Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9377–9381, September 1994
Biochemistry

Early changes in protein synthesis induced by basic fibroblast growth factor, nerve growth factor, and epidermal growth factor in PC12 pheochromocytoma cells

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Communicated by Robert L. Hill, June 13, 1994 (received for review May 9, 1994)

ABSTRACT **Nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) stimulate neuronal differentiation, whereas epidermal growth factor (EGF) promotes only mitogenic responses in PC12 pheochromocytoma cells. The early changes in protein synthesis induced by bFGF, NGF, and EGF in these cells have been determined by two-dimensional PAGE of [^{35}S]methionine-labeled proteins and computerized image analysis. The rate of synthesis of only 29 proteins (out of ≈1500 identified) was found to be modulated during the first several hours of growth factor stimulation. Individually, 12 were affected by EGF, 23 were affected by bFGF, and 20 were affected by NGF. Eight of these were regulated by all three growth factors, while 10 proteins were commonly induced by bFGF and NGF, in accordance with the essentially identical morphological responses induced by these two factors. In addition, the effects of bFGF and NGF were about equally divided between increases and decreases in the rate of synthesis of individual proteins, whereas EGF caused significantly more positive (increased) responses. All proteins modulated by NGF or FGF alone were negative in their response and those induced by only EGF were positive. Of particular interest, the rate of synthesis of two proteins of 55 kDa and pI 5.45 and 5.50 was dramatically and transiently induced during the first 2 hr of bFGF and NGF treatment and was not affected by EGF. This study indicates that all three factors elicit early increases and decreases in the synthesis of a quite limited number of proteins and provides molecular evidence for the specificity of a differentiative vs. a proliferative growth factor-induced signaling pathway in these cells.**

Polypeptide growth factors can promote proliferation or trophic stimulation of responsive cells, by mechanisms that are still incompletely described. Various transduction signals have been identified, but the same events are known to be implicated in mitogenic and differentiative growth factor signaling pathways, thus obscuring the specificity leading to the two responses (1, 2).

The rat pheochromocytoma cell line PC12 (3) is induced by nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) to reversibly differentiate into cells that morphologically and biochemically resemble sympathetic neurons (4–6). This neuronal differentiation of PC12 cells is accompanied by a number of rapid biochemical events similar to those induced by mitogenic agents in other systems, including changes in phospholipid hydrolysis, protein phosphorylation, ion fluxes across the plasma membrane, and early gene expression (for reviews, see refs. 1 and 7–9). In contrast to bFGF and NGF, epidermal growth factor (EGF) does not induce differentiation but rather acts as a mitogen for PC12 cells (10). However, most of the transduction signals reported for neurotrophic stimulation have also been described for EGF (11–21). Thus the molecular specificity of a differentiative vs. a mitogenic signaling pathway in PC12 cells has not yet been elucidated.

It is of considerable interest that the induction of neurite outgrowth in PC12 cells by NGF is blocked by inhibitors of transcription (22). This suggests that ongoing mRNA and protein synthesis are required for neuronal differentiation. Previous studies have demonstrated a modulation of protein synthesis after NGF stimulation. Garrels and Schubert (23) showed that 72 hr after its addition to the culture medium, NGF caused only quantitative changes in protein synthesis in PC12 cells. Approximately 25–30% of the proteins analyzed by two-dimensional (2D) PAGE was found to be altered in their rate of synthesis. Since during this period, PC12 cells have already grown neurites, these changes are clearly at least partly related to the neuronal phenotype (cytoskeletal and neurite stabilization). Tiercy and Shooter (24) have studied the effect of NGF on protein synthesis during the first hours of stimulation. These experiments, mostly done using one-dimensional PAGE, indicate that during this time, NGF increased the incorporation of [^{35}S]methionine into four bands with apparent molecular masses of 56, 50, 51.5, and 48 kDa. They also analyzed the proteins synthesized from 7 to 8 hr by 2D PAGE and determined the apparent pI values and molecular masses of several proteins that are modulated during this time period. However, modulation of protein synthesis after bFGF and EGF stimulation and a comparison to NGF have not been examined, particularly with respect to proteins affected by two or more growth factors.

To further understand the signaling pathways leading to PC12 cell proliferation or differentiation, the effect of bFGF, NGF, and EGF on the early synthesis of >1500 individual proteins using 2D PAGE and computerized image analysis was examined. Our results show an early modulation of protein synthesis induced by the three growth factors and provide evidence for specificity of the differentiative vs. proliferative pathways induced by growth factors in these cells.

