JCB Article

Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes

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A central tenet of nerve growth factor (NGF) action that is poorly understood is its ability to mediate cytoplasmic signaling, through its receptor TrkA, that is initiated at the nerve terminal and conveyed to the soma. We identified an NGF-induced protein that we termed Pincher (pinocytic chaperone) that mediates endocytosis and trafficking of NGF and its receptor TrkA. In PC12 cells, overexpression of Pincher dramatically stimulated NGF-induced endocytosis of TrkA, unexpectedly at sites of clathrin-independent macropinocytosis within cell surface ruffles. Subsequently, a system of Pincher-containing tubules mediated the delivery of NGF/TrkA-containing vesicles to cytoplasmic accumulations. These vesicles selectively and persistently mediated TrkA-erk5 mitogen-activated protein kinase signaling. A dominant inhibitory mutant form of Pincher inhibited the NGF-induced endocytosis of TrkA, and selectively blocked TrkA-mediated cytoplasmic signaling of erk5, but not erk1/2, kinases. Our results indicate that Pincher mediates pinocytic endocytosis of functionally specialized NGF/TrkA endosomes with persistent signaling potential.

Introduction

Nerve growth factor (NGF)* is the prototypic and best-studied member of the neurotrophin family of neuronal growth factors. NGF controls the survival and development of certain populations of peripheral neurons and influences neuronal phenotype throughout life (Levi-Montalcini, 1987). The principal site of NGF action is at the nerve terminal, where it is internalized and transported retrogradely to the neuronal cell body (for review see Reynolds et al., 2000). The retrograde transport of NGF, and/or an NGF-generated signal, has been postulated to mediate both neuronal survival and changes in gene expression (for review see Miller and Kaplan, 2001).

Although retrograde transport of NGF would appear to be important for NGF actions, the mechanisms for NGF internalization, formation of the transport vehicle, and its interaction with the transport machinery have been elusive. NGF is internalized after binding to its plasma membrane receptors, $p75^{NTR}$, and TrkA. NGF and TrkA have been reported to be associated with and/or stimulate different types of endocytic machinery in PC12 cells, including clathrin-coated vesicles (Grimes et al., 1996; Howe et al., 2001), ruffling and pinocytosis (Connolly et al., 1987), and calveolae (Huang et al., 1999). The means by which NGF and TrkA are internalized may involve any combination of these processes. As opposed to the well-defined modes for processing receptor-mediated endosomes (for review see Mellman, 1996), a remarkable feature of NGF-containing endosomes in neuronal terminals is that they can avoid degradation or recycling, and instead can be directed to the retrograde transport machinery.

How does the NGF received at the neuronal terminal mediate signaling at the cell body? It has been proposed that NGF filled endosomes with the capacity for receptor-mediated intracellular signal transduction function during and after retrograde transport (Halegoua et al., 1991). Putative signaling endosomes containing NGF and TrkA have since been identified and isolated from PC12 cells (Grimes et al., 1996). Although signaling pathways activated by retrogradely transported NGF and TrkA have only recently been investigated, TrkA signaling at the plasma membrane is well documented, and is mediated through multiple pathways initiated by specific TrkA autophosphorylation sites (for review see Kaplan and

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*Abbreviations used in this paper: GST, glutathione-*S*-transferase; HA, hemagglutinin; MAP, mitogen-activation protein; NGF, nerve growth factor; TOGA, total gene analysis; VAB, vesicle accumulating body.

Key words: neutrophin; membrane trafficking; signal transduction; EH domain; MAP kinase

Miller, 2000). The two best-studied signaling sites on TrkA, P-Y490 and P-Y785, bind to Shc and phospholipase C-γ (PLC-γ), respectively. Shc mediates stimulation of Ras-mitogen -activated protein (MAP) kinase and PI-3-kinase signaling pathways, whereas PLC-γ mediates signaling via phosphatidylinositol turnover. These signaling pathways are further branched, leading to differential control of gene expression (D'Arcangelo and Halegoua, 1993) and survival (for review see Kaplan and Miller, 2000). Retrograde axonally transported TrkA has also been shown to be autophosphorylated (Ehlers et al., 1995; Riccio et al., 1997) on the Shc-binding site Y490 (Bhattacharyya et al., 1997), and mediates CREB phosphorylation in the cell body (Riccio et al., 1997; Watson et al., 1999), which is in part necessary for neuronal survival (Riccio et al., 1999). Several studies have suggested that internalized TrkA may signal differently from the plasma membrane receptor, although a consensus on the different signaling parameters has not yet been reached (Saragovi et al., 1998; Zhang et al., 2000; Wu et al., 2001). A recent study has identified the erk5 MAP kinase as selectively stimulated in the cell body by retrogradely transported TrkA (Watson et al., 2001).

Understanding the mechanisms for the formation and processing of, and signaling from, the endosomal NGF/TrkA complex, have been hampered by a lack of tools with which to specifically manipulate internalization of the NGF/TrkA complex and generation of the signaling endosome. We have identified a new NGF-induced protein in PC12 cells termed Pincher that functions as a pinocytic chaperone for vesicles containing NGF and TrkA. Pincher function is necessary for both the NGF-induced internalization of TrkA by a pinocytic process, and the sorting of long-lived endosomal vesicles with NGF signaling capability. Pincher function may shed light on the process of retrograde endosomal NGF signaling.

Results

Identification and regulation of a new NGF-induced cDNA-encoding Pincher

We examined gene inductions hours after pulsatile (1 min) NGF treatment using the PCR-based gene expression profiling technology known as total gene analysis (TOGA) (Sutcliffe et al., 2000). One cDNA, HAL18, represented an mRNA that was induced three- to fivefold by NGF treatment of PC12 cells. Two mRNA species were identified at ~4 and 3 kilobases in size (Fig. 1 A). In over five induction experiments, both mRNAs were induced within 3 h of NGF treatment, reaching peak levels (between three- and fivefold) at 4 h, and diminishing to near baseline levels within 24 h (Fig. 1 A).

To assess the array of signaling pathways used by TrkA to induce the HAL18 gene, we exploited the PC12 nnr5–derived cell lines lacking TrkA, or expressing TrkA mutants in PLC-γ (P-Y785) and Shc (P-Y490) binding sites. HAL18 mRNAs were induced sixfold by NGF treatment of the PC12 nnr5 lines expressing TrkA, but not in the lines lacking TrkA (Fig. 1 B, T14 and nnr, respectively). However, lower levels of induction could be seen in cells expressing each of the TrkA single mutants (Fig. 1 B, 1.9-fold in Y490F and 1.5-fold in Y785). NGF-treated cells expressing the TrkA double mutant, Y490F/Y785F, did not show any HAL18 mRNA induction. Thus, as with neurite growth (Stephens et al., 1994), both TrkA autophosphorylation sites contribute to the mRNA induction.

A full-length cDNA for HAL18 was created from overlapping PC12 library cDNAs and the predicted open reading frame is shown in Fig. 1 C. The only difference noted between clones was the length of 5′ untranslated sequence, suggesting that the two mRNAs detected on Northern blots represent alternate splice forms. For reasons discussed below, this protein was called Pincher. An antibody raised to a glutathione-*S*-transferase (GST)–Pincher fusion protein immunoprecipitated a protein from PC12 cell extracts that was detected on Western blots probed with the same antibody (Fig. 1 D). No Pincher protein could be detected in blots prepared from immunoprecipitates using anti-Pincher antibody that was preblocked with GST–Pincher (unpublished data). Pincher was induced by NGF treatment of PC12 cells over a time course that corresponded in magnitude and followed the time of induction of the HAL18 mRNAs detected on Northern blots. The tissue distribution of Pincher expression was determined by probing Northern blots containing RNAs from various tissues. Expression of both Pincher mRNAs was observed in heart and lung, dorsal root ganglion peripheral neurons, kidney, and brain (Fig. 1 E). Protein was also detected immunocytochemically in various neuronal populations in brain and in sympathetic neurons (unpublished data).

The sequence of Pincher revealed that it was a member of a gene family containing four mammalian genes (human EHD-1 to EHD-4 and mouse mRme-1 genes) (Pohl et al., 2000; Lin et al, 2001) and a nematode gene (RME-1) (Grant et al., 2001). Pincher-coding cDNA sequence is most closely related to the human EHD-4 gene (90% nucleic acid identity) (Kuo et al., 2001). Analysis of the predicted Pincher amino acid sequence revealed several interesting domains (Fig. 1 C), including an EH membrane trafficking domain, a coiled-coiled domain, and a domain distantly related to AAA type ATPases containing an intact P-loop ATP/GTP-binding motif. Because of these structural features, and its induction by NGF, we decided to examine whether Pincher was involved in TrkA trafficking.

