Tyrosine Phosphorylation and Proteolysis

PERVANADATE-INDUCED, METALLOPROTEASE-DEPENDENT CLEAVAGE OF THE ErbB-4 RECEPTOR AND AMPHIREGULIN

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Enhancement of tyrosine phosphorylation in cells by the application of pervanadate, an extremely potent phosphotyrosine phosphatase inhibitor, provokes the rapid metalloprotease-dependent cleavage of ErbB-4, a transmembrane receptor tyrosine kinase. The pervanadate-induced proteolysis occurs in NIH 3T3 cells expressing transfected human ErbB-4 and in several cell lines that express endogenous ErbB-4. One product of this proteolytic event is a membrane-anchored molecule of approximately 80 kDa, which is heavily tyrosine phosphorylated and which possesses tyrosine kinase catalytic activity toward an exogenous substrate in vitro. This response to pervanadate is not dependent on protein kinase C activation, which has previously been demonstrated to also activate ErbB-4 cleavage. Hence, the pervanadate- and 12-O-tetradecanoylphorbol-13-acetate-induced proteolytic cleavage of ErbB-4 seem to proceed by different mechanisms, although both require metalloprotease activity. Moreover, pervanadate activation of ErbB-4 cleavage, but not that of 12-O-tetradecanoylphorbol-13-acetate, is blocked by the oxygen radical scavenger pyrrolidine dithiocarbamate. A second phosphotyrosine phosphatase inhibitor, phenylarsine oxide, also stimulates a similar cleavage of ErbB-4 but, unlike pervanadate, is not sensitive to pyrrolidine dithiocarbamate. Last, pervanadate is shown to stimulate the proteolytic cell surface processing of a second and unrelated transmembrane molecule: the precursor for amphiregulin, an epidermal growth factor-related molecule. Amphiregulin cleavage by pervanadate occurred in the absence of a cytoplasmic domain and tyrosine phosphorylation of this substrate.

ErbB-4 is a receptor tyrosine kinase member of the epidermal growth factor (EGF) receptor family (1) which can be activated by various heregulin/neuregulin isoforms (2, 3) plus the EGF receptor agonists betacellulin (4), heparin-binding EGF (5), or epiregulin (6). However, other EGF receptor agonists such as EGF or transforming growth factor α, are reported to not bind to ErbB-4 (7, 8). Following ligand binding and the initiation of signal transduction pathways, the EGF receptor is rapidly trafficked to coated pits, internalized, and ultimately degraded when endosomes fuse with lysosomes (9). This receptor trafficking pathway gives rise to down-regulation of the EGF receptor and is a feature of most all growth factor-activated receptor tyrosine kinases (10).

In contrast to the processing of the EGF receptor, the ErbB-4 receptor, as well as ErbB-3 and ErbB-2, are not subject to rapid internalization following ligand binding (11–13). The molecular basis for this difference is not clear; however, only the activated EGF receptor is able to associate with the adaptin AP-2 coated-pit molecule (12). Subsequently, it has been found that ErbB-4, but not other EGF receptor family members, is subject to proteolytic cleavage that produces a membrane-anchored 80-kDa cytoplasmic domain fragment and a 120-kDa ectodomain fragment (14, 15). Based on inhibitor studies, it is likely that this cleavage is due to a metalloprotease. Binding of an ErbB-4 ligand, however, does not alter the rate of proteolytic cleavage. Activation of protein kinase C, directly with TPA or indirectly by occupancy of the platelet-derived growth factor receptor, dramatically accelerates the rate of metalloprotease-dependent ErbB-4 cleavage (14). While the exact site of cleavage is not known, an ErbB-4 isoform, HER4 JM-b, containing a 13-residue sequence insertion within the ectodomain juxtamembrane region is resistant to TPA-induced cleavage (16). This suggests that the sequence insertion, a result of alternative mRNA splicing, has interrupted the metalloprotease cleavage site. Also, a chimeric ErbB-4 receptor in which the ectodomain is replaced by that of the EGF receptor is resistant to TPA-induced cleavage (14).