EXPERIMENTAL PROCEDURES

Materials. PC12 cells were obtained from D. Schubert (Salk Institute, San Diego). NGF-β (referred hereafter as NGF) was prepared by the method of Mobley *et al.* (25). EGF was prepared as described (26). bFGF was kindly provided

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Abbreviations: NGF, nerve growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; 2D, two dimensional.

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by Gary Fox (Amgen, Thousand Oaks, CA). [^{35}S]Methionine was from ICN.

PC12 Cell Culture. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories) supplemented with 5% (vol/vol) fetal calf serum (Irvine Scientific), 10% (vol/vol) horse serum (Cell Culture Laboratories, Cleveland), penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml). Stock cells were grown in a Belco tissue culture flask (T75 or T150). Cells were fed every 2 days, passaged weekly, and maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Cell Stimulation by Growth Factors and Labeling. PC12 cells were resuspended at 10^5 cells per ml in complete medium and plated on 35-mm dishes, precoated with rat tail collagen (27). When 50% confluence was reached, medium was replaced by DMEM containing 1% horse serum. After 48 hr in this medium, cells were stimulated by growth factors. The following concentrations were used: NGF, 100 ng/ml; bFGF, 5 ng/ml; EGF, 10 ng/ml. Cells were labeled for 2 hr with [^{35}S]methionine at 250 μCi/ml. Labeling was performed immediately after growth factor addition or after 4 hr of stimulation.

Sample Preparation for Electrophoresis. Samples were prepared as described (28) with some modifications. At the end of the labeling time, medium was removed, and cells were rinsed in ice-cold medium and scraped off with a rubber policeman. After low-speed centrifugation, cells were lysed with 0.3% SDS/1% 2-mercaptoethanol/4% (vol/vol) LKB Ampholines, pH 5–7, 5–8, 3–10. Within 30 sec, RNase (0.1 mg/ml) and DNase I (0.2 mg/ml) (Worthington) were added and mixed. After 20 sec, the sample was quickly frozen. Upon thawing, the samples were diluted 1:2 with 9.5% (wt/vol) urea/0.8% Nonidet P-40/10% (vol/vol) 2-mercaptoethanol/2% LKB Ampholines, pH 5–7, 5–8, 3–10, and mixed for 20 sec. All samples were quickly frozen on dry ice and stored at −70°C.

The 2D PAGE. Electrophoresis was performed in the Cold Spring Harbor Laboratory 2D Gel Laboratory Core Facility as described (28, 29). Briefly, for the first dimension, the polyacrylamide isoelectric focusing gel contained 9.5 M urea, 3% (wt/vol) acrylamide, 0.22% *N,N'*-methylene-bisacrylamide, 4% Nonidet P-40, and 2% ampholytes (pH 4–8; BDH). For the second dimension, 10% polyacrylamide gels were used. Typically, 10 μl of sample containing 200,000–300,000 trichloroacetic acid-precipitable cpm was applied to each gel. After electrophoresis, gels were processed for fluorography and exposed to Kodak XAR (10 × 12 inch size; 1 inch = 2.54 cm) and three film exposure times (typically, 2, 12, and 50 days) were used.

Computerized Image Analysis. The PDQuest system of 2D gel image analysis was used to quantify the gel autoradiographic images (Protein Databases, Huntington Station, NY). Spot intensity, expressed as parts per million (ppm), represents the amount of radioactivity contained within each spot relative to the total amount of radioactivity applied to each gel. The apparent isoelectric point and molecular mass of each protein were calculated based on spot position relative to proteins from the REF52 (rat) data base (30).

RESULTS

The synthesis of individual proteins induced by growth factors in PC12 cells was analyzed by 2D PAGE, after incorporation of [^{35}S]methionine for 2 hr. Incorporation was initiated immediately and 4 hr after addition of bFGF, NGF, and EGF to the culture medium. For each growth factor and time period studied, three sets of gels were obtained from three experiments and each gel was exposed to x-ray film for three different times.