Pincher overexpression enhances NGF-induced TrkA endocytosis

To determine the localization and effects of Pincher on the distribution of TrkA in PC12 cells, TrkA-PC12 cells that overexpress TrkA were transfected with a mammalian expression vector encoding a hemagglutinin (HA)-tagged Pincher. HA-Pincher expression was confirmed in Western blots of cell extracts from transfected cultures (Fig. 1 D). HA-Pincher localization was examined by confocal immunofluorescence microscopy using anti-HA antibody. As seen in Fig. 2 A, HA-Pincher is localized primarily to the plasma membrane, but can be occasionally visualized intracellularly. Plasma membrane localiza-

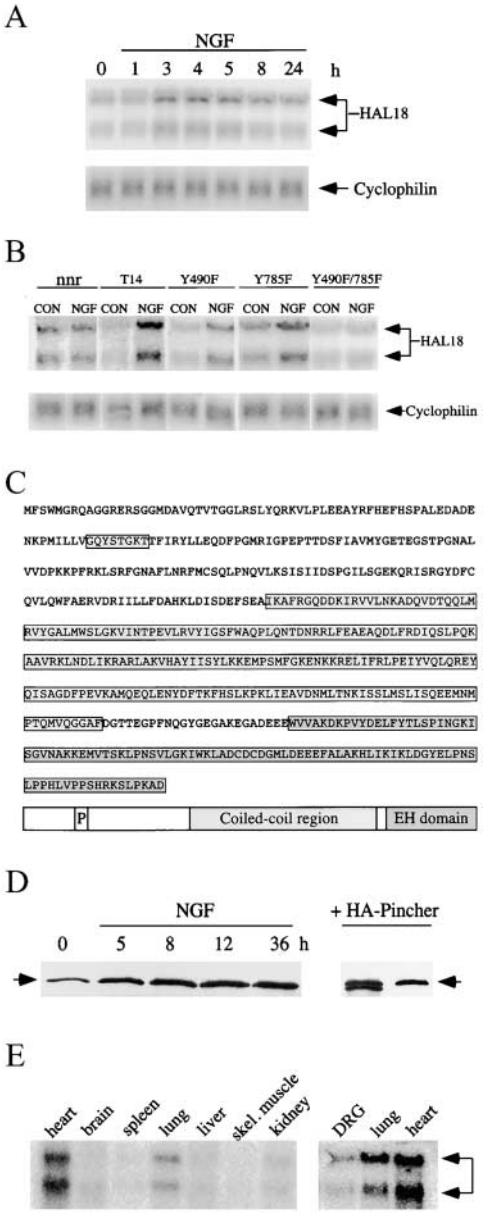


Figure 1. **HAL18 identifies a novel NGF-induced cDNA for Pincher that is upregulated through multiple TrkA signaling pathways.** (A) Total cellular RNA was isolated from PC12 cells treated with NGF for the times indicated, and subjected to Northern blotting using both ^{32}P-RNA HAL18 and cyclophilin probes. (B) PC12 nnr5 cells lacking TrkA and nnr5-derived transfectants expressing TrkA wild-type (WT), or Y490F, Y785F, or Y490F/Y785F TrkA mutants, were treated or not with NGF for 4 h. Cellular RNA was isolated and analyzed for HAL18 expression as in A. (C) The predicted Pincher protein sequence was derived from the cDNA sequence (EMBL/GenBank/DDBJ accession no. AY 094093). Shown below is a diagrammatic representation of Pincher domains of interest. P = P-loop (ATP/GTP-binding site). (D) Western blots of Pincher immunoprecipitated from cell extracts were probed with anti-Pincher or anti-HA antibodies. (Left) Blots from PC12 cells treated with NGF as indicated were probed with anti-Pincher antibody. (Right) Blots from PC12 cells transfected with a HA-tagged Pincher construct were first probed with anti-Pincher (left lane) and reprobed with anti-HA antibodies (right lane). The position of HA-Pincher is indicated. (E) Northern blots of RNAs obtained from the rat tissues indicated were probed with Pincher probe. Positions of Pincher mRNAs are indicated. (Left) CLONTECH blot of polyA-mRNAs. (Right) Blot of 10 μg total RNA in each lane.

tion was confirmed by costaining with wheat germ agglutinin, which showed a greatly overlapping staining pattern (unpublished data). The distribution of HA-Pincher did not appear to depend on the level of HA-Pincher expression as indicated by the similar pattern of staining for both dimly and brightly stained cells. TrkA, visualized in the same cells by double labeling using anti-TrkA antibody, was normally localized to the plasma membrane in a pattern that partially overlapped with that of HA-Pincher (Fig. 2 A). The small level of intracellular TrkA staining was presumably associated with the endoplasmic reticulum in the TrkA-PC12 cells. After NGF treatment, both HA-Pincher and TrkA migrated away from the peripheral plasma membrane in a time-dependent manner. After 2 min of NGF treatment, both HA-Pincher and TrkA became preferentially associated with NGF-induced ruffles and blebs at the plasma membrane (Fig. 2 A). Within 3 min of NGF treatment, cells were also seen in which HA-Pincher and TrkA were both dramatically associated with intracellular vesicle-like structures in largely overlapping patterns (Fig. 2 A). Although NGF treatment rapidly caused an apparent colocalization and internalization of HA-Pincher and TrkA, within 10 min of NGF treatment HA-Pincher and TrkA localization became differentially reorganized. HA-Pincher was associated predominantly with an unusual array of intracellular structures (Fig. 2 A, NGF 10 min). The Pincher-containing arrays were sorted away from a centralized collection of TrkA-containing structures. Pincher arrays were often found surrounding the TrkA-containing structures, as shown in the three-dimensional reconstruction of the cell in Fig. 2 C. Within 1 h of NGF-treatment, HA-Pincher was again found predominantly at plasma membrane and juxtamembrane locations, but rarely in internal structures, whereas TrkA was observed to have accumulated mainly in a dense collection of intracellular vesicle-like structures (Fig. 2 A), which were never seen to be associated with the nucleus. In many cells, as depicted in Fig. 2 A, TrkA was extensively internalized and was barely detected at the plasma membrane.

As described above, a massive internalization of TrkA, accumulation of intracellular TrkA-containing structures in the cytoplasm, and apparent depletion of TrkA from the plasma membrane, was seen after NGF treatment of Pincher-overexpressing cells. Although an overall similar reorganization of TrkA occurred in untransfected TrkA-PC12 cells in response to NGF, the reorganization of TrkA in Pincher-overexpressing cells was dramatically enhanced. Although not described previously, TrkA became concentrated at sites of ruffling plasma membrane within 2 min of NGF treatment (Fig. 2 A). However, the concentration of TrkA at ruffling membranes appeared to affect a greater proportion of the TrkA in Pincher-overexpressing cells (Fig. 2 A). The normal sequence of TrkA internalization after NGF treatment, as reported (Grimes et al., 1996) and as seen in the untransfected cells in the same slides (Fig. 2 A), affected a much smaller percentage of TrkA in the 1-h time frame, leaving TrkA predominantly plasma membrane associated. TrkA was internalized with vesicle-like structures formed after NGF treatment, but the accumula-

