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TACE is required for the activation of the EGFR by TGF-α in tumors

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The factors and mechanisms that transduce the intracellular signals sent upon activation of the receptor for the epidermal growth factor (EGFR) and related receptors are reasonably well understood and, in fact, are the targets of anti-tumor drugs. In contrast, less is known about the mechanisms implicated in sending the signals that activate these receptors. Here we show that when its proteolytic shedding is prevented, the transmembrane form of the transforming growth factor-α (proTGF-α) interacts with, but does not activate, the EGFR. Thus, shedding seems to control not only the availability of the soluble form of the growth factor (TGF-α) but also the activity of the transmembrane form. The activity of the protease responsible for the shedding of proTGF-α, tumor necrosis factor-α converting enzyme (TACE), is required for the activation of the EGFR *in vivo* and for the development of tumors in nude mice, indicating a crucial role of TACE in tumorigenesis. In agreement with this view, TACE is dramatically overexpressed in the majority of mammary tumors analyzed. Collectively, this evidence points to TACE as a promising target of anti-tumor therapy.

Keywords: EGFR/metalloproteases/shedding/TGF-α

Introduction

The epidermal growth factor (EGF) receptor (EGFR) is the prototype of a family of tyrosine kinases that participate in the control of differentiation, proliferation and cell survival, as well as in the development of tumors of epidermal origin (Yarden and Sliwkowski, 2001). The biochemical pathways that transduce the signals initiated upon EGFR-like receptors activation have been extensively characterized, are reasonably understood and, in fact, are the target of different anti-tumor drugs that are currently under different stages of development (Baselga, 2001). Much less is known about the mechanisms involved in sending the signals that activate the EGFR-like receptors, therefore many efforts are presently focusing in studying the biosynthesis and regulation of the ligands of these receptors in normal as well as in malignant cells. Conceivably, these studies will eventually lead to the discovery of novel targets of anti-tumor therapy.

In addition to EGF, several ligands bind and activate the EGFR via EGF-like motifs: the prototypical transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin (Prenzel *et al*., 2001) and the recently described epigen (Strachan *et al*., 2001). All of these are synthesized as transmembrane molecules that can release their extracellular domains containing the EGF-like motif through a specialized type of limited proteolysis, known as ectodomain shedding, which is regulated via protein kinase C (PKC) (Massagué and Pandiella, 1993). It is widely accepted that ectodomain shedding regulates the availability of soluble EGFR ligands that act in autocrine or paracrine fashion activating EGFRs in the very EGFR ligand-producing cell or in proximal cells, respectively. In contrast, the activity of the transmembrane forms of EGFR ligands is poorly understood. Early reports showed that the transmembrane form of TGF-α (known as proTGF-α) containing mutations which prevented its shedding had the ability to activate EGFR in adjacent cells (Brachmann *et al*., 1989; Wong *et al*., 1989). This type of signaling was named juxtacrine. Supporting a role of proTGF-α in the activation of the EGFR, it has been shown that the tetraspanin CD9 associates with proTGF-α, inhibits the shedding of the growth factor and enhances the level of cell surface proTGF-α, leading to a hyperactivation of the EGFR in juxtacrine assays (Shi *et al*., 2000). Furthermore, proTGF-α expressed by certain malignant cells apparently induces a higher phosphorylation of EGFR on the cell surface of adjacent cells than equivalent levels of TGF-α (Yang *et al*., 2000). In contrast to these reports, it has been shown that inhibitors of ectodomain shedding prevent the juxtacrine activation of EGFR (Dong *et al*., 1999), and that mice genetically deficient in the proteolytic activity responsible for the shedding of proTGF-α show a phenotype similar to that of TGF-α knock-out mice (Peschon *et al*., 1998). Thus, these reports indicate that in some contexts proTGF-α cannot substitute for soluble TGF-α and questions the juxtacrine mode of signaling for this growth factor under those circumstances.

The protease responsible for the shedding of several EGFR ligands, including proTGF-α, seems to be identical to tumor necrosis factor-α converting enzyme (TACE). Although TACE was initially identified as the metalloprotease responsible for the shedding of protumor necrosis factor-α (proTNF-α) (Black *et al*., 1997; Moss *et al*., 1997), it has been proposed that TACE is also necessary for the shedding of a wide variety of proteins that include proTGF-α (Peschon *et al*., 1998), proHB-EGF (Merlos-Suárez *et al*., 2001) and amphiregulin (Sunnarborg *et al*., 2002), because mouse embryonic fibroblasts established from TACE knock-out mice show apparent defects in the

shedding of several proteins, including these transmembrane growth factors. Moreover, Chinese hamster ovary (CHO) somatic cell mutants initially isolated for lack of proTGF-α shedding (Arribas and Massagué, 1995) are also defective in the shedding of different proteins including proHB-EGF (Merlos-Suárez *et al*., 2001), and have been recently shown to be specifically defective in the proteolytic activation of TACE (A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga and J.Arribas, manuscript submitted). Thus, genetic evidences strongly suggests a role of TACE in the shedding of different proteins that include several ligands of the EGFR, at least in the cells mentioned.

TACE belongs to the family of metalloprotease disintegrins (also known as ADAM or MDC family), which are modular transmembrane proteins with a Zinc-dependent catalytic domain (recently reviewed in Kheradmand and Werb, 2002). Metalloprotease disintegrins are synthesized as inactive precursors containing a prodomain that blocks the activity of the catalytic domain. As is the case of most metalloprotease disintegrins analyzed to date, the prodomain of TACE is removed during transit through the secretory pathway by furin-like proprotein convertases (A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga and J.Arribas, manuscript submitted). Metalloprotease disintegrins share several domains with the membrane-type matrix metalloproteases (MT-MMP), a subfamily of the MMPs that is also proteolytically activated by proprotein convertases. In fact, both metalloprotease disintegrins and MMPs form part of the superfamily of the metzincins. Numerous *in vitro* and *in vivo* studies have established that MMPs participate in the development of tumors as well as in invasion and metastasis (recently reviewed in Brinckerhoff and Matrisian, 2002). Given the similarities between MMPs and metalloprotease disintegrins, and the potential of the latter in regulating the activity of the ligands of EGFR, it is expected that TACE, and perhaps other metalloprotease disintegrins, are involved in the regulation of tumor progression; however, no studies aimed to elucidate this point have been published yet.

Results

Activation of EGFR by juxtacrine proTGF-α

To analyze the possible role of ectodomain shedding on the activation of the EGFR by TGF-α, we compared the juxtacrine activation induced by cells expressing proTGF-α with that induced by soluble TGF-α. To facilitate this study, we used a version of proTGF-α tagged at the N-terminus with the HA epitope that has been characterized elsewhere (Arribas and Massagué, 1995). In agreement with previous results (Brachmann *et al*., 1989; Wong *et al*., 1989), addition of CHO cells permanently transfected with proHA/TGF-α for a short period of time to the well-characterized A431 cells, which overexpress the EGFR, induces the activation of the EGFR (Figure 1A and B). This activation is higher than that induced by the soluble form of the growth factor produced by the same number of cells during the same period of time (Figure 1A and B). This result could be due to dilution or inactivation of TGF-α during its accumulation in the conditioned media or to the consumption of the growth factor due to autocrine binding to the EGFR expressed in the same cells (see e.g. Dempsey and Coffey, 1994). However, as previously shown (Arribas and Massagué, 1995), in pulse–chase experiments CHO cells secrete quantitative amounts of TGF-α, which are readily detected. Thus, the results shown in Figure 1 open the possibility that the juxtacrine activity of cells expressing proTGF-α is not exclusively due to the production of soluble TGF-α. As an additional control, we showed that addition of anti-HA antibodies to the CHO/proHA/TGF-α cells in juxtacrine assays or to the conditioned media of these cells prevents the activation of the EGFR (See Supplementary figure 1, available at *The EMBO Journal* Online).

