Regulation of TACE-Dependent TGF-α Shedding

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Abstract

Timothy Myers: Regulation of TACE-Dependent TGF-α Shedding
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The ErbB signaling network regulates many critical biological processes. This network consists of Epidermal Growth Factor Receptor (EGFR) and three related receptors and a superfamily of growth factor ligands. Signaling is initiated when EGF-like polypeptide ligands bind to EGFR, ErbB3 or ErbB4, causing receptor homo- or heterodimerization between the four related receptor tyrosine kinases, including the orphan receptor ErbB2. The EGF-like family of growth factors include: epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), heparin binding-epidermal growth factor (HB-EGF) betacellulin (BTC), epiregulin (EPR), epigen (EPI). The growth factors binding to ErbB receptors are produced as membrane anchored precursors that can be proteolytically cleaved in the extracellular juxtamembrane domain to release mature, soluble ligands, in a process termed ectodomain shedding. Metalloproteases, in particular the family of disintegrin and metalloproteases (ADAMs) have been identified as the proteases responsible for the shedding of diverse cell surface proteins. Efforts to understand the regulation of growth factor shedding led to the discovery of ADAM17, or tumor necrosis factor-α converting enzyme (TACE), as the major sheddase for the ErbB ligands. Evidence came from biochemical and *in vivo* studies, including mice lacking functional TACE due to a deletion in the metalloprotease domain. Homozygous TACE-deficient animals displayed perinatal lethality similar to EGFR-null mice and revealed subtle phenotypes that mimicked single growth factor null mice such as delayed eyelid closure like TGF-α deficient mice,

heart and lung defects seen in the absence of HB-EGF and mammary gland development issues found in AR null creatures. Despite the recognition of a key role for TACE in development, understanding of the regulation mechanisms are incomplete. Several findings point to a role for MAPK pathways, non-receptor tyrosine kinases, calcium and calcium-dependent kinases and reactive oxygen species. The work described here identifies a pathway initiated by ATP binding to the P2Y family of GPCRs that mediates TACE-dependent TGF-α shedding and concomitant EGFR activation. Transactivation offers a physiologically relevant pathway to examine the mechanism of TACE regulation, which I use to identify mitochondria as a source of the key signaling intermediate in TACE activation, reactive oxygen species.

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List of Abbreviations

ADAM……...A Disintegrin And Metalloprotease

AngII…….Angiotensin II

APP……..…….Amyloid Precursor Protein

APT……..Alkaline Phosphatase tagged TGF-α

AR……..Amphiregulin

ATP……...Adenosine Triphosphate

BAPTA-AM……..1,2-bis(2-aminophenoxy)-ethane-N,N,N’,
N’-tetraaceticacid acetoxymethyl ester

BCA……Bicinchoninate Acid

BIM-I……Bisindolylmaleimide I

BSA……...……...Bovine Serum Albumin

BTC…….…….Betacellulin

CADTK……..……..Calcium Dependent Tyrosine Kinase

cDNA…….Complimentary Deoxyribonucleic Acid

CHO……...……Chinese Hamster Ovary

COS…….simian CV-1, SV40 transformed cells

CRD……...……...Cysteine-Rich Domain

DAG……...……..Diacylglycerol

DGK……Diacylglycerol Kinase

DMSO……..Dimethyl sulfoxide

EC-2……....$TACE^{\Delta Zn/\Delta/Zn}$ mouse fibroblasts

EC-4…….Wild type mouse fibroblasts

EDTA……..…….....Ethylenediaminetetraacetic Acid

EGF……..Epidermal Growth Factor

EGFR……..Epidermal Growth Factor Receptor

EPI……....Epigen

EPR…….Epiregulin

ERK……Extracellular Regulated Kinase

FBS……Fetal Bovine Serum

GPCR…….G-protein Coupled Receptor

HB-EGF……..Heparin-Binding Epidermal Growth Factor

HER…….Heregulin

LPA……..….Lysophosphatidic Acid

M2 Cells……..TACE mutated CHO cells

MAPK……Mitogen Activating Protein Kinase

MEK……..MAPK/Erk Kinase

MMP…….Matrix Metalloprotease

MMTV…….Mouse Mammary Tumor Virus

MTR…….MitoTracker Red

NAC……...N-Acetyl-L-Cysteine

NADP...…….Nicotinamide Adenine Dinucleotide Phosphate

NCAM……..…….Neural Cell Adhesion Molecule

Nox…….NADP Oxidase

NRDc……..….. N-Arginine Dibasic convertase

NRG……..……...Neuregulin

PACSIN……..…….PKC And Casein Substrate In Neurons

PDK1…….Phosphoinositide-Dependent Kinase 1

PI-3K……..Phospho-Inositol-3 Kinase

PKC…….Protein Kinase C

PMA……..……..Phorbol 12-Myristate 13-Acetate

PTB…….Protein Binding Domain

PTPH1……..Protein Tyrosine Phosphatase H1

pTyr……...phospho-Tyrosine

RGD……..…Arg Gly Asp

RNA…….Ribonucleic Acid

ROS……...…….Reactive Oxygen Species

RT-PCR…….Reverse Transcriptase Polymerase Chain Reaction

SAP……...…….....Synapse Associated Protein

SCC……...Squamous Cell Carcinoma

SH……Src Homology

SHC……...Src Homologous and Collagen protein

SHP……Src-homolgy Phosphatase

SFM…….Serum Free Media

SVMP…….Snake Venom Metalloprotease

TACE…….TNF-α Converting Enzyme

TCC……..……Transitional Cell Carcinomas

TGF-α……..Transforming Growth Factor-α

THC……Tetrahydrocannabinol

TNF-α……...Tumor Necrosis Factor-α

TNFR……..Tumor Necrosis Factor Receptor

Chapter 1

Introduction

Activation of ErbB receptors by G-protein coupled receptors (GPCRs) regulates many essential biological processes and could play an important role in cancer progression and cardiovascular disease. The focus of this work is on the regulation of cleavage of the ErbB receptor activating growth factor ligand, TGF-α. TGF-α is cleaved from the extracellular segment of a cell surface precursor, proTGF-α, in a process termed ectodomain shedding. The disintegrin and metalloprotease (ADAM) proteins, and in particular ADAM17/TACE, are established mediators of this TGF-α shedding event. Shedding can be stimulated by signals from GPCRs, leading to the activation of the ErbB receptor, EGFR. This process, known as transactivation, allows us to examine regulatory signals and proteins that can control TACE-dependent TGF-α shedding.

The ErbB Signaling Network

Epidermal Growth Factor Receptor (EGFR/ErbB1) is the prototype of a family of receptors whose imperative functions regulate cell activities such as adhesion, differentiation, proliferation and survival (1-3). The ErbB superfamily is made up of four related, type-I receptor tyrosine kinases and their polypeptide growth factor ligands. Homologues of these receptors and growth factors are found throughout evolution (4). Highlighting the importance of this signaling network are key physiological roles in development and maintenance of the cardiovascular, respiratory and gastrointestinal systems (1). Moreover, several of these network components act as oncogenes in many different types of cancer (5,6). EGFR and ErbB2 are promising clinical targets and growth factor ligands are overexpressed in many neoplastic tissues. Thus, these receptors and their ligands are essential not only for normal development and adult cell maintenance, but are also key targets in the fight against malignant cancer.

The ErbB Receptors

The four related receptors of the ErbB family include: EGFR (ErbB1, mouse/HER1, human), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) (2,7). Each contains an ectodomain consisting of two cysteine-rich domains combined with two unique domains that function together as ligand binding regions. A single transmembrane domain connects the extracellular portion of the receptor to a cytoplasmic tail containing an intrinsic tyrosine kinase domain. The cytoplasmic region also contains numerous tyrosines and docking motifs that allow signaling molecules to bind when phosphorylated. ErbB3 lacks the active kinase domain, but maintains its signaling motifs and function through cross-phosphorylation in heterodimers with the other ErbB receptors.

ErbB receptor signaling requires the cooperation of the entire protein. Receptor activation occurs upon ligand binding which leads to dimerization with either the same receptor family member (homodimerization), or another ErbB receptor (heterodimerization) (8). Dimerization occurs after ligand binding to the extracellular domain causing a conformational change that exposes a bridging arm made up mostly of the first cysteine-rich domain; once exposed this arm interacts with the arm of another receptor (9). ErbB2, which does not have a known ligand, but imparts higher ligand affinities and longer, more robust signaling activities with heteromeric partners, appears to adopt this pro-interaction conformation constitutively based on the crystal structure of the extracellular domain (10). The ErbB2 receptor-interaction arm is unique in containing a negative electrostatic charge that deters ErbB2 homodimers, but strengthens heterodimer interactions, thus helping to explain why ErbB2 is the preferred heterodimer partner. The solution of the ErbB2 crystal structure also clarified why there may be no binding ligand for ErbB2, as key ligand binding residues differ from those of the other ErbB receptors and a smaller, enclosed binding pocket impedes ligand association (10). The extracellular ligand interaction and subsequent domain rearrangement of ErbB1, ErbB3 and ErbB4 receptors is also believed to allow for a

conformational change of the intracellular domain that triggers auto- and trans-phosphorylation of the cytoplasmic tyrosine kinase domain activation loop based on the well studied prototypes of non-receptor protein kinases (11). The kinase domain can then phosphorylate tyrosines on either receptor (auto- or trans- phosphorylation) which interact with signaling proteins containing Src homology 2 (SH2) or phosphorylated tyrosine binding (PTB) motifs.

The SH2 and PTB domain-containing signaling proteins link the receptors to canonical signaling pathways. Proteins that contain SH2 and PTB domains include adaptor proteins such as, Shc, Crk, Grb2, Grb7, Gab1, non-receptor src-like kinases Src, the common signaling protein PI3K and tyrosine phosphatases like SHP1 and SHP2 (12,13). Which signaling proteins bind and are activated depends on the receptor pair involved, since each ErbB receptor has overlapping as well as distinct docking motifs; it also depends on the pattern of tyrosine phosphorylation, which specifies the motifs available as docking sites (14,15). Initiation of biological effects is accomplished mainly through two major signaling pathways: Ras-Raf-MAPK and PI3K-Akt (1,16). The Mitogenic-Activated Protein Kinase (MAPK) pathways are targeted by every ErbB receptor. As with all ErbB initiated pathways, binding and phosphorylation of adaptor proteins leads to larger transient complexes that allow activation of more downstream signaling molecules. Activation of signaling molecules is accomplished by phosphorylation cascades that activate downstream kinases before ultimately targeting transcription factors for the alteration of gene expression patterns. The other major pathway, PI3K-Akt, is vital due its influence on not only gene expression patterns, but also cell survival and proliferation signals (17). PI3K can interact directly with the phosphorylated ErbB3/4 cytoplasmic tail via its p85 regulatory subunit, while adaptor proteins are required in the case of EGFR and ErbB2 (18). Growing evidence suggests that it is this activation of the PI3K pathway, required for tumorigenesis, which makes the EGFR family of receptors potent oncogenes and thus important targets for cancer therapies.

ErbB Ligands

Under basal conditions receptors require ligands to initiate efficient dimerization. The ligands, like their receptors, are members of a family, in this case polypeptide growth factors, that share similar structure and functions. The Epidermal Growth Factor (EGF)-like family is named after the first identified member, EGF (19). There are seven members of this family that can be further divided into two groups based on their binding specificity. Group 1 consists of EGF, Transforming Growth Factor-α (TGF-α), Amphiregulin (AR) and Epigen (EPI); this group binds with high affinity to only EGFR. Group 2 is made up of Betacelllulin (BTC), Heparin Binding-Epidermal Growth Factor (HB-EGF) and Epiregulin (EPR); members of this group interact strongly with both EGFR and ErbB4. A third group consists of the related growth factors termed neuregulins (NRG), which are ligands for ErbB3 and ErbB4. Neuregulins are related to the other EGF family growth factors by their EGF-like domain and receptor preferences, but considered unique due to their function in neuronal development and the use of alternative splicing to generate up to 14 different isoforms, including some that lack a cytoplasmic domain (20).

EGF-like growth factor family members in groups 1 and 2 are all produced as membrane anchored precursors with similar structure (Figure 1.1) (19). The signal peptide and pro-domain are followed by an N-terminal extension of variable length, except in the case of TGF-α and EPI, which have no intervening sequence. Prior to the EGF-like peptide, which forms a three-loop structure due to disulfide bonds between six cysteines, is the receptor-binding motif of the protein. The cysteines are embedded in a consensus sequence that allows for proper folding of the mature, receptor binding region. This EGF-like peptide, which sits a short distance from the membrane, can be proteolytically released from its transmembrane tail by cleavage in the juxtamembrane region to produce a mature, soluble peptide that is necessary and sufficient to interact with receptor. The N-terminal extension can also be proteolytically removed in a rapid cell surface event, though the identity and

regulation of this protease is unclear (21,22). The ectodomain of all family members appears to be highly glycosylated leading to variably sized precursor forms whose effect on function is not understood. The single-spanning transmembrane domain is followed by a cytoplasmic tail of variable length depending on the growth factor. The cytoplasmic tail of the precursor, while not necessary for receptor activation, may play a role in growth factor maturation and proper localization (23).

While all family members act as potent growth signals and can induce transformed growth, important distinctions exist (19). Despite the overall structure and EGF-like motif, minimal sequence homology is present among the ligands and the N-terminal pro-region and cytoplasmic domains often vary widely in their length and function (24). Lastly, while there appears to be some overlap in biological activity, each family member potentially may have distinct roles as well.

The precursor to the first growth factor discovered, proEGF, is a large protein with a greatly extended N-terminal sequence compared to the other growth factors (19). This extension is made up of eight separate EGF-like motifs in addition to the bona fide receptor binding EGF-like sequence (Figure 1.1). As with most family members, EGF is broadly expressed throughout life and a long list of functions has been attributed to this growth factor. EGF knockout mice display no overt phenotype (25), likely owing to redundancy of other EGF-peptides. However, EGF transgenic mice showed stunted growth and bone deformities, but no increase in tumorigenesis (26). As noted previously, EGF has a high affinity for EGFR and preferentially induces EGFR homodimers and EGFR:ErbB2 heterodimers, though there is now evidence for a low affinity interaction with ErbB3 (27,28).

Other type I EGF-like growth factors are TGF-α, AR and EPI. TGF-α is the best studied member of the family and possesses several prominent actions. TGF-α knockout mice are viable and fertile, but display epidermal defects including wavy hair and whiskers, abnormal patterning of hair follicles and delayed eyelid closure during embryonic development (29).

TGF-α transgenic mice revealed that overexpression produced abnormal growth in several organs and mammary tumors (30). Spontaneous, overexpressed AR has also been discovered in many malignancies, including those induced by a TGF-α transgenic model in the mammary gland (31). AR is a heparin binding, bifunctional ligand, so described because it could inhibit prolifereation of A431 carcinoma cells while stimulating proliferation of fibroblasts, through a relatively low affinity interaction with EGFR. Studies of mice bearing a targeted deletion in AR showed a critical role in mammary gland development (25). Ductal outgrowth was impaired in female mice lacking functional AR, hindering the proper quantity and quality of milk; this phenotype was further exacerbated when combined with TGF-α and EGF deficiencies in triple-null mice. Epigen is the most recently identified EGFR ligand and little is known about its function. Its low-affinity interaction with EGFR does not rule out interaction with other receptor family members, but the higher mitogenic potential than EGF with EGFR suggests EPI is compatible with this group of growth factors (32).

Group 2 of the EGF-like growth factors (HB-EGF, BTC and EPR) are capable of high affinity interactions with both EGFR and ErbB4. The best studied member of this group, HB-EGF, requires its heparin binding domain for optimal receptor binding and activity (24). This appears to be due to the availability, presentation and altered binding afforded the mature peptide by an interaction with extracellular heparin. As with all other family members, expression is detected throughout mammalian tissues. HG-EGF knockout mice produced a non-Mendelian ratio of genotypes from crosses of HB-EGF heterozygotes with 60% of HB-EGF$^{-/-}$ homozygotes dying before weaning (33,34). Survivors were viable and fertile, yet contained enlarged hearts with no discernable difference in body weights compared to wild type control mice. Death was likely due to cardio-pulmonary defects, including enlarged heart valves from persistent proliferation of mesenchymal cells during valvulogenesis and alveoli formation in the lung, likely due to dysregulated BMP signaling (33). Further evidence for the role of HB-EGF in heart development came from Iwamoto et al (34), who

confirmed the above findings and uncovered enlarged ventricular chambers and reduced cardiovascular function in their version of HB-EGF-null mice. Upregulation of HB-EGF expression occurs at sites of injury, suggesting a role in wound healing (24). Wound stimuli initiate an HB-EGF-shedding response and subsequent EGFR activation leading to migration of keratinocytes into the wound site (35). HB-EGF has also been found to be linked to the upregulation of an early immune response in skin wounds (36). During development, HB-EGF may promote implantation and growth of the embryo, as evidenced by HB-EGF expression in response to estrogen and progesterone (37), and central nervous system maturity due to cell proliferation, motility and survival (38,39). Finally, HB-EGF expression has also been detected in several types of tumors positioning HB-EGF as a potentially important EGFR family ligand in cancer (19).