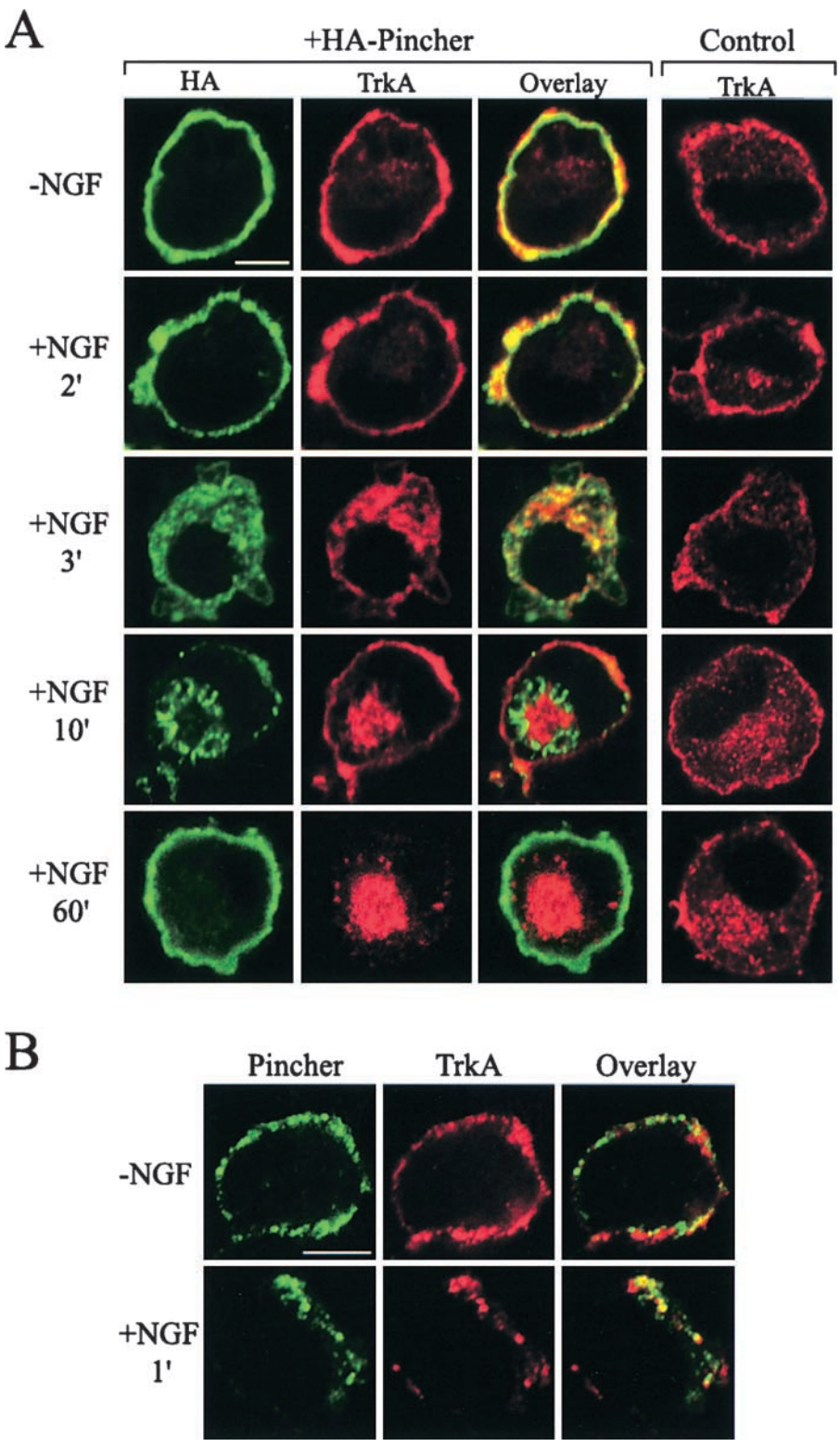
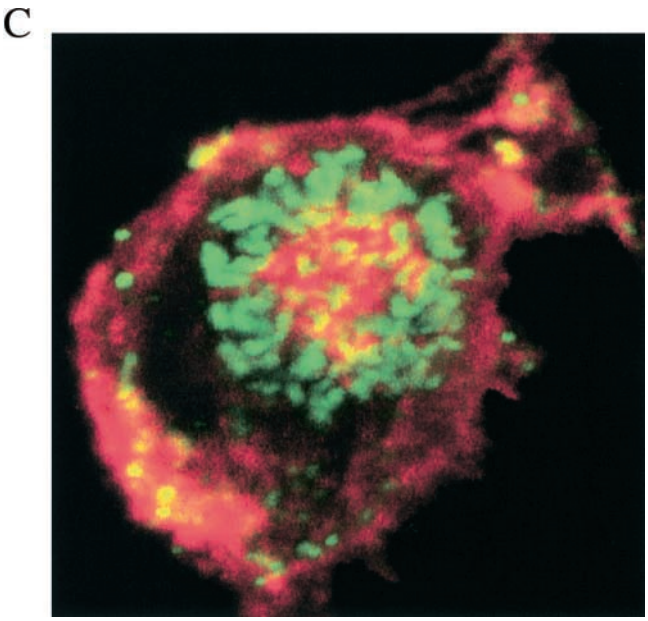


Figure 2. **Pincher overexpression enhances NGF-induced internalization and sorting of TrkA.** TrkA-PC12 cells were transfected with a CMV-HA-Pincher construct. Double immunofluorescence staining of fixed, permeabilized cells with anti-HA mAb (Alexa 488, green) and anti-TrkA antibody (Cy5, red) and confocal microscopy is described in Materials and methods. (A) Cells expressing HA-Pincher or not (control) are indicated. Cells were treated or not with NGF for the indicated times. HA-Pincher and TrkA are localized to the plasma membrane in untreated cells, concentrated in ruffles within 2 min. NGF, cointernalized within 3 min NGF, differentially sorted within 10 min NGF, and HA-Pincher is selectively recycled to the plasma membrane by 1 h NGF, whereas TrkA is accumulated in clusters of cytoplasmic vesicles. (B) Control, untransfected TrkA-PC12 cells were left untreated (top) or treated with NGF for 1 min (bottom). Double immunofluorescence staining and confocal microscopy was performed using anti-Pincher antibody (Cy5, green) and anti-TrkA mAb (Alexa 488, red) to observe the localization of the endogenous proteins. (C) 3-D reconstruction of the HA-Pincher expressing cell shown in the NGF 10-min overlay. Bars, 5 μm.

tions were relatively fewer in number, and distributed in smaller clusters throughout the cytoplasm (Fig. 2 A). Large accumulations of cytoplasmic TrkA seen in Pincher-overexpressing cells were quite stable and were often present even after 24 h of NGF treatment (see also Fig. 8 A). An apparent dosage effect of ectopic HA-Pincher expression on the NGF-induced pattern of reorganization of both TrkA and Pincher was observed. All patterns of TrkA reorganization were most dramatic in the brightly stained HA-Pincher–transfected cells, and were less dramatic in the lightly stained cells (unpublished data). Detection of endogenous Pincher using anti-Pincher antibody required a milder, acetone-based fixation method, with which double staining also using anti-TrkA antibody was carried out. In control, untreated cells, as seen with ectopic HA-Pincher, antigen-blockable (unpublished data) staining of endoge-



nous Pincher was seen with TrkA predominantly at the plasma membrane (Fig. 2, A and B, compare top panels). Within 1 min of NGF treatment, at the onset of internalization both Pincher and TrkA showed a similar pattern of redistribution. Punctate staining of both Pincher and TrkA was seen at ruffling plasma membrane and juxtamembrane locations, within which a subset of structures demonstrated discrete overlapping focal distributions (Fig 2 B, bottom).

The confocal observations on Pincher localization and translocation with NGF treatment were verified and extended by EM immunogold analyses of cells overexpressing Pincher. Pincher labeling could be well labeled only in Pincher-overexpressing cells, presumably due to the low antibody reactivity after gluteraldehyde-based fixation. Between 2 and 20 min of NGF treatment, as depicted in Fig. 3, immunogold-labeled Pincher was associated with plasma membrane-associated complex ruffling formations. The peripheral membrane localization corresponded well with the NGF-induced ruffles observed by confocal microscopy. Notably, these complex ruffling formations exhibited fibrous structures indicative of cytoskeletal arrangements (Fig. 3 B), which is consistent with the notion that macropinocytotic ruffling was being observed. The complex ruffling structures appear to be an infolded matrix of multiple ruffling membrane edges (Fig. 3 C). At 10 min of NGF treatment, similar complex structures were occasionally seen in the cytoplasm (Figs. 4 and 5). These internal structures would appear to be remnants of the complex ruffling structures, suggesting that they can be internalized en mass. Pincher was never found to be associated with clathrin-coated invaginations of the plasma membrane or vesicles, but was frequently associated with intracellular vesicles and tubules (Figs. 4 and 5). The Pincher-containing complex ruffling structures as well as the vesicular and tubular structures were seen to surround and associate with accumulations of large Pincher-free bodies filled with vesicles (Fig. 4 and 5). This pattern of Pincher staining was strikingly similar to the pattern seen by immunofluorescence confocal microscopy (Fig. 2 A, NGF 10 min). Electron microscopically, examples were seen in which the internalized Pincher-containing complex ruffling structures and tubules were fused with the vesicle-filled bodies and many of these ruffling structures and tubules were found to contain vesicular structures within them (Fig. 5). The pattern of vesicle-filled bodies also appeared to closely match that observed for TrkA-containing structures using confocal immunocytochemistry (Fig. 2 A). Immunogold EM analyses could not provide further substantiation, as we were not successful with the available anti-TrkA antibodies. At 1 h after NGF treatment, Pincher was again associated with the plasma membrane and with vesicles and tubules near the cell periphery (Fig. 6). That the tubules and their cores were electron lucent (Fig. 6) suggested they were contiguous with the extracellular fluid.

Pincher-generated vesicles contain extracellular fluid and NGF

The internalization of TrkA and Pincher, enhanced by Pincher overexpression, appeared to be caused by an NGF-induced, fluid-phase macropinocytotic process, occurring at membrane ruffles. To further test this possibility, we examined whether the fluid-phase uptake of media-soluble fluorescent Alexa488–10 kd–dextran was stimulated by NGF treatment of HA-Pincher–transfected TrkA-PC12 cell cultures. As seen in Fig. 7 A, dextran was not significantly taken up by the cells, whether or not they overexpressed Pincher after transfection. However,

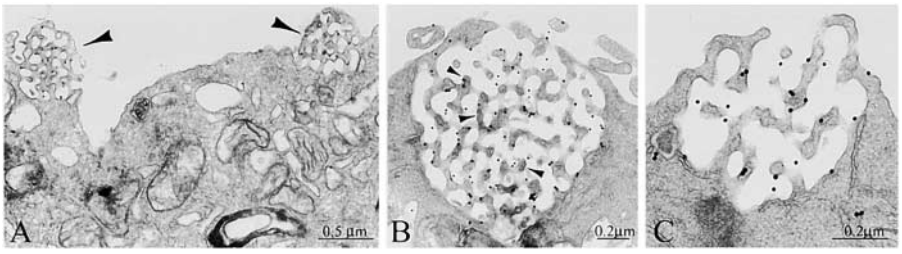


Figure 3. **Pincher is associated with extensive NGF-induced macropino cytosis at the ruffling surface membrane.** TrkA-PC12 cells were transfected with a CMV-HA-Pincher construct and treated with NGF as in Fig 2. Pincher immunogold electron microscopy is described in Materials and methods. (A) Immunogold-labeled Pincher associated with complex ruffling formations (arrowheads) localized at the peripheral membrane (20 min NGF treated). (B) Immunogold labeled Pincher associated with a complex ruffling formation at the peripheral membrane (10 min NGF). Arrowheads indicate cytoskeletal structures. (C) Immunogold-labeled Pincher associated with a complex ruffling formation at the peripheral membrane (10 min NGF). The shape suggests fusions of individual ruffling membrane events. Pincher-labeled complexes were seen within as early as 2 min NGF (unpublished data).

