Protein Kinase C-dependent Tyrosine Phosphorylation of p130\textsuperscript{cas} in Differentiating Neuroblastoma Cells

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The cell signaling docking protein p130\textsuperscript{cas} became tyrosine-phosphorylated in SH-SY5Y human neuroblastoma cells during induced differentiation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and serum or a combination of basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I). The differentiating cells develop a neuronal phenotype with neurites and growth cones and sustained activation of protein kinase C (PKC) and pp60\textsuperscript{src}. The TPA-induced p130\textsuperscript{cas} phosphorylation increased within 5 min of stimulation and persisted for at least 4 days, whereas bFGF/IGF-I-induced p130\textsuperscript{cas} phosphorylation was biphasic. However, in increase in tyrosine phosphorylation of p130\textsuperscript{cas} was not restricted to differentiation inducing stimuli. The phosphorylation was blocked by the specific PKC inhibitor GF 109203X, and transient transfection with a function for p130\textsuperscript{cas} is a recently identified docking protein that contains an SH3 domain and a region with several tyrosine residues that can become tyrosine-phosphorylated and constitutes putative SH2 binding domains (1). The protein structure suggests a function for p130\textsuperscript{cas} in assembling signaling complexes. The only identified kinases that directly phosphorylate p130\textsuperscript{cas} is pp60\textsuperscript{src} (2) and Abl (3). However, little is known about the signaling pathways that lead to induced tyrosine phosphorylation of p130\textsuperscript{cas} and thereby promote binding of SH2 domain containing proteins, and the downstream effects of this complex formation remain to be clarified. It is established that p130\textsuperscript{cas} becomes heavily tyrosine-phosphorylated after integrin stimulation (4–6) and that the protein is localized to focal adhesions (4, 7) and along stress fibers (4). p130\textsuperscript{cas} associates with focal adhesion kinase (FAK)\textsuperscript{1} (7, 8) and pp60\textsuperscript{src} (1, 9, 10), both proteins involved in focal adhesion regulation. Stimulation of PC12 rat pheochromocytoma cells with nerve growth factor or epidermal growth factor also induces phosphorylation of p130\textsuperscript{cas} (11). The finding that p130\textsuperscript{cas} can bind to SH2 domains of Grb2, phosphoinositide 3-kinase, Crk, Nck, and phospholipase C-γ (2), for example, suggests that p130\textsuperscript{cas} is a docking protein that integrates signals from growth factor receptors and adhesion molecules.

SH-SY5Y is a human neuroblastoma cell line that can be induced to differentiate into a neuronal phenotype when treated with 16 nm phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) in the presence of fetal calf serum (FCS) or a growth factor (12, 13) or with a combination of basic fibroblast growth factor (bFGF) and insulin-like growth factor I (IGF-I) in serum-free medium (14). The differentiated cells extended neurites with neurotransmitter containing varicosities and growth cones. The growth cones are the leading tips of the growing neurites and are mainly composed of actin filaments (reviewed in Ref. 15), and actin reorganization is the mechanism underlying growth cone motility. In SH-SY5Y cells, the activity of pp60\textsuperscript{src} increases during differentiation (16), and pp60\textsuperscript{src} is enriched and activated in growth cones (17). pp60\textsuperscript{src} binds to the growth cone cytoskeleton in an activity dependent manner (18). Protein kinase C (PKC) is a family of serine-threonine kinases that are subdivided into three classes based on activator and co-factor dependence. Classical PKCs (PKC-α, PKC-β, and PKC-γ) and novel PKCs (PKC-δ, PKC-ε, PKC-η, PKC-θ, and PKC-µ) are activated by TPA, but the endogenous activator is, for example, diacylglycerol that is generated after growth factor stimulation (reviewed in Refs. 19 and 20). SH-SY5Y cells express at least PKC-α, PKC-ε, and PKC-γ (21). A sustained PKC activity, measured as phosphorylation of myristoylated alanine-rich protein kinase C substrate (MARCKS), is detected during differentiation of SH-SY5Y cells (22), and treatment with a high concentration of TPA (1.6 μM) that down-regulates

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* This work was supported by grants from the Swedish Cancer Society and The Childrens' Cancer Foundation of Sweden (to S. P. and E. N.), HKH Kronprinsessan Lovisas förening for barnasjukvård, Hans von Kantzows Stiftelse, Crafoordska Stiftelsen, and the MAS University Hospital Research funds (to S. P.) and Göran Gustavssons och Magnus Bergvalls Stiftelser (to E. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: FAK, focal adhesion kinase, TPA, 12-O-tetradecanoylphorbol-13-acetate; FCS, fetal calf serum; bFGF, basic fibroblast growth factor; IGF-I, insulin-like growth factor I; PKC, protein kinase C; MARCKS, myristoylated alanine-rich protein kinase C substrate; dbcAMP, dibutyryl cyclic AMP; PAGE, polyacrylamide gel electrophoresis; PTP, protein-tyrosine phosphatase.

