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Neurogranin, a B-50/GAP-43-immunoreactive C-kinase substrate (BICKS), is ADP-ribosylated

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Neurogranin is a neurone-specific, B-50-immunoreactive C-kinase substrate that has limited homology to, but considerable biochemical similarity to B-50/GAP43. The most significant differences between these two proteins are their cellular localisation and molecular mass (Neurogranin, 7.5 kDA cytosolic; and B-50, 25 kDa membranal). An understanding of the similarities and differences between Neurogranin and B-50 may facilitate the elucidation of their hitherto elusive functions in the nervous system. The results of the present study demonstrate that, in common with B-50, Neurogranin is a substrate for ADP-ribosyltransferases. This finding is discussed with regard to the concept of molecular flexibility of B-50-like proteins as the basis of their putatively diverse roles in the nervous system.

ADP-ribosylation; B-50/GAP-43; Neurogranin; Neuronal protein; Post-translational modification; Protein kinase C

1. INTRODUCTION

The neuronal protein, B-50/GAP-43, is a major substrate for protein kinase C (PKC) and is associated with growth and regeneration in the nervous system [1,2]. However, the mechanisms by which B-50 contributes to these processes remain elusive despite extensive biochemical characterisation that includes the elucidation of the primary sequence and the identification of several functional domains within the protein, not least of these being the PKC phosphoacceptor site [3] and a calmodulin (CaM) binding domain [4]. In the course of experiments designed to further characterise the CaM binding properties of B-50, which are restricted to dephosphorylated (dp)-B-50 and occur only in the absence of calcium [4], we accidentally discovered a second CaM binding, B-50-immunoreactive, C-kinase substrate (BICKS) in bovine brain cytosol [5]. This BICKS protein is identical to Neurogranin (NG), a rat brain-specific, C-kinase substrate purified and sequenced by Baudier and colleagues [6]. While NG shares some common biochemical properties with B-50, sequence homology is restricted to a short domain of some 25 amino acids that include the PKC-phosphoacceptor site, and the CaM binding domain (that also binds acidic phospholipids [7]) [6]. Although having otherwise different sequences and molecular masses, B-50 and NG (25 kDa and 7.5 kDa, respectively) have the same unusual property of anomalous migration on SDS-PAGE (apparent molecular masses of 43 kDa and 16 kDa, respectively), which reflects their acidic nature (pI's of 4.5 for B-50, and an average of 5.4 for NG) [5,6]. They share acidic isoelectric points and anomalous migration on SDS-PAGE with MARCKS, a third major substrate of PKC in neuronal tissue [8].

We have recently demonstrated that B-50 is ADP-ribosylated [9] as well as phosphorylated. The correlations that can be made between these post-translational modifications and the spatial and temporal distribution of B-50 isoforms in the nervous system have been interpreted as evidence for a wider regulatory role of the protein in, at least, calcium, calcium/CaM, and G-protein-linked signal transduction [10–12]. Alternatively, B-50 may be subject to regulation by all major signal transduction mechanisms. Moreover, this molecular flexibility or adaptability may be at the heart of, and common to all, B-50-like proteins that only significantly differ in their molecular mass and cellular localisation. Therefore, in the present study we investigated whether NG is a substrate for endogenous ADP ribosyltransferases.

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Abbreviations: APAD, 3-acetylpyridine adenine dinucleotide; BICKS, B-50-immunoreactive C kinase substrate; CaM, calmodulin; DTT, dithiothreitol; dp-, dephosphorylated; GIDP, 5′-guanylimidodiphosphate; HPLC, high performance liquid chromatography; MARCKS, myristoylated alanine-rich C kinase substrate; NAD, nicotinamide adenine dinucleotide; NG, neurogranin; p-, kinase C phosphorylated; pI, isoelectric point; PKC, protein kinase C; RB, ADP-ribosylation buffer; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]NAD (1,000 Ci/mmol ^{32}P-adenylate) and [^{32}P]ATP (3,000 Ci/mmol) were purchased from Amersham Canada (Oakville, Ont.).

