neuroReport

NeuroReport 6, 969–972 (1995)

N-terminal cysteines essential for Golgi sorting of B-50 (GAP-43) in PC12 cells

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We have examined the subcellular distribution of the growth-associated protein B-50 (GAP-43) in pheochromocytoma (PC12) cells, using confocal microscopy. Proliferating PC12 cells contained very low levels of B-50 in the cytosol. Enhanced expression of B-50 in these cells, evoked by either nerve growth factor (NGF) treatment or transient transfection with rat B-50 cDNA, led to Golgi sorting and membrane targeting of the B-50 protein. Site directed mutagenesis of Cys_3Cys_4 to Ser_3Gly_4 in B-50 resulted in a cytosolic distribution. We conclude that Cys_3 and Cys_4 are essential for accumulation of B-50 both at the plasma membrane and in the Golgi apparatus of PC12 cells.

Key words: B-50; GAP-43; Neuromodulin; Golgi apparatus; Protein sorting; PC12 cells; Wheat germ agglutinin; Confocal microscopy; Mutation

Introduction

B-50 (also known as GAP-43, F1, p57, pp46 and neuromodulin) is a neuronal, calmodulin binding, PKC substrate that accumulates presynaptically during growth. It is thought to have a role in axonal growth, G-protein regulation and neurotransmitter release.[1,2] B-50 is synthesized as a highly hydrophilic protein on free ribosomes in the cytosol and associates with membranes rapidly after its synthesis.[3] Tight association of B-50 with membranes is thought to be accomplished via palmitoylation of two cysteine residues at positions 3 and 4.[3] Mutation of these cysteines prevented association of B-50 to the plasma membrane[4–6] as well as cell shape changes induced by transient over-expression of B-50 in a variety of non-neuronal cells.[7,8] In addition, these cysteines are essential for *in vitro* G_0 activation by B-50.[9]

B-50 immunoreactivity (BIR) has been detected in the perinuclear region of dissociated SCG neurones[10] and of cultured hippocampal neurones.[11] In electron microscopic studies, B-50 could be immunolocalized to the Golgi apparatus in PC12 cells[12] and in axotomized motor neurones of cranial nerve nuclei.[13] The demonstration of BIR on the cytoplasmic site of vesicles in the Golgi region, neuritic shaft and growth cones of cultured hippocampal neurones suggests that B-50 passes the Golgi apparatus before transport to target membranes.[14]

In this study, we investigated the effect of nerve growth factor (NGF) on the subcellular localization of BIR in PC12 cells. Administration of NGF leads to differentiation of PC12 cells to sympathetic neurone-like cells,[15] a process that is accompanied by enhanced B-50 expression.[16] To assess the importance of the N-terminal cysteine residues for B-50 localization in neuronal cells we compared the subcellular locations of BIR in PC12 cells, transiently transfected with wild type and Ser_3Gly_4 B-50 cDNA. Recently, Liu *et al*[17] demonstrated the importance of intact cysteine residues for accumulation of BIR in the Golgi apparatus of transiently transfected non-neuronal COS cells, employing normal fluorescence microscopy. However, this technique can hardly discriminate between cytosolic BIR and Golgi-associated BIR. We used the more sensitive confocal laser scanning microscope to examine the subcellular distribution of BIR in PC12 cells. The presented data demonstrate the absolute necessity of intact cysteine residues for accumulation of B-50 at the plasma membrane and in the Golgi apparatus.

Material and Methods

PC12 cells and PC-B2 cells[18] were grown essentially according to Greene *et al.*[19] Cells were maintained in a humidified atmosphere at 37°C and 7% CO_2 in Costar tissue culture flasks, coated with rat tail collagen (40 ng mm^{-2}), and containing 1 × RPMI [Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% horse serum (ICN), 5% fetal calf serum (Gibco) and penicillin/streptomycin (each 100 IU ml^{-1}; ICN)]. Proliferating PC12 cells were grown for 7 days in 1 × RPMI, fixed and immunostained with B-50 specific monoclonal antibodies (NM4,[20] 1:1000) and DTAF- or TRITC-conjugated goat anti-mouse

secondary antibodies (1:100). To assess the effect of NGF on the subcellular location of BIR, PC-B2 and PC12 cells were grown in chemically defined medium (N1) consisting of RPMI 1640 supplemented with transferrin (5 μg ml^{-1}; Greiner), progesterone (20 μg ml^{-1}; Sigma), putrescine (100 μM; Sigma) and selenium dioxide (30 μM; Sigma). Before use this medium was supplemented with β-NGF (10 ng ml^{-1}; Boehringer) and insulin (5 μg ml^{-1}; Sigma). Medium was replenished every other day up to 7 days when cells were triturated through a 21-gauge needle and replated on collagen coated coverslips. Cells were fixed 8.5 h after replating with 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer for 20 min at 4°C. Fixed cells were stored in 1% paraformaldehyde at 4°C before immunostaining.