Other Group 2 growth factors that share high affinity binding with EGFR and ErbB4 are BTC and EPR. These growth factors can also bind to ErbB3 in ErbB2:ErbB3 heterodimers making these pan-ErbB ligands (19). Both peptides are expressed in many different tissues, with enhanced expression in several neoplastic tissues. BTC knockout mice present no overt phenotype indicating overlap of other family growth factors (33). This redundancy was supported when BTC knockout mice were crossed with HB-EGF knockout mice to form the double knockouts that exacerbated the HB-EGF heart and mortality phenotype (33). Overexpression of BTC by transgenics however, produced early postnatal death and reduced body weight. Mortality was most likely due to pulmonary defects, such as thickened alveolar septa, accumulation of macrophages and alveolar hemorrhaging (40). EPR, like AR, is a bifunctional ligand that can stimulate and inhibit growth in different cell lines. EPR-null mice reveal no phenotype, but are vulnerable to cancer predisposing intestinal damage (19). No transgenic models yet exist.

The members of the EGF-like family of growth factors are similar, yet different. Structurally, the growth factors are almost identical, sharing a conserved protein fold

important for receptor binding. However, there is very little sequence homology and the precursor proteins can vary widely in size. In terms of signaling, there does appear to be some redundancy among growth factors, but individual knockout models also reveal independent activities that cannot be accounted for by related growth factor family members. Almost, all family members potentially function in transformed growth of neoplastic tissue making them important therapeutic targets for several types of human cancer. The details of signaling specificity must be elucidated to take advantage of these potent signaling ligands.

ErbB Signaling Characteristics

As presented above, the ErbB family of receptors and ligands play fundamental roles in development, growth and homeostasis in many different species as seen by lethality in mice lacking the ErbB receptors and disruption or delay of development of several tissues with the loss of growth factors. Genetically engineered models lacking growth factor activity indicates redundancy of the growth factor functions, underscoring the importance of these systems. Overlapping functions raise the question of how specificity is generated within the numerous and sometimes opposing responses that define a network. The ligands and receptors each offer characteristics with which potential degrees of specificity can be defined.

A central purpose of the ligands appears to be influencing the selection of dimer partners. While ErbB2 is always a possible partner, the ligands influence which of the remaining receptors are available for dimerization (41). This is important because downstream signals are determined by the receptor pairing due to the differences in signaling motifs present on each receptor and the affect of trans-phosphorylation on selection of targeted tyrosines (42). Ligand discrimination by the receptor dimers would also allow ligands to directly affect the tyrosine phosphorylation pattern of the receptors (43). The first evidence of ligand

differentiation was reported when EGF- and BTC-induced phosphorylation patterns were compared in mouse cells expressing EGFR or ErbB4 (44). The strongest evidence was seen using ErbB4 interactions with BTC NRG1β, NRG2β and NRG3. While bulk phosphorylation of the receptor cytoplasmic domain was increased with each ligand, different consequences were detected that affected proliferation and migration of a human T cell line, CEM, depending on the growth factor. Closer inspection of the phosphorylated tyrosines revealed a consistently unique pattern following stimulation with each ligand (45).

Another characteristic of ligands that affects signal specificity is binding affinity. The strength and interval of the interaction could have a profound effect on the final genetic program initiated by receptor signaling, as lower affinity ligands delay degradation of EGFR (46). Other features that also could affect ligand affinity include the N-terminal extension on some growth factors, as seen with the heparin binding domains of HB-EGF and AR, and distinctions in signaling between soluble and membrane anchored ligands (19). Though genetic evidence suggests the soluble growth factor is the essential form (33,47), signaling by membrane anchored TGF-α has been described (48,49). Lastly, the strength of the ligand:receptor interaction varies under diverse pH conditions for each ligand. For instance, TGF-α dissociates at endosomal pH while EGF, and to an even greater extent BTC, can withstand lysosomal pH ranges (50). This allows longer signal duration and possibly diverse signals, since EGFR has been shown to transmit distinct signals when localized to endosomes (51).

Along with specific docking sites, receptors have additional characteristics that can be utilized in specifying signal pathways. Localization of receptors in polarized cells, in membrane microdomains and to internal vesicles have all been demonstrated to influence potential signaling attributes of the ErbB receptors. As mentioned, EGFR signaling from endosomes is distinct from that initiated on the surface. Neuregulin induced expression of acetylcholine receptor requires MAPK signaling that only occurs following receptor

internalization and signaling from a clathrin-coated endocytic vesicle (52). Additionally, phospholipase-Cγ signaling from EGFR does not require endocytosis, while sustained EGF-induced MAPK signaling does, due to the necessary recruitment of scaffolding proteins by endosomal adaptor proteins (51). EGFR signaling based on membrane sorting has also been established. When channeled to the apical membrane of polarized epithelial cells EGFR activated SHC-dependent pathways and increased β-catenin phosphorylation, whereas on the basolateral surface, SHC pathways were combined with phospholipase-Cγ and focal adhesion kinase activity (53,54). Detergent-resistant membrane microdomains, called rafts, have been shown to contain various ErbB receptors prior to ligand binding and activation. Also, positioning in rafts prior to activation could influence dimer partners, depending on which receptors are present (41). Rafts could also function in assembling signaling complexes on activated receptors by enriching for particular signaling proteins, such as H-Ras on the cytoplasmic side of the raft (55). Beyond localization, extracellular interacting proteins could function in facilitating or obstructing ligand binding (43), while the carbohydrate modifications could also function in spatial and temporal alterations of ligand binding (41). Finally, recruitment of phosphatases to the receptor cytoplasmic region can influence the phosphorylation pattern and thus the duration and nature of signaling (56). Clearly there is much more work to be done in order to validate these potential layers of specificity.

The ADAM Family Proteins

Soluble EGF-like growth factors can mediate unique activities compared to anchored growth factors and soluble ligands are vital to proper development. Identification of the protease(s) responsible for cleavage is therefore critical to the understanding of regulation of

EGFR signaling. ADAM family proteases have since emerged as the chief enzymes responsible for cleavage of the EGF-like growth factors.

ADAM Structure

ADAM is short for A Disintegrin And Metalloprotease which describes the key domains of these family members that belong to the adamalysin group of the metzincin zinc protease superfamily (Figure 1.2). ADAMs are closely related to the snake venom metalloproteases (SVMP) in their shared extracellular domain arrangement and similar metalloprotease catalytic domain structure (57,58). Along with these features, these type I transmembrane glycoproteins are known for their conserved domain sequence: pro-, metalloprotease, disintegrin, a cysteine-rich juxtamembrane, a transmembrane and cytoplasmic domain. From the 40 family members currently recognized across all species, crucial functions have been identified in fertilization, neurogenesis, angiogenesis and ectodomain shedding of membrane anchored proteins (59,60). ADAM proteins can be generally grouped into two categories: testis specific and those that are more broadly expressed. In humans, the predominantly testis specific ADAMs are 2, 3, 6, 18, 20, 21, 29, 30, and 32. The remaining are somatically expressed and of these 8-10, 12, 15, 17, 19, 28 and 33 (predicted) are thought to be catalytically active (61). While activities and relevance have been shown for ADAMs without protease activity, especially concerning cell adhesion in spermatogenesis and fertilization, I will focus only on the protease active proteins. It is from these, specifically ADAMs 9, 10, 12, 15, 17 and 19, that potential sheddases for the ErbB ligands emerge.

The prodomain is a ~200 amino acid peptide that acts as an autoinhibitor of the metalloprotease domain to dampen protease function during transport through the cell. Most ADAMs utilize a “cysteine switch” mechanism, whereby a conserved cysteine in the prodomain helps to coordinate the essential catalytic zinc atom, thus inactivating the protease. A similar mechanism has been described for matrix metalloproteases (MMP) (61).

Alternatively, ADAM10 and 17 employ the cysteine switch only to protect against premature degradation (62). Instead, with these ADAMs, the protease is potently restrained when the cysteine-rich domain (CRD) helps position the prodomain, thus altering the native conformation of the metalloprotease domain (63). Prior to surface localization, all ADAMs are cleaved by proprotein convertases to detach the prodomain from the mature ADAM. Serine/Threonine proteases furin and PC7 have been identified as the enzymes responsible for this action on ADAM10 and 17, and most likely, for all other ADAMs (64-66), except ADAM28 which appears to autocatalytically remove its prodomain (67). Furin cleavage takes place in the Trans to Late Golgi Network, though the prodomain may remain bound in the metalloprotease substrate binding site until delivery to the surface, as the prodomain also acts as a chaperone for folding and secretion (62).

The metalloprotease domain directly follows the prodomain. Highlighting the dual behavior of some ADAMs is the fact that some lack functional protease domains. Twenty-five ADAMs are thought to be active proteases based on analysis of this domain, though a proven protease activity has only been shown for half of these. Activity is thought to correlate with the presence of a highly conserved consensus sequence found in all active proteases of the metzincin family: His-Glu-X-X-His-X-X-Gly-X-X-His. The three conserved histidine residues position the metal ion, usually zinc, which in turn coordinates the water molecules to allow hydrolytic proteolysis. Use of *in vitro* peptide assays to test individual domains of ADAM17 have established that the metalloprotease domain alone contains the necessary sequence for catalytic activity, though other domains may impart regulation (68,69). The metalloprotease domain is also subject to many active site binding inhibitors as a form of regulation. The endogenous tissue inhibitors of metalloprotease are broad proteinacious inhibitors of metalloproteases that block substrate binding in the catalytic pocket (70,71). Related to this, chemically synthesized inhibitors that are aimed at specific ADAM active sites have also been under development due to the role of ADAMs in disease

progression. The crystal structure for several active proteases in association with these inhibitors (58,72) will allow better design of the next generation of inhibitors (73,74).

The disintegrin domain was first identified in SVMPs that interact with platelet integrins to inhibit clotting in the victim. Integrin interactions have also been associated with this domain in ADAMs, yet the functional links have only recently been addressed (Table 1.1). Human ADAM15, which is the only known ADAM with the consensus integrin binding sequence Arg-Gly-Asp (RGD), can interact with the integrin receptors αVβ3 and α5β1 through the RGD sequence (75,76). The integrin α9β1 is often found in association with ADAMs, but associates independent of the RGD sequence. Instead, α9β1 requires the sequence RXXXXXXDLPEF found in the disintegrin domain of all interacting partners (77). Functionally, ADAM:integrin associations appear able to affect cell migration, both positively and negatively (78). The TACE:α5β1 interaction is found in focal adhesions and leading edges of CHO and HeLa cells and inhibits cell migration in wounding assays (79,80). Likewise ADAM12 appears to impair normal integrin function leading to decreased cell adhesion of adipocytes by α4β1 (81), and ADAM15 overexpression reduces ovarian carcinoma cell adhesion to vitronectin through αVβ3 (76). ADAM9 appears to be a promoter of cell invasion, since α6β1-expressing cells displays enhanced migration ability on ADAM9 coated plates versus control plates (76) and soluble ADAM9 provokes invasion of numerous cell lines through α6β4 and α2β1 (82). It appears as though ADAM, integrin interactions can disrupt the conventional integrin interactions leading to altered signaling and function. However, the majority of these studies were done using recombinant proteins, thus further investigation of the endogenous functions and interactions will be required to verify the validity of these findings.

Thus far the cysteine-rich domain (CRD) has only been found to complement the other domains. Beyond coordination of the prodomain as an inhibitor of metalloprotease activity,

the ADAM12 disintegrin/CRD promotes adhesion between fibroblasts and myoblasts dependent on the disulfide bonds formed between cysteines (83). Coated culture dishes of ADAM12 CRD led to adhesion and cell migration due to an interaction with the cell surface adhesion molecule snydecan-4. In the absence of this interaction cells did not adhere to the plate, suggesting the CRD may have an independent function in some ADAMs (84). Other ADAM family members offer examples of non-adhesion based interactions and will be covered below. In summary, these domains have been determined, thus far, to regulate the binding capabilities of the extracellular region.

Finally, the cytoplasmic domain varies in both length, sequence and function among the family members. A regulatory role for the cytoplasmic domain is suggested by the presence of potential binding motifs for signaling molecules. Most commonly they are Pro-X-X-Pro, that are considered SH2 or 3 binding motifs for signaling proteins. Interactions of signaling molecules with the cytoplasmic domains of ADAMs 9, 10, 12, 13, 15 and 17 have been reported, though limited functional studies have been performed and no physiological roles for these interactions have been established (Table 1.2). There are also several examples of induced phosphorylation of this domain. ADAM9 was phosphorylated following phorbol 12-myristate 13-acetate (PMA) stimulation (85), while Erk-dependent phosphorylation of ADAM17 in HeLa cells was also induced by PMA and was necessary for TACE surface localization (86). A second article challenged this finding in CHO cells, using a point mutation at Thr735 that did not diminish overall TACE phosphorylation levels, suggesting a different residue was phosphorylated. Serum-induced phosphorylation of TACE instead targeted Ser819 and was coupled with Ser791 dephosphorylation. However, this phosphorylation had no effect on the metalloprotease activity, as point mutations of these serines and a complete cytoplasmic truncation had no effect on TGF-α shedding (87). This is in agreement with Reddy and colleagues, who showed PMA stimulated TNF-α shedding was not dependent on the presence of the TACE cytoplasmic domain (88). The cytoplasmic

domain of several other ADAMs is also dispensable for protease function based on cell models and *in vitro* assays. Recently, ADAM10 was shown to mediate BTC shedding in response to calcium induced signals, despite the absence of its cytoplasmic domain (89). Evolution also suggests the expendable nature of the cytoplasmic domain. Naturally occurring alternative splice variants of ADAM9 and ADAM12 that are secreted due to truncations above the transmembrane domain maintain their protease activity towards laminin (82) and insulin-like growth factor binding protein-3 (71), respectively. Due to their low abundance in nature, the requirements and functional consequence of these alternatively spliced forms is still in doubt, especially under normal circumstances, as the short form of ADAM9 may only be produced in response to aberrant neoplastic signals for assistance in cell invasion by sequestering integrin interactions (82). Thus, while not necessary, the cytoplasmic domain may still function in localization, maturation and regulation of ADAMs.

Ectodomain Shedding of ErbB Ligands By ADAMs

After the critical discovery of ADAM17 as a TGF-α sheddase, other ADAM family members were implicated in cleavage of ErbB ligands. Overexpression experiments with ADAM9 in monkey kidney cells revealed a phorbol ester-induced increase in HB-EGF shedding (90). This event could be precluded with ADAM9 protease domain mutants. However, ADAM9 deficient mice displayed no overt phenotypes and fibroblasts isolated from these mice showed no deficiencies in basal, or PMA-induced levels of shed HB-EGF (91). ADAM12 has also been suggested as an HB-EGF sheddase since dominant negative ADAM12 transfected into cardiac cells inhibited GPCR-induced HB-EGF shedding (92). However, the ADAM12-null mice lacked any distinguishable phenotype and fibroblasts

derived from the null mice from one study were able to constitutively and PMA-inducibly shed HB-EGF in culture (93), while PMA-induced shedding was markedly decreased in another (94). Sensitivity differences between the two distinct assays could account for the conflicting observations. ADAM15 was implicated in TGF-α and AR shedding by knocking down ADAM15 expression (95,96), but shedding remained constant in fibroblasts from null mice (93). A role for ADAM19 in constitutive neuregulin cleavage was insinuated from mouse cells overexpressing wild type and dominant negative ADAM19 mutants, which increased and inhibited, respectively, shedding of NRG1β (97). Therefore while ADAM-9, -12, -15, -19 may be capable of cleaving ErbB ligands, genetic evidence does not support this as a primary responsibility.

ADAM10 and ADAM17 share unique characteristics that may separate them from other family members. As mentioned above, other ADAMs and MMPs employ the cysteine switch mechanism for inhibition of the catalytic activity during transport, while these ADAMs do not, relying on other determinants to alter the conformation of this domain (63,66). Comparison of the metalloprotease sequences of ADAM10 and ADAM17 predict surface protuberances found in the TACE structure that may function in substrate specificity and are not found on any other ADAMS (58). The primary function of these proteins appears to be as metalloproteases, with only minor roles as adhesion molecules since only two integrin interactions have been ascribed to either ADAM. Also, among the ADAMs, only knockouts of ADAM10, ADAM17 and ADAM19 caused lethality of homozygous mutant offspring, implying a requirement for these metalloproteases that cannot be compensated for by redundant functions of other ADAMs (47,98,99). ADAM10 knockout mice die due to central nervous system defects and insufficient development of the cardiovascular system (98). ADAM10 has been described as another HB-EGF sheddase (100,101), yet again embryonic fibroblasts from the knockout mice shed HB-EGF up to wild type levels. PMA-stimulated shedding of TGF-α, AR, EPR and EPI were also unaffected by the loss of ADAM10.

However, there was diminished constitutive and induced shedding of BTC and EGF (93,102). These levels returned to normal following overexpression of wild type, but not catalytically inactive ADAM10, validating ADAM10 as a key sheddase for these ligands. Along with its role in ErbB shedding, ADAM10 may also function as an α-secretase of amyloid precursor protein (APP) forming α-sAPP and opposing formation of β-sAPP, the key component of plaques found in Alzheimer's disease, and prion precursor proteins (103,104). ADAM10 may not be the primary, or only, protease for APP, since embryonic fibroblasts lacking ADAM10 are still able to produce soluble APP (98). Furthermore, ADAM10 targets the Notch receptor and its ligand Delta in *Drosophila* and ligands of the Eph tyrosine kinase receptors, both critical pathways in neuronal development and control of cell fate (105,106).