In agreement with published results (Dong *et al*., 1999), addition of BB-94, an hydroxamic acid-based metalloprotease inhibitor that blocks protein ectodomain shedding, prevents the activation of the EGFR by CHO cells expressing proHA/TGF-α (Figure 1A and B), indicating that the metalloprotease activity responsible for ectodomain shedding is necessary for the juxtacrine activity of the growth factor. As expected, due to the inhibition of proTGF-α ectodomain shedding, the conditioned media from cells treated with BB-94 had no effect (Figure 1A and B). As a control, we showed that BB-94 does not interfere with the activation of the EGFR by TGF-α, since the addition of the inhibitor does not affect the activation of the EGFR by the conditioned media of proHA/TGF-α expressing cells (data not shown). The lack of activity of CHO/proHA/TGF-α cells on A431 cells is not due to a lack of interaction between proHA/TGF-α and the EGFR, as shown by cell–cell interaction assays: while a very low percentage of parental CHO cells bind to A431 cells, a significant percentage of CHO/proHA/TGF-α cells bind to A431 cells (Figure 1C); the percentage is significantly higher in the presence of BB94 ($p < 0.001$, Mann–Whitney test; Figure 1C), probably because a greater number of proHA/TGF-α molecules are available in the absence of ectodomain shedding. The specificity of the binding between proHA/TGF-α and the EGFR was monitored using C225 (Figure 1C), a monoclonal anti-EGFR that prevents the binding of EGFR ligands (see e.g. Mendelsohn and Baselga, 2000). These results suggest that the juxtacrine activity of proTGF-α is higher than that of the soluble form of the growth factor and that ectodomain shedding is required for the activity of proTGF-α in juxtacrine assays. To confirm these results, we employed two additional independent methods to prevent the shedding of proTGF-α: the use of a somatic CHO mutant cell line, known as M2/proHA/TGF-α, defective in the shedding of several proteins including proTGF-α (Arribas *et al*., 1996), and the use wild-type CHO cells expressing shedding-resistant proHA/TGF-α deletion constructs

In agreement with previous results (Arribas and Massagué, 1995), treatment of M2/proHA/TGF-α cells with the well-characterized activator of ectodomain shedding PMA (a phorbol ester known to activate PKC) does not induce the typical downmodulation of cell surface proTGF-α observed in normal cells (Figure 2A). Confirming the results shown in Figure 1, despite the fact that M2 cells express a higher level of proHA/TGF-α

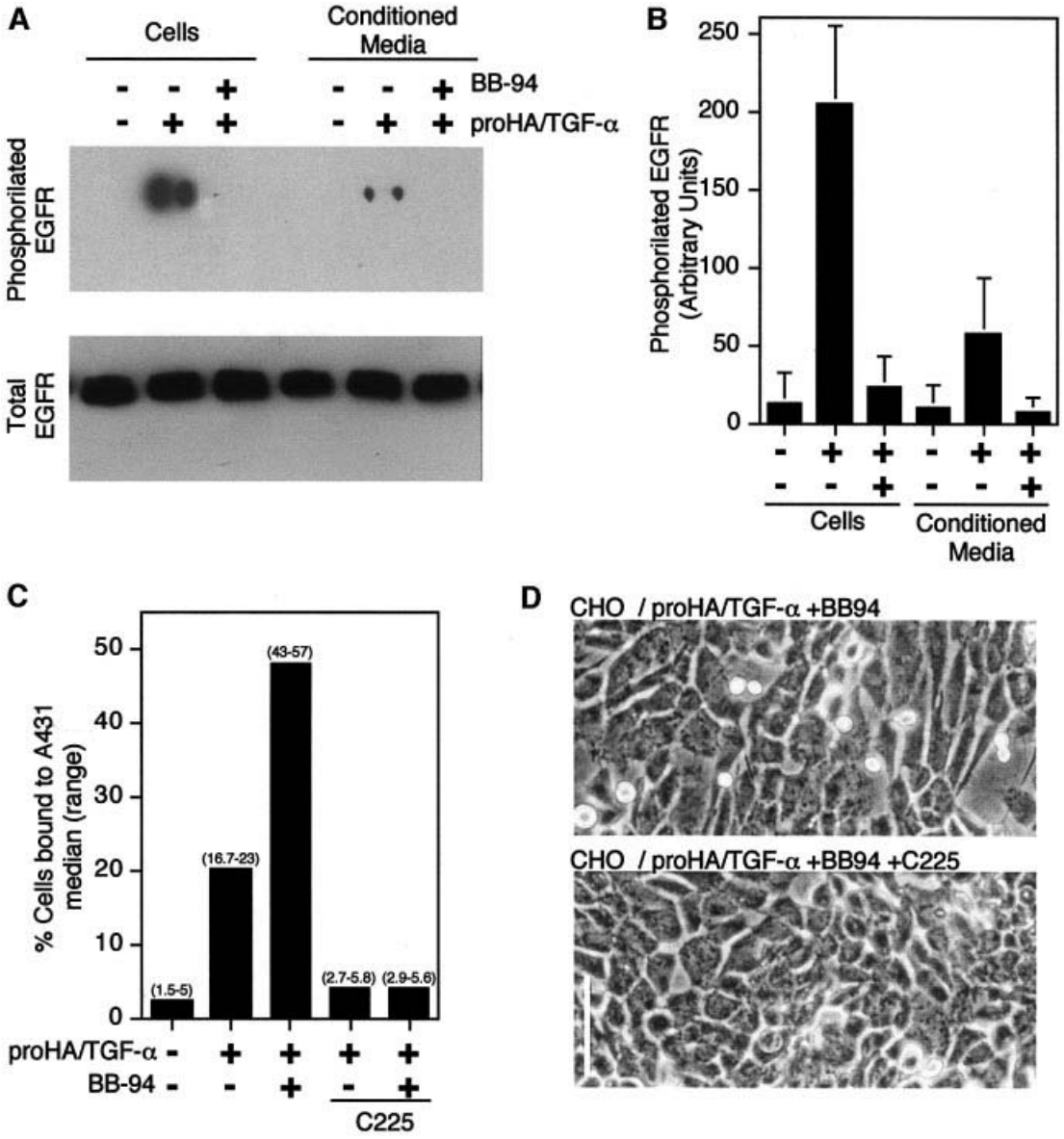


Fig. 1. Effect of BB-94 on the activation of EGFR by transmembrane proTGF-α or soluble TGF-α. (**A**) Subconfluent A431 cells were incubated with parental CHO cells, CHO/proHA/TGF-α cells or the conditioned media of these cells obtained in the absence or presence of BB-94 as indicated. Then, A431 cells were washed, lysed and the cell lysates analyzed by western blotting with monoclonal antibodies against phosphorylated EGFR or against total EGFR. (**B**) The results of three independent experiments performed as in (A) were quantified; the averages ± SD are shown. (**C**) Interaction between A431 cells and parental CHO or CHO/proHA/TGF-α cells. Confluent A431 cells were incubated with parental CHO or CHO/proHA/TGF-α cells with or without BB-94 and the monoclonal antibody C225 (which blocks the interaction between TGF-α and the EGFR) as indicated, and then cells not bound were counted. The percentage of cells bound to A431 was calculated by subtracting the number of cells recovered after the incubation from the number of cells added. The results are expressed as the medians of five independent determinations. (**D**) Confluent A431 cells were incubated with CHO/proHA/TGF-α in the presence of BB-94 with or without C225 as in (C) and gently washed with PBS. Photomicrographs of representative fields are shown. Bar = 0.1 mm.