Mutant B-50 cDNA, in which the cysteines at positions 3 and 4 were replaced by serine and glycine respectively, was constructed via oligonucleotide-directed *in vitro* mutagenesis (Amersham) with the synthetic oligonucleotide 5′-CACCATGCTGTC-CGGTATGAGAAG-3′. The mutations were confirmed by direct DNA sequencing. Wild type and Ser_3Gly_4-B-50 cDNAs were inserted into pCNDA1 (Invitrogen) and transfected into PC12 cells with lipofectin (Gibco-BRL) according to Greene *et al.*[19] Two days after transfection, cells were fixed and immunostained for B-50. In order to localize the Golgi apparatus, fluorescein-labelled wheat germ agglutinin (WGA; 30μg ml^{-1}; Sigma) was included with the secondary antibody. Cells were embedded with Dabco/Mowiol and observed with a Biorad MRC-600 confocal scanning unit mounted on a Zeiss Axioplan microscope. Co-localization of B-50 and WGA was studied by reading the confocal images in the red or green channel respectively before merging, resulting in a yellow colour in case of co-localization.

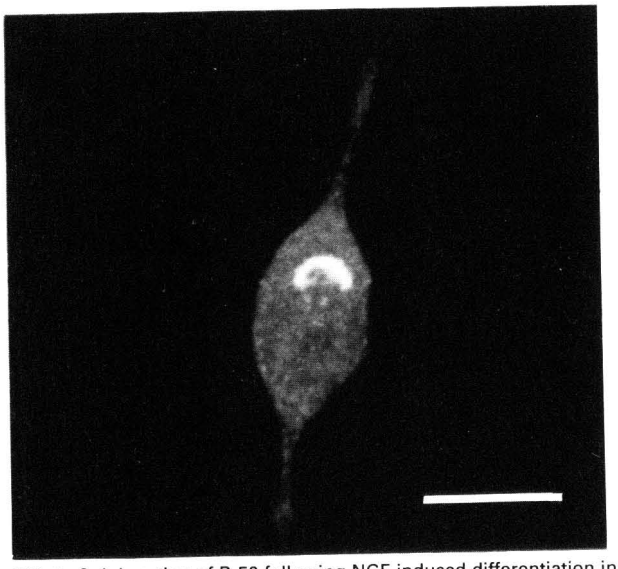

FIG. 1. Golgi sorting of B-50 following NGF-induced differentiation in PC-B2 cells. PC-B2 cells were primed with NGF for 7 days, triturated, replated and fixed after 8.5 h. B-50 immunostaining was observed by CLSM. Bar = 10μm.

Results

Following NGF-induced differentiation of PC-B2 cells, a PC12 clone virtually devoid of B-50 but still capable of growing out neurites,[17] we observed BIR in a perinuclear region (Fig. 1). The amount of BIR in proliferating PC-B2 cells was below the detection limit of the confocal laser scanning microscope.

We wondered whether this distribution of BIR could also be seen in wild type PC12 cells. After extensive signal enhancement, we detected low levels of BIR in the cytosol of proliferating PC12 cells, with no accumulation of BIR at the plasma membrane nor in a perinuclear region (Fig. 2a). NGF-induced differen-