Fig. 1 shows the 2D gel pattern obtained from control PC12 cells (i.e., cells unstimulated by growth factor). About 1500 spots are sufficiently intense and well defined to be compared on the different films. The variation in these spots, induced by growth factor treatment, has been measured using the PDQuest system. The reproducibility of this 2D gel analysis has already been described (31) and the average coefficient of variation for well-defined spots in 10 replicate gels has been found to be 21%. This level of reproducibility is exactly matched in these experiments, as demonstrated by the calculation of the coefficient of variation for 200 well-defined spots (which were found not to be regulated by any growth factor used in this study) in all of the gels analyzed. The results of this statistical analysis (Fig. 2) show that the coefficients of variation for the nonregulated proteins are between 12 and 39%, with an average value of 21.4%. A 2-fold variation in spot intensity corresponds to the 95% confidence level that the spot is growth factor regulated. A variation of 3-fold corresponds to the 99% confidence level (32). As an additional criteria, the variation in spot intensity had to be consistent through the three experiments.

On the basis of these data, any change in spot intensity >2-fold was considered a growth-factor-induced variation. Proteins numbered 1–29 in Fig. 1 were found to be reproducibly modulated after growth factor stimulation. The apparent isoelectric point, relative molecular mass, and quantification of these spots are shown in Table 1. A summary of the individual protein changes observed with each growth factor is displayed in Table 2. Table 3 summarizes the relative numbers of proteins affected by each growth factor. Most of the modulations are quantitative rather than qualitative in

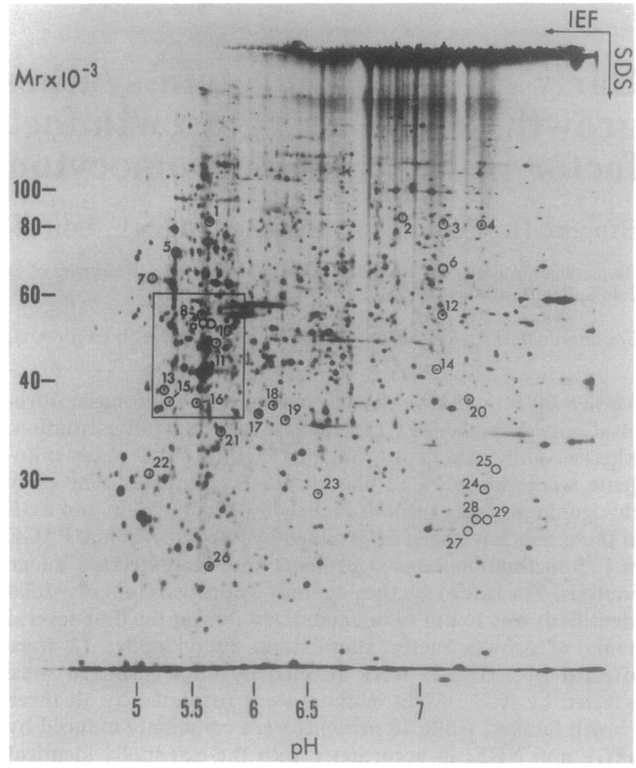

FIG. 1. Profile of [^{35}S]methionine-labeled proteins of unstimulated (control) PC12 cells in 2D PAGE gels. PC12 cells were ^{35}S-labeled and lysed, and the proteins were analyzed by 2D PAGE. Molecular masses and pI values were determined from the positions of proteins observed in the REF52 data base (13). Proteins numbered 1–29 were found to be regulated in their rate of synthesis after stimulation by growth factors. Characteristics and quantification of the regulation observed for the numbered proteins after growth factor treatment are reported in Table 1. The outlined area is enlarged in Fig. 3. IEF, isoelectric focusing.

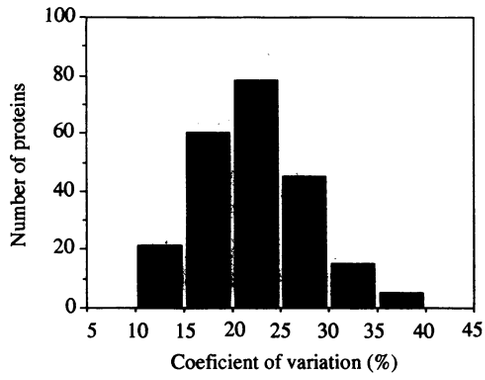

FIG. 2. Coefficient of variation for spots unregulated by growth factor. The coefficient of variation of 226 well-defined spots was calculated from the analysis of all categories of gels used (control, EGF for 2 hr and 6 hr, bFGF for 2 hr and 6 hr, and NGF for 2 hr and 6 hr).

nature—i.e., most of the corresponding proteins are also found in the unstimulated control cells, in keeping with the observations of Garrels and Schubert (23) at longer time points.

