Cleavage of the HER2 Ectodomain Is a Pervanadate-activable Process That Is Inhibited by the Tissue Inhibitor of Metalloproteases-1 in Breast Cancer Cells

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Abstract

HER2/neu, a Mγ 185,000 tyrosine kinase receptor that is overexpressed in breast cancer, undergoes proteolytic cleavage of its extracellular domain (ECD). In contrast with other membrane-bound proteins, including growth factor receptors, that are cleaved by a common machinery system, we show that HER2 cleavage is a slow process and is not activated by protein kinase C. Pervanadate, a general inhibitor of protein-tyrosine phosphatases, induces a rapid and potent shedding of HER2 ECD. The shedding of HER2 ECD is inhibited by the broad-spectrum metalloprotease inhibitors EDTA, TAPI-2, and batimastat. The tissue inhibitor of metalloproteases-1; an inhibitor of matrix metalloproteases that does not inhibit cleavage by the general protein kinase C-dependent shedding machinery, also inhibited HER2 ECD shedding, whereas tissue inhibitor of metalloproteases-2 did not. These data suggest that HER2 cleavage is a process regulated by an as-yet-unidentified distinct protease.

Introduction

The HER2/neu gene (also known as neu and as c-erbB-2) encodes a Mγ 185,000 transmembrane glycoprotein receptor (HER2, c-erbB-2) that has partial homology with the other members of the epidermal growth factor receptor family (1). HER2 is overexpressed in 25–30% of breast cancers, and it has been associated with a high risk of relapse and death (2). The HER2 ECD can be released and detected in the conditioned medium of HER2-overexpressing cells (3–6). HER2 ECD is also detected in the serum of cancer patients, indicating that HER2 secretion actually occurs in vivo (7–11). Several observations suggest that this process is of clinical importance. High serum levels of HER2 ECD correlate with a poor prognosis and decreased responsiveness to endocrine therapy and chemotherapy in patients with advanced breast cancer (9–11). Such findings may be due to the potentially enhanced signaling activity of amino-terminally truncated, cell-associated HER2 molecules that are predicted to appear upon HER2 cleavage. This view is suggested by studies showing that an engineered deletion of the ECD of HER2 increases the tyrosine kinase activity and transforming efficiency of the resulting amino-terminally truncated HER2 protein (12, 13). On the other hand, the released HER2 ECD has the capability to bind to anti-HER2 monoclonal antibodies and to induce resistance to their growth-inhibitory activity in the laboratory (6). In addition, in patients with breast cancer treated with the anti-HER2 antibody trastuzumab (Herceptin), high serum levels of HER2 ECD result in altered pharmacokinetics and possibly in resistance to therapy (7, 8).

Many cell surface transmembrane proteins, including growth factor receptors, can be released from the cell surface by a general shedding system activable by several independent mechanisms, the best characterized of which involves PKC (reviewed in Ref. 14). The proteolytic component(s) of the shedding machinery can be blocked by hydroxamic acid-derived metalloprotease inhibitors, such as TAPI (14). To date, only members of the metalloprotease-disintegrin family (also known as ADAMs; reviewed in Ref. 15) have been convincingly shown to participate in protein ectodomain shedding. Recently, TACE, a metalloprotease-disintegrin initially isolated by its ability to cleave proTNF-α, has been found to play a central role in protein ectodomain shedding because the shedding of a variety of structurally and functionally unrelated transmembrane molecules is deficient in cells genetically impaired in TACE (16).

Despite the existence of an alternatively spliced truncated form of HER2 that lacks the transmembrane and cytoplasmic domains (17), experiments with cultured cells suggested that HER2 ECD can arise by proteolytic cleavage of transmembrane full-length HER2 (3–5). However, the proteolytic activity responsible for this cleavage has been poorly characterized. We now report that, in contrast to the majority of transmembrane proteins susceptible of ectodomain shedding, the cleavage of cell surface HER 2 is slow, even in the presence of PKC activators, and is inefficiently blocked by the metalloprotease inhibitor TAPI. However, the cleavage of HER2 is enhanced by pervanadate, a general phosphotyrosine phosphatase inhibitor, suggesting that the shedding of HER2 ECD is regulated by tyrosine phosphorylation. In the cell lines tested, we found that metalloprotease activity is involved in the shedding of HER2 ECD because this process was inhibited by broad-spectrum metalloprotease inhibitors. Furthermore, BB-94, a synthetic compound initially designed to inhibit MMPs that has entered clinical trials (18), also blocks HER2 ECD shedding. Notably, the naturally occurring inhibitor of MMPs, TIMP-1, also inhibited HER2 cleavage, suggesting that the proteolytic activity might be different from TACE.

