Mol. Cells, Vol. 10, No. 2, pp. 169–179

Molecules and Cells

Sustained Formation of Focal Adhesions with Paxillin in Morphological Differentiation of PC12 Cells

Sangmyung Rhee[1], Kun Ho Lee[1], Daegun Kim[1], Yunhee Kim Kwon[2], Man-Sik Kang[1], and Hyockman Kwon[†,*]

Department of Molecular Biology, Dankook University, Seoul 140-714, Korea;

[1] Department of Molecular Biology and Research Center for Cell Differentiation, Seoul National University, Seoul 151-742, Korea;

[2] Department of Biology, Kyung Hee University, Seoul 130-701, Korea.

(Received on November 19, 1999)

Differentiation of PC12 cells triggered by nerve growth factor (NGF) is characterized by several well-defined events including induction of a set of neuron-specific genes, gain of membrane excitability, and morphological changes such as neurite outgrowth. Here we report that K252a, a protein kinase inhibitor, converts the proliferation signal of epidermal growth factor (EGF) into the morphological differentiation signal without inducing the sustained activation of ERK and the expression of neurofilament. Major effects of EGF/K252a, found also in the NGF-treated cells, are the sustained mobility shift of paxillin in SDS-PAGE and the promoted association of Crk-II with paxillin. These effects explain the prominent and robust development of peripheral focal adhesion assembly and stress fiber-like structures observed in the early stages of PC12 cell differentiation. These results suggest a model that cytoskeletal reorganization via focal adhesion assembly triggered by NGF provides a signal required for the morphological differentiation of PC12 cells.

Keywords: Differentiation; EGF; Focal Adhesions; K252a; Paxillin; PC12 Cells.

Introduction

PC12 cells have provided a useful model system for defining the growth factor signaling pathway leading to neuronal differentiation and proliferation. In the presence of NGF, PC12 cells differentiate into sympathetic-like neurons, characterized by outgrowth of axon-like neurites, increased electrical excitability, and induced expression of a set of neuron-specific genes (Greene and Tischler, 1976; 1982). NGF exerts its action through its bona fide receptor, trkA, a receptor tyrosine kinase (Barbacid, 1994; Klein *et al*., 1991). NGF binding to trkA triggers activation of a diverse signaling cascade, including phospholipase C-γ1, phosphatidylinositol 3-kinase, and extracellular signal regulated kinases (ERKs) (Carter and Downes, 1992; Kaplan *et al*., 1991; Kim *et al*., 1991; Ohmichi *et al*., 1991; Qui and Green, 1991). Numerous studies have been devoted to delineate the pathways responsible for PC12 cell differentiation among these signaling cascades

PC12 cells show rapid but unusually persistent increases in ERKs activity in response to NGF (Gotoh *et al*., 1990; Traverse *et al*., 1992). Based on a close correlation of PC12 cell differentiation with the sustained activation of ERKs in various circumstances, the sustained activation of ERKs has been proposed to be a major determinant of PC12 cell differentiation (reviewed by Marshall, 1995). A rapid and transient increase of ERKs activity induced by epidermal growth factor (EGF), for example, does not induce PC12 cell differentiation, but rather promotes proliferation (Chao, 1992; Huff *et al*., 1981). In addition, the ectopic expression of a constitutively active form of Ras, which causes sustained activation of ERKs, induces spontaneous neurite outgrowth in PC12 cells (Bar-Sagi and Feramisco, 1985; Noda *et al*., 1985). However, Ras-dependent ERK activation is quickly terminated in wild type PC12 cells, possibly by the ERK-induced phosphorylation of the Ras guanine-nucleotide-exchange factor, Sos (Porfiri and McCormick, 1996; York *et al*.,

† Present address: Department of Neuroscience, Graduate School of East-West Medical Science, Kyung Hee University, Seoul 130-701, Korea.

* To whom correspondence should be addressed.
Tel: 82-331-201-2182; Fax: 82-2-793-0176
E-mail: hmkwon@chollian.net

Abbreviations: EGF, epidermal growth factor; ERKs, extracellular signal regulated kinases; MBP, myelin basic protein; NGF, nerve growth factor.

1998). Therefore, the sustained activation of ERKs by NGF in PC12 cells appears to require additional Ras-independent pathways. Recently, Rap1, a small G protein, is shown to be responsible for the sustained phase of ERK activation by NGF (York *et al*., 1998). When a dominant-negative mutant of Rap1, RapN17, was expressed in PC12 cells, the sustained phase of ERK activation by NGF disappeared. In contrast, a dominant-negative mutant of Ras, RasN17, blocked the initial phase of ERK activation without inhibiting the sustained phase of ERK activation. These results have been explained by a model that NGF activates Ras and Rap1 in a parallel pathway, and Ras and Rap1 are responsible for the initial rapid phase and the sustained phase of ERK activation respectively. In contrast to complete inhibition by a dominant-negative mutant of Ras, however, the inhibitory effect of RapN17 was limited to increased electrical excitability and the expression of a set of neuron-specific genes, i.e. the ability of NGF to induce neurites in PC12 cells was not inhibited by RapN17. These results indicate that morphological differentiation of PC12 cells require a supplemental signaling pathway(s) in addition to the initial phase of ERKs activation but do not require the sustained phase of ERK activation.

To elucidate supplemental signaling pathways required for morphological differentiation of PC12 cells, we sought to find a molecule(s) that converts EGF into an inducer of morphological differentiation of PC12 cells. Here, we show that the differentiation of PC12 cells by the co-treatment of EGF and K252a (EGF/K252a) is limited to the morphological differentiation of PC12 cells. EGF/K252a induced active neurite outgrowth of PC12 cells, without inducing sustained activation of ERKs and expression of a neuron-specific gene, neurofilament. Major effects of K252a on the EGF-treated cells were found in the sustained mobility shift of paxillin in SDS-PAGE, the promoted association of Crk-II with paxillin, and robust formation of peripheral focal adhesions. We discussed these results using a model in which a signal for the morphological differentiation of PC12 cells is mediated by cytoskeletal reorganization.

Materials and Methods

Materials Culture media and supplements were obtained from Gibco BRL (Gaithersburg, MD, USA). Human recombinant EGF was obtained from Upstate Biotechnology (Lake Placid, NY, USA). K252a was from Calbiochem (San Diego, CA, USA). Isotope and enhanced chemiluminescence reagent (ECL) were purchased from Amersham Corp. (Arlington Heights, IL, USA). Rhodamine-labeled phalloidin was from Molecular Probes (Eugene, OR). Anti-phosphotyrosine monoclonal antibodies, PY20 (Transduction Laboratories., Lexington, KY, USA) and 4G10 (Upstate Biotechnologies Inc.) were used for immunoprecipitation and immunoblotting, respectively. Monoclonal antibodies to Crk, FAK, and paxillin were from Transduction Laboratories. All other chemical reagents were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Cell culture and neurite outgrowth PC12 cells were grown in RPMI 1640 medium containing 5% fetal bovine serum and 10% heat-inactivated horse serum. In neurite outgrowth experiments, serum-free RPMI 1640 medium supplemented with insulin (5 μg/ml), transferrin (100 μg/ml), sodium selenite (30 nM), and progesterone (20 nM) was used. The cells were plated on poly-L-lysine coated 35-mm culture dishes and incubated for 24 h before adding growth factors. After further incubation for 4 d, the cells were fixed with 2% glutaraldehyde, washed with phosphate-buffered saline (PBS), and observed under phase-contrast microscope. Cells with neurites three times longer than the cell body were counted as neurite bearing cells.