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PKC-α completely only induces poor differentiation (21, 23, 24). Furthermore, inhibition of PKC activity by specific inhibitors blocks differentiation induced by 16 nM TPA in FCS or the combination of bFGF and IGF-I with respect to both morphological and transcriptional events (24). PKC-α and PKC-ε are enriched in growth cones of SH-SY5Y cells (21, 24), and maintenance of growth cone structure appears to be PKC-dependent (24).

In this study, we have investigated tyrosine phosphorylation of p130\textsuperscript{CAS} in differentiating SH-SY5Y cells and its dependence on activation and inhibition of PKC and Src family kinases. We also studied p130\textsuperscript{CAS} phosphorylation in isolated growth cones. The data presented show that there are at least two signaling pathways in differentiating SH-SY5Y cells that promote tyrosine phosphorylation of p130\textsuperscript{CAS}—an initial PKC-dependent but pp60\textsuperscript{src}/Src-kinase family-independent pathway, and a second PKC and Src-kinase family-dependent pathway. A functional role for p130\textsuperscript{CAS} in regulating the assembly of signals that control growth cone function is suggested.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—The human neuroblastoma cell line SH-SY5Y (25, 26) was cultured in Eagle’s minimum essential medium supplemented with 10% FCS (Life Technologies, Inc.), 100 IU/ml penicillin, and 50 μg/ml streptomycin in an atmosphere of 5% CO\textsubscript{2} at 37°C. For experimental procedures, cells were plated at a density of 2.5 × 10\textsuperscript{4} cells/10-cm dish culture dish for 24 h. Where indicated, cells were serum-starved in SHTE medium (RPMI 1640 medium containing 30 nM selenium, 10 mg/ml hydrocortisone, 30 μg/ml transferrin, and 1 mg β-estradiol) for 24–36 h before additions to decrease the basal level of p130\textsuperscript{CAS} phosphorylation. 100 μM Na\textsubscript{2}VO\textsubscript{4} was included 15 min prior to harvest. GF 109203X (Calbiochem), Go 6976 (Calbiochem), and herbimycin A (Calbiochem) were dissolved in Me\textsubscript{2}SO. bFGF was purchased from Promega, dbcAMP was from Sigma, and IGF-I was a generous gift from Pharmacia Upjohn.

**Transient Transfections**—The activated PKC-ε-E159 cDNA cloned into a pMT2 COS cell expression vector was kindly provided by Dr. P. Nijhage. 2 × 10\textsuperscript{4} SH-SY5Y cells/10-cm dish were plated in FCS-containing Eagle’s medium 24 h before transfection and received fresh medium 3 h prior to transfection. The cultures were transfected with 30 μg of plasmid DNA using the calcium-phosphate precipitate method as described (27). 16 h after transfection, the cells were washed in phosphate-buffered saline and kept in Eagle’s/FCS for 48 h before harvest.

**Immunoprecipitation and Western Blotting**—Cells were lysed in RIPA (10 mM Tris-HCl, pH 7.2, 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EDTA, 10 μg/ml apotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na\textsubscript{3}VO\textsubscript{4}). The lysates were pre-clear by centrifugation for 30 min at 15,000 g. Equal amounts of protein were removed for immunoprecipitation after protein determination by the method of Bradford (28). Cell lysates were immunoprecipitated with 1 μg of a polyclonal anti-p130\textsuperscript{CAS} antiserum (Santa Cruz) and protein A-Sepharose (Phar-macia), and the proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (129) followed by blotting to Hybond C extra filters (Amersham Corp.) (30). Phosphorylated proteins were detected by PSSO and RC20 antibodies (Transduction Laboratories) diluted 1:2500. For immunodetection of p130\textsuperscript{CAS}, filters were incubated with an anti-p130\textsuperscript{CAS} antiserum (Transduction Laboratories) at 0.1 μg/ml. The immunoreactivity was detected by the enhanced chemiluminescence method (Amersham Corp.). Filters were scanned using a Umax super vista S-12 scanner, and the values were expressed in arbitrary units relative to the level in control cells or cell bodies.

**Src Kinase Assay**—Cells were lysed in RIPA as described above, and pp60\textsuperscript{src} was immunoprecipitated with mAb 327 monoclonal anti-Src antibody (kindly provided by Dr. J. Brugge) as described previously (31). Immune complexes pp60\textsuperscript{src} kinase assay was performed as described previously (16), and the phosphorylated products were separated by 10% SDS-PAGE, and the autophosphorylated protein was visualized by autoradiography.

