

# TrkA Receptor Ectodomain Cleavage Generates a Tyrosine-phosphorylated Cell-associated Fragment

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**Abstract.** The extracellular domain of several membrane-anchored proteins can be released as a soluble fragment by the action of a cell surface endoproteolytic system. This cleavage results in the generation of a soluble and a cell-bound fragment. In the case of proteins with signaling capability, such as tyrosine kinase receptors, the cleavage process may have an effect on the kinase activity of the cell-bound receptor fragment. By using several cell lines that express the TrkA neurotrophin receptor, we show that this receptor tyrosine kinase is cleaved by a proteolytic system that mimics the one that acts at the cell surface. TrkA cleavage is regulated by protein kinase C and several receptor agonists (including the TrkA ligand NGF), occurs at the ectodomain in a membrane-proximal region, and is in-

dependent of lysosomal function. TrkA cleavage results in the generation of a cell-associated fragment that is phosphorylated on tyrosine residues. Tyrosine phosphorylation of this fragment is not detected in TrkA mutants devoid of kinase activity, suggesting that phosphorylation requires an intact TrkA kinase domain, and is not due to activation of an intermediate intracellular tyrosine kinase. The increased phosphotyrosine content of the cell-bound fragment may thus reflect higher catalytic activity of the truncated fragment. We postulate that cleavage of receptor tyrosine kinases by this naturally occurring cellular mechanism may represent an additional mean for the regulation of receptor activity.

THE extracellular domain of several transmembrane proteins can be recovered as a soluble and bioactive fragment from the culture media of cells or animal fluids (14, 33). The generation of these soluble forms may occur by either alternative splicing of mRNA, or by proteolytic cleavage of the ectodomain. The latter largely depends on the activity of a cell surface endoproteolytic system, and affects a wide variety of physiologically relevant molecules, such as membrane-anchored growth factors, some of their receptors, cell adhesion proteins, ectoenzymes, molecules involved in immune responses, and other proteins whose function has not been fully elucidated (14, 25). The cleavage of these proteins will be expected to affect their biological function. For example, shedding of the neutrophil MEL<sup>14</sup> adhesion molecule has been implicated in the recruitment of these cells to inflammatory sites (20); and cleavage of membrane-anchored growth factors extinguishes the juxtacrine function of the membrane-bound form, allowing the free diffusion of the factor to neighboring tissues (24). In the case of receptors, released fragments may act as agonists, antagonists, or car-

rier molecules. Agonistic functions of soluble receptors have been demonstrated for the interleukin-6 receptor that, upon ligand binding, is able to interact with and activate the signal transducing subunit gp130 leading to a biological signal (19). Antagonistic and carrier functions are based on the capture of ligand molecules in situations in which soluble receptors are in excess with respect to the transmembrane counterparts (14, 33).

For proteins with signaling capability, cleavage of the ectodomain could have an effect on the functioning of the intracellular region. In the case of receptor tyrosine kinases, substantial evidence has demonstrated that the ectodomain acts as a negative regulator of the intracellular enzymatic activity. Some retroviral receptor oncogenes, such as the *v-erbB*, *v-kit*, and the *v-ros*, code for oncoproteins that lack most of the extracellular domain of their respective protooncoproteins and give rise to constitutively active receptor fragments (32). Moreover, *in vitro* deletion of the ectodomain of several receptor tyrosine kinases, such as *erbB2/neu*, the EGF receptor (EGFR),<sup>1</sup> or the insulin receptor, confers transforming potential to the mutated receptor. Genetic analyses carried out in *Drosophila* have corroborated the inhibitory role exerted by the ectodomain of receptor tyrosine kinases on its intracellular

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1. Abbreviations used in this paper: EGFR, EGF receptor; TNFR, tumor necrosis factor receptor.

catalytic activity. The sevenless receptor tyrosine kinase plays an essential role in fly eye development by dictating the fate of the R7 photoreceptor cell (34). Mutations that remove the sevenless ectodomain give rise to gain-of-function phenotypes characterized by the presence of supernumerary R7 photoreceptors (5).

In this report we show that the TrkA receptor tyrosine kinase is cleaved by a cell surface endoproteolytic system. This receptor belongs to the Trk family of neurotrophin receptors (3) that, together with the accessory molecule p75<sup>LNTFR</sup> (9) are involved in the development and survival of particular groups of neural cells (35). TrkA cleavage is rapidly induced by PKC activation, and by stimulation of receptors for neurotransmitters and growth factors, including NGF and EGF. The cleavage of TrkA results in the release of the extracellular domain to the culture medium, and the generation of a membrane-associated fragment whose phosphotyrosine content increases in wild type but not in kinase inactive TrkA mutants. This naturally occurring cellular mechanism may be responsible for the production of truncated, constitutively active forms of receptor tyrosine kinases, and may represent an additional mean for the regulation of receptor signaling.

## Materials and Methods

### Reagents

Tissue culture plasticware was purchased from Nunc (Roskilde, Denmark); cell culture media and G418, from GIBCO BRL (Gaithersburg, MD); and FBS from BioWhittaker (Walkersville, MD). NGF was from Quality Controlled Biochemicals (Hopkinton, MA), Inc.; and EGF from Collaborative Research (Waltham, MA). HRP conjugates of anti-rabbit IgG and anti-mouse IgG were from Bio-Rad Laboratories (Cambridge, MA). HRP conjugates of protein A and streptavidin were from Calbiochem-Behring Corp. (San Diego, CA); and protein A-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ). Immobilon-P membranes were from Millipore Corp. (Bedford, MA). Promix and Hyperfilm ECL were from Amersham Corp. (Arlington Heights, IL). Luminol, Triton X-114, PMA, bradykinin, and carbamylcholine were purchased from Sigma Chemical Co. (St. Louis, MO); and 4-iodophenol, from Aldrich Chem. Co. (Milwaukee, WI). All other chemicals were reagent grade, and were purchased from Sigma Chemical Co., Boehringer-Mannheim Biochemicals (Indianapolis, IN), or Merck, Sharpe, and Dohme (Rahway, NJ).

The monoclonal antibody MGR12 was obtained from Dr. S. Ménard (Istituto Nazionale dei Tumori, Milano, Italy). This mAb was obtained by immunizing mice with TrkA-transfected SKNBE neuroblastoma cells, and selection for its ability to immunoprecipitate the receptor (Dr. E. Tagliabue, Istituto Nazionale dei Tumori, Milano, Italy, personal communication). The polyclonal  $\alpha$ 203 antiserum raised against the 14 COOH-terminal residues of TrkA has been reported (23) and the anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

### Cell Culture and Transfection with Expression Vectors

All cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO<sub>2</sub>, 95% air. Cells were grown in DME containing high glucose (4,500 mg/l) and antibiotics (100 U/ml penicillin, 100  $\mu$ g/ml streptomycin) and supplemented with 5% FBS (parental CHO and derived clones), 10% FBS (PC12, Trk-PC12; A431<sup>TrkA</sup>), or 10% calf serum (106-63, E25-427).

The expression plasmid for the human non-neuronal form of TrkA has been described (23). The kinase-inactive mutant form of human TrkA (K<sup>538</sup>→A) was generated by standard M13-based site-directed mutagenesis (2), and expressed from a pCMV5-based expression vector (a gift of Dr. M. V. Chao, Cornell University, New York, NY).