Fig. 3 compares the boxed area of the gel presented in Fig. 1 for all three growth factor conditions (2 hr and 6 hr) as well as the control and is a visual representation of the quantitative data presented in Table 1, which is derived from a computer image that merges three or more calibrated x-ray film images into one image. This procedure allows one to maintain a quantitative relationship between spots that vary by four or five orders of magnitude in intensity. The montage (Fig. 3) was composed from the darkest x-ray film exposure, which was then photographed, printed, and assembled. These multiple photographic processes lose the grey scale information that was present in the original film and tend to present the spots as saturated black spots. Nevertheless, it is clear that spot 9 is absent in the control and EGF-treated cells and is strongly induced at the 2-hr points by both bFGF and NGF. Spot 10 is just at the limit of detection in the control and in EGF-treated cells and is strongly induced at the 2-hr points by both bFGF and NGF. The induction of spots 9 and 10 is transient and by 4–6 hr spot 9 has returned to control levels and spot 10 is greatly reduced in intensity.

DISCUSSION

As reported for NGF (10, 24), bFGF and EGF do not significantly change the overall rate of protein synthesis in PC12 cells and one-dimensional PAGE did not allow the reproducible detection of changes in the rate of synthesis of individual proteins for any of the factors (data not shown). However, the use of high-resolution 2D PAGE led to the identification of 29 proteins that are variously regulated during the first hours of growth factor action. Overall, these variations are approximately equally distributed between stimulation and inhibition (14 vs. 15 spots) of synthesis and this pattern is maintained for bFGF and NGF. In contrast, 9 of the 12 proteins regulated by EGF showed an increase in their rate of synthesis. Furthermore, all of the proteins affected by EGF alone were positive in their response while

Table 1. Quantification of the modulation of individual protein synthesis rates

Protein number	pI	Molecular mass, kDa	Protein synthesis rate, ppm						
				EGF		bFGF		NGF	
			Control	0–2 hr	4–6 hr	0–2 hr	4–6 hr	0–2 hr	4–6 hr
1	5.45	85	746	856	624	220	296	847	808
2	6.90	86	336	358	766	372	316	420	310
3	7.10	86	471	416	382	377	75	232	210
4	7.25	86	244	268	210	27	28	238	198
5	5.20	71	3264	3900	1192	1922	638	1447	2991
6	7.10	69	55	64	86	277	86	226	74
7	5.05	63	104	130	216	387	251	241	282
8	5.45	57	1477	1271	1379	206	415	602	420
9	5.45	55	ND	ND	ND	335	ND	950	ND
10	5.50	55	69	90	60	3950	225	5444	108
11	5.55	51	382	474	384	367	1368	1126	1602
12	7.10	59	328	484	396	377	270	444	ND
13	5.15	40	613	588	428	392	171	523	145
14	7.05	43	342	546	384	812	383	1469	315
15	5.20	39	336	310	980	637	915	723	976
16	5.35	39	484	478	230	212	136	532	152
17	5.90	38	1171	1030	1018	715	328	885	995
18	6.00	39	520	488	472	697	95	344	489
19	6.15	37	586	684	490	647	188	476	487
20	7.20	40	448	504	1062	490	325	382	489
21	5.60	35	775	714	354	562	78	600	330
22	5.10	31	434	434	944	797	1250	720	861
23	6.20	27	459	436	354	460	388	405	128
24	7.30	34	407	450	380	ND	ND	212	196
25	7.45	34	232	230	756	300	386	253	369
26	5.65	22	782	680	616	740	296	597	378
27	7.25	25	182	164	544	287	433	264	426
28	7.30	26	186	260	738	255	358	458	228
29	7.35	26	175	185	758	262	221	147	132

[^{35}S]Methionine-labeled proteins obtained from PC12 cells were separated by 2D PAGE and the fluorograms were analyzed using the PDQuest system. The values have been obtained from control cells and cells stimulated by EGF for 0–2 hr and 4–6 hr, bFGF for 0–2 hr and 4–6 hr, and NGF for 0–2 hr and 4–6 hr. The molecular mass and pI were determined by the positions of proteins observed in the REF52 data base (13). Underlined values represent those that differ significantly ($P < 0.05$) from the control value. ND, not detected.

