Synergistic Induction of Neurite Outgrowth by Nerve Growth Factor or Epidermal Growth Factor and Interleukin-6 in PC12 Cells*

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Native PC12 cells respond differentially to nerve growth factor (NGF) but not interleukin-6 (IL-6); PC12-E2 cells, a stable variant, respond to both stimuli (and more rapidly to NGF). Neither responds to epidermal growth factor (EGF). NGF primarily induces the RAS/extracellular signal-regulated kinase (ERK) pathway and IL-6 activates a JAK (Janus tyrosine kinase)/STAT (signal transducers and activators of transcription) response. EGF also stimulates RAS/ERK but in a transient manner. When either cell type is treated with combinations of NGF, EGF, and IL-6, at concentrations that produce modest or no response, a substantial augmentation of neurite outgrowth is observed. With PC12-E2 cells, a subthreshold concentration of IL-6 increases NGF response by ~2-3-fold after 1-2 days; the increase with EGF is more pronounced. Native PC12 cells show even greater synergistic effects with NGF and IL-6. The most dramatic effect was observed with low levels of EGF, where IL-6 increased the percentage of responsive cells from zero to ~60% after 3 days. In addition, two neural-specific transcripts, GAP-43 and SCG-10, are synergistically increased by the combinations of growth factors. Importantly, IL-6 does not enhance ERK phosphorylation in the presence of either NGF or EGF. In contrast, NGF and EGF, in the presence or absence of IL-6, cause mobility shifts of Stat3 that are consistent with serine phosphorylations. Although these modifications do not lead to activation and translocation by themselves, in the presence of the tyrosine phosphorylation induced by IL-6, they may play a role in the synergistic responses. These observations suggest a differentially regulated two-stage mechanism for the differentiative response of PC12 cells to NGF.

The rat PC12 cell line has become a widely used paradigm for studying the regulation of neuronal differentiation (1, 2). It can be induced to adopt a reversible neuronal-like morphology by neurotrophic factors such as NGF and bFGF. These factors bind to their respective tyrosine kinase receptors, resulting in activation of the receptor tyrosine kinase and the initiation of downstream signaling events including the sustained activation of the RAS/ERK pathway (3, 4). The activation of this pathway is necessary, but apparently additional signaling events are also required for induction of differentiation in this cell system (5-8). In a recent report, it has been shown that sustained activation of an independent signaling system, i.e. a JAK/STAT pathway, by IL-6 also leads to neurite outgrowth of E2 cells, a PC12 variant (9, 10), as well as primed native PC12 cells (10). The JAK/STAT pathway is involved in various cytokine-receptor signaling mechanisms; it is composed of one or more members of the JAK family of tyrosine kinases (Jak1, Jak2, Jak3, and Tyk2) and one or more of a family of latent cytoplasmic transcription factors (Stat1 through Stat6) (11-14). JAKs, associated with a transmembrane receptor and/or a signal transducing component(s), are activated after ligand-receptor interaction, leading to the tyrosine phosphorylation and activation of the STATs. This pathway has been implicated in the induction of diverse cellular functions by various cytokines in different cell types (11-14).

In contrast to induction of differentiation by NGF and bFGF, primarily via activation of the ERK pathway, the STAT pathway is insufficient to cause a significant neurite outgrowth in native PC12 cells. However, significant neurite outgrowth is induced by IL-6 in NGF-primed PC12 cells (10). The basis of this difference is not clear, but likely relates to the synthesis of a key protein(s), probably induced by the ERK pathway but not by the STAT pathway, that is preexistent in PC12-E2 cells or accumulates in primed PC12 cells. These results suggest that the NGF- and IL-6-activated signaling pathways are separate but share some common components (or at least common functions).

In the present study, the synergistic effects of NGF and EGF with IL-6 in parental PC12 and PC12-E2 cells has been examined. Specifically, it has been shown that the activation of the ERK pathway by NGF or EGF can complement the JAK/STAT pathway activated by IL-6 to produce a significant differentiation in native PC12 cells. Synergistic effects are also observed in PC12-E2 cells. The results suggest that the two pathways may, under these conditions, selectively affect the two stages of activation required to induce neurites in these cells.

