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Shedding of Soluble Epidermal Growth Factor Receptor (sEGFR) Is Mediated by a Metalloprotease/Fibronectin/Integrin Axis and Inhibited by Cetuximab

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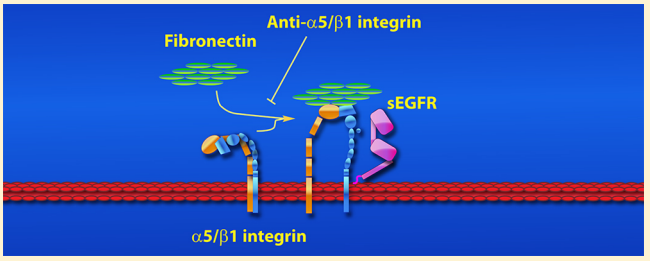
Supporting Information

ABSTRACT: Soluble epidermal growth factor receptor (sEGFR) is a circulating serum biomarker in cancer patients. Recent studies suggest that baseline serum sEGFR concentrations may predict responsiveness to EGFR-targeted therapy. Here, we demonstrate that sEGFR is generated through proteolytic cleavage of a cell surface precursor of an alternately spliced EGF receptor isoform and that sEGFR binds to EGF with high affinity. Proteolytic cleavage is stimulated by an anti-$\alpha5/\beta1$ integrin antibody and 4-aminophenylmercuric acetate, and inhibited by fibronectin. Two FDA-approved therapeutic anti-EGFR antibodies also inhibit shedding of sEGFR, thus implicating the cell surface precursor of sEGFR as a competing target for anti-EGFR antibodies in human tissues. These observations parallel trastuzumab regulation of HER2 shedding and have implications for patient stratification in future clinical trials of EGFR-targeted antibodies.



The human epidermal growth factor receptor (*EGFR/HER/ERBB*) gene family encodes four distinct receptor tyrosine kinases: EGFR (HER1/ErbB1), HER2 (Neu/ErbB2), HER3 (ErbB3), and HER4 (ErbB4).[1] In addition to full-length EGFR/HER isoforms, human and rodent tissues express soluble EGFR/HER isoforms (sEGFR/sHER) that lack the cytoplasmic and transmembrane domains of the receptor (reviewed in ref 2). These soluble receptor isoforms can be generated either by alternate splicing of EGFR/HER mRNAs or by proteolytic cleavage of full-length EGFR/HER isoforms. For example, sHER2 is derived by proteolytic cleavage of full-length HER2 by a metalloprotease, tentatively identified as ADAM10,[3] thus shedding a 105-kDa protein comprised of subdomains I–IV of the extracellular domain (ECD) of this receptor.[4−6] Similarly, tumor necrosis alpha transforming enzyme (ADAM17/TACE) catalyzes shedding of HER4 splice variants (JM-a and JM-b) that differ in their extracellular juxtamembrane region, resulting in their differential susceptibility to proteolytic cleavage.[7] While JM-b HER4 is not susceptible to ADAM17/TACE cleavage, ADAM17/TACE liberates a 120-kDa soluble fragment of JM-a HER4.[8] In contrast, full-length EGFR generally is not cleaved by metalloproteases;[8] however, exceptions can be found in certain malignant cell lines that highly overexpress EGFR.[9−11]

Although proteolytic cleavage of full-length EGFR is rare, an abundant circulating isoform of EGFR has been shown to have clinical utility as a cancer biomarker (see ref 12 for review and Figure 1). Tandem mass spectrometric analysis of purified serum EGFR shows that this protein is derived from an alternately spliced 3.0 kb *EGFR* transcript.[13−15] The protein product of this 3.0 kb *EGFR* transcript, termed soluble EGFR (sEGFR), encompasses ECD subdomains I–III, most of

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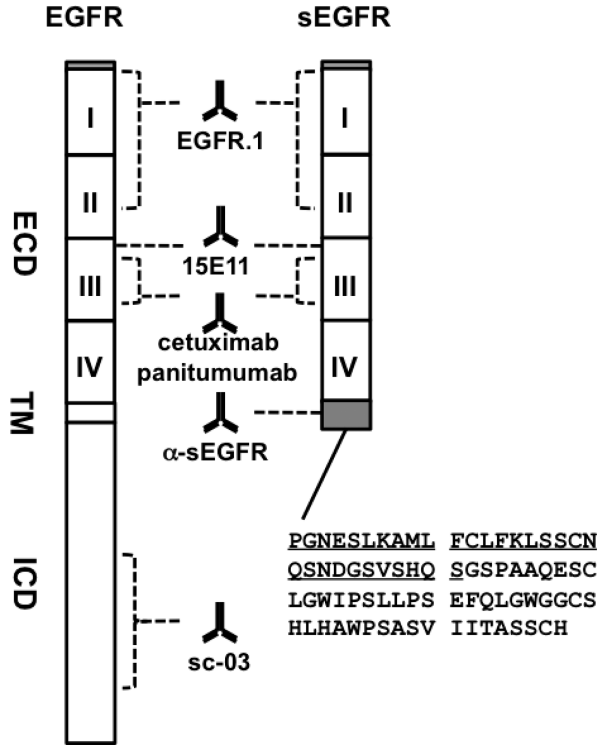

Figure 1. EGFR is compared sEGFR in this diagram. sEGFR shares identity with EGFR through extracellular subdomains I, II, III, and most of IV, which includes the antigenic epitopes for a number of antibodies such as mAb 15E11, mAb EGFR.1, cetuximab, and panitumumab. Near the carboxyl-terminus of subdomain IV, sEGFR diverges from EGFR and includes a unique sequence of 78 amino acids. Polyclonal antibody α-sEGFR is directed against the first 31 amino acids of sEGFR's unique 78 amino acid C-terminus (underlined sequence); this epitope does not exist in full-length EGFR. Conversely, polyclonal antibody sc-03 is directed against an epitope of the intracellular domain of EGFR, which is not found in sEGFR.

subdomain IV, and terminates in a unique 78 amino acid carboxy-terminal peptide (CTP) encoded by exon 15B.[13] Paradoxically, this 90/110-kDa sEGFR isoform lacks both the transmembrane and cytoplasmic domains of EGFR but is both a cell surface and secreted protein.[14]