Transfections were carried out by the calcium phosphate precipitation technique (2). After G418 selection (500 or 100  $\mu$ g/ml for CHO or A431, respectively), single colonies were isolated by ring cloning and analyzed

for TrkA expression by immunoprecipitation and Western blotting. In addition, G418-resistant colonies from a single plate were pooled and analyzed for their receptor content and response to phorbol esters.

### Metabolic Labeling

CHO<sup>TrkA</sup> cells were washed twice, 15-min each, with methionine- and cysteine-free DME, and incubated in this medium supplemented with 75  $\mu$ Ci/ml of a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 4 h. Monolayers were washed twice, 15-min each, with DME, and incubated for an additional 30-min period with or without PMA. Media samples were collected and immunoprecipitated with MGR12 antibody as described below, and cell lysates were immunoprecipitated with the  $\alpha$ 203 antiserum. The resulting immunoprecipitates were resolved in a 10% SDS-PAGE gel; and, after electrophoresis, gels were incubated in fixing solution, and then in salicylate. Bands in dried gels were detected by autoradiography.

### Immunoprecipitations and Western Blotting

Cells were washed once with cold PBS and lysed with 0.5–1 ml of ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40; 20 mM Tris, pH 8.0, 1 mM PMSF, 1 mM sodium orthovanadate). After scraping the cells from the dishes, samples were centrifuged at 10<sup>4</sup> g, and the supernatant was transferred to new tubes with the corresponding antibody and protein A-Sepharose. Immunoprecipitations were performed at 4°C for 2 h, and immune complexes were recovered by a short centrifugation, followed by three washes with 1 ml of cold lysis buffer. Samples were then boiled in 2 $\times$  electrophoresis sample buffer, and resolved in 10% SDS-PAGE gels. After electrophoresis and transfer to Immobilon-P membranes the filters were blocked for 1 h in TBST (20 mM Tris, pH 7.5, 1% BSA, 150 mM NaCl, 0.1% Tween 20) and then incubated for at least another hour with the anti-phosphotyrosine 4G10 antibody, or the anti-endodomain  $\alpha$ 203 antiserum. After washing three times, 5-min each, with TBST, filters were incubated with HRP conjugated to either a secondary antibody or protein A. Bands were visualized by a luminol-based detection system with *p*-iodophenol enhancement (2).

Immunoprecipitations of media supernatants were carried out by collecting culture media from cell monolayers metabolically labeled with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine, followed by the addition of the MGR12 mAb and protein A-Sepharose. Samples were incubated at 4°C for 2 h, and the beads containing the immunocomplexes were washed three times with immunoprecipitation lysis buffer. Samples were boiled in electrophoresis sample buffer, resolved in 10% SDS-PAGE gels, and visualized by autoradiography.

### Cell Fractionation

CHO<sup>TrkA</sup> cells from two 100 mm dishes (70% confluent) were incubated with or without PMA for 30 min, washed twice with 5 ml of PBS, and 2 ml of hypotonic PBS (19:1, distilled water/PBS) containing 1 mM PMSF and 10 mM EDTA added to the monolayers. Cells were then scraped, and manually disrupted using a glass Dounce homogenizer. Major cell debris was eliminated by centrifugation at 1,800 rpm at 4°C for 10 min, and the supernatants subjected to ultracentrifugation at 10<sup>5</sup> g for 30 min at 4°C. The supernatants were made 1% NP-40, and the pellet was lysed in immunoprecipitation lysis buffer. Both fractions were immunoprecipitated with the  $\alpha$ 203 antiserum, and proteins identified by Western blotting with the same antiserum.

### Triton X-114 Extraction and Protease Protection Experiments

Triton X-114 phase separation was performed as previously described (6) with some modifications. Briefly, cells were lysed for 60 min on ice in 0.5 ml of a buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1% (wt/vol) Triton X-114, 1 mM EDTA, and 1 mM PMSF. The lysate was collected and centrifuged at 10<sup>4</sup> g for 15 min at 4°C. The supernatant was incubated at 37°C for 5 min, centrifuged at 10<sup>4</sup> g for 5 min at room temperature, and the upper aqueous phase subjected to another round of phase separation. The aqueous, and the pooled detergent phases, were immunoprecipitated with the  $\alpha$ 203 antiserum and subjected to SDS-PAGE and Western blot.

For protease protection experiments with proteinase K, CHO<sup>TrkA</sup> cells were washed once with KRH buffer (that contained, in mmol/l: 140 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 6 glucose, 25 Hepes, pH 7.4), and incubated in this buffer supplemented with 200  $\mu$ g/ml of proteinase K for 90 min. After washing three times with PBS containing 2 mM PMSF, cells

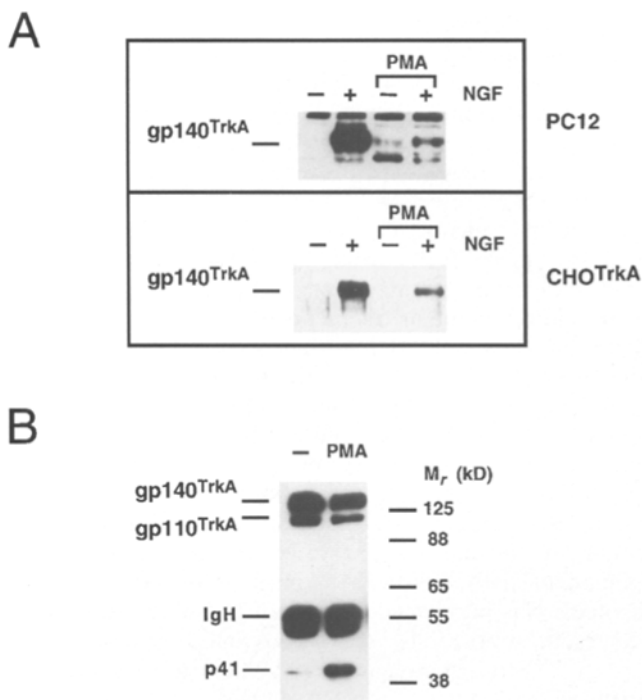
were lysed in 1 ml of immunoprecipitation buffer and analyzed for TrkA fragment generation by using the  $\alpha 203$  antiserum.