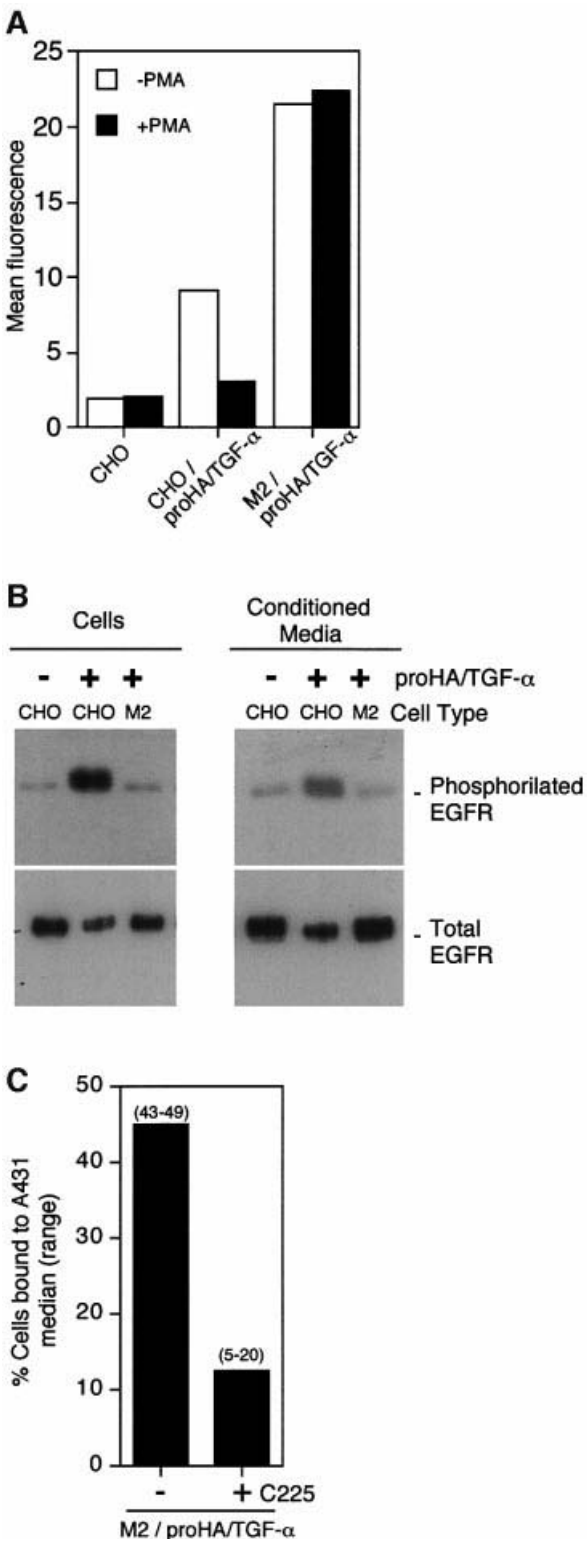
at the cell surface than CHO/proHA/TGF-α cells (Figure 2A), they are unable to activate the EGFR in juxtacrine assays (Figure 2B). This lack or activity is not due to a lack of interaction between proHA/TGF-α and EGFR, as shown by cell–cell interaction assays (Figure 2C).

It has been shown that small deletions surrounding the cleavage site prevent the shedding of several transmembrane proteins (reviewed in Arribas and Merlos-Suárez, 2002). Thus, we made a series of proTGF-α deletion constructs affecting the cleavage site (Figure 3A) and transfected them permanently into CHO cells. Analysis of the shedding of these proHA/TGF-α deletion constructs by flow cytometry showed that the Δ93–102 and Δ90–105 constructs are resistant to shedding (Figure 3B), while the shedding of Δ98–102 was only slightly affected. Thus, we used the shedding-resistant constructs to analyze the activity of uncleavable proTGF-α. As expected, these constructs showed a lack of EGFR-activating ability (Figure 3C), that was not due to a deficient binding to the EGFR (Figure 3D). Collectively, the results presented show that ectodomain shedding is a critical regulatory step that controls not only the paracrine but also the juxtacrine activity of proTGF-α.

Effect of ectodomain shedding in tumorigenicity assays

The importance of ectodomain shedding in the signaling mediated by proTGF-α in cells prompted us to determine the possible involvement of ectodomain shedding in the signaling mediated by proTGF-α, which leads to the development of tumors. It has long been known that overexpression of proTGF-α induces a variety of neoplasias (Jhappan *et al*., 1990; Sandgren *et al*., 1990). In agreement with these results, overexpression of proHA/TGF-α allows the vigorous growth of CHO xenografts in nude mice (Figure 4). In contrast, the size of the tumors induced by M2/proHA/TGF-α cells or wild-type CHO cells expressing the deletion construct Δ90–105 is significantly reduced (Student's *t*-test $P < 0.001$), indicating that the shedding of proTGF-α is necessary for the maximum growth of tumors in this model.

Histological analysis of the tumors showed no obvious difference between the tumors corresponding to the three different cell lines, except for a higher proportion of blood vessels in the tumors induced by CHO/proHA/TGF-α cells, as judged by immunohistochemistry with anti-CD34 (data not shown). To further characterize the tumors induced by wild type and shedding-defective mutant cell lines, we analyzed the expression of proHA/TGF-α and EGFR in samples from the different tumors by immunostaining with anti-HA and anti-EGFR antibodies. As shown in Figure 5 (upper panels), anti-HA immunoreactivity was readily detected in tumors derived from wild-type and mutant cells indicating that, as expected, both types of tumors express proHA/TGF-α. Also, the levels of EGFR, as determined with specific antibodies, were similar in specimens from both types of tumors (Figure 5, middle panels). However, using a monoclonal antibody that specifically recognizes the activated form of the EGFR (Albanell *et al*., 2002), a higher number of cells were stained in samples from tumors induced by CHO/proHA/TGF-α than in samples from tumors induced by shedding-defective mutant cells (Figure 5, lower panels). Thus, these results show that the level of activation of EGFR induced by normal cells expressing proTGF-α is higher than that induced by their shedding-defective counterparts *in vivo* and opens the possibility that ectodomain shedding regulates the activation of EGFR by proTGF-α in tumors.



Tumors induced by M2 mutant cells contain shedding-competent revertant cells

Although the levels of activated EGFR are clearly diminished in the small tumors induced by M2 cells as judged by immunhistochemistry, there is detectable activation of the receptor (see Figure 2B). These results indicate that *in vivo* transmembrane proTGF-α could activate the EGFR, albeit with lower efficiency than soluble TGF-α or, alternatively, that M2 cells regain the ability to shed proTGF-α in tumors. To distinguish

Fig. 2. Effect of proTGF-α expressed in shedding-defective mutant CHO cells (M2 cells) on the activation of the EGFR. (**A**) Flow cytometry analysis of parental CHO, CHO/proHA/TGF-α and M2 proHA/TGF-α cells immunostained with anti-HA antibodies. Cells were treated with or without PMA and the level of proHA/TGF-α was determined by flow cytometry. The quantitative results presented are the average of duplicate determinations. (**B**) Subconfluent A431 cells were incubated with CHO cells, CHO/proHA/TGF-α cells, M2/proHA/TGF-α or the corresponding conditioned media. Then, A431 cells were washed, lysed and the cell lysates analyzed by western blotting with monoclonal antibodies against phosphorylated EGFR or against total EGFR. (**C**) Interaction between A431 cells and M2/proHA/TGF-α cells. Confluent A431 cells were incubated with parental M2/proHA/TGF-α cells in the presence or absence of the monoclonal antibody C225 and cells not bound were counted. The percentage of cells bound to A431 was calculated by subtracting the number of cells recovered after the incubation from the number of cells added. The results are expressed as the medians of five independent determinations.

between these possibilities, we analyzed the shedding of proHA/TGF-α in cell lines established from five independent tumors of each type (we named these cell lines Mo1 to Mo5-CHO/proTGF-α and Mo1 to Mo5-M2/proHA/TGF-α cells, respectively). To select these cell lines, we used the selectable marker geneticin, which does not allow the growth of cells from mouse origin. The identity of the cell lines obtained was confirmed using a β-actin fragment amplified from cDNA obtained from Mo1 and Mo5-M2/proHA/TGF-α cells. Digestion of the fragment with the endonuclease *Sal*I showed the existence of such a restriction site, present in β-actin from hamster but not from mouse (Supplementary figure 2; data not shown). In addition, the sequence of the fragments was identical to that of hamster β-actin (data not shown).