**Subcellular Fractionation**—Differentiated SH-SY5Y cells were fractionated into growth cones and cell bodies as described previously (17). For optimal differentiation, cells were plated at 10\textsuperscript{6} cells/culture dish. The cells were kept in serum-containing medium until additions of TPA or growth factors, when the cultures receiving bFGF/IGF-I were changed to SHTE medium. Briefly, cells were homogenized in ice-cold EDTA buffer (0.54 mM EDTA, 137 mM NaCl, 10 mM NaH\textsubscript{2}PO\textsubscript{4}, 2.7 mM KCl, 0.15 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.4) and layered onto a 20% sucrose cushion before centrifugation for 4 min at 500 × g. The growth cones were recovered in the EDTA supernatant and the cell bodies were recovered with the pellet. Each fraction was pelleted for 20 min at 20,000 × g and lysed in RIPA. Equal amounts of protein were immunoprecipitated as described above.

**RESULTS**

**Identification of the Major 110–130-kDa Phosphotyrosine Proteins in Differentiating SH-SY5Y Cells**—During in vitro differentiation of SH-SY5Y neuroblastoma cells, a group of proteins with sizes between 110 and 130 kDa and with a major component at 130 kDa becomes heavily tyrosine-phosphorylated. Under such conditions, differentiation by 16 nM TPA in 10% serum (TPA) for example, an increase in tyrosine phosphorylation of the 110–130-kDa proteins was apparent within 4 h of treatment (Fig. 1A, left panel). The same proteins became phosphorylated during treatment with bFGF and IGF-I in serum-free medium (bFGF/IGF-I), an alternative method to differentiate SH-SY5Y cells (14) (Fig. 1A, right panel). In addition, a protein of approximately 190 kDa became heavily tyrosine-phosphorylated during the bFGF/IGF-I treatment (Fig. 1A, right panel). The molecular size of this substrate is larger than both the IGF-I receptor units and the receptors binding bFGF, and the identity of the band remains to be determined.

In an attempt to identify the 110–130-kDa tyrosine-phosphorylated proteins, anti-p130\textsuperscript{CAS} and anti-p125FAK antibodies, among others, were used. Immunoprecipitation of p130\textsuperscript{CAS} followed by immunoblotting with an anti-phosphotyrosine antibody revealed that in TPA-treated SH-SY5Y cells, p130\textsuperscript{CAS} tyrosine phosphorylation was induced within 5 min and remained elevated for at least 4 days (Fig. 1B, upper panel). The amount of p130\textsuperscript{CAS} protein in the loaded samples was determined by Western blot analysis against p130\textsuperscript{CAS} (Fig. 1B, lower panel). By taking into account that the total amount of p130\textsuperscript{CAS} protein was slightly higher in the samples from cells treated for longer times in this experiment (not a general finding), the net increase in p130\textsuperscript{CAS} tyrosine phosphorylation was comparatively low at day 4. Thus, the TPA-induced p130\textsuperscript{CAS} phosphorylation occurred during a period when the cells differentiate functionally into neuron-like cells (12, 16). Immunoreactive p130\textsuperscript{CAS} appeared as three bands with apparent sizes of 115, 125, and 130 kDa, respectively (Fig. 1B), as reported earlier (1). The 130-kDa protein was the most prominently phosphorylated member of the triplet (compare the two panels in Fig. 1B). Therefore, the lower 110-kDa phosphoprotein detected in the anti-phosphotyrosine blots (Fig. 1A) is most likely not related to p130\textsuperscript{CAS}. The three sizes of the protein has been suggested to depend on differences in phosphorylation status, although protein products generated from alternatively spliced CAS mRNA cannot be excluded (1).

Considering the molecular mass range (110–130 kDa) of the tyrosine-phosphorylated proteins shown in Fig. 1A, p125FAK could be among these proteins. Western blotting of total lysates showed that SH-SY5Y cells express p125FAK (not shown), as reported earlier (32). However, no tyrosine-phosphorylated p125FAK could be detected. It is therefore unlikely that phosphorylated p125FAK was one of the 110–130-kDa phosphoproteins.

**Tyrosine Phosphorylation of p130\textsuperscript{CAS} Was Not Strictly Correlated to Differentiation of SH-SY5Y Cells**—To test whether the increase in p130\textsuperscript{CAS} tyrosine phosphorylation correlated with the differentiated phenotype, SH-SY5Y cells treated with bFGF/IGF-I were analyzed. Also this treatment induced a rapid tyrosine phosphorylation of p130\textsuperscript{CAS}, but in contrast to
that after the initial peak around 30 min, the response decreased after 1 h, returned to basal levels after 2 and 4 h, and subsequently returned again after 6 h of stimulation (Fig. 1C and not shown). In contrast to the TPA-treated cells where the p130\textsuperscript{cas} tyrosine phosphorylation was rapid and sustained but started to decrease from day 1 to be significantly lower at day 4, the bFGF/IGF-I stimulated cultures showed a slower increase, a transient drop, and a later strong phosphorylation signal at day 4 (Fig. 1, B and C).