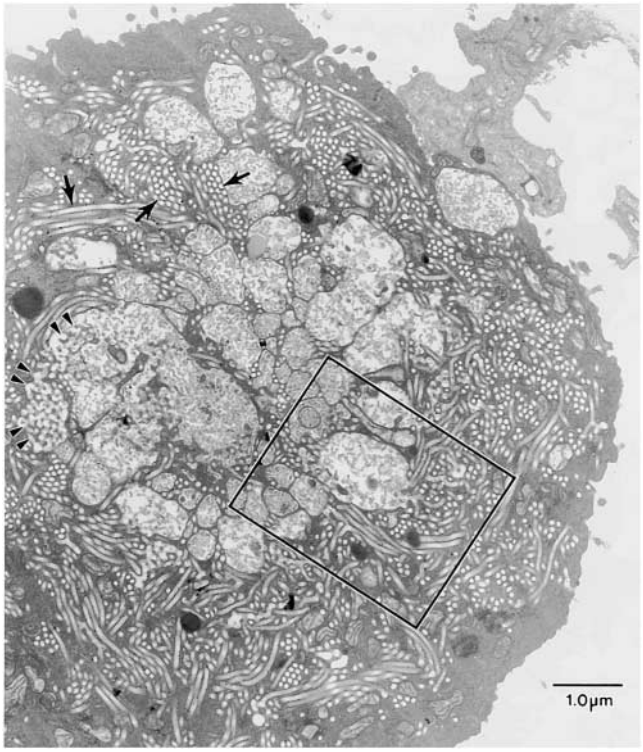


Figure 4. **Low power electron micrograph of a NGF-treated TrkA-PC12 cell overexpressing Pincher.** Arrowheads point to Pincher immunogold labeled structures that appear to be internalized complex ruffling formations. The small arrows point to examples of intracellular vesicles and tubules that are immunogold labeled for Pincher. The boxed region is shown at higher power in Fig. 5.

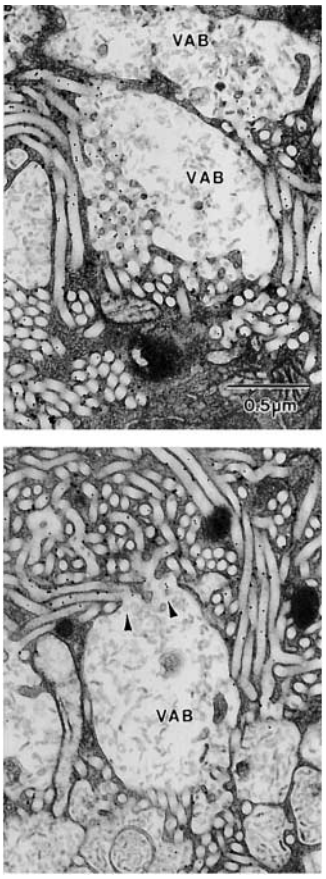


Figure 5. **Pincher is associated with tubular vesicle processing centers that are contiguous with vesicle accumulating bodies from which Pincher is excluded.** (Top) Internalized complex ruffling formation apparently delivering membrane-bound vesicles to a large vesicle accumulating body (VAB). Note that Pincher is absent from vesicles in the VAB. (Bottom) Higher power micrograph of the boxed region in Fig. 4, suggesting that immunogold Pincher-labeled tubules (arrowheads) may serve as trafficking structures that deliver vesicles to VABs. The sections in both panels are taken from Trk-PC12 cells overexpressing Pincher at 10 min NGF.

NGF treatment caused an uptake of dextran into cells. In cells that did not overexpress Pincher, an expected pattern of dextran uptake was observed after 15 and 30 min of NGF treatment, consisting of a scattered array of cytoplasmic vesicular structures containing dextran. However, in the cells overexpressing Pincher, a massive accumulation of dextran-containing cytoplasmic structures could be seen within 15 min NGF treatment, in large part surrounded by an array of Pincher containing tubular/vesicular structures (Fig. 7 A). The pattern of dextran surrounded by Pincher structures was similar to the pattern obtained with TrkA labeling described above. However, interestingly, the dextran localization also overlapped with the Pincher structures, suggesting that they also contained extracellular fluid. After a 30-min NGF treatment, Pincher recycled to the plasma membrane, leaving dextran accumulated in the cytoplasm. All of the dextran-positive, Pincher-overexpressing cells showed a similar pattern of cytoplasmic accumulation. To assess the effects of Pincher overexpression on clathrin-mediated endocytosis, the uptake of transferrin was examined (Mellman, 1996). Transferrin was taken up by the TrkA-PC12 cells as expected in an array of small clathrin-mediated vesicular structures (Fig. 7 B). In cells overexpressing Pincher, the punctate pattern of transferrin uptake did not appear to change, and Pincher did not relocalize from the plasma membrane to the transferrin containing intracellular structures (Fig. 7 B). However, the extent of transferrin uptake was inhibited by Pincher overexpression. In the 37.5% (n = 48) of the Pincher-overexpressing cells in which transferrin was taken up, the amount of transferrin labeling often appeared lower than the untransfected cells. In the remaining majority of the Pincher-overexpressing cells (62.5%), transferrin uptake was not clearly observed (Fig. 7 B). Thus, unlike TrkA and dextran internalization, the patterns of which are dramatically enhanced by Pincher overexpression, the vesicular pattern of transferrin internalization is not enhanced but is actually inhibited, confirming that Pincher overexpression enhances a clathrin-independent endocytic mechanism.

Because the NGF that is added to the culture medium is expected to be internalized together with TrkA, we examined the pattern of NGF uptake in Pincher-transfected cells. Myc-tagged NGF was added to TrkA-PC12 cell cultures that were transfected with a Pincher expression plasmid. Cells were treated with myc-NGF at 4°C to allow binding to TrkA without internalization. Under these conditions, as seen in Fig. 7 C, myc-NGF was not internalized, even in Pincher-expressing cells, and some

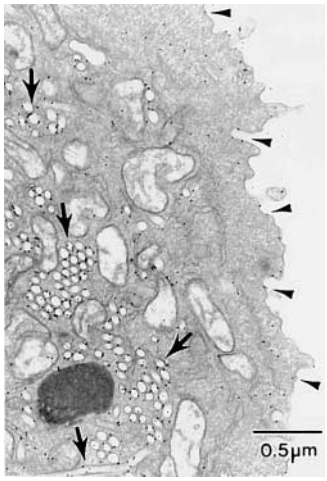


Figure 6. **Empty Pincher-containing vesicles and tubules recycle to the plasma membrane.** Electron micrograph showing immunogold labeling of Pincher associated with vesicles and tubules (arrows) and the peripheral membrane (arrowheads). The section is from a TrkA-PC12 cell overexpressing Pincher at 1 h NGF.

anti-myc staining could be seen at the cell surface. When myc-NGF treatment was carried out at 37°C, myc-NGF was found with Pincher at ruffling membrane blebs, and cointernalization could be seen within 5 min of treatment (Fig. 7 C). By 1 hr of treatment, myc-NGF was found to be concentrated in a dense accumulation of cytoplasmic vesicles (Fig. 7 C), as described above for TrkA and for dextran, whereas Pincher was localized to the plasma membrane. This pattern of myc-NGF staining contrasts with that seen in untransfected cells, in which NGF treatment resulted in a sparse distribution of intracellular punctate staining (Fig 7 C).

