Post-transcriptional Up-regulation of ADAM17 upon Epidermal Growth Factor Receptor Activation and in Breast Tumors*\(^5\)

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ADAM17 is a transmembrane metalloprotease involved in the proteolytic release of the extracellular domain of many cell surface molecules, a process known as ectodomain shedding. Despite its likely participation in tumor progression and its current consideration as a therapeutic target, very little is known about the regulation of the expression of ADAM17. Here we show that long term treatment with epidermal growth factor (EGF) leads to a marked increase in the levels of ADAM17. EGF receptor activation does not affect the levels of the mRNA that encodes for, or the rate of synthesis of, ADAM17 but increases its half-life. The effect of EGF is biologically relevant because it increases the shedding of several substrates of ADAM17, including the desmosomal cadherin Dsg-2. Analysis of protein and mRNA levels in mammary tumor samples shows that in vivo the levels of ADAM17 can also be controlled post-transcriptionally. Finally, we show that both the shed extracellular domains of Dsg-2 and ADAM17 are frequently expressed in tumors, further supporting the participation of the metalloprotease in malignant progression.

The ADAMs\(^2\) constitute a large family of transmembrane proteases involved in different physiological processes, including fertilization, heart development, angiogenesis, and neurogenesis (1, 2). Structurally, ADAMs are modular proteins containing the following recognizable domains: signal peptide, prodomain, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane, and cytoplasmic (3). The prodomain, which keeps the enzyme in an inactive state, is typically removed during transit through the secretory pathway; thus, the metalloprotease reaches the plasma membrane in an active state. At the cell surface, certain ADAMs participate in the proteolytic removal of the ectodomain of a significant number of membrane-anchored proteins. This specialized type of proteolysis is known as ectodomain shedding and affects different cell surface molecules, including growth factors, growth factor receptors, and cell adhesion molecules (4).

Increasing evidence points to a role of certain ADAMs, particularly the 17th member of the family, in the development of certain tumors (5). ADAM17, also known as tumor necrosis factor-α-converting enzyme, participates in the activation of the epidermal growth factor receptor (EGFR) and related receptors (6), which play a crucial role in the development of different tumors of epidermal origin (7). All the ligands for the EGFR are synthesized as transmembrane molecules (8), and the inhibition of the shedding of their extracellular domains, carried out by ADAM17 and other ADAMs, prevents the activation of the EGFR in different experimental settings (see for example Refs. 9–11). Therefore, metalloprotease inhibitors targeting ADAM17 have been proposed as anti-tumor drugs (reviewed in Ref. 12).

Despite its importance, the regulation of the expression of ADAM17 remains poorly characterized. Certain cytokines and growth factors up-regulate the transcription of ADAM17 in endothelial and human monocyte-like cells (13, 14). On the other hand, the expression of ADAM17 can be post-transcriptionally down-modulated by phorbol esters and interferons (15, 16). However, the mechanisms and factors that increase the transcription or those that mediate the down-modulation of ADAM17 are unknown and remain uncharacterized. Despite this lack of knowledge, the analysis of its expression in human tumors also points to the participation of ADAM17 in malignant progression (recently reviewed in Ref. 17). For example, ADAM17 mRNA levels are increased in different types of human tumors (18–23). Furthermore, although we did not analyze mRNA levels, we have recently found that ADAM17 protein levels are dramatically augmented in the majority of breast cancer samples analyzed (24).

Here we show that long term treatment of different cells with EGF leads to a marked increase in the levels of ADAM17. EGFR activation does not affect the levels of the mRNA or the rate of synthesis of ADAM17 but increases its half-life. The effect of EGF on ADAM17 is biologically relevant because it increases the shedding of different substrates of the metalloprotease, including the desmosomal cadherin desmoglein-2. Analysis of ADAM17 protein and mRNA levels in mammary tumor samples shows that in vivo the levels of the metalloprotease can also be controlled post-transcriptionally. Finally, we show that both...
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**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—BB-94 and recombinant EGF were obtained from British Biotech and R&D Systems, respectively. Iressa was purchased from AstraZeneca R&D (Wilmington, DE). [α-32P]dCTP (3000 Ci/mmol) was from Amersham Biosciences. Endoglycosidase H (Endo H) was from Roche Applied Science. The polyclonal antibodies against the intracellular domains of ADAM17 and ADAM10 have been described previously (25). The monoclonal antibody against the extracellular domain of Dsg-2 was from Abcam (Cambridge, UK). Anti-APP antibody was from Chemicon, and anti-ALCAM was from Abcam (Cambridge, UK). Anti-APP antibody was from Oncogene Research Products (San Diego, CA). Anti-phospho-MAPK and anti-anti-PKP were from Cell Signaling. Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences. All other chemicals were from Sigma. Furin inhibitor II was from Calbiochem.