NAD, 3-acetylpyridine adenine dinucleotide (APAD), 5'-guanylimidodiphosphate (GIDP), DTT, and Triton X-100 were from Sigma Chemical Co. (St. Louis, MO, USA). Electrophoresis reagents were from Bio-Rad Canada (Mississauga, Ont.) except ampholines, which were from Pharmacia Canada (Baie de Urfe, Que.). All other reagents, of analytical grade or better, were from local distributors (Fisher Scientific, and BDH). Fresh bovine brain (XL Beef Ltd., Calgary, Alba) was soaked in ice-water for 30 min to facilitate the removal of meninges, and frozen at −80°C in 100 g portions. The standard ADP-ribosylation buffer (RB) was 50 mM Tris-HCl, pH 7.5, containing 1% v/v Triton X-100, 2 mM APAD, 0.2 mM GIDP, and 10 mM DTT (all final concentration in the assay).

2.2. *Purification and PKC phosphorylation of bovine NG*

Bovine dp-NG was prepared as previously described [5]. The dp-NG-containing calcium eluate from the CaM–Sepharose affinity column was repurified by reverse-phase HPLC. Following three cycles of lyophilisation in water, an aliquot of the dp-NG was phosphorylated using a purified rat PKC preparation, re-purified on HPLC, and re-lyophilised, as previously described [5,13,14].

2.3. *Tissue preparation and ADP-ribosylation*

Fresh or frozen bovine brain was homogenised in 50 mM Tris-HCl, pH 7.5, immediately prior to use (glass Teflon, 0.25 mm clearance, 1 g/25 ml buffer), and diluted to a final protein concentration of 2 mg/ml. Protein concentration was determined by the method of Bradford [15]. Brain homogenate (100 μg total protein), plus or minus 5 μg NG, was ADP-ribosylated as previously described for rat brain homogenate plus or minus B-50 [9]. In brief, 50 μl of homogenate was mixed with 30 μl RB and preincubated for 5 mins at 37°C. NG was added, as appropriate, prior to the addition of NAD (20 μl RB containing 25 μCi [^{32}P]NAD and unlabelled NAD at a final concentration in the assay of 10 μM). The reaction continued for a further hour at 37°C and was terminated by rapid freezing and lyophilisation. Samples were prepared for first-dimension isoelectric focusing (pH 3.5–10.0), and second-dimension 15% SDS-PAGE, as previously described [9,16]. Following electrophoresis gels were Fast green- and silver stained, dried, and subject to autoradiography where appropriate (Kodak-X-Omat, −80°C). PKC-phosphorylated NG underwent identical two-dimensional electrophoresis and autoradiography.

3. RESULTS

CaM–Sepharose-purified dp-NG (referred to as BICKS in all the figures) appeared as a slightly asymmetric peak eluting after B-50, following final purification by reverse-phase HPLC (Fig. 1). While the UV chromatogram indicated some heterogeneity, 15% SDS-PAGE with Fast green staining showed a single, apparently homogenous, band of 16 kDa (Fig. 1, inset). However, two-dimensional electrophoresis with silver staining demonstrated that dp-NG preparations were often contaminated with trace amounts of B-50, despite extensive purification (Fig. 2, middle panel, spot 1). In contrast to the single spot for dp-B-50, bovine dp-NG was heterogeneous on 2D gels and comprised a single spot with a pI of approximately 6.0 (Fig. 2, spot 6), and an elongated, predominantly more acidic than basic band of pI 8.0–6.5 (Fig. 2, spot 7). While spot 6 was more distinct, spot 7 consistently represented the majority of the dp-NG. In contrast, the majority of p-NG (as revealed by autoradiography, Fig. 2, lower panel) occupied the same position as the relatively minor form of dp-NG, spot 6. In addition to the major form, decreasingly significant amounts of p-NG occupied increasingly acidic positions (Fig. 2, spots 5, 4, and 3), although there is no evidence to suggest that NG is polyphosphorylated by PKC in vitro, and the same results were obtained with every batch of p-NG tested.