Immunoprecipitation and immunoblotting Cells grown on 100-mm dishes to subconfluence (~10^7 cells/dish) were incubated with serum-free medium for 18 h before growth factor treatment was done. For EGF/K252a treatment, the pre-incubation with K-252a for 1 h was followed by EGF treatment. Cells were washed twice with ice-cold PBS and lysed for 1 h at 4°C in 1 ml of modified RIPA buffer containing 20 mM Hepes, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10 mM $Na_2P_2O_7$, 100 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μM leupeptin, 1.5 μM pepstatin, and 10 μg/ml aprotinin. Insoluble material was removed by centrifugation at 15,000 × *g* at 4°C for 15 min. Protein concentration in the supernatants was determined as described by Bradford (1976). For immunoblotting, cell lysates were added to Laemmli sample buffer (Laemmli, 1970), boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins in the gels were transferred onto polyvinylidine difluoride membranes. Immunoblotting was performed according to the method descried previously (Lee *et al*., 1997). For immunoprecipitation, 0.5–1.0 mg of cell lysate protein was incubated with appropriate antibodies for 3 h at 4°C, followed by additional incubation with protein A-sepharose beads for 2 h at 4°C. Beads were then washed three times with RIPA buffer to remove nonspecific binding proteins. Immunocomplexes were treated with Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis for immunoblotting with various antibodies.

***In vitro* kinase assay** Cell lysates were incubated with the antibody recognizing ERKs for 3 h at 4°C, followed by additional incubation with protein A-sepharose beads for 2 h at 4°C. The beads were then washed three times with cold washing buffer (0.25 M Tris·HCl, pH 7.5, 0.1 M NaCl). Immunocomplexes were resuspended in 40 μl of kinase assay buffer containing 10 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, 5 μCi [γ-^{32}P] ATP, and 10 mg of myelin basic protein (MBP), and incubated for 30 min at room temperature. Reaction was stopped by adding 4× Laemmli sample buffer and boiling for 3 min. Proteins were resolved by 15% SDS-polyacrylamide gel electrophoresis. The gels were dried and exposed to X-ray films. Incorporation of ^{32}P into MBP was also quantitated using an image scanner (Phospholmager 400: Molecular dynamics).

Immunocytochemistry PC12 cells grown on poly-L-lysine-coated glass coverslips were fixed with 3% paraformaldehyde for

10 min at room temperature and washed in PBS. They were then permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with 10% goat serum and 0.1% gelatin in PBS. The resulting cells were incubated with appropriate combinations of first antibodies in PBS containing 1% BSA for 1 h in a humidified chamber at 37°C and washed four times with 0.1% Triton X-100 in PBS. They were then stained with appropriate fluorophore-conjugated secondary antibody in PBS containing 1% BSA for 50 min at room temperature. Coverslips were washed with 0.1% Triton X-100 in PBS and mounted with 90% glycerol and 0.1% o-phenylenediamine in PBS. Coverslips were viewed on a Zeiss Axioplan II microscope equipped with a 63X (1.4 NA) planapochromat objective lens and filters for epifluorescence.

Production of anti-paxillin antibody Paxillin cDNA (Turner and Miller, 1994) was subcloned in-frame into pGEX-4T plasmid expression vector. The 91-kDa GST-paxillin fusion protein, which is equivalent to the sum of 29-kDa GST and 62-kDa paxillin fragment (i.e. 566 amino acids), was then purified from the IPTG-treated cells. To prepare the polyclonal antibody against paxillin, the purified fusion protein was injected into albino rabbits three times at 4-week intervals. Upon immunoblot analysis, the antiserum raised against the 91-kDa protein was found to specifically interact with the 91-kDa or 68-kDa protein bands in the lysates of *E.coli* and PC12 cells, respectively.

Results

In search of reagents that convert EGF into an inducer of PC12 cell differentiation, we recognized the ability of K252a to induce PC12 cell differentiation in the presence of EGF (Isono *et al.*, 1994; Wu and Howard, 1995). Although K252a in combination of EGF (EGF/K252a) has been claimed to induce a full range of differentiation events, we closely re-examined the effects of EGF/K252a on PC12 cells. In agreement with previous observations, EGF/K252a led to neurite outgrowth of PC12 cells (Fig. 1A). The extent of neurite outgrowth obtained by EGF/K252a was comparable with that induced by NGF (Fig. 1B). Next we examined the kinetics of tyrosine phosphorylation of ERK1 and ERK2 in NGF-, EGF-, or EGF/K252a-treated PC12 cells (Fig. 2A). ERKs in the NGF- and EGF-treated cells were rapidly tyrosine phosphorylated within 5 min. Thereafter, the tyrosine phosphorylation of EGF-treated cells precipitously decreased while that of NGF-treated cells remained robust: a similar level of tyrosine phosphorylation of ERKs was observed even after 60 min. In contrast to the previous report (Isono *et al.*, 1994), however, the tyrosine phosphorylation of ERKs in the EGF/K252a-treated cells was not sustained as the NGF-treated cells but mimicked the kinetics of tyrosine phosphorylation observed in the EGF-treated cells. In order to test whether this contradiction is due to variation in PC12 cell isolates, we examined several different PC12 cell isolates and obtained similar results with all of the isolates: sustained activation

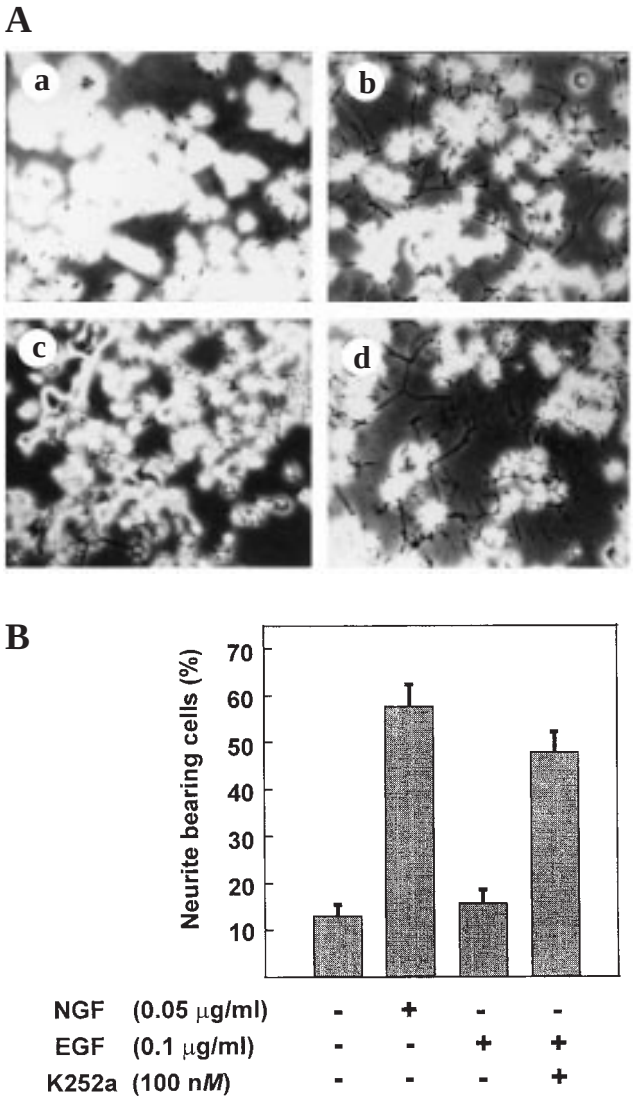

Fig. 1. Stimulation of PC12 neurite outgrowth by combination of EGF and K252a. **A.** Cells were incubated with NGF, EGF or EGF/K252a in serum-free media. Phase-contrast photographs were taken at five days after the treatment. (a) Untreated; (b) 50 ng/ml NGF; (c) 100 ng/ml EGF; (d) 100 ng/ml EGF + 100 nM K252a. **B.** Cells were treated as in (**A**) and scored if neurites were three times longer than cell body. Bars represent mean ± SEM (n = 3), and > 200 cells were counted at each treatment.