## Results

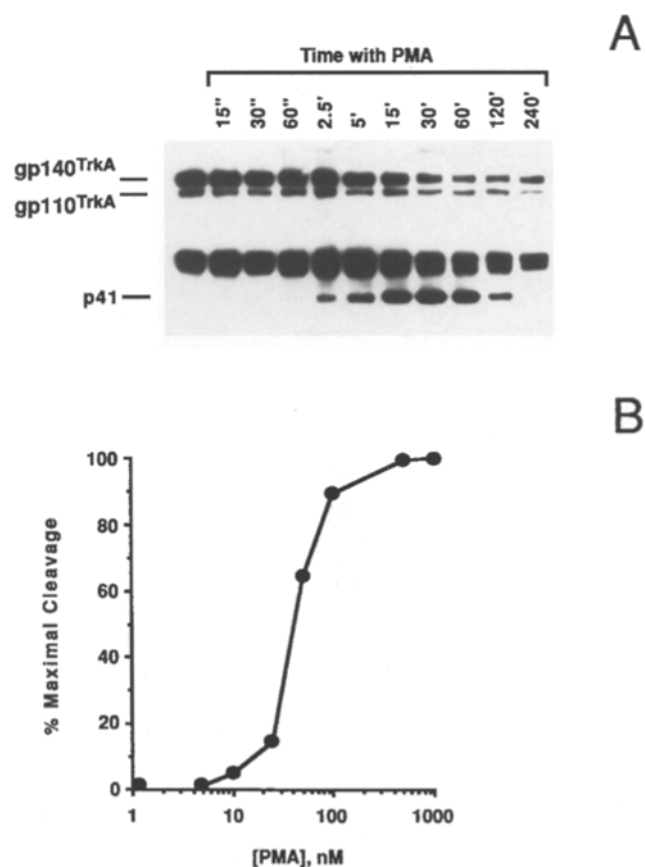
### PKC Activation Induces Cleavage of TrkA

As a screening method for the identification of receptor tyrosine kinases sensitive to regulated cleavage, we analyzed ligand-induced receptor autophosphorylation under conditions known to stimulate the activity of the cell surface endoproteolytic system (28, 29). In PC12 cells, a rat pheochromocytoma cell line that endogenously expresses the TrkA neurotrophin receptor tyrosine kinase, these exploratory studies showed that the PKC pathway had a significant inhibitory role on NGF-induced TrkA autophosphorylation (Fig. 1 A). To test whether this effect was a general cell biological phenomenon (and not a particular characteristic of PC12 cells), and to facilitate its study, we used CHO cells expressing human TrkA (CHO<sup>TrkA</sup> cells). As previously reported for other fibroblastic cells transfected with the TrkA cDNA (23), CHO<sup>TrkA</sup> cells con-

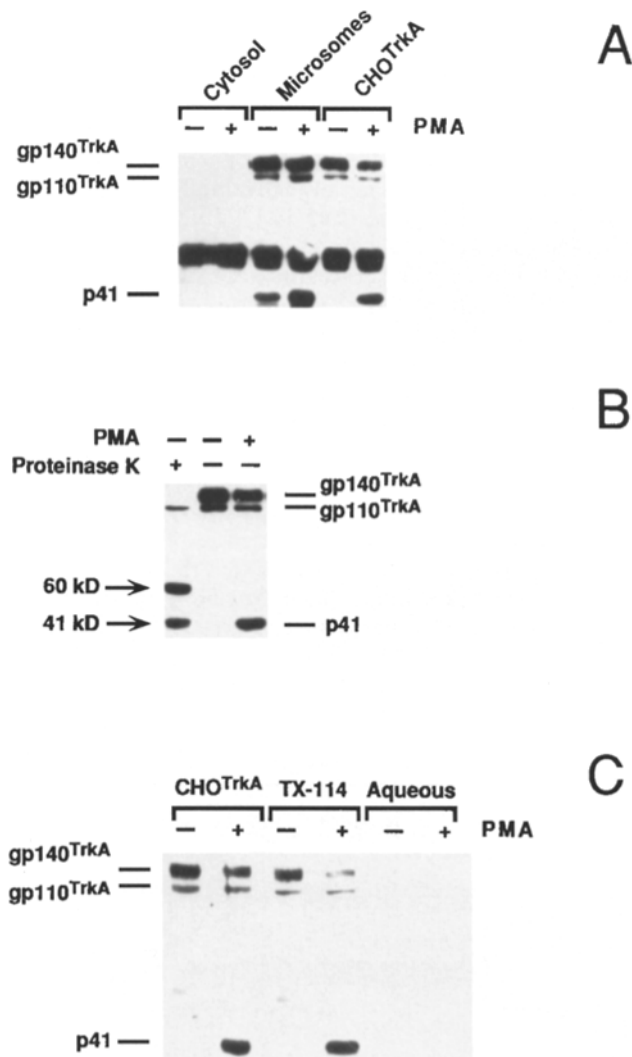
tained two major glycosylated forms of TrkA: gp140<sup>TrkA</sup>, that is the predominant cell surface form and responds to NGF (Fig. 1 A); and gp110<sup>TrkA</sup>, that is an intracellular immature precursor of the cell surface form (Fig. 1 B). Addition of saturating concentrations of NGF to CHO<sup>TrkA</sup> cells rapidly increased gp140<sup>TrkA</sup> receptor phosphotyrosine content, and this effect was largely prevented by pretreatment with the PKC-activating drug PMA (Fig. 1 A). Western blot analysis with the anti-endodomain antiserum  $\alpha 203$  showed that phorbol ester treatment resulted in a decrease in gp140<sup>TrkA</sup> content (Fig. 1 B). In addition to the two glycosylated TrkA forms, a polypeptide of 41 kD (p41) was recognized by the  $\alpha 203$  antiserum in Western blots of immunoprecipitates from cells treated with PMA. gp140<sup>TrkA</sup>, gp110<sup>TrkA</sup>, and p41 were undetectable by Western blotting when immunoprecipitations were carried out in the presence of the competing peptide against which the  $\alpha 203$  antiserum was raised (data not shown). The fact that p41 was both immunoprecipitated and recognized by the  $\alpha 203$  antiserum in Western blots, indicated that this protein corresponded to a TrkA intracellular fragment containing the epitope identified by that antiserum.



**Figure 1.** Treatment with phorbol esters induces cleavage of TrkA. (A) Effect of phorbol esters on TrkA tyrosine phosphorylation. PC12 (100 mm dish) or CHO<sup>TrkA</sup> (60 mm dish) cells were preincubated with or without 1  $\mu$ M PMA for 30 min, and then treated with NGF (100 ng/ml) for 5 min where indicated. Cell lysates were immunoprecipitated with anti-Trk endodomain antiserum ( $\alpha 203$ ), and tyrosine-phosphorylated Trk identified on Western blots with an anti-phosphotyrosine antibody. (B) Preincubation of CHO<sup>TrkA</sup> cells with PMA induces the generation of a 41-kD fragment that is recognized by the  $\alpha 203$  anti-endodomain antiserum. CHO<sup>TrkA</sup> cells were incubated with or without PMA (1  $\mu$ M) for 30 min, cell lysates immunoprecipitated with  $\alpha 203$  antiserum, and Western blots probed with the same antiserum. IgH, Ig heavy chain. The migration of the relative molecular mass (M<sub>r</sub>) markers is indicated at the right side.



**Figure 2.** Time course and dose-response of the PMA effect on TrkA cleavage. (A) CHO<sup>TrkA</sup> cells were incubated with PMA (1  $\mu$ M) for the indicated times, cell lysates immunoprecipitated with the  $\alpha 203$  antiserum, blotted, and detected with the same antiserum. (B) CHO<sup>TrkA</sup> cells were incubated with the indicated PMA concentrations for 30 min, and TrkA and the p41 fragment identified by immunoprecipitation and Western blotting with the  $\alpha 203$  antiserum.