As shown in Figure 6A and B, a considerable fraction of Mo1-M2/proHA/TGF-α cells recovered the ability to shed the ectodomain of proTGF-α, indicating that reversion to the wild-type phenotype is concomitant with the tumor growth and further supporting a role of the shedding of proTGF-α in the development of tumors in this model. Similar results were observed when the shedding of proHA/TGF-α was analyzed in the Mo2-, Mo3-, Mo4 and Mo5-M2/proHA/TGF-α cells lines (data not shown).

It has been shown recently that the defect in M2 cells specifically affects the intracellular trafficking and activation of TACE (A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga,J. and J.Arribas, manuscript submitted). While, as judged by pulse–chase experiments, in wild-type cells

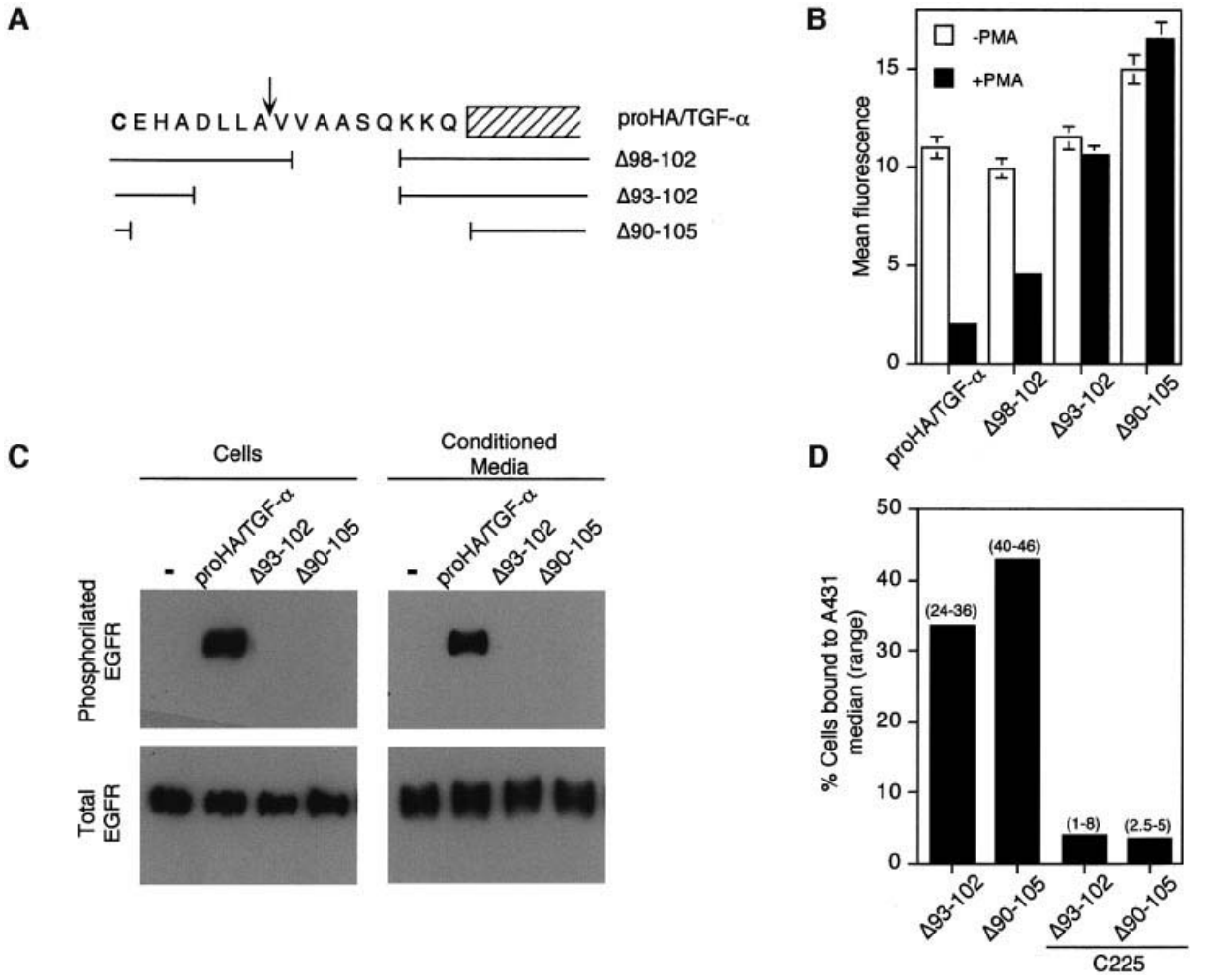


Fig. 3. Effect of proTGF-α deletion constructs on the activation of the EGFR. (**A**) Schematic showing the juxtamembrane region of proTGF-α indicating the cleavage site (arrow) and different deletion constructs. The transmembrane domain is shown as a hatched box and the C-terminal cysteine that participates in the EGF motif is shown in bold. (**B**) Flow cytometry analysis of parental CHO/proHA/TGF-α and CHO cells permanently transfected with the deletion constructs shown in (A). Cells were treated with or without PMA and the level of cell surface anti-HA immunreactivity was determined by flow cytometry. The quantitative results presented are the average of triplicate determinations ± SD. (**C**) Subconfluent A431 cells were incubated with CHO cells, CHO/proHA/TGF-α cells or CHO cells expressing the indicated deletion constructs. Then, A431 cells were washed, lysed and the cell lysates analyzed by western blotting with monoclonal antibodies against phosphorylated EGFR or against total EGFR. (**D**) Interaction between A431 cells and cells expressing proTGF-α deletion constructs. Confluent A431 cells were incubated with CHO cells permanently transfected with the indicated deletion constructs in the presence or absence of the monoclonal antibody C225 and cells not bound were counted. The percentage of cells bound to A431 was calculated by subtracting the number of cells recovered after the incubation from the number of cells added. The results are expressed as the medians of five independent determinations.

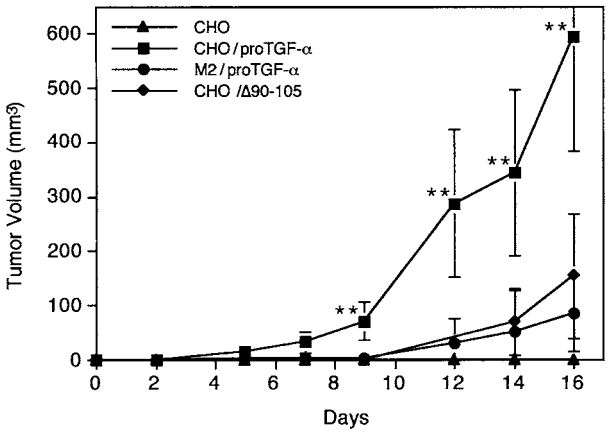


Fig. 4. Analysis of the tumors induced by parental CHO, CHO/proTGF-α, CHO/Δ90–105 and M2/proTGF-α in nude mice. The indicated cells were subcutaneously injected into nude mice. Tumor volumes were measured at the indicated days after injection. Each point represents the mean of six individual determinations ± SD [**Student's *t*-test values ($P < 0.01$) comparing tumors induced by CHO/proHA/TGF-α to those induced by CHO/Δ90–105 or M2/proHA/TGF-α].

TACE is processed to a form that lacks the prodomain and is transported to the cell surface (Figure 6C; A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga and J.Arribas, manuscript submitted), in M2 cells TACE accumulates intracellularly as an inactive form (A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga and J.Arribas, manuscript submitted). In agreement with these findings, in wild-type cells TACE can be detected as a full-length molecule containing the prodomain and also as processed forms devoid of it (Figure 6D; data not shown). Note that the processed form of TACE migrates as a broad band in western blots (Figure 6D). This is particular to CHO cells, and is likely due to the fact that an unknown post-translational modification alters the electrophoretic migrations of processed TACE in these cells, as shown at long time-points in pulse–chase experiments (Figure 6C). In M2 cells, TACE migrates as a single form containing the prodomain (Figure 6D; A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga and J.Arribas, manuscript submitted).

