

UNCLASSIFIED

AD NUMBER
ADB281601
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2001. Other requests shall be referred to US Army Medical Research and Materiel Comd., 504 Scott St., Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 8 Jan 2003

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-98-1-8193

TITLE: Engineering of Specific Tissue Inhibitors to Block ADAM
Type Metalloprotease-Mediated Mammary Neoplasia

PRINCIPAL INVESTIGATOR: Yibing Yan, Ph.D.
Zena Werb, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government
agencies only (proprietary information, Jul 01). Other requests
for this document shall be referred to U.S. Army Medical Research
and Materiel Command, 504 Scott Street, Fort Detrick, Maryland
21702-5012.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20020814 207

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8193
Organization: University of California, San Francisco

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

K. Dale Jr.

7/15/02

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 98 - 30 Jun 01)	
4. TITLE AND SUBTITLE Engineering of Specific Tissue Inhibitors to Block ADAM Type Metalloprotease-Mediated Mammary Neoplasia		5. FUNDING NUMBERS DAMD17-98-1-8193	
6. AUTHOR(S) Yibing Yan, Ph.D. Zena Werb, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, San Francisco San Francisco, California 94143-0962 E-mail:		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Communication between different signaling pathways enables cells to coordinate the responses to diverse environmental signals. Activation of the transmembrane growth factor precursor plays a critical role in this communication and often involves in metalloproteases-mediated proteolysis. Stimulation of G protein-coupled receptors (GPCR) transactivates the epidermal growth factor receptors (EGFR), which occurs via a metalloprotease-dependent cleavage of heparin-binding epidermal growth factor (HB-EGF). However, the metalloprotease mediating the transactivation remains elusive. We show that the integral membrane metalloprotease Kuzbanian (KUZ, ADAM10), which controls Notch signaling by cleaving Notch and its ligand Delta in <i>Drosophila</i> , stimulates GPCR transactivation of EGFR. Upon stimulation of the bombesin receptors, KUZ increases the docking and activation of adaptors SHC and Gab1 on the EGFR, and activation of Ras and Erk. In contrast, transfection of a protease-domain deleted KUZ (K \cdot MP) or blocking endogenous KUZ by morpholino antisense oligonucleotides suppresses the transactivation. The effect of KUZ on shedding of HB-EGF and consequent transactivation of the EGFR depends on its metalloprotease activity. GPCR activation enhances the association of KUZ and its substrate HB-EGF with tetraspanin CD9. Thus KUZ regulates the relay between GPCR and EGFR signaling pathways.			
14. SUBJECT TERMS metalloprotease, membrane-type inhibitors, neoplastic growth			15. NUMBER OF PAGES 31
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments	24
Reportable Outcomes	24
Conclusions	24
References	25
Appendices.....	27

Introduction

Cells sense diverse extracellular signals with different types of receptors on cell surface, and transduce these signals into cells through distinct signal pathways. But there is also significant crosstalk between the signaling pathways. In many cases, crosstalk occurs at the level of sharing components in the pathway, such as MAP kinases and other intracellular kinases. Crosstalk also occurs directly between membrane receptors; for example, stimulation of G protein-coupled receptors (GPCR) leads to activation of epidermal growth factor receptors (EGFR) (Carpenter, 1999; Daub et al., 1996; Pierce et al., 2001a). This transactivation of EGFR by GPCR occurs via a release of heparin-binding epidermal growth factor (HB-EGF) which is blocked by metalloprotease inhibitor (Prenzel et al., 1999).

Metalloproteases regulate cell behavior by modifying both the macro- and microenvironment of cells during the normal growth and development (Werb, 1997; Werb and Yan, 1998). Misregulation of metalloprotease activities contributes to many pathological processes, including tumorigenesis (Sternlicht et al., 1999). The integral membrane metalloproteases with a disintegrin domain (ADAMs) cleave various membrane-bound proteins, including ligands, receptors and ligand-receptor complexes (Black and White, 1998; Blobel, 2000). Among the well studied of ADAMs are TNF- α -converting enzyme (TACE, ADAM17), which not only cleaves TNF- α but also converts pro-TGF α precursors to active TGF α (Peschon et al., 1998), and Kuzbanian (ADAM10), which is a key regulator of Notch signaling pathways in *Drosophila* because it cleaves Notch receptor and its ligand Delta (Pan and Rubin, 1997; Qi et al., 1999). Thus, proteolysis by ADAMs can change the active state of surface molecular complexes, affecting the signaling pathways inside cells.

Metalloprotease-mediated release of EGFR ligands not only contributes to normal development process, as revealed with the metalloprotease-deficient mice, but also plays important roles in abnormal growth of tumor cells. Inhibition of metalloprotease-mediated the EGFR ligand shedding reduces the proliferation and migration of breast cancer cells (Dong et al., 1999). Moreover, so-called autocrine growth of tumor cells is often EGFR-dependent, and GPCR ligands such as bombesin also act as growth factors. In PC3 prostate cancer cells, the metalloprotease inhibitor BB94 inhibits bombesin and TPA -induced EGFR transactivation (Prenzel et al., 1999). Thus metalloproteases are an integral part of this EGFR-dependent autocrine growth pathway. However, the understanding of pathway from GPCR to EGFR activation has been limited by the lack of knowledge about the metalloprotease involved. Several

metalloproteases are capable of cleaving EGFR ligands, but there is no evidence that they support the EGFR transactivation by GPCR. For example, ADAM9 cleaves the HB-EGF when PKC δ is activated, but neither the wildtype, nor the dominant-negative ADAM9, affects the EGFR transactivation (Izumi et al., 1998; Prenzel et al., 1999). Here we report that the metalloprotease KUZ (ADAM10), described initially as the regulator of Notch signaling, supports the GPCR – induced transactivation of EGFR signaling pathway.

Body

To identify the metalloprotease that mediates the transactivation of EGFR, we transfected COS7 cells with mouse KUZ and other metalloprotease-disintegrins (ADAMs), and examined the EGFR activation after activating the transfected cells with GPCR ligands. Stimulation of COS7 cells with the GPCR ligands lysophosphatidic acid (LPA) and bombesin significantly increased EGFR activation, confirming that, indeed, there were strong interactions between the GPCR and EGFR signaling pathways (Fig. 1, A and B). Transfection of KUZ further augmented GPCR-induced EGFR phosphorylation. However, transfection of evolutionarily related ADAMs 9, 15, and TACE (ADAM17) did not elevate the EGFR activation by GPCR in COS7 cells (data not shown). These results suggest that KUZ enhances EGFR transactivation by GPCR.

In contrast to transfection of wild type KUZ, transfection of COS7 cells with a KUZ mutant, which lacks the metalloprotease domain ($K\Delta MP$) and acts as a dominant-negative mutation in *Drosophila*, reduced the GPCR-induced EGFR phosphorylation. In fact, after bombesin treatment, the level of EGFR phosphorylation in the $K\Delta MP$ -transfected cells was lower than that in cells transfected with a vector plasmid, indicating that $K\Delta MP$ blocks the endogenous metalloprotease-mediated EGFR transactivation in COS7 cells. In cells transfected with KUZ, the phosphorylation of EGFR in the presence of GPCR ligand was effectively inhibited by CRM197, an inactivated diphtheria toxin that specifically binds to HB-EGF, and the EGFR kinase inhibitor AG1478, confirming that effect of KUZ on increased EGFR phosphorylation is due to activation of EGFR ligand HB-EGF by GPCR signaling (Fig. 1 C). Thus KUZ appears to function as an intermediate between the activated GPCR and EGFR phosphorylation.

To access the effects of KUZ on signaling pathways downstream of transactivated EGFR, we investigated the docking and phosphorylation of proteins known to associate with phosphorylated EGFR. Transfection of KUZ increased, and transfection of $K\Delta MP$ decreased the bombesin-dependent phosphorylation of all three isoforms of the adapter protein SHC (Fig. 2 A) and another adapter Gab1 (Fig. 2 B). KUZ, but not $K\Delta MP$, increased amount of phosphorylated EGFR co-precipitated with

SHC upon bombesin treatment (data not shown), indicating that KUZ facilitated the direct recruitment of SHC by activated EGFR. Thus KUZ increases activation of signaling components docked on activated EGFR upon stimulation of GPCR.