Ectodomain cleavage occurs frequently among cell surface transmembrane proteins including several that are involved in the control of cell proliferative behavior: receptor tyrosine kinases (14, 15, 17–23), growth factor precursors (24–27), cell adhesion molecules (27–29), and protein tyrosine phosphatases (30). In a few cases, such as ErbB-4 (15), i-selectin (27), cadherin (29), heparin-binding EGF (24), transforming growth factor α (27), EGF (26), amphiregulin (31), and tumor necrosis factor (32, 33), metalloproteases are implicated in the proteolytic cleavage. In some cases, the physiological significance of these cleavage events has been demonstrated to be biologically significant; the release of diffusible growth factors, reduced cell adhesion, or increased migration. Depending on the cell type, ErbB-4 is thought to function as either an initiator of cell proliferation or differentiation (1). Hence, significant loss of ErbB-4 receptor could influence cell behavior in several direc-
tions. Targeted disruption of the ErbB-4 gene in mice leads to impaired trabeculation of the developing heart and embryonic lethality after 10 days gestation (34). However, ErbB-4 expression is limited compared with that of other receptors in this family and its essential functions beyond embryonic heart development are not known.

Although ErbB-4 ligands do not accelerate cleavage of this receptor, it is possible that other tyrosine kinases influence its fate on the surface either by modification of ErbB-4 or modification of basal protease activity. Hence, we have examined the influence of pervanadate, a potent phosphotyrosine phosphatase inhibitor, on ErbB-4 processing at the cell surface.

**Experimental Procedures**

**Materials**—Polyclonal IgG to the carboxyl terminus (residues 1291–1308) of ErbB-4 were purchased from Santa Cruz Biotechnology. Serum was raised against the carboxyl-terminal sequence 1108–1264 of ErbB-4 was generously supplied by Dr. Matthias Kraus, Istituto Europeo di Oncologia, Milan. Antiserum to PLC-γ1 was described previously (35). Anti-phosphotyrosine purified IgG and horseradish peroxidase-conjugated Protein A were purchased from Zymed Labs, Inc. Mouse monoclonal antibody 6R1C2.4 to human amphiregulin was a generous gift of Dr. Gregory Plowman (Sugen). Affinity-purified rabbit antiserum to mouse amphiregulin was purchased from Cappel Laboratories. TPA, phenylarsine oxide (PAO), fibronectin, pyrrolidine dithiocarbamate (PDTC), Protein A-Sepharose, and enhanced chemiluminescence (ECL) reagents were obtained from Sigma. Sulfo-NHS-LC-biotin and Protein A-agarose were products of Pierce. 125I-labeled Protein A was a product of ICN and 125I-labeled streptavidin was from Amersham. Immobilon-P membranes were from MCI. The metalloprotease inhibitor BB-94 (1308) was a generous gift of Dr. Lynn Matsurita, Vanderbilt University, Nashville, TN. Pervanadate was freshly prepared by each experiment as follows: 1 mM solutions of vanadate and H2O2 in phosphate-buffered saline were prepared and mixed to give a 0.5 mM solution of pervanadate. As pervanadate is unstable, this was diluted into cell cultures, at the indicated final concentration of 100 μM, within 20 min of preparation.

**Cell Culture**—T47-D11 cells, transfected NIH 3T3 cells that overexpress human ErbB-4 (approximately 1 × 10^6 receptors per cell), have been described elsewhere (12). This cell line was routinely grown in 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) containing 20 mM Hepes, pH 7.4, 50 μM gentamicin, and 10% calf serum. When required, cells were grown on fibronectin (10 μg/ml) pre-coated plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates. Atrial tumor myocytes, AT-1 cells, derived from T antigen transplanted plates.

**Immunoprecipitation and Immunoblotting of ErbB-4**—Cell lysates were obtained as described previously (15). Briefly, after overnight starvation in DMEM and 0.5% serum, monolayers were incubated for the indicated times at 37 °C in basal medium (DMEM, 0.1% bovine serum albumin, and 20 mM Hepes, pH 7.2) with indicated additions, i.e., pervanadate, inhibitors, etc. The cells were washed with calcium, magnesium-free PBS and solubilized for 20 min at 4 °C in TGH buffer (1% Triton X-100, 10% glycerol, 20 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM sodium orthovanadate). Lysates were clarified by centrifugation (14,000 × g, 10 min) at 4 °C and protein concentration was determined by the method of Bradford. The ErbB-4 protein was immunoprecipitated by adding approximately 1 μg of ErbB-4 antibody per 200 μg of cell lysate for 2 h at 4 °C and then incubating (1 h, 4 °C) with Protein A-Sepharose CL-4B. Subsequently the immunocomplexes were extensively washed with TGH buffer and resuspended in 1 × Laemmli buffer.

