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Spontaneous morphological changes by overexpression of the growth-associated protein B-50/GAP-43 in a PC12 cell line

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In order to study the direct effects of B-50 on neural cell morphology, rat B-50 cDNA was transfected into a PC12 cell line (PC-B2) exhibiting neurite outgrowth independent of the expression of endogenous B-50. The morphological changes were visualized by confocal scanning laser microscopy using fluorescence labelling for B-50 and for F-actin. The transfected cells exhibited filopodia and/or blebs on the plasma membrane, containing most of the B-50 immunoreactivity. No spontaneous neurite outgrowth was observed. Following NGF treatment transfected and non-transfected PC-B2 cells extended F-actin positive filopodia and neurites with a striking colocalisation of B-50 and F-actin. Our data show that the presence of B-50 can influence cell surface morphology independent of the presence of NGF. The colocalisation of B-50 and F-actin in the filopodial protrusions but not in the blebs might be indicative for a role of B-50 in actin polymerization and depolymerization.

The neural-specific protein B-50 (= GAP-43, F1 or neuromodulin) is a presynaptic protein kinase C substrate, implicated in signal transduction. The protein consistently shows high expression during periods of neuronal differentiation, suggesting a role in neurite outgrowth [17, 26]. In the peripheral nervous system, regeneration is accompanied by enhanced expression of B-50 mRNA and protein, while in the CNS with its limited regenerative capabilities such a correlation is less clear [7, 27]. In neuroblastoma cells, enhancement of neurite stability after transfection with B-50 cDNA has been reported [13, 21].

Rat pheochromocytoma (PC12) cells form a well-characterized model for neuritogenesis [9, 10]. On the one hand, NGF-induced neurite outgrowth appeared to be dependent on an enhanced B-50 expression and translocation to the membrane [28]: (i) transfection with B-50 cDNA enhanced NGF-induced neurite outgrowth [31]; (ii) antisense oligonucleotides to B-50 interfered with neurite outgrowth [12]. On the other hand, subclones deficient in B-50 expression were selected that, nevertheless, exhibited NGF-induced neurite outgrowth [1, 24]. Apparently, in PC12 cells, B-50 expression can be either conditional for neurite outgrowth or not. This situation might contain similar clues as are involved in the different role of B-50 in peripheral and central regeneration.

So far, direct evidence for the role of B-50 in neuritogenesis has been deduced from studies interfering with its expression in neuronal cells [refs. above, 25] or inducing the neural-specific B-50 in non-neuronal cells [30, 32]. In the latter case, however, the lack of other neuronal features may prohibit the full action of B-50.

In the present study, we used a PC12 clone (PC-B2) devoid of B-50, but still capable to exhibit neurite outgrowth upon NGF-induction [24]. Under these conditions, we investigated whether the introduction of B-50 will change cell morphology into the direction of neuritogenesis. It will be shown that B-50 per se (thus without NGF) induces both normal and abnormal protrusions of the cell membrane, most probably by interfering with submembrane polymerisation/depolymerisation of actin.

PC-B2 cells, grown on collagen-coated coverslips, were transfected with rat B-50 cDNA [23] under the control of a strong viral promoter (CMV; expression vector pCDNA1, Invitrogen, USA) using lipofectin (Gibco-BRL), essentially according to Greene [10] except for an additional change of medium 16 h before transfection, and cultured in RPMI1640 with 10% horse and 5% fetal calf serum. To relate the morphological phenomena to those after NGF-induction, in some experiments trans-