**Figure 3.** Membrane association of the p41 TrkA fragment. (A) Subcellular fractionation of CHO<sup>TrkA</sup> cells. CHO<sup>TrkA</sup> cells incubated with or without PMA (1  $\mu$ M) were homogenized in a hypotonic buffer, and the microsomal and cytosolic fractions separated by differential ultracentrifugation. (B) Protease protection experiments with proteinase K. CHO<sup>TrkA</sup> cells were treated with proteinase K (200  $\mu$ g/ml) for 90 min, washed in the presence of PMSF, lysed, immunoprecipitated with the same antiserum, and Western blots of the samples probed with the  $\alpha$ 203 antiserum. To avoid interference of the heavy Ig chains in the detection of fragments of TrkA generated by proteinase K treatment, detection of the primary antibody in this blot was done with HRP-conjugated protein A. (C) Triton X-114 extraction of TrkA and the p41 fragment. CHO<sup>TrkA</sup> cells were treated with or without PMA (1  $\mu$ M) for 30 min, and lysed on ice in the Triton X-114 lysis buffer. Lysates were directly immunoprecipitated with  $\alpha$ 203 antiserum (CHO<sup>TrkA</sup>), or subjected to phase separation into detergent (TX-114) or aqueous (Aqueous) phases. The procedure for immunoprecipitation and Western blotting with the  $\alpha$ 203 antiserum was essentially as described in the Materials and Methods section.

The p41 cell-associated band was detected within 2.5 min of treatment with PMA, continued to increase for up to 30 min, and turned over almost completely between 2 and 4 h of treatment (Fig. 2 A). As expected from a precursor-product relationship, the progressive increase in

the intensity of the p41 band was paralleled by a concomitant decrease in gp140<sup>TrkA</sup>. The effect of PMA on the generation of p41 was dose-dependent (Fig. 2 B), with a half maximal concentration of 40 nM, and full effect at concentrations above 100 nM. Taken together, these data suggest that TrkA is proteolytically cleaved by a regulated mechanism involving PMA-induced PKC activation.

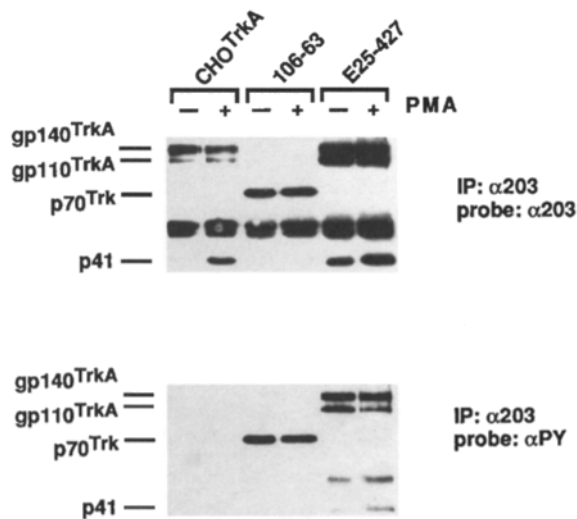
### Cleavage of TrkA Occurs at the Ectodomain

The relative molecular mass of the p41 TrkA fragment suggested that the cleavage site should be close to the transmembrane region, but did not distinguish whether the site was located in the ecto- or endodomain. To more precisely define the region where TrkA cleavage occurred, several types of experiments were carried out. First, we tested whether p41 was membrane associated. CHO<sup>TrkA</sup> cells treated with or without PMA were homogenized, and the cytosolic and microsomal fractions separated by differential ultracentrifugation. Both fractions were subjected to immunoprecipitation and Western blotting with the  $\alpha$ 203 antiserum. The crude microsomal pellet fraction contained the p41 fragment as well as gp140<sup>TrkA</sup> and gp110<sup>TrkA</sup>, while none of them were detected in the cytosolic fraction (Fig. 3 A). A significant amount of p41 was associated to the pellet of untreated cells, and increased upon PMA treatment. The p41 present in the microsomal fraction from untreated cells could originate from either enrichment of a small proportion normally produced by resting cells, or by proteolysis during the manipulation of the samples.

In protease protection experiments (Fig. 3 B), treatment of CHO<sup>TrkA</sup> cells with proteinase K produced the disappearance of gp140<sup>TrkA</sup>, while gp110<sup>TrkA</sup> was largely resistant to digestion by this protease. The gp140<sup>TrkA</sup> was converted into two major fragments of  $\approx$ 60 and  $\approx$ 41 kD, the latter having a mobility indistinguishable, by conventional electrophoresis, from that of the p41 PMA-induced fragment. The p41 fragment generated by treatment with proteinase K of intact cells could not be recovered when the incubation with the protease proceeded in the presence of 0.1% Triton X-100 (data not shown).

To determine whether membrane association of the p41 fragment was due to conservation of the transmembrane domain of TrkA, Triton X-114 phase separation of cellular proteins was performed (Fig. 3 C). After two sequential detergent extractions, the glycosylated gp140<sup>TrkA</sup> and gp110<sup>TrkA</sup> forms as well as the p41 fragment were predominantly found in the Triton X-114 phase.

To define whether correct targeting of TrkA as an integral transmembrane protein was essential for its regulated cleavage, we analyzed the effect of PMA on a form of TrkA that is targeted to the cytosolic compartment. This form (p70<sup>Trk</sup>) was generated by a fusion between nonmuscle tropomyosin, and the transmembrane and cytosolic domains of TrkA (22). Addition of PMA to NIH3T3 cells transformed by the *TrkA* oncogene (106-63 cell line) had no apparent effect on the p70<sup>Trk</sup> oncoprotein (Fig. 4 A), and did not induce the generation of p41. NIH3T3 cells contain the machinery necessary for cleavage of TrkA, as demonstrated by the cleavage of transmembrane gp140<sup>TrkA</sup> (Fig. 4 A, E25-427 cell line; 23). In the latter cell line, TrkA overexpression was accompanied by a significant



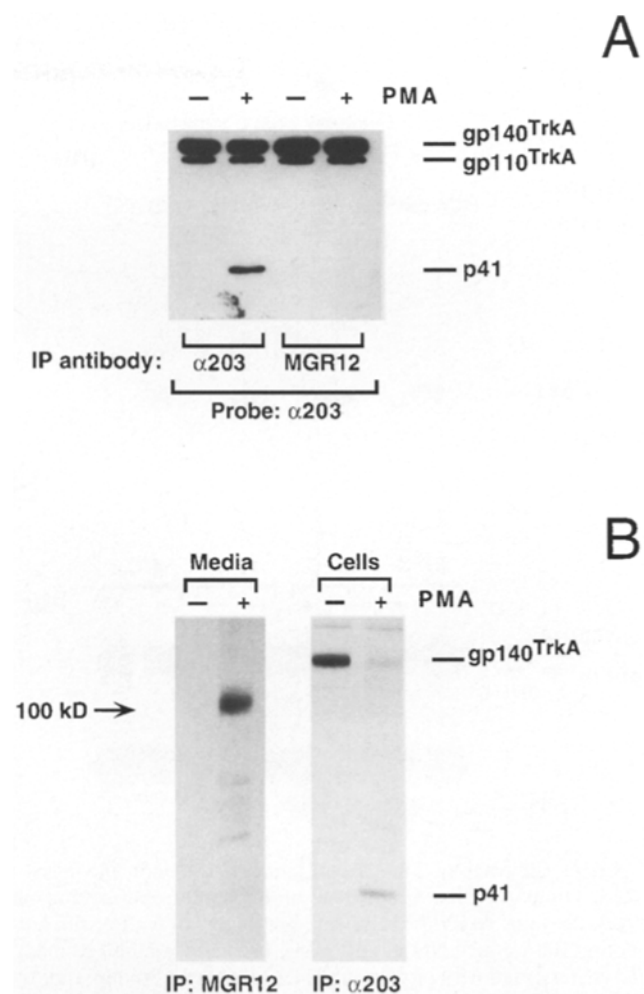
**Figure 4.** Cytosolic versions of TrkA are resistant to cleavage. NIH3T3 cells transfected with either a genomic sequence that codes for a cytosolic chimera between nonmuscle tropomyosin and Trk (106-63 cells), or the cDNA coding for the human TrkA protooncogene (E25-427 cells), were treated with PMA (1  $\mu$ M) for 30 min, immunoprecipitated with  $\alpha$ 203 antiserum, and blots of the samples probed with the  $\alpha$ 203 antiserum (A), or with anti-phosphotyrosine (B,  $\alpha$ PY) antibodies.

