1, through its ability to bind multiple heterologous receptors, may also transactivate EGFR (52, 54, 55). Of course this would not account for the activity of isolated E123. Finally, one or more TSP1 domains might also antagonize EGFR activation by the EGF-like repeats. In fact, our data indicate that the TSP1 EGF-like repeats co-expressed with either the flanking third type I repeat or the type III Ca$^{2+}$-binding repeats each increased EGFR Tyr-1068 phosphorylation to lower levels than did the EGF-like repeats alone (Fig. 2B, lane 4 versus lanes 3 and 5).

EGFR tyrosine autophosphorylation is coupled to recruitment of signaling elements and cellular responses, including proliferation, differentiation, survival, and motility (27, 63). Our studies show that TSP1 and its EGF-like repeats increase phosphorylation of Tyr-992 and Tyr-1173 (Fig. 4), binding sites for PLCγ (27). PLCγ participates in EGFR-driven cell motility (63). In our studies, TSP1 and its EGF-like repeats increased PLCγ activation (Fig. 5, A, B, and E) and cell motility (Figs. 6 and 7). TSP1/E123-induced increases in PLCγ activation and cell motility were completely blocked by prior EGFR-selective PTK inhibition (Fig. 5C and Fig. 6) or siRNA-mediated EGFR depletion (Fig. 5, D and E, and Fig. 7). Although E123 also increased cell migration, it was 50% less active than native TSP1 (Fig. 6). Whether one or more domain(s) outside the EGF-like repeats is required for an optimal migratory response is unclear. Our combined data indicate that the EGF-like repeats of TSP1 can increase cell motility through EGFR and possibly through PLCγ activation.

The ability to activate EGFR, and possibly other co-expressed ErbB receptors, together with the numerous signaling elements to which they are coupled, may elucidate relatively unexplained aspects of TSP1 biology, especially apropos to the epithelial cell. In TSP1 null mice, multilineage epithelial hyperplasia with thickening and ruffling of selected epithelia was prominent (22). In human epithelium-derived cancer cells, EGF increases TSP1 expression (21). TSP1 expression is increased during tissue repair and wound healing (7, 64) where it accelerates re-epithelialization (8). In a murine model of corneal abrasion, exogenous TSP1 stimulated epithelialization, whereas anti-TSP1 antibodies inhibited it (8). In A431 cells, EGFR is co-expressed with ErbB2. TSP1 and its EGF-like repeats, through the ability to activate EGFR, transactivates its preferred heterodimerization partner ErbB2. Co-activation of EGF/Erbb2 heterodimers appears to be key in epithelial tumor cell biology (65). TSP1 is known to influence tumor cell adhesion, migration, invasion, and metastasis both in vitro and in vivo (20, 65, 66). Whether TSP1 influences tumor cell behavior through EGFR/ErbB2 biology had not been tested. We now have demonstrated for the first time that increased migration of a human epidermoid carcinoma (A431) cell line, in response to TSP1, is blocked by prior EGFR-selective PTK inhibition (Fig. 6) or siRNA-induced knockdown (Fig. 7). Our findings on TSP1, TSP2, and TSP4, and the work of others on laminin-5 (34) and tenascin-C (35), collectively suggest that the phylogenetically ancient EGF-like repeat that has been conserved across a large and diverse group of proteins (33) may contribute to their bioactivity.

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