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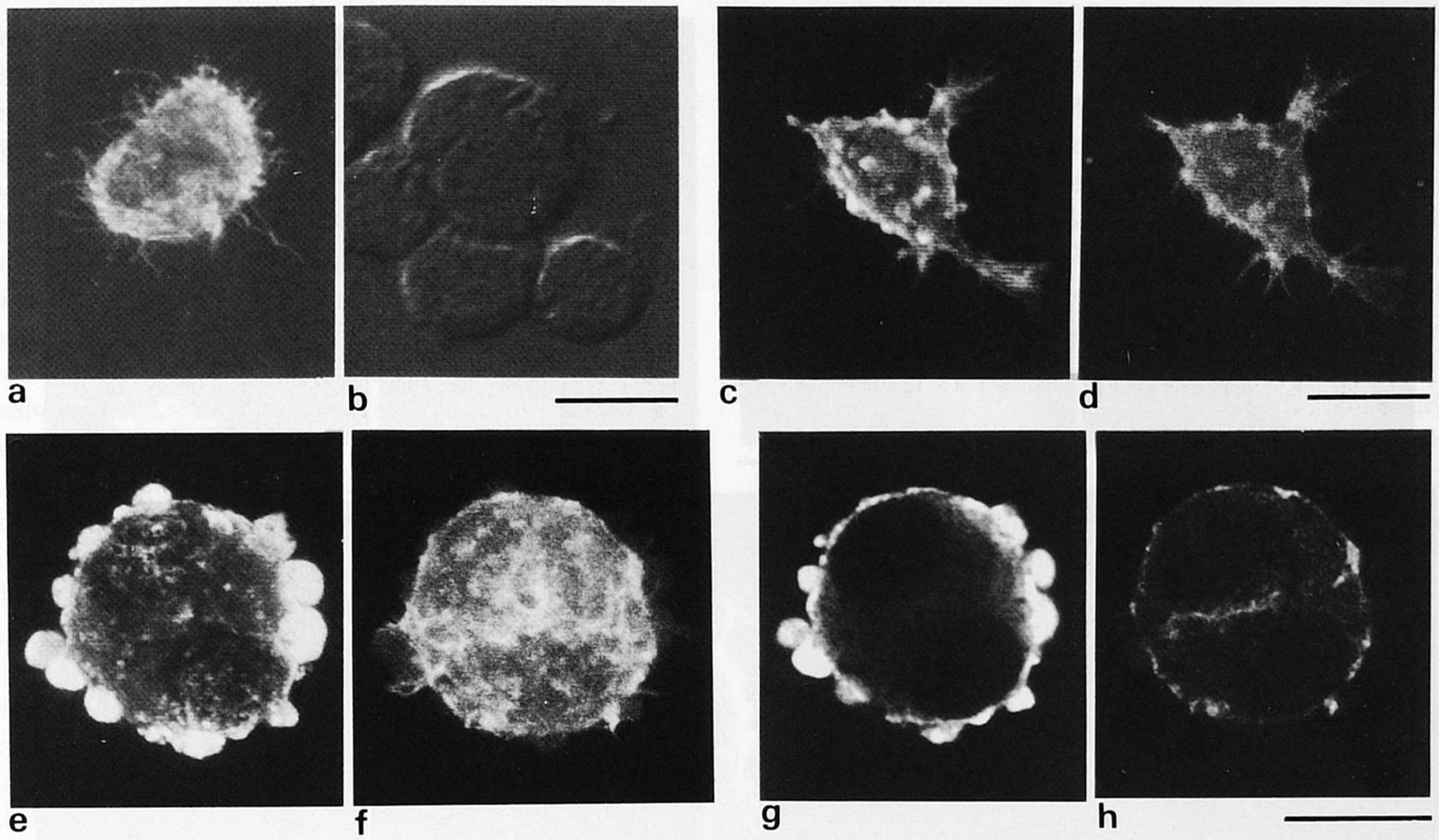

Fig. 1. A PC12 cell line with reduced expression of endogenous B-50 (PC-B2) was transfected with B-50 cDNA. After 2 days cells were fixed, stained for B-50 (a,c,e,g) with specific monoclonal antibodies, and for F-actin (d,f,h) with fluorescein-phalloidin, and analyzed by confocal scanning laser microscopy (using Nomarski optics (b) as well). Unless indicated otherwise a projection of all confocal planes is shown. (a) and (b) are pictures from the same cells. Note, that 1 out of a cluster of 6 cells in (b) has been effectively transfected, bearing B-50 positive microspikes (a). (c) and (d) represent a transfected cell with a more irregular shape bearing extensions positive for B-50 and F-actin, respectively. (e–h) represent a rounded transfected cell bearing blebs filled with B-50, but not with F-actin, as is shown most clearly in one of the confocal planes (g) and (h), respectively. Bars = 10 μm.

fected cells were cultured in N1 (serum-free RPMI1640) with 10 ng/ml β-NGF (Boehringer). Following fixation with 4% paraformaldehyde in PBS at 4°C for 20 min, cells were stored in 1% paraformaldehyde at 4°C. After immunostaining with B-50 specific monoclonal antibodies (NM4 [19]; secondary antibody goat anti-mouse IgGs conjugated to Texas Red, Jackson), cells were embedded in Dabco/Mowiol and observed with a Zeiss Axioplan equipped with a Biorad MRC600 unit for confocal scanning laser microscopy.