ADAM17 was first identified by its ability to cleave Tumor Necrosis Factor-α (TNF-α), hence its more widely known designation TNF-α Converting Enzyme (TACE), but is also the major EGF-like growth factor sheddase. Mice deficient in TACE activity, due to removal of the exon that encodes the zinc binding domain (TACE ΔZn/ΔZn), exhibited perinatal lethality in a majority of pups, that could not be attributed to a lack of TNF-α, since TNF-α and TNF receptor null mice survived to adulthood (107,108). TACE ΔZn/ΔZn newborns that survived displayed several epithelial defects that resembled the phenotypes found in both EGFR and TGF-α null mice, namely: open eyelids, stunted vibrissae, wavy hair and impaired development of the respiratory and digestive tracts (29,47). Embryonic fibroblasts derived from these mice were impaired in shedding of TGF-α, HB-EGF and AR. Shedding could be partially rescued by transfection of wild type TACE into the cells (47,89,109). This data was further supported by *in vitro* peptide cleaving assays that demonstrated purified TACE could cleave TGF-α, AR and HB-EGF at the correct juxtamembrane site on each growth factor (109) and later through comparison of AR and HB-EGF null phenotypes to TACE null mice (33,110). TACE has recently been reported to also cleave EPR and EPI in cell culture

assays. (93,111). TACE may also be able to cleave several isoforms of the neuregulin family of growth factors (112). These cumulative findings suggest that TACE is the major sheddase for ErbB family ligands.

Regulation of ADAM-Dependent Ectodomain Shedding

In addition to ErbB ligands, numerous substrates for the catalytic activity of ADAMs have been reported, including cytokines, adhesion proteins and ligand receptors indicating the need for regulation in substrate selection (61,113). Low levels of ADAM-dependent ectodomain shedding occurs constitutively, but can be rapidly induced by a host of stimulants including other growth factors (FGF, PDGF), non-physiological phorbol esters (PMA), calcium ionophores, the phosphatase inhibitor pervanadate and signals from other receptors such as G-protein coupled receptors (GPCRs), suggesting multiple pathways for activation of ADAM protease activity (61). The stimulated cleavage of EGF-like ligands allows GPCRs to harness EGFR downstream signaling pathways and is the central step of a triple membrane spanning signal required for transactivation of ErbB receptors (114) (Figure 1.3). This has been seen with GPCR activation of EGFR-regulated pathways such as ERK activation and cell proliferation (115,116). Stressing the importance of soluble growth factors, metalloprotease inhibitors have been shown to block transactivation (117). MMP2 and 9 are implicated in HB-EGF mediated EGFR transactivation in gonadotropic cells (118), while MMP7 in response to phenylephrine can cleave HB-EGF in rat arteries (119). Since ADAMs are implicated as the major proteases responsible for ErbB ligand cleavage, it has been hypothesized they could play a key role in transactivation. Accordingly, several ADAMs are directly implicated in transactivation of EGFR in various cell lines and with numerous GPCR stimulants (120). ADAM10 shed HB-EGF and AR in gastric cancer cells when the GPCR agonist interleukin-8 was used to activate EGFR (121). ADAM10-dependent HB-EGF shedding was also detected in response to lysophosphatidic acid (LPA)

and bombesin stimulation of kidney and prostate cancer cell models, respectively (96,101). ADAM12 and ADAM15 are also implicated in EGFR transactivation. ADAM15 mediated LPA-stimulated cleavage of TGF-α and AR in bladder carcinoma cells (95,96), while ADAM12 stimulation of cardiac myocytes, shed HB-EGF in response to phenylephrine (92). The evidence for TACE as a mediator of transactivation is abundant. GPCR-induced TACE-dependent shedding of TGF-α and HB-EGF was observed in response to angiotensin II (AngII) treatment of vascular smooth muscle cells, kidney cells and COS7 cells (95,122-124), while AR was shed from squamous cell carcinoma cells in response to LPA and an active ingredient in marijuana, THC (114, 254). Thus, ADAMs and in particular, TACE, are key regulators of EGFR transactivation.

Mechanisms of Regulation

Despite their central role, little is known about the specificity and upstream regulation of ADAMs. Cell type (or tissue) along with stimulant appear to be important determinants in targeting growth factors for cleavage, but this alone does not address the protease activation mechanism or issues of substrate selectivity. The prodomain inhibition of ADAMs is one level of regulation, but with removal believed to occur prior to surface localization it is not believed to be the only layer for regulation of protease activity (125). The cytoplasmic domain of ADAMs can be phosphorylated, interact with proteins and have recognized signaling motifs that may allow them to regulate inside-out signaling. While the decisive regulation mechanism for TACE activity has not been determined, several reports identify potential regulatory mechanisms that include substrate presentation, activation by phosphorylation, participation in protein interactions, stimulation of translocation, a role for common signaling pathways and second messenger production. With the diversity of substrates and abundant stimulants, a complex assortment of mechanisms may be required to determine substrate specificity and regulation of function.

Influence of the Substrate

The target substrate of ADAM-directed cleavage could have a profound effect on the ability of metalloproteases to function. It has been repeatedly proven that the primary sequence of the substrate cleavage site for TACE and ADAM10 has no bearing on selection. Instead it appears to be a combination of the distance from the membrane along with conformation and surrounding residues that permit access of the protease active site to the cleavage site (89,126-129). Proper localization and presentation of the substrate may also play a part in stimulated shedding. For example, in transitional cell carcinoma (TCC) bladder cells a reserve of pro-HB-EGF has been found in the nucleus. Upon stimulation with the known inducer of shedding, hydrogen peroxide, the ligand translocates from the nucleus to the plasma membrane and is then shed. It was further shown that HB-EGF was deposited in lipid rafts at the cell surface possibly allowing greater access to activated ADAMs (see below) (130). HB-EGF shedding can also be stimulated by the GPCR agonist bombesin through ADAM10 proteolysis (101). Bombesin also enhances both the ADAM10 and HB-EGF association with the tetraspanin CD9 (101). This interaction, which also occurs with TGF-α (131), could help target ErbB ligands for shedding by positioning the substrate and enzyme in close proximity. Presentation of the ADAM10-substrate ephrin-A5 plays a major role in regulating cleavage of this ligand. ADAM10 constitutively associates with the ephrin-A5 receptor EphA3 and following membrane anchored ligand binding to the Eph receptor in trans (ligand and receptor on opposite cells) a new interacting site is generated in the ADAM10 CRD positioning the metalloprotease in such a way that cleavage is achieved (132). However, an *in vitro* cell culture experiment revealed a requirement for TACE and shedding when a non-cleavable form of TGF-α did not activate EGFR (133). Also, no interaction between TACE and EGFR has been reported and this contradicts earlier evidence that TGF-α can activate EGFR in trans (48,49), raising doubts about the feasibility

of juxtacrine signaling by ErbB ligands. Further evidence that the disintegrin and CRD of ADAMs may be responsible for substrate presentation to the metalloprotease domain comes from studies of the development of frogs. *Xenopus* ADAM13 chimeras containing the ADAM10 metalloprotease domain functions the same as wild type ADAM13 in developing animals. However, when the ADAM10 CRD was substituted into ADAM13, function was lost (134). These examples, in conjunction with the role of the CRD in prodomain coordination and inhibition of ADAM10 and TACE (63), demonstrate the importance of the extracellular adhesion domains in regulation of the protease, likely through substrate selection and presentation.

Role of Localization

ADAMs may also be regulated by spatial effects. One potential mechanism for regulation of ADAMs is compartmentalization of the protease. The ADAM10 substrate CD171 is constitutively cleaved in membrane-enclosed secretory vesicles, called exosomes, followed by extracellular release of soluble product from ovarian carcinoma cells (135). Production of soluble CD171 and another cytokine, CD44, was induced in an ADAM10-dependent manner by an increase in intracellular calcium levels, also from exosomes (136). TACE, however, stimulated by the global activator of shedding PMA in this same study, shed CD171 and CD44 only at the cell surface. Conversely, the TACE substrate TNFR has also been found to be cleaved and released from exosome-like vesicles (137). This implies different ADAMs can function in distinct compartments of the cell depending on the stimulus or substrate.

Micro-compartments on the cell surface comprise another region where ADAM activity is found. Rafts are detergent-insoluble membrane regions rich in cholesterol and signaling proteins. In cholesterol depleting experiments, which disrupt raft formation, ADAM10-dependent amyloid precursor protein shedding was increased in neural cell lines (138), while ADAM10 and ADAM17 activity towards the IL-6 receptor was enhanced in COS-7

cells. Shedding of the ErbB ligand NRG-β1 by ADAM19 was found to occur in rafts of neuronal cells (139). TACE activity is partitioned in rafts during transport through the Golgi network and the mature form of TACE and its protease activity is limited to rafts in endothelial cells (140). These results demonstrate the importance of rafts as negative regulators of ADAM protease activity by amassing ADAMs during transport through the cell and sequestering their activity away from potential substrates until stimulation leads to translocation of the substrate or ADAM.

Trafficking and maturation of ADAMs may also play a role in regulating activity. The prodomain acts as both an inhibitor and a chaperone during passage of ADAMs through the secretory pathway (141). SH3PX1 and endophilin 1 are SH3 containing proteins that utilize SH3 binding domains in the cytoplasmic domains of ADAM9, ADAM15 and ADAM19 to potentially regulate these proteins (248). Interestingly, these proteins preferentially bind the pro-form of ADAM-9 and -12 over the mature forms and since endophilin is associated with endocytosis at synapses and SH3PX1 contains a phospholipid interacting domain, these may play a role in processing or transport of ADAMs prior to the removal of the prodomain. PKC and casein substrate in neurons (PACSINs) contain one SH3 domain and are believed to function in vesicle formation and transport. PACSIN2 is an ADAM13 binding partner (142), while PACSIN3 interacts with ADAM-9, -10, -12, -15, and -19 (143). Furthermore, PACSIN3 overexpression enhanced HB-EGF shedding in response to phorbol esters and the GPCR agonist AngII, while knockdown of PACSIN3 inhibited ADAM12 dependent HB-EGF shedding (143). This suggests that PACSIN3 is necessary for the shedding activity of some ADAMs, possibly through regulation of the translocation of these ADAMs.

Protein Interactions

Along with integrins and other extracellular interactions, cytoplasmic binding partners that take advantage of the SH3 binding motifs may directly regulate ADAM protease activity

(Table 1.2). ADAM12 also associates with the regulatory subunit of PI3K through SH3 motifs (144). This association is believed to be part of the regulatory mechanism of PI3K by mediating recruitment of this enzyme to the plasma membrane in differentiating myoblasts. Grb2 is a well known adaptor protein that can bind to activated EGFR to initiate Ras-MAPK signaling pathway. ADAM12 and ADAM15 also interact with Grb2, but while the Grb2 binding to ADAM15 can be mediated by phorbol ester induced phosphorylation, the effect of these interactions on ADAM function is unknown (145,146).

In contrast, the TACE cytoplasmic binding partners appear to effect protease activity. Eve-1 is a protein with no enzymatic domain, but numerous proline-rich and SH3 motifs that allow docking with signaling proteins (147). It was shown through immunoprecipitation assays that ADAM-9, -10, -12 and -15 interact with Eve-1 via the ADAM SH3 binding domains (147). When Eve-1 expression was knocked down, TPA stimulated HB-EGF, TGF-α, AR and EPR shedding were all diminished. Likewise, N-arginine dibasic convertase (NRDc) potentiates PMA-induced TACE protease activity towards HB-EGF (148). NRDc is an active endopeptidase, but mutations to the catalytic domain had no effect on HB-EGF shedding, ruling out any effect of the endopeptidase function in HB-EGF shedding, or TACE binding (148). Instead NRDc was shown to also interact specifically with HB-EGF, but not other ErbB ligands, providing another example of how substrate specific interactions may influence shedding (149). Another interacting protein lacking enzymatic domains is a Synapse Associated Protein (SAP97) that binds TACE at the very C-terminus at a novel PDZ domain (150). These two proteins also colocalize early in the secretory pathway, suggesting SAP97 may function in TACE maturation. The interaction also regulated TACE protease activity. Overexpressed SAP97 inhibited TACE-dependent shedding of TNF-α and TNFR, whereas overexpression of a SAP97 mutant incapable of binding TACE had no effect. The TACE interaction and phosphatase activity of protein tyrosine phosphatase H1 (PTPH1) negatively regulates TACE cleavage of TNF-α, although this affect has not been

examined on ErbB ligands (151). While the mechanism of regulation is unknown and the relationship or function of these interactions has not been tested following GPCR stimulation, these results demonstrate that interactions can influence protease activity and that interaction partners may specify unique substrates for cleavage.

Signaling Intermediates Implicated in Activation of ADAMs

Many signaling intermediates have been implicated in pathways that lead to the activation of ADAM metalloprotease activity. These pathways include MAPK proteins, non-receptor Src family tyrosine kinases, PKC, and signaling molecules such as calcium and reactive oxygen species.

The MAPK signaling pathway is an intermediate in many signaling schemes including those directly downstream of GPCR signaling that can lead to ectodomain shedding. However, it is difficult to differentiate between MAPK signaling initiated directly by GPCRs versus MAPK signaling activated by EGFR. As expected, GPCR transactivation leads to EGFR-dependent Ras/Raf/MEK/ERK activation (117). TACE knockdown, but not ADAM12 knockdown, in head and neck squamous cell carcinoma cells (SCC-9) led to loss of EGFR phosphorylation and downstream signaling induced by LPA and carbachol (114). However, MAPK pathways may also be directly activated by GPCRs and involved in ADAM regulation. The stress response pathway comprising the p38 MAPK can mediate ADAM-dependent shedding of HB-EGF and TGF-α in a variety of cell types (152-154). Stress-inducing reagents elevated p38 phosphorylation and subsequent HB-EGF shedding that also could be induced with constitutively active p38 mutants and blocked by dominant negative p38 mutants (152). Interestingly, phorbol ester and LPA induced shedding were not affected by p38 inhibitors indicating specific signals that utilize p38 in activating shedding (154). For TGF-α, p38 functioned only in constitutive shedding as growth factor stimulation was able to induce production of soluble TGF-α (153). Fan and Derynck (153) also reported a role for

another MAPK protein, MEK1, in inducible, but not constitutive TGF-α shedding stimulated by other receptor tyrosine kinases. Only when inhibitors of both pathways were combined was fibroblast growth factor-induced shedding completely abolished (153). Pervanadate directed ADAM10 shedding of the neural cell adhesion molecule (NCAM) through ERK1/2 (155) and PMA stimulated shedding of the TACE substrates L1 and HB-EGF that was dependent on the active kinase MEK (135,156). MEK is an upstream activator of ERK and could induce the Erk:TACE interaction and subsequent phosphorylation that regulates TACE transport to the cell surface following PMA stimulation (86). Montero et al. (157) have also reported on the role of MAPK in TGF-α shedding from CHO cells. In this report, MAPK inhibition using the same reagents described above only partially blocked PMA stimulated TGF-α shedding demonstrating a MAPK-independent pathway by phorbol esters. Further supporting the idea of dual pathways, the GPCR agonist LPA requires MEK activity to mediate HB-EGF shedding by an unidentified ADAM in Vero-H cells, while phorbol ester-induced HB-EGF shedding was independent of MEK signaling (158). This is, however, the only example of Ras-Raf-MEK signaling mediating GPCR-induced EGF-like shedding. Taken together, it appears that MAPK pathways do have a role in regulation of ADAMs depending on the signal and cell type, but only in response to certain conditions. This does not rule out alternative pathways that may also lead to shedding and leaves the exact situations for which MAPK pathways are applied to be determined.

Cytosolic nonreceptor tyrosine kinases, like Src family proteins, have been implicated in EGFR transactivation (159-163), though only recently has their precise role been scrutinized. HB-EGF shedding following activation of andrenergic GPCRs was sensitive to c-Src inhibitors (115), while carbachol stimulated TGF-α shedding was dependent on Src-like CADTK/PYK2 nonreceptor tyrosine kinase activity (164). Src-family kinases can also directly interact with the SH3 domains of several ADAMs (Table 1.2). Src interacts with ADAM-9, -12, -13, and-15; Abl interacts with ADAM-12, -13, and -15; Yes can bind

ADAM12; Lck associates with ADAM-10 and -15; Fyn, and Hck, so far, only bind to ADAM15. The ADAM15 interactions with Hck and Lck were dependent on ADAM15 cytoplasmic phosphorylation, though the kinase responsible is not known (145). The ADAM12 interaction with v-Src leads to phosphorylation of ADAM12 in muscle cells, and although this interaction activates Src, the affect on ADAM12 function is unknown (146,165). Providing the most comprehensive study of Src signaling to date, Zhang et al. (166) showed that Src association with TACE is part of a mechanism that activates PI3K and causes phosphoinositol dependent kinase-1 (PDK1) to directly phosphorylate serines and threonines in TACE, leading to AR shedding and activation of EGFR. Finally, the Src substrate Fish is a scaffolding protein that binds ADAM-12, -15, and -19 (167) and could mediate substrate presentation, or protease regulation, of these ADAMs through formation of complexes with secondary binding sites. Thus, Src proteins can function upstream of ADAMs in some scenarios, though their function is not well understood on a mechanistic level.