To investigate which components in the differentiation protocols stimulated p130\textsuperscript{cas} phosphorylation, serum-starved SH-SY5Y cells were treated for 5 and 30 min with TPA, FCS, and growth factors alone or in combinations. Five min of treatment with 10% FCS induced a weak tyrosine phosphorylation of p130\textsuperscript{cas}, whereas TPA or the combination of FCS and TPA had more pronounced effects (Fig. 1D). After 30 min of stimulation, tyrosine phosphorylation had increased as a result of all three treatments, and the strongest effect was obtained with TPA alone (Fig. 1D). Also bFGF and IGF-I added individually for 5 or 30 min induced p130\textsuperscript{cas} phosphorylation (Fig. 1E). bFGF had a slightly stronger effect than IGF-I which was most apparent after 30 min. The effect of the combination of factors seemed to be additive (Fig. 1E). Also after 4 days of stimulation, bFGF and IGF-I separately induced p130\textsuperscript{cas} phosphorylation, and again, the effect of the combination was additive (Fig. 1F).

Thus, 10% FCS, bFGF, IGF-I, and 16 nM TPA added separately to serum-free cultures, which all are treatments that fail to induce a well-developed differentiated phenotype in SH-SY5Y cells (13, 14), induced phosphorylation of p130\textsuperscript{cas}. Therefore, the initial phosphorylation of p130\textsuperscript{cas} seemed not to be strictly correlated to activation of a differentiation program. Since the differentiation protocols studied, as well as the stimulation by phorbol esters, IGF-I, and bFGF, all result in the activation of PKC (14, 22), the detected increase in tyrosine phosphorylation of p130\textsuperscript{cas} might be a PKC-dependent reaction.

**PKC-dependent p130\textsuperscript{cas} Tyrosine Phosphorylation**—The specific PKC inhibitor GF 109203X (33) was used to test further a role for PKC in the phosphorylation of p130\textsuperscript{cas}. Previous studies have shown that 2 \textmu M GF 109203X prevents TPA- and bFGF/IGF-I-induced differentiation and TPA-induced phosphorylation of the endogenous PKC substrate MARCKS in SH-SY5Y cells (24). Phosphorylation of p130\textsuperscript{cas} induced by 30 min treatment with TPA was partially prevented by 1 \textmu M PKC inhibitor, and at 4 \textmu M the phosphorylation remained almost at the control level (Fig. 2A). In addition, the basal level of p130\textsuperscript{cas} tyrosine phosphorylation in unstimulated cultures was reduced in a dose-dependent manner (Fig. 2A). bFGF/IGF-I-induced phosphorylation of p130\textsuperscript{cas} was also largely inhibited by 2 \textmu M GF 109203X (Fig. 2B, right panel), as well as the basal phosphorylation in the corresponding serum-starved control cultures (Fig. 2B, left panel).

GF 109203X inhibits the activity of both classical and novel isoforms of PKC through interaction with the ATP-binding site. Its effect on atypical isoforms, e.g., PKC-\textepsilon, has not been conclusively described. Another inhibitor, Go 6976, blocks preferentially the activity of classical PKCs (34). In light of our previous finding that mainly PKC-\textepsilon seems to be crucial for differentiation, including growth cone formation and neurite outgrowth of SH-SY5Y cells (24), we compared the effect of GF 109203X with that of Go 6976. In contrast to GF 109203X, Go 6976 repeatedly did not prevent but rather augmented both TPA and bFGF/IGF-I-induced tyrosine phosphorylation of p130\textsuperscript{cas} (Fig. 2, C and D). These data suggested that enhanced PKC activity promoted tyrosine phosphorylation of p130\textsuperscript{cas} and that this was predominantly mediated by novel isoforms of PKC.

**Constitutively Active PKC-\textepsilon Induced Tyrosine Phosphoryla-**
PKC-dependent p130as Tyrosine Phosphorylation

**FIG. 2. PKC-dependent tyrosine phosphorylation of p130as.** In all experiments, cell lysates normalized for total protein were immunoprecipitated with anti-p130as serum, followed by immunoblotting with an anti-phosphotyrosine antibody. A, serum-growing cells were pretreated for 30 min with the indicated concentrations of the PKC inhibitor GF 109203X (GF) prior to addition of vehicle (c) or TPA (TPA) for 30 min. B, serum-starved SH-SY5Y cells were pretreated with 2 μM GF 109203X (+) or vehicle (−) for 30 min. Control cells (c) in the left panel were left untreated, and cells in the right panel were stimulated with a combination of 2 μM bFGF and 5 μM IGF-I (bFGF/IGF-I) for 30 min before harvest. C, cells grown in serum-containing medium were preincubated with (+) 2 μM Go 6976 (Go) or vehicle (−) before addition of 16 μM TPA for 30 min. D, serum-starved cells pretreated with 2 μM Go 6976 (+) or vehicle (−) were stimulated with bFGF/IGF-I for 30 min.