After boiling, proteins in the samples were electrophoretically separated on reducing 7.5% PAGE-SDS gels and transferred to nitrocellulose membranes for Western blotting. Membranes were blocked with 5% milk in PBS containing 0.05% Tween for 1 h prior to blotting with antibodies to anti-ErbB-4. Prior to anti-phosphotyrosine blots, membranes were blocked by incubating the blots with 3% bovine serum albumin in TBST buffer (0.05% Tween, 150 mM NaCl, 50 mM Tris, pH 7.4). Membranes were then incubated with the appropriate antibody for 2 h at room temperature and washed with PBS or TBST buffer, incubated with 125I-Protein A for 1 h at room temperature, and after five washes with PBS or TBST buffer, visualized by autoradiography (Kodak X-Omat AR film). Where indicated, bound antibody was detected with horseradish peroxidase-Protein A and ECL.

**In Vitro Kinase Assay**—T47-D11 cells overexpressing ErbB-4 were washed and the cell monolayers solubilized at 4 °C in TGH buffer with Na3VO4. Equal aliquots of cell lysates (100 μg of protein) were immunoprecipitated by adding 0.5 μg of antibody to ErbB-4. After a 2-h incubation at 4 °C, Protein A-Sepharose was added for 1 h. The immunocomplexes were then washed twice with TGH buffer without Na3VO4 and twice with kinase buffer (20 mM Hepes, pH 7.4, 3 mM MgCl2, 20 mM MnCl2, 50 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM Na3VO4). The immunocomplexes were resuspended in 50 μl of kinase buffer containing 20 μM cold ATP and 4 μg of recombinant phospholipase C-γ1 (PLC-γ1) (39) was added. The reaction mixtures were incubated at room temperature for the indicated times, stopping the reaction by adding 50 μl of 2 × Laemmli buffer and boiling for 5 min. Proteins were subsequently separated on a 7.5% PAGE-SDS gel and analyzed for phosphotyrosine content by Western blotting with antibody to phosphotyrosine. The amount of tyrosine-phosphorylated PLC-γ1 was quantitated by densitometric scanning. For each phosphorylation sample, a parallel aliquot of lysate was analyzed for ErbB-4 protein by immunoprecipitation and Western blot, as described above.

**Analysis of Amphiregulin Processing**—Transfected MDCK cells were incubated with sodium butyrate (5 mM) for 16 h to induce amphiregulin expression. The monolayers were washed with serum-free DMEM and twice with PBS containing 0.1 mM CaCl2 and 1.0 mM MgCl2 (PBS–) before cell surface biotinylation (30 min at 4 °C) with 1.5 mg/ml sulfo-NHS-LC-biotin in PBS7. Following biotinylation in PBS–, the cells were washed twice with PBS– and once with serum-free DMEM. To measure processing of surface biotinylated amphiregulin, cells were incubated for the indicated times with serum-free DMEM (supplemented with 1-glutamine, penicillin/streptomycin, non-essential amino acids) in the absence or presence of pervanadate. At each indicated time the media was collected and cell lysates were prepared as described elsewhere (31, 36). Subsequently, total cell lysates and conditioned media samples were incubated overnight with 0.20 and 0.05 μg/ml anti-amphiregulin, respectively. The immune complexes were then precipitated by adding affinity-purified rabbit anti-mouse IgG for 1 h followed by a 50% slurry of Protein A-agarose for 2 h. The Protein A-agarose was then pelleted and washed stringently. Proteins in the complexes were separated on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose. These membranes were subsequently rinsed in Tris-buffered saline containing 0.5% Tween 20, blocked for 1 h in the same buffer containing 3% bovine serum albumin, and incubated with 125I-labeled streptavidin. After extensive washing, amphiregulin precursor and processed forms were detected by autoradiography, using Kodak X-Omat film.