Here, we show that sEGFR is a cell surface membrane protein that is proteolytically released (shed) via a regulated mechanism. sEGFR shedding requires metalloprotease activity, which includes ADAM17/TACE as a component of the sEGFR shedding pathway. Shedding of sEGFR is inhibited by fibronectin and stimulated by an antibody directed against $\alpha5/\beta1$ integrin. In addition, we show that two EGFR-targeted therapeutic antibodies (i.e., cetuximab and panitumumab) can inhibit sEGFR shedding, thus implicating the cell surface precursor of sEGFR as a competing target for these antibodies.[16] Although sEGFR shedding shares mechanistic elements in common with both sHER2 and sHER4 shedding,[3,7] sEGFR lacks both canonical transmembrane and cytoplasmic domains. We speculate that regulation of sEGFR shedding has important functional implications for EGFR/integrin-mediated signal transduction, as well as for patient selection in EGFR-targeted cancer therapy.

MATERIALS AND METHODS

Cell Lines and Reagents. Chinese hamster ovary (CHO) parental cells and CHO cells stably expressing EGFR (CHO-EGFR) or sEGFR (CHO-sEGFR) have been previously described.[17] CHO-M1 cells were a kind gift from Dr. Joan Massagué. Drosophila Schneider 2 (S2) cells were purchased from Invitrogen (Carlsbad, CA). Porcine, equine, caprine, human, bovine, and fetal bovine sera were purchased from Atlanta Biologicals (Lawrenceville, GA). Lipofectamine was purchased from Invitrogen. EZ-link Sulfo-NHS-LC-Biotin and protein A/G beads were purchased from Pierce/Thermo Scientific (Rockford, IL). Human serum fibronectin, blue-4-agarose, 4-aminophenyl mercuric acetate (APMA), EGFR, and fraction V bovine serum albumin (BSA) were purchased from Sigma Chemical Co (St. Louis, MO). Mouse monoclonal antibody (mAb) 15E11, which recognizes both EGFR and sEGFR, and affinity purified rabbit polyclonal anti-sEGFR antibody, which recognizes sEGFR but not EGFR, have been previously described.[17,18] Anti-EGFR mAb EGFR.1 was purchased from NeoMarkers (Fremont, CA), and rabbit polyclonal anti-EGFR intracellular domain antibody sc-03 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). $\alpha5/\beta1$ integrin-inhibitory ascites (MAB1969) was purchased from Millipore (Billerica, MA). Anti-Shc rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Ras mouse ascites (146−03E04) was purchased from American Type Culture Collection (Manassas, VA). Goat antimouse horseradish peroxidase-conjugated secondary antibody and ECL substrate were purchased from Thermo Scientific. Alexa 568-conjugated goat antimouse secondary antibody and Prolong antifade reagent were purchased from Molecular Probes (Eugene, OR). IMAC Sepharose columns were purchased from GE Healthcare (Piscataway, NJ). Microcon (10-kDa cutoff) and Centricon (30-kDa cutoff) centrifugal filtration units were purchased from Millipore. All cell culture reagents were purchased from Mediatech (Manassas, VA), except G418, which was purchased from Invitrogen. Cetuximab (ImClone/Bristol-Myers Squibb) and panitumumab (Amgen) were obtained from the Yale New Haven Hospital.

CHO Cell Culture and Plasmid Transfection. CHO parental cells were maintained in Ham's F12 media supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). CHO-EGFR and CHO-sEGFR cells were maintained in the above media, supplemented with 800 μg/mL G418 (growth media). For sEGFR shedding studies, cells were cultured in growth media without FBS (serum free assay media). CHO-M1 cells were maintained in histidine-free minimal essential media (MEM) supplemented with 10% dialyzed FBS, 0.5 mM histidinol, 50 U/mL penicillin, 50 μg/mL streptomycin. Subconfluent cultures of CHO-M1 cells were transiently transfected with vector encoding sEGFR[17] using Lipofectamine, following the manufacturer's instructions.

Indirect Immunofluorescence Microscopy. CHO-sEGFR cells were cultured on glass coverslips at 37 °C for 24 h. To observe cell surface sEGFR localization, cells were chilled to 4 °C for 20 min, washed gently with cold phosphate buffered saline (PBS), and incubated with blocking buffer (5% goat serum, 1% glycerol, 0.1% bovine serum albumin, and 0.1% gelatin in PBS) for 10 min at 4 °C. Cells were then incubated with 5 μg/mL mAb EGFR.1 diluted in blocking buffer for 30 min at 4 °C. Cells were washed with PBS and incubated with Alexa 568-conjugated goat antimouse secondary antibody diluted 1:800 for 30 min at 4 °C, washed with PBS, and fixed with 4% paraformaldehyde for 10 min at 4 °C, followed by

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30 min at room temperature. Cells were washed with PBS, counterstained with 0.1 mg/mL DAPI, mounted with Prolong antifade reagent, and viewed using a confocal microscope (Carl Zeiss, model 510) equipped with a 100× objective.

Cell Surface Biotin-Labeling. CHO cells were plated into 35-mm^2 culture dishes and transiently transfected with the plasmids pcDNA3 (Invitrogen) or pcDNA2241[17] containing the cDNA encoding sEGFR using Lipofectamine reagent. Cell surface proteins were reacted with a membrane impermeable biotin cross-linker 48 h post-transfection. Briefly, cell monolayers were chilled to 4 °C and washed with 10 mL cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ four times. Cells were then incubated with 0.5 mg/mL EZ-link Sulfo-NHS-LC-Biotin for 10 min on ice at 4 °C to prevent the internalization of biotin cross-linked proteins. Unbound biotin cross-linker was aspirated and the labeling reaction was terminated by the addition of quenching buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 100 mM glycine). Cells were rinsed twice with cold quenching buffer, incubated with quenching buffer for 10 min at 4 °C, and then rinsed twice more with the same buffer. Cells were lysed with cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 2 mg/mL aprotinin). Cell lysates were immunoprecipitated with either anti-EGFR mAb EGFR.1 or with anti-Shc polyclonal antibody and protein A/G beads (Pierce). Immunoprecipitated proteins were resolved by SDS-PAGE in 8% acrylamide gels[19] and transferred to Immobilon polyvinylidene difluoride (PVDF) membrane for Western immunoblotting using a semidry graphite electroblotter system (Millipore Corp.) according to the manufacturer's instructions. Immunoblots were probed with either 0.1 μg/mL horseradish peroxidase-conjugated streptavidin (Pierce) or anti-Shc antibody followed by peroxidase-conjugated secondary antibody as described below.