**TABLE I** Phosphorylation of p130as in cells transiently transfected with activated PKC-e and pp60src compared with mock-transfected cells

| Plasmid       | Experiment | Mean |
|---------------|------------|------|
| Mock          | 1.0        | 1.0  |
| PKC-e+        | 1.6        | 2.0  |
| Src+          | 1.7        | 2.0  |

The amount of tyrosine-phosphorylated p130as in cells transiently transfected with plasmids encoding constitutively active PKC-e (PKC-e+) or functional pp60src (src+), empty vector was quantified by densitometry. Values were expressed relative to mock (set to 1.0) in each experiment. The results of four independent experiments are shown.

**FIG. 3. Tyrosine phosphorylation of p130as increased in cells transiently transfected with active PKC-e and src.** SH-SY5Y cells were transiently transfected for 16 h with plasmids encoding constitutively active PKC-e (PKC-e+) or src (src+) or mock-transfected (−), respectively. Forty-eight h after transfection, the cells received 2 μM GF 109203X (GF), 3 μM herbimycin A (HA), or vehicle for 1 h. Tyrosine phosphorylation of p130as was analyzed by immunoprecipitation of p130as from samples containing the same amount of total protein followed by immunoblotting with the PY20 anti-phosphotyrosine (α-PY) antibody. Enhanced chemiluminescence signal was quantified by densitometry scanning of the film, and the numbers under the blot show detected p130as tyrosine phosphorylation relative to the signal in control mock-transfected cells, expressed in arbitrary units. The numbers represent the mean of two experiments.

An opposite effect was found on mock-transfected cells where p130as phosphorylation was slightly augmented in the presence of herbimycin A, indicating that the resting level of phosphorylated p130as in unstimulated cultures was independent of an Src-related kinase activity (see for comparison the effect on GF 109203X described above). GF 109203X prevented the enhanced tyrosine phosphorylation of p130as in the src+ transfected cultures (Fig. 3). Thus it seems that active pp60src can promote phosphorylation of p130as and that this effect was dependent on functional PKC.

In previous studies on SH-SY5Y cells, we have shown that no increase in the specific pp60src activity could be detected until 6 h after addition of TPA (16). Thus, the initial stimulation of phosphorylation of p130as after addition of TPA (Figs. 1 and 2) did not coincide with any detectable up-regulation of the pp60src activity. In addition, in vitro kinase assays with immunoprecipitated pp60src from cells treated for 5 and 30 min with bFGF/IGF-I showed that an immediate activation of pp60src could not be found under those conditions (Fig. 4A, left panel). But similar to TPA treatment, prolonged stimulation with bFGF/IGF-I led to increased pp60src activity measured after 24 h (Fig. 4A, right panel) (16). Thus, tyrosine phosphorylation of p130as could be induced in the absence of a simultaneous detectable increase in pp60src activity.

Pretreatment of SH-SY5Y cells with 3 μM herbimycin A did not prevent the basal or the initially induced (30 min) phosphorylation of p130as seen after addition of TPA or bFGF/IGF-I (Fig. 4, B and C). On the contrary, a weak enhancement was noted. As this concentration of herbimycin A during the same incubation time inhibited src-induced p130as phosphorylation (Fig. 3), the results do not favor the involvement of...
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active pp60<sup>src</sup> in unstimulated cells or during the early phosphorylation response. To evaluate further the effect of herbi-
mycin A on basal and induced pp60<sup>src</sup> activity, the in vitro kinase assay was performed on cells preincubated with various concentrations of the inhibitor followed by stimulation with TPA for 20 h. 3 μM herbinycin A completely blocked the TPA-induced pp60<sup>src</sup> activity, and 5 μM also blocked the basal activity (not shown). This higher concentration employed on control cells and cells treated with TPA for 30 min did not block p130<sup>cas</sup> phosphorylation (not shown), and the results confirmed that basal and early induced p130<sup>cas</sup> phosphorylation could occur in cells with greatly reduced pp60<sup>src</sup> activity. At later time points, e.g., 1–4 days of stimulation with TPA, the activity of pp60<sup>src</sup> increased in SH-SY5Y cells (16). At this later time point, addition of herbinycin A decreased phosphorylation of p130<sup>cas</sup> within 5 min, indicating a pp60<sup>src</sup>- or Src kinase family-dependent phosphorylation (Fig. 4D).

