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Regulated ADAM17-dependent EGF family ligand release by substrate-selecting signaling pathways

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Contribution by Harvey F. Lodish, April 28, 2013 (sent for review December 6, 2012)

Ectodomain cleavage of cell-surface proteins by A disintegrin and metalloproteinases (ADAMs) is highly regulated, and its dysregulation has been linked to many diseases. ADAM10 and ADAM17 cleave most disease-relevant substrates. Broad-spectrum metalloprotease inhibitors have failed clinically, and targeting the cleavage of a specific substrate has remained impossible. It is therefore necessary to identify signaling intermediates that determine substrate specificity of cleavage. We show here that phorbol ester or angiotensin II-induced proteolytic release of EGF family members may not require a significant increase in ADAM17 protease activity. Rather, inducers activate a signaling pathway using PKC-α and the PKC-regulated protein phosphatase 1 inhibitor 14D that is required for ADAM17 cleavage of TGF-α, heparin-binding EGF, and amphiregulin. A second pathway involving PKC-δ is required for neuregulin (NRG) cleavage, and, indeed, PKC-δ phosphorylation of serine 286 in the NRG cystolic domain is essential for induced NRG cleavage. Thus, signaling-mediated substrate selection is clearly distinct from regulation of enzyme activity, an important mechanism that offers itself for application in disease.

epidermal growth factor receptor | transactivation

The ectodomains of many cell surface proteins are shed from the surface (i.e., “ectodomain shedding”) by metalloproteases. Ectodomain shedding generates many diverse bioactive cytokines and growth factors, and governs important cellular processes in the developing and signaling organism, including the control of growth, adhesion, and motility of cells (reviewed in refs. 1–3). EGF receptor activation generates signals for cell proliferation, migration, differentiation, or survival. The 12 EGF family members are synthesized as cell surface transmembrane precursors. The active growth factors are released by A disintegrin and metalloproteinases (ADAMs) and activate specific heterodimeric EGF receptors on the cell surface connected to diverse intracellular signaling pathways (4, 5). Increased shedding of EGF ligands has been linked to different clinical pathologic processes (6–10); hence, therapeutic control of ligand release would be beneficial. Of the 12 functional ADAMs encoded in the human genome (5) only two—ADAM10 and ADAM17—handle most of the numerous ADAM substrates, in particular, the EGF ligands. However, broad-spectrum metalloprotease inhibitors tested for clinical use have failed as a result of indiscriminate blockade of substrate cleavage, leading to clinical side effects (11). Even recently developed selective ADAM inhibitors still affect the cleavage of many substrates (12). Modulation of the release of specific ADAM substrates has been impossible to date because it is unknown how cleavage specificity is regulated on the molecular level. It is therefore necessary to identify key signals that determine substrate specificity of cleavage.

Ectodomain cleavage is made specific by a number of intracellular signals; e.g., by calcium influx, by activation of G protein-coupled receptors, and the release of diacylglycerol (reviewed in refs. 3, 13). Several distinct mechanisms that modulate cleavage on the level of ADAM17 have been described, including regulation of ADAM17 expression, maturation, trafficking to the cell surface (reviewed in ref. 13), and posttranslational modifications on the ADAM17 ectodomain (14, 15) or its C terminus (16, 17). However, modulation of activity of the relatively few available ADAMs does not suffice to explain substrate-specific regulation of cleavage (18, 19), and none of the referenced studies has addressed how specificity of cleavage is achieved. Transgenic overexpression of ADAM17 in mice does not lead to overactivity of ADAM17 or increased ADAM17 substrate release, emphasizing the importance of posttranslational control of cleavage (20). Most reports on induced shedding (reviewed in refs. 5, 21) have only used monitoring of substrate cleavage as a surrogate measure of protease activity. However, only few studies unequivocally document induced changes of protease activity, and those were small. A tight-binding ADAM17 inhibitor interacts with the catalytic site of ADAM17 only after 12-O-tetradecanoylphorbol-13-acetate (TPA; i.e., phorbol ester) stimulation (12), suggesting regulation of the catalytic site. Another convincing example of regulated enzyme activity has been based on observed effects of oxidation on several putative disulphide bonds in the ADAM17 ectodomain that result in a structural change. This involves the interaction with an extracellular redox regulator, protein disulphide isomerase (PDI). PDI down-regulation enhanced TPA-induced shedding of heparin-binding (HB) EGF, addition of exogenous PDI decreased it, and PDI addition to recombinant ADAM17 reduced basal cleavage of a fluorescence resonance energy transfer (FRET) peptide. These changes correlated with altered topology of antibody epitopes outside of, but not within, the catalytic domain (14). However, induced HB-EGF cleavage could have also resulted from enhanced interaction of the substrate with ADAM17 via the altered topology outside of the catalytic domain without requiring changes in protease activity. Neither study determined protease activity independent of substrate cleavage, still leaving us with uncertainty whether induced substrate cleavage truly requires enhanced protease activity. By using stopped-flow X-ray spectroscopy and other techniques, Solomon et al. showed that ADAM17 activity is primed by enzyme conformational changes induced by the substrate before proteolysis (22). Novel exosite inhibitors of ADAM17 activity that bind ADAM17 outside of the catalytic site and likely interfere with the binding of glycosylated moieties of the substrate have been developed (23). Both studies further support regulation of proteolysis on the substrate level.