EXPERIMENTAL PROCEDURES

Materials—Reagents and materials were obtained as described previously (10).

Cell Culture—PC12 cells were obtained from Dr. D. Schubert (Salk Institute, San Diego, CA), and the variant E2 cells were isolated as described previously (9). Cells were maintained as described previously (10).

Neurite Outgrowth—The neurite outgrowth assay was performed as described previously (9). For the synergistic induction of neurite outgrowth, cells were grown in complete media for 6 h and changed to low serum media (Dulbecco’s modified Eagle’s medium containing 1% horse
serum and 1% penicillin-streptomycin) with the addition of growth factor(s) for 1–3 days.

Immunoprecipitation and Immunoblot Analyses—Preparation of cell lysates, immunoprecipitation of ERK, and immunoblot analyses were performed essentially as described previously (10).

Northern Blot Analysis—PC12 cells were treated with NGF or combinations of NGF and IL-6 for 2 days. The concentrations of IL-6 used are 0 (●), 0.1 (○), 0.3 (△), 1.0 (▲), 3.0 (■), and 10.0 (□) ng/ml, respectively. Vertical bars represent S.E. (n = 4–5).

RESULTS

Synergistic Stimulation of Neurite Outgrowth by IL-6 and NGF—The neurite regeneration effect of IL-6 in NGF-prime PC12 cells (10) suggests that there is a potential interaction between NGF- and IL-6-activated signaling pathways. To determine this possibility, the combined actions of these factors on PC12 and PC12-E2 cells were examined. As described previously (9), NGF or IL-6 alone stimulate a rapid and dose-dependent outgrowth of neurites in PC12-E2 cells. Interestingly, the effect of NGF (0.03–1 ng/ml) in the presence of a subthreshold concentration of IL-6 (0.1 ng/ml) is markedly enhanced as compared with the effect of NGF alone (Fig. 1A). For example, when cells were treated with NGF at 0.3 ng/ml for 1 day, the presence of IL-6 increased the responsive cells from 18% to 50%. This interactive effect between IL-6 and NGF on neurite outgrowth is even more demonstrable in native PC12 cells (Figs. 2A and 3A). Low concentrations of NGF (1 ng/ml) or IL-6 (0.1–10 ng/ml) alone produce no morphological changes within 2 days in native PC12 cells. However, combination of both factors potently promotes the neurite outgrowth in a dose-dependent manner (ranges from 2 to 68%). In addition, when cells were treated with NGF at a concentration as low as 3 ng/ml, instead of taking 6 or more days to reach a fully differentiative state, they are fully differentiated within 2 days in the presence of IL-6 (10 ng/ml) (Figs. 2A and 3A).

Synergistic Stimulation of Neurite Outgrowth by IL-6 and NGF—EGF is mildly mitogenic for both PC12 and PC12-E2 cells, and PC12-E2 cells grown with EGF alone display short spikes. Native cells do not produce morphological changes with this stimulus. However, 60% of PC12-E2 cells bear neurites extending longer than 2 cell bodies when treated with a combination of EGF (3 ng/ml) and subthreshold concentration of IL-6 (0.1 ng/ml) for 2 days (Fig. 1B) and more than 50% of native cells are responsive when treated with a similar combination (0.3 ng/ml EGF and 10 ng/ml IL-6) for 3 days (Figs. 2B and 3B). It is interesting to note that, unlike stimulation with NGF or a combination of IL-6 and NGF, combinations of subthreshold concentrations of IL-6 and EGF were only able to produce a maximal neurite outgrowth of about 60% in both cells. Furthermore, increasing the concentration of EGF resulted in a decreased number of responsive cells at all concentrations of IL-6 (with both cell types) (Figs. 1B and 2B).