**RESULTS**

**Pervanadate Cleavage of ErbB-4**—Previous data have established that ErbB-4 is cleaved by a metalloprotease activity and that this reaction is not influenced by ligand binding to the receptor tyrosine kinase (15). To assess whether the tyrosine phosphatase activity of other molecules might influence this proteolytic event, cells were treated with pervanadate, a potent tyrosine phosphatase inhibitor (40), which raises the tyrosine phosphate content of many intracellular proteins. As shown in Fig. 1A, a 30-min exposure to 1 mM pervanadate, but not 1 mM vanadate, 1 mM H2O2, resulted in the appearance of a 90-kDa molecule that is reactive with antibody 6R1C2. This 90-kDa band is present in ErbB-4 and is, therefore, equivalent to the 80-kDa ErbB-4 fragment previously detected (14, 15). Also, the appearance of the 80-kDa ErbB-4 fragment coincides with a decrease in the level of the native 180-kDa ErbB-4 receptor. In the experiment shown in Fig. 1B, cells were exposed for increasing periods of time to 100 μM pervanadate, an optimal concentra-
were incubated with no additions, 1 mM Na3VO4, 1 mM pervanadate (PV), or 1 mM H2O2 for 30 min at 37 °C. The cell monolayers were then washed with Ca2+-, Mg2+-free PBS and lysed in TGH buffer. Approximately 100 μg of cell lysate were subjected to SDS-PAGE and, following transfer to nitrocellulose, probed with antibody to phosphotyrosine. Bound antibody was detected separated by SDS-PAGE and, following transfer to nitrocellulose, subjected to precipitation with anti-ErbB-4. The precipitates were then washed with Ca2+-(data not shown), and the cell lysates were precipitated with anti-ErbB-4 and subsequently probed with anti-phosphotyrosine. The data show that pervanadate induces within 10 min the tyrosine phosphorylation of the native 180-kDa ErbB-4 receptor and concomitantly its hydrolysis to yield the 80-kDa fragment which accumulates as a heavily tyrosine-phosphorylated molecule.

Requirements for ErbB-4 Cleavage by Pervanadate—While the mechanisms of pervanadate influence on all cellular functions at the molecular level are not completely understood, activated oxygen species formed from the combination of H2O2 and vanadate oxidize an essential thiol at the active site of phosphotyrosine phosphatases and thereby inhibit enzymatic activity (40, 41). It has been demonstrated that the oxygen radical scavenger PDTC can block some but not all cellular responses to pervanadate (41). We have tested this possible mechanism by pretreating cells with PDTC, before adding pervanadate. This experiment, shown in Fig. 2, employed two cell types NIH 3T3 cells that express the transfected human ErbB-4 receptor (panel A) and AT-1 rat cardiac myocytes that express endogenous ErbB-4 receptors (panel B). As the latter cell line has a lower number of ErbB-4 receptors, the receptors in those lysates were concentrated by immunoprecipitation prior to blotting with anti-ErbB-4. The results show that pervanadate in each cell type induces the cleavage of ErbB-4 and that PDTC effectively blocks this cleavage. Additionally, pervanadate induces a similar cleavage of endogenous ErbB-4 in two human carcinoma cell lines, OVCAR-3 and T47D, derived, respectively, from ovarian and mammary tumors (data not shown).

Previous data have shown that TPA induces cleavage of the ErbB-4 receptor to a 80-kDa fragment, containing the transmembrane and cytoplasmic domains, and a 120-kDa ectodomain fragment requires metalloprotease activity (14, 15). The specific metalloprotease inhibitor BB-94 (Batimastat) was utilized to determine whether the pervanadate-induced cleavage of ErbB-4 requires metalloprotease activity. The results, shown in Fig. 3, clearly show that preincubation of cells with BB-94 effectively blocks pervanadate-induced ErbB-4 cleavage, demonstrating that the pervanadate-induced accumulation of intracellular phosphotyrosine and ErbB-4 cleavage is dependent on metalloprotease activation.