Here we identify pathway components that distinguish substrates of ADAM17 and parse substrate selection from regulation of protease activity.


The authors declare no conflict of interest.

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Results

shRNA Screen for Regulators of TGF-α Cleavage by ADAM17. Phorbol ester (i.e., TPA) stimulates most PKC isoforms (α, β, γ, δ, ε, η, θ, and µ), and is a commonly used cleavage stimulus in shedding studies. ADAM17 is the physiological effector of TPA-induced signals, whereas ADAM10 primarily responds to calcium signals (24, 25). To identify novel genes that regulate shedding downstream of PKC, we carried out a lentiviral shRNA knockdown screen targeting most human kinases and phosphatases and some of their associated components, probing their effect on TPA-induced cleavage of TGF-α, a classical ADAM17 substrate (24). Cleavage was measured with an extensively validated high-throughput 96-well FACS assay (18, 19) (Fig. S1A). We screened 3,500 unique lentiviral shRNAs carrying puromycin resistance for selection at >3x coverage with biological duplicates in human Jurkat cells expressing HA–TGF-α–EGFP. Genes were targeted with three to five individual shRNAs per gene and selected with puromycin. A shRNA targeting lacZ, a protein not present in mammalian cells (control-shRNA) was used as a control. After stimulation with TPA for 2 to 5 min, mean geometric red and green fluorescence of the cells was measured by FACS and normalized across samples by using z-scores. Cleavage was induced to approximately 50% of maximum to allow detection of cleavage activation or inhibition in the same screen. Assuming that most tested shRNAs would not affect cleavage, we selected targeted genes with fluorescence (ectodomain) reporters product ratio of at least 2 z-scores (for best shRNA) and 1.5 z-scores (for other shRNAs) above or below the mean z-score of all samples. A positive z-score identifies inhibitory shRNAs (cleavage-activating genes) and a negative z-score activating shRNAs (cleavage-inhibiting genes). A distribution of all shRNA red/green fluorescence z-score is shown in Fig. S1B. shRNAs that showed reproducible effects on TGF-α cleavage were retested after subcloning into an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible lentiviral expression vector.

PKC-α and Protein Phosphatase 1 Inhibitor 14D Regulate Induced Cleavage of only Specific EGF Ligand ADAM17 Substrates, Including TGF-α, but Not Neuregulin. Our screen identified positive and negative regulators of TPA-induced TGF-α cleavage, including the cleavage activating genes PKC-α and protein phosphatase 1 (PP1) inhibitor 14D (PPP1R14D), a PP1 inhibitor that is activated by PKC phosphorylation (26, 27). Fig. L4 shows representative screen FACS plots of HA–TGF-α–EGFP-expressing Jurkat cells. The elliptical marker was added to highlight changes in the relevant plot area. In control-shRNA-expressing cells, the red fluorescence (ectodomain) by TPA compared with control-treated cells is dramatic, whereas green fluorescence (C terminus) is roughly maintained. In PKC-α and PPP1R14D knockdown cells (Fig. L8 shows Western blot confirmation), this fluorescence shift is largely absent, indicating maintained HA–TGF-α–EGFP ectodomain fluorescence on the cell surface, a result of blocked cleavage. The GFP signal as measured by FACS is slightly different between the cell lines as a result of effects on basal expression or basal cleavage of the reporter. This does not affect cleavage detection by red/green fluorescent ratio as it is highly linear over a wide range of reporter expression (18, 19). We also showed the effect of PKC-α or PPP1R14D knockdown in FACS time-course experiments (Fig. 1C) and in whole cell lysates using anti-GFP Western blots (Fig. 1D). In Fig. 1D, the Western blot shows two bands, an upper full-length HA–TGF-α–EGFP and a lower C-terminal cleavage product. In control shRNA-expressing Jurkat cells, the full-length band strongly diminishes (to approximately 10% of control) over 5 and 15 min, whereas the C-terminal cleavage product accumulates over the same time frame, suggesting strong cleavage. The full-length band appears to slightly recover at 15 min compared with 5 min of TPA, but the C-terminal cleavage product continues to accumulate at 15 min, further decreasing the full-length:C-terminal product ratio. A similar result can be seen in the FACS time-course experiments in Fig. 1C that plot the red:green ratio of HA–TGF-α–EGFP-expressing cells.