of ERKs was not observed in the EGF/K252a-treated PC12 cells (data not shown). To assess if the prolonged phosphorylation of ERK1 and ERK2 reflected sustained enzymatic activation, activity of ERKs was analyzed by an *in vitro* kinase assay (Fig. 2B). After 30 min from the treatments, the activity of ERKs was maintained only in the NGF-treated cell, but not in the EGF- or EGF/K252a-treated cells.

Sustained activation of ERKs is essential for the biochemical differentiation characterized by electrical

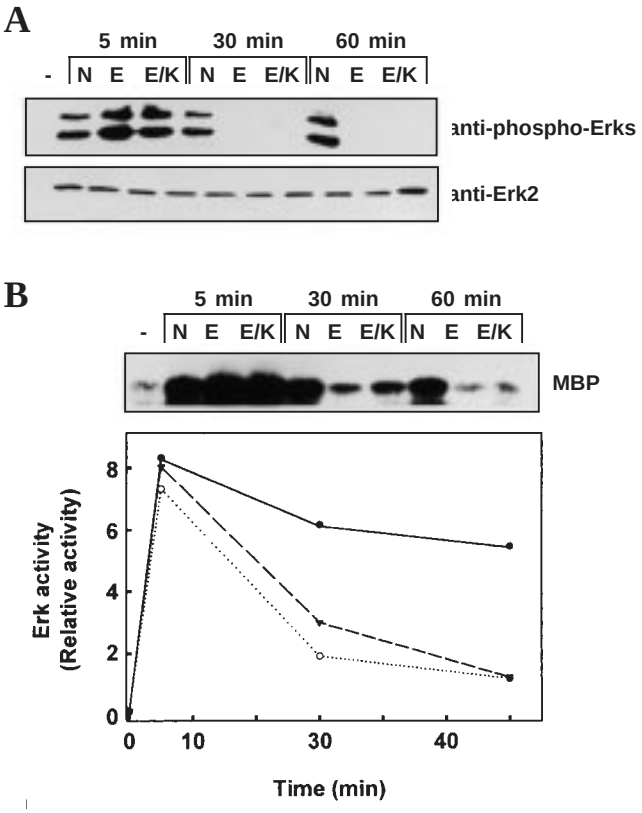

Fig. 2. EGF/K252a does not induce sustained activation of ERKs. Cells were treated with 50 ng/ml NGF (N), 100 ng/ml EGF (E), or 100 ng/ml EGF + 100 nM K252a (E/K) for up to 1 h. **A.** Equivalent amounts of cellular protein were resolved electrophoretically and probed with phospho-ERKs antibodies to detect the activated ERKs (upper panel). Equivalent loading of the protein was confirmed by reprobing the blots with anti-ERK2-specific antibodies (lower panel). **B.** Equivalent amounts of cellular protein were immunoprecipitated with anti-ERK antibodies. Precipitates were assayed for ERK activity using [^{32}P] ATP and MBP as a substrate. Data are representative of three separate experiments.

excitability and induction of a set of neuron-specific genes (reviewed by Marshall, 1995). If the tyrosine phosphorylation of ERKs in the EGF/K252a-treated cells were not sustained as shown in here, the biochemical differentiation would not be induced in spite of the apparent morphological differentiation. In order to investigate this possibility, we examined the accumulation of neurofilament in the neurites of EGF/K252a-treated PC12 cells (Fig. 3). Neurofilaments are neuron-specific intermediate filaments and accumulated in the neurites of PC12 cells treated with NGF. Thus, the accumulation of neurofilaments in neurites can serve as a marker of neuron-specific gene expression (Lazarides, 1980; Yao and Osada, 1997). When we visualized neurofilaments by staining with an anti-neurofilament antibody, NF200, neurites in the NGF-treated cells were heavily stained, indicating that neurofilaments accumulated along the neurites. In contrast, PC12 cells treated with EGF/K252a failed to show an increase in neurofilament accumulation in spite of the dramatic morphological response. This result is consistent with the inability of EGF/K252a to induce the sustained activation of ERKs. Taken together, we concluded that K252a in the presence of EGF is able to elicit the signal pathway that leads to the morphological, but not the biochemical differentiation of PC12 cells.

Since K252a is a protein kinase inhibitor, we examined the effects of K252a on tyrosine phosphorylation by EGF in PC12 cells. Immunoblot analysis using the anti-phosphotyrosine antibody 4G10 following immunoprecipitation with the anti-phosphotyrosine antibody PY20 revealed two clusters of tyrosine-phosphorylated proteins, with approximate sizes of 115–130 kDa and ~70 kDa, in the untreated cells (Fig. 4A). Although no new tyrosine-phosphorylated protein except their cognate receptors appeared after exposure to NGF or EGF, a close

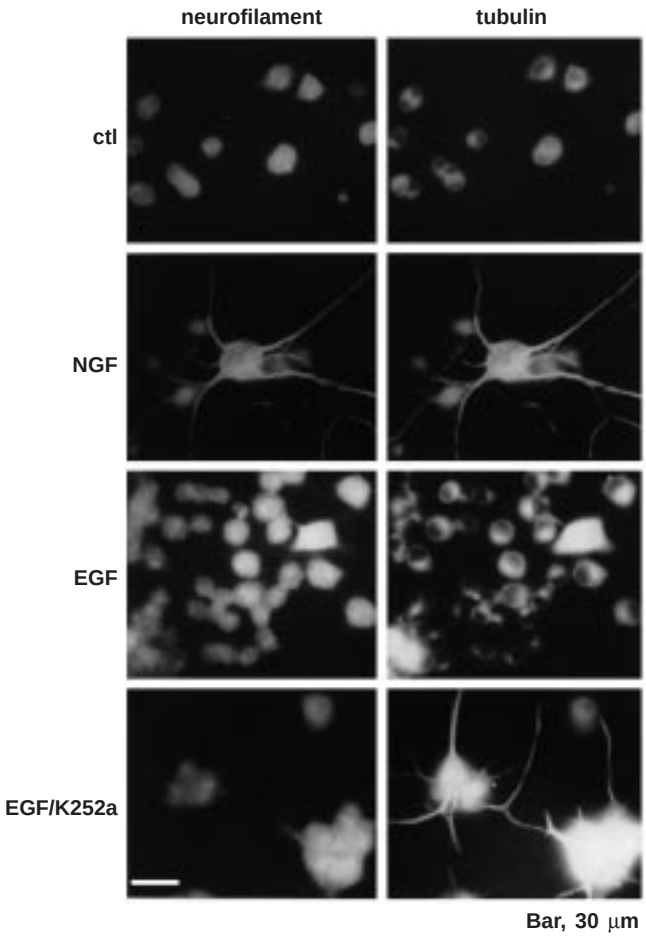

Fig. 3. Neurites induced by EGF/K252a do not accumulate neurofilament. Cells were incubated with 50 ng/ml NGF, 100 ng/ml EGF, or 100 ng/ml EGF + 100 nM K252a for 5 d. They were then fixed and stained with the anti-tubulin antibody or the anti-neurofilament antibodies, NF200. Bar, 30 μm.