Table 2. Distribution of proteins affected by growth factors in PC12 cells

Growth factor(s)	Map position	Effect
EGF	2	+
	20	+
	25	+
	29	+
bFGF	1	−
	4	−
	17	−
	18	−
	19	−
NGF	12	−
	13	−
bFGF/NGF	6	+
	9	+
	10	+
	11	+
	14	+
	3	−
	8	−
	13	−
	24	−
	26	−
EGF/bFGF/NGF	7	+
	15	+
	22	+
	28	+
	5	−
	16	−
	21	−

Map positions refer to the map positions given in Fig. 1. + or − indicates direction of effect relative to zero time control (proteins affected by more than one factor always showed the same direction of response).

the opposite is true for the proteins affected by NGF or bFGF alone (Table 2). This striking difference may indicate that EGF, a mitogenic factor, stimulates the synthesis of proteins that are concerned with cell growth and division and are already activated in the transformed PC12 cells. In contrast, neurotrophic factors may induce neuronal differentiation, in part, by turning down (or off) the rate of synthesis of crucial proteins implicated in the mitogenic process. Such a model is in accordance with the observed down-regulation of the EGF receptor by NGF (10, 33). We have also shown that none of the proteins for which synthesis is activated by EGF is inhibited by NGF and bFGF. This could be related to the transformed character of PC12 cells in which crucial proteins implicated in mitogenesis are presumably activated prior to stimulation by growth factors.

Several proteins were found to be regulated by all of the factors. These proteins had apparent molecular masses of 71 kDa (pI 5.2; spot 5), 63 kDa (pI 5.05; spot 7), 39 kDa (pI 5.2; spot 15), 39 kDa (pI 5.35; spot 16), 35 kDa (pI 5.6; spot 21), 31 kDa (pI 5.1; spot 22), 25 kDa (pI 7.25; spot 27), and 26 kDa (pI 7.30; spot 28) (see Table 1). Induction of early gene transcription has been reported after PC12 stimulation by

Table 3. Summary of proteins affected by growth factors during 2–6 hr of incubation

Growth factor	No. affected/total no.	Direction of effect, no. +	−
EGF	12/29	9	3
bFGF	23/29	10	13
NGF	20/29	10	10

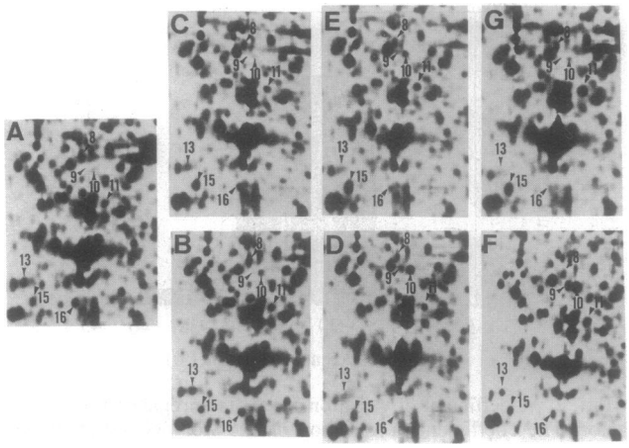

FIG. 3. Enlargement of the outlined area in Fig. 1 obtained from unstimulated cells (*A*), EGF-stimulated cells at 2 hr (*B*) and 6 hr (*C*), bFGF-stimulated cells at 2 hr (*D*) and 6 hr (*E*), and NGF-stimulated cells at 2 hr (*F*) and 6 hr (*G*).

growth factors, as exemplified by increases of myc, fos, jun, src, and other TIS gene transcripts during the first 2 hr of both NGF and EGF stimulation (11, 13, 15, 34).

Common effects in protein synthesis induced by EGF and aFGF have been described in cultured rat astroblasts (35). These authors showed that acidic FGF and EGF modulate the synthesis of 48 and 44 proteins, respectively, and that 41 of these proteins are commonly regulated by both growth factors. In rat astroblasts, EGF and acidic FGF have a similar effect—i.e., both stimulate cell proliferation. In the experiments reported here, only 5 proteins were found to be commonly regulated by EGF and FGF, which may reflect the difference between the mitogenic effect of EGF and the neuronal differentiation induced by bFGF in these cells. Interestingly, despite the fact that increases in the transcription of the β-actin gene have been reported during the first 2 hr of EGF and NGF stimulation (13), no difference in the rate of β-actin synthesis even after 6 hr of stimulation by these growth factors was observed. However, an increase in mRNA transcription does not necessarily indicate an increase in protein translation and a concomitant change in the turnover rate of β-actin could result in an apparent stability in its rate of synthesis.