The red signal stems from surface-stained full-length HA–TGF-α–EGFP, and the green signal stems from the C-terminal GFP fusion. The red signal is lost after cleavage (ectodomain lost in supernatant before FACS stain is carried out; Fig. S1A), whereas the GFP signal migrates with the C terminus after cleavage, as is also seen in the Western blot. Hence, the low red:green ratio at 15 min of the FACS plot mirrors the results from the Western blot when full-length:C-terminal product ratio is taken into account. In PKC-α or PPP1R14D knockdown Jurkat cell Western blots, the full-length band does not diminish by 5 min and shows some decrease at 15 min. This is reflected in the accumulation of C-terminal cleavage product particularly in the PPP1R14D knockdown cells. Our knockdown westerns show 90% to 100% knockdown for PKC-α and approximately 80% knockdown for PPP1R14D in these experiments (Fig. 1B). This could explain why PPP1R14D knockdown was less effective than PKC-α knockdown in blocking cleavage. Of note, the C-terminal cleavage product is already present in control-treated cells, reflecting basal cleavage, also seen in the control shRNA-expressing cells. We confirmed our results in HEK293T cells overexpressing the G protein-coupled angiotensin II (AngII) type 1 receptor known to activate PKC (28). Broad-spectrum inhibition of PKC isoforms by bisindolylmaleimide 1 (BIM1) indeed strongly inhibited TPA- and AngII-induced TGF-α cleavage as measured by FACS (Fig. 2A). PKC-α or PPP1R14D down-regulation (Fig. 2B) had the same effect as BIM1 in inhibiting TGF-α cleavage (Fig. 2C, 1 and 2). In the latter experiment, we used cell-surface anti-HA immunoprecipitation (IP) to detect full-length HA–TGF-α–EGFP because detection of the small cleaved cell surface fraction of TGF-α was difficult in whole-cell lysates containing a large fraction of uncleaved intracellular TGF-α. However, we have been able to observe TPA-induced
accumulation of C-terminal cleavage products in control shRNA-expressing cells that is significantly blocked in PKC-α or PPP1R14D knockdown cells (Fig. 2C, 3). Importantly, knockdown of either gene did not affect TPA-induced cleavage of neuregulin (NRG) in HEK cells (Fig. 2D), also an ADAM17 substrate (Fig. 2E–G). We therefore hypothesized that PKC-α and PPP1R14D may act in the regulation of only a subset of ADAM17 substrates. We confirmed this by using specific ELISAs to detect different endogenous protein ectodomains cleaved by ADAM17 (Fig. S1 C and D) in the same sample supernatant of cells that express various cleaved metalloprotease substrates, including EGF ligands. In MDA-MB-231 breast cancer cells that depend on EGF ligand cleavage for proliferation and express TGF-α, PKC-α, and PPP1R14D, PKC-α and PPP1R14D are indeed required for TPA-induced ADAM17 cleavage of TGF-α, HB-EGF, and amphiregulin (AR), but not for the basal cleavage of TNF receptor 1 (Fig. S2). In 12Z cells (which do not express TGF-α), both genes were required for the cleavage of HB-EGF (Fig. S3). Cleavage of the ADAM10 substrate c-Met was unaffected in both cell lines (Figs. S1–S3), suggesting that regulation by either gene is specific for ADAM17 substrates. We were unable to detect NRG cleavage with several different ELISAs in MDA-MB-231 cells.

PKC-δ-Dependent C-Terminal Serine Phosphorylation Regulates Induced NRG Cleavage. The broad-spectrum PKC inhibitor BIM1 blocks TPA- and AngII-induced NRG cleavage in HEK293T cells (Fig. 3A, 1 and 2). In contrast, and consistent with the lack of effect of PKC-α down-regulation (Fig. 2G), G66976, an inhibitor of only PKC-α and -δ, did not block NRG cleavage (Fig. 3A, 3). We confirmed these results for BIM1 and G66976 detecting NRG cleavage by FACS (Fig. 3A, 4 and 5). We therefore asked which other PKC-regulated PKC isoforms might be responsible. PKC-δ down-regulation strongly blocked TPA-induced NRG cleavage (Fig. 3B, 1 and 2), whereas the knockdown of other PKC isoforms had no effect. By using a phospho-specific PKC consensus site antibody, we previously showed that TPA induces a serine phosphorylation on the C terminus of NRG before cleavage, which is sensitive to BIM1 inhibition (18). Serum 286 (S286) is contained in the only sequence in the NRG C terminus matching the consensus PKC phosphorylation site. Indeed, mutation of S286 to alanine blocked TPA-induced NRG cleavage as measured by FACS (Fig. 3C). We confirmed these findings by immunofluorescence and Western blot. The TPA- and AngII-induced S286 phosphorylation was prevented by BIM1, but not by the PKC-δ inhibitor Gö6976 (Fig. 3D, 1 and 2), consistent with their respective effects on NRG cleavage (Fig. 3A, I–5). In contrast, PKC-δ knockdown abolished AngII- or TPA-induced S286 phosphorylation (Fig. 3D, 3), suggesting that PKC-δ activity establishes S286 phosphorylation.

TPA and AngII Induce Inhibition of PP1. Phosphorylation of serine 58 in PPP1R14D by PKC activates the PP1 inhibitor by inducing a conformational change (26, 27), allowing binding of the inhibitor to a specific PP1 complex. In IP experiments, PP1-α but not PP1-β or PP1-γ was coprecipitated strongly with Flag-PPP1R14D from TPA-treated Jurkat cells and TPA- or AngII-treated HEK293T cells compared with controls (Fig. 4A and B), suggesting that PP1α is the downstream effector of PPP1R14D. We confirmed the inhibitory effect of activated PPP1R14D in a biochemical PP1-α assay in vitro, by using a FRET peptide substrate that only reveals its fluorescence when dephosphorylated. Only an exogenously applied PP1 inhibitor (inhibitor-2) or endogenous PPP1R14D immunoprecipitated from TPA- or AngII-treated cells significantly inhibited PP1-α activity; however, PPP1R14D precipitated from control cells did not (Fig. 4C). In contrast to knockdown of PPP1R14D, a regulator of only specific PP1-α-containing complexes, PKC-α knockdown itself was not well tolerated in Jurkat or HEK293T cells, leading to significantly decreased cell proliferation and increased cell death. PP1-α carries out many cellular dephosphorylation reactions, affecting multiple cellular functions (29).