level of p41 that was, as the receptor itself, tyrosine phosphorylated in the absence of any treatment (Fig. 4 B). The oncogenic protein, that is also overexpressed in 106-63 cells and whose kinase is constitutively active, was also tyrosine phosphorylated under resting conditions, but no p41 band was detected (Fig. 4 B).

#### **Cleavage of TrkA Results in the Solubilization of the Ectodomain, and Occurs Outside the Endolysosomal Pathway**

If cleavage of gp140<sup>TrkA</sup> occurred close to the transmembrane region by a unique cutting event, then two fragments should be generated: a cell-associated fragment containing the transmembrane and cytosolic regions of TrkA (the above described p41); and a soluble fragment of  $\approx$ 100 kD corresponding to the ectodomain. To test for the presence of the latter in culture supernatants, we used the anti-ectodomain mAb MGR12. To confirm that mAb MGR12 interacted with TrkA from CHO<sup>TrkA</sup> cells, lysates from this cell line were immunoprecipitated with this mAb, and Western blots of the samples probed with the  $\alpha$ 203 antiserum. Fig. 5 A shows that MGR12 immunoprecipitated gp140<sup>TrkA</sup> and gp110<sup>TrkA</sup>, but, as expected from an anti-ectodomain antibody, failed to immunoprecipitate p41 (Fig. 5 A). In addition, in immunofluorescence experiments using unpermeabilized CHO<sup>TrkA</sup> cells, i.e., exposing only ectodomain epitopes, this mAb stained the cell periphery (N. Cabrera, and A. Pandiella, unpublished observations).

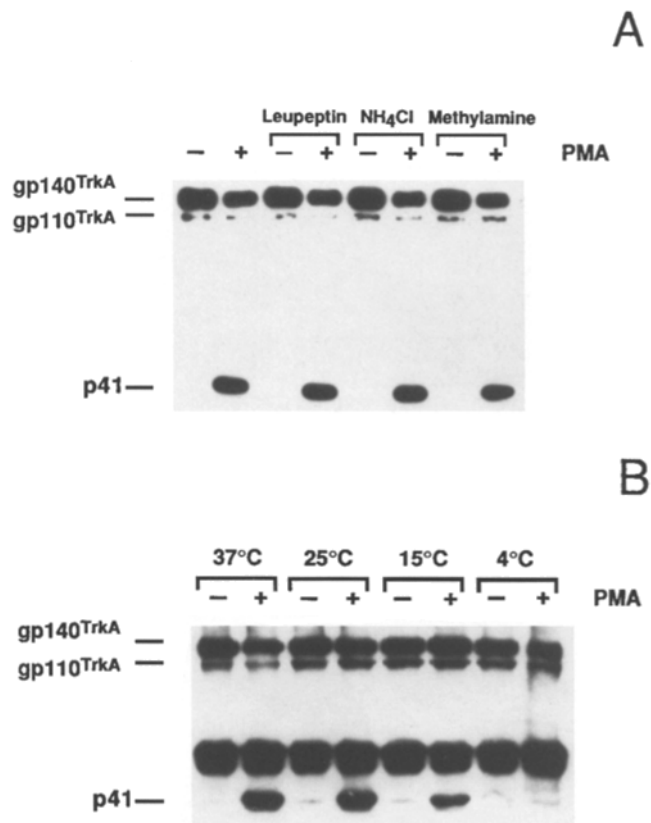
When media samples from metabolically labeled CHO<sup>TrkA</sup> cells were immunoprecipitated by the MGR12 mAb, a band of 100 kD was detected in the culture media from cells incubated with the PKC activator PMA (Fig. 5 B, left). Immunoprecipitation of the cell lysates with the  $\alpha$ 203



**Figure 5.** The TrkA ectodomain can be recovered as a soluble fragment. (A) The anti-ectodomain mAb MGR12 immunoprecipitates gp140<sup>TrkA</sup>, but not p41. CHO<sup>TrkA</sup> cells were treated with or without PMA (1  $\mu$ M), and cell lysates were immunoprecipitated with either  $\alpha$ 203 antiserum, or the MGR12 antibody. The blot was probed with the  $\alpha$ 203 antiserum. (B) PMA induces solubilization of the TrkA ectodomain. Metabolically labeled CHO<sup>TrkA</sup> cells were treated with or without PMA (1  $\mu$ M), culture media was collected and immunoprecipitated with mAb MGR12, and cell lysates were immunoprecipitated with  $\alpha$ 203 antiserum. Immunoprecipitates were resolved in 10% SDS-PAGE gels, and bands were visualized by fluorography.

antiserum showed that gp140<sup>TrkA</sup> amount decreased in PMA-treated cells, and this was followed by the appearance of p41 (Fig. 5 B, right). The 100-kD band could not be detected in cell lysates from PMA-treated CHO<sup>TrkA</sup> cells immunoprecipitated by MGR12 (data not shown).

Since the endosomal-lysosomal pathway participates in the proteolytic degradation of cell surface proteins (16, 36), including receptor tyrosine kinases (38), we tested its possible involvement as a site for regulated TrkA cleavage. Preincubation of CHO<sup>TrkA</sup> cells with drugs targeted to disrupt lysosomal enzyme activity such as leupeptin, ammonium chloride, or methylamine, did not inhibit p41 generation in response to PMA (Fig. 6 A). Treatment of cells at low temperatures has been used to prevent membrane