Culture Conditions for sEGFR Shedding Assay. CHO-sEGFR cells were seeded into 24-well plates and cultured to near-confluence. CHO-sEGFR cells were rinsed three times with PBS and incubated with serum free assay media for 72 h, unless otherwise indicated. Cells were examined microscopically for viability and the conditioned culture media (CCM) was collected from each well of CHO-sEGFR cells.

Western Immunoblot Shedding Assay. Following clarification by centrifugation (15000*g* for 30 min at 4 °C), CCM samples were resolved by SDS-PAGE in 7.5% acrylamide gels[19] and transferred to PVDF membrane by semidry method and blocked with Tris-buffered saline (TBS; 10 mM Tris HCl, 150 mM NaCl, pH 7.4) containing 5% nonfat dry milk for 1 h. Membranes were rinsed six times for 10 min each with TBS containing 0.1% Tween 20 (TBS-TW20) and incubated with TBS containing 1% BSA and mAb 15E11 (1:10 dilution of culture medium) overnight at 4 °C. Membranes were rinsed six times for 10 min each with TBS-TW20 and incubated with goat antimouse horseradish peroxidase conjugated secondary antibody (Pierce, 1:4000 dilution) for 1 h at room temperature. Membranes were rinsed six times for 10 min each with TBS-TW20, reacted with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), and visualized using NucleoVISION chemiluminescence detection system.

Dot Blot Shedding Assay. CHO-sEGFR CCM samples, harvested as described above, were applied to nitrocellulose membrane in triplicate using a 96-well dot blot apparatus (Bio-Rad, Hercules, CA); CCM of CHO-sEGFR cells cultured with FBS was added to parallel wells as a negative control. Following gravity filtration of the CCM samples through the nitrocellulose membrane, the membrane was processed for Western immunoblotting as described above. Dot blot and Western immunoblot of SDS-PAGE resolved samples yielded comparable standard dose response curves with CHO-sEGFR CCM (data not shown).

Expression of sEGFR in Insect Cells for Ligand Binding Assay. Using pcDNA2241 as a source vector, sEGFR cDNA was cloned into the pMT/BiP/V5/HisA expression plasmid (Invitrogen) and transfected into Drosophila S2 cells by calcium phosphate precipitation as described by the manufacturer. S2 cells were grown in complete Schneider's Drosophila medium (SDM) supplemented with heat-inactivated FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin until the cells reached a density of 2×10^6 cells/mL in a 28 °C nonhumidified incubator.

Purification of sEGFR. Expression of sEGFR was induced by rinsing pMT/BiP/V5/HisA/sEGFR-transfected S2 cells with serum-free SDM and incubating for six days in serum-free SDM supplemented with 0.5 mM $CuSO_4$. Cell suspensions were briefly centrifuged to collect CCM, which was supplemented with protease inhibitors leupeptin, pepstatin, aprotinin, and PMSF, filter sterilized, and loaded onto a Sepharose IMAC column. The IMAC column was washed extensively with PBS, then eluted stepwise with 10, 50, and 250 mM imidazole in 50 mM Tris, pH 8.0. sEGFR eluted preferentially with 50 mM imidazole. Fractions containing sEGFR were pooled, concentrated by Centricon YM-30 ultrafiltration, and assesed for protein purity by SDS-PAGE with Coomassie stain. sEGFR content was confirmed by Western immunoblot with mAb 15E11.

Quantification of Ligand Binding to EGFR and sEGFR. mAb EGFR.1 was conjugated to scintillation proximity assay (SPA) beads at a concentration of 0.4 mg/mL overnight at 4 °C with shaking. EGFR.1-conjugated SPA beads (SPA-R.1) were washed twice with SPA buffer (20 mM HEPES, pH 7.4, 0.1% BSA), then resuspended in SPA buffer supplemented with 40% glycerol at 20 mg beads/mL. SPA-R.1 beads were conjugated with EGFR (Sigma) or purified sEGFR (26 nM/reaction). EGF binding studies were performed overnight at room temperature with 0.15 mCi of ^{125}I-labeled EGF and competing concentrations of unlabeled EGF (0−500 nM), and 1 mg SPA-R.1 beads at a final volume of 200 mL. Samples were analyzed by scintillation counting with GraphPad Prism software. To confirm EGFR and sEGFR binding to mAb EGFR.1, SPA-R.1 beads were washed three times with SPA assay buffer, and boiled with 2× Laemmli sample buffer to solubilize bound proteins, which were resolved by SDS-PAGE and probed by Western immunoblot with mAb 15E11.

RESULTS

sEGFR Is a Cell Surface Membrane Protein. Heterologous expression of sEGFR in CHO cells, which do not express endogenous EGFR (or sEGFR), has been reported by us previously and results in no obvious morphologic changes in this cell line.[14,17] We were surprised, however, to observe that sEGFR is localized to the cell surface of CHO-sEGFR cells by indirect immunofluorescence microscopy (Figure 2A) since sEGFR lacks a canonical transmembrane domain.[13] This localization pattern was confirmed in intact CHO-sEGFR cells by cell surface biotin labeling (Figure 2B) and also by studies demonstrating that sEGFR is a constituent of