Effect of Dibutyryl cAMP on p130<sup>cas</sup> Phosphorylation—To evaluate if activation of cyclic AMP-dependent protein kinase, in addition to PKC, promotes p130<sup>cas</sup> phosphorylation, SH-SY5Y cells were stimulated with 1 mM dibutylryl cAMP (dbcAMP) for 5 min to 2 h. In SH-SY5Y cells, dbcAMP potentiates TPA-induced differentiation but lacks a differentiating effect when added alone (39). An increase in p130<sup>cas</sup> tyrosine phosphorylation was seen 1 to 2 h after stimulation, but the effect was somewhat weaker and delayed compared with stimulation with TPA (Fig. 5).

PKC-dependent Phosphorylation of p130<sup>cas</sup> in Differentiated Cells and Isolated Growth Cones—Growth cones isolated from SH-SY5Y cells differentiated with TPA or bFGF/IGF-I, respectively, were analyzed for p130<sup>cas</sup> protein content. With both differentiation protocols, p130<sup>cas</sup> protein was enriched about 2-fold in the growth cones compared with the cell bodies when measured as immunodetectable p130<sup>cas</sup> per total protein in each fraction (Fig. 6A). We have previously shown that in bFGF/IGF-I-differentiated SH-SY5Y cells, the growth cones start to retract their filopodia within seconds after PKC inhibition with GF 109203X (24). The same result was obtained with TPA-differentiated cultures (not shown), and p130<sup>cas</sup> phosphorylation was now studied under identical conditions. Addition of GF 109203X to cells differentiated with TPA for 4 days reduced the p130<sup>cas</sup> phosphorylation to a level comparable to undifferentiated control cells within 5 min after addition of the inhibitor (Fig. 6B). The suppression of p130<sup>cas</sup> phosphorylation remained for at least 2 h, although the phosphorylation signal had started to slowly recover after 1 h. In similar experiments using bFGF/IGF-I-differentiated cells, a more transient dephosphorylation of p130<sup>cas</sup> was seen after 5 min of treatment with GF 109203X, with the effect reversed after 1–2 h (Fig. 6C).

Thus, the initial decrease in tyrosine phosphorylation of p130<sup>cas</sup> after application of the PKC inhibitor correlated well in time with the retraction of growth cone filopodia.

Tyrosine phosphorylation of p130<sup>cas</sup> was further investigated in growth cones isolated from TPA-differentiated cells. Relatively more (3.5-fold) phosphorylated p130<sup>cas</sup> was detected in the growth cones compared with the cell bodies, although a more exact quantification was difficult due to the small amount of material. After 30 min of treatment with GF 109203X prior to harvest, the phosphorylation signal was reduced to close to that in the cell body fraction (Fig. 6D). These results suggest also that the late and presumably more pp60<sup>src</sup>-dependent phosphorylation of p130<sup>cas</sup> that occurred in differentiated cells required active PKC. The rapid suppression of p130<sup>cas</sup> phosphorylation observed in GF 109203X-treated cells suggested an efficient turnover rate of phosphate on the tyrosine residues.

DISCUSSION

We here report a PKC-dependent increase in tyrosine phosphorylation of p130<sup>cas</sup> in differentiating SH-SY5Y neuroblasto-
toma cells. Src kinase and herbinycin A data suggested an initial p130<sup>cas</sup> tyrosine phosphorylation pathway independent of pp60<sup>src</sup> and a later pathway in differentiating cells involving pp60<sup>src</sup> or related herbinycin A-sensitive kinase(s). We also propose a role for p130<sup>cas</sup> in assembling signals that regulate the functional growth cone in axons and dendrites.