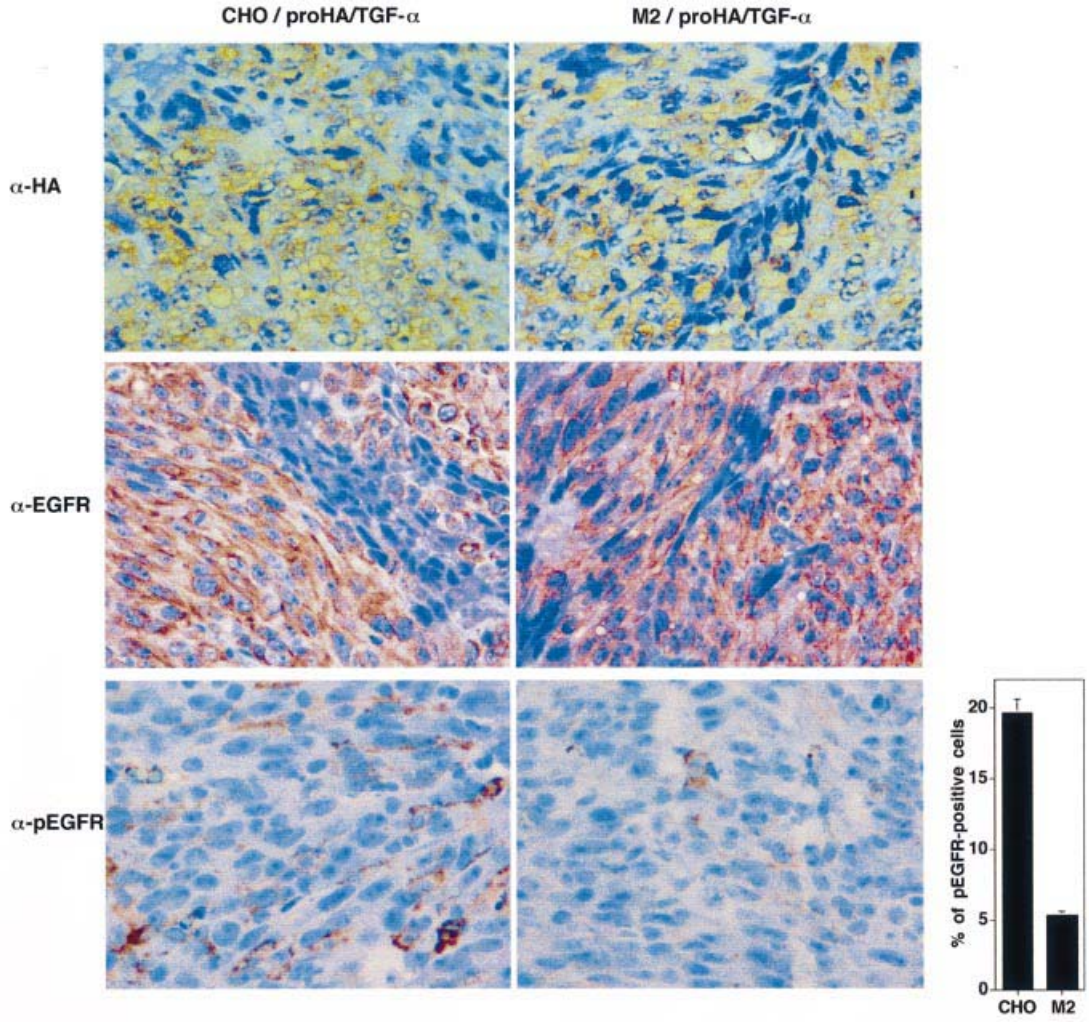


Fig. 5. Analysis of the EGFR in tumor xenografts. Immunocytochemical staining with anti-HA, anti-total EGFR or anti-phosphorylated EGFR. The number of cells positively stained with the anti-phosphorylated EGFR were counted in 10 different fields, the data shown are the average ± SD.

Analysis of TACE species in Mo5-M2/proTGF-α showed that the partial recovery of the capability to shed proTGF-α was concomitant with a partial regaining of the ability of process proTACE to the active form devoid of prodomain (Figure 6D) and a partial recovery of the activation of EGFR in culture cells (Figure 6E). Similar results were obtained with Mo2-Mo5-M2/proHA/TFG-α cell lines (data not shown). Therefore, these results strongly support a crucial role of the shedding of proTGF-α mediated by TACE in the activation of the EGFR in tumors.

Expression of TACE and activation of EGFR in breast tumor samples

Considering that TGF-α and the EGFR are commonly expressed in breast cancers, we hypothesized that TACE might play a role in the biology of this type of tumor and, therefore, we analyzed the expression of TACE in 36 breast tumors and paired histologically normal tissues. As judged by western blot with two indedependently generated antibodies against the cytoplasmic tail of TACE, the majority of tumors analyzed overexpressed the metalloprotease (Figure 7; data not shown); Figure 7 (upper panel) contains a representative group of samples showing low (sample 21), medium (samples 18 and 22) and high (samples 19 and 20) expression of TACE. Note that although TACE was detected in normal samples (data not shown), the concentration of TACE is so high in tumor samples that the signal corresponding to TACE in normal samples lies below detection threshold in the western blot shown in Figure 7A. Surprisingly, the predominant TACE species in tumors apparently corresponds to the processed form and little or no full-length TACE can be detected (Figure 7A). This is in contrast with TACE expressed by