Pincher-generated vesicles mediate NGF/TrkA signaling

The above results indicated that NGF was internalized with TrkA; thus, we expected that the internalized TrkA might remain activated and autophosphorylated (Bhattacharyya et al., 1997). To test this possibility, we used an anti–phospho-Y490TrkA antibody in confocal immunofluorescence microscopy of Pincher-transfected, TrkA-PC12 cell cultures. As expected, before NGF treatment, Pincher- overexpressing cells detected with anti-HA antibody did not stain well with anti–phospho-Y490TrkA antibody (Fig. 8 A). However, after NGF treatment, a time-dependent pattern of anti–phospho-Y490TrkA staining was observed that was remarkably similar to that described above using anti-TrkA antibody. Within 2 min of NGF treatment, anti–phospho-Y490TrkA staining could be seen at the plasma membrane, concentrated together with Pincher at membrane ruffles and blebs (Fig. 8 A). Pincher overexpression was found to increase both the appearance of phospho-TrkA in ruffles after NGF treatment (Fig. 8 A), and the level of TrkA autophosphorylation. Staining of phosphorylated TrkA was seen in 67% of the Pincher-overexpressing cells (n = 70) compared with 28% (n = 100) of the cells not overexpressing Pincher, when assayed at 10 min of NGF treatment. Between 5 and 15 min of NGF treatment, a massive internalization of both HA-Pincher and phospho-Y490TrkA was seen accumulated in the cytoplasm in both overlapping and nonoverlapping patterns. As seen with TrkA staining described above, at 15 min of NGF treatment, cells could be seen in which the accumulation of internalized phospho-TrkA was surrounded by HA-Pincher-labeled tubule-like structures (Fig. 8 A). Cytoplasmic staining of internalized phospho-Y490TrkA was seen in 89% of those Pincher-overexpressing cells, compared with

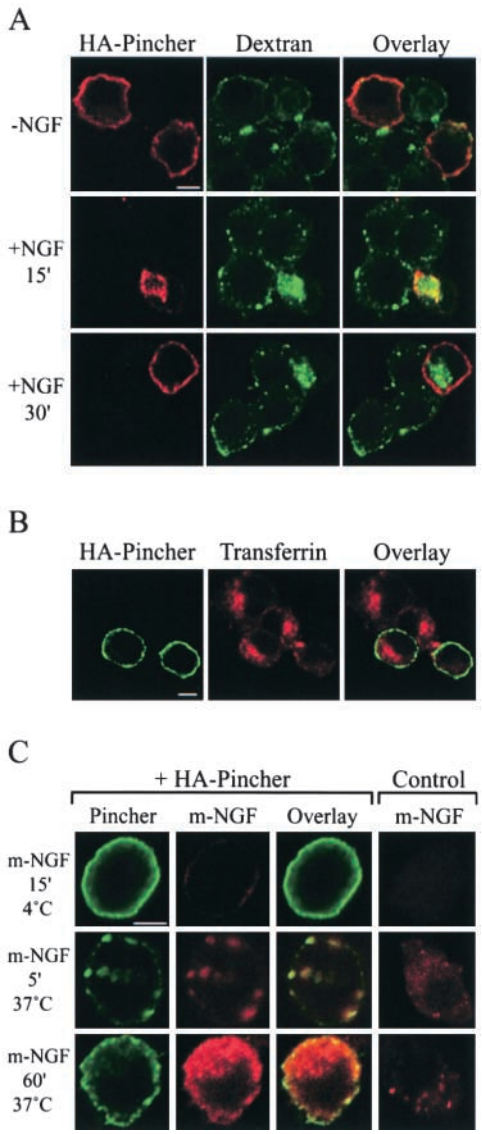


Figure 7. **Pincher overexpression enhances fluid-phase uptake of NGF in PC12 cells.** TrkA-PC12 cells were transfected with a CMV-HA-Pincher construct as in Fig 2. (A) (Fluid-phase uptake) Cells were incubated with media containing fluorescent Alexa488-dextran (green) for 15 min without (−NGF) or with NGF for 15 or 30 min, and were stained with anti-HA mAb (Alexa 546, red). (B) (Clathrin-mediated transferrin internalization) Cells were treated with Alexa633-transferrin (red) for 15 min and stained with anti-HA mAb (Alexa 488, green). (C) (NGF internalization) Cells treated with myc-tagged NGF (m-NGF) for the indicated times at 4°C (to prevent uptake) and at 37°C were stained with anti-myc mAb (Alexa 546, red) and anti-Pincher antibody (Alexa 488, green). Cells expressing HA-Pincher or not (control) are indicated. Bars, 5 μm.

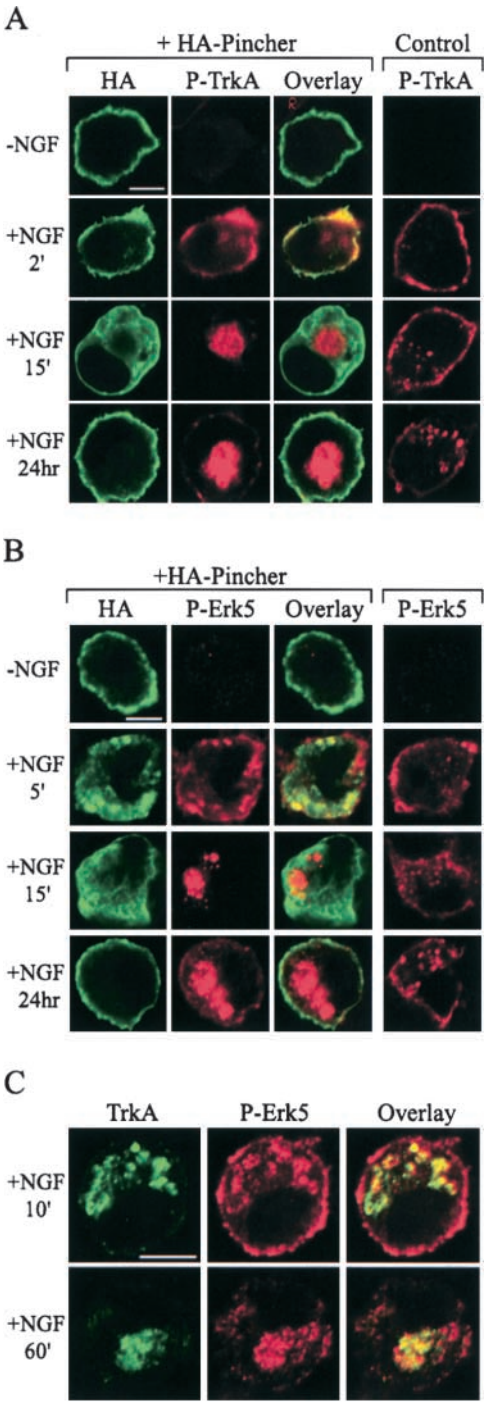


Figure 8. **Pincher overexpression enhances NGF-stimulated internalization and sorting of autophosphorylated TrkA associated with activated erk5 kinase.** TrkA-PC12 cells were transfected with a CMV-HA-Pincher construct as in Fig. 2 and treated with NGF as indicated. (A) Double immunofluorescence staining was performed using anti-HA mAb (Alexa 488, green) and anti-phospho-Y490TrkA antibody (Cy5, red). Cells expressing HA-Pincher or not (control) are indicated. In transfected cells, HA-Pincher (HA) and phospho-TrkA (P-TrkA) are initially localized to the plasma membrane, concentrated at ruffles within 2 min NGF, internalized and differentially sorted within 15 min NGF, and HA-Pincher is selectively recycled to the plasma membrane by 1 h NGF, whereas P-TrkA remains for up to 24 h. Bar, 5 μm. (B) Immunofluorescence staining using anti-HA mAb (Alexa 488, green) and anti–phospho-erk5 kinase antibody (Cy5, red). Phospho-erk5 kinase (P-Erk5) showed bright staining only after NGF treatment. HA-Pincher–expressing cells showed bright P-Erk5 staining with intracellular Pincher (NGF 5′), bright P-Erk5 staining clustered within a surrounding pattern of Pincher staining (NGF 15′), and Pincher returned to the plasma membrane (NGF 24 h). Cells not expressing HA-Pincher (control) showed bright clustered P-Erk5 staining. Bar, 5 μm. (C) Double immunofluorescence staining of HA-Pincher–transfected cells using anti-TrkA mAb (Alexa488, green) and anti–phospho-erk5 antibody (Cy5, red), showed overlapping staining patterns at 10 and 60 min NGF treatments. Bar, 5 μm.

34% of the phospho-TrkA stained cells not overexpressing Pincher. In addition, the extent of internalization per positive cell appeared dramatically higher in the Pincher-overexpressing cells, compared with untransfected cells (Fig. 8 A). After 1 h and for as long as 24 h of NGF treatment, Pincher was found predominantly on the cell surface, whereas phospho-Y490-TrkA staining was bright and in a concentrated array of cytoplasmic vesicles in a distribution that was not seen in untransfected cells (Fig. 8 A), as was seen for TrkA and myc-NGF. In many cells, as shown in Fig. 8 A, little phosphorylated TrkA was seen at the cell surface, suggesting that the internalization was sufficient to significantly deplete activated TrkA from the cell surface.