Taken together, our findings strongly suggest (i) that regulation of substrate cleavage by modulating protease activity may...
not suffice to explain the observed specificity of cleavage, (ii) that the identified regulators may act in intracellular signaling pathways that address the C terminus of substrates to regulate cleavage, and (iii) that ADAM17 substrate cleavage is regulated and made specific by distinct PKC isoforms, PPP1R14D and PP1-α.

**PKC-α and PPP1R14D Regulate ADAM17 Cleavage Without Significantly Affecting Protease Activity.** Given our finding that ADAM17 substrates are specifically selected, does this require, in addition, regulation at the level of protease activity? We measured basal and TPA-induced protease activity by using proteolytic activity matrix analysis (PrAMA). PrAMA allows measurement of particular protease activities independently of measuring specific physiological substrate cleavage (30). This technique uses a panel of FRET-based peptide substrates that emit fluorescence upon cleavage. Peptide substrates can be incubated directly with live cells, with generated fluorescence measured in a plate reader. Specific protease-associated cleavage profiles were developed and validated with purified proteases in vitro and in ADAM-KO cells in vivo. We used five different FRET substrates that vary in their specificity for ADAMs and matrix metalloproteinases to infer the activity of ADAM17. Knockdown of ADAM17 indeed strongly reduced PrAMA-inferred ADAM17 protease activity in MDA-MB-231 cells (Fig. 5A). Knockdown of ADAM17 (approximately 80%) for both shRNAs used was confirmed by Western blot (Fig. 5D). However, baseline live-cell ADAM17 protease activity was only mildly reduced by 0% to 20% in PKC-α or PPP1R14D MDAMB-231 knockdown cells (significant in only one of two shRNAs tested per gene) compared with control (Fig. 5B). Even more surprisingly, 30 min TPA stimulation, although able to induce substantial substrate cleavage in control cells (Figs. 1–3 and Figs. S2–S4), did not increase protease activity under any condition, but rather decreased it to a small degree (Fig. 5B). We obtained similar results by using immunoprecipitated cell-surface ADAM17 and one relevant FRET substrate for cleavage detection. TPA or anisomycin, an inducer of cell stress, did not increase activity of cell surface ADAM17 at 5, 15, or 30 min, but a small decrease in protease activity was detected at 30 min in TPA-stimulated samples (Fig. 5C). Basal cell surface ADAM17 activity showed only small differences in control and knockdown cells, similar to live-cell PrAMA (Fig. S4). Overall levels of mature ADAM17 were unchanged by PKC-α or PPP1R14D knockdown in MDA-MB-231 cells, with only minor changes in the ratio of pro- to mature form (Fig. 5D), arguing against transcriptional down-regulation or reduced ADAM17 maturation as explanations for the small reductions in protease activity. Cell surface levels of ADAM17, another factor that could influence cell surface protease activity, were equal in control and PKC-α knockdown cells and were moderately reduced with PPP1R14D down-regulation (Fig. 5E). As basal protease activity in PPP1R14D knockdown cells is not significantly affected (Fig. 5B)},
formed in triplicate. Assays, we show the mean of at least three independent experiments per-independent experiments. ADAM17/tubulin ratios were determined by ADAM17 by FACS (ADAM17 ectodomain antibody). Cells were treated as final ADAM17 Western blot and (a) pro-ADAM17 (100 kDa) and mature ADAM17 (75 kDa) expression levels measured by C-terminal ADAM17 Western blot and (b) by measuring cell surface levels of ADAM17 by FACS (ADAM17 ectodomain antibody). Cells were treated as indicated. For all Western blots, we show one representative of three to five independent experiments. ADAM17/tubulin ratios were determined by densitometry of three independent Western blots. For the protease activity assays, we show the mean of at least three independent experiments performed in triplicate.

suggests that surface levels of active ADAM17 are in saturation. Nonetheless, PPP1R14D may regulate ADAM17 surface levels. In summary, our results indicate that the predominant effects of PKC-α and PPP1R14D on substrate cleavage are not mediated by changes in ADAM17 expression, maturation, or cell surface expression, nor are they caused by significant regulation of ADAM17 protease activity. The implication is that PKC-α and PPP1R14D (and PPI-α) directly or indirectly act on the substrate or on a third interacting protein required for cleavage regulation.

Discussion

Our results support at least two principal conclusions, as noted below.