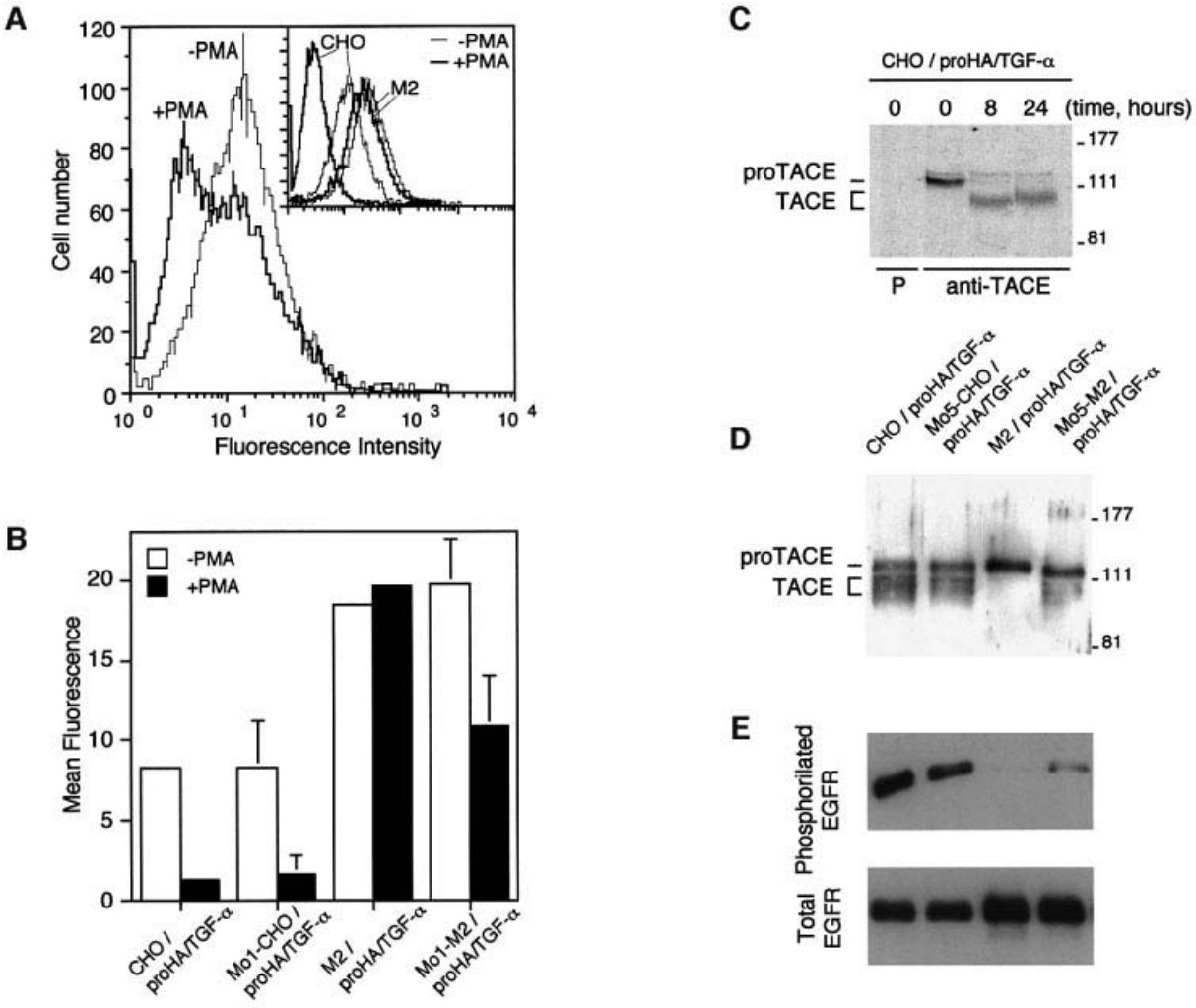


Fig. 6. Characterization of CHO/proTGF-α and M2/proTGF-α cells recovered from tumor xenografts. (**A**) CHO/proHA/TGF-α, M2/proHA/TGF-α (inset) or Mo1-M2/proHA/TGF-α cells, obtained from a tumor induced by M2/proHA/TGF-α cells, were treated with or without PMA and analyzed by flow cytometry using anti-HA antibodies. (**B**) The results of three independent experiments performed as in (A) using CHO/proHA/TGF-α, Mo1-CHO/proHA/TGF-α cells, M2/proHA/TGF-α and Mo1-M2/proHA/TGF-α cells were quantified, the average ± SD are shown. (**C**) CHO/proHA/TGF-α cells were pulsed for 1 h with S^{35}-translabel and chased in complete medium for the indicated times. Cell lysates were immunoprecipitated with antibodies against the cytoplasmic domain of TACE or preimmune serum (P) and immunoprecipitates were resuspended in sample buffer and analyzed by SDS–PAGE and fluorography as described in the Materials and methods. (**D**) Cells obtained from individual xenografts (Mo5-CHO/proHATGF-α and Mo5-M2/proHATGF-α) were lysed and cell lysates were analyzed by western blot with antibodies directed against the cytoplasmic tail of TACE. CHO/proHA/TGF-α and M2/proHA/TGF-α were used as a control. (**E**) Activation of EGFR by Mo5-CHO/proHA/TGF-α and Mo5- M2/proHA/TGF-α cells. The assay was performed as described in Figure 1. CHO/proHA/TGF-α and M2/proHA/TGF-α were used as a control.

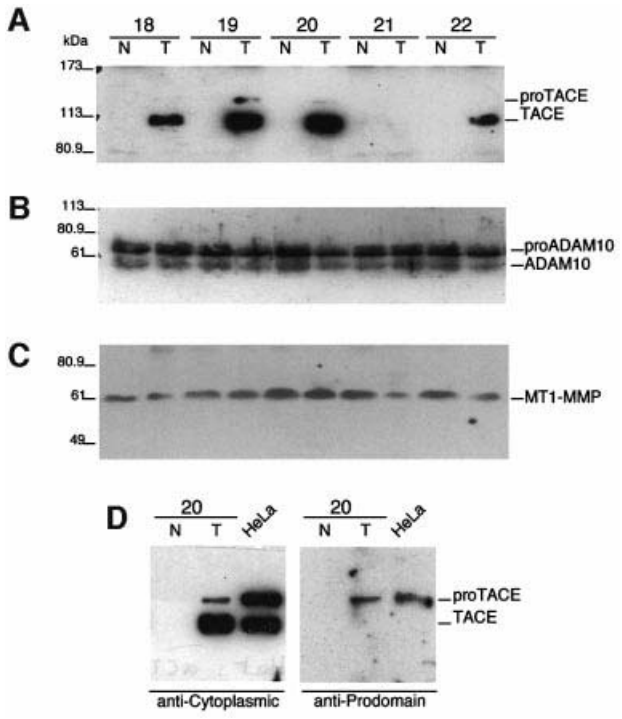


Fig. 7. Analysis of the expression of TACE (**A**), ADAM10 (**B**) and MT1-MMP (**C**) in tumor samples and paired normal tissue. Clarified homogenates of the corresponding tissues were analyzed by western blot with antibodies against the cytoplasmic tail of TACE or ADAM10 or monoclonal antibodies against the metalloprotease domain of MT1-MMP as indicated. (**D**) Samples from tumor number 20, paired normal tissue and cell lysates from HeLa cells were subjected to western blot analysis with polyclonal antibodies against the prodomain of TACE and against the cytoplasmic domain of TACE, respectively.

CHO (Figure 6E) or HeLa cells (Figure 7D) where the TACE species containing the prodomain is readily detected. To confirm the identity of the bands detected in tumor samples, a polyclonal antibody against the prodomain of human TACE was used; as expected, this antibody recognizes only the full-length form of TACE (Figure 7D). In contrast with the overexpression of TACE, other metalloproteases putatively involved in ectodomain shedding such as ADAM10 (also known as kuzbanian) or MT1-MMP are expressed at the same levels in normal and tumors samples (Figure 7B and C). No evident relationships were observed between the expression levels of TACE and the size of the tumors or the presence of regional axilary lymph node metastasis (data not shown). However, to rule out potential associations between TACE and tumor size or metastasis, a larger group of breast cancer patients would need to be studied.

To determine whether the expression of TACE was related to that of TGF-α, EGFR and/or to the activation of the EGFR, we analyzed samples from each tumor by immunohistochemistry as previously described (Albanell *et al*., 2002). TGF-α was detected regardless of the level of TACE (Table I); however, there was a high rate of activation of the EGFR in tumors with high TACE levels compared with those tumors with low TACE expression (Table I; $P < 0.05$ by the χ^2 test). This finding provides support for a link between TACE overexpression and EGFR activation in human breast cancers.

Table I. Expression of TACE, TGF-α, EGFR and activated-EGFR human breast tumor samples

TACE expression[a]	+TGF-α[b]	+EGFR[b]	+Act-EGFR[b]
Low (n = 9)	7 (78)	3 (33)	2 (22)
Medium (n = 17)	9 (53)	5 (29)	5 (29)
High (n = 10)	7 (70)	6 (60)	6 (60)
Total (n = 36)	23 (64)	14 (39)	13 (36)

The number of cells expressing TGF-α, EGFR and activated-ERGF was determined as described previously.
[a]The expression of TACE was analyzed by western blot in tumor samples and compared with that in paired normal tissues. The expression of TACE in tumor samples was considered low, medium or high when overexpressed between 1- to 5-fold, 5- to 10-fold or 10- to 20-fold, respectively.
[b]Number of tumors expressing the indicated marker, with the percentage in parentheses.