Transfected cells (2–4% of the total cell population) were easily identified by their intense immunostaining for B-50, indicating abundant B-50 expression by the CMV promoter (Fig. 1a). The nontransfected cells served as controls (Fig. 1b, the same picture visualized by Nomarski optics). The transfected cells showed various shapes. About 25% of the transfected cells was rounded up and the intense B-50 staining was localized at the plasma membrane extending into spontaneously formed filopodial structures (Fig. 1a). This localization of B-50 was even more obvious in the separate confocal planes (not shown). The nature of the filopodia and microspikes was analyzed using double fluorescent staining for B-50 and F-actin (phalloidin-FITC, Sigma). Transfected cells, like the one shown in Fig. 1c and d (about 40%), displayed extensions at the cell base attached to the substrate. A striking colocalisation of B-50 with filamentous (F-) actin in these extensions and in the subplasma membrane region was observed. Besides the growth cone-like structures, these cells also displayed small rounded protrusions (blebs) at the apical cell part enriched in B-50. Blebs were observed by phase contrast microscopy on 25–40% (depending on the experiment) of the transfected cells, but not on control cells. About 5% of the transfected cells showed exclusively blebs, filled with most of the B-50 (Fig. 1e). In those cells, actin filaments were distributed unevenly at the submembrane region and did not enter the blebs (Fig. 1f). Careful examination of 1 μm thick optical sections (e.g. Fig. 1g,h) revealed that the B-50 staining was found in the blebs, whereas no F-actin staining was observed within the blebs. Monoclonal anti-tubulin antibodies showed a diffuse distribution of tubulin both throughout the cytoplasm and the blebs (data not shown), indicating the differential staining for B-50 and F-actin is no artefact. The spontaneous morphological changes as a result of transfection with B-50 cDNA