ADP-ribosylation of bovine brain homogenate in vitro demonstrated that a bewilderingly large but select number of proteins are subject to this post-translational modification. A spot corresponding to endogenous B-50 was readily identifiable in the silver-stained gel and

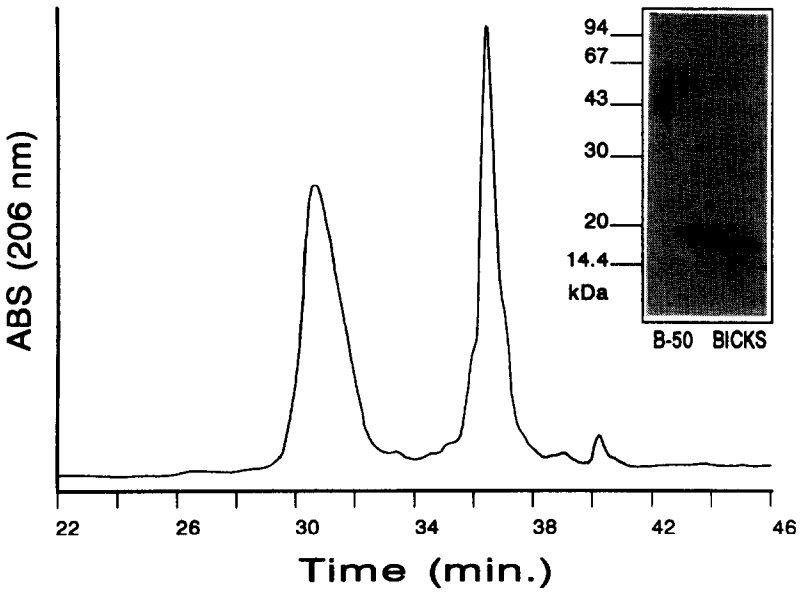

Fig. 1. Reverse-phase HPLC of dp-B-50 and BICKS (NG). CaM–Sepharose purified B-50 and BICKS (approximately 0.5 mg of each, 2.0 AUFS, 206 nm) were re-purified by HPLC. A slightly asymmetric peak of BICKS (16 kDa) eluted after B-50 (25 kDa) but both proteins appeared to be homogeneous as determined by 15% SDS-PAGE with Fast green staining (inset).