GPCRs such as bombesin receptor activate growth signaling through both Ras-dependent and independent pathways. To test whether KUZ-mediated transactivation of EGFR is an integral part of the mechanism that GPCR activates Ras pathway, we next examined the effect of KUZ on the transactivation of Ras, which links the activated EGFR to MAP kinase activation. Precipitation of activated Ras from the COS7 cell lysates with the fusion protein containing the Ras-binding domain of Raf1 showed that bombesin treatment significantly increased Ras activation (Fig. 2 C). The expression of KUZ, but not Δ KMP further elevated the increase of activated Ras. The results demonstrate that the metalloprotease KUZ contributes to the GPCR activation of EGFR, which leads to activation of the Ras-dependent signaling pathway.

Antagonists of bombesin inhibit tumor growth in nude mice seeded with PC3 cells and their effects are EGFR-dependent, suggesting EGFR is involved in bombesin-induced tumor growth (Heasley, 2001; Plonowski et al., 2000). Interestingly, the expression of ADAM10, the human homologue of KUZ significantly increases with androgen treatment in PC3 cells, while TACE expression is inhibited (McCulloch et al., 2000). To assess whether endogenous KUZ was involved in GPCR transactivation of EGFR signaling pathway, we then investigated the effect of KUZ on activation of MAP kinase (Erk1/2) in PC3 prostate cancer cells. Inhibition of EGFR kinase with AG1478 and neutralization of HB-EGF with CRM197 both reduced Erk1/2 phosphorylation by bombesin, suggesting that transactivation of EGFR is required, at least in part, for bombesin to activate Erk signaling in PC3 cells (Fig. 2 D).

To determine whether PC3 cells require KUZ for this transactivation, we used two methods to inhibit endogenous KUZ and tested the response of the cells to bombesin. First, transfecting Δ KMP suppressed transactivation of Erk1/2 by bombesin (Fig. 2 E). Second, introduction of the specific antisense morpholino oligonucleotide against ADAM10 inhibited the bombesin-mediated transactivation of Erk1/2, while a control oligonucleotide with same nucleotide sequence but in a reverse orientation did not (Fig. 2 F), nor did the anti-sense oligonucleotides against ADAMs 9, 15, and 17 (data not shown). These results show that blocking KUZ inhibits transactivation, implicating the endogenous KUZ is a critical player in GPCR transactivation of EGFR signaling.

The effect of KUZ on the EGFR transactivation required its metalloprotease activity. KUZ was involved in shedding of HB-EGF from COS7 cells. Stimulation of cells with bombesin increased the amount of shed HB-EGF in the medium. KUZ enhanced the ability of bombesin to release HB-EGF into medium (Fig. 3 A). In contrast, Δ KMP

blocked the release of HB-EGF. These data show that KUZ is required to shed HB-EGF. Inhibition of metalloprotease using a broad-spectrum inhibitor TAPI also inhibited bombesin-induced EGFR and SHC phosphorylation (Fig. 3 B). A single E385A mutation causes the loss of KUZ metalloprotease activity. When this mutant K(E-A) was introduced in COS7 cells, EGFR and SHC activation did not increase upon bombesin treatment (Fig. 3 C). Therefore, metalloprotease activity of KUZ is responsible for EGFR transactivation.

However, the ability to cleave HB-EGF alone is not sufficient to cause the transactivation; an additional signaling step is needed. Indeed, ADAM9 cleaves the HB-EGF under the regulation of PKC, yet neither the wild type nor the dominant-negative ADAM9 affects the GPCR-induced EGFR phosphorylation (Izumi et al., 1998; Prenzel et al., 1999). Stimulation of GPCR unleashes an array of signaling mediators that may activate KUZ to support the EGFR transactivation. Interestingly, only G_i and G_q -coupled GPCRs are associated with transactivation of EGFR and these GPCRs activate p44/42 Erk. Whether Erk directly regulates the cleavage of proTGF α (Fan and Derynck, 1999) and c-Met (Nath et al., 2000) in response to extracellular signals is unresolved, because dominant negative MEK1 does not block the transactivation resulted from HB-EGF shedding (Pierce et al., 2001b). However, inhibition of Src kinase has a profound effect on transactivation (Pierce et al., 2001b). It is conceivable that kinases, such as Src activated by GPCR, can phosphorylate cytoplasmic domain of ADAMs. The mechanism by which this phosphorylation activates metalloprotease remains elusive because the cytoplasmic domain of TACE is dispensable in PMA induced shedding (Reddy et al., 2000).

For shedding to occur *in cis* would require binding of KUZ to its substrate proHB-EGF. We therefore hypothesized that GPCR affects KUZ activity by regulating the formation of such a complex. Indeed, we found that the antibody to CD9, a tetraspan-transmembrane protein, precipitated not only HB-EGF, but also KUZ from cell lysates, suggesting they co-exist in the same molecular complex on the cell membrane (Fig. 4). Precipitation of CD9 co-precipitated with two forms of HB-EGF, the long form of about 24 kD and a short form of 18 kD. While CD9 binding of the long form appeared to be constitutive, the complex of short form with CD9 increased in the presence of transfected KUZ and with bombesin treatment. Therefore GPCR regulates KUZ-dependent activation of HB-EGF by promoting the binding of KUZ to the molecular complex centered around CD9. Interestingly, the potency of HB-EGF in stimulating cell growth correlates with its binding to CD9. CD9 also associates with ADAM2 and regulates interaction of ADAM2 with $\alpha 6\beta 1$ integrin (Chen et al., 1999; Shi et al., 2000).

We have shown that metalloprotease KUZ defines a control point in the relay between the GPCR and the EGFR signaling pathways. The identification of KUZ as the mediator of transactivation reveals an evolutionarily conserved role of KUZ in coordinating cell behavior. EGFR transactivation occurs not only in the same cell where both KUZ and EGFR reside, but also affects the neighboring cells that sense released HB-EGF. By relaying the extracellular signal from GPCR to EGFR, KUZ not only propagates signal laterally on the cell membrane, but also coordinates the response of surrounding cells with cleaved HB-EGF. Interestingly, two other well defined KUZ functions also involve precise control of signals between adjacent cells: in lateral inhibition where KUZ mediates cleavage of Notch and its ligand Delta (Pan and Rubin, 1997; Qi et al., 1999), and in interaction and cleavage of ephrin-A2 upon binding to its receptor Eph in the contacting cell (Hattori et al., 2000). In all cases, KUZ functions at the point of cell-cell contact. Elucidation of the mechanism of GPCR activation of KUZ increases our understanding of how metalloprotease-mediated shedding regulate signaling pathways.

Figures:

Figure 1 KUZ mediates GPCR transactivation of EGFR. (A) KUZ, but not K Δ MP, stimulates the LPA induced EGFR phosphorylation. (B) KUZ, but not K Δ MP, stimulates bombesin induced EGFR phosphorylation. EGFR was immunoprecipitated from GPCR stimulated or control cells transfected with KUZ, KDMP and vector DNA. Activation of EGFR was detected in immunoblots as phosphorylation of EGFR with anti-phosphotyrosine antibody 4G10 and amount of EGFR was detected with goat anti-EGFR antibody against EGFR. (C) bombesin-induced EGFR phosphorylation depends on HB-EGF and EGFR kinase. COS7 cells transfected with bombesin receptor and KUZ were pretreated for 20 min with CRM197 (lanes 3 and 4) or specific EGFR kinase inhibitor AG1487 (lanes 5 and 6) before treatment with Bombesin. EGFR was precipitated from cell lysates. EGFR activation was detected in immunoblots with anti-phosphotyrosine antibody 4G10 and amount of EGFR was detected with goat anti-EGFR antibody in the same blot.