**Cell Culture and Treatments with EGF**—A431 and T47D cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium:F-12 medium (1:1) containing 10% (v/v) fetal bovine serum and 2 mM l-glutamine. MCF7 Tet-Off cells (BD Biosciences) were stably transfected with the pUHD10-3 Hyg vector containing mouse ADAM17 under a doxycycline-responsive promoter. Mouse embryonic fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum on 0.25% gelatin precoated cell culture plates.

For EGF treatments, A431 and T47D cells were plated at a density of 2 × 10^5 cells/cm^2. After 24 h, cells were serum-starved for an additional 24 h and then treated with 50 ng/ml EGF, 1 μM Iressa (also known as ZD1839), 10 μM BB-94, or 20 μg/ml cycloheximide as indicated for different periods of time. The cells were then lysed as indicated below. When indicated, cells were also treated with 100 μM chloroquine, 10 μM MG132, or 10 μM BB-94 for different times. Potassium depletion was performed as described in Ref. 26.

**Immunoblotting**—Cells or tissue samples were lysed in lysis buffer (50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% Triton X-100, 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 15,000 × g for 15 min, and proteins in the supernatant were quantified. For Dsg-2 fractionation analysis, Triton-insoluble pellets were solubilized in 10 mM Tris–HCl, pH 7.5, 1% SDS, 8 M urea. Equal amounts of total protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes for Western blot analysis. Conditioned media were collected and concentrated with wheat germ agglutinin–agarose beads (Vector Laboratories, Burlingame, CA) for 2 h at 4 °C, which were eluted directly in SDS-PAGE sample buffer for Western blot analysis. Endoglycosidase H treatments were performed as described in Ref. 27.

**Biotinylation**—Serum-starved A431 cells were treated with 50 ng/ml EGF for different times, shifted to 4 °C, and labeled at the same temperature during 30 min with 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce). The cells were then lysed or washed three times with a quenching solution (phosphate-buffered saline containing 1 mM CaCl2, 0.5 mM MgCl2, and 100 mM glycine) and incubated at 37 °C for different times in the absence or presence of 50 ng/ml EGF and lysed. Biotinylated proteins were isolated from cell lysates with neutravidin beads and analyzed by anti-ADAM17 immunoblotting.

**Reverse Transcription-PCR**—Total RNA was isolated from cultured cells or tissue samples using RNeasy midi kit (Qiagen) according to the manufacturer’s instructions. RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Ambion). Human ADAM17 cDNA was amplified with the following primers: GCACAGGTAATAGCAGTGAGT (forward) and CTCAGCATTTGCAGCTGACTG (reverse). Human β-actin cDNA was amplified using the following primers: CCTGAGCCGGCTGGCTAC (forward) and GAAGCATTTGCGGTGGACG (reverse).

**Northern Blot**—6 μg of total RNA were electrophoresed on a 1% (w/v) agarose gel containing 2.2 M formaldehyde. The RNA was then transferred onto nylon membranes (Hybond-N; Amersham Biosciences). The blots were prehybridized at 65 °C for 2 h in Rapid-hyb buffer (Amersham Biosciences) before hybridization for 16 h at 65 °C with [α-32P]dCTP-labeled DNA probe produced using Ready-to-Go DNA labeling beads (Amersham Biosciences). After stringent washing steps, the blots were exposed to a PhosphorImaging screen.

**Metabolic Labeling and ADAM17 Immunoprecipitation**—2 × 10^4 cells/cm^2 exponentially growing A431 cells were serum-starved and treated with EGF for different times, washed twice with methionine- and cysteine-free medium, and incubated for 30 min in medium supplemented with 1 mCi/ml Tran35S-label (Biolink 2000; Arlington Heights, IL). The cells were then washed and lysed in lysis buffer. Lysates were counted and normalized. Equal counts were precleared by incubation with protein A-agarose for 1 h at 4 °C. Precleared extracts were immunoprecipitated overnight at 4 °C with polyclonal anti-ADAM17 antibody, followed by a 1-h incubation at 4 °C with protein A-agarose. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

**Tumor Samples and Immunohistochemistry**—The breast tissue samples used in this study were surgical resection specimens obtained at the Vall d’Hebron University Hospital following institutional guidelines. For immunohistochemistry analysis, tumor samples were fixed in 10% neutral formalin following the standard procedures and stained with a polyclonal antibody against the cytoplasmic tail of ADAM17.