Protein Kinase C (PKC) is another family of signaling proteins involved in ADAM regulation via Ser/Thr phosphorylation as a regulatory modification. PKC can be activated by phorbol esters, which are general activators of shedding. Though a non-physiologic stimulus, the mechanism by which PMA-activated PKC regulates ADAM activity is nevertheless of interest. ADAM9 associates with PKC-δ and that this phorbol ester-induced interaction can regulate ADAM9 dependent HB-EGF shedding (90). ADAM9 was phosphorylated in response PMA (85), but whether or not this is a direct result of PKC binding, or its affect on ADAM9 activity, is not known. ADAM12 can also associate with PKC-δ and PKC-ε (92,168). PKC-ε regulates ADAM12 translocation from a perinuclear compartment to the cell surface upon stimulation with PMA (168). This event was dependent on the cytoplasmic binding and kinase activity of PKC-ε, though direct phosphorylation of ADAM12 was not investigated. PKC was identified upstream of EGFR transactivation by carbachol in human

embryonic kidney 293 cells (169) and again in hepatic C9 cells in response to AngII (170). PKC-δ, using specific PKC inhibitors, was later situated in between upstream calcium and Src signaling and downstream EGFR transactivation by phenylephrine in neurons and 293 cells (171). PKC isoforms are modulated by diacylglycerol (DAG), which in turn are regulated by DAG kinase (DGK) (172). Thus, it was of great interest when DGK-δ knockout mice displayed EGFR and TACE-like open-eye phenotypes and enhanced activation of PKC and PKC substrates (173). This suggests DGK can influence EGFR signaling, but whether this is dependent on shedding is under investigation. Together, this evidence proves a necessary role for PKC, but also supports the contention that multiple signaling pathways can converge on ADAMs to activate shedding.

Second messengers are diffusible signaling molecules commonly used as intermediates for signal transduction utilized by GPCRs and other receptor initiated pathways. Calcium is a common downstream target of G-proteins and participates in EGFR transactivation (174-176). Extracellular calcium uptake, which initiates signaling, can be reproduced using an ionophore to transport calcium across the membrane and this has been shown in several independent reports to specifically trigger ADAM10-dependent shedding. This led to cleavage of the cell adhesion molecule CD44 by ADAM10, whereas TACE-dependent CD-44 shedding was regulated by PKC (177). Calcium also regulated shedding of the ADAM10 specific substrates EGF and BTC (89,102). TACE substrates TGF-α, AR, HB-EGF and EPR could also be shed by intracellular calcium increases, but independent of TACE activity, implicating other metalloproteases and demonstrating how various stimuli can target different proteases and substrates.

Calcium has been reported upstream of TACE-dependent HB-EGF shedding in COS7 cells. Using phospholipase-C activators, a calcium ionophore and dominant negative TACE, Mifune et al. (123) elucidated an AngII pathway that utilized phospholipase C-Ca-ROS-TACE-HB-EGF in transactivation of EGFR. How calcium influences TACE activity may be

explained through involvement of another second messenger, reactive oxygen species (ROS). ROS are byproducts of oxidative phosphorylation in the mitochondria and the reduction of NADPH by its oxidase in the cytoplasm of most cells along with a host of other oxidases such as xanthine oxidase, cyclooxygenase, lipoxygenase and nitric oxide (178). They are known to promote a variety of cellular events through their stimulated production following signaling by GPCRs, cytokines and stress inducing agents. The ROS, hydrogen peroxide, can stimulate the shedding of TGF-α, AR, TNFR and L-selectin by TACE (100,179,180). HB-EGF shedding mediated by ADAM10, TACE and to a lesser extent ADAM9 (152), utilized the p38 MAPK pathway, however in rat prostate cancer cells HB-EGF shedding involving ROS was independent of p38 signaling (181). ROS activation of TACE and ErbB ligand shedding is important in many physiologically relevant transactivation events, too. Insulin growth factor-1 receptor and endothelin-1 receptors can transactivate EGFR through ROS-stimulated ADAM-directed shedding of HB-EGF (182,183). Transactivation of EGFR regulates mucin expression in airway epithelial cells relying on TACE shedding of TGF-α initiated by ROS production from NADPH oxidase (Nox) to propagate the signal (179). In vascular smooth muscle cells, phenylephrine and angiotensin II induce proliferation through ROS-ADAM-HB-EGF-EGFR and have become useful models for cardiovascular disease (123,180). Angiotensin II (184) and phenylephrine (180), along with endothelin I (185), appear to utilize Nox complex as the main source of ROS by activating Rac-1 (186), which is responsible for the phosphorylation and translocation of the cytoplasmic subunits of Nox to the membrane where they interact with and form a functioning complex (187). Several reports suggest that Src kinases may play a role in Nox activation and ROS production following GPCR activation (171,186,188). Zhang et al. (189) showed that hydrogen peroxide might directly activate TACE metalloprotease by removing a peptide mimic of the TACE prodomain in an *in vitro* peptide cleavage assay. This corresponds with the ability of ROS to oxidize cysteine residues as a modification. ROS

may also regulate ADAM activity through oxidation of lipid moieties, or targeting of cholesterol, which may alter lipid raft integrity. Thus, calcium and ROS, or even calcium upregulating ROS, are positioned as key determinants in selection of substrate and activation of ADAMs, though exactly where and how are undetermined.

The importance of the ErbB network, and by extension ligand shedding, has been established through the studies concerning development and the role of disregulated signaling in disease progression. Since GPCRs appear to routinely utilize EGFR to integrate and amplify hormone signaling, transactivation of EGFR may also play an important role in development of cardiovascular diseases, lung fibrosis and asthma, and Alzheimer's disease. The dependence on soluble ligand indicated by the requirement of metalloproteases from Prenzel et al. (117) reveals how indispensable shedding is and how regulation of this central process could be crucial. ADAMs have been implicated in this shedding event through genetic and biochemical studies of TACE and ADAM10 knockout mice and in cell culture models, yet substrate specificity and enzymatic regulation are still not fully understood. The evidence so far points to many different levels of regulation that could cooperate to control ADAM and ectodomain shedding. Signaling molecules and pathways, such as calcium, ROS and MAPK, translate external ligand binding to ADAM activation by yet unknown mechanisms. Other signals, such as those transmitted by PKC and Src-like kinases, may alter localization and protein interactions that regulate associations of enzyme with its substrate. Still for all that has been discovered, contradictions remain and there remains much to learn. Thus, refining and improving our understanding of ADAM regulation mechanisms is the current focus of this field with the hope that each advance in our knowledge of ADAM-dependent shedding brings us closer to understanding and development of novel treatments for any number of diseases.

Table 1.1 ADAM, Integrin Interactions

ADAM	Integrin	Reference
ADAM1	α9β1	77, 234
ADAM2	α4β1, α6β1, α9β1	77, 234, 235
ADAM3	α4β1, α6β1, α9β1	77, 234, 236
ADAM7	α4β1, α4β7, α9β1	237
ADAM8	α9β1	238
ADAM9	α2β1, α3β1, α6β1, α6β4, α9β1, αvβ5	82, 77, 239, 240, 241
ADAM12	α4β1, α9β1, α3β1, α7β1, β1, β3	77, 79, 81, 83, 242, 243, 244
ADAM15	α5β1, α9β1, αIIβ3, αvβ3	75, 76, 77, 245
ADAM17	α5β1	80
ADAM19	α4β1, α5β1	79
ADAM23	αvβ3	246
ADAM28	α4β1, α4β7, α9β1,	11, 237
ADAM33	α4β1, α5β1, α9β1	79, 247

Table 1.2 Selected ADAM Protein Interactions

ADAM	Interacting Protein	Identifying Technique	Reference
ADAM9	Enophilin-1	Y2H, GST-pd	248
	Eve-1	tIP	147
	Fish	tIP	167
	MAD2β	Y2H, GST-PD	249
	PACSIN3	Y2H	143
	PKCδ	FW, tIP	90
	SH3PXI	Y2H, GST-PD	248
	Src	FW	250
ADAM10	Eve-1	tIP	147
	Lck	GST-PD	145
	MAD2	GST-PD	145
	PACSIN3	Y2H	143
ADAM12	Abl	GST-PD	146
	Actinin-1	FW, GST-PD, tIP	251
	Actinin-2	Y2H, GST-PD, tIP	252
	Eve-1	Y2H, tIP	147
	Fish	tIP	167
	Grb2	GST-PD, eIP	146
	MAD2β	Y2H	249
	PACSIN3	Y2H, GST-PD, tIP	143
	p85-PI3K	GST-PD, tIP	144
	PKCδ	Y2H	92
	PKCε	eIP	168
	Yes	GST-PD	146
ADAM15	Abl	GST-PD	145
	Endophilin-1	Y2H, GST-PD	248
	Eve-1	tIP	147
	Fish	tIP	167
	Fyn	GST-PD	145
	Grb-2	GST-PD	145
	Hck	GST-PD, tIP, FW	145
	Lck	GST-PD, eIP, FW	145
	MAD2β	Y2H	249
	PACSIN3	Y2H	143
	SH3PX1	Y2H, GST-PD	248
	Src	GST-PD	145
ADAM17	ERK	GST-PD, eIP	232
	Eve-1	tIP	147
	MAD2	GST-PD	249
	NRDc	tIP	148
	PTPH1	Y2H, GST-PD, tIP	151
	SAP97	Y2H, GST-PD, eIP	150
ADAM19	ArgBP1	Y2H, GST-PD	253
	b-Cop	Y2H	253
	Endophilin-1	Y2H	253
	Fish	PD, GST-PD, tIP	167
	PACSIN3	Y2H	143
	Ubiquitin	Y2H	253

Technique key: eIP, endogenous immunoprecipitation; tIP, transfected immunoprecipitation; FW, far western; GST-PD, glutathione-S-transferase pulldown; PD, phage display; Y2H, yeast two-hybrid.

Figure 1.1 *The EGF-Like Growth Factors.* Schematic of the seven EGF-like growth factors. Epidermal growth factor possesses an N-terminal extension consisting of eight EGF-like motifs (dashed lines). All other N-terminal extensions are indicated by solid lines. The N-terminal cleavage sites are designated by black arrows, while the C-terminal cleavage sites are illustrated with grey arrows. These polypeptide ligands begin as membrane anchored precursor proteins. They all contain an approximately 50 amino acid receptor binding region that is folded into a three loop structure, formed by disulfide bonds between six conserved cysteines. This region is found in the ectodomain of the protein, a short distance above the membrane.

Figure 1.1

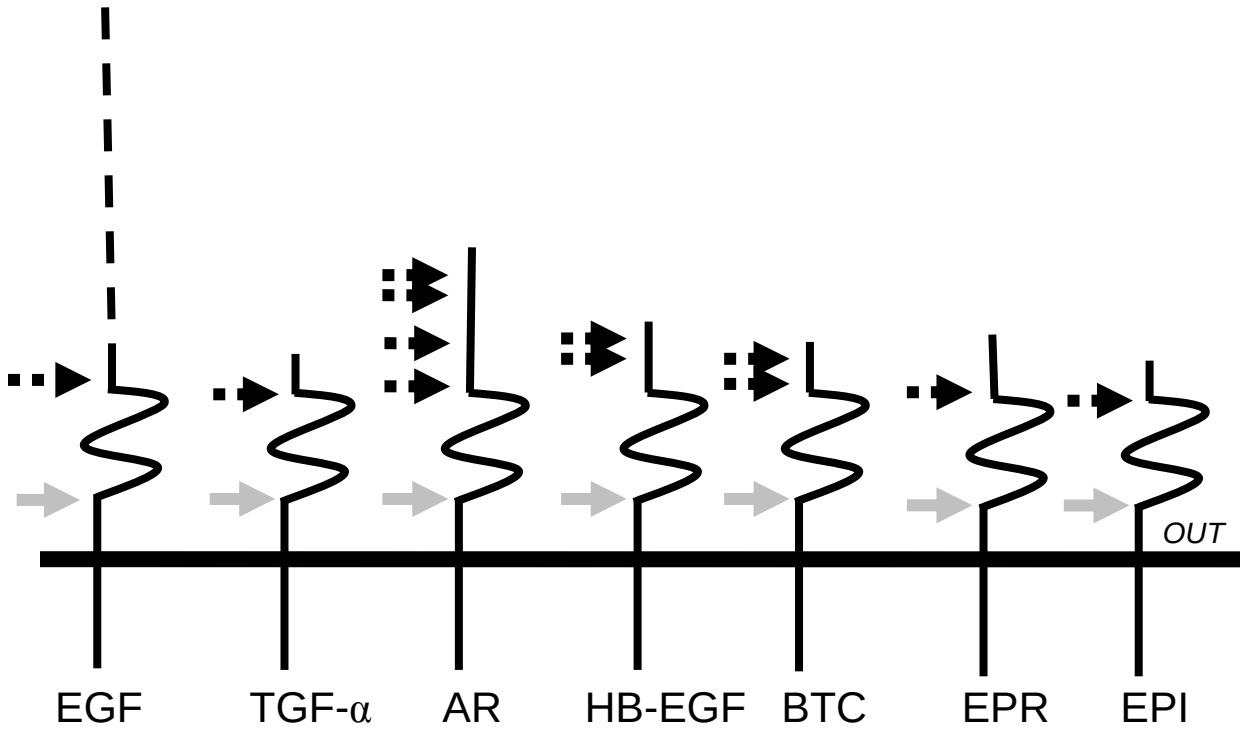


Figure 1.2 *ADAM Domain Sequence.* A schematic of disintegrin and metalloproteases (ADAMs) and their relationship to snake venom metalloproteases and matrix metalloproteases. Note matrix metalloproteases may include domains such as hemopexin-like and proline-rich regions that participate in substrate selection and protein interactions, C-terminal to the catalytic domain. SP, signal peptide; Pro, prodomain; MP, metalloprotease; Dis, disintegrin; CRD, cysteine-rich domain; TM, transmembrane domain; Cyto, cytoplasmic domain.

Figure 1.2

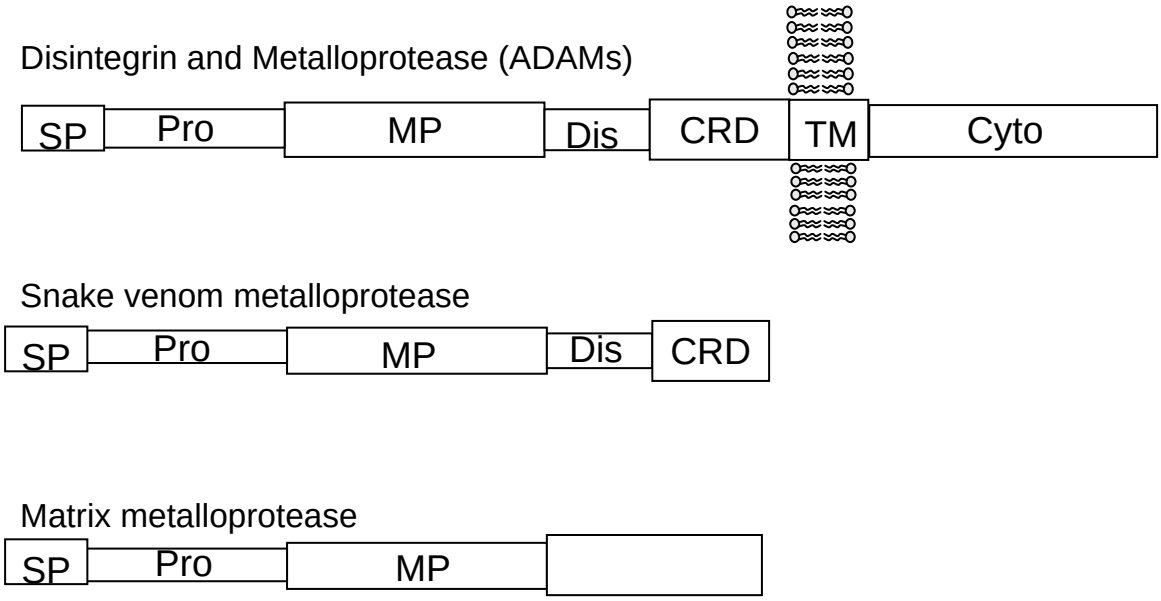
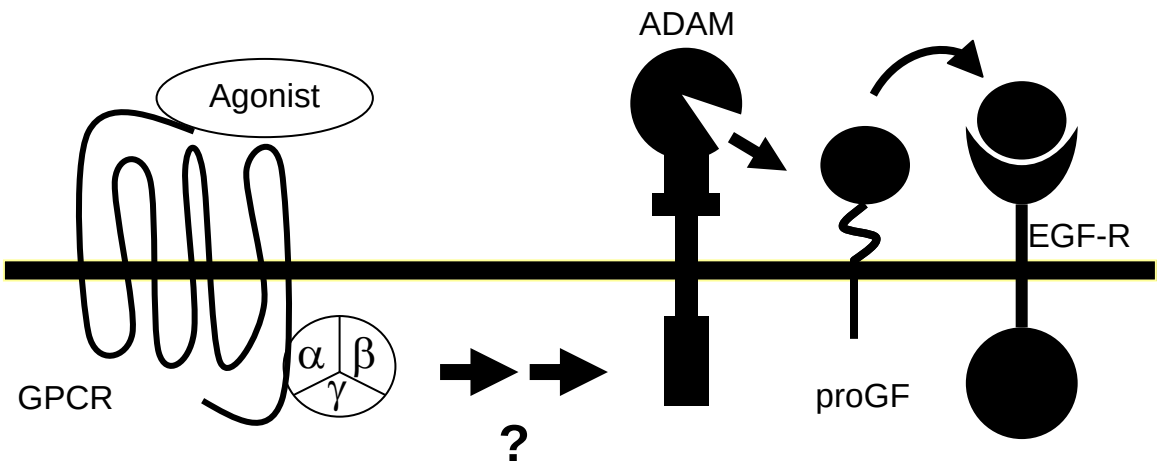

Disintegrin and Metalloprotease (ADAMs)
SP
Pro
MP
Dis
CRD
TM
Cyto
Snake venom metalloprotease
SP
Pro
MP
Dis
CRD
Matrix metalloprotease
SP
Pro
MP

Figure 1.3 *Triple Membrane Passing Transactivation Pathway.* Paradigm for transactivation of EGFR following GPCR stimulation. A GPCR agonist binds and stimulates G-protein signaling that eventually leads to the activation of ADAM metalloproteases, which is possibly dependent on the cytoplasmic domains of ADAMs. The protease cleaves membrane anchored growth factors, which bind EGFR and initiate downstream signaling pathways

Figure 1.3



Chapter 2

Purinergic GPCRs Utilize Mitochondrial ROS to Mediate TACE-Dependent TGF-α Shedding

This work has been submitted to the American Society of Microbiology for publication in the journal Molecular and Cellular Biology. Myers, T. J., Brennaman, L. H., Stevenson, M., Higashiyama, S., Russell, W. E., Lee, D. C. and Sunnarborg, S. W.