Figure 2 KUZ stimulates, and blocking endogenous KUZ inhibits the transactivation of signaling pathway downstream of EGFR. (A) Activation of all three forms of SHC by GPCR is stimulated by transfecting KUZ, but not protease-deleted K Δ MP. (B) GPCR-induced Gab1 phosphorylation are stimulated by KUZ, but not K Δ MP. SHC or Gab1 in COS7 cells was immunoprecipitated with the polyclonal

antibody to SHC or Gab1. Activation of SHC or Gab1 was detected in immunoblots as phosphorylation of SHC or Gab1 with anti-phosphotyrosine antibody 4G10. (C) KUZ elevates GPCR-induced Ras activation. The activated Ras in cell lysates was selectively absorbed onto beads coated with RBD domain of Raf1. The amounts of activated Ras in the cells were detected in the immunoblot with anti-Ras antibody. The upper band non-specifically reactive to the anti-Ras antibody is shown as the loading control. (D) Bombesin induces Erk1/2 activation that is partly due to transactivation of EGFR and depends on HB-EGF release. PC3 cells were treated with CRM197 or AG1487 and then stimulated with bombesin. Erk1/2 activation in treated or control PC3 cells were detected with anti-phospho-Erk1/2 in immunoblots of cell lysates. (E) Transfecting K Δ MP suppresses Erk1/2 activation in PC3 cells. Bombesin treated or untreated cells PC3 cells transfected with vector or K Δ MP were lysed and active Erk1/2 were detected in immunoblots with anti-phospho-Erk1/2 antibody. (F) Bombesin-induced Erk1/2 activation can be inhibited by an anti-sense morpholino oligo against ADAM10. Morpholino anti-ADAM10 oligos (Anti-KUZ) and control oligos with the same base composition but in reverse order (Reverse-KUZ) were introduced into PC3 cells. Erk1/2 phosphorylation was detected in lysates of PC3 cells treated with bombesin. The endogenous ADAM10 in antisense oligo-treated cells was detected with polyclonal antibody to ADAM10 (KUZ).

Figure 3 Metalloprotease activity of KUZ is responsible for the GPCR

transactivation EGFR signaling pathway. (A) Transfection of wild-type KUZ, but not K Δ MP, stimulates the release of soluble HB-EGF into the medium. The HB-EGF in the medium of cells transfected with HA-tagged HB-EGF and KUZ or K Δ MP was analysed by collecting the heparin-binding proteins with heparin-agarose following stimulation with bombesin. The HA-tagged HB-EGF in collected protein pool was detected with anti-HA antibody 12CA5. (B) Metalloprotease inhibitor TAPI blocks bombesin-induced transactivation of EGFR and SHC. Cells pre-incubated either with TAPI or solvent DMSO before stimulation with bombesin. EGFR or SHC was immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody 4G10 to reveal the activated EGFR and SHC. The total EGFR and SHC were equivalent in each lane (not shown). (C) Catalytically-inactive KUZ (K(E-A)) does not support the EGFR and SHC transactivation. EGFR or SHC were precipitated from cleared cell lysates and immunoblotted with anti-phosphotyrosine antibody 4G10 to reveal the activated EGFR and SHC. The total EGFR and SHC were equivalent in each lane (not shown).

Figure 4 **GPCR activation regulates the formation of a complex of CD9 with KUZ and HB-EGF.** COS7 cells were transfected with Flag-tagged KUZ and HA-tagged HB-EGF. CD9 was precipitated from CHAPS lysates of control or bombesin-treated cells with a polyclonal anti-CD9 antibody. Proteins from immunoprecipitation were blotted with monoclonal antibodies against Flag epitope, HA epitope, and CD9, respectively