examination revealed a discernible change in the migration of the 70 kDa tyrosine-phosphorylated protein on SDS-PAGE. When exposed to NGF or EGF, the mobility of the 70 kDa protein was rapidly reduced within 5 min. After that a clear difference between the NGF- and EGF-treated cells was observed in the kinetics in which the slowly migrating 70 kDa protein regained its original mobility of untreated cells. In the cells treated with EGF alone, the mobility of the 70 kDa protein regained its original value swiftly: most of the 70 kDa protein showed migration of untreated cells at 6 h. On the other hand, the mobility shift of the 70 kDa protein was maintained for 6 h and more in the cells treated with NGF (Fig. 4 and data not shown). This difference in the kinetics is somehow related to the prolonged activation of ERKs by NGF compared to EGF. More importantly here, the mobility shift of the 70 kDa protein induced by EGF/K252a followed the kinetics of the NGF-treated cells. Since K252a converted the proliferation signal of EGF into a morphological differentiation signal, this correlation suggests a possible link between 70 kDa protein and the differentiation promoting effect of K252a.

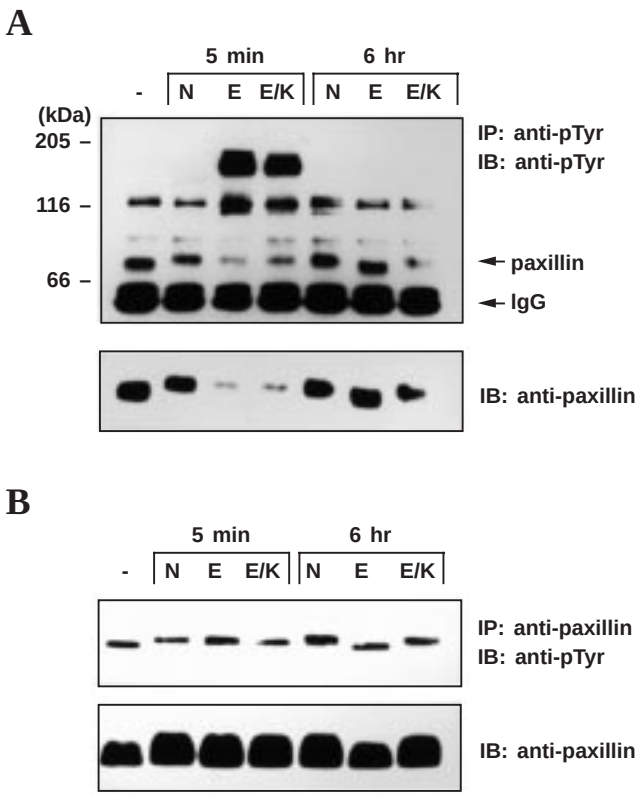

Fig. 4. Effect of NGF, EGF or EGF/K252a on the mobility shift of paxillin in SDS-PAGE. PC12 cells were incubated in the absence or presence of 50 ng/ml NGF (N), 100 ng/ml EGF (E), or 100 ng/ml EGF + 100 nM K252a (E/K) for the indicated periods. **A.** Mobility shift of tyrosine-phosphorylated paxillin was analyzed by a combination of immunoprecipitation (IP) using PY20, an anti-phosphotyrosine antibody and immunoblotting (IB) with 4G10, another anti-phosphotyrosine antibody (upper panel). The same blot was stripped off and immunoblotted with anti-paxillin monoclonal antibodies (lower panel). **B.** Cell lysates were immunoprecipitated with anti-paxillin polyclonal antibody and analyzed by anti-phosphotyrosine antibodies, 4G10 (upper panel). The same blot was stripped off and reprobed with anti-paxillin monoclonal antibody (lower panel).

Previous studies showed that the phosphorylation of paxillin in response to NGF is accompanied by the appearance of slower migrating forms (Khan *et al*., 1995). Since the size of paxillin is about 70 kDa, we stripped off the anti-phosphotyrosine antibodies from the membrane and subjected it to immunoblotting assay using a specific antibody to paxillin. The 70-kDa protein cross-reacted with the anti-paxillin antibody (Fig. 4A lower panel). The reciprocal experiments, in which the precipitates with the anti-paxillin antibody were subjected to immunoblotting with the anti-phosphotyrosine antibody, confirmed the identification of the 70 kDa protein as paxillin (Fig.4B). It also clearly demonstrated that the mobility shift of tyrosine-phosphorylated paxillin in the NGF- or EGF/K252a-treated cells maintained for a longer period than in the EGF-treated cell although the overall extent of tyrosine phosphorylation of paxillin appeared similar in both cases.

The over-expression of v-Crk has been shown to induce the differentiation of EGF-treated PC12 cells (Hempstead *et al*., 1994). Furthermore, v-Crk interacts with paxillin in the v-Crk over-expressing PC12 cells. Therefore, we examined whether the opposing effects of NGF, EGF/K252a, and EGF on the kinetics in the mobility shift of paxillin and eventually neuritogenesis might be explained by the differential interactions of cellular Crks with paxillin. Since the difference in the mobility of paxillin became prominent at 6 h after the exposure to growth factors, we treated PC12 cells with NGF, EGF or EGF/K252a for 6 h, prepared total cell lysates, and subjected them to immunoprecipitation assay with the anti-paxillin antibody (Fig. 5). As expected, the treatment of cells with NGF or EGF/K252a resulted in increased association of paxillin with endogenous Crk-II, whereas the increased association of paxillin with Crk-II was not observed in the EGF-treated cells. In contrast with Crk-II, FAK remained associated with paxillin up till this time regardless of whether the PC12 cells were treated with NGF, EGF or EGF/K252a (see below). Taken together, these results suggest that the interaction of paxillin with Crk-II may play a pivotal role in the morphological differentiation of PC12 cells.