Induced Shedding May Not Require Significant Changes in ADAM17 Protease Activity. In the present study, live-cell PrAMA studies with immunoprecipitated cell surface ADAM17 showed a reduction of only 0% to 20% of basal protease activity with PKC-α or PPP1R14D down-regulation compared with control cells and small decreases in protease activity in response to 30-min TPA stimulation. Decreases of ADAM17 surface levels in response to TPA stimulation, either by endocytosis or possibly cleavage, have been reported before (14, 31). More importantly, even without increases in protease activity, strong TPA-induced substrate cleavage (1.5- to fivefold vs. control) was observed. PKC-α or PPP1R14D down-regulation blocked this proteolysis without inducing significant changes in protease activity, at least not as measured with currently available techniques that allow determination of protease activity independent of measuring physiological substrate cleavage. This indicates that these signaling components regulate substrate cleavage independently of a major effect on protease activity. In live-cell PrAMA experiments using 30-min TPA stimulations that we published previously, TPA also enhanced ADAM17 protease activity only slightly (30), yet substrate cleavage was increased much more significantly (18). In contrast to our studies, the application of cell stress including by anisomycin for 60 min (16) increased protease activity by 20% to 25% (measured with immunoprecipitated ADAM17 and one FRET-peptide substrate). Concurrently, cell stress produced a small to moderate increase of ADAM17 surface levels not observed in our study that likely account for the observed small increases in protease activity after 60 min of stimulation. However, despite only small changes in protease activity in this study, cell stress-induced TGF-α cleavage was increased five to 10 fold vs. control cells. These findings further support the notion that substrate cleavage is regulated independently of a major change in protease activity.

Different Signaling Modules Select Distinct Substrates for ADAM17 Cleavage. PKC-α and PPP1R14D act only in ADAM17 cleavage of TGF-α, HB-EGF, and AR, and possibly act in a signaling cascade by inhibiting PI1-α. PKC-8 is part of a different signaling module that regulates ADAM17 cleavage of NRG. PKC-α and PKC-8 are both activated by TPA, yet the signaling modules they drive are different. This would lend a satisfying explanation to the conundrum of how specific substrate cleavage in response to the same cleavage stimulus, TPA, could be achieved by only one metalloprotease, ADAM17. Additionally, different stimulus-specific responses in substrate cleavage (e.g., osmotic stress, G protein-coupled receptor activation) could be explained by stimulus-dependent engagement of different signaling modules that act on distinct subsets of substrates and/or act in different ways on the same substrate, e.g., by posttranslational modifications. Cleavage of substrates depends, of course, on the level of mature enzyme on the cell surface. This level is regulated by ADAM gene expression, transport, and maturation, as well as by internalization. Signal transduction pathways affect all these processes (e.g., ref. 32). The rapid choice of substrates for cleavage must, however, be defined by their posttranslational modifications. Such selection likely involves modifications of the C terminus of substrates, as shown here for NRG, or, alternatively, of the C terminus of yet unidentified regulatory proteins that interact with the substrates. In contrast to many substrates, the C terminus of ADAM17 is not required for induced or basal cleavage (25), although conflicting results on the regulatory role of a C-terminal threonine 735 phosphorylation have been reported (16, 17, 32). To date, a number of C-terminal regulatory events on substrates that regulate shedding and could be influenced by intracellular signaling have been described. Phosphorylated serine residues and linkage to the actin cytoskeleton via ezrin/radixin/moesin proteins are important for PKC-dependent shedding of L-selectin (33). Calmodulin is constitutively bound to the cytoplasmic tails of L-selectin and angiotsin-converting enzyme, and its dissociation induced by calmodulin kinase inhibitors or TPA activates their shedding (34, 35). Shedding of CD44, EGF, betacellulin, N-cadherin, and IL-6R are inducible by calmodulin kinase inhibitors and calcium ionophores (36-38). Regulation of substrate cell surface levels also affects cleavage of certain substrates. As examples, the APP homologue APLP1 binds to APP through a conserved motif in the cytoplasmic domain and increases the shedding of APP by reduction of its endocytosis (39). Monoubiquitination of AR’s C terminus induces its immediate endocytosis and thereby rapidly blocks its ecto-domain cleavage by ADAM17 (40). However, not all substrates require their C terminus for shedding and not all C-terminal phosphorylation events induced by shedding stimuli regulate cleavage (e.g., IL-6 receptor (41), TNF-α receptor II (42), HB-EGF (43)). Because ADAM17 and ADAM10 also do not require their C-termini for stimulus-induced cleavage (25), these findings suggest the existence of other third regulatory proteins that receive signaling input, and/or that C termini of some substrates contain negative regulatory signals for cleavage that are missing yet substrate cleavage was increased much more significantly (18). In contrast to our studies, the application of cell stress including by anisomycin for 60 min (16) increased protease activity by 20% to 25% (measured with immunoprecipitated ADAM17 and one FRET-peptide substrate). Concurrently, cell stress produced a small to moderate increase of ADAM17 surface levels not observed in our study that likely account for the observed small increases in protease activity after 60 min of stimulation. However, despite only small changes in protease activity in this study, cell stress-induced TGF-α cleavage was increased five to 10 fold vs. control cells. These findings further support the notion that substrate cleavage is regulated independently of a major change in protease activity.
involved in cleavage regulation, but none clearly distinguished whether protease or substrates were the target of regulation [e.g., PKC-ε/TNF-α (44), PKC-δ/EHB-EFG (45), PKC-δ and PKC-ε/IIL-6R (46)]. In concordance with our study, PKC-ε siRNA knocked-down blocks TPA-induced HE-EFG cleavage (47), and PKC-δ and phosphorylated C-terminal serine residues regulate cleavage of chicken NGRI in neuronal cells (48).