This work was a collaborative effort. I collected conditioned media for analysis by RIA following pretreatment with NAC in Table 2.1 and Figure 2.3, RT-PCR experiments for murine and CHO cells (Figures 2.2 and 2.4), all AP shedding assays in Figures 2.4 and 2.5 along with measurements of ATP induced mitochondrial ROS in Figure 2.5. All RIA media analysis was performed by Mary Stevenson under William E. Russell. Detection of EGFR phosphorylation was performed by Susan Sunnarborg, while collection of conditioned media for RIA analysis in Figures 2.1, 2.2 and Table 2.1 were performed by Susan Sunnarborg and Leann Brennaman.

Abstract

EGFR transactivation by GPCRs has been shown to regulate many essential biological processes. ADAM metalloprotease activity has been implicated as a key step in transactivation, yet the regulation mechanisms are not fully understood. Here, we investigate the regulation of TGF-α shedding through the ATP-dependent activation of the P2Y family of GPCRs. We report that ATP stimulates TGF-α proteolysis with concomitant EGFR activation and that this process requires ADAM17/TACE activity in both murine and CHO cells. ATP-induced TGF-α shedding was independent of Src family kinases, PKC and MAPK signaling, but could be stimulated by a calcium ionophore. Moreover, ATP-induced TGF-α shedding was completely inhibited by scavengers of reactive oxygen species (ROS) and stimulated by hydrogen peroxide, while stimulation with the calcium ionophore was partially inhibited by reduction of ROS in the cell. We also found that the cytoplasmic NADPH oxidase complex was not required for ATP-induced shedding. Instead, mitochondrial ROS production increased in response to ATP and mitochondrial oxidative complex activity was required to activate TACE-dependent shedding. This study reveals the requirement for mitochondrial ROS in regulating GPCR-induced growth factor shedding.

Introduction

Epidermal growth factor receptor (EGFR) has long been recognized as a critical component of cellular signal transduction machinery (2). The recent discovery of the essential role of EGFR in propagating signals generated by G-protein coupled receptor (GPCR) agonists indicates that EGFR may function as a central signal integration point for stimuli impacting the cell surface (59,120). A wide variety of GPCR agonists, including lysophosphatidic acid (LPA), phenylephrine and carbachol, can harness EGFR to promote ERK activation leading to physiological and patho-physiological processes (95,101,114,180). Until recently, GPCR crosstalk was thought to consist of entirely intracellular signaling pathways that led to EGFR phosphorylation, independent of ligand binding and dimerization. However, elucidation of a rapid, metalloprotease-dependent growth factor cleavage step leading to EGFR activation (117) revealed the importance of regulated proteolysis in GPCR-EGFR transactivation.

The seven known members of the EGF family of growth factors, amphiregulin (AR), betacellulin, EGF, epigen, epiregulin, heparin-binding EGF (HB-EGF), and transforming growth factor alpha (TGF-α), are all initially synthesized as type I transmembrane precursors, containing the growth factor moiety in the ectodomain (19). Several studies have demonstrated the biological activity of the non-cleavable, membrane anchored precursor molecules (48,49), but the metalloprotease dependence of transactivation, the loss of EGFR signaling in cells inhibited with metalloprotease inhibitors (133,190), and the convergence of phenotypes of growth factor, growth factor receptor and protease knockout models indicate that proteolytic cleavage of the growth factors is an important and regulatable step in most contexts (33, 34, 47, 110).

A variety of *in vitro* and *in vivo* evidence points to the ADAM family member Tumor Necrosis Factor Alpha Converting Enzyme (TACE/ADAM17) as the critical convertase for TGF-α, AR and HB-EGF. ADAMs (A Disintegrin And Metalloprotease), along with

matrix metalloproteases, belong to the metzincin family of zinc-dependent proteases. When mice lacking active TACE were compared to TGF-α, AR and HB-EGF knockouts, they were found to share epidermal defects with homozygous TGF-α null mice (47), loss of mammary gland branching as in mice lacking AR (110), and heart and lung defects with HB-EGF -/- animals (33,34). Fibroblasts derived from the TACE-deficient mice were impaired in shedding of TGF-α, HB-EGF and AR, but shedding could be partially rescued by transfection of wild type TACE into the cells (93,109). In cell culture, knockdown of TACE expression can also have an inhibitory effect on growth factor-dependent transactivation by LPA and angiotensin II stimulation (96,123). The overlapping phenotypes of mice lacking these growth factors and those lacking TACE/ADAM17, along with the *in vitro* results, support a critical role for the soluble forms of the growth factors and highlight the importance of their proteolysis as a regulatory step.

Despite its presence at a critical signaling juncture, the regulation of ADAM metalloprotease activity is still not fully understood. ADAMs are type I transmembrane proteins that possess an archetypal organization, including the metalloprotease and disintegrin domains along with a cytoplasmic domain often rich in SH3 binding sites that could potentially regulate ADAM inside-out signaling (59,61). Recently, several signaling components have been implicated in GPCR-initiated TACE activation. Src-like non receptor tyrosine kinases are accepted as intermediates in EGFR transactivation and have been found in association with several ADAMs leading to phosphorylation of ADAM cytoplasmic domains (61,115,166,191). Elevation of intracellular calcium was also found to stimulate the release of ErbB ligands in an ADAM-dependent manner (89,102,123), while PKC is suspected to play a role in ADAM activation because of the ability of PMA to trigger PKC signaling and stimulated ectodomain shedding. MAPK proteins have also been implicated both prior and subsequent to EGFR activation (114,117,152,158).

Lastly, reactive oxygen species (ROS) can function as a second messenger in response to G-protein signaling (189,192). ROS has been shown to be necessary for Angiotensin II and endothelin-1 mediated TACE activation and HB-EGF shedding, possibly through direct modification of a cysteine that coordinates the binding of the inhibitory prodomain (123,185,189). The wide variety of possible regulatory mechanisms suggests substrate- or cell type-specific pathways dependent on the stimulant, or intended signal effect.

In this study we characterize a P2Y-initiated pathway that, in response to ATP, stimulates TACE-dependent TGF-α proteolysis and EGFR phosphorylation, independent of Src, PKC and MAPK signaling. We report for the first time the requirement for mitochondrially-derived ROS, whose production is stimulated by ATP, for regulation of GPCR-stimulated growth factor shedding. Collectively these results offer a model that can be utilized to further our understanding of the activation mechanisms of TACE.

Materials and Methods

Materials – RC-20 antibody was purchased from Transduction Laboratories and MF9 anti-human TGF-α came form Lab Vision Corporation. The rabbit anti-EGFR, antibody, ERCT, was a generous gift from H.Shelton Earp (193). Horseradish peroxidase-conjugated secondary antibodies were from Roche Molecular Biochemicals. Adenosine Triphosphate was purchased from Amersham. N-Acetyl-L-Cysteine, rotenone and myxothiazol were from Sigma and all other chemicals were from Calbiochem.

Cell Lines, Transfections, and Stable Clones – EC-4 (*Tace*$^{+/+}$) and EC-2 (*Tace*$^{\Delta Zn/\Delta Zn}$) fibroblasts (88) and their derivatives were maintained in Dulbecco's modified Eagle's medium/F-12, 1% FBS with antibiotic. Wild type and M2 CHO cells and their derivatives were maintained in Dulbecco's modified Eagle's medium, 10% FBS, 1x non-essential amino acids and antibiotic. Transfections were performed using lipofectAMINE

(Invitrogen). The human EGFR cDNA (194) was subcloned into modified pCEP4 (Invitrogen) and stably expressed in all cell lines following selection with 800 µg/mL hygromycin B (Roche). Alkaline phosphatase-tagged TGF-α (35) was kindly sent by Dr. Carl Blobel. Stable expression of AP-TGF-α in wild type CHO cells was achieved by selecting clones in 500 µg/mL of G418 (Sigma). For stable expression in M2 cells that had already been subjected to G418 selection, AP-TGF-α was subcloned into pcDNA 3.1 Hygro vector (Invitrogen) using a PCR-generated fragment (Hind III-capped forward PCR primer 5'-AAGCTTGTTCTAGCGGCACCGC-3'; Kpn I capped reverse PCR primer 5'-GGTACCCGTTCTTACAGCAAAAGGC-3') and the TOPO cloning kit (Invitrogen). Stable clones were selected in 800 µg/mL hygromycin B.

Harvesting, Immunoprecipitation and Western Blot Analysis – Confluent cells were washed twice in serum free medium (SFM) and then starved for 4 hours at 37 ^{0}C in SFM. Following a 5 minute stimulation, conditioned media was collected and cells washed in phosphate buffered saline and lysed in 1% Triton X-100 50 mM Tris, pH 7.4, 150 mM NaCl with 10 µg/mL leupeptin, 20 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluride, 2 µM sodium orthovanadate, 10 mM sodium fluoride and 5 mM sodium molybdate. Protein concentration was determined with a BCA assay kit (Pierce). For some experiments media was concentrated using Sep-Pak C-18 reverse phase cartridges (Waters) and TGF-α amounts in both lysates and media measured with a specific radioimmunoassay (RIA) (196). For immunoprecipitations, equal amounts of protein were incubated for 30 minutes in primary antibody at 4 ^{0}C followed by rProtein Agarose G (Invitrogen) at 4 ^{0}C for 1 hour. Beads were washed three times in 20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40 and protein was eluted in Laemmilli's sample buffer. Proteins were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride (Millipore). Membranes were blocked in Tris-

buffered saline, 0.1% Tween 20, 5% nonfat milk or 3% BSA for pTyr immunoblots. Bands were visualized using Pierce SuperSignal West Pico chemiluminescent system for autoradiography.

Expression of P2Y Receptors by RT-PCR – RNA was extracted from wild type EC-4 or CHO cells grown in 10-cm dishes using RNeasy Mini Kit (Qiagen). Total RNA was used to prepare cDNA with MMTV Reverse Transcriptase (Invitrogen). PCR amplification used primers for mouse $P2Y_2$ or $P2Y_4$ corresponding to analogour regions to yield the same size products. Primers for $P2Y_2$, 5'-ACGGTGCTCTGCAAGCTGGTGC -3'; 5'-GTAGAGGGTGCGCGTGACGTGG-3'; $P2Y_4$, 5'-TACTACTATGCTGCCAGAAACCAC-3'; 5'-AGCAAAGACAGTCAGCACCACAGC-'3.

Alkaline Phosphatase Assay – Cells were plated in 24-well dishes at a density of 2.5 x 10^5 for 18 hours. Cells were washed twice in SFM and starved in fresh SFM for 4 hours at 37 ^{0}C. Vehichle (DMSO) or inhibitors were added directly to cells for 30 minutes: NAC 5 mM, apocynin 1 mM, rotenone 25 μM, myxothiazol 1 μM. After inhibition, media was changed to fresh SFM containing inhibitor and stimulant for 5 minutes. Media was then collected, centrifuged for 30 minutes, 14,000 xg at 4 ^{0}C. Media aliquots were mixed with an equal volume of 2x AP buffer (0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 5.4 mM p-nitrophenylphosphate) and allowed to develop at room temperature. Absorbance at 405 nm was measured to quantify alkaline phosphatase activity.

Measurement of Mitochondrial ROS – Cells were plated in 24-well dishes at a density of 2.5 x 10^5 for 18 hours. Cells were washed twice in SFM and starved in fresh SFM for 4 hours at 37 ^{0}C. Cells were loaded with 250 nM reduced MitoTracker Red (MTR) (Molecular Probes) for 15 minutes at 37 ^{0}C away from direct light. Media was aspirated

and cells washed twice in phenol free serum free media supplemented with 2 mM L-glutamine and left in washing media. Cells were then stimulated for 5 minutes and excited at 544 nm and emission detected at 612 nm.

Statistical Analysis – Statistical analysis was performed with assistance from the UNC Lineberger Comprehensive Cancer Center's Biostatistics Shared Resources Group using the Wilcoxon rank sum test for pairwise group comparisons.

Results

In order to study the regulation of proteolytic growth factor cleavage induced by GPCR stimulation, we chose TACE/ADAM17 cleavage of proTGF-α as a model system. Stimulation of EC-4 transformed fibroblasts stably expressing EGFR with the purinergic receptor agonist ATP led to the phosphorylation of receptor, as did treatment with exogenous EGF (Figure 2.1A). Therefore, these cells possess the endogenous components to accomplish GPCR-induced EGFR transactivation.

Since EC-4 cells constitutively release low levels of TGF-α (109), we asked if ATP stimulation would lead to rapid release of soluble TGF-α. ATP stimulates the murine purinergic $P2Y_2$ and $P2Y_4$ receptors with an EC50 of 100 μM (195). Using a highly specific TGF-α radioimmunoassay (RIA) (196), we detected a greater than 2-fold increase in endogenous TGF-α shedding 5 minutes after addition of 100μM ATP (Figure 2.1B). The effect of ATP was not dependent on hydrolysis, as indicated by comparable results with the non-cleavable ATP analog, ATPγS and was comparable to shedding induced by the known EGFR transactivator, thrombin. These results indicate that GPCR

stimulation via purinergic receptors leads to rapid release of soluble TGF-α and EGFR transactivation.

The paradigm for GPCR-induced EGFR transactivation requires a metalloprotease-dependent growth factor cleavage step (117). We verified the dependence on metalloprotease activity in this model system by treating cells with the metalloprotease inhibitor TAPI-2. In the presence of inhibitor, ATP transactivation of EGFR was abolished, while EGF was still able to activate the receptor (Figure 2.2A). Therefore, purinergic stimulation of EGFR requires metalloprotease activity.

In astrocytoma cells, ATP stimulated the release of amyloid precursor protein through $P2Y_2$ receptors by activating both ADAM10 and TACE (197). In our system, ATP stimulated TGF-α release, and as proTGF-α does not appear to be a substrate for ADAM10 (89,93), we hypothesized that TACE was responsible for the ATP-induced TGF-α release and EGFR transactivation that we have observed. To test this hypothesis, we used TACE-deficient EC-2 fibroblasts (88) stably expressing EGFR. While EGF activated its receptor in wild-type and TACE-deficient cells, ATP was unable to stimulate phosphorylation of EGFR in the EC-2 cells compared to the wild type EC-4 cells (Figure 2.2B). Similar to our previous observations for constitutive shedding (109), induction of TGF-α release by treatment with 100 μM ATP was abolished in the absence of TACE (Figure 2.2C). To ensure that the lack of TGF-α shedding in EC-2 cells was not attributable to a defect in coupling of purinergic receptors to downstream signaling, we monitored inositol phosphate metabolism following ATP stimulation. EC-2 and EC-4 cells incorporated comparable levels of ^{3}H-inositol in response to 100 μM ATP and UTP (data not shown). Thus, TACE is the crucial protease responsible for ATP-induced TGF-α shedding in mouse fibroblasts.

We also wished to determine the purinergic receptor subtype responsible for this shedding event. ATP and UTP stimulated the same level of ^{3}H -inositol incorporation over a range of concentrations (data not shown), a response indicative of mouse $P2Y_2$ or $P2Y_4$ receptors. Semi-quantitative reverse transcriptase-PCR on RNA from EC mouse fibroblasts revealed expression of both receptor subtypes (Figure 2.2D), with higher levels of $P2Y_2$ present. Therefore, both receptors may contribute to the ATP response in our cell model.

In order to elucidate the regulatory steps of TACE-dependent shedding, we used chemical inhibitors to ask which pathways contributed to the release of TGF-α following ATP stimulation in EC-4 cells. Pathways involving the protein kinase C family, calcium, and src-family non-receptor tyrosine kinases have been implicated in shedding of a number of EGF family members, including GPCR-induced shedding (89,90,102,115,164,177). As shown in Table 2.1, reagents that target these pathways, including the broad spectrum PKC inhibitor bisindolylmaleimide (BIM-1), intracellular calcium chelator BAPTA-AM, as well as the Src family inhibitor PP2, showed no significant inhibition of ATP-stimulated TGF-α shedding. MAPK pathways have also been associated with growth factor shedding following stress or other stimuli (152,153,156,158,188). However, we observed no inhibition of TGF-α release when ERK activity was blocked, or with either of two inhibitors of p38 MAPK signaling. This indicates use of an alternative route for purinergic signaling.