Previous studies showed that the translocation of paxillin from cytoplasm into the adhesion complex is accompanied by the mobility shift in SDS-PAGE (Brown *et al*., 1996; 1998; Burridge *et al*., 1992; Turner, 1994). As shown here, an early and major change was observed in the kinetics of the mobility shift of paxillin when K252a converted the proliferation signal of EGF into the

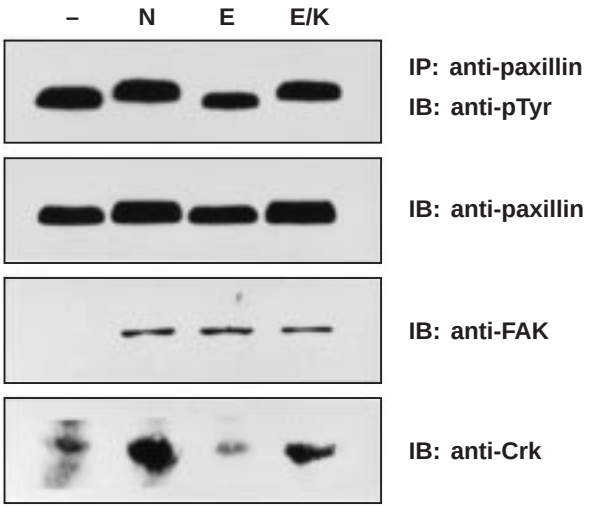

Fig. 5. Association of Crk with paxillin is stimulated by NGF or EGF/K252a, but not by EGF alone. Cells were treated with 50 ng/ml NGF (N), 100 ng/ml EGF (E), or 100 ng/ml EGF + 100 nM K252a (E/K) for 6 h. The cell lysates were immunoprecipitated with anti-paxillin antibodies, and the precipitates were analyzed by anti-phosphotyrosine antibodies (top panel). The presence of paxillin, FAK, and Crk in the precipitates was analyzed by immunoblotting the same blot with anti-paxillin, anti-FAK, or anti-Crk-II antibodies respectively (three lower panels).

morphological differentiation signal. Therefore, we hypothesized that the translocation of paxillin is an early event possibly leading to the morphological differentiation. In order to examine the translocation of paxillin by immunofluorescence analysis, the PC12 cells were treated with NGF, EGF, or EGF/K252a, and then stained with the anti-paxillin antibody (Fig. 6). In the cells treated with NGF, EGF, or EGF/K252a, focal adhesions were detected as early as within 5 min, although they were small and not well developed. In the EGF-treated cells, the size and shape of focal adhesions remained unchanged until 6 h after the treatment, consistent with the result that FAK remained associated with paxillin until this time point in the EGF-treated cells (Fig. 5). In a dramatic contrast, however, focal adhesions in the NGF or EGF/K252a treated cells started growing from the initial time point of treatment and formed obvious dash-like structures at 1 h. These complexes maintained and even increased their sizes up to 6 h after the treatment. These dash-like complexes are likely to be peripheral focal adhesions because vinculin, another focal adhesion protein, was co-localized with these areas (data not shown). Therefore, we observed a close correlation between the maintenance of mobility shift of paxillin and the continuous growing of focal adhesions in the differentiating PC12 cells. Based on the

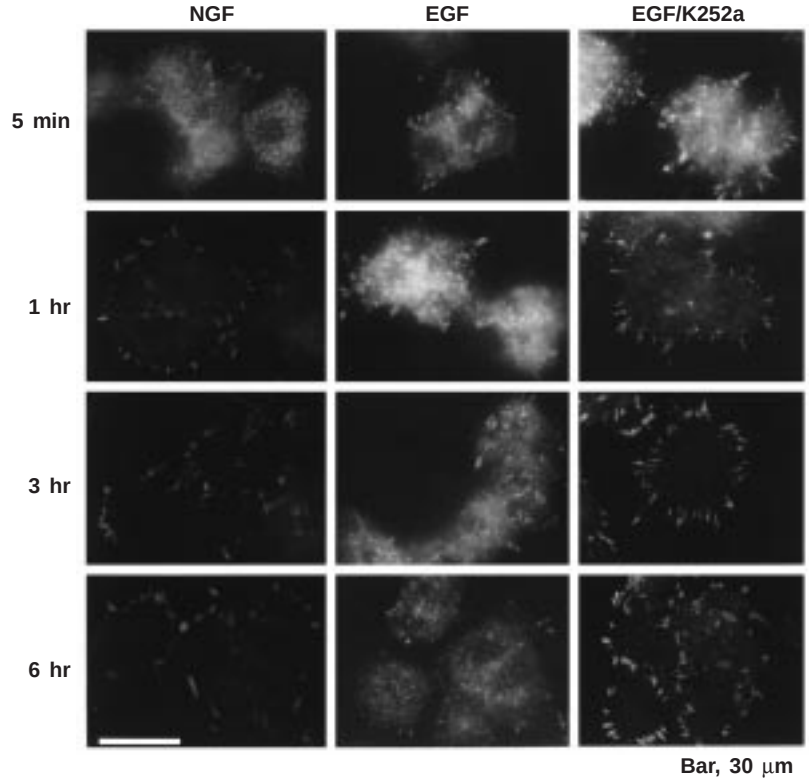

Fig. 6. Effects of growth factors on development of focal adhesion in PC12 cells. PC12 cells were incubated with 50 ng/ml NGF, 100 ng/ml EGF, or 100 ng/ml EGF + 100 nM K252a for the indicated period. They were then fixed and stained with anti-paxillin monoclonal antibodies and FITC-conjugated anti-mouse IgG antibodies. Bar, 30 μm.

observation that pretreatment with actinomycin D or cycloheximide before the treatment of NGF had no effect on the NGF-induced formation of peripheral focal adhesions (Fig. 7), a new gene activation or protein synthesis seems to be unnecessary for the NGF-induced formation of peripheral focal adhesions.

Focal adhesions include a number of cytoskeletal proteins and signaling molecules and associate actin cables on the inner face of plasma membrane (Burridge *et al*., 1988; Turner and Burridge, 1991). Therefore, dramatic formation of peripheral focal adhesions implies massive reorganization of the cytoskeletal structure. To examine actin cytoskeletal reorganization in the early stage of PC12 cell differentiation, PC12 cells treated with NGF, EGF, or EGF/K252a were stained with rhodamine-phalloidin (Fig. 8). Upon treatment with NGF for 1 h, the actin filaments developed from membrane area and started to stretch out in longitudinal direction. These actin filaments appeared as discernible stress fiber-like structures at 3 h and became more abundant up to 6 h. Similar formation of stress fiber-like structures was also observed in the EGF/K252a-treated cells. On the other hand, actin filaments were randomly directed and poorly accumulated in the EGF-treated cells. These results indicate that the strong and robust development of peripheral focal adhesions lead to massive reorganization of cytoskeletal structure in the differentiating PC12 cells.

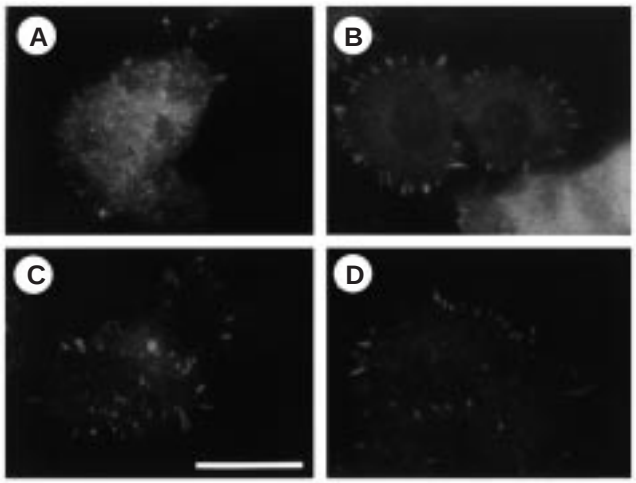

Fig. 7. Development of focal adhesion by growth factors was not suppressed by inhibition of transcription or translation. PC12 cells were pre-treated with 1 μg/ml actinomycin D or 10 μg/ml cycloheximide for 30 min, and further incubated with 50 ng/ml NGF for 3 h. They were then fixed and stained with anti-paxillin monoclonal antibodies and FITC-conjugated anti-mouse IgG antibodies. **A**, control; **B**, NGF; **C**, NGF + Actinomycin D; **D**, NGF + Cycloheximide. These results are representative of three separate experiments.