FIG. 1. Pervanadate cleavage of ErbB-4. Panel A, T47-14 cells were incubated with no additions, 1 mM Na3VO4, or 1 mM H2O2 for 30 min at 37 °C. The cell monolayers were then washed with Ca2+-, Mg2+-free PBS and lysed in TGH buffer. Approximately 100 μg of cell lysate were subjected to SDS-PAGE and, following transfer to nitrocellulose, Western blotted with anti-ErbB-4. Bound antibody was detected with 125I-labeled Protein A and autoradiography. Panel B, T47-14 cells were either untreated or exposed to 100 μM pervanadate for 30 min, pretreated for 30 min with 100 μM PDTC prior to the addition of 100 μM pervanadate for an additional 30 min, or, as controls, treated with the same concentration of PDTC alone for 60 min. After treatment cell monolayers were washed and lysed in TGH buffer. Approximately 100 μg of each lysate from T47-14 cells was subjected to SDS-PAGE, while 500 μg or each AT-1 lysate was precipitated with anti-ErbB-4 prior to SDS-PAGE. Proteins were then transferred to nitrocellulose and blotted with anti-ErbB-4. Bound antibody was detected with 125I-labeled Protein A and autoradiography for T47-14 samples (Panel A) or ECL for AT-1 samples (Panel B).

FIG. 2. Capacity of PDTC to influence pervanadate-induced cleavage of ErbB-4. Either T47-14 cells (Panel A) or AT-1 cardiac myocytes (Panel B) were either untreated, exposed to 100 μM pervanadate (PV) for 30 min, pretreated for 30 min with 100 μM PDTC prior to the addition of 100 μM pervanadate for an additional 30 min, or, as controls, treated with the same concentration of PDTC alone for 60 min. After each treatment, the cells were lysed in TGH buffer and 100 μg each lysate was subjected to SDS-PAGE, while 500 μg or each AT-1 lysate was precipitated with anti-ErbB-4 prior to SDS-PAGE. Proteins were then transferred to nitrocellulose and blotted with anti-ErbB-4. Bound antibody was detected with 125I-labeled Protein A and autoradiography for T47-14 samples (Panel A) or ECL for AT-1 samples (Panel B).

FIG. 3. Role of metalloprotease activity in pervanadate-induced ErbB-4 cleavage. T47-14 cells were either untreated or incubated with pervanadate (PV) (100 μM) for 30 min, preincubated with BB-94 (5 μM) for 30 min prior to the addition of pervanadate (100 μM) for 30 min, or, as control, incubated with BB-94 (5 μM) for 60 min. After each treatment, the cells were lysed in TGH buffer and 100 μg each lysate was subjected to SDS-PAGE, transferred to nitrocellulose, and Western blotted with anti-ErbB-4. Bound antibody was detected with 125I-labeled Protein A.
FIG. 4. Protein kinase C and pervanadate-induced ErbB-4 cleavage. Panel A, influence of protein kinase inhibition. T47-14 cells were pretreated (30 min) with the protein kinase C inhibitor GF 109203X (5 \(\mu M\)) prior to the addition of TPA (100 ng/ml) or pervanadate (PV) (100 \(\mu M\)) for an additional 30 min. Controls were single treatments with the same concentrations of TPA or pervanadate for 30 min or GF 109203X for 60 min. Aliquots (100 \(\mu g\)) of each lysate were then subjected to SDS-PAGE and Western blotted with anti-ErbB-4. Bound antibody was detected with \(^{125}\)I-labeled Protein A and autoradiography. Panel B, influence of PDTC on TPA-induced ErbB-4 cleavage. T47-14 cells were untreated, exposed to TPA (100 ng/ml) for 30 min, preincubated with PDTC (100 \(\mu M\)) for 30 min prior to the addition of TPA for an additional 30 min, or, as a control, incubated with only PDTC for 60 min. Thereafter cells were solubilized and 100 \(\mu g\) of each lysate subjected to SDS-PAGE. Following transfer to nitrocellulose, proteins were blotted with anti-ErbB-4 and bound antibody detected by ECL.

There are several reports demonstrating that various stimuli, including TPA and \(H_2O_2\), can produce the tyrosine phosphorylation of protein kinase C \(C_5\) (42–50). However, the functional role of this modification in regard to protein kinase C activity or substrate selectivity is unclear. Nevertheless, we have tested the hypothesis that the pervanadate-induced cleavage of ErbB-4 may be mediated by protein kinase C. The results, shown in Fig. 4A, demonstrate that the protein kinase C inhibitor GF 109203X blocks ErbB-4 cleavage by TPA, as previously demonstrated (14), but has no capacity to attenuate ErbB-4 hydrolysis induced by pervanadate. Similarly, prolonged TPA-induced down-regulation of protein kinase C levels prevents subsequent TPA-induced ErbB-4 cleavage, but not that induced by pervanadate (data not shown).