Figure 1

A

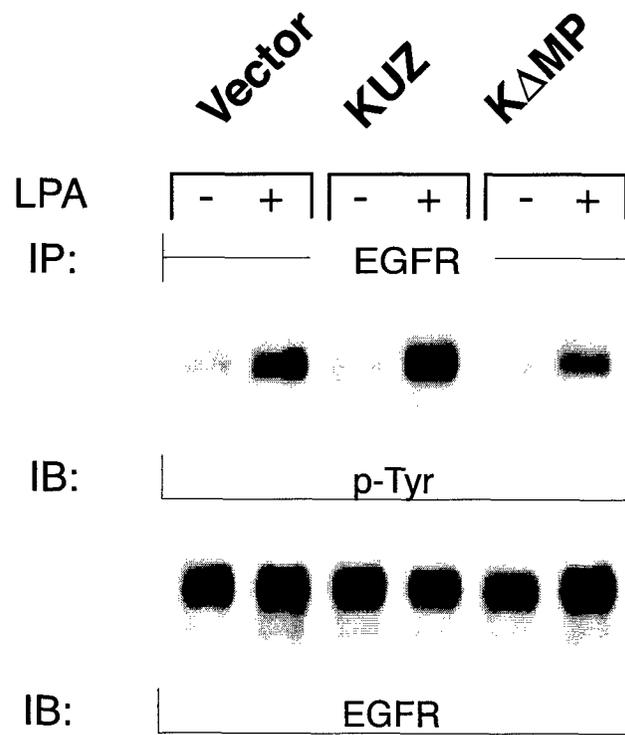


Figure 1

B

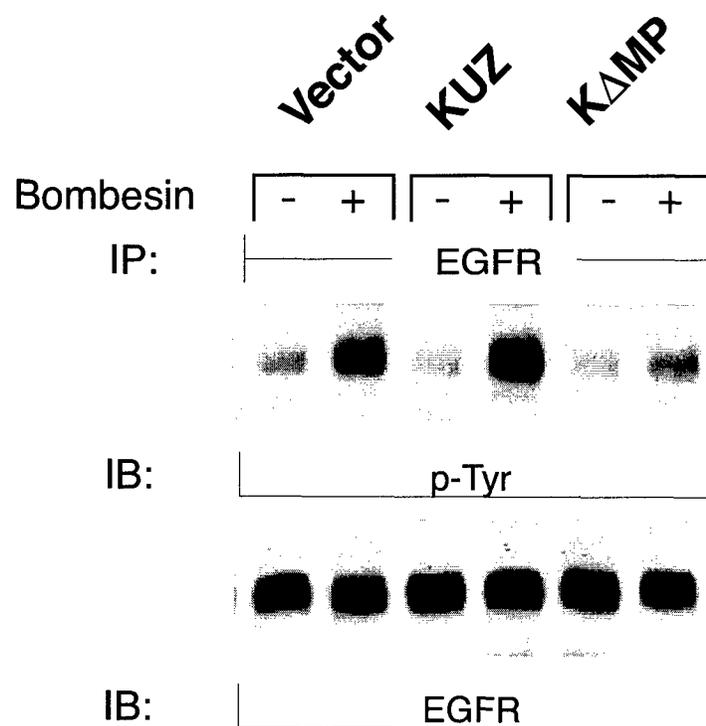


Figure 1

C

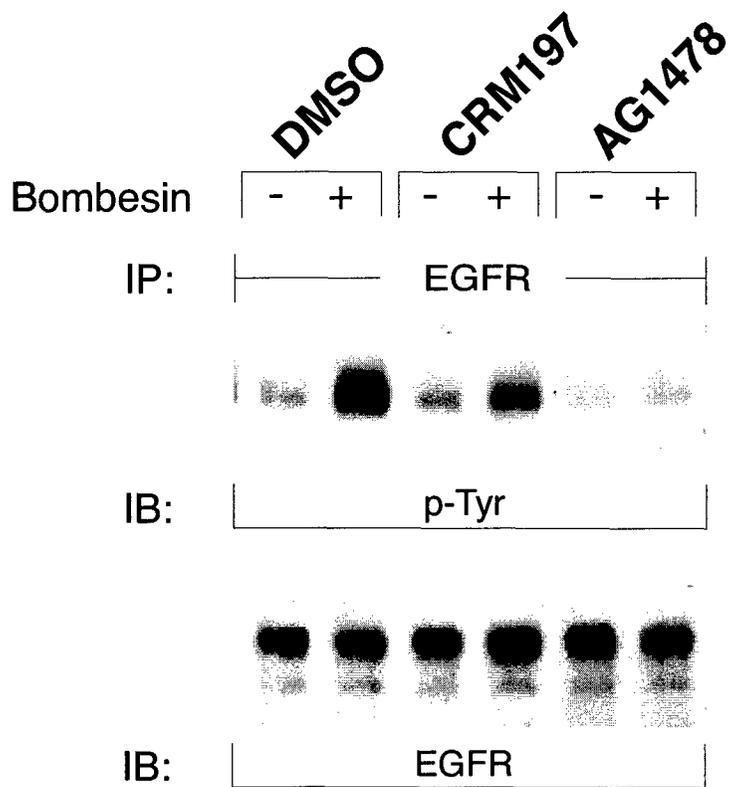


Figure 2

A

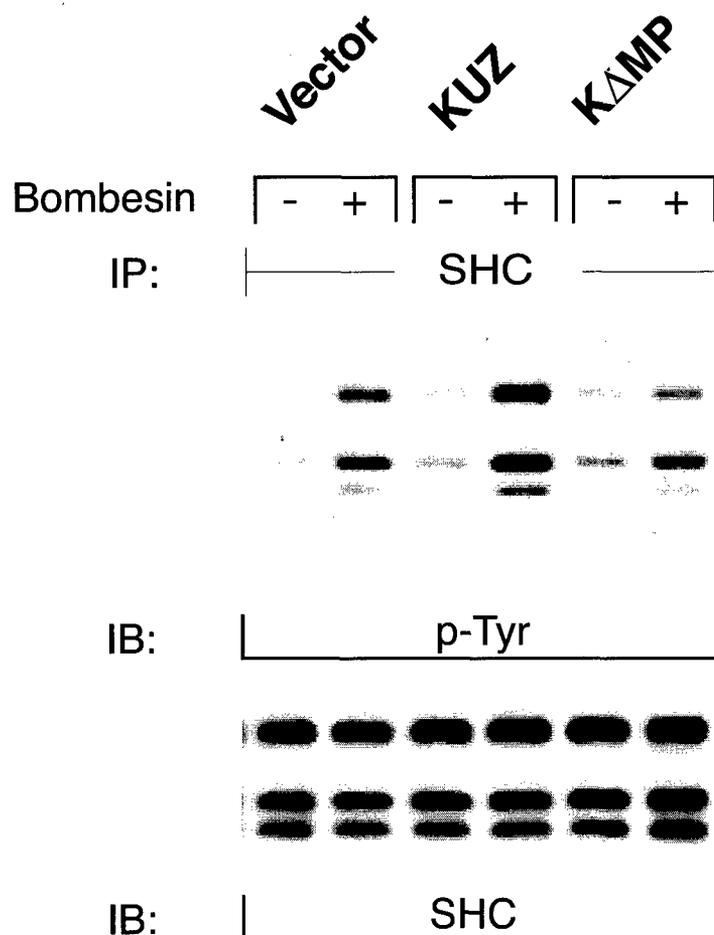


Figure 2

B

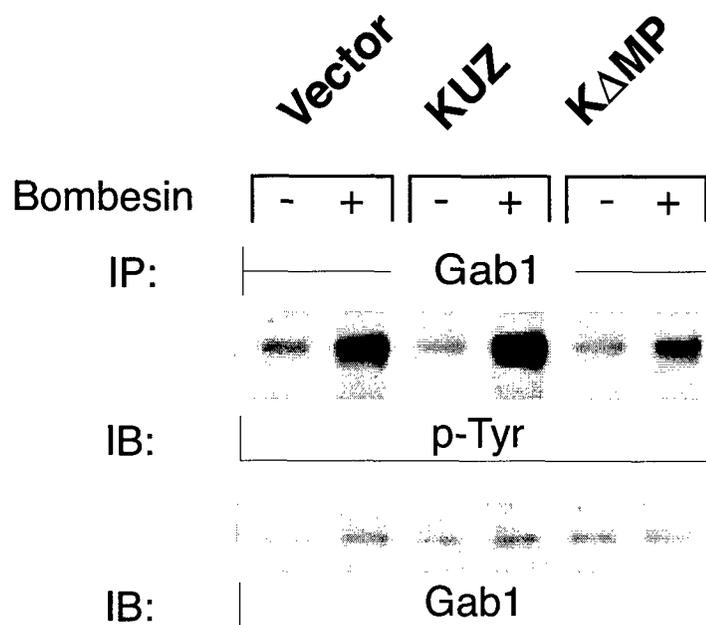


Figure 2

C

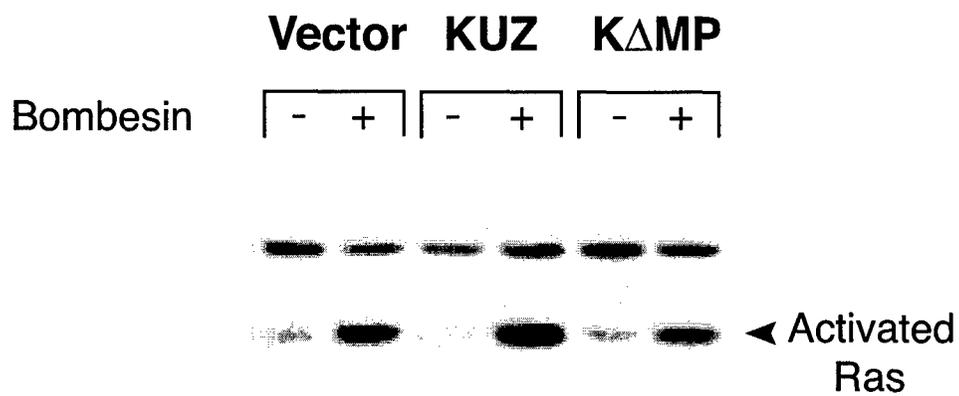


Figure 2

D

Bombesin	-	+	+	+