In summary, we show that the regulatory proteins PKC-ε, PPP1R14D, and PKC-δ specifically affect the cleavage of only select ADAM17 substrates without significantly affecting protease activity. This observation offers itself as a new avenue for therapeutic intervention independent of the protease and possibly specific for a particular disease-causing substrate.

Materials and Methods

**SI Materials and Methods** describes materials, retrovirus/lentivirus production, retroviral packaging, IP, Western blotting and ELISAs, PP1 biochemical assay, immunoprecipitated cell-surface ADAM17 protease assay, FACS, and shRNA FACS screen.

**P.R.A.M.A.** For live-cell P.R.A.M.A., IPTG-induced MDA-MB-231 human breast cancer cells were seeded in a 384-well clear-bottom black-walled plate. The next day, samples were stimulated with internally quenched FRET substrates (5 μM) alone or with TPA (1 μM) for 30 min. Substrates were also added to no-cell (negative control) and trypsin (positive control). Fluorescence readings were obtained every 10 min for 2 h at 37 °C.

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Supporting Information

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SI Materials and Methods

Reagents. Reagents were from the following vendors: FuGENE 6 (Roche); RPMI medium 1640, DMEM, and DMEM/F12 (Cellgro); FCS (Invitrogen); puromycin and acid-extracted collagen I (BD); isopropyl β-D-thiogalactopyranoside (Roigien); Polybrene, procidium iodide, tetradeacetyl phorbol acetate, angiotensin II (AngII), and protein A + G Sepharose (Sigma); batimastat (Tocris); bisindolylmaleimide I, G06976, A disintegrin and metalloproteinase (ADAM) 17 fluorescence resonance energy transfer substrate, and recombinant ADAM17 (Calbiochem); pro-ADAM9 and pro-ADAM10 (BioZyme); and protein phosphatase 1 (PP1) and inhibitor 2 (NEB).

Antibodies. Antibodies were from the following vendors: anti-HA11.1 [1:100 FACS or immunoprecipitation (IP); Covance]; rat anti-HA [1:1,000 Western blot (WB); Roche]; APC-coupled goat anti-mouse (1:150 FACS; BD); anti–PKC-α (1:1,000 WB; Cell Signaling); anti–PKC-δ (1:1,000 WB) and phospho-(Ser) PKC-β (shown is sh1PKC-αδ NG Sepharose (Sigma); batimastat (Tocris); bisindolylmaleimide I, G06976, A disintegrin and metalloproteinase (ADAM) 17 fluorescence resonance energy transfer substrate, and recombinant ADAM17 (Calbiochem); pro-ADAM9 and pro-ADAM10 (BioZyme); and protein phosphatase 1 (PP1) and inhibitor 2 (NEB).

cDNA Constructs. Retroviral. pB–HA–TGF-α–EGFP, pB–Flag–NRG–EGFP (1), HA–TGF-α were provided by J. Arribas (Vall d’Hebron Institute of Oncology, Barcelona, Spain); Flag–NRG construct was provided by Carie Lai (University of California, San Diego, CA); and pB–Flag–NRGS286A–EGFP was constructed by mutagenesis.

Lentiviral. Puromycin-selectable pLKO-shRNA library covering all human kinases and phosphatases (n = 5 shRNAs per gene; Broad Institute, Cambridge, MA); control-shRNA (targeting lacZ); and sh1–2–PKC-α or sh1–2–PP1R14D were cloned into plKO-904, pLKO–sh1–2–PKC-δ (shown is sh1–PKC-δ or shRNA (Dharmacon) were used for PKC-δ knockdown.

Cell Lines. Cell lines were as follows: Jurkat WT [ATCC; RPMI 10% (vol/vol) FCS], HEK293T [ATCC; DMEM 10% (vol/vol) FCS], MDA-MB-231 [ATCC; DMEM 10% (vol/vol) FCS], 12Z [provided by Anna Starzinski-Powitz (Frankfurt, Germany) and Steve Palmer (EMD Serono)]; DMEM/HAMsF12 10% (vol/vol) FCS), and HEK293T–AngII type 1 receptor (gift from Issie Komuro, Chiba University Graduate School of Medicine, Japan; DMEM 10% (vol/vol) FCS). Knockdown cell lines were generated by lentiviral infection.

Immunoprecipitation and Western Blotting. Cell were induced with isopropyl β-D-thiogalactopyranoside for 7 d and control-treated with DMSO or stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA; i.e., phorbol ester; 1 μM) or AngII (1 μM). After stimulation, cells were dissociated from the plate with cold PBS solution/EDTA (5 mM) and spun down at 10,000 × g for whole-cell lysate Western blots, cells were lysed with 0.1% Triton lysis buffer (plus protease/phosphatase inhibitors), cleared via centrifugation, and resolved by SDS/PAGE and Western blotting after determining protein levels (Bradford protein assay). After transfer to a nitrocellulose membrane, the membrane was blocked with 5% milk/Tris-buffered saline solution (TBST; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton) for 1 h at room temperature. The primary antibody was incubated overnight at 4 °C in 5% BSA/TBST. The membranes were washed three times in TBST and then incubated with the respective secondary antibody (1:6,000) for 1 h at room temperature in 5% (wt/vol) milk/TBST. For surface staining immunoprecipitation dissociated cells were first stained with antibody (1:100) in PBS solution/FCS (3%) to capture only cell-surface protein. Afterward, cells were washed and lysed on ice with 0.1% Triton lysis buffer (plus protease/phosphatase inhibitors) as described earlier. Lysates were cleared via centrifugation, and immunocomplexes were precipitated with protein A or G agarose for subsequent SDS/PAGE and Western blotting.