Discussion

Since the mechanisms that transduce the signals initiated by the EGFR and related receptors are reasonably well known, many researchers are currently focusing on the elucidation of the mechanisms that regulate the activity of the EGFR ligands. The transport to the cell surface of one of the prototypic EGFR ligands, proTGF-α, exemplifies a first level of regulation and is mediated by proteins that contain a protein–protein interaction domain of the PDZ type (Fernández-Larrea *et al*., 1999; Kuo *et al*., 2000). A second level of regulation occurs at the cell surface; the availability of soluble TGF-α is controlled by ectodomain shedding mediated by the metalloprotease disintegrin TACE (Peschon *et al*., 1998). It has been shown recently that the transport to the cell surface of Spitz, one of the orthologs of proTGF-α in *Drosophila*, is regulated by Star (Lee *et al*., 2001), a transmembrane protein of unknown activity. Rhomboid, a putative intramembrane protease that belongs to a novel family, releases the extracellular domain of Spitz (Urban *et al*., 2001). Thus, the regulatory steps in the biosynthesis of EGFR ligands are conserved during the evolution. However, the factors mediating these mechanisms do not seem to be conserved since Star is not a PDZ-containing protein and metalloprotease disintegrins do not seem to be involved in the shedding of Spitz (Lee *et al*., 2001).

Early publications indicated that the shedding of proTGF-α, and probably that of other EGFR ligands, represented the conversion between two active forms of the ligands: the transmembrane form that activates the EGFR in cells adjacent to the proTGF-α-expressing cell and soluble TGF-α would possibly act on the EGFR of cells located at a distance of the signal-producing cell (reviewed in Massagué and Pandiella, 1993). Although recent reports confirm this conclusion and even suggest that proTGF-α activates the EGFR more potently than TGF-α (Shi *et al*., 2000; Yang *et al*., 2000), others have challenged this view by showing that transmembrane proTGF-α does not seem to be biologically active (Peschon *et al*., 1998; Dong *et al*., 1999). The results presented here provide an explanation for these paradoxical observations and also show, using different approaches, that although transmembrane proTGF-α binds the EGFR this interaction does not lead to activation

of the EGFR in the absence of ectodomain shedding. Therefore, ectodomain shedding not only regulates the availability of soluble TGF-α that can act in a paracrine or autocrine fashion, but also controls the juxtacrine activity of proTGF-α. One explanation to these results is that juxtacrine transduction of signaling mediated by proTGF-α would occur only after the shedding of the growth factor takes place. A similar mechanism has been recently shown for the Eph receptor and its ligand, ephrin-A2 (Hattori *et al*., 2000). Eph receptor binding triggers ephrin-A2 shedding in a localized reaction specific to the cognate ligand (Hattori *et al*., 2000). The signal transduced by Eph results in axon withdrawal, and inhibition of the shedding of ephrin-A2 delays axon withdrawal, indicating that the signaling transduced by Eph depends on the shedding of ephrin-A2.

Several reports indicate that different metalloprotease disintegrins are involved in the shedding of the ectodomain of certain ligands of the EGFR-like receptors. ADAM9 and ADAM12 have been implicated in the shedding of proHB-EGF since dominant-negative forms of these metalloprotease disintegrins inhibit the shedding of the growth factor proHB-EGF (Izumi *et al*., 1998; Asakura *et al*., 2002), and an inhibitor relatively specific for the shedding of proHB-EGF binds to ADAM12 (Asakura *et al*., 2002). In addition, dominant-negative forms of ADAM19 prevent the shedding of proneuregulin-β1 and 4, two ligands of HER3 and HER4 (Shirakabe *et al*., 2001). In contrast to these reports, the characterization of TACE$^{-/-}$ cells and shedding-defective cell mutants indicates that TACE has a role in the shedding of the ligands of the EGFR-like receptors tested to date [proTGF-α (Peschon *et al*., 1998), proHB-EGF (Merlos-Suárez *et al*., 2001), pro-amphiregulin (Sunnarborg *et al*., 2002) and proneuregulin-α-2C (Montero *et al*., 2000)]. On the other hand, cells established from ADAM9 knock-out mice show no apparent defects in the shedding of proHB-EGF (Weskamp *et al*., 2002). Thus, although other metalloproteases may also be involved in the shedding of the ligands of EGFR, genetic evidence indicates that TACE plays a central role in the shedding of these ligands.

Components of the EGFR signaling pathway have been implicated in various forms of human cancer and are used both as prognostic markers and/or as therapeutic targets. Humanized monoclonal antibodies directed against the ectodomain of the EGFR or specific tyrosine kinase inhibitors are currently under different stages of development. The characterization of novel mechanisms that modulate the activity of the EGFR may lead to the expansion of the repertoire of drugs to counteract the signals conveyed by the EGFR-like receptors and their cognate ligands. Prompted by the *in vitro* data, we asked whether the shedding of the ectodomain of proTGF-α is necessary for the development of tumors. The results presented here clearly show that ectodomain shedding is required for activation of the EGFR and for maximal tumor growth *in vivo*. Furthermore, we found that the small tumors induced by shedding-defective cell mutants contained revertants that had regained the ability to proteolytically activate TACE, thus the ability to shed proTGF-α and other EGFR ligands and, hence, the competence to activate the EGFR. Although reversion of shedding-defective cell mutants is frequent after long periods in culture (see Arribas and Massagué, 1995), previous characterization of the clone injected into nude mice (M2) showed that it is particularly stable because it remained unchanged after 1 year in culture. Thus, highlighting the relevance of the shedding of proTGF-α, it appears that in tumors there is a considerable growth advantage of spontaneous shedding-competent revertants. It has been shown recently that overexpression of α 1-PDX, an inhibitor of the proprotein convertase furin, results in significant decrease of the tumorigenicity of head and neck squamous carcinoma cells in nude mice (Bassi *et al*., 2001). Since the proteolytic activation of TACE can be inhibited by α 1-PDX (Brou *et al*., 2000; A.Borroto, S.Ruíz-Paz, M.Borrell-Pagès, T.Villanueva de la Torre, A.Merlos-Suárez, C.P.Blobel, J.Baselga and J.Arribas, manuscript submitted), the effect of this inhibitor could be partially due to a blockade in the ability of TACE to shed transmembrane growth factors.

Since TACE controls the activity of proTGF-α, and probably that of other ligands of the EGFR family of receptors, it can be considered as a target of anti-tumor therapy. However, inhibitors of Zn^{2+}-dependent metalloproteases known to inhibit MMPs and metalloprotease disintegrins have been tested in different clinical trials with little or no success (for a recent review, see Brinckerhoff and Matrisian, 2002). One recurrent suggestion to improve the therapy aimed at inhibiting metalloproteases is the need for specificity. Metalloproteases form large families of highly similar components with different functions, to prevent undesired side effects, ideally the inhibitors should target one metalloprotease. In addition, to shed light on the role of individual metalloproteases, targeted genetic disruptions could help to determine whether efforts to develop specific inhibitors are worthwhile. For example, using mice genetically deficient in MMP-9 it has been shown that MMP-9 is involved in the regulation of the early stages of tumor progression in a mouse model of skin tumorigenesis (Coussens *et al*., 2000) and plays a role in establishing the tumor vasculature, probably through the regulation of the accesibility of angiogenic factors to endothelial cells (Bergers and Coussens, 2000). The results presented here strongly suggest that TACE is also crucial in the development of certain tumors and encourages the search for specific inhibitors.

Materials and methods

Cell lines

CHO cells permanently transfected with proTGF-α tagged at the N-terminus with the HA epitope using resistance to geneticin as selection (CHO/proHA/TGF-α cells), and the shedding-defective mutant cell line M2/proHA/TGF-α have been previously characterized (Arribas *et al*., 1996, 1997). To recover CHO/proHA/TGF-α and M2/proHA/TGF-α cells from tumor xenografts, tumors were surgically removed, minced and cultured in DME containing 20% of FBS and 500 μg/ml of geneticin during 2 weeks. The identity of the cells was then assessed by flow cytometry using the HA monoclonal antibody. A431 cells were purchased from the ATCC.