CRM197	-	-	+	-
AG1487	-	-	-	+



IB: pErk1/2



IB: Erk1/2

Figure 2

E

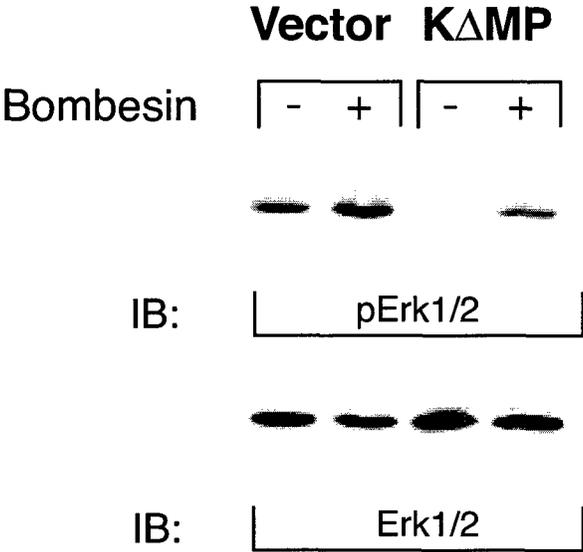


Figure 2

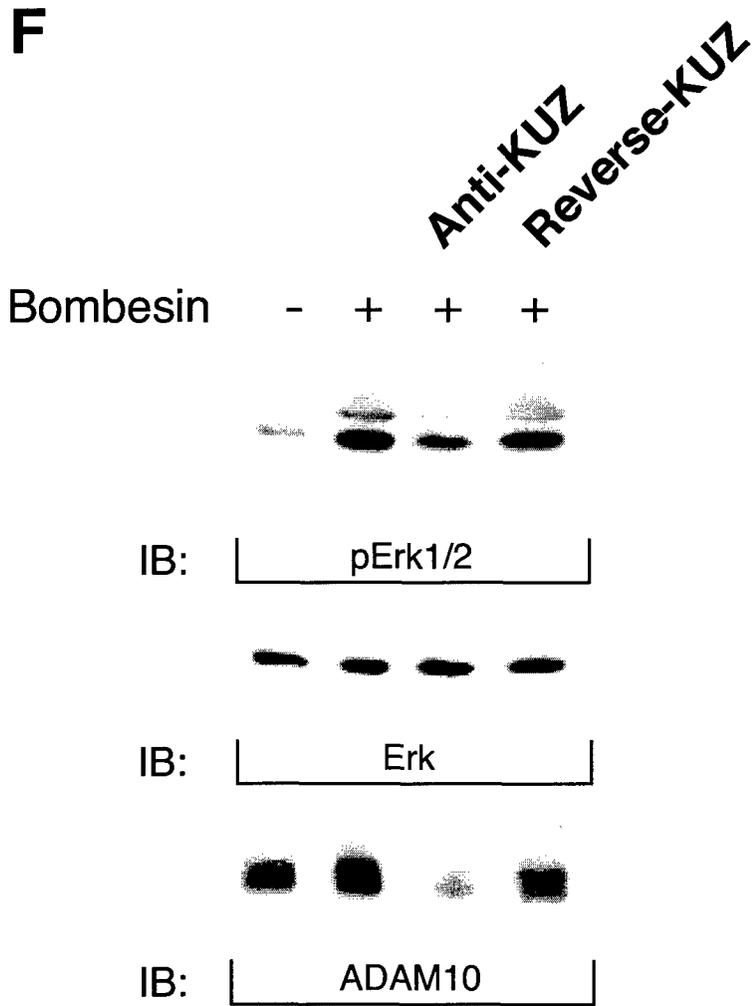


Figure 3

A

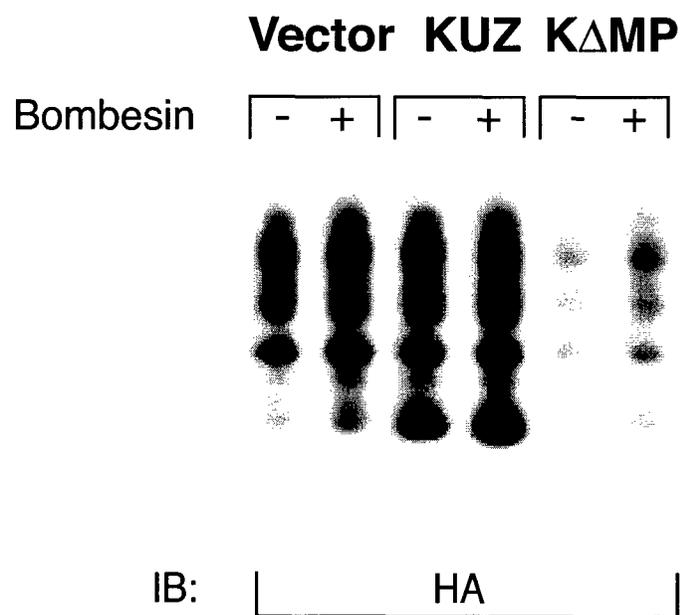


Figure 3

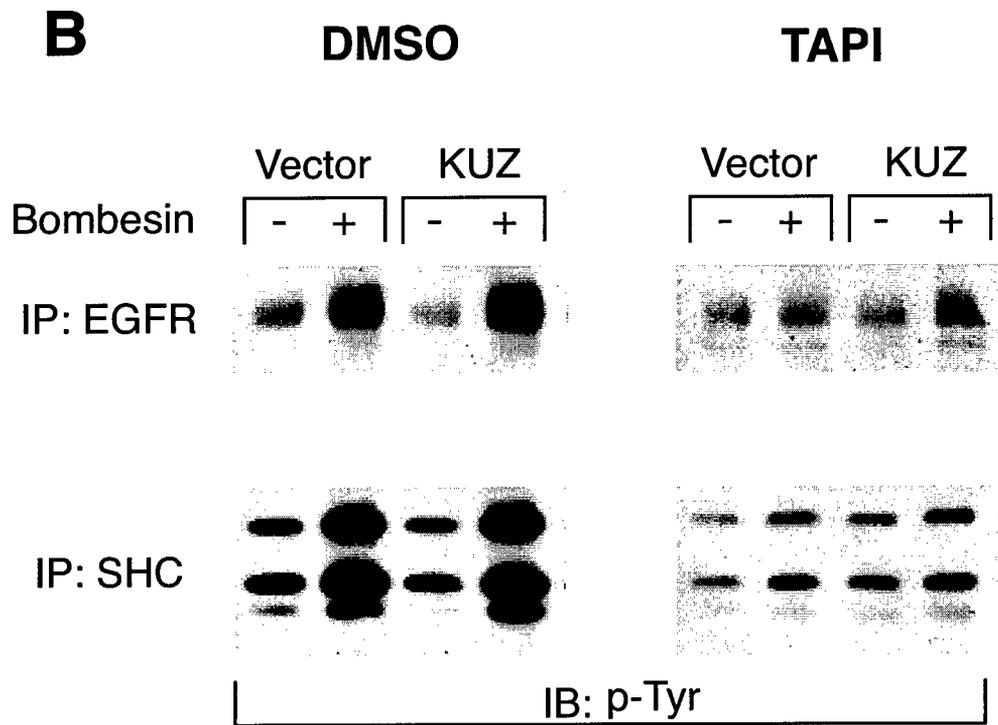


Figure 3

C

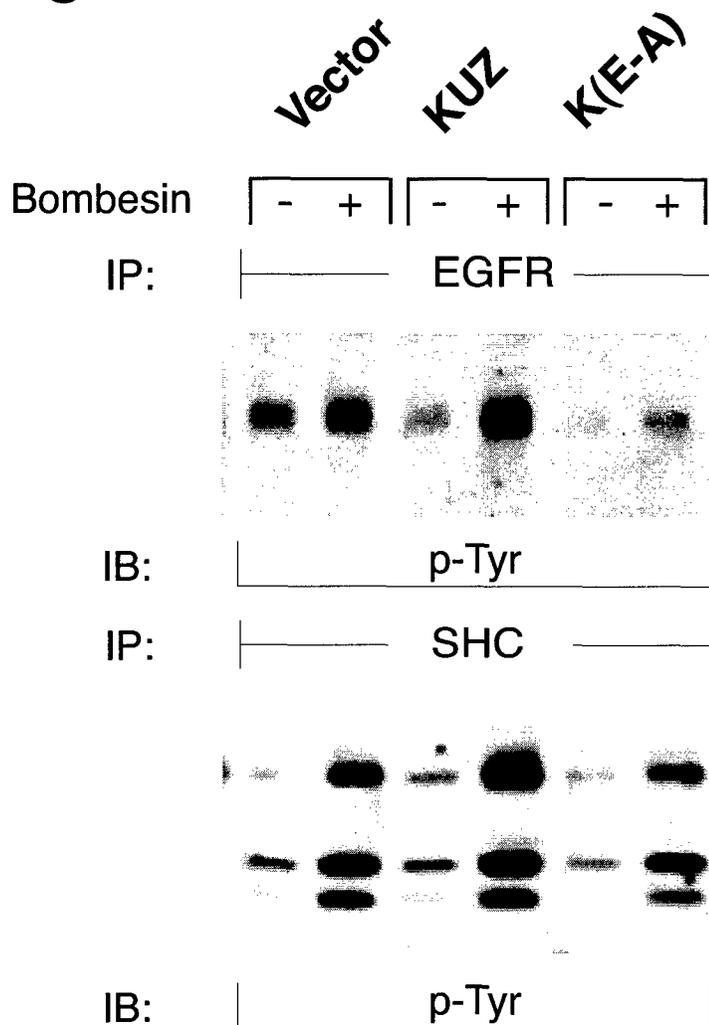
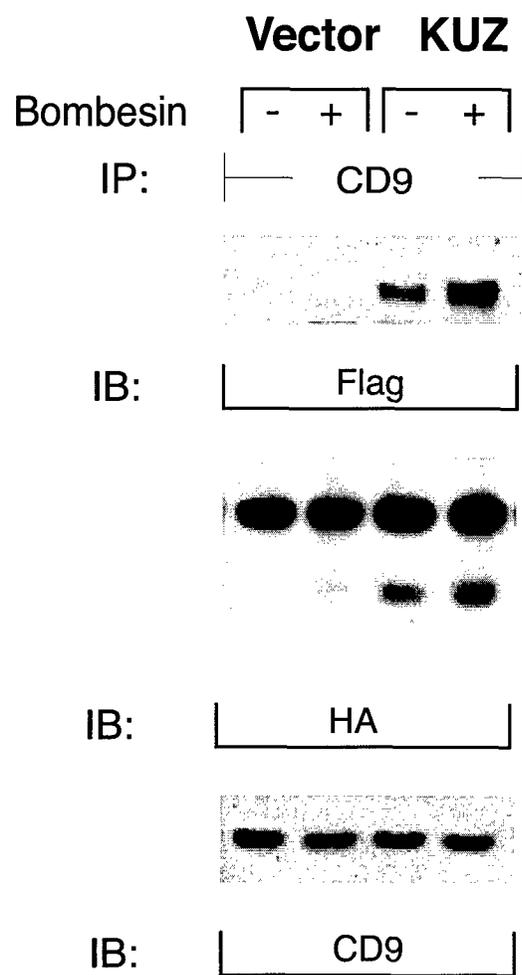