The persistence of TrkA autophosphorylation in intracellular vesicles suggested that they would have the capacity to mediate pertinent downstream signaling events in the cytoplasm. To test this possibility, we examined the distribution of NGF-activated MAP kinases, whose activation is mediated through phospho-Y490 TrkA signaling. Phosphorylation of the erk MAP kinases is persistently stimulated in PC12 cells, and erk5 kinase is selectively activated in neuronal soma by retrograde axonal NGF signaling (Watson et al., 2001). Activated erk5 was examined using an anti–phospho-erk5 kinase antibody in immunofluorescence staining of TrkA-PC12 cells transfected with a Pincher expression plasmid. Before NGF treatment, Pincher overexpressing cells detected with anti-HA antibody, showed low level staining with the anti–phospho-erk5 kinase antibody (Fig. 8 B). However, after NGF treatment, a time-dependent pattern of phospho-erk5 kinase staining was observed that was remarkably similar to that described above using the anti–phospho-Y490TrkA antibody. Within 5 min of NGF-treatment, bright phospho-erk5 kinase staining could be seen at the plasma membrane concentrated with Pincher, and colocalized with Pincher upon internalization (Fig. 8 B). By 15 min of NGF-treatment, phospho-erk5 kinase staining was seen in large concentrated punctate formations in the cytoplasm. As was seen with phospho-TrkA staining, the bright cytoplasmic staining of phospho-erk5 kinase was often surrounded by the Pincher arrays (Fig. 8 B). Within 1 h and for as long as 24 h of NGF treatment, Pincher was again seen associated predominantly with the plasma membrane, whereas phospho-erk5 kinase remained concentrated in the cytoplasm (Fig. 8 B). As exemplified in Fig. 8 B and similar to the distribution of phospho-TrkA, although bright staining of phospho-erk5 kinase was seen in a diffuse punctate pattern throughout the cytoplasm of nontransfected cells, the patterns of concentrated formations of phospho-erk5 kinase were only seen in Pincher-overexpressing cells. To determine if the distribution of phospho-erk5 overlapped with

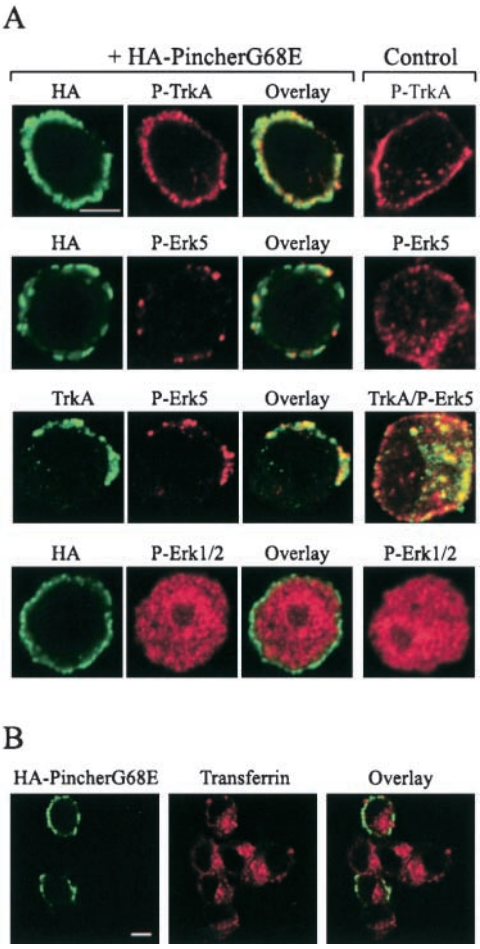


Figure 9. **Expression of mutant PincherG68E inhibits cointernalization of activated TrkA and erk5 kinases, with no effect on distribution of activated erk1/2 kinases or clathrin-mediated internalization.** (A) PC12 cells (second and fourth panels) or TrkA-PC12 cells (first and third panels) expressing HA-PincherG68E or not (control) were treated with NGF for 15 min. Double immunofluorescence staining used anti-HA mAb (Alexa 488, green), together with either anti–phospho-Y490TrkA antibody (first panel, P-TrkA, Cy5, red), or anti–phospho-erk5 antibody (second panel, P-Erk5, Cy5, red), or anti–phospho-erk1/2 antibody (fourth panel, P-Erk1/2, Cy5, red). Double staining also performed for TrkA using anti-TrkA mAb (Alexa488, green) together with anti-phospho-erk5 (Cy5, red) is shown in third panel. P-TrkA and P-Erk5 that were cointernalized in control cells, were clustered at the plasma membrane in cells expressing HA-PincherG68E. Cytoplasmic and nuclear staining of P-Erk1/2 was unaffected by HA-PincherG68E. (B) Clathrin-mediated transferrin internalization was seen using Alexa633-transferrin (red) as in Fig. 7 B. Cells were stained with anti-HA mAb (Alexa 488, green), illustrating that the internalization was unaffected by expression of HA-PincherG68E.

that of TrkA, Pincher-transfected cells were double labeled with monoclonal anti-TrkA and polyclonal anti–phospho-erk5 antibodies. As shown in Fig. 8 C, the intracellular staining patterns of TrkA and phospho-erk5 largely overlapped at both 10 and 60 min NGF treatments. Unlike the punctate distribution of phospho-erk5, the pattern of phospho-erk1/2 kinase staining after NGF treatment of PC12 cells, as recently reported (Wu et al., 2001), was diffusely distributed throughout the cytoplasm and nucleus (Fig. 9 A). A similarly diffuse pattern of phospho-erk1/2 staining was

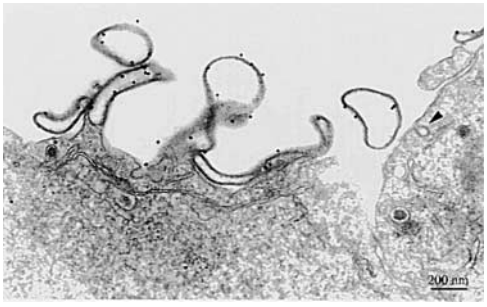


Figure 10. **PincherG68E is defective in the formation of complex structures at membrane ruffles.** TrkA-PC12 cells were transfected with a CMV-HA-PincherG68E construct, NGF treated for 10 min, and Pincher immunogold electron microscopy performed as in Fig. 3. Immunogold-labeled Pincher is associated with membrane blebs that are devoid of complex ruffling formations, and contain neither membrane fusion events nor cytoskeletal elements seen with Pincher overexpression (Fig. 3). The arrowhead points to an unlabeled, clathrin-coated pit.

generally seen in HA-Pincher–overexpressing cells at all times of NGF treatment (unpublished data). At 24 h of NGF treatment, when the greatest number of cells showed large aggregates of cytoplasmic phospho-TrkA staining, 79% ($n = 33$) of the anti–phospho-erk5–stained cells showed similar large aggregate staining patterns (Fig. 8 B), whereas only 15% ($n = 68$) of cells showed a similar pattern with anti–phospho-erk1/2 staining, with the vast majority (85%) of the cells demonstrating diffuse phospho-erk1/2 staining (unpublished data).

NGF/TrkA endosomal signaling is blocked by dominant negative Pincher G68E

To assess the requirement of Pincher in mediating TrkA endocytosis and endosomal signaling, we created a mutant form of Pincher in which the P-loop ATP-binding site is destroyed by mutation G68E, and transfected the mutant construct into PC12 cells. As expected, expression of PincherG68E resulted in a plasma membrane staining pattern similar to Pincher, although the surface appeared more ruffled. NGF treatment did not result in any detectable changes in cell morphology or PincherG68E localization at time points between 2 and 60 min (Fig. 9). When the distribution of activated TrkA was examined, although bright phospho-TrkA staining was seen in NGF-treated cells overexpressing PincherG68E, it almost exclusively remained with PincherG68E at the plasma membrane at all times after NGF treatment (Fig. 9 A). Although the PincherG68E did not enhance the internalization of phospho-TrkA, it did not completely inhibit internalization of phospho-TrkA. Within 15 min of NGF treatment, a small number of punctate structures were weakly stained with the anti–phospho-TrkA antibody. However, unlike with the untransfected cells (Fig. 8 A), neither the number of intracellularly stained phospho-TrkA structures, nor the level of staining, increased with further NGF treatment (unpublished data).

The distribution of activated erk5, seen by anti–phospho-erk5 staining, indicated that like phospho-TrkA, phospho-erk5 remained at or very near the plasma membrane during NGF treatment of PincherG68E expressing cells (Fig. 9 A).

This contrasts with the NGF-induced cytoplasmic staining pattern of phospho-erk5 normally seen in untransfected cells (Figs. 8 B and 9 A), or in cells overexpressing Pincher (Fig. 8, B and C), which largely colocalizes with TrkA (Figs. 8 C and 9 A). As expected, in PincherG68E-expressing cells, double labeling with both TrkA and phospho-erk5, indicated greatly overlapping patterns (Fig. 9 A). The pattern of phospho-erk5 staining differed from that of phospho-TrkA in that intracellular phospho-erk5 staining was never seen in cells overexpressing PincherG68E. In contrast to the pattern of phospho-erk5, phospho-erk1/2 displayed a normal distribution pattern after NGF treatment of cells expressing PincherG68E. Phospho-erk1/2 staining was bright and diffuse throughout the cytoplasm and nucleus after 15 min of NGF treatment (Fig. 9 A), and at all later times examined. Thus, the expression of Pincher G68E selectively prevented cytoplasmic signaling by erk5, but not by erk1/2.

To determine if clathrin-mediated endocytosis still occurred in cells expressing PincherG68E, the internalization of transferrin was examined in these cells. As shown in Fig. 9 B, PincherG68E did not change its plasma membrane localization upon treatment of cells with transferrin, and its expression had no effect on the extent or pattern of transferrin internalization.