Studies of ADAM regulation, and specifically TACE, have recently focused on the ability of reactive oxygen species (ROS) to act as a signaling intermediate. *In vitro* experiments have shown that hydrogen peroxide can lead to TACE activation and shedding of both HB-EGF and TGF-α (179,180,198). ROS have also been found to act downstream of the angiotensin II receptor and P2Y purinergic receptors (123,199,200). We therefore hypothesized that ROS may also play a role in ATP-induced TGF-α

shedding. To test this prediction, we treated mouse fibroblasts with the ROS scavenger N-Acetyl-L-Cysteine (NAC). Shedding of TGF-α was dramatically reduced in the presence of NAC, pointing to a significant role for ROS in the regulation of TGF-α shedding (Table 2.1, Figure 2.3).

Further investigation of the source and role of ROS in the activation of TGF-α shedding required an assay system with high-throughput capabilities. Alkaline phosphatase-tagged EGF-like growth factors are an established method of monitoring the growth factor cleavage by quantifying alkaline phosphatase activity in conditioned media (35,89,93,147,152,156). To facilitate transfection and culturing, we shifted our cell model to Chinese Hamster Ovary cells (CHO), which have been used previously to study the regulation of growth factor shedding (156,201). While lacking endogenous EGF- family growth factors, they express TACE (201,202), and have been reported to respond to purinergic stimulation (203). Reverse transcriptase-PCR of CHO RNA revealed strong expression of $P2Y_2$, although $P2Y_4$ was undetectable even with elevated cycle numbers (Figure 2.4A). Thus, CHO cells should provide a suitable model for further study of GPCR-induced TGF-α shedding.

To test the cells and assay system we prepared wild type CHO clones that stably express alkaline phosphatase-tagged TGF-α (AP-TGF-α) or empty vector (35). We next determined the effect of ATP on AP-TGF-α shedding. As in mouse cells, a 5 minute stimulation with 100 μM ATP caused a significant increase of alkaline phosphatase activity in the media compared to mock stimulation (Figure 2.4B). Metalloprotease-dependence was verified for ATP-induced shedding by pre-treatment of the cells with TAPI-2. When these cells were stimulated with water or ATP, shedding was almost completely abrogated (Figure 2.4B). We next confirmed that TACE was responsible for AP-TGF-α shedding in CHO cells by using M2 CHO cells that carry mutations in each TACE allele that prohibits TACE activity (201,202). Following ATP treatment, alkaline

phosphatase activity was negligible in M2-APT cells stably expressing AP-TGF-α, compared to wild type cells (Figure 2.4D). These results confirm the presence of a $P2Y_2$-TACE-TGF-α pathway in CHO cells.

We then tested the requirement for ROS signaling in the CHO cell model. Hydrogen peroxide stimulated a greater than 2-fold increase in shedding over basal levels (Figure 2.4B). This ROS-induced increase in shedding was also dependent on metalloprotease activity since the inhibitor TAPI-2 prevented shedding (Figure 2.4B). When the cells were pre-incubated with the scavenger NAC, ATP-induced shedding was completely lost as seen in the EC-4 cells (Figure 2.4C). Thus, ROS regulation of TGF-α shedding appears to be a conserved pathway of P2Y receptor activation.

Calcium is a common signaling molecule utilized by GPCRs, especially P2Y receptors. Calcium has also been demonstrated to play a role in other shedding and transactivation studies. We therefore tested for calcium in our pathway using a calcium ionophore, A23187, which mimics extracellular calcium influx by coupling to and transporting extracellular calcium across the membrane. Stimulation with the calcium ionophore A23187 led to a significant increase in shedding over control levels (Figure 2.4E). A23187-induced shedding was mainly dependent on TACE activity, as shedding was markedly reduced in the TACE deficient M2-APT CHO cells (Figure 2.4D). A23187-stimulated shedding was also partially inhibited by the ROS scavenger, NAC, in wild type CHO-APT cells (Figure 2.4E). These data suggest that extracellular calcium influx can function upstream of ROS to activate TACE-dependent shedding, but can also stimulate a lesser ROS and TACE independent shedding pathway.

Several sources of ROS exist within non-phagocytic cells, including the cytoplasmically bound NADPH oxidase, the mitochondrial electron transport chain, nitric oxide and several other oxidases (178). In most non-phagocytic cells the NADPH oxidase produces the majority of cytoplasmic superoxide, which is then rapidly dismutated into

hydrogen peroxide (187). To test whether the ROS required for TGF-α shedding is dependent on NADPH oxidase activity, we treated CHO-APT cells with apocynin, which blocks formation of the complete NADPH oxidase complex, thus blocking ROS production by this complex. CHO cells pre-incubated with 1 mM apocynin and then stimulated with water, or ATP, showed no signs of inhibition (Figure 2.5A). Comparable results were obtained using a range of concentrations as low as 30 μM (data not shown). Because several reports present evidence of a role for the NADPH oxidase in GPCR transactivation of EGFR (100,179,180,183,204), we also tested apocynin on endogenous TGF-α shedding from EC-4 mouse fibroblasts (data not shown). In fibroblasts as well, apocynin had no effect on ATP-stimulated TGF-α shedding, indicating an NADPH-oxidase independent mechanism of ROS production.

The electron transport chain of the mitochondrion is another major source of cellular ROS in cells. To determine if the mitochondrially-derived ROS contributes to the regulation of TGF-α shedding, we examined two independent inhibitors of the electron transport chain: rotenone, which inhibits transfer of electrons from iron-sulfer centers to ubiquinone in Complex I, and myxothiazol, which inhibits transfer of electrons between cytochrome b and cytochrome c_1 in Complex III. Treatment with myxothiazol had no effect on basal shedding, but partially inhibited ATP-induced shedding. A consistent 30% decrease was measured compared to ATP alone (Figure 2.5B). Likewise, rotenone, did not affect basal shedding, but it inhibited ATP-stimulated of TGF-α shedding by more than 65% (Figure 2.5C). Individually these inhibitors were unable to inhibit shedding to the same extent as the scavenger NAC. However, when used in tandem, rotenone and myxothiazol reduced shedding as efficiently as NAC (Figure 2.5D). These results strongly suggest that ROS produced in combination from mitochondrial Complex I and Complex III are an essential component of the signaling pathway for GPCR-induced TGF-α shedding.

To determine whether ATP signaling generated increased levels of mitochondrial ROS we directly measured ROS using the CM-H_2XRos MitoTracker Red. This fluorescent dye is specifically sequestered in mitochondria and remains non-fluorescent until oxidized. Cells stimulated with ATP displayed a significant, rapid increase in fluorescent intensity over control cells, as measured by a fluorescent platereader (Figure 2.5E). This increase in ROS-dependent fluorescence was inhibited by pretreatment with the combination of mitochondrial inhibitors rotenone and myxothiazol. Taken together, these results indicate that ATP-induced signaling pathways can induce production of ROS from mitochondria oxidative complexes, which is an essential step in regulating TGF-α shedding.

Discussion

Transactivation of EGFR via GPCR signaling has emerged as an important event for signal integration and amplification in many biologically essential processes, and typically requires a metalloprotease-mediated growth factor cleavage event (59,120). This study examines a transactivation pathway from the P2Y family of GPCRs that utilizes ADAM17/TACE metalloprotease activity to cleave TGF-α, followed by activation of EGFR. Our goal was to further elucidate the signaling events leading to TACE-mediated growth factor cleavage following stimulation with GPCR agonists. We report here the first observation of mitochondrially produced ROS as a critical step in regulated growth factor proteolysis.

Extracellular nucleotides, such as ATP, are released from many cell types in response to stress, mechanical or biochemical stimulation (205) and can function as a paracrine signal for the P2Y family of purinergic receptors. In this study we identified a P2Y-

dependent pathway leading to TGF-α shedding and transactivation of EGFR. More prominent expression of $P2Y_2$ in mouse cells along with the lack of detectable $P2Y_4$ expression in CHO cells suggests a favored role for the $P2Y_2$ subtype in this transactivation pathway. $P2Y_2$ receptors have been previously linked to EGFR transactivation in PC12 cells and rat fibroblasts (206,207), consistent with the ability of $P2Y_2$ receptors to stimulate proliferation of epidermal keratinocytes (208) and smooth muscle cells (209). $P2Y_2$ receptors are also important for regulating ion channel function in epithelial cells (205). Activation of $P2Y_2$ can promote mucin release in airway cells, an event that has also been tied to TACE-dependent TGF-α shedding and EGFR transactivation (210,211). Thus, elucidation of the mechanisms responsible for regulating EGFR transactivation pathways could have important physiological and therapeutic implications.

A recent study showed that ATP stimulation through $P2Y_2$ receptors led to both ADAM10 and TACE metalloprotease activity towards amyloid precursor protein in astrocytoma cells (197). Here, we show that ATP stimulated TACE-dependent TGF-α shedding in mouse fibroblasts, consistent with previous evidence for TACE as the major constitutive and PMA-stimulated TGF-α sheddase (47,89,93,109,128). A variety of studies have implicated roles for other ADAM proteases in TGF-α processing under specific conditions (89,96,201,212); however, the lack of TGF-α release and of EGFR transactivation in TACE-deficient cells indicates that TACE is the predominant TGF-α sheddase in this transactivation model as well.

In this study, we undertook a further examination of the signaling pathways leading to TACE activity. Reactive oxygen species (ROS) have recently gained attention as second messengers in GPCR signaling pathways (213). P2Y family receptors have been specifically linked to ROS production in eosinophils and prostate tumor cells (200,214). Here we use the ROS scavenger N-acetyl-cysteine to establish a critical role

for ROS in P2Y receptor-dependent TGF-α shedding. Other studies have implicated ROS in EGFR transactivation initiated via other GPCRs (180,183,215). These observations are also consistent with reports that exogenous hydrogen peroxide stimulated the shedding of TGF-α and other EGF family members such as HB-EGF, amphiregulin, and betacellulin (100,152,179,181,183).

The cytoplasmic NADPH oxidase (Nox) complex, which assembles at the plasma membrane following cell stimulation, has been implicated as the main source of ROS in EGFR transactivation pathways stimulated by angiotensin II, phenylephrine, endothelin-1 and tobacco smoke (100,180,183,204). We investigated the involvement of the Nox complex in our system by using the Nox inhibitor apocynin, which directly blocks formation of the Nox complex. We observed that at apocynin concentrations ranging from 30 μM up to 1 mM, ATP-induced TGF-α shedding was unaffected, ruling out the NADPH oxidase complex in this pathway.

To further examine the source of ROS for regulation of P2Y dependent TGF-α shedding, we used specific inhibitors of Complex I and Complex III of the mitochondrial electron transport chain to gauge the contributions of mitochondrial ROS. The inhibitors myxothiazol and rotenone partially decreased shedding when used individually, but when used together inhibited fully, as did the general ROS scavenger NAC, indicating that the functional ROS generation in this pathway can be entirely attributed to mitochondrial sources. Mitochondrial ROS have been implicated in aging and cell fate, cell responses to hypoxia and many aspects of cardiovascular remodeling and disease (216), as well as in hydrogen peroxide-induced EGFR activation (217) and as a downstream signal of EGFR (218-220). Here, we present evidence here for mitochondrial ROS as a signaling intermediate for regulation of P2Y-induced, TACE-dependent growth factor shedding. This novel observation of mitochondrial ROS in

regulating shedding reveals another possible mechanism that cells may use to generate specificity.

ROS-induced TACE shedding may also involve other signaling molecules that have been previously implicated in EGFR transactivation. Increases in intracellular calcium concentrations is a well-characterized result of GPCR activation (224) and has been shown to stimulate ROS production (123,199,200,204,223). In our system, a calcium chelator had no effect on media TGF-α levels following ATP stimulation, indicating that an increase in calcium may not be a requirement for shedding, though it has been implicated in other $P2Y_2$-dependent events (199). However, a calcium ionophore did stimulate shedding that was largely dependent on TACE activity and was partially inhibited by the ROS scavenger NAC. Since the ionophore A23187 has been shown in CHO BQ1 cells to induce higher levels of extracellular calcium influx than with the physiological ATP event (255) it may be that ionophore-induced calcium levels led to mitochondrial ROS production in our system. This is consistent with reports demonstrating ATP, AngII and lysophosphatidylcholine stimulation of extracellular calcium influx and subsequent increases in ROS (123,199,200,204,223), though more work will be required to establish a role for calcium as a physiological, intermediate in ATP-induced mitochondrial ROS production. The Ca^{2+}-ionophore-induced residual shedding seen in M2 and NAC-treated wild type CHO cells may indicate a ROS- and TACE- independent shedding event, like that shown in mouse cells lacking functional TACE (89). Taken together, these observations hint at the complexity of regulating specific downstream circuits following receptor stimulation.

PKC has been widely implicated in regulation of ectodomain shedding based on the well-established ability of phorbol esters to stimulate TACE-dependent TGF-α shedding (89,93,155). A previous report implicated the PKC-δ isoform in regulating stimulated shedding of HB-EGF (90). Inhibition of PKC with bisindolylmaleimide-1 (BIM-1,

GF190203X) did not affect ATP-stimulated TGF-α release in our system. PKC has also been positioned upstream of Nox-dependent ROS production following PMA, lysophosphatidylcholine and ATP, but not angiotensin II stimulation (179,198,199,222). However, it may be that this pathway does not function in regulating the shedding response to GPCR stimulation.

Src family kinases have also been implicated in several GPCR-mediated transactivation cascades upstream of growth factor shedding, including the direct association of Src with TACE, that led to TACE phosphorylation and increased shedding of AR (166). Other examples of $P2Y_2$ transactivation of EGFR have demonstrated a role for Src including direct association with $P2Y_2$ receptors via SH3 motifs (225). There is also evidence suggesting ROS may modulate Src function in transactivation schemes (188,204). In contrast, Camden et al. (197) demonstrated Src-independent activation of TACE and ADAM10 following ATP stimulation. We show here that activation of TGF-α shedding is independent of the Src family members Src, Fyn, Hck and Lck. This further underscores the selective use of available regulatory pathways for particular physiological events.

In summary, we present evidence for the first time of regulation of ATP-stimulated, TACE-dependent shedding of TGF-α regulated by mitochondrially derived ROS. The discovery of a second source of ROS for the activation of TACE-dependent shedding reveals the necessary complexity of signaling that could define specificity in EGFR transactivation.

Acknowledgements

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Table I
ATP-Stimulated TGF-α Shedding Uniquely Requires ROS

Inhibitor	Target	TGF-α Shedding
PP2	Lck, fyn, hck	+
BIM-I	PKC	+
BAPTA	Ca^{2+} Chelator	+
SB202190	p38	+
SB203580	p38	+
U0126	MEK	+
N-Acetyl-Cysteine	ROS	--

Four replicates of EC-4 cells were pretreated for 30 minutes with the following concentration of inhibitors: 10 μM PP2; 20 nM BIM-I; 10 μM BAPTA; 10 μM SB202190; 10 μM SB203580; 5 μM U0126; 5mM NAC; or vehicle (DMSO). Following five minute stimulation with 100 μM ATP, conditioned media was collected and analyzed for TGF-α using a radio-immunoassay (196). TGF-α levels were compared to mock inhibited samples treated with ATP. Results are the average of a minimum of two experiments with at least three replicates.

Figure 2.1 GPCR stimulation induces EGFR transactivation and TGF-α shedding. (A) EC-4 EGFR cells were serum starved for 4 hours and treated with vehicle, 100 ng/mL EGF or 100 μM ATP for 5 minutes. EGFR was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibodies. Representative results from three separate experiments are shown. (B) EC-4 cells were serum starved for 4 hours, then stimulated for 5 minutes with vehicle, 100 μM ATP, 100 μM ATPγS or 2 Units/mL mouse thrombin (Thr). Media and lysates were harvested and concentrated as described in Materials and Methods, and analyzed for TGF-α content by specific RIA. Data is presented as picograms of TGF-α in conditioned media per milligram of total lysate from three separate experiments with at least three replicates. *, $p < 0.02$ as compared to the control.