Figure 4



Key Research Accomplishments

Demonstrate that the integral membrane metalloprotease KUZBANIAN (ADAM10) mediates the signal relay between G-protein coupled receptors and EGF receptors, which may play a key role in sustaining the autocrine growth of late stage breast cancer cells.

Reportable Outcomes

Publication: Werb Z., and Yan Y. 1998. A cellular striptease act. *Science*. 282:1279-80.

Two selected oral presentation:

42th Annual Meeting of American Society of Cell Biology. 2001, San Francisco.
Gordon Research Conference in Matrix Metalloproteinases, 2001, Il Ciocco, Italy,
Also invited to give a speech at the Frontier of Metalloproteases and TIMPs
Research in Tokyo, Japan, Oct. 2001 (the event was cancelled due to the War
on Terrorism.)

Three transgenic mice lines:

TRE-DNK (dominant-negative KUZ),
TRE-TIMP3cs (TIMP3 inactive mutant), and
TRE-INT3 (constitutively active Notch4 mutant).

Conclusions

The proposed research seeks to validate the concept of blocking integral membrane proteases Kuzbanian to suppress neoplastic growth of mammary cells. We have shown that metalloprotease KUZ defines a control point in the relay between the GPCR and the EGFR signaling pathways. EGFR-mediated autocrine signaling plays a key role in neoplastic growth of breast cancer cells, where many GPCR ligands act as autocrine growth factors. By relaying the extracellular signal from GPCR to EGFR, KUZ not only propagates signal laterally on the cell membrane, but also coordinates the response of surrounding cells with cleaved HB-EGF. The identification of KUZ as the mediator of EGFR transactivation not only reveals an evolutionarily conserved role of KUZ in coordinating cell behavior but also unveils the key step in the autocrine growth of tumor cells through metalloprotease-mediated cleavage of EGFR ligand HB-EGF.

The results of complete research validates the integral membrane metalloprotease ADAM10 (KUZ) as a target for control the growth of unregulated breast cancer cells, and directly suggest that a specific inhibitor of ADAM10 (KUZ) may be valuable in suppressing late stage breast cancer growth.

References

- Black, R.A., and J.M. White. 1998. ADAMs: focus on the protease domain. *Curr Opin Cell Biol.* 10:654-9.
- Blobel, C.P. 2000. Remarkable roles of proteolysis on and beyond the cell surface. *Curr Opin Cell Biol.* 12:606-12.
- Carpenter, G. 1999. Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J Cell Biol.* 146:697-702.
- Chen, M.S., K.S. Tung, S.A. Coonrod, Y. Takahashi, D. Bigler, A. Chang, Y. Yamashita, P.W. Kincade, J.C. Herr, and J.M. White. 1999. Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization. *Proc Natl Acad Sci U S A.* 96:11830-5.
- Daub, H., F.U. Weiss, C. Wallasch, and A. Ullrich. 1996. Role of transactivation of the EGF receptor in signalling by G-protein- coupled receptors. *Nature.* 379:557-60.
- Dong, J., L.K. Opresko, P.J. Dempsey, D.A. Lauffenburger, R.J. Coffey, and H.S. Wiley. 1999. Metalloprotease-mediated ligand release regulates autocrine signaling through the epidermal growth factor receptor. *Proc Natl Acad Sci U S A.* 96:6235-40.
- Fan, H., and R. Derynck. 1999. Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *Embo J.* 18:6962-72.
- Hattori, M., M. Osterfield, and J.G. Flanagan. 2000. Regulated cleavage of a contact-mediated axon repellent. *Science.* 289:1360-5.
- Heasley, L.E. 2001. Autocrine and paracrine signaling through neuropeptide receptors in human cancer. *Oncogene.* 20:1563-9.
- Izumi, Y., M. Hirata, H. Hasuwa, R. Iwamoto, T. Umata, K. Miyado, Y. Tamai, T. Kurisaki, A. Sehara-Fujisawa, S. Ohno, and E. Mekada. 1998. A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *Embo J.* 17:7260-72.
- McCulloch, D.R., M. Harvey, and A.C. Herington. 2000. The expression of the ADAMs proteases in prostate cancer cell lines and their regulation by dihydrotestosterone. *Mol Cell Endocrinol.* 167:11-21.
- Nath, D., P.M. Slocombe, A. Webster, P.E. Stephens, A.J. Docherty, and G. Murphy. 2000. Meltrin gamma(ADAM-9) mediates cellular adhesion through alpha(6)beta(1) integrin, leading to a marked induction of fibroblast cell motility. *J Cell Sci.* 113:2319-28.
- Pan, D., and G.M. Rubin. 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. *Cell.* 90:271-80.
- Peschon, J.J., J.L. Slack, P. Reddy, K.L. Stocking, S.W. Sunnarborg, D.C. Lee, W.E. Russell, B.J. Castner, R.S. Johnson, J.N. Fitzner, R.W. Boyce, N. Nelson, C.J. Kozlosky, M.F. Wolfson, C.T. Rauch, D.P. Cerretti, R.J. Paxton, C.J. March, and R.A. Black. 1998. An essential role for ectodomain shedding in mammalian development. *Science.* 282:1281-4.
- Pierce, K.L., L.M. Luttrell, and R.J. Lefkowitz. 2001a. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene.* 20:1532-9.
- Pierce, K.L., A. Tohgo, S. Ahn, M.E. Field, L.M. Luttrell, and R.J. Lefkowitz. 2001b. Epidermal growth factor receptor dependent ERK activation by G protein- coupled receptors: A Co-culture system for identifying intermediates upstream and downstream of HB-EGF shedding. *J Biol Chem.* 4:4.
- Plonowski, A., A.V. Schally, J.L. Varga, Z. Rekasi, F. Hebert, G. Halmos, and K. Groot. 2000. Potentiation of the inhibitory effect of growth hormone-releasing hormone antagonists on PC-3 human prostate cancer by bombesin antagonists indicative of interference with both IGF and EGF pathways. *Prostate.* 44:172-80.