Discussion

Differentiation of PC12 cells by NGF involves striking morphological and biochemical changes. Interestingly, the morphological and biochemical differentiation of PC12 cells can be induced independently of each other under certain circumstances. For example, a dominant negative form of Rap1, RapN17, cannot inhibit neurite outgrowth induced by NGF, although RapN17 effectively blocks the biochemical differentiation such as electrical excitability and neuron-specific gene expression (York *et al*., 1998). As a similar situation, we show here that the co-treatment of EGF and K252a promotes neurite outgrowth without inducing the biochemical differentiation. These phenomena strongly support a model in which activation of multiple and parallel signal pathways is required for the full range of PC12 cell differentiation. Activation of the Ras-ERK pathway, which can be diagnosed by a rapid and transient increase of ERK activity, is absolutely necessary for both morphological and biochemical differentiation (Teng *et al*., 1995; York *et al*., 1998). However, activation of the Ras-ERK pathway is not sufficient for either morphological or biochemical differentiation (Hwang *et al*., 1997). Recently, a small G protein, Rap1, was identified as a mediator for PC12 cell differentiation, but its action is limited only to the biochemical differentiation (York *et al*., 1998). Rap1 appears to induce the sustained phase of ERK activation. Together with Ras activation, the activation of Rap1 by NGF can produce a rapid and sustained activation of ERKs, which has been considered as a hallmark of NGF-induced differentiation. On the other hand, these results led to the unexpected conclusion that the morphological differentiation of PC12 cells does not require the sustained phase of ERK activation, but requires another signal pathway which has not been identified yet. This conclusion is further supported by the results observed here that the sustained phase of ERK activation is not accompanied when the morphological differentiation of PC12 cells is induced by the treatment of EGF and K252a. Supporting this conclusion, low levels of cAMP or depolarization of membrane induced by high concentration of KCl has been previously shown to induce the morphological differentiation of EGF-treated PC12 cells without the sustained phase of ERK activation (Hilborn *et al*., 1997; Mark and Storm, 1997; Mark *et al*., 1995). However, the mechanism underlying these phenomena remains elusive.

What signal pathways would be required for the morphological differentiation? As an initial effort to identify this signal pathway, we looked for the signal pathway activated rapidly by NGF and EGF/K252a, but not by EGF alone. One signal pathway that satisfied this criterion was the signal that accompanied the formation of peripheral focal adhesions and massive cytoskeletal reorganization. Compared with the weak formation of focal adhesions in the EGF-treated cells, a strong and robust

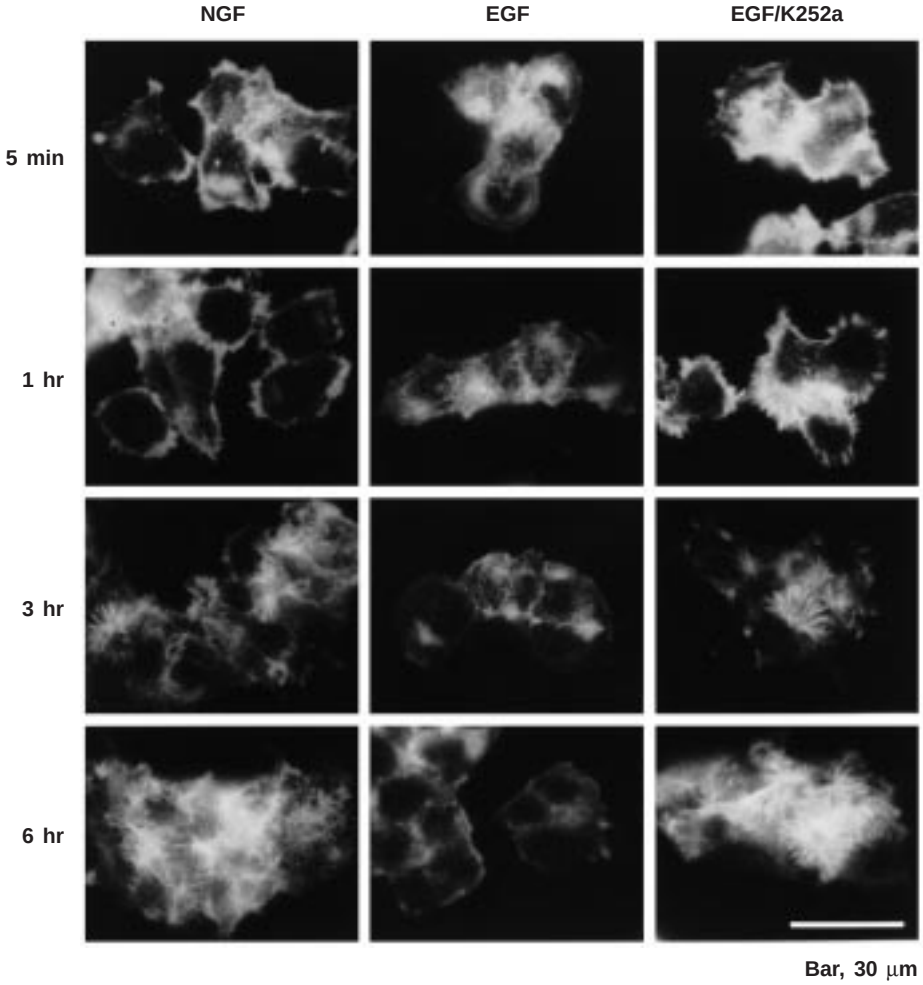

Fig. 8. Effects of growth factors on development of actin filament in PC12 cells. PC12 cells were incubated with 50 ng/ml NGF, 100 ng/ml EGF, or 100 ng/ml EGF + 100 nM K252a for the indicated period. They were then fixed and stained with rhodamine-conjugated phalloidin. Bar, 30 μm.

formation of peripheral focal adhesions was observed in the NGF- or EGF/K252a-treated cells. The formation of peripheral focal adhesions appeared to be an early but sustained event. Immunofluorescent staining of paxillin and vinculin showed that peripheral focal adhesions kept growing from the initial time point of treatment, were detected as early as within 5 min and developed into obvious dash-like structures at 1 h. Furthermore, the early phase of peripheral focal adhesion formation did not require a new round of gene activation or protein synthesis, although it might not be sustained for longer durations. Persistent development of peripheral focal adhesions was also accompanied by a massive reorganization of the cytoskeletal structure. Actin filaments were observed as developing from membrane area, possibly from peripheral focal adhesions, at 1 h after NGF- or EGF/K252a-treatment. These actin filaments continued to grow into stress fiber-like structures and remained persistent up to 6 h, the last time point we measured. Although it remains speculative whether the persistent development of peripheral focal adhesions and its accompanying reorganization of cytoskeletal structure provide a signal for the morphological differentiation of PC12 cells, we found several examples where dynamic rearrangement of the cytoskeleton controls discrete morphological developmental processes in the late stages of neuronal differentiation. In a growing axon, differential regulation of actin and microtubule assembly plays an important role in growth cone movement (Goslin *et al*., 1989; Gundersen and Barrett, 1979; Seeley and Greene, 1983; Tanaka and Kirschner, 1995). Furthermore, local actin instability in the growth cone area has been suggested to provide a signal specifying a single growth cone as an axon-bound neurite, as distinguished from the rest of neurites which are dendrite-bound ones (Bradke and Dotti, 1999). Therefore, the results presented here suggest an interesting possibility

that peripheral focal adhesion, whose formation is initiated by differentiation inducing agents such as NGF or EGF/K252a, provide a signal for morphological differentiation at the initial stage of differentiation and some of them continue to provide sites for neurite elongation. Local actin instability may provide a determinant for elongation sites for neuronal outgrowth.