Retrovirus and Lentivirus Production and Infection. For virus production, we used the following protocol: 800,000 HEK293T cells were seeded in a 6-cm plate on day 0. On day 1, 50% confluent cells were transfected with 1 mg viral plasmid of choice, 0.9 mg of VSVG, and 0.1 mg pUVMV (retroviral polymerase) or 0.1 mg deltaPVR (lentiviral polymerase) using 6 μL of FuGENE 6 (all premixed in serum-free medium and incubated 20 min at room temperature). On day 2, 18 h after transfection, medium was changed to 4 mL medium containing 30% (vol/vol) FCS in DMEM. On day 3, 48 h after transfection, viral supernatant was harvested and syringe-filtered through a 0.4-Mm filter directly onto cells to be infected (50% confluent at point of infection). Polybrene 4 mg/mL was added to the virus, and infection was done by spin-infection at 750 × g for 30 to 60 min.

PP1 Biochemical Assay. The PP1 biochemical assay (ProFlouor Set/Thr PPase assay; Promega) uses an internally quenched PP1 peptide substrate resistant to cleavage by aminopeptidase when phosphorylated. Aminopeptidase cleavage of a non–phosphorylation-dependent control FRET substrate is used to monitor potential aminopeptidase inhibition by tested parameters. Cells were treated as indicated. Cells were then washed with cold PBS solution and lysed on ice with lysis buffer (0.1% Triton, protease/phosphatase inhibitors, and 100 nM okadaic acid). Okadaic acid at this concentration inhibits PP1 and other phosphatases. Lysates were cleared via centrifugation, and 6 mg protein was incubated with 1.7 μL anti–PP1RI14D no. 1 antibody at 4 °C overnight. Immunocomplexes were precipitated with protein A or G agarose for 3 h and washed three times with modified lysis buffer (0.1% Triton, protease inhibitors, no phosphatase inhibitors), and beads were dried with a syringe and subjected to the phosphatase assay in accordance with the manufacturer’s instructions. After spinning, supernatants were transferred into a 384-well black-walled plate, and fluorescence was measured with a plate fluorimeter (Molecular Devices). PP1-α 0.5 U (positive control) and 1 μM inhibitor-2 (positive control for inhibition) were used as controls.

Immunoprecipitated Cell-Surface ADAM17 Protease Assay. IPTG-induced MDA-MB-231 cells were treated with control, TPA (1 μM), or anisomycin (1 μM). Cells were washed with cold PBS solution and stained for 2 h with ADAM17 ectodomain antibody. After washing with PBS solution, cells were lysed on ice (0.1% Triton, protease/phosphatase inhibitors). Lysates were cleared via centrifugation, and 750 μg protein was used to precipitate immunocomplexes with protein G agarose for 2 h. After three washing steps with lysis buffer, agarose beads were dried.
with a syringe, and 100 μL of an ADAM17 FRET substrate was added (1:200 dilution in assay buffer; JA9121; Calbiochem). After incubation for 24 h at 37 °C, beads were spun down, and supernatants were transferred to a 96-well black-walled plate for fluorescence reading. Recombinant ADAM17 was used as a positive control.

**ELISAs.** Ninety percent confluent 12Z and MDA-MB-231 cells were treated for 24 h with control medium or medium containing TPA (1 mM). Experiments were carried out in the presence or absence of cetuximab (mab225; 10 mg/mL), an antibody that inhibits binding of ligands to heregulin 1 (HER1) and thereby their internalization. Cetuximab was preincubated 30 min before treatment. Collected supernatant was clarified (300 × g, 5 min) before freezing, and cells were counted by using Vi-Cell (Beckman Coulter). All ELISAs followed the manufacturer’s guidelines (Duo-set kits; R&D Systems). For the 30-min amphiregulin (AREG) measurement, supernatants were concentrated using Amicon Ultra centrifugal filters (Millipore) before performing the ELISA.

**FACS.** FACS analysis was performed as described extensively in previous reports (2, 3).

**shRNA Screen by FACS.** On day 0, 20,000 Jurkat/HA–TGF-α–EGFP cells were seeded in 50 μL medium (V-bottom 96-well plate). On day 1, cells were spin-infected (750 × g for 30–60 min) with 1 μL of library virus by using robotics. On days 2 to 5, puromycin selection occurred. On days 6 and 7, puromycin was removed. On day 8, the FACS assay (FACSCalibur HTS robot; BD) was performed after stimulation with TPA (1 μM) for 2 to 5 min. Samples were compared by using z-scores calculated for each fluorescence channel (red fluorescence, ectodomain; GFP fluorescence, C-terminal GFP fusion). Z-score was calculated as the mean fluorescence of duplicate sample minus the plate mean fluorescence divided by the SD of fluorescence across the plate. Samples were compared with the plate mean (assuming that most shRNAs tested in the screen would have no effect on cleavage) and also to control shRNAs to detect effects on the GFP channel, the red channel and on red:green z-score ratios (the latter two report cleavage).