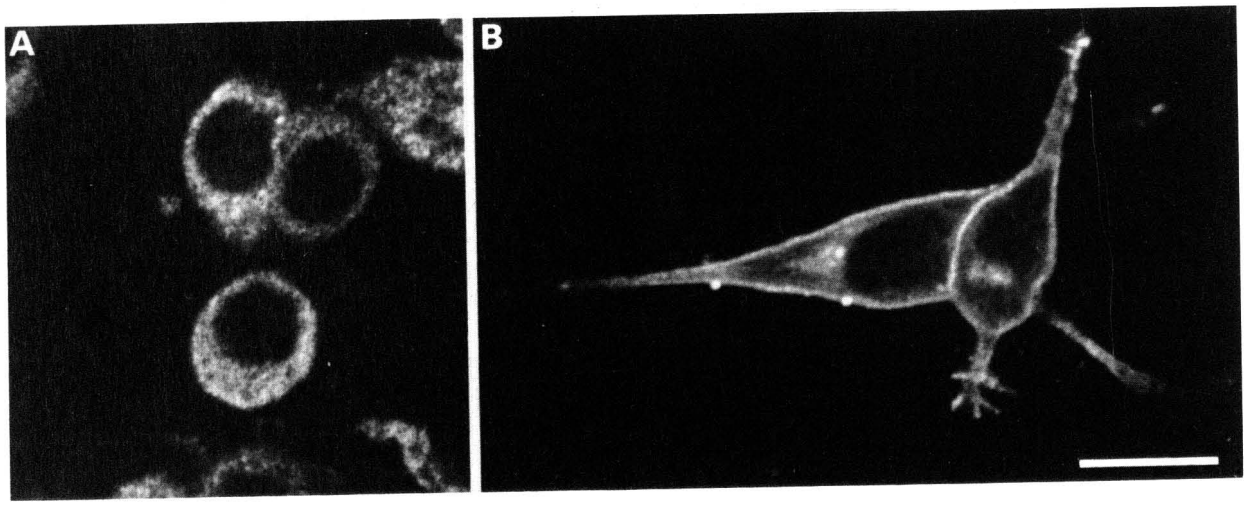

FIG. 2. Golgi sorting and membrane targeting of B-50 in PC12 cells following NGF-induced differentiation. (**a**) PC12 cells grown for 7 days in RPMI, fixed and immunostained for B-50. (**b**) PC12 cells treated as PC-B2 cells in Fig. 1. Bar = 10μm.

tiation resulted in neurite outgrowth and accumulation of BIR in a perinuclear region as well as at the plasma membrane (Fig. 2b).

Since NGF is known to augment B-50 expression in PC12 cells,[16] the question arose whether the observed perinuclear accumulation of BIR was caused by the enhanced B-50 expression. To answer this question we transfected proliferating PC12 cells with rat B-50 cDNA under the control of the CMV promoter. Transfected cells (about 10% of the total cell population) displayed clear BIR in a perinuclear region and along the plasma membrane (Fig. 3a). Double labelling of these cells with the Golgi marker WGA–FITC[21] (Fig. 3b) revealed that this perinuclear region completely coincided with the Golgi apparatus (Fig. 3C, yellow). WGA–FITC was also detected at the plasma membrane because of the presence of WGA binding glycoproteins at this site.

The B-50 protein is thought to be attached to the plasma membrane via fatty acylation at cysteines 3 and 4.[3] After transfection of PC12 cells with Ser_3Gly_4 B-50 cDNA, BIR was located in the cytosol and could not be detected at the plasma membrane (Fig. 3d). Moreover, BIR appeared to be completely absent (Fig. 3d) from the site of the WGA–FITC stained Golgi apparatus (Fig. 3e). Therefore no co-localization was found (Fig. 3f, no yellow).

Discussion

NGF-induced differentiation of PC12 cells causes enhanced B-50 expression[16] accompanied by translocation of B-50 from intracellular organelles to the plasma membrane.[12] In the present study, we found a striking accumulation of BIR in a perinuclear location of PC-B2 cells, after NGF-induced differentiation (Fig. 1). In wild type PC12 cells, NGF-induced differentiation led to sorting of BIR from the cytosol (Fig. 2a) to both a perinuclear region and the plasma membrane (Fig. 2b). This phenomenon was not dependent on the presence of NGF, since proliferating PC12 cells transfected with wild type B-50 cDNA displayed a similar distribution of BIR (Fig. 3a). Double labelling of B-50 transfected cells with anti-B-50 antibodies and WGA–FITC clearly showed that perinuclear BIR was associated with the Golgi apparatus (Fig. 3c).