Antibodies

The anti-EGFR (non-phospho-Y1173) and the anti-phospho-EGFR (Y1173, clone 9H2) monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY), the specificity of these antibodies has been previously reported (Albanell *et al*., 2001). C225 antibodies were kindly provided by Imclone Systems. Polyclonal antibodies against

a polypeptide contained in the cytoplasmic tail of TACE (CPKLDHQRMDTIQEDPSTDSHA) were obtained using standard procedures. Characterization of this antibody showed that it recognized bands identical to those recognized by a previously characterized antibody directed against the cytoplasmic tail of TACE (Schlöndorff *et al*., 2000). Anti-TACE polyclonal antibodies directed against the prodomain of TACE were kindly provided by Dr Carl Blobel. Anti-ADAM10 and anti-MT1-MMP antibodies were generous gifts from Dr Alain Israel and Dr Alicia Arroyo, respectively.

Analysis of the activation of the EGFR

To analyze the juxtacrine activation of the EGFR by proTGF-α, 5×10^5 A431 cells were incubated in the presence or absence of 25 μM BB-94 or 10 μg of anti-HA antibodies as indicated, for 20 min at 37°C with 2.5×10^5 CHO, CHO/proHA/TGF-α or M2/proHA/TGF-α cells (detached from plates with PBS containing 10 mM EDTA at 4°C). To analyze the activation of EGFR by soluble TGF-α, the same number of A431 cells were incubated with the media conditioned with 2.5×10^5 CHO, CHO/proHA/TGF-α or M2/proHA/TGF-α cells treated with or without 25 μM BB-94 or 10 μg of anti-HA antibodies, for 20 min at 37°C. Treated A431 cells were washed with PBS at 4°C and lysed in buffer A (20 mM sodium phosphate pH 7.4, 150 mM sodium chloride, 50 mM EDTA, 1% Triton X-100, 1 mM magnesium chloride, 10 mM orthovanadate and 10 mM 1,10 phenanthroline, 1 mM leupeptin, 1 mM aprotinin) and spun at 15 000 *g* for 15 min at 4°C. Cell lysates were electrophoresed in 7% polyacrilamide gels and subjected to western blotting with anti-EGFR or anti-phospho-EGFR. Signals were detected with Super Signal West Dura Extended Duration Substrate (Pierce). Films were digitized and quantified using MacBAS software (Fujifilm).

Cell–cell interactions mediated by proTGF-α/EGFR

5×10^5 parental CHO, CHO/proHA/TGF-α or M2/proHA/TGF-α cells were detached with PBS containing 10 mM EDTA and incubated with moderate rocking with 10^6 confluent A431 cells for 20 min at 37°C in the presence or absence of 25 μM BB-94 and/or 400 nM of the monoclonal antibody C225, which prevents the interaction between EGFR and its ligands. Then, non-adherent cells were recovered and counted.

Construction and expression of proTGF-α cDNA deletion constructs

Using the cDNA encoding rat proHA/TGF-α (Arribas and Massagué, 1995) as a template, deletions encompassing amino acids 98–102, 93–102 or 90–105 were introduced by PCR and appropriate oligonucleotides using standard techniques. The resulting deletion constructs were subcloned into the pVAO expression vector, co-transfected with a selectable plasmid as previously described (Arribas and Massagué, 1995). Stable transfectants were selected in 500 μg/ml geneticin and subcloned.

Metabolic labeling and immunoprecipitation of TACE products

To analyze TACE processing, ~2×10^6 CHO or M2 cells were metabolically labeled with 1 mCi/ml [^{35}S]Translabel (Biolink 2000, Arlintong Heights, IL) for 1 h in methionine- and cysteine-free medium at 37°C and chased for variable periods of time in complete medium. Cells were then lysed in lysis buffer (PBS containing 1% NP-40, 10 mM 1,10 phenanthroline, 5 mM EDTA and 20 μM BB-94) and the cell lysates were immunoprecipitated with 4 μl of antibodies directed against the cytoplasmic tail of TACE. Immune complexes were collected with protein A–Sepharose, washed three times with washing buffer (PBS containing 0.1% Triton X-100 and 0.1% SDS), diluted 1:50 with lysis buffer and incubated with concanavilin A (ConA)–Sepharose to remove non-glycosylated background proteins as previously described (Schlöndorff *et al*., 2000).

Tumorigenicity assay in nude mice

Cultures of subconfluent CHO, CHO/proTGF-α and M2/proTGF-α cells were trypsinized and centrifuged at 1000 *g* for 5 min. Then, cells were resuspended in serum-free DME and subcutaneously injected into 6- to 8-week-old female nude mice (BALB/c nu/nu; Charles River Laboratories, Wilmington, MA). In each experiment, a total volume of 0.1 ml containing 5×10^6 cells of each type were injected into six mice. Injected mice were examined every 2–3 days for tumor apparition, and mean tumor volume was calculated as described previously (Baselga *et al*., 1993).

Histological analysis of tumors

Tumor xenografts and paired tumor and histologically normal breast were analyzed for each patient ($n = 40$). The breast tissues used in this study were surgical resection specimens obtained at the Vall d'Hebron Hospital following Institutional Guidelines. Specimens were fixed in 10% neutral formalin, dehydrated and embedded in paraffin. Tissue sections of a thickness of 4 μm were placed on poly-lysine-coated glass slides, deparaffined in xylene and rehydrated in graded alcohols. Endogenous peroxidase was blocked by immersion in 0.03% hydrogen peroxide for 15 min. After incubation with a 1/500 dilution of anti-HA, anti-total EGFR or anti-phosphorylated EGFR for 2 h, peroxidase-labeled polymer conjugated to goat anti-rabbit antibodies was applied to the slides. Sections were visualized using 3,3′-diaminobenzidine as a chromogen and lightly counterstained with Mayer's hematoxylin. For quantitative analysis, the percentage of stained cells was scored from representative sections in 10 high-power fields (400×), and the average percentage of cell staining was calculated in every sample.

Western blot analysis of tissue specimens

The breast tissues used in this study were surgical resection specimens obtained at the Vall d'Hebron Hospital following Institutional Guidelines. Paired tumor and histologically normal breast were analyzed for each patient ($n = 35$). Approximately 0.2–0.4 g of tissue was fresh-frozen and stored at –70°C. Frozen tissues were minced on dry-ice and homogenized in buffer A (20 mM sodium phosphate pH 7.4, 150 mM sodium chloride, 50 mM EDTA, 1% Triton X-100, 1 mM magnesium chloride, 10 mM orthovanadate and 10 mM 1,10 phenanthroline) and spun at 15 000 *g* for 10 min at 4°C. The protein concentration in the supernatant was determined by the Lowry assay. Proteins (5–25 μg) from high-speed supernatants of tissue homogenates or cell lysates were directly analyzed or incubated with ConA–Sepharose (Pharmacia) for 1 h at 4°C to concentrate glycoproteins. ConA–Sepharose beads were washed twice with buffer A, resuspended in sample buffer, boiled, electrophoresed in 7% polyacrilamide gels and subjected to western blotting analysis using 1/1000 of the anti-cytoplasmic domain antibodies directed against TACE or 1/500 anti-Kuzbanian polyclonal antibodies or anti-MT1-MMP, as indicated. Signals were detected with Super Signal West Dura Extended Duration Substrate (Pierce). Films were digitized and quantified using MacBAS software (Fujifilm).

RT–PCR and sequencing of β-actin

Double-stranded cDNAs from NIH 3T3, CHO and Mo5-M2/proHA/TGF-α cell lines were synthesized using the Amersham-Pharmacia kit according to the manufacturer's instructions. To sequence α-actin, a 546 bp fragment was amplified using aliquots of the cDNA from the different cell lines and the following oligonucleotides: CCTGACCG-AGCGTGGCTAC and GAAGCATTTGCGGTGGACG. The PCR fragments were purified from agarose gels and sequenced using BigDyeTM Terminator Cycle Sequencing Ready Reaction DNA Sequencing Kit from Pharmacia, or digested with *Sal*I. The products of the digestion were analyzed in agarose gels.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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