The above results suggested that expression of PincherG68E blocked NGF-induced endocytosis at an early step. To gain insights into the stage at which internalization was blocked, we examined the distribution of PincherG68E by immunogold electron microscopy of NGF-treated cells. As shown in Fig. 10, although PincherG68E was found only at blebs at the cell surface, no complex ruffling structures were seen that were indicative of macropinocytic endocytosis. However, examples could be seen of clathrin-coated pits formed at the cell surface (Fig. 10).

Discussion

We have identified an NGF-induced protein that we termed Pincher, and shown that it dramatically enhances NGF-induced internalization, sorting, and trafficking of TrkA-containing vesicles. Pincher-enhanced internalization is initiated at the ruffling plasma membrane at sites where pinocytic structures are formed. At later stages, Pincher is associated with tubular structures in which the sorting and trafficking of TrkA-containing vesicles takes place. The TrkA vesicles accumulated in Pincher-overexpressing cells contain NGF, and the TrkA is continually activated for up to at least 24 h. NGF/TrkA vesicles also remain selectively associated with phosphorylated, activated erk5 kinase for hours, indicating that the vesicles persistently mediate the TrkA signaling pathway. A dominant inhibitory Pincher mutant prevents NGF-induced TrkA internalization and selectively blocks cytoplasmic endosomal signaling through erk5 kinase. We suggest that Pincher is a pinocytic chaperone for TrkA that mediates pinocytic endocytosis of NGF signaling endosomes.

The regulation of gene expression by NGF is mediated through multiple signaling pathways derived from TrkA. NGF-induced gene expression changes are mediated through TrkA by either triggered PLC-γ signaling (Choi et al., 2001), or by Shc- and Ras-mediated signaling that requires persistent stimulation (Segal and Greenberg, 1996). Unlike other genes thus far studied, Pincher induction by NGF is mediated through a combination of these TrkA-stimulated pathways. Mutation of the TrkA binding sites for PLC-γ or for Shc resulted in partial inhibition of Pincher induction, whereas mutation of both sites completely blocked induction of Pincher by NGF. This is much like the paradigm seen with NGF-induced neurite growth that can be stimulated through both PLC-γ and Shc binding sites on TrkA (Stephens et al., 1994), a mechanism that has been suggested to ensure the elicitation of important events by NGF. Increased Pincher production thus might ensure continued and perhaps even increased endosomal signaling with persistent NGF exposure.

Pincher facilitates the internalization and sorting of intracellular vesicles containing NGF and TrkA. Pincher would appear to function early in the internalization process because it was found primarily on the plasma membrane and was rapidly distributed, together with NGF and TrkA, to sites of membrane ruffling and pinocytosis after NGF treatment. The massive array of pinocytic structures at ruffling membrane blebs was found only in NGF-treated cells overexpressing Pincher, suggesting that Pincher drives the macropinocytotic process. Because Pincher-stimulated TrkA internalization required NGF-treatment, and ATP/GTP binding site (P-loop) mutants of Pincher blocked NGF-induced receptor internalization, it is likely that Pincher is a mediator of NGF-induced pinocytic internalization of TrkA. The time course of Pincher localization offered insights into the stages of the internalization process. The large complexes of pinocytic ruffling structures were internalized en mass, and developed into Pincher-containing tubular structures, concomitant with the appearance of vesicles within the tubules. In key respects the Pincher pinocytic process resembled receptor-induced macropinocytosis (Swanson and Watts, 1995) in that Pincher was enriched in NGF-induced ruffling membrane blebs where pinocytosis occurred, and the pinocytic vesicles were filled with extracellular fluid, as evidenced by the inclusion of media-soluble dextran. Although the appearance of large masses of pinocytic structures was atypical, it may reflect an unusually high degree of coupling of pinocytosis to ruffles caused by Pincher overexpression. Interestingly, TrkA was concentrated at NGF-induced ruffles and blebs of the plasma membrane, which resulted in an enrichment of TrkA in the internalized membranes. The specificity for receptor-enriched membrane internalization provided by the Pincher-driven internalization of TrkA, suggests a unique mode of receptor-mediated pinocytic endocytosis.

A late stage of Pincher-stimulated pinocytic endocytosis is the sorting of vesicles containing NGF and TrkA from the tubules, and recycling of the Pincher-containing tubules to the plasma membrane. This process was dramatically enhanced in Pincher-overexpressing cells, allowing visualization of intermediates that have not been described previously. A surprising finding was the presence of vesicles within many of the Pincher-containing tubules and the connections of tubules to vesicle accumulating bodies. The tubules may represent endosome/pinosome processing cen-

ters providing a unique generating, sorting and delivery system for endosomal vesicles to the cell interior. It is not clear at present how the vesicles are formed within the tubules, or how and when TrkA is sorted differentially from the Pincher-containing membrane. Pincher recycling to the plasma membrane occurs via recycling of Pincher-containing tubules. After delivery of NGF/TrkA vesicles to vesicle accumulating bodies, the tubules become EM lucent and could at these late stages be seen to be contiguous with the plasma membrane. The NGF/TrkA vesicles that are accumulated in the cytoplasm persist for periods of as long as 24 h, indicating that the process is distinct from endocytic events associated with lysosomal degradation or receptor recycling. In concordance with this idea, we also did not observe significant co-staining of Pincher with either clathrin or with the late endosomal marker LBPA at any times after NGF treatment (unpublished data). Pincher-mediated NGF and TrkA internalization appear to be mediated through a clathrin independent process. The differential effects of Pincher in stimulating, and PincherG68E in blocking, TrkA versus clathrin-mediated transferrin internalization best illustrate this. Consistent with these results, Pincher does not associate with and PincherG68E does not prevent, clathrin coated pits seen electron microscopically. However, the decrease in clathrin-mediated transferrin uptake in Pincher-overexpressing cells may be an indication that the Pincher and clathrin endocytotic machineries might share common components. That the unique mode of Pincher-mediated pinocytic endocytosis may provide for the formation and segregation of vesicles with long lifetimes is an intriguing possibility.

Recent reports have implicated Pincher family members in events underlying receptor-mediated endocytosis. The mouse mRME-1 gene product has been suggested to mediate recycling of receptor-containing endocytic vesicles (Lin et al., 2001). This function is unlike that for Pincher, which did not mediate recycling of the TrkA-containing vesicles that persisted in the cytoplasm. Furthermore, mRME-1 overexpressed in TrkA-PC12 cells did not act like Pincher to enhance NGF-induced TrkA internalization (unpublished data). Also, unlike Pincher, EHD-1 was recently suggested to be involved in clathrin-mediated endocytosis of IGF-1 (Rotem-Yehudar et al., 2001). The differences between Pincher and mRME-1 or EHD-1 suggest that the different family members may have different functions in vesicle transport. It is tempting to speculate that a common functionality of Pincher and other family members lies in the ability to pinch membrane, which in the case of mRME-1 results in the formation of recycling vesicles and in the case of Pincher results in macropinocytotic structures. The tissue distribution of Pincher mRNA is similar to that of mRME-1 and EHD-1, with high levels of expression in heart and lung, suggesting that Pincher may also be involved in a broader spectrum of receptor-mediated pinocytic events. The ability of Pincher to mediate internalization of receptors for neurotrophins and growth factors other than NGF remains to be determined.

The intracellular vesicles that accumulated after NGF-treatment of Pincher-overexpressing cells had signaling capability for NGF, similar to those mediating axonal retrograde signaling in neurons. This conclusion was supported not only by the cointernalization of NGF and TrkA, but also by the intracellular accumulation of TrkA autophosphorylated at its Shc binding site as seen in neurons (Ehlers et al., 1995; Bhattacharyya et al., 1997), and the preferential association of the internalized vesicles with an activated form of erk5 kinase, a critical neuronal retrograde signaling effector (Watson et al., 2001). The expression of PincherG68E completely and selectively blocked the cytoplasmic appearance of erk5 over erk1/2 kinases, although it had no effect on clathrin-mediated transferrin internalization, and allowed only a limited, low level of phospho-TrkA internalization. These results raise the possibility that the clathrin-independent internalization of NGF and TrkA by Pincher may selectively mediate the formation of the critical NGF/TrkA signaling endosome. The persistence of intracellular vesicles containing phospho-TrkA and phospho-erk5 kinase in Pincher-overexpressing PC12 cells, even after 24 h of NGF treatment, indicated that the internalized, activated NGF/TrkA complex was remarkably stable. This is a requisite characteristic for NGF/TrkA signaling endosomes in neurons, which must remain active during and after transport from the nerve terminal to the cell body. Pincher is expressed in peripheral neurons and brain, making Pincher an attractive candidate for mediating retrograde endosomal signaling in neurons.

Materials and methods

Cell lines and cell culture

Growth of PC12 and derived stable transfectant TrkA-PC12, and the PC12 mutant nnr5 and derived stable transfectants, T14, Y490F, Y785F, and Y490F/Y785F cell lines, have been described previously (Greene and Tischler, 1976; Hempstead et al., 1992; Stephens et al., 1994).