Expression of GAP-43 and SCG10—GAP-43 (growth-associated protein) and SCG10 are neural-specific proteins expressed mainly during the neuronal development and regeneration, which are localized in growth cones and synapses (18, 19). In primary neuronal cultures and in NGF-treated PC12 cells, GAP-43 expression correlates with the induction of differentiation as judged by the extension of neurites and increased localization of the protein in the growth cones.

To determine if the expression of these two delayed response genes are also synergically increased in PC12 cells treated with combination of factors, Northern analyses were performed. As shown in Fig. 4, GAP-43 mRNA is not appreciably...
made in unstimulated cells and IL-6 treatment (10 ng/ml) has little effect. Both NGF and EGF induce message synthesis, and both are significantly enhanced at all concentrations by IL-6. In contrast, SCG10, which is expressed as two transcripts of 1.1 and 2.2 kilobases, occurs at a considerably higher level in unstimulated cells. Neither IL-6 or EGF alone significantly enhances this expression (after normalization to the Chob standard). However, combinations of IL-6 and either EGF or NGF significantly increase the expression of both transcripts. As with GAP-43, the effect is more pronounced with NGF than EGF.

Mechanistic Responses: Tyrosine Phosphorylation of ERK—Both NGF and EGF stimulate the tyrosine phosphorylation of the ERKs in PC12 cells, although the latter is transient and does not lead to neurite proliferation (20, 21). Similar responses are observed in PC12-E2 cells (9); IL-6, in contrast, produces only a very small phosphorylation of ERKs in either cell type (10). As shown in Fig. 5, neither cell type shows a significant increase in ERK phosphorylation when treated with IL-6 plus NGF or EGF over that observed with NGF or EGF alone. Thus, the enhancement effects observed morphologically are not caused by amplification of the ERK pathway.

Mechanistic Responses: Phosphorylation of Stat3—IL-6 produces both tyrosine and serine phosphorylation of Stat3, a cytoplasmic transcription factor, that is translocated to the nucleus following tyrosine phosphorylation. As shown previously (10), this occurs in both PC12 and PC12-E2 cells, although only the latter produce neurites. NGF does not cause tyrosine phosphorylation or the consequent SIE binding necessary for transcriptional regulation (Ref. 10; see also below). EGF does give a weak transient tyrosine phosphorylation of Stat3 but does not produce any detectable increase in the SIFA, -B, or -C binding complexes.

Serine phosphorylation of Stat3 retards its mobility on SDS-PAGE, resulting in a slower migrating band (Stat3s) and a faster one (Stat3f) on the Stat3 immunoblot (Fig. 6). IL-6 causes this modification, which peaks at 15 min and gradually returns to basal level at 1 h (data not shown). Interestingly, NGF and EGF also possibly cause serine phosphorylation (as judged by the same mobility changes) of Stat3 with the effect peaking at 5 and 2 min, respectively (Fig. 6). The combined effect of IL-6 and either NGF or EGF leads to a greater enhancement of Stat3 serine phosphorylation than IL-6 but does not result in significantly increased levels over the NGF or EGF alone. No detectable mobility shift of Stat1 was detected under the same conditions when the blot was reprobed with both anti-Stat1 and anti-Stat3 antibodies (Fig. 6).

Stimulation of SIE Binding Activity—To determine if modification of Stat3 by NGF or EGF affects the transcriptional binding activity of Stat3 stimulated by IL-6, EMSA analyses of nuclear extracts, treated with either NGF or EGF alone for 15 min or 24 h, were performed. There was no increase in SIE binding activity (Fig. 7). As reported previously (10), IL-6 treatment predominantly activates Stat3 and causes formation of SIFA (homodimers of Stat3) and SIFB (heterodimers of Stat1 and Stat3). The induction of SIFC complex (homodimers of Stat1) is weak and transient. Clearly, the presence of NGF or EGF does not significantly affect the amount or the kinetics of formation of SIFA and SIFB, but a slight increase in the amount of SIFC complex was noticed when cells were treated with a combination of either NGF and IL-6 or EGF and IL-6 (Fig. 7).