Materials and Methods

Materials. PMA, sodium orthovanadate, EDTA, EGTA, phosphoramidon, diprotin A, diprotin B, bestatin, amastatin, aprotinin, and pepstatin were purchased from Sigma Chemical Co. (St. Louis, MO). Pervanadate was freshly prepared for each experiment by mixing vanadate (1 mM) and H2O2 (1 mM) and was used within 20 min of preparation. E-cadherin (Ab-2) monoclonal antibody was purchased from Oncogene Research Products (Cambridge, MA). Recombinant human TIMP-1, and recombinant human TIMP-2 were purchased from Fuji Chemical...
were chased for different periods of time, shifted to 4°C, and incubated with 5 mM EDTA (lysis buffer). Aliquots from the cell lysates and the media were immunoprecipitated with the monoclonal antibodies trastuzumab or Ab-2, both directed against the ectodomain of HER2. Immune complexes were collected by incubation of cell lysates and media samples with protein A and G-Sepharose for 45 min at 4°C, washed three times with PBS containing 0.1% Triton X-100 and 0.1% SDS, and analyzed by SDS-PAGE. The shedding of proTGF-α was analyzed by immunoprecipitation or FACS analysis, as described previously (20).

For immunoprecipitation of cell surface HER2, metabolically labeled cells were chased for different periods of time, shifted to 4°C, and incubated with 5 μg/ml Herceptin in PBS containing 5% BSA for 1 h at 4°C. Cells were then extensively washed with PBS to eliminate any Herceptin unbound to cell surface HER2 and lysed as described above. Immune complexes were collected and analyzed as described above.

Western Blotting. Western blotting using monoclonal antibodies against the ECD of βAPP (22C11) was performed using standard techniques.

Flow Cytometry Analysis. CHO cells stably expressing proHA/TGF-α, BT-474, or SK-BR-3 were treated with or without 1 μM PMA for variable periods of time. PMA-treated cells were washed three times with PBS, detached with PBS containing 10 mM EDTA, and incubated with 10 μg/ml anti-hemagglutinin monoclonal antibodies or the monoclonal antibody Ab-2 directed against the ectodomain of HER2 for 30 min at 4°C. Cells were then washed and incubated with 1:100 goat antimouse FITC under the same conditions. Flow cytometry was done using a FACS instrument and software (Becton Dickinson).

Construction and Transfection of HER2-Tα juxt. The HER2-Tα juxt construct was generated by inserting the sequence encoding amino acids 98–107 corresponding to the juxtamembrane domain of rat proTGF-α (20) into the unique Ac7 III site of human HER2, using standard techniques. The final construct was confirmed by sequencing, subcloned into the expression vector pDNA 3.1 Zeo+ (Invitrogen), and cotransfected in CHO cells with the selectable plasmid pREP4 at a DNA ratio of 1:25 using the calcium phosphate precipitation method. Stable transfecants were selected in 600 μg/ml hygromycin (Life Technologies, Inc.) and subcloned. The expression of HER2 was analyzed by flow cytometry.

Results

Constitutive Cleavage of Cell Surface HER2. To analyze the kinetics of HER2 ECD production, pulse-chase experiments were performed using human breast cancer cell lines BT-474 and SK-BR-3, because these cell lines have been previously shown to overexpress HER2. In agreement with previous results (5), immunoprecipitation using specific antibodies shows the presence of a soluble M, 110,000 form of HER2 in the conditioned medium of BT-474 and SK-BR-3 (Fig. 1, A and B; data not shown). Concomitantly, the decrease in the cell-associated M, 110,000 form of HER2 is appreciable (Fig. 1, A and B), indicating a precursor-product relationship between both forms of HER2 and suggesting that the M, 110,000 form arises by proteolytic cleavage of the M, 185,000 form. The soluble M, 110,000 form of HER2 very likely corresponds to the HER2 ECD because it can be immunoprecipitated with different antibodies against HER2 ECD