We have also tested whether oxygen radical scavengers prevent the TPA-induced cleavage of ErbB-4. The results, shown in Fig. 4B, show that PDTC does not influence the capacity of TPA to induce cleavage of ErbB-4. When the PDTC concentration was increased to 300 \(\mu M\) (data not shown) the results were the same as those shown for 100 \(\mu M\) (Fig. 4B). Hence, TPA-induced cleavage of ErbB-4 can be differentiated from pervanadate-induced cleavage by their relative sensitivities to PDTC. Pervanadate-induced cleavage is completely blocked by 100 \(\mu M\) PDTC (Fig. 2A), while TPA-induced cleavage is insensitive to 300 \(\mu M\) PDTC.

ErbB-4 Cleavage by Phenylarsine Oxide—The data presented thus far indicate that a potent phosphorysine phosphatase inhibitor leads to the proteolytic hydrolysis of ErbB-4. To further substantiate the hypothesis of pervanadate in this activity, the phosphorysine phosphatase inhibitor PAO was employed (51–53). PAO is more toxic to cells than pervanadate; however, plating T47-14 cells on fibronectin decreased the observable toxicity of this inhibitor. The results of this experiment are shown in Fig. 5A. The data show that exposure of T47-14 cells to PAO does produce the cleavage of ErbB-4 and, unlike the cleavage initiated by pervanadate, the influence of PAO is not blocked by the oxygen radical scavenger PDTC. When PAO-treated samples were immunoprecipitated and blotted with phosphorysine antibody, it is clear that the 80-kDa ErbB-4 fragment has a substantial level of phosphorysine (Fig. 5B). Also, the data in this experiment show that the PAO-induced tyrosine phosphorylation of ErbB-4 is insensitive to PDTC. Hence, activated oxygen species are not involved in either the PAO-induced phosphorylation or cleavage of ErbB-4.

Pervanadate Generation of Active ErbB-4 Tyrosine Kinase—The data in Fig. 1B show that following pervanadate treatment, a heavily tyrosine-phosphorylated ErbB-4 cytoplasmic domain fragment is produced. To determine whether this fragment, which contains the cytoplasmic tyrosine kinase domain, is an active tyrosine kinase, ErbB-4 immunoprecipitates tyrosine kinase assays of exogenous substrate phosphorylation were performed. After treatment of intact cells with or without pervanadate, lysates were incubated with antibody to ErbB-4. The immunoprecipitates were then assayed, as described under “Experimental Procedures,” for their capacity to tyrosine phosphorylate recombinant PLC-\(\gamma_1\), a physiological tyrosine kinase substrate (54). The results (Fig. 6A), assessed by Western blotting with anti-phosphotyrosine, show that ErbB-4 immunoprecipitates from both control and pervanadate-treated cells extensively tyrosine phosphorylated PLC-\(\gamma_1\). Identical aliquots of each lysate were also assayed, by immunoprecipitation and Western blotting, for the relative levels of native ErbB-4 and the 80-kDa ErbB-4 fragment. The results, shown in Fig. 6B, indicate comparable levels of ErbB-4 immunoreactive molecules in each sample. More importantly, these data show that the lysate from pervanadate-treated cells does not contain a detectable level of native ErbB-4. Hence, the tyrosine kinase activity detected in ErbB-4 immunoprecipitates from this lysate is attributable only to the 80-kDa fragment.

Influence of Pervanadate on Amphiregulin Processing—A substantial number of cell surface molecules are cleaved by controlled proteolysis to generate biologically significant products. One of these is amphiregulin, an EGF-like growth factor (55), which is synthesized as a transmembrane precursor and