Figure 2.1

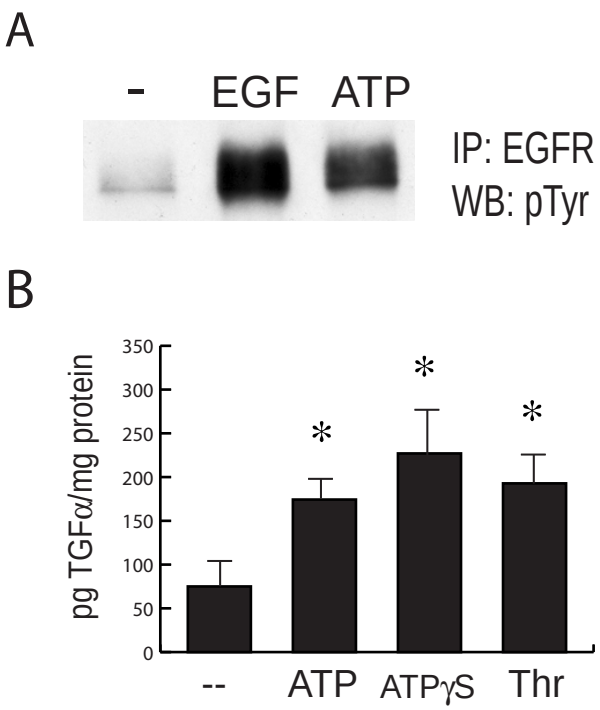

A
-
EGF
ATP
IP: EGFR
WB: pTyr
B
pg TGFα/mg protein
350
300
250
200
150
100
50
0
*
*
*
--
ATP
ATPγS
Thr

Figure 2.2 ADAM17/TACE is required for P2Y GPCR-stimulated TGF-α shedding and EGFR transactivation. EC-4 EGFR cells were serum starved for 4 hours and pretreated with vehicle, or 50 μM TAPI-2 for 15 minutes where indicated. Cells were then stimulated with vehicle, 100 ng/mL EGF, or 100 μM ATP for 5 minutes. EGFR was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibodies. Representative results of three separate experiments are shown. (B) EC-4 EGFR or EC-2 (*Tace*$^{\Delta Zn/\Delta Zn}$) EGFR cells were serum starved for 4 hours then treated with vehicle, 100 ng/mL EGF, or 100 μM ATP for 5 minutes. EGFR was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibodies. Representative results of three separate experiments are shown. (C) EC-4 and EC-2 cells were serum starved for 4 hours then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media and lysates were harvested and concentrated as described in Materials and Methods, and analyzed for TGF-α content by specific RIA. Data is presented as picograms of TGF-α in conditioned media per milligram of total lysate from three separate experiments with at least three replicates. *, $p < 0.05$ as compared to the control. (D) Total RNA from EC-4 cells was subjected to first strand cDNA synthesis with, or without, reverse transcriptase and amplified for 28 cycles to detect the expression of $P2Y_2$ and $P2Y_4$ receptors.

Figure 2.2

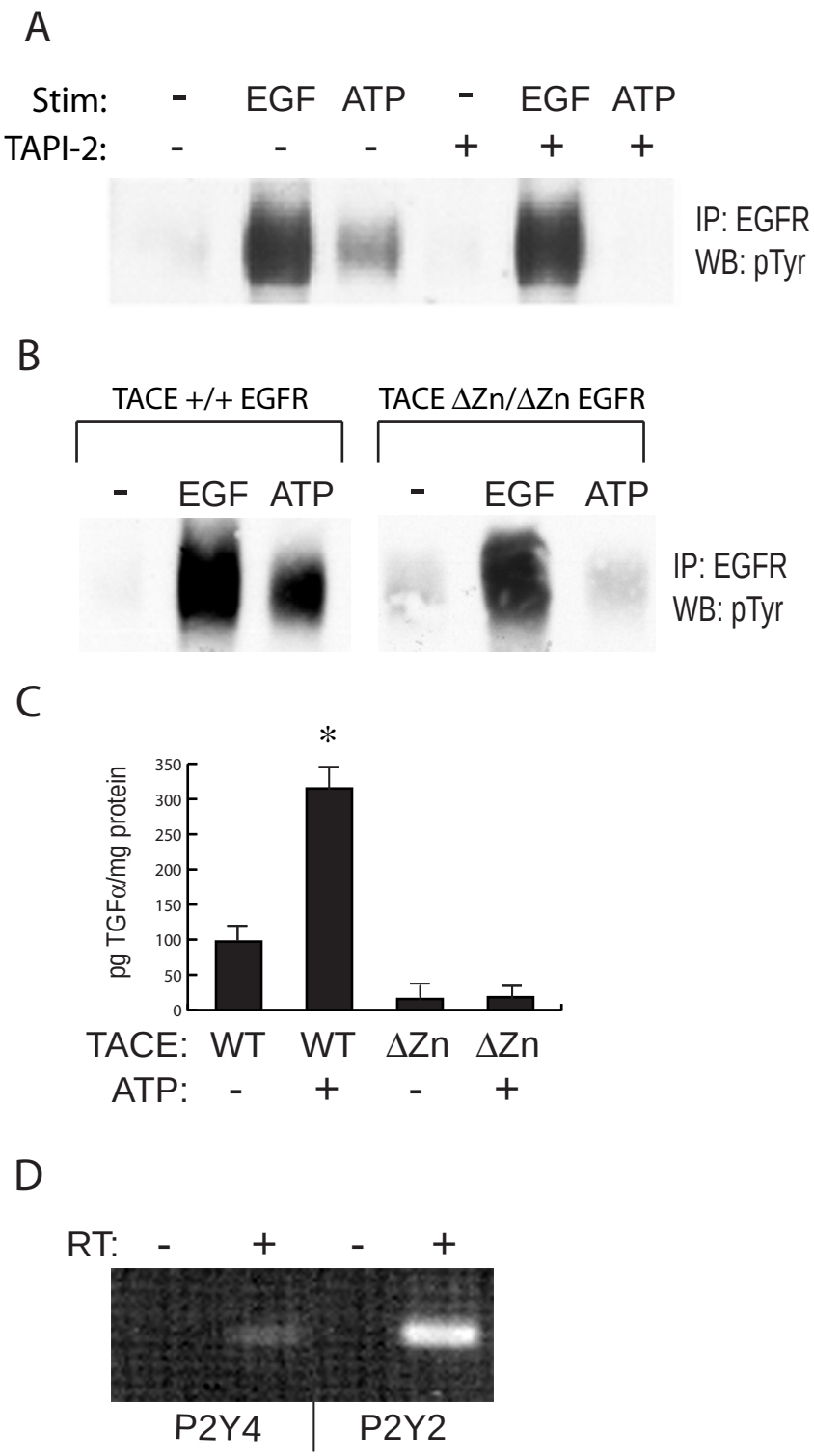

A
Stim: - EGF ATP - EGF ATP
TAPI-2: - - - + + +
IP: EGFR
WB: pTyr
B
TACE +/+ EGFR
TACE ΔZn/ΔZn EGFR
- EGF ATP - EGF ATP
IP: EGFR
WB: pTyr
C
pg TGFα/mg protein
350
300
250
200
150
100
50
0
*
TACE: WT WT ΔZn ΔZn
ATP: - + - +
D
RT: - + - +
P2Y4
P2Y2

Figure 2.3 ROS mediate ATP-induced TGF-α shedding. EC-4 cells were serum starved for 4 hours then pretreated with vehicle, or 5 mM NAC for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media and lysates were harvested and concentrated as described in Materials and Methods, and analyzed for TGF-α content by specific RIA. Data is presented as picograms of TGF-α in conditioned media per milligram of total lysate from three separate experiments with at least three replicates. *, $p < 0.001$ as compared to the stimulated control.

Figure 2.3

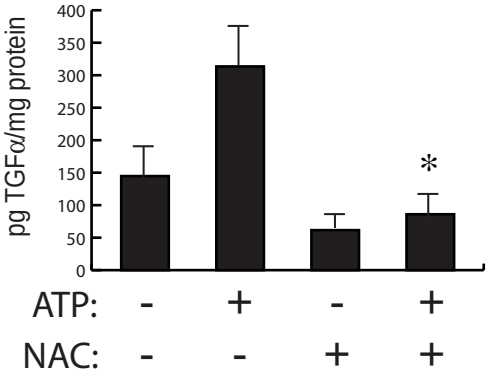


Figure 2.4 CHO cells demonstrate $P2Y_2$-TACE-TGF-α shedding. (A) Total RNA from CHO cells was subjected to first strand cDNA synthesis with, or without, reverse transcriptase and amplified over 28 cycles to detect the expression of $P2Y_2$ and $P2Y_4$ receptors. Comparable sized products are expected as seen in Figure 2.2D. (B) CHO-APT cells were serum starved for 4 hours and pretreated with vehicle, or 25 μM TAPI-2 for 30 minutes where indicated. Cells were then stimulated for 5 minutes with vehicle, 100 μM ATP, or 30 μM hydrogen peroxide (HP). Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least three replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. *, $p < 0.001$ as compared to control. (C) CHO-APT cells were serum starved for 4 hours and pretreated with vehicle, or 5 mM NAC for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least 3 replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. *, $p < 0.001$ as compared to stimulated control. (D) Mutant M2 CHO-APT clones, which lack functional TACE, were serum starved for 4 hours. Cells were then stimulated for 5 minutes with vehicle, 100 μM ATP, or 10 μM of the calcium ionophore A23187 (A23). Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least 3 replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. (E) Wild type CHO-APT cells were serum starved for 4 hours and pretreated with vehicle, or 5 mM NAC for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, 100 μM ATP, or 10 μM A23187. Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least 3 replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. *, $p < 0.002$ as compared to A23187 stimulated control.

Figure 2.4

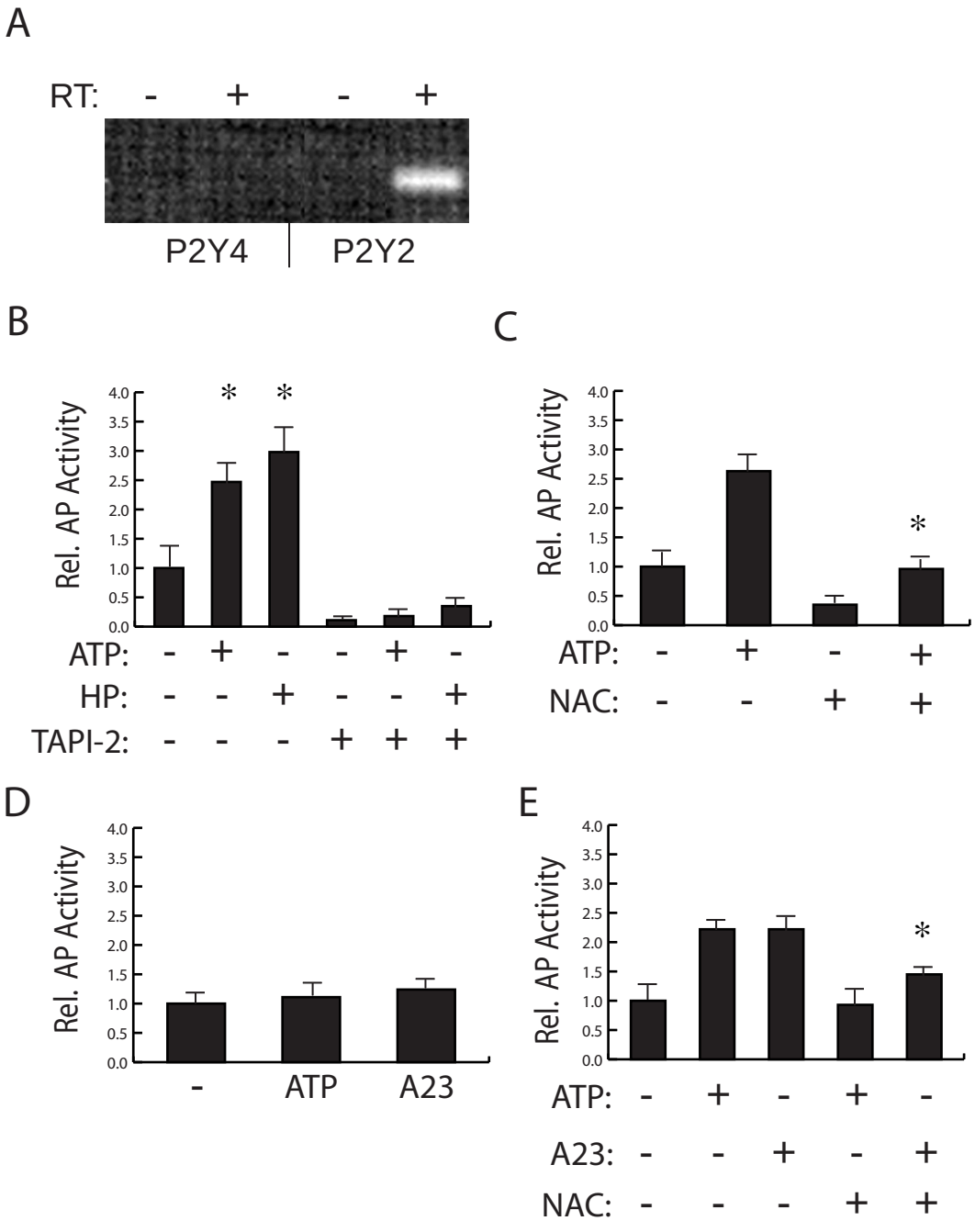
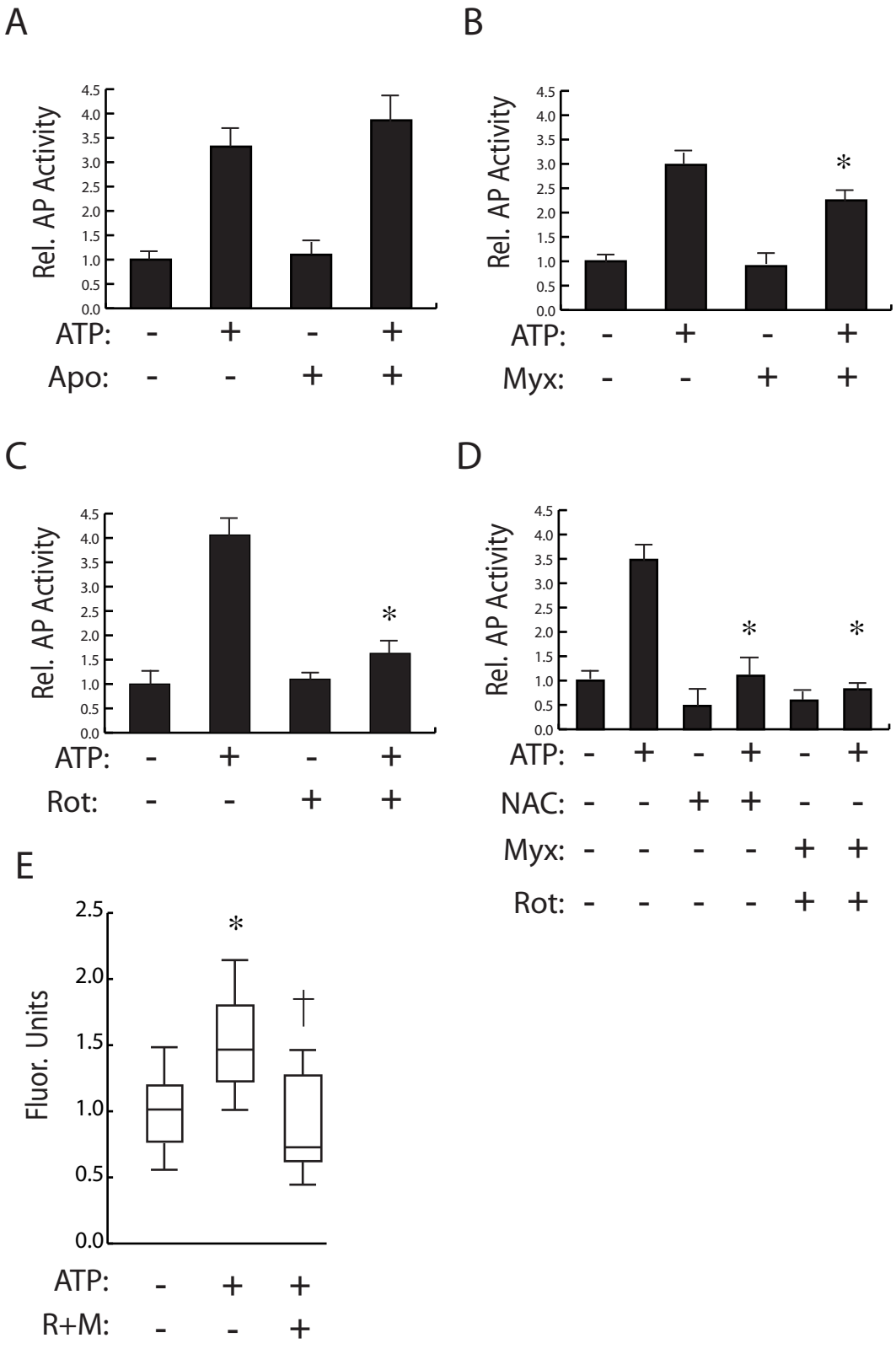

A
RT: - + - +
P2Y4
P2Y2
B
Rel. AP Activity
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
ATP: - + - - + -
HP: - - + - - +
TAPI-2: - - - + + +
C
Rel. AP Activity
*
ATP: - + - +
NAC: - - + +
D
Rel. AP Activity
- ATP A23
E
Rel. AP Activity
*
ATP: - + - + -
A23: - - + - +
NAC: - - - + +

Figure 2.5 Mitochondrially-derived ROS is required for ATP-induced TGF-α shedding. (A) CHO-APT cells were serum starved for 4 hours and pretreated with DMSO, or 1 mM apocynin for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least 3 replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. (B) CHO-APT cells were serum starved for 4 hours and pretreated with DMSO, or 1 μM myxothiazol for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least 3 replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. *, $p < 0.001$ as compared to stimulated control. (C) CHO-APT cells were serum starved for 4 hours and pretreated with DMSO, or 25 μM rotenone for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media was collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least 3 replicates. Alkaline phosphatase activity is presented relative to the unstimulated control. *, $p < 0.001$ as compared to stimulated control. (D) CHO-APT cells were serum starved for 4 hours and pretreated with DMSO, 5 mM NAC, or 1μM myxothiazol and 25 μM rotenone for 30 minutes. Cells were then stimulated for 5 minutes with vehicle, or 100 μM ATP. Media was collected and analyzed for alkaline phosphatase activity. All Alkaline Phosphatase assays are an average of three separate experiments with at least three replicates and presented relative to the unstimulated control samples. *, $p < 0.001$ as compared to stimulated control. (E) CHO cells were serum starved for 4 hours then pre-treated with vehicle, or 1 μM myxothiazol and 25 μM rotenone for 30 minutes. During pre-treatment, cells were also loaded with 250 nM MitoTracker Red and treated as described in Materials and Methods. Cells were stimulated with vehicle, or 100 μM ATP. Data represents three separate experiments of at least 4 replicates relative to

the unstimulated control. *, $p < 0.03$ as compared to the unstimulated control. †, $p < 0.001$ as compared to the stimulated control.