TOGA analysis

TOGA was performed as described previously (Sutcliffe et al., 2000), herein using PC12 cytoplasmic polyA (+) RNA isolated from cells at various times after 1 min of NGF treatment.

Northern blot analysis

Total cellular RNA was isolated from cells, and Northern blot analysis was carried out as previously described (D'Arcangelo and Halegoua, 1993). Rat multitissue mRNA blots (CLONTECH Laboratories, Inc.) and total RNA blots from rat dorsal root ganglia, lung, and heart tissues were probed with UTP[α-^{32}P]-labeled antisense RNA probes using pHAL18, encoding 700 bp of 3′ UTR region of Pincher mRNA and PIB15, encoding cyclophilin. HAL18/Pincher mRNA bands were analyzed using a PhophorImager and ImageQuant software (Molecular Dynamics). All values were normalized to the level of cyclophilin mRNA.

cDNA library construction and screening

PC12 Poly(A)+ mRNA isolated from PC12 cells 5 h after NGF treatment was also used to construct both random primed and oligo (dT)-primed λ ZAPII cDNA libraries (Stratagene). A pooled library consisted of 1.0×10^6 independent clones prior to amplification. Screening of 10^6 recombinants with a ^{32}P-labeled HAL18 cDNA probe (oligo labeling kit; Amersham Pharmacia Biotech) resulted in the isolation of 15 positive clones. All 15 clones were sequenced and the overlapping sequences revealed a single full-length coding region for Pincher.

Recombinant cDNA constructs, expression and isolation of recombinant proteins, and generation of antibodies

A mammalian expression vector encoding HA-tagged Pincher was generated by subcloning full-length Pincher cDNA into the pCGN-HA vector between Xba1 and Kpn1 restriction sites. PincherG68E was generated by PCR-based mutagenesis, using primers designed to contain the mutation site and allow insertion (between Xba1 and BamH1 sites within Pincher) to generate pCGN-HA-PincherG68E. A bacterial expression vector encoding GST-

Pincher was generated by subcloning full-length Pincher cDNA into pGEX-3× vector at the EcoR1 and BamH1 sites. GST-Pincher was purified from XL1-Blue (Stratagene) cells using glutathione-agarose beads (Sigma-Aldrich). Rabbit anti–GST-Pincher polyclonal antibody was generated by Research Genetics, Inc. Myc-NGF was prepared from media of COS cells transfected with a myc-tagged NGF construct (Moller et al., 1998), 72 h after transfection and concentrated tenfold using ultra-free-15 Biomax-10k (Millipore) at 4°C. Cells were treated with myc-NGF at a concentration equivalent to 100 ng/ml NGF, as determined by bioassay. Cells were transfected using Lipofectamine 2000 (GIBCO BRL). 24 h after transfection, cells were plated on coverslips coated with 25μg/ml poly-L-lysine (Sigma-Aldrich) and 10 μg/ml laminin (BD Sciences Inc.). 5 h later, the medium was replaced with DME containing 1% horse serum and the cells cultured overnight.

Immunoprecipitation and Western blot analysis

Cells were washed in PBS and then lysed with lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml leupeptin, 5 mM benzamidine, 1% NP-40). The lysates were clarified by centrifugation, and Pincher was immunoprecipitated from 500 μg of cell lysate for 4 h at 4°C using 5 μg of anti-Pincher antibody. The immunoprecipitates were subjected to SDS-10% PAGE and analyzed by Western blotting. Blots were probed with anti-Pincher (1:5,000) or anti-HA (1:200) antibodies and subsequently probed with secondary donkey anti–rabbit or donkey anti–mouse IgG antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) followed by ECL detection.

Immunofluorescence staining and imaging

After appropriate treatments as indicated in the text, cells were fixed and then subjected to double immunofluorescence staining. Cells were fixed in PBS containing 3.7% formaldehyde and 0.12 M sucrose for 10 min at room temperature for double staining using monoclonal anti-HA (1 μg/ul; Santa Cruz Biotechnology, Inc.) together with either anti-TrkA (1:250; Hempstead et al., 1992), or anti-phospho-erk5 (1:500; BioSource), or anti–phosho-erk1/2 (1:250; New England Biolabs); or anti-Pincher (1:2,000) with monoclonal anti-myc (1 μg/ul, Sigma-Aldrich). Fixation in 4% paraformaldehyde was used for staining of anti-HA with anti–P-Y490 (1:100; Bhattacharyya et al., 1997). Acetone fixation was used for anti-Pincher antibody with anti-TrkA mAb (1:100, MCTrkA; Santa Cruz Biotechnology, Inc.). Methanol fixation was used for anti-TrkA mAb with anti–phospho-erk5. After fixation, cells were rinsed with PBS, blocked and permeabilized in Blotto (PBS containing 5% nonfat milk, 0.1% Triton) for anti-HA with anti-TrkA or anti–P-Y490TrkA (also containing 1 mM vanadate), and for anti-Pincher with monoclonal anti-myc, and then incubated with primary antibody at 4°C overnight. Staining for monoclonal anti-TrkA, anti–phospho-erk5 and anti–phospho-erk1/2 was done according to New England Biolabs protocol, except that all solutions contained 1 mM vanadate. A collection of conjugated secondary antibodies were used including goat anti–mouse Alexa 488 or Alexa 546 (Molecular Probes), donkey anti–rabbit-Cy5 (Jackson) or goat anti–rabbit-Alexa 488. To visualize dextran internalization, cells were treated with Alexa 488–10 kd–dextran (Molecular Probes) in DME at 1.25 mg/ml. For transferrin internalization, cells were treated with Alexa 633–conjugated transferrin (25 μg/ml; Molecular Probes) in DME. Confocal images were obtained using a Zeiss LSM 510 laser scanning confocal microscope. Images were processed using Photoshop 6.0 software.

Electron microscopy and immunogold labeling

Fixation, dehydration, and embedding. At 24 h posttransfection, TrkA-PC12 cells were further grown on ACLAR (Ted Pella) for 24 h. After NGF treatment, the cells were immersion fixed for 15 min in a cold mixture of 0.1 M phosphate buffer (PB, pH 7.4) containing 2% paraformaldehyde and 2% gluteraldehyde. Cells were rinsed 3 × 10 min in PB, osmicated (2% OsO4 for 1 h), rinsed 2 × 10 min in PB, rinsed 2 × 10 min in dH_2O, en bloc stained with aqueous 1% uranylacetate for 1 h, rinsed 2 × 10 min in dH_2O, dehydrated through an ascending series of ethanols, and embedded in Durcupan (Fluka) between sheets of ACLAR.

Postembedding immunogold labeling of Pincher. Serial ultrathin sections (60–90 nm) were cut on a Reichert Ultracut E ultratome and picked up on formvar-coated nickel slot grids. Postembedding immunogold labeling was done using a modification of the protocol of Phend et al. (1995). Sections were rinsed for 2 min in 0.1 M TRIS (pH 7.6) and 0.005% Tergitol NP-10 (Sigma-Aldrich), hereafter referred to as TRIS/T), followed by 5-s immersion in saturated (10%) sodium metaperiodate. Sections were rinsed 3 × 10 min in TRIS/T, incubated for 1 min at room temperature in 1% sodium borohydride, and rinsed 3 × 5 min in TRIS/T, and treated overnight with anti-Pincher antibody (TRIS/T, 1:30,000). Sections were rinsed 2 × 5 min and 1 × 30 min in TRIS/T, rinsed 5 min in TRIS/T at pH 8.2, and incubated for 1 h in goat anti–rabbit IgG-conjugated to 15 nm gold (Amersham Pharmacia Biotech), 1:25, TRIS/T, pH 8.2. Sections were rinsed 2 × 5 min in TRIS/T, rinsed 2 × 5 min dH_2O, stained with 1% methanolic uranylacetate (10 min) and 0.3% aqueous lead citrate (5 min), and examined with a JEOL 1200 EX electron microscope (JEOL). In all control runs where either the primary or secondary antibody incubation step was skipped, all tissue specific anti-Pincher, immunogold labeling was eliminated.

We thank J. Speh for guidance with confocal imaging and G. Mandel for providing use of the confocal microscope. We thank Drs. P. Brehm, D. Brown, D. Ginty, and G. Mandel for valuable discussions. We thank R. Segal (Harvard Medical School, Boston, MA) for providing anti-TrkA and anti–P-Y490TrkA antibodies, E. Shooter (Stanford University, Stanford, CA) for providing the myc-NGF construct, F. Maxfield (Cornell Medical School, New York, NY) for providing the mRme-1 construct, J. Gruenberg (University of Geneva, Geneva, Switzerland) for providing the anti-LBPA antibody, D. Kaplan (McGill University, Montreal, Canada) for the TrkA-PC12 cells, and D. K. Zhen for making the GST–Pincher fusion protein.

This work was supported by grants from the National Institutes of Health (NS18218 to S. Halegoua, and HL24103 to J.B. Cabot).

Submitted: 15 January 2002
Revised: 22 March 2002
Accepted: 1 April 2002

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