The pattern of protein synthesis induced by the two neurotrophic growth factors, bFGF and NGF, appears to be very similar. Only a few proteins, five for bFGF and two for NGF, are differently modulated by each growth factor (Tables 1 and 2). These differences could reflect the slight morphological differences in neuritogenesis induced by bFGF and NGF. Neurite outgrowth, although initially appearing similar, has a different time course for each; after NGF stimulation, neurites continue to elongate and arborize, but with bFGF, process formation tends to stop after 2–3 days (6, 19). Of the 23 and 20 proteins found regulated by bFGF and NGF, respectively, 18 proteins are commonly regulated by both factors. After subtraction of the spots also regulated by EGF, 10 proteins remain that are specifically regulated by the two neurotrophic factors that may be specific for neurodifferentiative signaling. The detection of such a subset of proteins, specifically modulated by neurotrophic factors, clearly demonstrates the specificity of differentiative vs. mitogenic signaling pathways.

Although most of the changes observed were quantitative, one spot was newly and transiently synthesized after stimulation by bFGF and NGF. This spot (number 9) of 55 kDa and pI 5.45 is not present in the gels corresponding to the control and EGF samples and appears in the bFGF- and NGF-related

gels only during the first 2 hr of growth factor stimulation. Although spots 9 and 10 are clearly distinct, they may correspond to the same protein with a different level of phosphorylation. Thus, protein 9 would be the more heavily phosphorylated of the two.

The identity of these spots is intriguing. Immunoblots indicate that they are apparently not Shc (D. Thomas and R.A.B., unpublished experiments), a protein that has been identified as being involved in growth factor responses (36, 37). There are, however, also other possible identities for these proteins, including nuclear protooncogenes (13). However, the 0- to 2-hr incubation before the first time point may, in some cases, be too long to see immediate early transient inductions in growth factor regulation. Indeed, the proteins identified in this study are probably more likely to represent primarily secondary transcriptional responses. Ornithine decarboxylase, as representative of this group, can also be excluded because (*i*) it has a significantly more basic pI (38), (*ii*) has a different time course of induction (14, 39), and (*iii*) is also induced by EGF (14). Tiercy and Shooter (24) showed a marked increase in the rate of biosynthesis of two nuclear 56-kDa proteins with pI values of 5.7 and 5.8 after 8 hr of NGF action; bFGF and EGF were not used in this study. Since these proteins have been detected after 8 hr of NGF action and their pI values are different, it is unlikely that they correspond to proteins 9 and 10 identified in this study.

The description of early protein synthesis induced by bFGF, NGF, and EGF shows a very similar pattern of modulation for bFGF and NGF, which is somewhat distinct from the EGF pattern. These observations have allowed the detection of several rapidly and specifically induced proteins and provide direct evidence for molecular specificity of proliferative vs. differentiative signaling pathways. Nonetheless, these findings do not specifically address the mechanisms underlying these two responses, and recent evidence (refs. 40–42 and S. Raffioni and R.A.B., unpublished data) suggests that the differences may be largely quantitative—i.e., EGF can stimulate the differentiative pathway but is unable to maintain the amplitude of the signal for a long enough time to allow neurite proliferation. In such a model, mitosis represents the default situation. If so, the differences observed here would primarily reflect the consequences of the sustained vs. transient activation of the p21 Ras pathway (43). The identification of these proteins should help to clarify further the nature of these differences.

We thank Mrs. Gale Trudeau for her excellent assistance in the preparation of this manuscript and Neena Sareen and Nick Bizios of the Cold Spring Harbor Laboratory 2D Gel Laboratory Core Facility for their excellent technical assistance. This work was supported by research grants from the Public Health Service AG09735 (R.A.B.), American Cancer Society BE41 (R.A.B.), the Amgen Corporation (R.A.B.), National Institutes of Health P41-RR02188 (C.S.M.), and National Institutes of Health P30-CA455088 (S.D.P.).

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