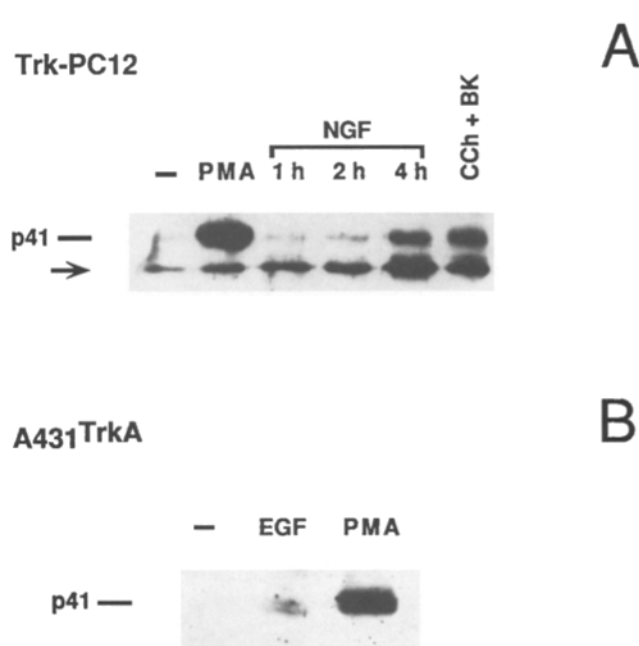


**Figure 6.** Cleavage of TrkA occurs outside the endosomal-lysosomal compartments. (A) Effect of lysosomotropic agents on TrkA cleavage. CHO<sup>TrkA</sup> cells were preincubated for 1 h with leupeptin (100  $\mu$ g/ml), NH<sub>4</sub>Cl (10 mM), or methylamine (10 mM), and then treated with or without PMA (1  $\mu$ M) for 30 min. Cell lysates were immunoprecipitated with  $\alpha$ 203 antiserum, Westerns probed with the same antiserum, and antibodies bound to TrkA or p41 detected by HRP-conjugated protein A. (B) Effect of decreasing incubation temperature on TrkA processing. CHO<sup>TrkA</sup> cells were treated with or without PMA (1  $\mu$ M) for 30 (37°C), 120 (25°C and 15°C), or 240 (4°C) min at the temperatures indicated. TrkA and p41 were detected by immunoprecipitation and Western blotting with the  $\alpha$ 203 antiserum as described in the Materials and Methods section.

trafficking in animal cells (21). Internalization of ligands and their receptors is inhibited below 10°C; proceeds between 15° and 20°C, but the internalized molecules are not delivered to the lysosomes, and they accumulate in a pre-lysosomal compartment. Cleavage of TrkA induced by PMA treatment was strictly dependent on the incubation temperature: it was observed at temperatures from 37° to 15°C, although the efficiency of the cleavage decreased at low temperatures, and further lowering the temperature to 4°C completely prevented TrkA cleavage (Fig. 6 B).

#### Physiological Regulation of TrkA Cleavage

To analyze whether physiological agonists were able to provoke TrkA ectodomain cleavage, we used two different cellular systems. One was derived from PC12 cells; the other from a human cell line known to possess high amounts of another receptor tyrosine kinase, the EGFR. Due to the low complement of TrkA receptors present in wild type



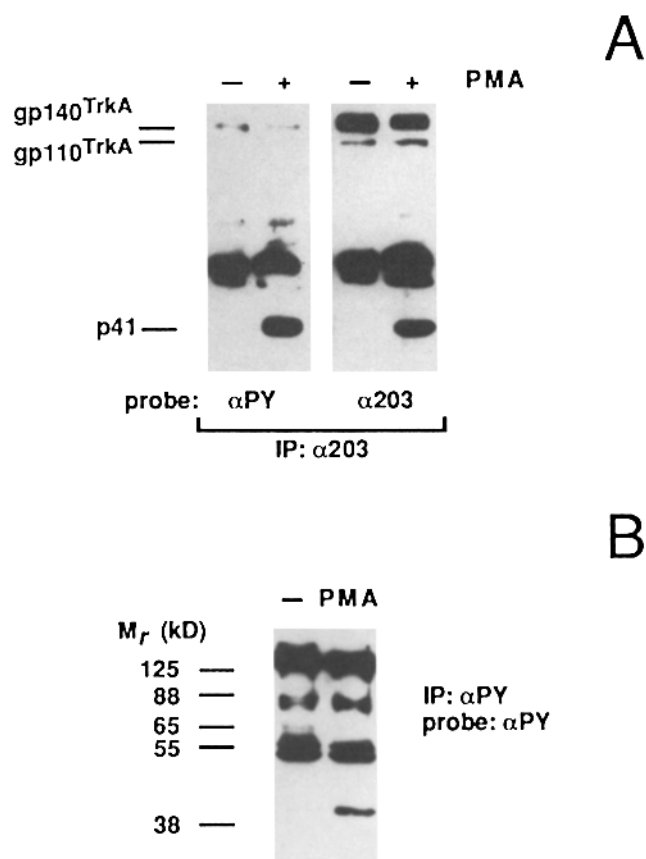
**Figure 7.** TrkA cleavage can be induced by first messengers. (A) Cleavage of TrkA in Trk-PC12 cells. Trk-PC12 cells were incubated with PMA (1  $\mu$ M, 30 min), NGF (100 ng/ml), or a mixture of carbamylcholine (CCh, 100  $\mu$ M, 30 min) and bradykinin (BK, 100 nM, 30 min), and cell lysates were analyzed for the presence of p41 by immunoprecipitation and Western blotting with the  $\alpha$ 203 antiserum. (B) Cleavage of TrkA in A431<sup>TrkA</sup> cells. A431<sup>TrkA</sup> cells were treated with EGF (10 nM), or PMA (1  $\mu$ M) for 60 min, and p41 identified as described above.

PC12 cells, we used a PC12 clone (named Trk-PC12 cells; 18) that overexpresses this receptor. PMA treatment of Trk-PC12 cells induced the generation of the p41 fragment (Fig. 7 A). The p41 fragment was also seen when different agonists were added to Trk-PC12 cells. Activation of cholinergic and BK2 bradykinin receptors, that are endogenously expressed by PC12 cells, had a remarkable effect on the generation of the p41 fragment (Fig. 7 A). NGF treatment also increased the generation of the p41 fragment, although less efficiently, and with a more prolonged time course. In addition to the p41 fragment, a band of lower relative molecular mass was also induced by several of these agonists (Fig. 7 A, arrow), and was specifically recognized by the  $\alpha$ 203 antiserum.

The potential regulation of TrkA cleavage following the activation of other receptor tyrosine kinases was next analyzed by using A431 human epidermoid carcinoma cells transfected with the TrkA cDNA (A431<sup>TrkA</sup>). Addition of EGF to these cells (that endogenously express high amounts of the EGF receptor) stimulated the cleavage of TrkA (Fig. 7 B). The effect of this growth factor was much less efficient than PMA treatment, probably due to rapid EGF-induced EGFR down-regulation.

#### Ectodomain Cleavage of TrkA May Regulate Tyrosine Phosphorylation of the p41 Cell-associated Fragment

Molecular studies of several receptor tyrosine kinases have shown that their extracellular domains may act as negative regulators of the cytoplasmic kinase activity (32).



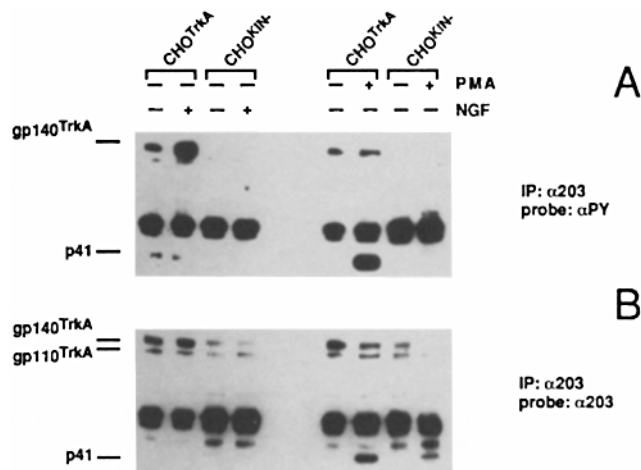
**Figure 8.** Tyrosine phosphorylation of p41. (A) Presence of phosphotyrosine in p41. CHO<sup>TrkA</sup> cells treated with or without PMA (1 μM) were immunoprecipitated with the α203 antibody and the Western blot of the samples probed with anti-phosphotyrosine antibodies (*left*). The blot was then stripped and reprobed with α203 antiserum (*right*). (B) Immunoprecipitation of p41 with anti-phosphotyrosine antibodies. CHO<sup>TrkA</sup> cells were incubated with or without PMA (1 μM) for 30 min, and cell lysates immunoprecipitated with anti-phosphotyrosine antibodies, followed by probing of blots with the same antibody.