The observation that NGF or EGF/K252a induces the sustained mobility shift of paxillin in SDS-PAGE is quite interesting in light of the fact that the sustained activation of ERKs is a major determinant of biochemical differentiation. Furthermore, the ability of the ectopic expression of constitutively active form of Ras to induce spontaneous morphological differentiation of PC12 cells suggests the possibility that the kinetics of Ras-activated events are a determinant of morphological differentiation. Therefore, the role of the Ras-independent pathway required for morphological differentiation would be to make a specific Ras-activated event sustainable analogous to the role of Rap1 for ERKs activation to induce biochemical differentiation. Our results suggest that K252a activates this Ras-independent pathway. Also, the induction of sustained mobility shift of paxillin observed in the differentiating PC12 cells strongly implies a possible connection between paxillin and the morphological differentiation of PC12 cells.

Phosphorylation of tyrosine residues may account for the reduced mobility of paxillin in SDS-PAGE. However, we consistently observed that the regaining of original mobility in the EGF-treated cells did not go with any reduction in the intensity of phosphotyrosine signal. Therefore, it is not likely that the mobility shift of paxillin is due to phosphorylation of tyrosine residues. Recently serine/threonine phosphorylation of paxillin LIM domain has been suggested to regulate the availability of paxillin to integrate into focal adhesions upon adhesion to fibronectin (Brown *et al*., 1996; 1998). With its ability to reduce mobility of protein in SDS-PAGE, phosphorylation of serine/threonine residue appears to be the most likely reason for the mobility shift of paxillin. However, we cannot discount the involvement of other kinds of protein modification such as ubiquitination and farnesylation.

What would be the nature of signals elicited from peripheral focal adhesions? We observed the increased association of paxillin with Crk accompanying the formation of peripheral focal adhesions induced by NGF or EGF/K252a. The Crk proteins are adaptor proteins containing the SH2 and SH3 domains (reviewed by Birge *et al*., 1996). There is an oncogenic product of the Crk proteins, v-Crk, which is expressed as a fusion protein of viral Gag sequences with the SH2 and the first SH3 domains of the c-Crk (Mayer *et al*, 1988; Reichman *et al*., 1992). The involvement of Crk in the differentiation of PC12 cells has been suggested by a demonstration that the ectopic expression of v-Crk augmented the NGF-induced differentiation of PC12 cells and converted EGF into a differentiating factor (Hempstead *et al*., 1994; Teng *et al*., 1995). However, the effect of v-Crk is more general than that of K252a in this regard. Treatment of EGF to the v-Crk over-expressing PC12 cells resulted in the sustained activation of ERKs, the induction of neuron-specific gene expression as well as neurite outgrowth (Hempstead *et al*., 1994). Interestingly, the SH2 mutants of v-Crk compromised the NGF- and EGF-dependent neurite outgrowth without any effect on the sustained activation of ERKs and the induction of neuron-specific gene expression (Teng *et al*., 1995). These results suggest that the ectopic expression of v-Crk activate multiple signal pathways. The pathway activated by the SH2 domain of v-Crk appears to be responsible for neurite outgrowth, whereas the SH3 domain seems to be required for the biochemical differentiation (Teng *et al*, 1995; 1996). This idea is consistent with the suggestion that Crk adaptor proteins are involved in the activation of Rap1, which is claimed to be responsible for the sustained activation of ERKs and the biochemical differentiation (York *et al*., 1998). The association of v-Crk or c-Crk with paxillin at focal adhesions has long been recognized (Altun-Gultekin *et al*., 1998; Birge *et al*., 1993; Ribon and Saltiel, 1996; Teng *et al*., 1996). Furthermore, the SH2 mutants of v-Crk showed a defect in the co-localization with paxillin at the tips of growth cones in the late stage of PC12 cell differentiation (Teng *et al*., 1996). We demonstrated here that the morphological differentiation by EGF/K252a showed a strong correlation with the differential association of endogenous Crk-II with paxillin, probably mediated by modification of paxillin, at the early stage of differentiation. Collectively, these results suggest that the formation of peripheral adhesions, where Crk associates with paxillin, is a critical step for morphological differentiation and they presumably modulate growth cone dynamics.

Acknowledgments This work was supported by GRANT No. 961-0501-004-2 from the Korea Science and Engineering Foundation (to H. K.) and the Academic Research Fund (GE97-019-D00035) of the Ministry of Education, Republic of Korea (to Y. K. K.).

References

Altun-Gultekin, Z. F., Chandriani, S., Bougeret, C., Isizaki, T., Narumiya, S., Graaf, P., Hengouwen, P. V. B., Hanafusa, H., Wagner, J. A., and Birge, R. B. (1998) Activation of Rho-dependent cell spreading and focal adhesion biogenesis by the v-Crk adaptor protein. *Mol. Cell. Biol.* **18**, 3044–3058.

Barbacid, M. (1994) The Trk family of neurotrophin receptors. *J. Neurobiol*. **25**, 1386–1403.

Bar-Sagi, D. and Feramisco, J. R. (1985) Microinjection of the ras oncogene protein into PC12 cell induces morphological differentiation. *Cell* **42**, 841–848.

Birge, R. B., Fajardo, E., Reichman, C., Shoelson, S. E., Songyang, Z., Cantley, L. C., and Hanfusa, H. (1993) Identification and characterization of a high-affinity interaction between v-Crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. *Mol. Cell. Biol*. **13**, 4648–4656.

Birge, R. B., Kundsen, B. S., Besser, D., and Hanafusa, H. (1996) SH2 and SH3-containing adaptor proteins: redundant or independent mediators of intracellular signal transduction. *Genes to Cells* **1**, 595–613.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Bradke, F. and Dotti, C. G. (1999) The role of local actin instability in axon formation. *Science* **283**, 1931–1934.

Brown, M. C., Perrotta, J. A., and Turner, C. E. (1996) Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell Biol.* **135**, 1109–1123.

Brown, M. C., Perrotta, J. A., and Turner, C. E. (1998) Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin. *Mol. Biol. Cell* **9**, 1803–1816.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. E. (1988) Focal ahesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* **4**, 484–525.

Burridge, K., Turner, C. E., and Romer, L. H. (1992) Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* **119**, 893–903.