- Prenzel, N., E. Zwick, H. Daub, M. Leserer, R. Abraham, C. Wallasch, and A. Ullrich. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. 402:884-8.
- Qi, H., M.D. Rand, X. Wu, N. Sestan, W. Wang, P. Rakic, T. Xu, and S. Artavanis-Tsakonas. 1999. Processing of the notch ligand delta by the metalloprotease Kuzbanian. *Science*. 283:91-4.
- Reddy, P., J.L. Slack, R. Davis, D.P. Cerretti, C.J. Kozlosky, R.A. Blanton, D. Shows, J.J. Peschon, and R.A. Black. 2000. Functional analysis of the domain structure of tumor necrosis factor- alpha converting enzyme. *J Biol Chem*. 275:14608-14.
- Shi, W., H. Fan, L. Shum, and R. Derynck. 2000. The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. *J Cell Biol*. 148:591-602.
- Sternlicht, M.D., A. Lochter, C.J. Simpson, B. Huey, J.P. Rougier, J.W. Gray, D. Pinkel, M.J. Bissell, and Z. Werb. 1999. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell*. 98:137-46.
- Werb, Z. 1997. ECM and cell surface proteolysis: regulating cellular ecology. *Cell*. 91:439-42.
- Werb Z., and Yan Y. 1998. A cellular striptease act. *Science*. 282:1279-80.

Appendices

Reprint Series
13 November 1998, Volume 282

SCIENCE

A Cellular Striptease Act

Zena Werb and Yibing Yan

A Cellular Striptease Act

Zena Werb and Yibing Yan

The cell surface is a dynamic place. During its life history the cell alters the repertoire of proteins displayed on its surface many times. Membrane-anchored adhesion molecules, receptors, ligands, and enzymes are removed and replaced as the cell proceeds through development and as its activation state changes.

Enhanced online at
www.sciencemag.org/cgi/content/full/282/5392/1279

How is this wholesale refurbishing of the cell membrane orchestrated? One key mechanism is proteolytic processing of the ectodomain (extracellular domain) of such membrane proteins. Cleavage or shedding of the ectodomains of plasma membrane proteins—widely observed in cells in culture—is blocked by inhibitors of metalloproteinases (1, 2). This result suggests that transmembrane and soluble metalloproteinases, such as matrix metalloproteinases (MMPs) and their relatives, are rate-limiting for cleavage and shedding. Other evidence also implicates serine proteinases in these processing events (3, 4).

The first such “shedase” characterized was the tumor necrosis factor- α (TNF- α) converting enzyme (TACE) (5). The study by Peschon and colleagues (6) on page 1281 of this issue now points to TACE’s essential role in the shedding of ectodomains during mouse development. The surprise comes from the observation that mice lacking TACE do not show a phenotype indicative of a lack of TNF- α availability. Rather, they show the same phenotype as mice engineered to be without the epidermal growth factor (EGF) receptor—because TACE-mediated proteolysis makes available ligands for the EGF receptor,

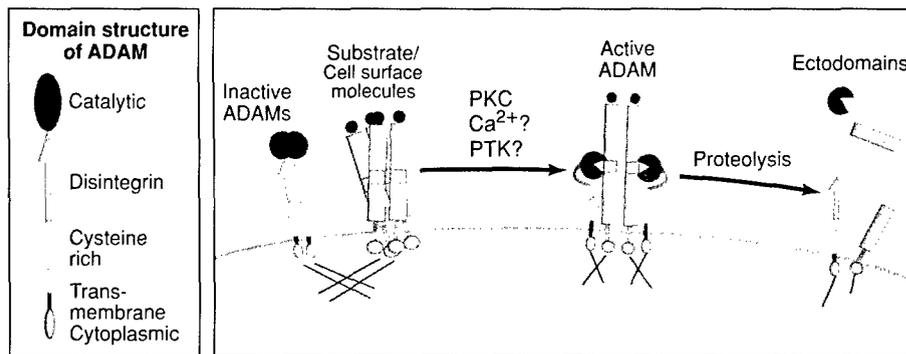
particularly transforming growth factor- α (TGF- α).

TACE turns out to be a membrane-anchored proteinase that is a member of the ADAM (a disintegrin and metalloproteinase) domain family of proteins that combines features of both cell surface adhesion molecules and proteinases (8). ADAMs all have a common domain organization, which endows these proteins with several potential functions—proteolysis, adhesion, signaling, and fusion (see figure below). The proteolytically competent ADAMs, such as TACE (ADAM17), are zinc-dependent metalloproteinases, closely related to the MMP family with which they share small molecule inhibitors and even one tissue inhibitor, TIMP-3 (9, 10). Several newly discovered MMPs appear to be hybrids of both MMP and ADAM domains (11), indicating that these two types of enzymes are part of one, larger family.

The ADAM proteinases are themselves targets of proteolytic events that ultimately strip off the catalytic domains (5, 8). This action could be a mechanism of functionally blunting the effects of the proteinases (see the figure on the next page). These

soluble ADAMs may have proteolytic activity, as is the case for snake venom enzymes (8), but soluble TACE is much less active than membrane-bound enzyme (5, 6). The residual adhesive domains of ADAMs left after cleavage may have regulatory or adhesive functions. In support of this idea, a catalytic domain-deleted mutant of *Kuz* (ADAM10/SUP17), first identified as being required for cleavage of Notch during neural development in *Drosophila*, exerts a dominant negative effect (8, 12). During sperm maturation fertilin, a heterodimeric ADAM essential for sperm-egg interaction (13), also loses its catalytic domains by proteolytic processing. The remaining adhesive disintegrin domain is then competent to bind integrins.

How does TACE act? TACE is widely expressed in the animal. Mutation of the catalytic domain of TACE (6) reveals several distinct functions for this ADAM in development. Ligands for the EGF receptor, which is essential for epithelial development (7), are usually made and used locally (14). Although the growth factor precursors may have some biological activity (15), the new results imply that the membrane-anchored forms are essentially inactive precursors (6). TACE also cleaves ectodomains of other receptors and ligands, such as TNF- α , the p75 TNF receptor, and L-se-



Activation of sheddases. The ADAM proteases (as dimers) and substrates are anchored apart in the plane of the membrane. Upon activation (via protein kinases and other pathways) they are brought together and proteolysis takes place, leading to free ectodomains.

The authors are at the Department of Anatomy, University of California, San Francisco, CA 94143-0452, USA. E mail: zena@itsa.ucsf.edu; yan@cgl.ucsf.edu

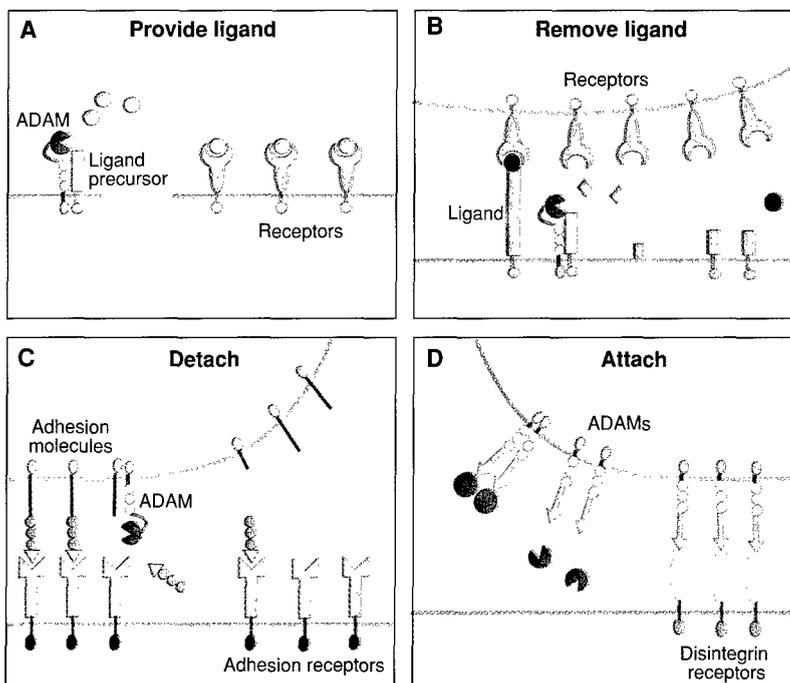
leucin, and thus participates in inflammatory and pathological reactions (6).