Fig. 1. Biosynthesis of HER2 and kinetics of HER2 ECD release. A and B, kinetics of HER2 ECD secretion. Metabolically labeled BT-474 cells were chased for the indicated times in complete medium at 37°C. Cell lysates and media supernatants were immunoprecipitated with Herceptin, a monoclonal antibody against the ECD of HER2. The immune complexes were precipitated with protein A and G-Sepharose, analyzed by SDS-PAGE, quantified using a PhosphorImager, and represented. For presentation purposes, the gel containing the cell-associated HER2 was exposed for 12 h, whereas the gel containing HER2 ECD was exposed for 1 week. C; metabolically labeled BT-474 cells were chased for 1 h in complete medium at 37°C. At the indicated times, cells were shifted at 4°C, lysed, and immunoprecipitated with Herceptin (top panel) or incubated with 5 μg/ml Herceptin for 1 h at 4°C, washed, and lysed. Immune complexes were precipitated with protein A and G-Sepharose and analyzed by SDS-PAGE and by using a PhosphorImager.
(Fig. 1A; data not shown) but not with antibodies against the intracellular domain of HER2 (data not shown). A low level of HER2 ECD is detected 30 min after labeling (data not shown). However, the shedding of HER2 ECD is slow, reaching a maximum level at 48 h, and inefficient, because only 5% of HER2 molecules labeled after the pulse undergo shedding of their ectodomain in the cell lines examined (Fig. 1B; data not shown). For presentation purposes, the gel corresponding to the HER2 ECD has been overexposed (see the legend of Fig. 1).

The majority of cell surface transmembrane molecules that undergo ectodomain shedding have been found to be cleaved at the cell surface (14). A possible explanation for the slow kinetics of HER2 ECD shedding could be a slow intracellular trafficking of HER2. Thus, we analyzed the kinetics of HER2 transport to the cell surface by pulse-chase experiments, followed by immunoprecipitation of total or cell surface HER2 as described in “Materials and Methods.” As shown in Fig. 1C, HER2 reaches the cell surface within 2 h after synthesis, indicating that the slow kinetics of ectodomain shedding are not due to slow intracellular trafficking of HER2.

**HER2 ECD Shedding Is Regulated by Tyrosine Phosphorylation.** The cleavage of the vast majority of proteins susceptible to ectodomain shedding, including growth factor receptors, is rapidly activated via PKC (14). To determine whether the shedding of HER2 ECD is also activable via PKC, we analyzed the effect of PMA, a potent activator of PKC, on the shedding of HER2 ECD in BT-474 cells. PMA induces a rapid down-modulation of several substrates of the general shedding system (see Ref. 20); in contrast, the levels of cell surface HER2 remain unchanged after PMA treatment as judged by FACS analysis (Fig. 2A), indicating that the shedding of HER2 ECD is not activable via PKC. This result is not due to a deficiency in the PKC-activated shedding machinery of BT-474 or SK-BR-3 cells, because the shedding of endogenous βAPP, a well-characterized...
PKC-activated process, is augmented six to eight times by PMA in these cells and is kinetically indistinguishable from the shedding of βAPP in CHO cells (data not shown).

It has been previously shown that short juxtamembrane sequences of substrates of the PKC-activated shedding system, such as pro-TGF-α or βAPP, are sufficient to endow transmembrane molecules with the ability to be cleaved by the PKC-activated shedding system (19). To determine whether HER2 is accessible to the PKC-activated shedding machinery, we introduced a short juxtamembrane segment of pro-TGF-α into HER2 and analyzed the shedding of the resulting HER2-Tα jux construct. As shown in Fig. 2B, PMA induced the typical loss of cell surface immunoreactivity that follows the activation of the shedding machinery by PKC in CHO cells transfected with HER2-Tα jux but not in CHO cells transfected with wild-type HER2. These results show that HER2 is accessible but is not a substrate of the PKC-activated shedding system and suggest the existence of an unidentified proteolytic activity not activated by PKC that acts on HER2.

Next, we analyzed whether the shedding of HER2 ECD could be regulated by phosphorylation/dephosphorylation using pervanadate, a general phosphotyrosine phosphatase inhibitor that increases the phosphotyrosine content of many intracellular proteins. Exposure of cells to pervanadate induced a potent shedding of HER2 ECD, and this effect was specific, because 1 mM vanadate or 1 mM H2O2 did not activate HER2 ECD shedding (Fig. 2C). Exposure of cells for various time points (30 min to 8 h) to a wide range of concentrations (50–1000 μM) of pervanadate resulted in the appearance of the M, 110,000 HER2 ECD form in a time- (Fig. 2D) and dose-dependent manner (data not shown). Taken together, the results presented indicate that the proteolytic activity that sheds HER2 ECD is regulated by phosphorylation/dephosphorylation but not by PKC, a modulator of the shedding of the ectodomain of many transmembrane molecules.