Previously, we have demonstrated that TPA- and to a large extent bFGF/IGF-I-dependent neuronal differentiation (growth cone formation, outgrowth of neurites, and gene expression) of SH-SY5Y cells is blocked by PKC inhibitors. However, the cells retain their capacity to differentiate in response to bFGF/IGF-I when classical PKCs (such as PKC-α) are down-regulated by prolonged treatment with 1.6 μM TPA (24). Under these conditions, PKC-ε is still present in substantial amounts. We have therefore proposed that PKC-ε or other novel isoforms are vital for neuronal differentiation in this cell system. We have now shown that under conditions where either of TPA and growth...
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**Fig. 6. Effect of inhibition of PKC on phosphorylation of p130\(^{cas}\)** in whole cells and growth cones isolated from differentiated SH-SYSY. A, SH-SYSY cells differentiated with either TPA or bFGF/IGF-I for 96 h were fractionated into cell bodies (CB) and growth cones (GC). Equal amount of protein from each fraction was separated by SDS-PAGE and analyzed for p130\(^{cas}\) content by immunoblotting with a monoclonal anti-p130\(^{cas}\) antibody. The bands were quantified by scanning densitometry. The numbers below the panel show the relative amount of p130\(^{cas}\)/total protein expressed as arbitrary units. B and C, cells were differentiated for 96 h in the presence of 16 \(\mu\)M TPA in serum-containing medium (B) or with bFGF/IGF-I in serum-free medium (C). The cells were treated with 2 \(\mu\)M PKC inhibitor GF 109203X (GF) for the indicated length of time and were harvested. p130\(^{cas}\) was immunoprecipitated (i.p.) from aliquots containing the same amount of protein, and the proteins were separated by SDS-PAGE and detected with the antibody. Immunoprecipitates from non-differentiated cultures were included as controls (left lane). D, cells differentiated with TPA for 96 h were incubated with or without 2 \(\mu\)M GF 109203X (TPA/GF) and TPA, respectively, for 30 min before cell fractionation and isolation of growth cones. Tyrosine-phosphorylated p130\(^{cas}\) in the cell body and growth cone fractions was detected by immunoprecipitation of p130\(^{cas}\) followed by immunoblotting (i.b.) with the antibody. The amount of starting material for immunoprecipitation in this experiment was approximately 50 times lower than that used in B and C, thus explaining the weak signal in E. The bands were quantified by scanning densitometry, and the amount of phosphorylated p130\(^{cas}\) in each fraction expressed as arbitrary unit is shown below the panel. The values for cell bodies are set to 1.0.

Factors induce differentiation, tyrosine phosphorylation of p130\(^{cas}\) is stimulated. This response is not entirely associated with the development of a differentiated phenotype, but rather with the activation of PKC, since a number of protocols inducing PKC activation but not differentiation also induced p130\(^{cas}\) tyrosine phosphorylation. We have previously demonstrated that both bFGF and IGF-I activates PKC in these cells, measured as in vivo phosphorylation of MARCKS, and that the combination of the factors was additive, whereas 16 \(\mu\)M TPA was twice as efficient (14). This order of magnitude was also true for p130\(^{cas}\) phosphorylation, speaking in favor of a PKC-dependent signaling step in growth factor- and TPAtreated cells. The use of specific inhibitors for classical and novel PKCs (GF 109203X and Go 6976) demonstrated that this phosphorylation seemed to be dependent on active novel isoforms of PKC. Also the maintenance of an elevated p130\(^{cas}\) phosphory-
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Fig. 7. Model for PKC-dependent p130<sup>Cas</sup> phosphorylation in SH-SY5Y cells. In unstimulated cells, the balance between unphosphorylated p130<sup>Cas</sup> (CAS) and phosphorylated p130<sup>Cas</sup>-P is exerted by the actions of one or several unknown protein-tyrosine kinases (PTK) and protein-tyrosine phosphatases (PTP). Activation of PKC, mediated by growth factor receptor or TPA stimulation, alters the balance toward a hyperphosphorylated state, whereas inactivation of PKC by GF 109203X decreases p130<sup>Cas</sup> phosphorylation. The function of PKC could be either activation of the protein-tyrosine kinase (shown to the left) or inhibition of the protein-tyrosine phosphatase (shown to the right). After 1–4 days of treatment with TPA or bFGF/IGF-I, the specific activity of PKC and pp60<sup>src</sup> (src) is increased as a consequence of differentiation. Under these conditions, tyrosine phosphorylation of p130<sup>Cas</sup> is blocked by the Src family inhibitor herbimycin A, indicating a function for pp60<sup>src</sup> or a related kinase in p130<sup>Cas</sup> phosphorylation in differentiating cells. However, the protein-tyrosine kinase(s) or phosphatase(s) that mediates the basal p130<sup>Cas</sup> phosphorylation might still be active.

and pp62<sup>yes</sup> kinases should be sensitive to herbimycin A. These kinases might therefore contribute to tyrosine phosphorylation of p130<sup>Cas</sup> at later time points but are unlikely to promote the initial signal. (v) Overexpression of functional pp60<sup>src</sup> caused increased tyrosine phosphorylation of p130<sup>Cas</sup> measured 40 h after transfection, and this phosphorylation was largely abolished by short term treatment with herbimycin A or GF 109203X, indicating the involvement of a PKC-dependent step also under these conditions. Another kinase, p125<sup>FAK</sup>, that also interacts directly with p130<sup>Cas</sup> (7, 8) and can be regulated by PKC (44) is not likely to significantly contribute to the phosphorylation of p130<sup>Cas</sup> in SH-SY5Y, since no active, i.e. phosphorylated, p125<sup>FAK</sup> was detected in cells stimulated with TPA. The cytosolic tyrosine kinase Abl has been shown to phosphorylate p130<sup>Cas</sup> <i>in vitro</i>. The phosphorylation is enhanced by binding of Crk to Abl (3). An <i>in vivo</i> function for Abl in p130<sup>Cas</sup> phosphorylation remains to be investigated in SH-SY5Y and other cells.