Processing membrane proteins by the ADAMs and other sheddases requires both the membrane-anchored enzyme and its substrate to be present in cis on the same cell (6, 8, 12). This presents several interesting problems. How are the active cell surface proteinases kept separate from their cell surface substrates until shedding is triggered? How do you exert selectivity for only certain ectodomain targets, out of many transmembrane proteins displayed on the cell surface? And how are the proteinase and substrate brought together in a coordinated manner so that all the cell surface substrate molecules

can be removed within seconds, as occurs for the adhesion molecules L-selectin and syndecans (4, 16)?

Despite nonconserved cleavage sites that may be adjacent to the membrane or further out on the molecule, there are clues that a common strategy may operate in most cases. First, all ectodomain shedding is inhibited in a single mutant cell line (1). Second, the proteolysis is regulated in different cell types by activation of protein kinase C (PKC), calcium/calmodulin kinases, or receptor tyrosine kinases (1, 17). A model that accounts for these observations requires the processing proteinases and their transmembrane substrates both to be anchored in distinct domains of the plasma membrane, probably through cytoskeletal interactions (see figure on previous page). Upon cell activation, the attachments become coclustered and can interact. Alternatively, the signaling cascade could modify the cytoplasmic domains of the proteinases or substrate, producing a conformational change that either activates the enzyme or makes the cleavage site available.

Although activation of the shedding reaction appears to control the rapid and complete removal of cell surface molecules such as L-selectin (an adhesion molecule involved in leukocyte rolling and extravasation into inflammatory sites) for most processing reactions there appears to be a constitutive level of ectodomain shedding. Processing is necessary to make available



Versatile shedding. Sheddases can supply or down-regulate ligands for receptors. Cleavage of adhesion molecules on cell surface or exposure of the disintegrin domain of ADAM regulate cell-cell and cell-extracellular matrix interactions.

paracrine growth and survival factors such as TGF- α , EGF, HB-EGF, the kit ligand, and amphiregulin (18). This makes sense to allow for the consistent supply of growth factors (see figure above).

Endogenous inhibitors allow even finer control of the action of the shedding enzymes. Recently TACE was shown to be inhibited by TIMP-3, but not by the three other TIMPs that also inhibit MMPs (10). If TACE liberates a survival factor, then the presence of TIMP-3 could lead to cell death. This may explain why TIMP-3, but not other TIMPs, induce apoptosis (19).

Proteolysis of the ectodomains of growth factor coreceptors such as syndecan provide a second mechanism for regulating growth factor availability. Shedding the ectodomain of syndecan converts it to a potent inhibitor of FGF-2 (20). Just as shedding can make growth factor ligands available and control proliferation and survival, cleavage can also control cell death. Membrane-bound Fas ligand induces apoptosis by binding to the Fas receptor. Proteolysis functionally down-regulates the ligand and short-circuits apoptosis in lymphoid cell (21).

Cell surface adhesive molecules can also be regulated by proteolysis. An emerging paradigm is that cleavage of adhesive molecules not only alters adhesion, but completely revamps cell signaling. In the case of Notch, cleavage by *Kuz* is required to make it functional as a receptor, promoting adhesion, signaling, and cell lineage choices (12). Shedding of L-selectin by

TACE or related enzymes inhibits leukocyte rolling and blunts their extravasation to inflammatory sites (16). The shedding of the ectodomains of E-cadherin (22) and transmembrane protein tyrosine phosphatases such as LAR have profound effects on cell-cell adhesions and also on important signaling pathways (17). These changing adhesion receptors and ligands may also be part of the apparatus for pathfinding in the nervous system.

Cells use a limited number of strategies to remodel their microenvironments. It is clear that the shedding process is an ancient, conserved, and fundamental pathway present from worms to humans. Thus, proteolysis by cell surface shedding enzymes provides a mechanism by which the wardrobe of externally displayed molecules can be changed or discarded. Spatial restriction of the enzymes and their substrates allows for either instant action or sustained activity.

References and Notes

1. J. Arribas, F. Lopez-Casillas, J. Massague, *J. Biol. Chem.*, **272**, 17160 (1997); A. Merlos-Suarez *et al.*, *ibid.*, **273**, 24955 (1998).
2. B. Walcheck *et al.*, *Nature* **380**, 720 (1996); L. Lum and C. P. Blobel, *Dev. Biol.*, **191**, 131 (1997); M. Suzuki *et al.*, *J. Biol. Chem.* **272**, 31730 (1997).
3. F. Logeat *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8108 (1998).
4. S. V. Subramanian, M. L. Fitzgerald, M. Bernfield, *J. Biol. Chem.* **272**, 14713 (1997).
5. R. A. Black *et al.*, *Nature*, **385**, 729 (1997); M. L. Moss *et al.*, *ibid.*, p. 733.
6. J. J. Peschon *et al.*, *Science* **282**, 1281.
7. P. J. Miettinen *et al.*, *Nature* **376**, 337 (1995); D. W. Threadgill *et al.*, *Science* **269**, 230 (1995); M. Sibilia and E. F. Wagner, *ibid.*, p. 234.
8. K. Maskos *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3408 (1998); C. P. Blobel, *Cell* **90**, 589 (1997); R. A. Black and J. M. White, *Curr. Opin. Cell Biol.* **10**, 654 (1998).
9. Z. Werb, *Cell* **91**, 439 (1997).
10. A. Amour *et al.*, *FEBS Lett.* **435**, 39 (1998).
11. R. Gururajan *et al.*, *Genomics* **52**, 101 (1998).
12. D. Pan and G. M. Rubin, *Cell* **90**, 271 (1997).
13. C. Cho *et al.*, *Science* **281**, 1857 (1998).
14. R. Derynck, *Adv. Cancer Res.* **58**, 27 (1992); P. J. Demsey *et al.*, *J. Cell Biol.* **138**, 747 (1997).
15. R. Brachmann *et al.*, *Cell* **56**, 691 (1989).
16. J. Kahn *et al.*, *Cell*, **92**, 809 (1998).
17. B. Aicher *et al.*, *J. Cell Biol.* **138**, 681 (1997); S. M. Dethlefsen *et al.*, *J. Cell. Biochem.* **69**, 143 (1998); M. Vecchi *et al.*, *J. Biol. Chem.* **273**, 20589 (1998).
18. Y. Tajima *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 11903 (1998); R. Sadhukhan *et al.*, *ibid.*, p. 138.
19. M. Ahonen, A. H. Baker, V. M. Kahari, *Cancer Res.* **58**, 2310 (1998); A. H. Baker, A. B. Zaltsman, S. J. George, A. C. Newby, *J. Clin. Invest.* **101**, 1478 (1998); M. R. Smith *et al.*, *Cytokine* **9**, 770 (1997).
20. M. Kato *et al.*, *Nat. Med.* **4**, 691 (1998).
21. M. Tanaka, T. Itai, M. Adachi, S. Nagata, *ibid.*, p. 31.
22. A. Lochter *et al.*, *J. Cell Biol.* **139**, 1861 (1997).
23. Supported by grants from NIH (CA72006, HD26732)



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

8 Jan 2003

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed. Request the limited distribution statement for the enclosed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

A handwritten signature in black ink, appearing to read "Phyllis M. Rinehart".

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

ADB265840

ADB266633

ADB282069

ADB279138

ADB251763

ADB265386

ADB264578

ADB281601

ADB282057

ADB281679

ADB258874

ADB258251

ADB281645

ADB281773

ADB264541

ADB261128

ADB281660

ADB241630

ADB261339

ADB259064

ADB281924

ADB273096

ADB266141

ADB281663

ADB281681

ADB281664

ADB281659

ADB259637

ADB258830

ADB256645

ADB266029

ADB262441

ADB281668

ADB281674

ADB259834

ADB281771

ADB266075

ADB281612

ADB281661