**Metalloprotease-dependent Shedding of HER2 ECD.** To determine the type of protease responsible for HER2 ECD shedding, we analyzed the effects of a series of protease inhibitors on HER2 ECD shedding. In pulse-chase experiments, cells were incubated in the presence of various protease inhibitors for 8 h (Table 1). Serine, cysteine, and aspartic protease inhibitors failed to inhibit the release of HER2 ECD to the medium. In contrast, several metalloprotease inhibitors significantly reduced the release of HER2 ECD to the medium in a dose-dependent fashion (Table 1, Fig. 3). Interestingly, TAPI-2, a hydroxamic acid-based inhibitor, had a limited inhibitory effect on the shedding of HER2 ECD (Table 1) at concentrations that block the shedding of most proteins tested thus far (20). The involvement of metalloprotease activity in the shedding of HER2 ECD was further suggested by experiments performed with BB-94, a synthetic compound initially designed to inhibit MMPs that has been tested in cancer clinical trials as an antitumor agent (18). BB-94 was found to inhibit the shedding of HER2 ECD at nanomolar concentrations. Pretreatment of cells with BB-94 also inhibited the pervanadate-induced shedding of HER2 ECD (data not shown).

Hydroxamic acid-based inhibitors such as TAPI or BB-94 have been found to block the activity of metalloproteases of the ADAM family as well as of metalloproteases of the MMP family. In contrast, the MMP inhibitors TIMP-1 and TIMP-2 do not inhibit the shedding of molecules cleaved by members of the ADAM family (21, 22). Therefore, we next addressed the question of whether the protease activity responsible for the cleavage of HER2 ECD could be inhibited by TIMP-1 and TIMP-2. As shown in Fig. 3C, TIMP-1 inhibits the shedding of HER2 ECD in the micromolar range, whereas TIMP-2 is without effect in the same range (Fig. 3C). These results indicate that the protease responsible for the shedding of HER2 ECD is a metalloprotease that does not have the characteristics of the ADAM pro-

<table>
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<th>Percentage of inhibition</th>
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*Shedding of HER2 ECD was tested in BT-474 cells as described in “Materials and Methods.” The results are expressed as percentages relative to untreated cells and are the averages of at least duplicate determinations.

**Discussion**

These studies demonstrate that the release of HER2 ECD in human breast cancer cells with endogenous overexpression of HER2 is a regulated process. This process, however, seems to be regulated in a different fashion than the shedding of most transmembrane proteins tested to date. Whereas activators of PKC, which are known to accelerate the shedding of many transmembrane molecules, failed to activate HER2 ECD release, a general phosphatase inhibitor potently induced the shedding of HER2 ECD, indicating that the shedding machinery that acts on HER2 is regulated by the phosphotyrosine content of an unidentified component. The proteolytic activity responsible for the cleavage of HER2 was blocked by an inhibitor of MMPs, TIMP-1. To our knowledge, this is the first study that involves TIMP-1 in the inhibition of the shedding of a transmembrane protein.

The PKC-activated shedding system was found intact in the cell lines tested in the present study, because the constitutive and PMA-induced shedding of βAPP was kinetically and quantitatively indistinguishable from that of other cell lines in which the shedding machinery has been thoroughly characterized, such as CHO cells. However, our data suggest that an unidentified shedding system that differs from the PKC-activated system is involved in the release of HER2 ECD. The kinetics of shedding of HER2 ECD are slow, as determined by the observation that less than 10% of cell surface HER2 molecules undergo shedding of their HER2 ECD at 48 h in the absence of activators. Studies on the trafficking of HER2 indicate that the slow shedding of HER2 ECD is not due to a slow transport of HER2 to the cell surface. The slow constitutive shedding of HER2 ECD was not activated by treatment with the PKC activator PMA. This finding is in agreement with a previous report in murine fibroblasts transfected with HER2 in which PKC activation failed to induce the shedding of the HER2 ECD (23).

Although PKC does not activate HER2 ECD shedding, our experiments indicate that phosphorylation content of one or more of the molecules regulates the shedding of HER2 ECD. Pervanadate (complexes of vanadate and hydrogen peroxide), a general inhibitor of protein-tyrosine phosphatases, that raises the tyrosine phosphate content of many intracellular proteins, induced a rapid shedding of HER2 ECD in our cells. The pervanadate-induced shedding was inhibited by pretreatment of the cells with BB-94, suggesting that this activated process is metalloprotease dependent. In recent reports, it has been shown that the shedding of the ectodomain of other transmembrane proteins can be activated by the general tyrosine phosphatase inhibitor...
pervanadate. These proteins include the HER4 receptor, which belongs to the epidermal growth factor receptor family (23), pro-amphiregulin, a precursor growth factor (23), syndecan-1, a heparan sulfate proteoglycan (24), and βAPP (25). Whereas the shedding of all these molecules is also activated by PKC stimulation, our studies on HER2 show that the activated cleavage of this receptor differs in the lack of PKC-induced cleavage. Overall, the data presented here provide the first evidence showing that HER2 ECD shedding is an activable process regulated by the phosphotyrosine content of one or more molecules.