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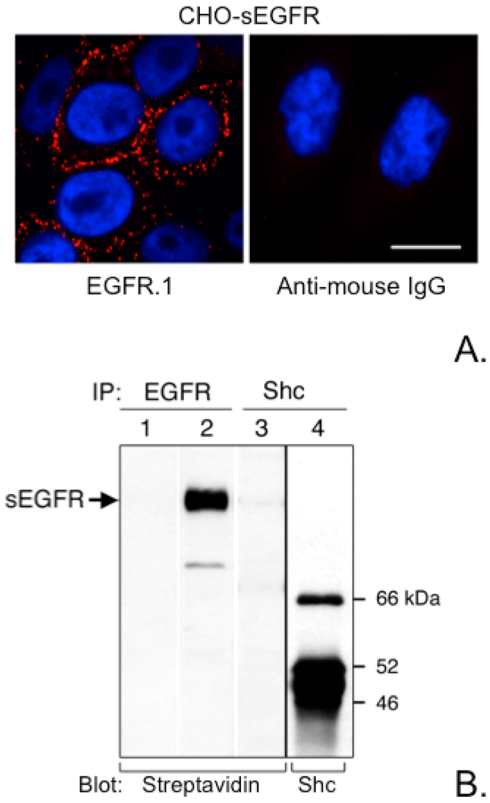

Figure 2. sEGFR localizes to the cell surface. Panel A. CHO-sEGFR cells were labeled at 4 °C with anti-EGFR mAb EGFR.1 (left panel) and an antimouse IgG secondary antibody conjugated to Alexa Fluor 568 (red). Nuclei were stained with DAPI (blue). No staining was observed when mAb EGFR.1 was omitted (right panel). Images were captured at 100× magnification using a confocal microscope. Bar = 10 mM. Panel B. Nontransfected CHO cells (lanes 1, 3, and 4) or CHO-sEGFR cells (lane 2) were labeled with a membrane-impermeable biotin cross-linker. Cell lysates were immunoprecipitated with either anti-EGFR (lanes 1 and 2) or anti-Shc (negative control; lanes 3 and 4) antibody and reacted with streptavidin-HRP. This blot demonstrates that sEGFR, but not Shc, was cross-linked to biotin and, therefore, localized on the cell surface.

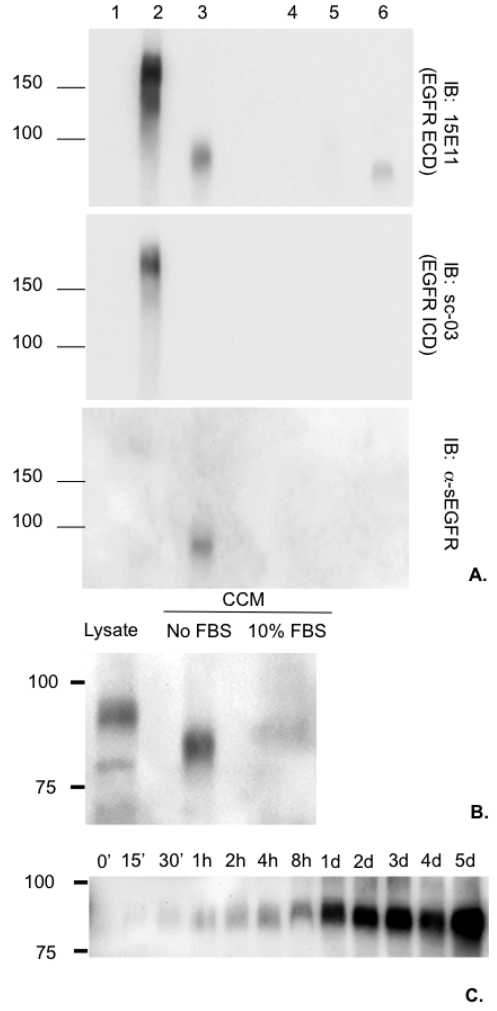

Figure 3. sEGFR, but not EGFR, is released from the cell surface of transfected CHO cells. Panel A. Immunoblot of CHO parental (lane 1), CHO-EGFR (lane 2), and CHO-sEGFR (lane 3) whole cell lysates, and serum free conditioned culture media (CCM) obtained after 72 h of growth from CHO parental (lane 4), CHO-EGFR (lane 5), and CHO-sEGFR (lane 6) cells is shown. mAb 15E11 immunoblot detects cell-associated EGFR (170-kDa) and sEGFR (95-kDa), as well as an 80-kDa sEGFR band in CCM from CHO-sEGFR cells, indicating proteolytic release of sEGFR from the cell surface. Polyclonal antibody sc-03 immunoblot detects cell-associated p170 EGFR only. Polyclonal antibody a-sEGFR immunoblot detects cell-associated p95 sEGFR only. Panel B. Immunoblot analysis of CHO-sEGFR whole cell lysate reveals a primary sEGFR band of 95-kDa. A sEGFR band of 80-kDa is detected in the conditioned culture media (CCM) of cells grown in serum free assay media, but not in CCM of cells grown with 10% FBS. Panel C. Immunoblot analysis of a sEGFR shedding time course is shown. CHO-sEGFR serum free CCM withdrawn at the indicated time points was analyzed by immunoblot.

radiolabeled cell membrane preparations.[14] Further scrutiny of the unique carboxy-terminal sequence of sEGFR reveals a short stretch of hydrophobic amino acids as predicted by modeling algorithms (Figure S1). The localization of sEGFR to the cell surface is dependent on the presence of its carboxy-terminal sequence, since deletion of this sequence results in efficient secretion (unpublished results; refs 20−24).

sEGFR Is Proteolytically Released from the Cell Surface. Since sEGFR is detected in human serum,[15] we investigated the mechanism by which sEGFR is released from the cell surface using CHO cells as a model system. This well-established cell model has been used to elucidate the shedding of diverse cell surface proteins, including HER ligands.[25−28] CHO-EGFR and CHO-sEGFR cells express EGFR or sEGFR at levels detectable by immunoblot analysis as bands migrating at predominantly 170-kDa and 90-kDa, respectively (Figure 3A). Antibodies specific for an intracellular epitope of EGFR (sc-03; Figure 3B) or for an epitope located in sEGFR's unique CTP (α-sEGFR; Figure 3C) immunoblot these same proteins in CHO-EGFR and CHO-sEGFR lysates, respectively. Unexpectedly, a shed sEGFR band of 80-kDa was detected by immunoblot with EGFR ECD-specific antibody (15E11) in conditioned culture media (CCM) of CHO-sEGFR cells cultured in the absence of serum, but not in the CCM of either parental CHO or CHO-EGFR cells cultured under the same conditions (Figure 3A, lane 6; and Figure 3B). Serum from other mammalian species (including equine, caprine, porcine, and notably, human serum) also inhibited p80 sEGFR shedding (Figure S2). This 80-kDa band also is detected by immunoprecipitation with another ECD-specific anti-EGFR antibody (EGFR.1; data not shown) but not with an anti-sEGFR antibody. These observations suggested that a serum-

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regulated proteolytic event liberates p80 sEGFR from the surface of CHO-sEGFR cells.