**RESULTS**

**Post-transcriptional Up-regulation of ADAM17 by EGF**—Despite the very high levels of expression of ADAM17 in the majority of breast tumors analyzed (24), the mechanism of up-regulation has not been characterized. In search for a model cell line for characterizing the regulation of ADAM17 expression, we used T47D and A431 cells because, compared with a panel of cell lines, they express moderate and low levels of the metalloprotease, respectively (25). Treatment with different agonists, including vitamin D₃, retinoic acid, estradiol, phorbol esters,
Post-transcriptional Up-regulation of ADAM17

Dexamethasone, or transforming growth factor-β, lead to little or no variations in the levels of ADAM17 as judged by Western blotting (data not shown). In contrast, prolonged treatment with EGF induced a 2-fold and a 5-fold increase in the levels of ADAM17 in T47D and A431 cells, respectively (data not shown and Fig. 1A). Because the levels of EGFR in A431 cells are very high compared with those in T47D cells (28), this result suggests that, indeed, the activation of the EGFR could lead to the up-regulation of ADAM17. To confirm this conclusion, we treated cells with EGF and Iressa, a specific tyrosine kinase inhibitor that targets the EGFR. As shown in Fig. 1B, Iressa effectively prevented the up-regulation of ADAM17 by EGF. As a control we analyzed the levels of phospho-MAPK, a well characterized downstream target of activated EGFR (7).

Because most, if not all, shedding events carried out by ADAM17 take place at the cell surface (29), intracellular ADAM17, even when processed, is likely biologically inactive. To analyze the subcellular localization of ADAM17 in cells treated with EGF, we performed biotinylation experiments. In agreement with previous reports (27), only mature ADAM17 can be detected at the cell surface (Fig. 1C); pro-ADAM17 is intracellular and localizes largely to the early secretory pathway. As shown in Fig. 1C, treatment with EGF leads to a marked increase of ADAM17 at the cell surface, where it can cleave its substrates.

EGFR activation frequently leads to the transcriptional activation of target genes (7). Therefore, we analyzed the steady-state levels of the mRNA encoding ADAM17 in cells treated with EGF. Analysis, both by Northern blotting and reverse transcription-PCR, showed a slight increase in the levels of the mRNA encoding ADAM17 at 60 h of treatment with EGF (Fig. 2A and data not shown). However, because the increase in protein levels is readily apparent at 24 h (see Fig. 1A), we conclude that the up-regulation of ADAM17 by EGF does not primarily affect the levels of mRNA and therefore is post-transcriptional. To confirm this conclusion, we analyzed the outcome of increasing the expression of ADAM17. As shown in Fig. 2B, induction of mRNA expression by treating with doxycycline a cell line transfected with ADAM17 under the control of a repressible promoter (Tet-Off) leads to the accumulation of pro-ADAM17 and little or no increase of ADAM17 (Fig. 2B). This result is in agreement with recent results showing that transcription of ADAM17 in different cell lines, including A431, leads to the specific accumulation of the proform of the metalloprotease in the early secretory pathway (25, 27, 30). In contrast, treatment with EGF leads to the predominant accumulation of processed ADAM17 (Fig. 2B), further supporting that the mechanism involved is not transcriptional.

EGF Does Not Affect the Rate of Synthesis of ADAM17 but Increases Its Half-life—To further characterize the effect of EGF, we first analyzed the rate of synthesis of ADAM17 in the presence of this growth factor. Thus, we treated cells with or without EGF and followed the levels of the mRNA encoding ADAM17 (Fig. 2B), further supporting that the mechanism involved is not transcriptional.