The tyrosine phosphatase PTP-PEST was recently shown to dephosphorylate specifically p130<sup>Cas</sup> in several cell lines (41) and to bind directly to the SH3 domain of p130<sup>Cas</sup> (45). PTP-PEST can be phosphorylated on serine residues <i>in vitro</i> by both PKC and cyclic AMP-dependent kinase, and the same residues are phosphorylated in TPA-stimulated HeLa cells. This phosphorylation negatively regulates the activity of the phosphatase by reducing the substrate affinity (46). It is feasible that PKC-evoked p130<sup>Cas</sup> phosphorylation in SH-SY5Y cells could be mediated by inactivation of PTP-PEST. The finding that activation of cyclic AMP-dependent kinase with dbcAMP also promoted tyrosine phosphorylation of p130<sup>Cas</sup> in agreement with a PTP-PEST-regulated p130<sup>Cas</sup> phosphorylation, but the possible involvement of PTP-PEST needs further studies.

Little is known about the physiological function of p130<sup>Cas</sup>. Its localization to growth cones could imply that p130<sup>Cas</sup> takes part in the regulation of growth cone function. Both PKC-α and PKC-ε are enriched in intact growth cones (21), and PKC activity (presumably PKC-ε) is necessary for maintenance of the growth cone structure (24). We now suggest that p130<sup>Cas</sup> could be a downstream target of PKC in the functional growth cone based on the following: (i) tyrosine-phosphorylated p130<sup>Cas</sup> was present in growth cones of differentiated SH-SY5Y; (ii) p130<sup>Cas</sup> in the growth cone fraction as well as in whole unfractinated cells was rapidly dephosphorylated after addition of a PKC inhibitor, a treatment that induces retraction of growth cone filopodia; and (iii) the time course of the initial dephosphorylation of p130<sup>Cas</sup> measured in whole cell extracts correlated closely to filopodia retraction. Unlike treatment with GF 109203X, exposure to herbimycin A did not lead to rapid changes of growth cone morphology, but prolonged exposure over several days caused cell detachment from the culture dish. p130<sup>Cas</sup> phosphorylation decreased in these cells but not as rapid as after GF 109203X treatment. Thus, p130<sup>Cas</sup> phosphorylation in growth cones did not seem to correlate closely to growth cone structure but rather to PKC activity.

An explanation for the functionally different effects of GF 109203X and herbimycin A on growth cone structure would be if PKC has a more complex role regulating and integrating a number of vital activities in the growth cone. For example, GAP-43 is a well characterized PKC substrate located in growth cones of differentiated SH-SY5Y cells (17) and with an important role in neuronal sprouting and growth cone migration (47, 48). Although the results discussed above point to an important role of PKC in signaling through p130<sup>Cas</sup> in growth cones, neither p130<sup>Cas</sup> phosphorylation nor PKC activation are sufficient for neurite extension (see for instance PKC-ε transfections). Experiments with PC12 cells have previously demonstrated that introduction of v-src or mutated active <i>ras</i> leads to neurite outgrowth and differentiation (49, 50) and that the two oncogenes have a synergistic effect (for a review see Ref. 51). Introduction of active Ras (Val-12 Ras) in SH-SY5Y cells also leads to neurite formation, clearly demonstrating signaling events leading to neurite formation which cannot solely be explained by activation of PKC or p130<sup>Cas</sup>.

SH-SY5Y cells undergoing an active process of differentiation have to coordinate signals mediating neurite outgrowth and other cytoskeletal events as well as altered gene expression. Also, survival and maintenance of the differentiated cell probably demands other signaling activities or combinations of signals than unstimulated or acutely stimulated SH-SY5Y cells. It is feasible that p130<sup>Cas</sup> has different or partially non-overlapping functions and is differently regulated during early and late phases of differentiation. The rapid phosphorylation of p130<sup>Cas</sup> in cells induced to differentiate might be an early event

<sup>2</sup>A.-K. Olsson and E. Nånberg, manuscript in preparation.
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during the dynamic phase of cytoskeleton rearrangement that precedes the formation of growth cones and neurite sprouting. The acute effects of TPA, FCS, and growth factors added separately might also lead to the same actin reorganization. Since p130<sup>as</sup> is the target of both growth factor and integrin stimulation (see Introduction for references) and is a signal transduction docking protein, p130<sup>as</sup> may sense and integrate different signals in growth cones, thus being a member of the tightly regulated machinery needed for axon growth and path finding.

Acknowledgments—We thank Ira Johansson for excellent technical assistance. We also thank Dr. Sara Courtneidge for the src construct, Dr. Peter Parker for the PKC-e159 construct, and Dr. Joan Biedler for the SH-SY5Y cells.

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