Kinetic analysis of sEGFR shedding demonstrated that p80 sEGFR could be detected in CCM within 15 min following serum withdrawal (Figure 3C). While shed p80 sEGFR continued to accumulate in the CCM for up to five days following serum withdrawal, microscopic observation predictably revealed increased numbers of rounded, detached, and dying CHO-sEGFR cells under serum free conditions over time (data not shown); therefore, unless otherwise indicated, 72 h was used as the end point for all subsequent sEGFR shedding experiments.

Identification of Serum Factors that Attenuate sEGFR Shedding. We hypothesized that sEGFR shedding is regulated by a component(s) of serum, which we named "attenuator of sEGFR release" (ASER) for its biological activity. Porcine serum (PS) was used as an economical substitute for FBS to identify the underlying protein component(s) of ASER.

As a first step toward purifying ASER activity, serum proteins were precipitated and resuspended from 40%, 40−50%, or 50−60% ammonium sulfate saturated PS, hereafter termed AS40-PS, AS50-PS, and AS60-PS fractions, respectively (Figure S3). Although ASER activity was detected in both AS40-PS and AS50-PS preparations (Figure S3A,B), AS50-PS contained significantly more total protein relative to ASER activity than AS40-PS (data not shown). We, therefore, used AS40-PS to further purify ASER activity.

We established a dot-blot assay to facilitate analysis of ASER in a high-throughput format. Several chromatographic techniques were employed to purify ASER from resuspended AS40-PS; blue-4-agarose affinity chromatography efficiently purified ASER activity by elution with a NaCl gradient (Figure S3C). Tandem ammonium sulfate fractionation and blue-4-agarose affinity purified proteins with ASER were pooled, concentrated, and analyzed by SDS-PAGE as shown in Figure S3D. Precipitation of PS with 40% ammonium sulfate saturation substantially purified ASER activity from albumin (~65 kDa), the most abundant protein constituent of serum (Figure S3D, lane 2). Blue-4-agarose chromatography of resuspended AS40-PS further purified ASER activity, resulting in nine prominent Coomassie-stained SDS-PAGE gel bands (Figure S3D, lane 3). Remarkably, eight of these bands were identified by liquid chromatography electrospray ionization mass spectrometry (LC ESI MS/MS) as fibronectin (Table S1).

sEGFR Shedding Is Regulated by Fibronectin and Anti-$\alpha5/\beta1$ Integrin. To test whether fibronectin has ASER activity, we evaluated sEGFR shedding in CHO-sEGFR cells grown in serum free assay media supplemented with purified human serum fibronectin. Fibronectin inhibited sEGFR shedding in a dose-dependent manner (Figure 4A,B) at concentrations within the normal range observed in sera of healthy adults.[29] Notably, fibronectin does not completely inhibit sEGFR shedding at the concentrations tested, suggesting that additional serum components also may be involved in regulating sEGFR shedding. No other morphologic or phenotypic changes besides inhibition of sEGFR shedding were observed in sEGFR expressing CHO cells following treatment with fibronectin.

Since fibronectin is the major extracellular matrix (ECM) ligand of the heterodimeric receptor $\alpha5/\beta1$ integrin,[30] we examined the potential relationship between $\alpha5/\beta1$ integrin and sEGFR shedding. CHO-sEGFR cells were treated with an