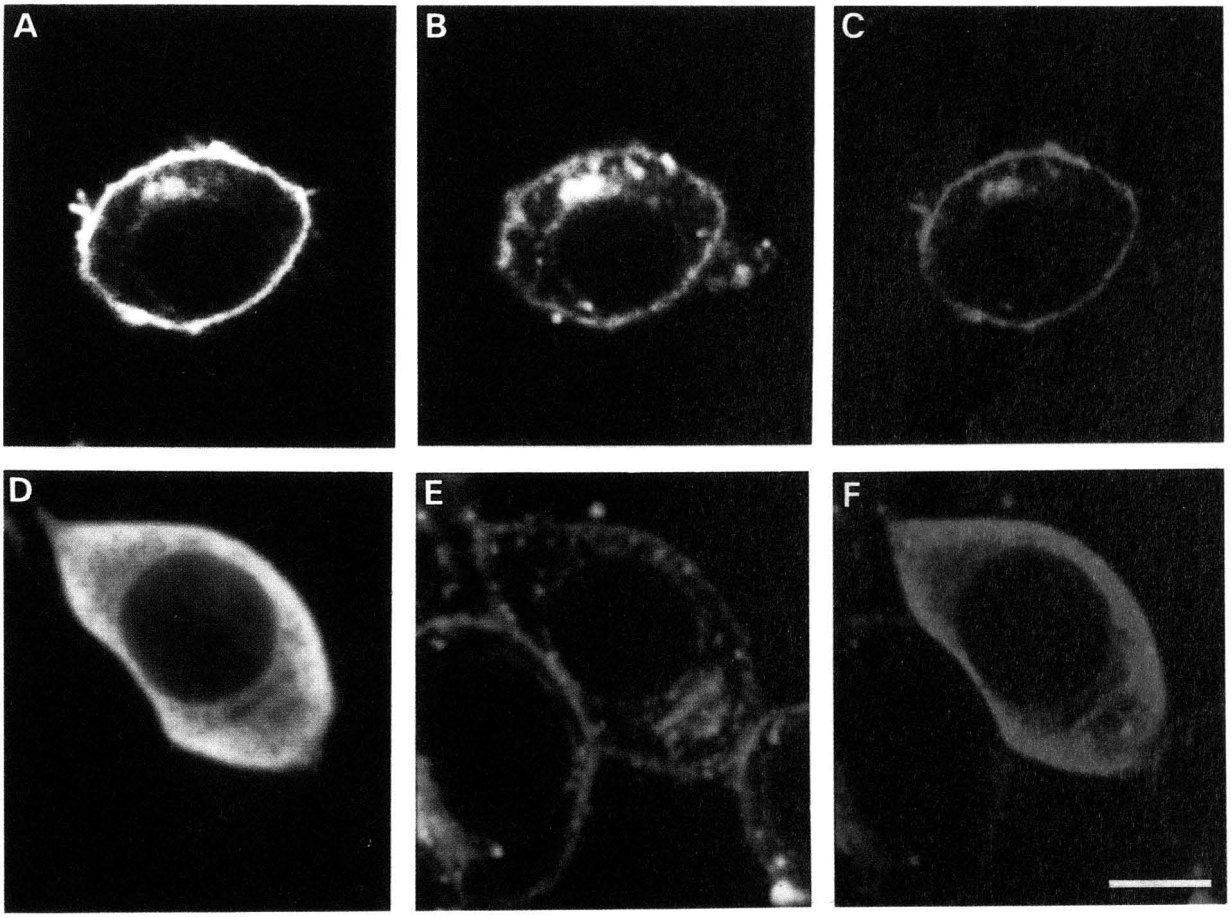

FIG. 3. Mutation of N-terminal cysteines abolishes Golgi sorting and membrane association of B-50 in PC12 cells. PC12 cells were transfected with wild type (**a, b, c**) or Ser_3Gly_4 (**d, e, f**) rat B-50 cDNA and stained for B-50 (a and d) or WGA–FITC (b and e) as described in Materials and Methods. Confocal images were read into the red (B-50) and green (WGA–FITC) channels and merged (c and f) resulting in a yellow colour in case of co-localization. Bar = 5 μm.

Introduction of B-50 in various non-neuronal cell types[7,8,22,23] and in PC12 cells[18] led to the spontaneous formation of microspikes and filopodial protrusions, characteristic for the start of neurite outgrowth. (These processes cannot be seen in the optical section depicted in Fig. 3a merely because they are outside the confocal plane of the Golgi apparatus.) We hypothesize that enhanced B-50 expression in PC12 cells leads to sorting of BIR to the Golgi apparatus and subsequent targeting to the plasma membrane, followed by initiation of neuritogenesis. This enhanced expression of B-50 can be evoked by either NGF treatment or transfection with B-50 cDNA.

Several authors have shown that the cysteine residues in the N-terminus are essential for association of B-50 with the plasma membrane.[3–6] Recently, Liu *et al* suggested that these cysteines are also important for Golgi accumulation in COS cells.[17] In this study we used a confocal laser scanning microscope that enabled us to focus on the microscopic plane at the height of the Golgi apparatus. Our data unequivocally show that accumulation of B-50 in the Golgi apparatus of PC12 cells depends on the integrity of Cys_3Cys_4 (Fig. 3d, e, f). The mechanism for Golgi sorting of B-50 remains unclear. It could be that the N-terminal primary sequence, comprising the cysteine residues, is responsible for Golgi targeting. Fatty-acyl transferases have been localized to the Golgi apparatus[24] and could couple B-50 to vesicles originating in this region via fatty acylation at its cysteines, followed by transport to the cell periphery.

Conclusion

Enhanced expression of the neuronal phosphoprotein B-50 in PC12 cells, induced by either NGF administration or transfection with B–50 cDNA, results in sorting of B-50 to the Golgi apparatus and the plasma membrane. This process depends entirely on the presence of cysteine residues at positions 3 and 4 of the B-50 molecule.

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ACKNOWLEDGEMENTS: This work was supported by the Netherlands Organization for Scientific Research (MW-NWO grant 903-48-143) and by the 'Prinses Beatrix Fonds'.

Received 13 February 1995; accepted 23 February 1995