If cleavage of TrkA were a natural activating mechanism, p41 would be expected to have an increased phosphotyrosine content with respect to the resting gp140<sup>TrkA</sup> receptor. To test this point we analyzed whether p41 was tyrosine phosphorylated. Immunoprecipitation of lysates from CHO<sup>TrkA</sup> cells with the α203 antiserum, followed by probing of blots with the anti-phosphotyrosine antibody, showed that gp140<sup>TrkA</sup> was slightly tyrosine phosphorylated under resting conditions (Fig. 8 A, *left*), and PMA treatment resulted in the appearance of tyrosine phosphorylated p41 (Fig. 8, A and B). Remarkably, the phosphotyrosine content of p41 was apparently higher than that of gp140<sup>TrkA</sup> of resting CHO<sup>TrkA</sup> cells (Fig. 8 A). Reprobing of the blot shown in Fig. 8 A (*left*) with anti-endodomain antiserum showed that the increased phosphotyrosine content in p41 was not due to higher amounts of this fragment with respect to the holoreceptor, but to a higher number of phosphorylated tyrosine residues per molecule (Fig. 8 A, *right*). Immunoprecipitation of cell lysates with the anti-phosphotyrosine antibody, followed by probing of

the blots with the same antibody, showed that PMA treatment of CHO<sup>TrkA</sup> cells induced the appearance of a tyrosine-phosphorylated band with a relative molecular mass indistinguishable, by SDS-PAGE, from p41 (Fig. 8 B). Reprobing of this blot with α203 antiserum showed that this band corresponded to p41 (data not shown).

We next analyzed whether p41 tyrosine phosphorylation represented an autophosphorylation event, or depended on an indirect mechanism. For this purpose, a CHO cell line expressing a kinase inactive version of TrkA (CHO<sup>KIN-</sup> cells) was generated. Fig. 9 A shows that the mutant TrkA receptor of CHO<sup>KIN-</sup> was unable to autophosphorylate in response to NGF. In addition, and besides corroborating that p41 had an increased phosphotyrosine content with respect to resting gp140<sup>TrkA</sup>, this experiment indicated that p41 tyrosine phosphorylation was close to that induced on gp140<sup>TrkA</sup> by NGF addition (that was used as a control for TrkA activation). It is noteworthy, however, that p41 tyrosine phosphorylation rarely surpassed the autophosphorylation induced in the holoreceptor by NGF, and depended on the degree of receptor cleavage (data not shown).

While gp140<sup>TrkA</sup> and p41 were tyrosine phosphorylated in CHO<sup>TrkA</sup> cells, the mutant form of the receptor and the p41 fragment were not recognized by the anti-phosphotyrosine antibodies. However, the mutated receptor present in CHO<sup>KIN-</sup> cells was sensitive to cleavage, as demonstrated by reprobing of the blot shown in Fig. 9 A with the α203 antiserum: gp140<sup>TrkA</sup> amount decreased upon phorbol ester treatment, and this was followed by the appearance of p41 (Fig. 9 B). Therefore, an intact kinase is not essential for regulated TrkA cleavage, but is required for regulated p41 tyrosine phosphorylation. Furthermore, these results exclude that phosphorylation of p41 is an indirect phenomenon caused, for example, by a change in overall ty-



**Figure 9.** The kinase activity of the TrkA endodomain is essential for tyrosine phosphorylation of p41. Where indicated, CHO<sup>TrkA</sup> or CHO<sup>KIN-</sup> cells were incubated with NGF (100 ng/ml) or PMA (1 μM) for 5 or 30 min, respectively. Lysates were immunoprecipitated with α203 antiserum and the Western blot probed with anti-phosphotyrosine antibodies (A), or, after stripping of the latter, with α203 antiserum (B).

rosine phosphatase activity, or by an intermediate itinerant tyrosine kinase activated by PKC.

## Discussion

In this study we show that the TrkA receptor tyrosine kinase is susceptible to endoproteolytic cleavage, and this results in a cell-associated fragment that is phosphorylated on tyrosine. Cleavage of TrkA generated two fragments: one (p100) that corresponded to the ectodomain and could be recovered from the culture media; and another (p41) that contained the transmembrane and cytosolic domains and remained membrane-bound. The characteristics of this cleavage, such as its regulated nature, kinetics, location of the cleavage site in a juxtamembrane region, and cellular compartment where it occurs, closely follow the properties of the cell surface proteolytic system that is responsible for the regulated cleavage of a heterogeneous group of membrane proteins (1, 25).

The cleavage of TrkA is highly regulated. A number of natural agonists and pharmacological agents can induce rapid cleavage of TrkA in several cell lines, suggesting that this mechanism may be widespread and may play a role in physiological processes involving TrkA expressing cells. These effectors act at the cell surface by a receptor mediated mechanism (NGF, EGF, bradykinin, carbamylcholine), or directly activate second messenger systems, such as PKC (phorbol esters). The latter is probably the most universal means for the regulation of the activity of the cell surface proteolytic system (14, 25), and has been shown to participate in controlling the balance between membrane-anchored and soluble forms of the ectodomain of several membrane proteins. Although any of these treatments may also lead to increased gene expression, the rapid kinetics of p41 generation, and the results obtained by incubations at 15°C (a temperature that blocks bulk flow of proteins from the *trans*-Golgi network to the plasma membrane; 7, 15) suggest that TrkA cleavage did not require protein synthesis.

Several experimental data demonstrated that TrkA cleavage occurred at the ectodomain in the juxtamembrane region. The p41 cell-associated fragment contained the TrkA transmembrane domain, since it cofractionated with the microsomal pellet obtained in crude cell fractionations, and was extracted by Triton X-114. That the cleavage occurred in a juxtamembrane location is supported by the characteristics of the soluble TrkA ectodomain fragment. The extracellular domain of human TrkA consists of 411 amino acids (including the signal peptide), with 13 potential sites of N-linked glycosylation (23). The expected relative molecular mass of the extracellular domain is  $\approx$ 100 kD (the contributions of the amino acid skeleton and the sugar chains are 45 and 55 kD, respectively; 23). Endoproteolytic cleavage of TrkA resulted in the generation of a 100-kD soluble fragment that could be recovered from the culture media. The electrophoretic migration, and the presence of extracellular epitopes demonstrated that this fragment corresponds to the ectodomain of TrkA. Precise topological location of the cleavage site will require analysis of the NH<sub>2</sub>- or COOH-terminal sequences of p41 or the soluble fragment, respectively.