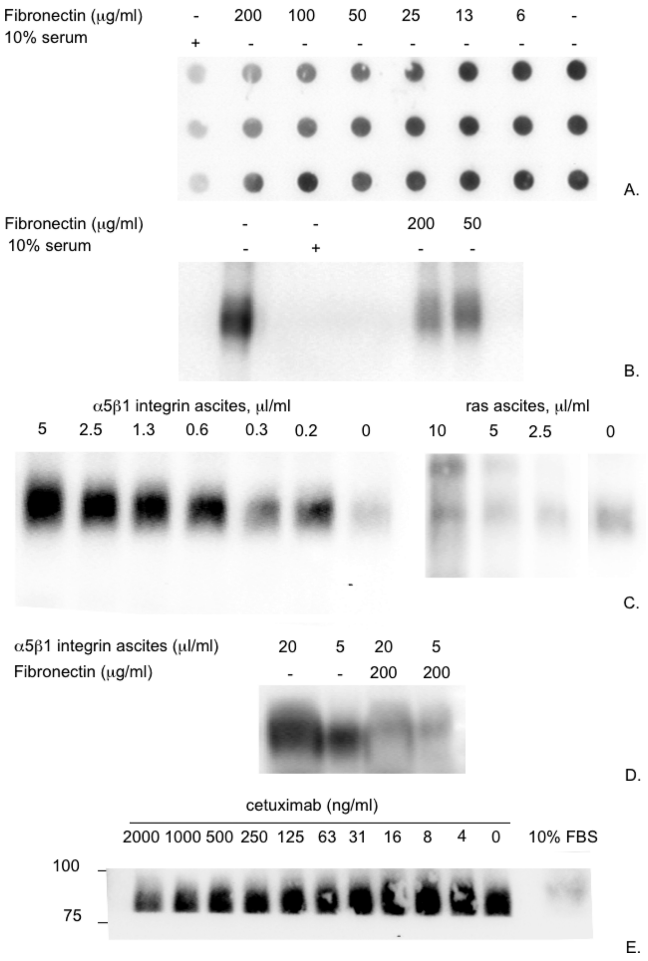

Figure 4. Fibronectin, anti-$\alpha5/\beta1$ integrin antibody, and EGFR-targeted inhibitory antibodies inhibit sEGFR shedding. Panel A. Dot blot analysis (in triplicate) of CCM of CHO-sEGFR cells cultured in the presence of fibronectin demonstrates a dose-dependent decrease in CCM concentration of an sEGFR species reactive with antibody 15E11. Panel B. Immunoblot analysis of sEGFR shedding in CHO-sEGFR cells treated with human serum fibronectin. Lane 1, serum free CCM; lane 2, CCM supplemented with 10% FBS; lane 3, serum free CCM supplemented with 200 μg/mL human serum fibronectin; lane 4, serum free CCM supplemented with 50 μg/mL human serum fibronectin. Panel C. Immunoblot analysis of sEGFR shedding in CHO-sEGFR cells treated with anti-$\alpha5/\beta1$ integrin antibody ascites fluid, or control idiotype-matched, anti-Ras antibody ascites fluid is shown. Note a decreased ECL exposure time to accommodate relatively intense signal of CCM from CHO-sEGFR cells treated with anti-$\alpha5/\beta1$ integrin. Panel D. Immunoblot analysis of sEGFR shedding in CHO-sEGFR cells treated with anti-$\alpha5/\beta1$ integrin antibody ascites fluid and 200 μg/mL human serum fibronectin in shown. Panel E. Immunoblot analysis of sEGFR shedding in CHO-sEGFR cells treated with a 1:1 dilution series of 2 μg/mL (2000 ng/mL) cetuximab. Cetuximab effectively inhibited sEGFR shedding at antibody concentrations $\geq$ 1 μg/mL ($\geq$1000 ng/mL).

inhibitory antibody directed against $\alpha5/\beta1$ integrin.[31] In contrast to our observations with purified fibronectin, we observed that anti-$\alpha5/\beta1$ integrin induces sEGFR shedding in a dose-dependent manner (Figure 4C). Moreover, anti-$\alpha5/\beta1$ integrin-induced sEGFR shedding can be antagonized by fibronectin treatment (Figure 4D).

EGFR ECD-targeted Antibodies Inhibit sEGFR Shedding. The HER2-targeted therapeutic antibody trastuzumab inhibits cell surface shedding of sHER2,[32] which has been

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hypothesized to be one of the major mechanisms of trastuzumab antitumor activity (reviewed in ref 33). While we previously have demonstrated that shed sEGFR is recognized by cetuximab and panitumumab,[16] the ability of therapeutic EGFR-targeted antibodies to regulate the shedding of sEGFR has not yet been examined. Accordingly, CHO-sEGFR cells grown in serum free media were treated with either cetuximab or panitumumab. Physiologically relevant concentrations (≥1 μg/mL) of both cetuximab (Figure 4E) and panitumumab (data not shown) inhibit sEGFR shedding. These observations have implications for the therapeutic mechanism of action of these antibodies, as well as for the appropriate selection of patients for treatment with these EGFR-targeted drugs.

sEGFR Shedding Is Catalyzed by a Metalloproteinase. Because sEGFR in CCM and also in serum (unpublished results) is of a lower apparent molecular mass than cell-associated sEGFR, we hypothesized that cell surface release of sEGFR is mediated by proteolytic cleavage. Studies by others have shown that there is a common mechanism of cell surface protein cleavage for other HER family members, such as HER2 and HER4, that involves proteolytic activities sensitive to metalloprotease activators and inhibitors.[34] Here, we show that one such proteolytic activator, 4-aminophenyl mercuric acetate (APMA), induces shedding of sEGFR in a dose-dependent manner under serum free conditions from CHO-sEGFR cells (Figure 5A). This observation suggested that sEGFR is actively shed from the cell surface by a regulated metalloprotease.

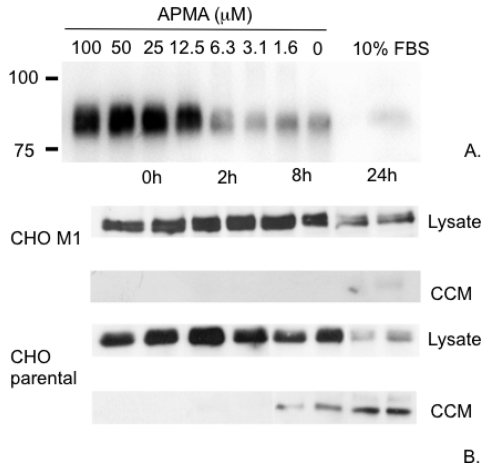

Figure 5. sEGFR shedding is regulated by a metalloprotease. Panel A. Immunoblot analysis of CCM of CHO-sEGFR cells grown in serum free assay media supplemented with a 1:1 dilution series of 100 mM 4-aminophenyl mercuric acetate (APMA) is shown. CHO-sEGFR CCM supplemented with 10% FBS is included as a control (far right lane). Note a decreased ECL exposure time to accommodate a relatively intense signal of CCM from CHO-sEGFR cells treated APMA. Panel B. Immunoblot analysis of serum free CCM and whole cell lysate of CHO-M1 cells, which represent CHO-M1 cells transiently transfected to express sEGFR. Cell surface-associated sEGFR, but not shed sEGFR, was detected in this TACE-deficient cell line.

Because tumor necrosis factor alpha converting enzyme (ADAM17/TACE) has been implicated in the shedding of HER4 ECD,[7] we tested the hypothesis that ADAM17/TACE also might be required for the shedding of sEGFR with a CHO cell line deficient in ADAM17/TACE cell surface localization (CHO-M1).[35] We observe that CHO-M1 cells which transiently express sEGFR, do not release sEGFR into the culture medium, suggesting that ADAM17/TACE may be involved in cell surface shedding of sEGFR (Figure 5B). However, this observation does not preclude sEGFR as a substrate for other metalloproteases, potentially as part of a more complex shedding pathway.