FIG. 5. PAO-induced ErbB-4 cleavage and tyrosine phosphorylation. Panel A, T47-14 cells, grown on fibronectin-coated culture dishes, were pretreated for 30 min with or without PDTC (100 \(\mu M\)) prior to the addition of PAO (10 \(\mu M\)) or pervanadate (PV) (100 \(\mu M\)) for an additional 30 min. Cells exposed to PDTC only were incubated for 60 min. Aliquots (100 \(\mu g\)) of each cell lysate were subjected to SDS-PAGE and Western blotted with anti-ErbB-4. Bound antibody was detected by ECL. Panel B, T47-14 cells grown as above were pretreated without or with PDTC (100 \(\mu M\)) for 30 min prior to the addition of PAO (10 \(\mu M\)), as indicated, for an additional 30 min. The cells were then lysed and aliquots (100 \(\mu g\)) of each lysate were precipitated with anti-ErbB-4. After SDS-PAGE and transfer to nitrocellulose, the filters were Western blotted with anti-phosphotyrosine. Bound antibody was detected by ECL. The band designated N.S is nonspecific and was not detected when the blots were stripped and reprobed with anti-ErbB-4, which did detect the native and 80-kDa ErbB-4 species.
amphiregulin, by Western blotting with anti-phosphotyrosine, have been negative (data not shown). To eliminate the potential role of amphiregulin tyrosine phosphorylation in proteolysis, the capacity of pervanadate to induce cleavage of a cytoplasmic domain truncation mutant ARTL (38) was examined. This truncation removes 25 residues from the carboxyl terminus of amphiregulin, leaving only two arginine residues in the cytoplasmic domain. The results of this experiment are shown in Fig. 8. In panel A, the pervanadate-induced accumulation of amphiregulin cleavage products into the media is presented, while in panel B the loss of cell surface amphiregulin species is shown. These data demonstrate that pervanadate does induce cleavage of this amphiregulin mutant and, furthermore, that this cleavage is blocked by the metalloprotease inhibitor BB-94. Hence, the cytoplasmic domain of amphiregulin, including the two tyrosine residues, are not required for pervanadate-induced, metalloprotease-dependent cleavage.

**DISCUSSION**

Tyrosine phosphorylation is well known as a mediator of intracellular signal transduction pathways leading to cell proliferation through the activation of G proteins, serine/threonine protein kinases, transcription factors, and phospholipid kinases and lipases. The ultimate targets of these signaling elements are intracellular response points such as the nucleus and gene expression, membrane-localized activation of transporters and ion channels, and the cytoskeleton and adhesion molecules regulating cell shape. Enhanced proteolysis, however, is not generally recognized as a target of tyrosine phosphorylation pathways. The data presented in this article show that elevation of intracellular tyrosine phosphate by the inhibition of tyrosine phosphatases, using either pervanadate or PAO, leads to the metalloprotease-dependent proteolytic cleavage of ErbB-4, a growth factor receptor tyrosine kinase, as well as that of pro-amphiregulin, a growth factor precursor. Pervanadate-induced cleavage of ErbB-4 is demonstrated in transfected cells as well as several cell lines, including two human carcinomas, that express endogenous ErbB-4. This indicates that the pervanadate-induced cleavage of ErbB-4 is a general property of cells that express this growth factor receptor.

It has previously been demonstrated that there is a low rate of basal cleavage of ErbB-4 that can be stimulated by direct or...
indirect activation of protein kinase C (14, 15). In both instances the cleavage occurs within the ectodomain but close to the transmembrane region, producing an 80-kDa cleavage product representing the transmembrane and cytoplasmic domains. This is analogous to the cleavage product shown to accumulate following pervanadate treatment. While the exact cleavage sites are not known for the 80-kDa fragment produced under basal, TPA-stimulated, or pervanadate-stimulated conditions, their similar size and antibody reactivity suggests a common or closely related cleavage site, mediated in each instance by metalloprotease activity. The expected cleavage site is further suggested by the resistance of an ErbB-4 insertion mutant to TPA-induced cleavage (16). This insertion occurs within the juxtamembrane region of the ErbB-4 ectodomain and likely perturbs the protease cleavage site. Also, it is a common property for metalloproteases to cleave a variety of cell surface molecules within the juxtamembrane ectodomain (59).

The mechanism by which pervanadate provokes cleavage of ErbB-4 or other cell surface proteins is unclear. There are several reasons to believe that pervanadate-induced phosphorylation of the substrate cytoplasmic domain is not involved. In the case of ErbB-4, growth factor agonists which induce tyrosine phosphorylation of ErbB-4 produce no detectable cleavage of this receptor (15). Also, the addition of TPA induces a similar cleavage of ErbB-4, based on the sizes of the cytoplasmic domain fragments, but does not enhance tyrosine phosphorylation of ErbB-4. However, the capacity of pervanadate to induce cleavage of amphiregulin without the detectable presence of phosphotyrosine and to promote cleavage of a cytoplasmic domain truncation mutant of amphiregulin is direct evidence that covalent modification of the substrate is not a prerequisite for its cleavage following pervanadate exposure.