DISCUSSION

The RAS/ERK and JAK/STAT pathways represent two of the major intracellular signaling mechanisms activated by...
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Fig. 5. Protein tyrosine phosphorylation of ERK1 and 2 by combinations of IL-6 and NGF or IL-6 and EGF in PC12 and PC12-E2 cells. A, immunoprecipitates of ERK1 and ERK2 from PC12 cells stimulated with no factor (lane C), 10 ng/ml IL-6 (15 min), 10 ng/ml NGF, 5 ng/ml EGF, or combination of factors for the indicated time were separated by 12.5% SDS-PAGE, immunoblotted with anti-phosphotyrosine antibody (4G10), and detected by ECL. The phosphotyrosine blot was stripped and reprobed with affinity-purified anti-ERK polyclonal antibody (4G10), and detected by ECL. The phosphotyrosine blot was stripped and reprobed with affinity-purified anti-ERK polyclonal antibody as shown on the lower panel. B, 1 immunoprecipitates of ERK1 and ERK2 from PC12-E2 cells, stimulated with no factor (lane C), 10 ng/ml IL-6 (15 min), 1 ng/ml NGF, 5 ng/ml EGF, or combination of factors for the indicated time, were separated by 12.5% SDS-PAGE, immunoblotted with anti-phosphotyrosine antibody, and detected by ECL. The phosphotyrosine blot was stripped and reprobed with affinity-purified anti-ERK polyclonal antibody as shown on the lower panel. The bands above ERK1 on the phosphotyrosine blot are the nonspecific bands.

Fig. 6. NGF and EGF-induced mobility shifts of Stat3. PC12 cells were treated with IL-6 (10 ng/ml), NGF (100 ng/ml), EGF (5 ng/ml), a combination of IL-6 and NGF, or a combination of IL-6 and EGF for 0–15 min as indicated. 20 μg of total cell extract was separated by a 7.0% SDS-PAGE, and immunoblotted with anti-Stat3 (upper sections of panels A and B), or anti-Stat1 and anti-Stat3 together (lower sections of panels A and B). The slower and faster migrating forms of Stat3 are designated Stat3s and Stat3f, respectively.

Polypeptide growth factors and cytokines. Although there are some indications that individual factors can activate both types (14, 20), they are presently viewed as independent means for activating gene sets that lead to response-specific phenotypes.

In PC12 cells, evidence has accumulated that growth factors causing substantial and prolonged activation of the RAS/ERK pathway produce the neuronal differentiation usually judged by significant neurite proliferation (21, 22). However, these factors, exemplified by NGF, also activate other pathways as well, and recent evidence using mutant receptors suggests that the RAS/ERK pathway may be essential but not sufficient. PC12 cells overexpressing a mutated PDGF receptor required the persistent stimulation of PLCγ or Src in addition to the sustained activation of ERK for differentiation (8); mutated TrkA receptors that were unable to induce the phosphorylation of the SNT protein, but were still able to stimulate Shc, PLCγ and induce ERK tyrosine phosphorylation, did not allow NGF-induced neurite outgrowth, when stably transduced into a mutant PC12 line not expressing native NGF (TrkA) receptors (6). Importantly, NGF does not activate any of the known STAT factors in PC12 cells, so that it may be inferred that these factors are not involved in the NGF response.

It is also well appreciated that even factors that can produce the same essential end point in PC12 cells do not give exactly the same response (23). Thus, NGF and bFGF stimulation give overlapping but unique protein expression profiles that presumably reflect different receptor specificities leading to different IEG profiles (24). Similarly, compounds such as CAMP derivatives can induce neurites, albeit the morphological characteristics they produce distinguish them from those produced by NGF and bFGF (25). These observations suggest that substantially different pathways can still produce an apparently related response.

Cytokines, a broad group of substances that are important for but not limited to the regulation of the immune system, generally do not have tyrosine kinase effectors as an intrinsic part of the receptor, but instead recruit cytoplasmic kinases that associate with the ligand binding entities located in the membrane (26–28). These receptor complexes are usually composed of two or three polypeptide species that associate noncovalently. The cytoplasmic kinases bind to dimers of the linking proteins, e.g. gp130 and LIFR, and tyrosine phosphorylate a number of protein substrates. Chief among these are the STAT proteins, which function, upon activation and translocation, as transcription factors in the nucleus. They also undergo serine phosphorylation (29–31).