Shed sEGFR Binds to EGF with High Affinity. sEGFR contains all of the necessary ligand-binding subdomains.[13,36] To measure protein−protein interactions between sEGFR and EGF, fluoromicrosphere beads conjugated with EGFR.1 antibody were incubated with either purified EGFR or sEGFR (His-tagged, expressed in Drosophila S2 cells) and with ^{125}I-labeled EGF at various concentrations of unlabeled EGF. As shown in Figure 6, EGF binds to purified EGFR and

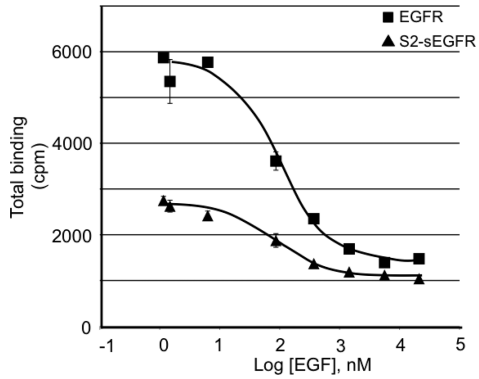

Figure 6. Shed sEGFR binds EGF with high affinity. Binding of EGF to EGFR and sEGFR was quantified using a scintillation proximity assay. Fluoromicrospheres were coated with EGFR.1 antibody and incubated with solubilized EGFR or sEGFR. ^{125}I-EGF was added at the indicated concentrations. Only ^{125}I-EGF bound to receptor generates luminescence via fluoromicroshperes. Both sEGFR and EGFR bind to EGF with high affinity.

to sEGFR with similar kinetics; however, an ~2.5 fold lower B_{max} was observed for binding to sEGFR compared to full-length EGFR. Similar results have been observed with recombinant EGFR ECD proteins, which share all of the EGFR ECD sequence, but which lack the unique CTP of sEGFR (unpublished results and ref 24).

DISCUSSION

We show here that sEGFR shedding is catalyzed by a metalloprotease (including but not necessarily limited to ADAM17/TACE) using a well-established CHO cell model system, which previously has been used by others for cell surface shedding studies. This shedding process is regulated by α5/β1 integrin and its ECM ligand, fibronectin, as summarized in Figure 7. While this is the first report demonstrating a relationship between α5/β1 integrin, the ECM, and sEGFR, associations between EGFR signaling, integrin, and attachment-mediated survival signaling have been studied extensively.[37−43]

In previously published studies, we have shown that the unique carboxy-terminal sequence (78 amino acids) of sEGFR shares an epitope of significant sequence homology with the conformationally active hinge region (i.e., calf-1 domain) of the α5 subunit of α5/β1 integrin and that antibodies directed against this sEGFR epitope promote cell cohesion.[44] This observation is particularly relevant in light of recent studies that demonstrate that integrin itself has direct growth factor-binding capacity[45] and also colocalizes with ADAM17 on the cell surface.[46] In spite of the implied functional relationships among

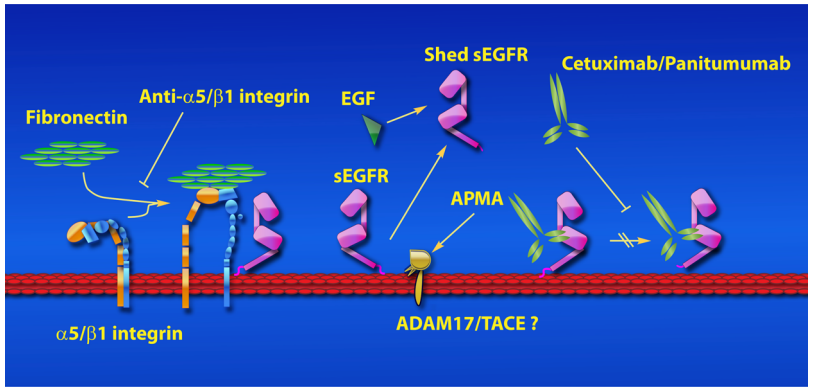

Figure 7. Preliminary model of sEGFR shedding. sEGFR is localized to the cell surface and is constitutively shed via metalloprotease-catalyzed proteolysis. A metalloprotease activator, APMA, stimulates sEGFR shedding. sEGFR may be a substrate of ADAM17/TACE, but ADAM17/TACE is not necessarily the only protease involved in sEGFR shedding. Engagement of the ECM by integrins (i.e., fibronectin binding to $\alpha5/\beta1$ integrin) inhibits basal sEGFR shedding, an effect antagonized by an inhibitory antibody directed against $\alpha5/\beta1$ integrin. EGFR-directed antibodies that also recognize sEGFR (i.e., cetuximab and panitumumab) also inhibit basal sEGFR shedding.

integrins, EGFR, and now sEGFR and perhaps ADAM17/TACE, the structural basis for interactions between these cell surface proteins remains poorly defined.

One important aspect of understanding the relationship between sEGFR shedding and integrin mediated signaling will be to determine the mechanism by which sEGFR associates with the cell membrane. Structural analyses suggest that sEGFR may associate with the plasma membrane via sequences within its unique carboxy-terminus, because various recombinant forms of EGFR that encompass the ECD, but which lack the unique CTP of sEGFR, are secreted.[20−24] Sequences within the CTP of sEGFR may promote intrinsic membrane interactions (Figure S1), or alternatively may result in cell surface localization via interactions with other cell surface proteins, and/or via novel post-translational modification(s) that have yet to be determined.

While it is not yet known what sequences dictate substrate specificity for either sEGFR or EGFR cleavage, previous studies examining EGFR shedding using chimeric receptors provide some insight. In these studies, chimeric EGF receptors embodying only portions of the ECD were used to demonstrate that the ECD, alone, is not efficiently shed from the cell surface,[8,47] suggesting that sequences within the ECD provide sheddase specificity. Here we propose that the sequence divergence between EGFR and sEGFR in the distal region of subdomain IV may explain why sEGFR, but not EGFR, is constitutively shed from the surface of CHO cells.