To assay the half-life of ADAM17, we performed a pulse-chase experiment. Unfortunately, even in the presence of EGF, labeled ADAM17 could not be detected after 6 h of labeling (data not shown), probably because of the low affinity of the antibodies available. This technical problem is complicated by the low levels of expression of ADAM17 in A431 cells (25). As an alternative method to analyze the half-life of ADAM17, we...
used cycloheximide, a compound that inhibits protein synthesis and therefore allows us to monitor the fate of proteins by Western blot. As shown in Fig. 3B, in cells treated with cycloheximide, the half-life of pro-ADAM17 is ~4 h, and the levels of ADAM17 are reduced ~20% during the same period. These results are in agreement with the half-life of ADAM17 as described previously in COS and Chinese hamster ovary cells (27, 31). Treatment of cells with EGF and cycloheximide did not have any effect on the half-life of pro-ADAM17 but prevented the degradation of ADAM17. This result clearly indicates that the effect of EGF is because of an inhibition of the degradation of ADAM17. Unfortunately, this analysis could not be performed at longer time points because of the toxic effects of cycloheximide.

To confirm these results with an independent technique, we monitored the levels of cell surface ADAM17 labeled with biotin in a time course experiment. Consistent with published reports (15), the half-life of biotin-labeled ADAM17 in untreated cells is ~24 h (Fig. 3C). Treatment with EGF clearly increased the half-life of biotinylated ADAM17, further supporting that the increase in the levels observed is because of an inhibition of the degradation of this metalloprotease.

**EGF Activates the Shedding of Different ADAM17 Substrates**—To determine whether the effect of EGF is functionally relevant, and results in the activation of the proteolytic activity of ADAM17, we analyzed the levels of the cell-associated and soluble forms of several substrates of this metalloprotease. We chose APP, ALCAM, and Dsg-2 because they are endogenously expressed by A431 cells (25, 32). ADAM17 is responsible for the shedding of ALCAM (25), whereas both ADAM17 and ADAM10 are involved in the shedding of APP and Dsg-2 (25, 33–35). Concomitantly with the increase in the levels of ADAM17 (Fig. 4D), treatment of A431 cells with EGF leads to an increase in the levels of the soluble forms of Dsg-2, ALCAM, and APP (Fig. 4, A–C). As expected, the increments in the production of soluble extracellular domains are readily prevented by treatment with the metalloprotease inhibitor BB-94 (Fig. 4, A–C), indicating that they are because of increased levels of ectodomain shedding. Despite the fact that ADAM10 can also act on APP and Dsg-2, the increase in the shedding of these molecules is likely attributable to ADAM17, because EGF has no effect on the levels of ADAM10 (Fig. 4D). These results indicate that the up-regulation of ADAM17 by EGF leads to an increase in the shedding of the substrates analyzed.

**Expression of ADAM17 mRNA in Breast Cancer Samples**—Despite the very high levels of expression of ADAM17 in some breast tumors (Fig. 5, A and B), the mechanism of up-regulation is unknown (24); to characterize it, we analyzed the levels of the mRNA encoding the metalloprotease by reverse transcription-PCR and Northern blot in 15 tumor samples and matched normal tissue. Both techniques showed little or no increase of ADAM17 mRNA in tumor samples (Fig. 5, C and D and data not shown). Because of the low availability of normal tissue, note that the corresponding lanes are underloaded in the Northern blot shown in Fig. 5D; however, normalization of the quantitative data confirmed little or no increase in the levels of ADAM17 mRNA in samples from mammary tumors. Thus, as in the case of the effect of EGF, these results showed that an increase in the mRNA levels is not the main mechanism of up-regulation of ADAM17 in the mammary tumors analyzed.

**Expression of Dsg-2, an ADAM17 Substrate, in Breast Cancer Samples**—To characterize the biological meaning of ADAM17 overexpression, we analyzed the levels of Dsg-2 in tumor samples. We chose Dsg-2 because the membrane-anchored and the soluble forms of this desmosomal cadherin can be identified because of their different electrophoretic mobilities. Full-length Dsg-2 migrates as a 180-kDa band (Fig. 6A, see also Fig.
Post-transcriptional Up-regulation of ADAM17

Despite its probable importance in tumor progression, current knowledge on the regulation of the expression of ADAM17 is incomplete and fragmentary. Few studies have addressed this issue in vitro showing that certain agonists transcriptionally up-regulate the levels of this metalloprotease, whereas others can down-regulate the metalloprotease post-transcriptionally (13–16). On the other hand, several authors have analyzed the levels of ADAM17 mRNA in tumor samples, and others have analyzed its protein levels, and both types of studies have concluded that ADAM17 is frequently overexpressed in human tumors (18–20, 22–24, 37). Therefore, taken together, the current published results indicate that the up-regulation of ADAM17 is because of an increased level of transcription and that such a mechanism could justify the up-regulation observed in tumors. However, in this study we show that in vitro, the levels of ADAM17 can be up-regulated post-transcriptionally up to 5-fold. In mammary tumor samples and matched controls, we do not observe a correlation between ADAM17 mRNA and protein levels, leading us to conclude that the levels of active ADAM17 can be post-transcriptionally up-regulated in vivo.