Recently, a stable line of PC12 cells was subcloned from a native population (9). This line, designated E2, is characterized by rapid responses to NGF and fibroblast growth factor and, most importantly, slower, but still robust responses to IL-6 (10). Although the limitations of the PC12 cell paradigm as a model for studying neuronal differentiation are well known, the demonstration that IL-6 can induce responses in these cells under specific conditions is consistent with other neuronal actions of this cytokine. Both IL-6 and its receptors are expressed and localized in discrete areas of the central nervous system in response to specific stimuli, suggesting a potential regulatory function for IL-6 in the nervous system (32, 33). Marked increases in expression of several cytokines including IL-6 have been observed in rats within hours of brain damage. Low concentration of IL-6 is expressed in the axotomized and

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embryonic sensory neurons (34). Moreover, several cytokines induce an increased production and secretion of biologically active NGF by astrocytes. Thus, IL-6 released after nerve injury or other insults may be able to interact with local neurotrophic factors and act directly on the nervous system (35, 36). It may sustain the state of neuronal differentiation or initiation of neuronal regeneration, as suggested by the effect of IL-6 on the PC12 cell system. It has been reported that simultaneous treatment with a combination of neurotrophic factors and cytokines are necessary to rescue degenerated motor neurons (37).

In both native and E2 cells, IL-6 induces Stat3 activation and nuclear translocation. Thus, in PC12-E2 cells, the neurite induction by IL-6 is probably the result of STAT protein activation (as opposed to RAS/ERK). Although the phenotypic characterization of PC12-E2 cells, relative to native PC12 cells, is incomplete, these and other observations suggest a testable model for growth factor-induced differentiation of PC12 cells (Fig. 8). In this model, the key element is a mitotic suppressor protein (MSP), not found in native PC12 cells, that is induced by NGF and fibroblast growth factor, but not by IL-6 (or related cytokines). It is also potentially induced by EGF but only leads to neurite proliferation under conditions of enhanced expression of EGF receptor when other activations also occur (7, 38). Judging by the normal time course of response of PC12 cells to NGF, this protein (for simplicity, denoted as a single entity, but it could be manifested in more than one gene product, working in concert) is probably a secondary gene product, i.e. is expressed following the translation of IEG, which in turn induces the transcription of the MSP gene(s). As diagrammed, it is proposed that the MSP is induced directly by activated ERK. Although this is the likely explanation for the dependence of NGF and other differentiative factors on this pathway, it cannot be formally ruled out that it is induced by other pathways known to be activated by NGF.

Although the nature of the MSP(s) involved in the NGF response is presently unclear, there are some possible candidates (39–41). Yan and Ziff (39) and Decker (40) have shown that NGF stimulation of PC12 and NIH-3T3 cells, the latter expressing stable transfected TrkA, respectively, leads to the production of p21\(^{Cip1/WAF1}\), a protein that inhibits CDK/cyclin activity. Interestingly, both also showed an increase in cyclin D1 levels, as did Dobashi et al. (41).

A second key aspect of this model is the "enabling" of the MSP function leading to cell arrest and/or the "activation" of the differentiative response. In native PC12 cells stimulated by NGF (or bFGF), this is most likely a transcription-independent event as judged by primed or PC12-E2 cell responses. Thus, it does not involve the production of IEG or secondary gene products, but rather the modulation of existing proteins, and it may be related to the RAS/ERK pathway or may be independent of it. Importantly, it cannot be distinguished at this junction whether this pathway is also related to the inhibition of the cycle cell ("enabling") or is involved in initiating the differentiative process ("activation"). The dephosphorylation of pRB is a possible candidate of the former type of response (39). It is also important to note that this function can apparently be achieved in different ways; IL-6 seems to induce it through STAT proteins, and this is transcriptionally dependent. Therefore, it is unlikely to be the same pathway induced by NGF.
This model provides a plausible explanation for the responses of PC12 and PC12-E2 cells, including temporal considerations, to both classes of factors. Principally, the model predicts that PC12-E2 constitutively express MSP, and that primed native cells that were induced to synthesize the protein(s) also retain sufficient amounts to bypass the necessity to synthesize more, at least initially. It is possible that the MSP activity generated by NGF differs from that constitutively expressed by PC12-E2 cells. The fact that both types of cells still must be stimulated in order to begin differentiation indicates the existence of the second pathway; however, it also explains the immediate response (1–2 h) of PC12-E2 cells to stimulation by NGF.