It is likely that the endoprotease responsible for TrkA cleavage acts at the cell surface. Analogously to other membrane proteins that undergo shedding of their ectodomain, such as the  $\beta$ -amyloid precursor protein (17) or proTGF- $\alpha$  (7), treatments that inhibit plasma membrane-endosomal trafficking or lysosomal function did not affect regulated TrkA cleavage, excluding those compartments as sites of action of the proteolytic system. In addition, the intracellular gp110<sup>TrkA</sup> precursor was largely resistant to regulated cleavage. Even though the ectodomain of this precursor is expected to remain luminal and be cotransported with the protease, the environment of the intracellular compartments does not seem to be favorable for the functioning of the endoproteolytic system. Incubation at 4°C, a treatment used to prevent membrane trafficking, did, however, inhibit p41 generation. It is unlikely that the regulated endoproteolytic system is completely inactive at this temperature, since under this condition it has been shown to cleave (although very marginally, and with slower kinetics) proTGF- $\alpha$  (7) and the Axl receptor (26). The reason for the failure to cleave TrkA at 4°C is unknown at present, but mimics the resistance of other receptors, such as p55 tumor necrosis factor receptor (TNF-R), to regulated cleavage at low temperatures (8). In this respect it should be noted that TrkA is less efficiently cleaved than other membrane-anchored proteins that undergo regulated release of their ectodomains, and this resistance to cleavage may be more pronounced at low temperatures. In fact, at 37°C cleavage of proTGF- $\alpha$  is complete within a few minutes of PMA treatment (7, 28, 29), while a significant proportion of TrkA is resistant to cleavage at this temperature. The reason for this different behavior of TrkA with respect to other membrane proteins is presently under study.

The kinase activity of TrkA was found to be dispensable for regulated cleavage. This is especially interesting since an intact kinase is essential for several receptor tyrosine kinase properties, such as signalling (37, 38). The susceptibility of kinase-inactive TrkA to regulated cleavage was not appreciably different from that of wild type TrkA. In addition, a cytosolic oncogenic version of Trk, whose kinase is constitutively active, is resistant to cleavage upon PMA treatment. These observations suggest that subcellular location, rather than the kinase activity, makes TrkA susceptible to cleavage. However, this does not necessarily exclude that TrkA kinase activity may play a role in ectodomain cleavage. In fact, there is a correlation between the amount of receptor in different overexpressing clones, the tyrosine phosphorylation of Trk holoreceptor, and the resting level of p41. This receptor activation in the absence of any exogenous treatment may mimic ligand-mediated increase in tyrosine kinase activity, and this may result in the triggering of mechanisms that finally activate the cell surface protease. In support for a role of TrkA receptor activation in the regulation of its cleavage are the results obtained with NGF. Treatment of Trk-PC12 cells with NGF resulted in a delayed, but substantial, formation of the p41 fragment. Although the mechanisms involved in the NGF-induced effect have not been elucidated yet, this factor (as well as EGF, carbamylcholine, and bradykinin) has been reported to increase the hydrolysis of membrane polyphosphoinositides (10, 27), that results in increased in-



tracellular  $\text{Ca}^{2+}$  and PKC activity. The notable effect of these messengers in membrane protein ectodomain cleavage (25) suggests that they may participate in first messenger-induced TrkA cleavage, or even in p41 generation that results from TrkA overexpression.

In addition to TrkA, the extracellular domains of the colony stimulating factor-1 receptor (13), c-met (30), Axl (26), and erbB2 (31, 39) receptor tyrosine kinases have been shown to be clipped by a regulated endoproteolytic system. The effect of this cleavage on the biochemical and biological properties of the cell-associated fragment has received limited attention. In the case of the colony stimulating factor-1 receptor, phorbol ester treatment did not apparently change the phosphorylation of either the holoreceptor, or that of the proteolytic fragment (13). Cleavage of the Axl receptor induced by phorbol esters has been shown to generate a cell-associated fragment whose phosphotyrosine content is apparently lower than that of the holoreceptor (26). Analysis of the phosphotyrosine content of TrkA showed that PMA treatment increased the phosphorylation of the p41 fragment when compared to the intact receptor. Several mechanisms could be responsible for the increased phosphorylation of p41 upon PMA treatment, including: (a) inhibition of tyrosine phosphatase activity; (b) indirect phosphorylation mediated by a membrane-bound or itinerant cytosolic tyrosine kinase whose activity would be up-regulated by PKC; or (c) autophosphorylation by the intrinsic tyrosine kinase activity of the p41 fragment. Data obtained with the kinase inactive mutant of TrkA showed that the kinase activity of the intracellular domain was essential for p41 tyrosine phosphorylation, thus excluding the participation of an intermediate kinase, or an overall decrease in tyrosine phosphatase activity. Therefore, the increased phosphotyrosine content of the p41 fragment probably reflects increased kinase activity of p41 when compared to that of the holoreceptor. Preliminary in vitro studies had shown that p41 is able to autophosphorylate, and exhibits tyrosine kinase activity against exogenous substrates (Cabrera, N., and A. Pandiella, unpublished results). Definitive proof of whether ectodomain cleavage represents an activating mechanism will require an exhaustive biochemical study of the enzymatic properties of p41.

Soluble forms of neurotrophin receptors have been detected in the culture media of neuronal (12) and nonneuronal (4, 40) cells. These soluble forms most likely derive from the  $\text{p75}^{\text{LNTR}}$ , because of their relative molecular mass and immunoreactivity with reagents specific for the low affinity receptor. Several mechanisms have been implicated in the generation of truncated  $\text{p75}^{\text{LNTR}}$ . In human melanoma A875 cells, processing of  $\text{p75}^{\text{LNTR}}$  into a soluble fragment was found to be dependent on endocytosis, and the mechanism of generation probably involves lysosomal compartments (40). However, truncation of  $\text{p75}^{\text{LNTR}}$  in Schwann cells probably occurs at the cell surface by a tightly bound membrane metalloprotease, since there is no evidence for internalization of  $\text{p75}^{\text{LNTR}}$  in these cells, lysosomotropic agents do not affect truncation, cleavage occurs in isolated membrane preparations, and the process is blocked by classical metalloprotease inhibitors (11). Interestingly, these metalloprotease inhibitors accelerate peripheral nerve regeneration in vivo, and this effect has

been linked to an increase in local NGF concentrations (11). Clearly, the mechanism of TrkA cleavage differs from that responsible for the solubilization of  $\text{p75}^{\text{LNTR}}$  in A875 cells, and present evidence is still incomplete to define whether the metalloprotease activity that cleaves  $\text{p75}^{\text{LNTR}}$  in Schwann cells could induce TrkA cleavage. Studies to elucidate whether  $\text{p75}^{\text{LNTR}}$  cleavage is also a regulated process; analysis of the effect of a panel of protease inhibitors on TrkA regulated cleavage; and reconstitution of the regulated cleavage of TrkA in membrane preparations, are now in progress.

What is the significance of TrkA receptor cleavage? Besides serving as an outlet of the solubilized ligand binding domain, proteolytic cleavage may represent a natural mechanism of receptor activation. In the case of other receptor tyrosine kinases, elimination of sequences coding for their extracellular domain has been reported to produce truncated molecules with constitutive enzymatic activity that resemble, structurally and functionally, the oncogenic products of certain transforming retroviruses (32, 38). If TrkA cleavage is an activating mechanism, it may act alone or in concert with ligand binding to initiate or potentiate receptor signaling in neuronal cells bearing TrkA. Development of adequate models to test the importance of truncation of extracellular sequences of membrane proteins on their biological properties may unravel a potential signaling capability of proteins with unknown function, or whose ligands have not been isolated yet.

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