Carter, A. N. and Downes, C. P. (1992) Phosphatidylinositol 3-kinase is activated by nerve growth factor and epidermal growth factor in PC12 cells. *J. Biol. Chem*. **267**, 14563–14567.

Chao, M. V. (1992) Growth factor signaling; where is the specificity? *Cell* **68**, 995–997.

Goslin, K., Birgbauer, E., Banker G., and Solomon, F. (1989) The role of cytoskeleton in organizing growth cone: a microfilament-associated growth cone component depend upon microtubules for its localization. *J. Cell Biol*. **109**, 1621–1631.

Gotoh, Y., Nishida, E., Yanashita, T., Hoshi, M., Kawakami, M., and Sakai, H. (1990) Microtubule-associated-protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. Identity with the mitogen-activated MAP kinase of fibroblastic cells. *Eur. J. Biochem*. **193**, 661–669.

Greene, L. A. and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428.

Greene, L. A. and Tischler, A. S. (1982) PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* **3**, 373–414.

Gundersen, R. W. and Barrett, J. N. (1979) Neuronal chemotaxis: chick dorsal root axons turn toward high concentrations of nerve growth factor. *Science* **206**, 1079–1080.

Hempstead, B. L., Birge, R. B., Fajardo, J. E., Glassman, R., Mahadeo, D., Kraemer, R., and Hanafusa, H. (1994) Expression of the v-crk oncogene product in PC12 cells results in rapid differentiation by both nerve growth factor- and epidermal growth factor-dependent pathway. *Mol. Cell. Biol*. **14**, 1964–2970.

Hilborn, H. D., Rane, S. G., and Pollock, J. D. (1997) EGF in combination with depolarization or cAMP produces morphological but not physiological differentiation in PC12 cells. *J. Nuerosci. Res*. **47**, 16–26.

Huff, K., End, D., and Guroff, G. (1981) Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. *J. Cell Biol*. **88**, 189–198.

Hwang, J.-J., Kwon, J.-H., and Hur, K. C. (1997) Insulin activates Ras in the PC12 cell line. *Mol. Cells* **7**, 438–443.

Isono, F., Widmer, H. R., Hefti, F., and Knüsel, B. (1994) Epidermal growth factor induces PC12 cells differentiation in the presence of the protein kinase inhibitor K-252a. *J. Neurochem*. **63**, 1235–1245.

Kaplan, D. R., Martin-Zanca, D., and Parada, L. F. (1991) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* **350**, 158–160.

Khan, M. A., Okumura, N., Okada, M., Kobayashi, S., and Nakagawa, H. (1995) Nerve growth factor stimulates tyrosine phosphorylation of paxillin in PC12h cells. *FEBS Lett*. **362**, 201–204.

Kim, U.-H., Fink, D. Jr., Kim, H. S., Park, D. J., Contreras, M. L., Guroff, G., and Rhee, S. G. (1991) Nerve growth factor stimulates phosphorylation phospholipase Cγ in PC12 cells. *J. Biol. Chem*. **266**, 1359–1362.

Klein, R., Jing, S. Q., Nanduri, V., O'Rourke, E., and Barbacid, M. (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* **65**, 189–197.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. *Nature* **283**, 249–256.

Lee, K. H., Kim, D. G., Shin, N. Y., Kwon, H., Chung, C. H., and Kang, M. S. (1997) NF-kB-dependent expression of nitric oxide synthase is required for membrane fusion of chick embryonic myoblasts. *Biochem. J.* **324**, 237–242.

Mark, M. D., Liu, Y., Wong, S. T., Hinds, T. R., and Storm, D. R. (1995) Stimulation of Neurite outgrowth in PC12 cells by EGF and KCl depolarization: a Ca^{2+}-independent phenomenon. *J. Cell Biol.* **130**, 701–710.

Mark, M. D. and Storm, D. R. (1997) Coupling of epidermal growth factor (EGF) with the antiproliferative activity of cAMP induces neuronal differentiation. *J. Biol. Chem.* **272**, 17238–17244.

Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185.

Mayer, B. J., Hamaguchi, M., and Hanafusa, H. (1988) A novel viral oncogene with structural similarity of phospholipase C. *Nature* **332**, 272–275.

Noda, M., Ko, M., Ogura, A., Liu, D., Armano, T., Takano, T., and Ikawa, Y. (1985) Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature* **318**, 73–75.

Ohmichi, M., Decker, S. J., Pang, L., and Saltiel, A. R. (1991) Nerve growth factor binds to the 140 kd trk proto-oncogene product and stimulates its association with the src homology domain of phospholipase Cγ1. *Biochem. Biophys. Res. Commun.* **179**, 217–223.

Porfiri, E. and McCormick, F. (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1. *J. Biol. Chem*. **271**, 5871–5877.

Qui, M.-S. and Green, S. H. (1991) NGF and EGF rapidly activate p21ras in PC12 cells by distinct, convergent pathways involving tyrosine phosphorylation. *Neuron* **7**, 937–946.

Reichman, C. T., Mayer, B. J., Keshav, S., and Hanafusa, H.

(1992) The product of the cellular crk gene consists primarily of SH2 and SH3 regions. *Cell Growth Differ*. **3**, 451–460.

Ribon, V. and Saltiel, A. R. (1996) Nerve growth factor stimulates the tyrosine phosphorylation of endogenous Crk-II and augments its association with p130Cas in PC12 cells. *J. Biol. Chem*. **271**, 7375–7380.

Seeley, P. J. and Greene, L. A. (1983) Short-latency local actions of nerve growth factor at the growth cone. *Proc. Natl. Acad. Sci. USA* **80**, 2789–2793.

Tanaka, E. and Kirschner, M. W. (1995) The role of microtubules in growth cone turning at substrates boundaries. *J. Cell Biol*. **128**, 127–137.

Teng, K. K., Lander, H., Fajardo, J. E., Hanafusa, H., Hempstead, B. L., and Birge, R. B. (1995) v-Crk modulation of growth factor-induced PC12 cell differentiation involves the Src homology 2 domain of v-Crk and sustained activation of the Ras/Mitogen-activated protein kinase pathway. *J. Biol. Chem*. **270**, 20677–20685.

Teng, K. K., Courtney, J. C., Henegouwen, P. B., Birge, R. B., and Hempstead, B. L. (1996) Dissociation of NGF induced signal transduction from neurite elongation by expression of a mutant adaptor protein v-Crk in PC12 cells. *Mol. Cell. Neurosci*. **8**, 157–170.

Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J*. **288**, 351–355.

Turner, C. E. (1994) Paxillin: a cytoskeletal target for tyrosine kinases. *Bioessays* **16**, 47–52.

Turner, C. E. and Burridge, K. (1991) Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr. Opin. Cell Biol*. **3**, 849–853.

Turner, C. E. and Miller J. T. (1994) Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125FAK-binding region. *J. Cell Sci.* **107**, 1583–1591.

Wu, C. F. and Howard, B. D. (1995) K252a-potentiation of EGF-induced neurite outgrowth from PC12 cells is not mimicked or blocked by other protein kinase activators or inhibitors. *Brain. Res. Dev.* **26**, 217–226.

Yao, R. and Osada, H. (1997) Induction fo neurite outgrowth in PC12 cells by γ-lactam-related compounds via RAS-MAP kinase signaling pathway independent mechamism. *Exp. Cell Res.* **234**, 233–239.

York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**, 622–626.