The response of PC12-E2 cells to IL-6 suggests that the STAT pathway activator can produce all of the essential products necessary for differentiation except MSP. Interestingly, these cells do not respond immediately to IL-6, first requiring the production of a new protein(s) (indicates as X in Fig. 8). The onset of response (6–8 h) is consistent with the view that X is an IEG product(s). A prediction of this model is that primed PC12 cells should respond to IL-6 (with the same time of onset) as PC12-E2 cells, which is in fact the case (10). A second prediction is that primed PC12 cells should eventually lose their responsiveness to IL-6 (since they cannot sustain the levels of MSP), and this has also been demonstrated (10).

The synergistic responses described in this report further amplify the nature of the NGF response in terms of the "two-stage" model described in Fig. 8. Clearly, subthreshold doses of NGF can induce levels of MSP that can act in concert with IL-6 (via the STAT pathway through the production of X) and then lead to the production of neurites but are unable to stimulate sufficiently the transcription-independent activation/cell effects. At low concentrations of EGF (0.3 ng/ml), where no activity is observed, native PC12 cells can be made to extensively (~60% responsive cells) react by the addition of 10 ng/ml or more of IL-6. This suggests that at modest levels of EGF alone, MSP is induced significantly but, unlike NGF, that the mechanism leading to cell arrest is totally inadequate (or absent). At higher levels of EGF, the down-regulation (leading to the loss of signal) becomes predominant and the ability to synergistically interact with IL-6 is reduced.

The putative phosphorylation of Stat3 by NGF and by EGF may also play a role in the synergistic responses of NGF/EGF and IL-6. As noted in Fig. 8, the relatively low levels of ERK activation induced by subthreshold amounts of NGF may be augmented by a broader range of gene responses by STAT factors that have been modified by both serine and tyrosine phosphorylation. However, the former is unlikely to be of consequence in the absence of the latter.

The model described is clearly consistent with a variety of specific known responses of PC12 cells (and various mutants) to different stimuli and is generally consistent with concepts of cell cycle regulation and differentiation. It further emphasizes the importance of quantitative as well as qualitative descriptions in evaluating the contribution of various pathways to signaling responses. The extent to which this model (or components of it) can be extrapolated to NGF-responsive neurons in vivo remains to be demonstrated. These cells are clearly convenient and relatively easily manipulated but do not precisely mimic any real neuronal system. Nonetheless, they have already provided considerable insight into growth factor-induced signaling mechanisms and resulting phenotypic responses that have found applicability in other systems, and it may be assumed that the model described, implied by these (and other results), will be of similar value in in vivo systems.

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FIG. 8. A schematic representation of a “two-stage” mechanism of PC12 cell response (and differentiation) to selected polypeptide growth factors. The major pathways (RAS/ERK and JAK/STAT), shown in highly abbreviated form, lead to the production of MSP and X, respectively. MSP represents the first stage (inducible by NGF and probably by EGF and present in primed and PC12-E2 cells); a second pathway (--- --- ---), transcription independent, is required to “enable” MSP and/or “activate” the cell cycle arrested cells to become differentiated. X can perform the same functions. Possible cross-influences by RAS/ERK pathway components with JAK/STAT components are indicated by broken arrows (---). TF, transcription factor; PM, plasma membrane; NM, nuclear membrane; P, phosphate; X, IL-6-induced factor capable of inducing PC12 differentiation in the presence of MSP.
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