Figure 2.5


A
Rel. AP Activity
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ATP: - + - +
Apo: - - + +
B
Rel. AP Activity
*
ATP: - + - +
Myx: - - + +
C
Rel. AP Activity
*
ATP: - + - +
Rot: - - + +
D
Rel. AP Activity
* *
ATP: - + - + - +
NAC: - - + + - -
Myx: - - - - + +
Rot: - - - - + +
E
Fluor. Units
2.5
2.0
1.5
1.0
0.5
0.0
*
†
ATP: - + +
R+M: - - +

Chapter 3

Discussion

The work presented here describes the characterization of a transactivation pathway consisting of purinergic G-protein coupled receptors (GPCRs) mediating the protease activity of TACE for TGF-α shedding and activation of EGFR signaling modules. This transactivation pathway offers a physiologically relevant model to examine the mechanism of TACE regulation of EGF-like growth factor shedding. I present evidence that mitochondrial ROS is a previously unrecognized source of ROS for the regulation of TACE-dependent P2Y-induced shedding. This pathway is also independent of the previously reported signaling intermediates Src, PKC, MAPK and intracellular calcium stores.

TACE mediates P2Y-stimulated TGF-α Shedding

EGF-like growth factor shedding by a zinc-dependent metalloprotease is necessary for GPCR-stimulated EGFR transactivation. Multiple metalloproteases have been implicated in this critical growth factor shedding step and specifically in TGF-α shedding (89,212,226). $P2Y_2$ receptors were recently shown to activate ADAM10 and TACE activity towards amyloid precursor protein in astrocytoma cells (197). To identify the metalloprotease responsible for P2Y-initiated TGF-α shedding and activation of EGFR, we tested TACE-deficient cells for their ability to transactivate EGFR following ATP stimulation. EGFR phosphorylation was detected after the addition of exogenous EGF, however without functional TACE, ATP-induced EGFR activation was not detected. Furthermore, when we directly assessed ATP-induced TGF-α shedding in TACE-deficient murine cells, almost all shedding was lost. We also demonstrated a requirement for TACE in TGF-α shedding from CHO cells using cells carrying point mutations in each TACE allele that disrupts either function, or folding (M2 cells) (201,202). We showed a complete loss of TGF-α shedding in response to ATP stimulation in M2 cells, yet when TACE was transiently expressed in the M2 cells, shedding was partially restored, though not to the same extent as in wild type cells due to expression of TACE in a small population of cells (data not shown). These results

demonstrate TACE as the major protease required for TGF-α shedding after P2Y receptor stimulation and confirm previous results from our lab, and many others, that TACE is the major sheddase for TGF-α, along with the other EGFR ligands, AR, HB-EGF, EPI and EPR (33,34,47,89,93,109-111,128). The loss of EGFR transactivation in cells inhibited with metalloprotease inhibitor, or lacking functional TACE, also underscores the necessity of proteolytic processing of EGFR ligands in this pathway.

P2Y Family Receptors Transactivate EGFR

The P2Y family of receptors binds extracellular nucleotides as hormone ligands; these nucleotides are released from millimolar intracellular stores in response to stress and stimulation (205). The $P2Y_2$ and $P2Y_4$ receptors specifically respond to ATP, or UTP, while other P2Y receptors utilize GTP, ADP and/or UTP. $P2Y_2$ and $P2Y_4$ receptors are expressed in muscle (smooth muscle cells), heart (cardiomyocytes), brain (astrocytes and astrocytomas), spleen and lung, among other tissues (205). We show by semi-quantatative RT-PCR that $P2Y_2$ receptors have elevated expression over $P2Y_4$ receptors in murine fibroblast cells and confirm that CHO cells also express $P2Y_2$, but lack $P2Y_4$ expression; given the presence of an intact transactivation loop in both cell types, this suggests a prominent role for $P2Y_2$ receptors as the initiator of ATP-induced EGFR transactivation. However, we cannot exclude a contribution by low-abundance $P2Y_4$ receptors in the mouse cell line.

Our work reveals an alternative model to the Src-dependent intracellular signaling in P2Y-stimulated EGFR transactivation pathways. $P2Y_2$ receptors have been established as transactivators of EGFR in rat fibroblasts, PC12 and astrocytoma cells, activating the MAPK pathways through EGFR-dependent signaling (206,207,225). $P2Y_2$–induced transactivation was shown to require both, intracellular calcium and PKC-dependent activation of the kinase CADTK (RAFTK, PYK2) for activation of EGFR in PC12 cells (206,207). $P2Y_2$ –dependent

transactivation was later shown to involve Src-dependent association between EGFR and $P2Y_2$ receptors in astrocytoma cells (225). Src can also directly phosphorylate EGFR and EGFR-binding proteins Shc and Grb2 (159,227), suggesting a ligand-independent activation model. We examined the role of the triple membrane passing model for P2Y transactivation of EGFR (114). This model was first described by Prenzel et al. (117) and utilizes metalloprotease activity to activate EGFR in a ligand dependent manner. Indeed, growth factor shedding was increased in response to ATP and protease activity was required for EGFR activation. Due to the previous emphasis on Src as an intermediate in GPCR-EGFR transactivation pathways, we used inhibitors to demonstrate that this pathway was independent of Src family tyrosine kinases. The existence of two distinct pathways for EGFR transactivation, one independent and one dependent on Src activity, could allow for increased signal module specificity. This idea is supported by the report that CADTK and Src combine to transactivate EGFR by direct phosphorylation following GPCR stimulation, but are not required for activation of MAPK pathways upon EGFR activation by LPA in mouse embryonic fibroblasts (163). Taken together, these reports, and our work, suggest that EGF-like growth factor binding could be key to activating EGFR-dependent MAPK pathways following GPCR stimulation complementing Src-activated EGFR downstream pathways.

Activation of TACE-mediated TGF-α Shedding Requires Mitochondrial ROS

Besides Src tyrosine kinases, many other distinct signaling pathways and molecules have been implicated in the activation of TACE-dependent shedding (120). Since transactivation allows for a physiologically relevant and rapid mechanism to study the activation of TACE, we tested previously identified signaling pathways in this system. We were surprised to discover that ATP-stimulated TACE activity is not regulated by PKC, MAPK, or intracellular calcium release. Our results are similar to those found for ATP-stimulated, TACE-

dependent APP shedding, which also did not rely on PKC, MAPK, or intracellular calcium (197). However, previous results, from numerous reports in distinct cell types with each of these signaling molecules, suggest that there are multiple signaling schemes that lead to TACE-dependent shedding, based upon cell type and cell treatment.

Calcium is a common second messenger utilized by GPCRs (224). Calcium can be found downstream of ATP signaling and is known as a key component for EGFR transactivation and in shedding activation schemes (123,174,199,200,204). We have used the calcium ionophore A23187, which couples extracellular calcium to transport the ions through the membrane, independent of channels or pores, to assess the role of extracellular calcium in activation of shedding. Stimulation with this reagent in wild type CHO cells led to a significant increase in TGF-α shedding similar to results seen with shedding of HB-EGF (123). Using CHO cells lacking functional TACE (M2), A23187-induced shedding was markedly decreased compared to wild type cells, but still displayed a minimal increase of TGF-α shedding over control treated M2 cells. This is consistent with a report that a different ionophore can stimulate TGF-α, HB-EGF and AR shedding in the absence of TACE activity (89). Based on these results and residual shedding from mouse cells lacking TACE, we conclude the major sheddase for TGF-α must be TACE, but it is not the only protease capable of cleaving TGF-α. The redundant activity could be important for tissues where TACE is not highly expressed, or cannot function due to disease or mutations.

Reactive oxygen species (ROS) gained increasing attention for their important role as a second messenger and in TACE activation. ROS can be generated in many cell types in response to a host of stimuli including cytokines, growth factors and cellular stress, and are then utilized as signaling intermediates in redox sensitive pathways (213). Exogenous addition of the ROS, hydrogen peroxide, which mimics oxidative stress, has been shown to stimulate shedding of TGF-α, AR, HB-EGF and EGFR activation (100,152,179,181). ROS have also been identified as intermediates of GPCR-induced shedding by angiotensin II,

endothelin-1, phenylepherine, lipopolysaccharide and lysophosphatidylcholine (179,180,185,198,222). P2Y receptors have also been tied to ROS production in eosinophils, prostate and thyroid tumor cells (199,200,214). We examined the possibility that ROS may therefore mediate TGF-α shedding in response to ATP in our system. Using the ROS scavenger NAC, we show that induced shedding is completely dependent on the presence of ROS in both murine and CHO cells. This evidence supports ROS signaling as a requirement for induced, TACE-dependent EGF-like growth factor shedding.

To further examine the source of ROS acting in TACE activation, we tested the inhibitor apocynin in ATP stimulated cells. The role of the NADPH oxidase (Nox) complex is a low level homologue of the phagocytic oxidase and one of the major sources of ROS in non-phagocytic cells. Using the inhibitor apocynin, which blocks formation of Nox complex, we found that over a range of concentrations from 30 μM to 1 mM, TGF-α shedding was unaffected ruling out the function of Nox in this pathway. Thus, we have identified a requirement for a source of ROS, distinct from Nox, for P2Y-induced TGF-α shedding.

A second major source of ROS that could be utilized as a second messenger is superoxide anion formation as a by-product of oxidative phosphorylation in the mitochondria (216). Mitochondrial ROS signaling has been implicated in cellular metabolism through JNK pathways (228) and linked to cardiovascular remodeling and disease, aging and cell fate determinations (216). Mitochondrial ROS are also implicated in GPCR-dependent lysophosphatidylcholine activation of MAPK pathways (223). It is tempting to presume this GPCR-stimulated activation of MAPK pathways could depend on EGFR transactivation, especially when considering that mitochondrial ROS can stimulate EGFR activation. Exogenous hydrogen peroxide added to COS-7 cells led to EGFR activation that was further dependent on mitochondrially-derived ROS (217). To test for the possible role of mitochondrially-derived ROS in our system, we used two separate inhibitors of the electron transport chain to block ROS production. Myxothiazol, which inhibits Coenzyme Q:

cytochrome c oxidoreductase (Complex III), and rotenone, which inhibits NADH dehydrogenase (Complex I); each partially inhibited ATP-induced TGF-α shedding when used separately. When added together the inhibitors completely blocked induced TGF-α shedding, matching the results with the non-specific scavenger NAC. Furthermore, we directly measured an increase in ROS within mitochondria, which was also inhibited by treatment with the combination of mitochondrial protein complex inhibitors. We offer evidence that P2Y receptors couple to mitochondrial ROS as a signaling intermediate for regulation of P2Y-induced EGFR transactivation and report for the first time that mitochondrial derived ROS regulates GPCR-induced growth factor shedding.

Our findings, implicating the mitochondria as a source of ROS following GPCR signaling, differ from previously reported GPCR-induced shedding results. Stimulation with angiotensin II, endothelin-1, lipopolysaccharide, lysophosphatidylcholine and phenylephrine utilized Nox dependent ROS production for HB-EGF and TGF-α shedding (179,180,185,204,222,229) based on experiments that either knocked down expression of Nox subunits. For P2Y-induced ROS production, use of the inhibitor diphenylene iodonium (DPI) implicated the cytoplasmic Nox complex (199,200). DPI acts by binding flavoproteins to inhibit ROS production such as the flavoprotein cytochrome b558 in the cytoplasmic Nox complex. However, DPI may also inhibit the NADP dehydrogenase complex of the mitochondrial electron transport chain at low concentrations, so DPI inhibition is not limited to Nox-mediated signaling (230). It is also possible that Nox may complement mitochondrial ROS in activation of shedding depending on the intended signal.

The Mechanism of ROS Signaling for the Activation of TACE-Dependent Shedding

The question remains as to how GPCR activation can induce increases in ROS, especially from the mitochondria. The Nox complex is dependent on Rac-1 phosphorylation of several cytoplasmic subunits prior to complex aggregation, which allows for known GPCR signaling

pathways to link to Nox through Rac-1 kinase activity (187). ROS production has been tied to Src, PKC and calcium signaling in certain cell systems, similar to their roles in EGFR transactivation (179,186,199,200). Despite their demonstrated role in alternative transactivation pathways, we have ruled out a role for Src, PKC and intracellular calcium stores as regulators of P2Y stimulated shedding and presumably as inducers of ROS for the same purpose. However, our findings that calcium ionophore induced shedding depends on ROS suggests that high concentrations of extracellular calcium influx may stimulate mitochondrial ROS production. Mitochondrially-derived ROS has previously been shown to respond to changes in intracellular calcium concentrations induced by stimuli (231). However, we also measured residual TGF-α shedding that was independent of ROS, pointing to a distinct pathway for extracellular calcium-induced shedding. These collective observations hint at the complexity of regulating stimulated TACE-dependent EGF-like growth factor shedding.

The underlying mechanism for ROS-dependent, TACE activation is also still undergoing investigation, though potential methods have been proposed. ROS are known to mediate protein kinase and phosphatase activity through oxidation of thiol groups (198). TACE is cytoplasmically phosphorylated in response to growth factors, serum stimulation and PMA treatment, on both serine and threonine residues (86,87,232). Our work has also shown phosphorylation of TACE that is dependent on the presence of the cytoplasmic domain, but is independent of ATP stimulation. Phospho-amino acid analysis revealed elevated serine phosphorylation following ATP treatment compared to tyrosine, or threonine, suggesting ATP may alter the residues that are modified following stimulation if not the overall level of phosphorylation of the protein. Erk kinase has been reported to phosphorylate TACE on threonine (86,232), while Phosphatidylinositol-Dependent Kinase-1 (PDK1) has also been shown to phosphorylate TACE (166,232). Both of these kinases require an activating phosphorylation event and thus, could be involved in pathways regulated by ROS.

However, the cytoplasmic domain of TACE may not be required for regulation of TACE activity. Our work demonstrates that the ATP-induced increase in shedding in TACE deficient CHO cells transfected with TACE lacking the cytoplasmic domain matches shedding in wild type transfected cells (data not shown). This is consistent with several other reports in which a TACE cytoplasmic deletion mutant lacking functional TACE displayed no changes in shedding in response to PMA and growth factor stimulation when transfected into murine cells (87,88). These results suggest that TACE may be inducibly phosphorylated, but requires a mechanism independent of the cytoplasmic domain for activation of the protease domain.

The prodomain of TACE, which is believed to be an inhibitor of metalloprotease function that must be removed prior to activation, could be the key. The prodomain inhibition has been shown to depend on coordination with the cysteine-rich domain to alter the native conformation (63). ROS can modify cysteine thiol groups by oxidation, thus potentially disrupting the prodomain masking of the catalytic zinc ion. Zhang et al. and colleagues (189) proposed this model after showing inhibition with a prodomain peptide that mimicked the native domain could be moderated by exogenous hydrogen peroxide in an *in vitro* cleavage assay. Thus, ROS, and especially hydrogen peroxide, which can freely diffuse through membranes, could act directly on the TACE protein to remove the self-imposed inhibition by post-translational modification of cysteine thiol moieties.

We have provided evidence for the P2Y-dependent transactivation of EGFR through TACE-mediated shedding of TGF-α. The P2Y family of receptors has been shown to function in cardiovascular remodeling and in protection of airway epithelial cells through mucin production (210,233). Both of these effects have been suggested to rely on EGFR signaling, revealing new potential therapeutic targets for the treatment of cardiovascular disease and cystic fibrosis. We have also presented novel evidence for the use of

mitochondrial ROS in regulating TACE-dependent shedding. The mounting evidence for ROS in TACE-mediated shedding events supports the hypothesis that ROS may be the point of convergence for activation of TACE by many distinct pathways. However, the discovery of a second source of ROS in shedding activation, independent of Nox and known transactivation intermediates PKC, Src and MAPK pathways foretells the complexity still confronting the comprehensive elucidation of TACE activation and EGFR regulation scheme(s). Alternatively, use of distinct pathways by individual GPCR pathways could also indicate necessary levels of signaling specificity that are required for *in vivo* ADAM function and substrate selection. Future work will focus on reconciling the usage of varying pathways and signaling molecules in different cell types and for different substrates. The differences between sources of ROS and the identification of the proximal signaling events required for induced ROS production will also be important, as these appear to be key intermediates in the activation of TACE-dependent shedding. These improvements in our understanding of TACE regulation mechanisms is the current focus of this field with the hope that each advance in our knowledge of TACE-dependent shedding brings us closer to understanding and development of novel treatments for any number of diseases.

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