In addition, several recent reports suggest that EGFR and proteolytic EGFR fragments can be exported from the cell via an exosome-mediated process.[9,48] In support of this observation, our results and those of others suggest that certain cell lines derived from malignancies that overexpress EGFR on the cell surface (e.g., BT-20 and MDA-MB-468, $\sim2 \times 10^6$ EGFR/cell) can release EGFR fragments into media.[49,50] Indeed, while we were writing this manuscript, Adamcyzk and colleagues reported a comparison of EGFR peptides present in "secreted" vs exosome-associated compartments from a series of pancreatic cancer cell lines.[48] While the secreted compartment included full-length EGFR (suggesting an exosomal contribution to these "secretome" samples), there also were notable differences between the EGFR-related peptides present in the "secretome" vs exosome compartments. While both compartments were reported to contain full-length EGFR, as well as a p110 EGFR ECD protein, that was presumed to be proteolytically derived, only the exosome compartment contained a 65 kDa EGFR-related protein derived from the intracellular domain.[48] Together, these studies demonstrate a previously unanticipated level of complexity for EGFR cell surface shedding, which may be reflected by an equally complex repertoire of sEGFR isoforms in blood and interstitial fluids, particularly in diseases such as cancer.

By using cell lines engineered to express a single EGFR isoform, we have been able to identify one potential mechanism of sEGFR isoform shedding. Here we demonstrate that ADAM17/TACE is required for sEGFR release from the cell surface; however, the direct site of sEGFR cleavage has not yet been identified. While we speculate that sEGFR may be a direct substrate for ADAM17/TACE, alternative interpretations remain plausible. We speculate that a ADAM17/TACE-dependent amino acid sequence, which permits proteolytic cleavage, may be located within the unique CTP of sEGFR. Our observations that the apparent molecular weight of sEGFR is reduced during shedding and that neither sEGFR shed from CHO cells (Figure 3) nor serum sEGFR (unpubl. results) is recognized by an antibody specific for the unique carboxyl-terminal sEGFR sequence support this hypotesis. Furthermore, sEGFR purified from human blood contains the sequence "AMLFCLFK",[15] which is located in the proximal region of sEGFR's unique CTP. Together, these observations suggest that the proteolytic cleavage of sEGFR occurs on the carboxyl-terminal side of "AMLFCLFK", thus potentially leaving a cell surface associated sEGFR "stub". Although the stability and fate of this putative (membrane-associated) carboxyl-terminal sEGFR "stub" have not yet been determined, this unique sequence is high in cysteine content and contains two novel consensus N-linked glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc). Furthermore, ADAM17/TACE may be a necessary but not sufficient component of an sEGFR shedding pathway. Other shed cell surface proteins such as E-cadherin, CD44, and transforming growth factor alpha require overlapping and/or multiple metalloprotease cleavage events.[51−54]

While numerous studies have measured serum "EGFR" concentrations in diverse human cancers and other diseases,[12] the molecular identity (and origin) of the EGFR isoform(s) that circulates in human blood has not yet been conclusively

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established. We have used mass spectrophotometric methods to show that at least one major constituent of serum EGFR is, in fact, derived from cell surface sEGFR.[15] While our observations do not preclude the existence of other EGFR isoforms in human blood, EGFR is not a classical metalloprotease substrate. In particular, previous reports have used EGFR as a negative control in comparative studies on the metalloprotease-mediated shedding of other cell-surface associated proteins such as tumor necrosis factor receptor and HER4.[8,47] In support of these findings, our studies on CHO cells also suggest that EGFR is not constitutively released from the cell surface;[10] we and others have shown, however, that there may be pathologic circumstances (e.g., breast cancer) under which shedding of full-length EGFR occurs.[9,10,55]

The potential clinical importance of sEGFR shedding is underscored by previous studies, which suggest that trastuzumab inhibits tumor cell growth by blocking sHER2 shedding,[32,33] at least in part, by modulating the density of full-length cell surface HER2 receptors.[56] Here we speculate that the CHO-sEGFR cell line is an accurate representation of the cell surface cleavage of sEGFR that occurs in human tissues, including its release into blood. While the tissue(s) of origin for serum sEGFR and sHER2 have not yet been identified, sEGFR has been shown to circulate in healthy subjects within a fairly defined range.[57] The results presented here are consistent with the notion that the serum pool of sEGFR is contributed primarily by normal human tissue(s) as opposed to malignant tissues[12] and further suggest that administration of EGFR-targeted therapeutic antibodies may have pleiotropic effects, because they target both EGFR and sEGFR on the cell surface and circulating in the blood.[16] The relevance of these findings to the mechanism of cetuximab and panitumumab action will require further study, but given trastuzumab's effect on sHER2 shedding, these studies imply that EGFR-targeted therapeutic antibodies may also affect sEGFR shedding in cancer patients. Consistent with this hypothesis, high pretreatment concentrations of serum sEGFR have been correlated with cetuximab and gefitinib responsiveness, as measured by improved overall survival in colorectal (Spindler et al., 2009 ASCO Annual Meeting)[59] and endometrial cancer patients (Leslie et al., in press),[58] respectively.

In conclusion, shed sEGFR, an emerging serum biomarker, is derived from a cell surface precursor of an alternate sEGFR isoform via a proteolytic cleavage event mediated by a metalloprotease. This shedding process is regulated by a fibronectin/integrin axis and can be inhibited by therapeutic EGFR-targeted antibodies. These studies demonstrate the existence of a highly regulated mechanism of sEGFR shedding, which is consistent with clinical correlative studies on serum sEGFR concentrations in healthy subjects[57] and analogous to the regulated proteolytic release of both sHER2 and sHER4. Together, these results have important physiological as well as clinical implications and suggest that future studies using EGFR-targeted antibodies for the treatment of diverse diseases, including cancer, should consider the potential for pleiotropic effects of these drugs on alternate EGF receptor isoforms.

■ ASSOCIATED CONTENT

Supporting Information

Additional information regarding the purification and identification of ASER as fibronectin, demonstration that sera from numerous mammalian species can inhibit sEGFR shedding, and a putative transmembrane domain in the CTP of sEGFR. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Drs. Maihle and Baron are cofounders of a biotechnology company that holds intellectual property rights to soluble epidermal growth factor receptor (sEGFR).

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