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Fig. S1. (A) FACS cleavage assay: TGF-α ectodomain is recognized by a red fluorescent antibody, and green fluorescence represents the C-terminal EGFP fusion. A decrease in the red:green fluorescence ratio reports cleavage. (B) Z-score distribution of all 3,500 tested shRNAs. Fluorescence data were compared using z-scores that allow comparison of samples by normalizing their SDs to the mean (SI Materials and Methods). Positive z-scores identify TGF-α cleavage-activating; negative z-scores identify TGF-α cleavage-inhibiting genes. (C and D) TGF-α, heparin-binding (HB) EGF, amphiregulin (AR), TNF receptor 1 (TNFR1), and NRG are ADAM17 substrates. Cell culture supernatants were divided for use in specific ELISAs detecting the presence of different cleaved ectodomains in the same sample supernatant. In the case of TGF-α, detection required the use of cetuximab (mab225; CTX), an antibody that inhibits binding of ligands to HER1 and thereby their internalization with the receptor. Shown are determinations of the mean of at least three independent experiments performed in triplicate. (C) ADAM17 shRNA knockdown: effect on TGF-α, HB-EGF, AR, and TNFR1 (all ADAM17 substrates) or c-Met (ADAM10 substrate) cleavage. (D) ADAM17 knockdown in MDA-MB-231 cells was monitored by Western blot.
**Fig. S2.** PKC-α and PPP1R14D regulate the cleavage of only specific ADAM17 substrates in MDA-MB-231 cells. Cell culture supernatants were divided for use in specific ELISAs detecting the presence of different cleaved ectodomains in the same sample supernatant. In the case of TGF-α, detection required the use of cetuximab (mab225; CTX), an antibody that inhibits binding of ligands to HER1 and thereby their internalization with the receptor. We show the effect of control-shRNA (sh-Co) or PKC-α and PPP1R14D shRNAs (sh1/2–PKC-α and sh1/2–PPP1R14D) on absolute values of the measured cleaved analytes in control or 24-h TPA-treated cells (Left) and on TPA-induced changes (Right): (A) TGF-α (1 and 2), HB-EGF (3 and 4), AR (5 and 6), TNFR1 (7 and 8), and c-Met (9 and 10).

Shown are determinations of the mean from at least three independent experiments performed in triplicate. (B) PKC-α or PPP1R14D knockdown in MDA-MB-231 cells as monitored by Western blot.
A. Endogenous ligands - ELISA
ADAM17 substrates  CTX = cetuximab

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ADAM10 substrate

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B. PKCα + PPP1R14D-Knockdown

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C. Endogenous ligand - ELISA 30min

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Fig. S3. PKCα and PPP1R14D regulate HB-EGF cleavage in 12Z cells. Cell culture supernatants were divided for use in specific ELISAs detecting the presence of different cleaved ectodomains in the same sample supernatant as described for Fig. S2. We show the effect of control-shRNA (sh-Co) or PKCα and PPP1R14D shRNAs (sh1/2–PKCα and sh1/2–PPP1R14D) on absolute values of the measured cleaved analytes in control or 24-h TPA-treated cells (Left) and on TPA-induced changes (Right). (A) HB-EGF (1 and 2) and c-met (3 and 4). (B) PKCα or PPP1R14D knockdown verification by Western blot. (C) PKCα or PPP1R14D knockdown: effect on AR cleavage in response to 30 min treatment with TPA. Significant effects (P < 0.05, pooled t test) are indicated by asterisks.
A. ADAM17 protease activity - cell surface ADAM17 (MDA-MB-231 cells)

**Fig. S4.** (A) PKC-α and PPP1R14D regulate ADAM17 cleavage without significantly affecting protease activity. We measured basal and TPA (1 μM) or anisomycin (10 μM)-induced protease activity in control-shRNA-expressing cells compared with PKC-α (sh1–PKC-α) or PPP1R14D (sh1–PPP1R14D) knockdown cells by using immunoprecipitated cell-surface ADAM17 and one relevant fluorescence resonance energy transfer substrate. (B) Schematic model of induced ADAM17 EGF ligand cleavage: AngII or phorbol ester activate different PKC isoenzymes that regulate distinct signaling pathways that select specific ADAM17 substrates for cleavage. According to our model, induced cleavage may not require significant changes in protease activity. Rather, substrates are selected by C-terminal modification for cleavage by a “largely” constitutively active protease. This may involve specific linker proteins that couple ligands to ADAM17 and could also be a target of regulation. Induced ADAM17 cleavage does not require its C terminus. However, there is evidence for regulation of the activity state of the metalloprotease. Prodomain removal upon maturation and presentation on the cell surface are initial critical steps. Regulated cleavage may involve structural changes on the ectodomain of the metalloprotease that could be induced by redox regulation or the substrate itself (see text). AT1R, AngII type 1 receptor.