In an attempt to characterize the proteolytic activity responsible for the constitutive shedding of HER2, we analyzed a series of protease inhibitors. Three metalloprotease inhibitors of broad-spectrum tested, EDTA, TAPI-2 and BB-94, inhibited the cleavage of HER2. It is important to note that the concentration of TAPI-2 (25 μM) that completely blocks the shedding of several transmembrane molecules that are substrates of the PKC-activated shedding system (20) has a modest effect on the shedding of HER2 ECD. TAPI and BB-94 have a similar potency to inhibit the metalloprotease-disintegrin TACE in vitro (26), the only metalloprotease that has a demonstrated role in the shedding of several transmembrane molecules. These data, and the greater activity of BB-94 over TAPI to inhibit the shedding of HER2 ECD observed in our study, suggest that TACE is not involved in the constitutive release of HER2 ECD.

The activities of all known MMPs can be inhibited with different specificities by TIMPs (reviewed in Ref. 27). To date, four members (TIMP-1, -2, -3, and -4) of the family of naturally occurring TIMPs have been characterized. TIMP-3 (but not TIMP-1, -2, and -4) has been found to potentially inhibit TACE in vitro (22) and modulates the shedding of TNF receptor, interleukin 6 receptor, TNF-α, and L-selectin (Ref. 22 and references therein and Refs. 28 and 29). In contrast, TIMP-1 did not affect the shedding of the interleukin-6 receptor (2 μg/ml; Ref. 28), TNF-α receptors (100 μg/ml; Ref. 29), or L-selectin (30 μg/ml; Ref. 30). Recently, TIMP-2 was found to inhibit the shedding of TNF-α receptors in a human colon cancer cell line (29). In the present study, we found that the shedding of HER2 ECD can be inhibited by TIMP-1 but not TIMP-2. The inhibition of HER2 ECD shedding by TIMP-1, which does not inhibit TACE, opens the possibility that a MMP may be involved in the proteolytic cleavage of HER2.

Because high levels of HER2 ECD bind to anti-HER2 antibodies and neutralize their antitumor effects (6), it is tempting to speculate that the exposure of HER2-overexpressing tumor cells to inhibitors that block HER2 ECD shedding (such as TIMP-1, BB-94, or deri-

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Fig. 3. Effect of metalloprotease inhibitors on the shedding of HER2 ECD. Metabolically labeled BT-474 cells were chased for 8 h at 37°C in the presence of the indicated concentrations of EDTA (A), BB-94 (B), TIMP-1, or TIMP-2 (C). Media supernatants were immunoprecipitated with Herceptin, and immune complexes were precipitated with protein A and G-Sepharose, analyzed by SDS-PAGE, quantified, and represented. Results are the average ± SD of triplicate determinations.
vates) before therapy with anti-HER2 monoclonal antibodies such as trastuzumab (Herceptin) may help to counteract this problem. In experimental models, endogenous MMP inhibitors such as TIMP-1 (reviewed in Ref. 27) and synthetic inhibitors such as BB-94 have suppressive activities on tumor growth, invasion, and metastasis (Ref. 18 and references therein). The use of MMP inhibitors in cancer patients appears to be safe, suggesting that these compounds have the potential to be developed clinically. In a Phase I clinical trial, the i.p. administration of BB-94 to patients with malignant ascites that resulted in plasma levels of BB-94 above those shown to be effective in animal models was well tolerated. Encouraging clinical activity was also seen, despite the fact that the therapeutic activity of BB-94 was not a goal of the study (18). Hence, because the shedding of HER2 ECD can be inhibited by compounds with clinical potential, together with the fact that elevated levels of shed HER2 ECD result in increased resistance to anti-HER2 antibodies, we propose that a combined therapeutic approach with inhibitors of HER2 cleavage and Herceptin may be of interest.

In summary, we show that a proteolytic system that is regulated by phosphorylation/dephosphorylation but not by PKC is responsible for the shedding of HER2 ECD. The process is regulated as demonstrated by the ability to induce a rapid and potent HER2 ECD shedding after exposure to the general phosphatase inhibitor pervanadate. The protease responsible for HER2 ECD shedding seems to be a metalloprotease different from TACE, which can be inhibited by TIMP-1. Further understanding of this process could have potential therapeutic applications for the therapy of patients with breast cancer and high levels of HER2 shedding.

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References

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