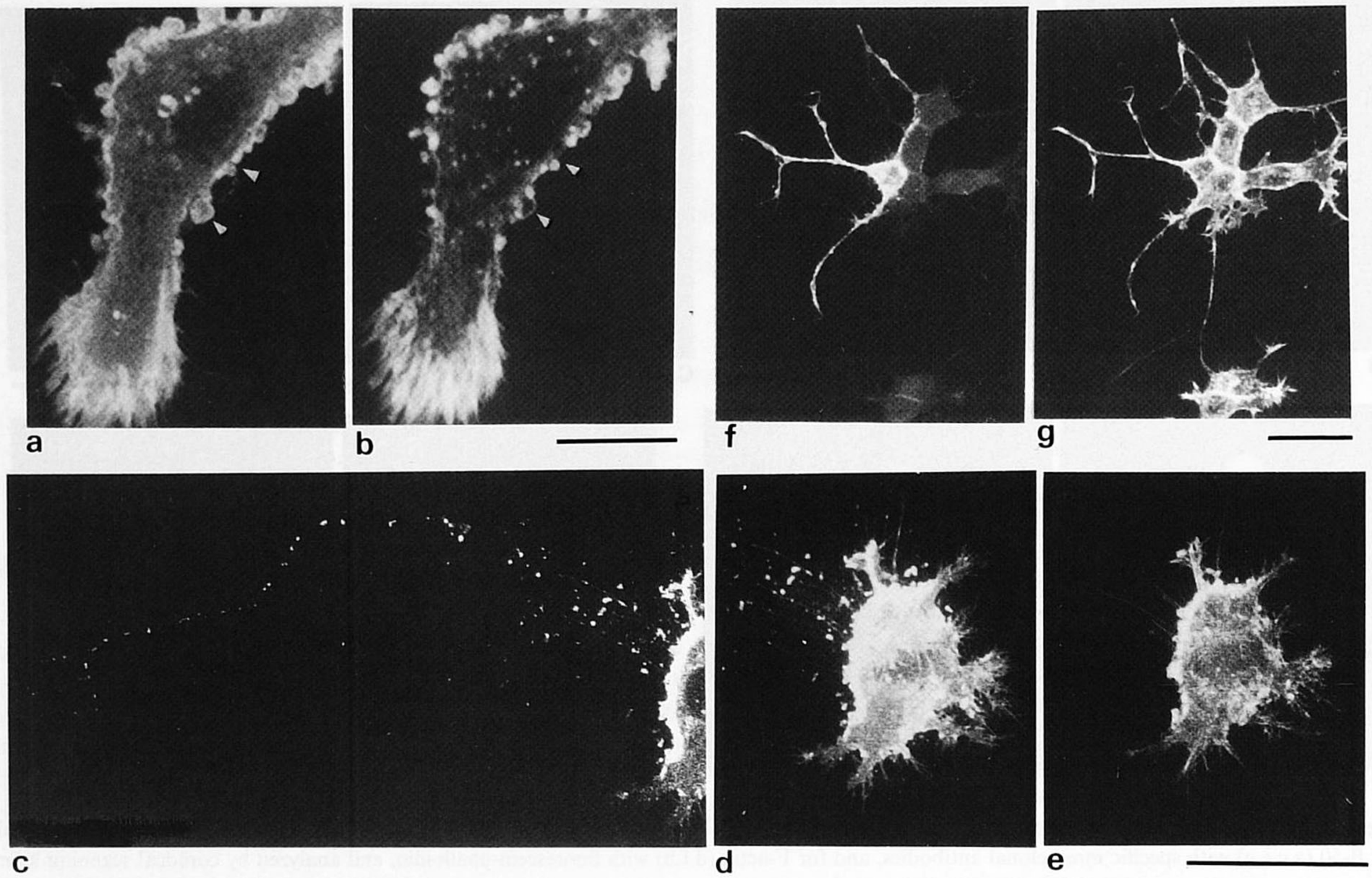

Fig. 2. PC-B2 cells transfected with B-50 cDNA and cultured in the presence of NGF for 2 (a–e) or 7 days (f,g) and stained for B-50 (a,c,d,f) and for F-actin (b,e and g), as described for Fig. 1. (a) and (b) show colocalisation of B-50 and F-actin, respectively, except for some bleb-like extensions as indicated with the 2 arrowheads. (d) and (e) show an example of a cell bearing numerous B-50 and F-actin positive blebs and microspikes with additional patchlike B-50 immunoreactivity; (c) is an extension of (d) taking 4 out of 8 confocal planes. (f) and (g) show colocalisation of B-50 with F-actin in the transfected cell (1 out of 7) after 7 days of NGF. Bars = 10 μm (a,b); 25 μm (c–g).

were observed in three independent experiments and also in the parental PC12 cell line (data not shown). Thus, the morphological phenomena described appear not to be dependent on any aberration of the PC-B2 cells other than those caused by the B-50 cDNA transfection.

Formation of microspikes or filopodia on the cellbody is normally only observed in PC12 and PC-B2 cells upon NGF treatment preceding neurite outgrowth. In the present study, overexpression of B-50 as a result of transfection with B-50 cDNA induced formation of filopodia in naive, NGF-untreated PC-B2 cells as well, in this respect mimicking the action of NGF. However, no spontaneous elongation to neurite-like extensions was observed.

To compare the effect of B-50 overexpression with that of NGF-induction and to study the interaction of both, cells were treated with NGF for 2 to 7 days, starting 16 h after transfection. After two days of NGF treatment, PC-B2 cells have formed neurite-like extensions, and both transfected and non-transfected cells showed numerous F-actin rich microspikes on their soma and extensions (Fig. 2b). Transfected cells, initially, often displayed rather short, broad extensions of irregular shape, different from the average 'normal' neurite in non-transfected cells. Neurite length of B-50 positive cells was compared to that of neighbouring non-transfected cells: in 21 out of 35 cell pairs the transfected cell bore shorter extensions as visualized by F-actin staining. Moreover, most of the transfectants were covered with small, B-50 containing blebs as well (Fig. 2a,b). In these cells, B-50 and F-actin staining were colocalised to a great extent in the submembrane region of the soma and in the filopodial extensions of the growth cone. The blebs showed varying intensities of F-actin staining, which was often confined to the subplasma membrane region, whereas B-50 staining appeared throughout the blebs (Fig. 2a,b, arrowheads).

In 1–5% of the transfected cells punctuate B-50 immunostaining was found in extensions with a remarkable length (up to 100 μm; Fig. 2c,d). The extensions, however, were stained for F-actin only over a short distance proximal to the cell body, no F-actin staining was detectable at or in between the more distal B-50 immunoreactive dots (Fig. 2e). Phase contrast nor differential inter-

ference contrast (Nomarski optics) did visualize any further connections between the B-50 immunoreactive dots, indicating that either such a connection has been lost or that the connecting structures are much thinner than an average neurite. Neurite outgrowth involves motile target searching with the growth cones extending or retracting on environmental cues [8,16]. The B-50 immunoreactive dots of Fig. 2c may represent membrane patches of withdrawn or collapsed neuritic extensions. The presence of B-50 in substrate-bound membrane areas of cultured cells has been reported previously by Meiri and Gordon-Weeks [20].