Metalloproteases exist as either secreted or transmembrane proteins, the topography of the latter offering a possible site for inside-out signaling stimulated either by pervanadate or TPA, which has also been shown to stimulate the cleavage of a number of cell surface molecules (59). It seems likely that pervanadate may directly or indirectly modulate the activity of a membrane-localized metalloprotease by altering the protease cytoplasmic domain. Interestingly, the cytoplasmic domains of many members of the disintegrin metalloprotease family contain SH3 domain consensus binding sites (61). SH3 domains are found in some tyrosine kinases, such as those in the Src family, as well as several tyrosine kinase substrates. The SH3 domain of Src, but not Abl, has been shown to associate with the cytoplasmic domain of disintegrin MDC9 (62). In addition there is evidence that the transmembrane/cytoplasmic domain of the membrane type 1-matrix metalloprotease may regulate protease activity by controlling its spacial organization in the membrane relative to substrates (63). Hence, there are several possible mechanisms by which tyrosine phosphate-based signals might interact with metalloprotease cytoplasmic domains and regulate enzyme function.

There are two previous examples of pervanadate enhanced cleavage of transmembrane molecules, the amyloid precursor protein (57) and syndecan 1 (58). However, the nature of the protease involved was not identified. The data herein indicate that ErbB-4 cleavage is mediated by a metalloprotease, as pervanadate-induced proteolysis of this molecule is blocked by BB-94 (Batimastat). Metalloprotease have been implicated in the processing of numerous cell surface molecules (59). However, none of these have been shown to be stimulated by pervanadate. The data reported in this article, therefore, represent the first indication that tyrosine phosphorylation may modulate metalloprotease activity. While the number of metalloproteases and the number of cell surface substrates are both large, there is little data regarding which metalloproteases cleave which cell surface substrates. Also, little is known regarding the regulation of these events. Perhaps the most well defined example is the processing the tumor necrosis factor α precursor, which has been shown recently to be cleaved in vivo by a specific disintegrin metalloprotease (32, 33). Tumor necrosis factor-α processing is stimulatable by TPA; however, the mechanism or site of action of TPA is not known. Whether pervanadate can also modulate the processing of tumor necrosis factor α has not yet been tested.

The metalloprotease-cleavage of ErbB-4 generates a soluble ectodomain of 120 kDa (14) and a cytoplasmic-transmembrane domain fragment of 80 kDa, which functions as an active tyrosine kinase at least in vitro (15). While most attention and physiological significance has been directed toward the ectodomain fragments of cleaved receptors (60), it seems likely that active tyrosine kinase cytoplasmic domains fragments may also be produced. The potential significance of these kinase fragments have yet to be defined.

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REFERENCES

1. Plowman, G. D., Culouaciu, J.-M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1746–1750
2. Plowman, G. D., Green, J. M., Culouaciu, J.-M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) Nature 366, 473–475
3. Tzahar, E., Levkowitz, G., Karungrongan, D., Yi, L., Peles, E., Lavri, S., Chang, D., Liu, X., Yayon, A., Wen, D., and Yarden, Y. (1994) J. Biol. Chem. 269, 25226–25233
4. Riese, D. J., II, Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996) Oncogene 12, 345–353
5. Elenius, K., Paul, S., Allison, G., Sun, J., and Klagesmun, M. (1997) EMBO J. 16, 1268–1278
6. Komurasaki, T., Toyoda, H., Uchida, D., and Morimato, S. (1997) Oncogene 15, 2841–2848
7. Beeri, R. R., and Hynes, N. E. (1996) J. Biol. Chem. 271, 6071–6076
8. Beeri, D. J., II, Kim, E. D., Elenius, K., Buckley, S., Klagesmun, M., Plowman, G. D., and Sterl, D. F. (1996) J. Biol. Chem. 271, 20047–20052
9. Carpenter, G., and Cohen, S. (1976) J. Cell Biol. 71, 159–174
10. Sorkin, A., and Waters, C. M. (1993) BioEssays 15, 375–382
11. Sorkin, A., Di Fiore, P. P., and Carpenter, G. (1993) Oncogene 8, 3021–3026
12. Baulida, J., Kraus, M. H., Alimand, M., Di Fiore, P. P., and Carpenter, G.