Even a modest increase in the levels of ADAM17 mRNA leads to the specific accumulation of the proform of the metalloprotease (see this report and Refs. 27 and 30). Therefore, there seems to exist a mechanism that restricts the levels of active ADAM17, even when the transcription of the gene is up-regulated. This mechanism is not likely the regulation of processing because that of ADAM17 does not seem limiting; different proprotein convertases can efficiently process the metalloprotease even in the absence of furin (27) or when furin is inhibited (supplemental Fig. 1B). Conceivably, the mechanism that restricts the levels of active ADAMs affects its trafficking and includes a saturable component necessary for the progression of the metalloprotease to the trans-Golgi network, where the prodomain is removed (27, 38). In agreement with this hypothesis, all pro-ADAM17 in transfected cells is located in the early secretory pathway as judged by endo-H sensitivity (supplemental Fig. 2).

EGF acts by stabilizing the levels of processed ADAM17 at the cell surface, allowing the accumulation of the active metalloprotease and resulting in increased levels of ectodomain shedding. This mechanism of action (i.e. the stabilization of...
ADAM17 at the cell surface) provides an explanation for the long period of time required to detect the effect of EGF. ADAM17 is a long lived protein (t<sub>1/2</sub> = 110 ± 12–24 h, depending on the cell line analyzed); therefore, the increase in the levels of the metalloprotease by EGF requires a time equivalent to its half-life to be detectable.

The long period of EGF treatment required to detect the accumulation of ADAM17 makes it difficult to characterize the mechanism involved. A plausible possibility is that EGFR activation (31) stabilizes cell surface ADAM17 by decreasing the rate of endocytosis of the metalloprotease. To test this possibility we depleted potassium from cells, a commonly used procedure to inhibit clathrin-mediated endocytosis. However, this treatment resulted in detachment of cells after 2 h (data not shown), precluding the analysis of the effect of EGF. An alternative approach, the introduction of cis-acting mutations and/or deletions interfering with the internalization of ADAM17 cannot currently be used because of the accumulation of transfected ADAM17 in the early secretory pathway (see above). The future characterization of the machinery required to transport ADAM17 to the cell surface will probably allow the use of this approach.

It has been shown previously that ADAM17 can cleave its own cytoplasmic tail (31), opening the possibility that the levels of ADAM17 can be regulated by autolysis. However, treatment with BB-94, which efficiently inhibits ADAM17, has no effect on the levels of the metalloprotease in the presence or absence of EGF (supplemental Fig. 1A), arguing that no metalloprotease inhibited by BB-94 participates in the turnover of ADAM17.

Because mammary tumor samples overexpress the processed form of ADAM17, it can be concluded that the overexpression of ADAM17 in tumors cannot be the result of a mere up-regulation of the transcription of the metalloprotease. In agreement with this conclusion, the mRNA levels of ADAM17 are not increased in the samples analyzed. It is tempting to speculate that, by analogy with the effect of EGF in vitro, in mammary tumors where the EGFR and related receptors are frequently overactivated, the overexpression of ADAM17 is because of a stabilization of the metalloprotease. In turn, the overexpression of ADAM17 likely contributes to establish a feedback loop in mammary tumors because of its role in EGFR ligand shedding.

Given the remarkable promiscuity of ADAM17, the biological consequences of its overexpression are not restricted to the EGFR activation. The analysis of the shedding of different cell adhesion molecules shows that increased ADAM17 expression leads to increased shedding of the ectodomains of Dsg-2, ALCAM, and APP. Because these molecules support cell-cell adhesion, the shedding of their extracellular domain likely con-
tributes to weaken cell-cell contacts, a likely requirement for epithelial cell invasion. The finding of the soluble form of Dsg-2 in human tumor samples that overexpress ADAM17 seems to support this hypothesis.

In summary, the results presented here show that the stabilization of ADAM17 is a likely mechanism of up-regulation of the metalloprotease in vitro and in tumors and further support the consideration of this metalloprotease as a useful anti-tumor target.

Acknowledgment—We thank Ines Teixeira for technical help.

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