In cultures treated with NGF for 7 days, there was still abundant B-50 expression in transfected cells (Fig. 2f,g). Blebs were observed only occasionally. At this stage of differentiation most cells have formed neurites of considerable length. Neurite length of cells with high B-50 expression was compared to that of their neighbouring non-transfected cells. Average total neurite length per cell at day 7 of B-50 overexpressing cells was 5.5 ± 3.1 ($n = 26$) vs. that of control cells 4.3 ± 2.4 ($n = 33$) times the cell body diameter. No significant differences were observed in this respect. In the transfected cells, B-50 immunoreactivity was localized in the submembrane region and in filopodial extensions (Fig. 2f) and largely colocalised with F-actin (Fig. 2g). No striking differences in F-actin staining between transfected and non-transfected cells were observed (Fig. 2g).

The morphological effects of the overexpression of B-50 are most prominent in PC-B2 cells without NGF-induction. It has been reported for non-neuronal cells that the induction of B-50 expression induces microspikes or filopodial-like protrusions [30, 32]. Those phenomena, however, appeared to be marginally and temporarily. Recently, the induction of B-50-positive microspikes has been reported in mouse neuroblastoma cells transfected with B-50 cDNA [19]. Our present data with respect to the spontaneous formation of B-50-positive filopodial structures in a PC12 cell line corroborate these findings. Moreover, the double staining for F-actin confirms that these structures resemble the filopodial backbone that is formed by F-actin. Thus, large concentrations of B-50 at the plasma membrane are able to initiate morphological similar changes as are normal for the start of neurite outgrowth. We observed before that in normal PC12 cells, upon NGF induction, one of the first events is the translocation of B-50 to the membrane and to filopodial protrusions. The present data show that such a translocation might be causal related to the following morphological changes. This is in agreement with our observations that inhibition of the enhanced B-50 expression by antisense oligomers during the first 24 h of NGF induction blocked the NGF-induced neurite outgrowth [12].

A striking feature of cells transfected with B-50 was the formation of so-called blebs. Membrane blebbing is implicated in cell motility [14] and is a characteristic morphological feature of hypoxic or toxic conditions [2–4, 15]. In our cultures, however, there was a normal survival of cells with B-50 overexpression, including those with blebs; Using either B-50 cDNA or a control construct expressing β-galactosidase, a similar percentage of transfected cells was found after 2 days. Moreover, within one experiment the % of transfected cells with B-50 overexpression did not change between day 2 and day 7 post transfection. The blebs reported here resemble those found in a number of NGF-nonresponsive PC12 clonal cell lines [5], and in PC12 cells overexpressing β/A4 amyloid [18]. Changes in cytoskeletal organization have been implicated in the appearance of blebs [2, 14, 15]. The local decrease in F-actin accompanying the bleb formation in B-50 overexpressing PC12 cells is also indicative for an interference with cytoskeletal organization. A modulatory role for B-50 in cytoskeletal architecture has been proposed before [6, 8, 16, 20]. Changes in morphology of non-neuronal L6 cells transfected with B-50 cDNA were also accompanied by rearrangements of the actin skeleton [30]. Ultrastructural studies show B-50 is mainly localized at the cytoplasmic face of the cell membrane [29]. Cell fractionation studies indicate that a significant part of B-50 is tightly bound to the actin-rich submembrane cytoskeleton [20, 22]. Recently, B-50 was shown to bind to F-actin in vitro [11, 27] but no indications for an effect of B-50 on actin dynamics were found [11]. The present study shows that in PC12 cells with overexpression of B-50 the distribution of B-50 and F-actin largely overlaps. However, after 7 days of NGF treatment non-transfected PC-B2 cells with very low B-50 expression showed a similar morphology and pattern of F-actin staining, indicating that under these conditions B-50 is not a prerequisite for the formation of neurites and F-actin rich filopodia. The blebs in transfected cells before NGF treatment appear to accumulate B-50 without enrichment in F-actin, suggesting that an excess of B-50 induces local dissociation of the plasma membrane and cortical actin skeleton. Whether an in vivo interaction of B-50 with actin-polymerization exists or whether B-50 plays a role in the association of cytoskeleton and membrane remains to be established. This study indicates that in neuronal cells B-50 per se can evoke membrane protrusions, that may precede neurite outgrowth by growthfactors like NGF, retinoic acid and others.

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