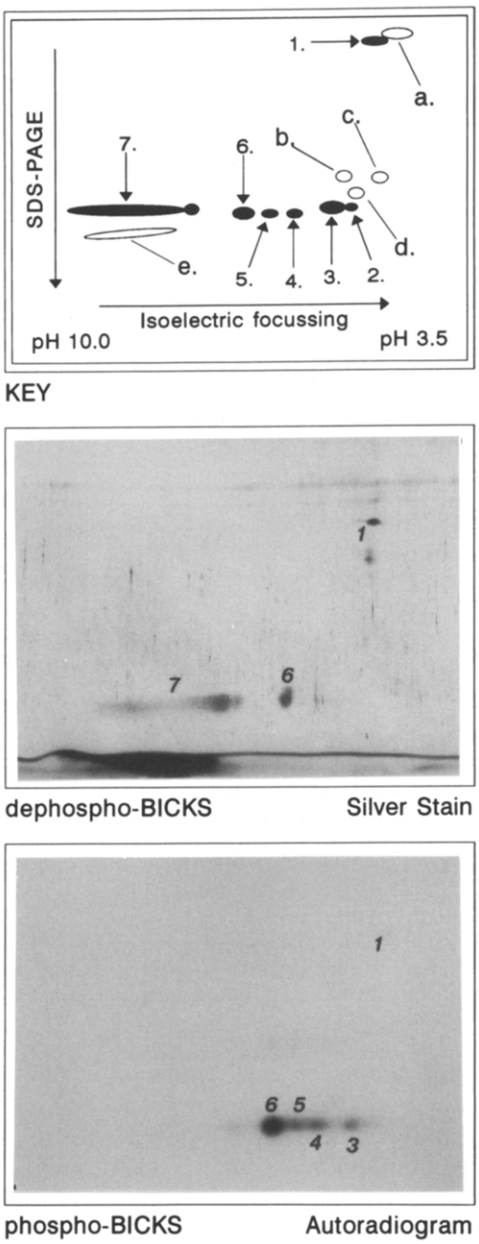

Fig. 2. Two-dimensional electrophoresis of BICKS (dp-NG and p-NG). Dephosphorylated and p-BICKS were separated on 2D gels (1st dimension isoelectric focusing pH 3.5–10.0, and 2nd dimension 15% SDS-PAGE) and subject to silver-staining and autoradiography (12 h, −80°C) as appropriate. The labels used in the key refer to this figure and Fig. 3. Spots labelled a–e inclusive are readily identifiable silver-stained landmark bands from bovine brain homogenate, some of which are ADP-ribosylated. The spot labelled 1 corresponds to endogenous B-50, spot 2 is a minor form of ADP-ribosylated BICKS (NG, pI 4.6), spots 3, 4, and 5 are minor forms of p-BICKS, spot 6 corresponds to a minor form of dp-BICKS which is indistinguishable from the major form of p-BICKS (pI 6.0), and spot 7 is a smeared band of dp-BICKS (pI 8.0–6.5).

accompanying autoradiogram (Fig. 3, spot 1), and yet, no spots corresponding to either dp- or p-NG were visible when bovine brain homogenate was ADP-ribosylated in the absence of added NG, the slight exception being a very diffuse spot that occupied the position of a less prominent form of p-NG (Fig. 3, spot 3). In contrast, additional spots were readily detectable following ADP-ribosylation in the presence of added NG. More specifically, silver-stained spots corresponding to spots 7 and, to a lesser extent, spot 6 of the pure dp-NG were present, in addition to a prominent, and more acidic spot 3 (Fig. 3). Spot 3 (pI 4.6) was the major ADP-ribosylated form of NG (Fig. 3), and occupied the same position as a minor form of p-NG. To re-emphasise, the ADP-ribosylation protocol is incompatible with protein phosphophorylation [17], which is readily substantiated by a comparison of the isoelectric points of ADP-ribosylated and the major form of phosphorylated NG – pI's of 4.6 and 6.0, respectively.

4. DISCUSSION

The results demonstrate that purified dp-NG is a substrate for endogenous ADP-ribosyltransferase activity in bovine brain homogenate. In contrast to B-50, which is also ADP-ribosylated [9], a spot corresponding to ADP-ribosylated NG was not routinely visible in 2D gels of bovine brain homogenate incubated in the absence of added NG. This relates to the smeared distribution of NG over a wide isoelectric range that renders low concentrations of endogenous protein difficult, if not impossible, to detect (this study and [6]). In contrast, the silver-stained and autoradiographic spot for ADP-ribosylated exogenous NG (spot 3) was the most clearly visible isoform, and readily distinguishable from the major form of PKC-phosphorylated NG (spot 6). This eloquently demonstrated that PKC-mediated phosphorylation, however unlikely [17], was not being confused for ADP-ribosylation.

The region of exact sequence homology between NG and B-50 is restricted to a KIQASFRGH peptide, which contains the PKC phosphoacceptor site of both proteins (Ser_{34} and Ser_{41}, respectively), while the remaining homology in this region is due to single or clustered basic residues [6]. Since the phosphorylation sites are identical, and in homologous regions, it is reasonable to suggest that the ADP-ribosylation sites may also be identical, assuming ribosylation occurs in this region. The 20 residues centred around the phosphorylation sites of NG and B-50 each contain seven putative ADP-ribosylation sites (four lysine, two arginine, one histidine) [6]. In addition, both NG and B-50 have Cys residues that, while not strictly homologous, are in generally analogous locations in the extreme amino-terminus of each protein [6]. Palmitoylation of these Cys residues in B-50 are probably involved in the attachment of the protein to the cytosolic face of the plasma membrane [18], and consequently, ADP-ribosylation could have the functional implications of directing newly synthesised B-50 towards its correct subcellular localisation, or at attenuating the attachment of B-50 in situ. This argument is less compelling in the case of NG the subcellular local-

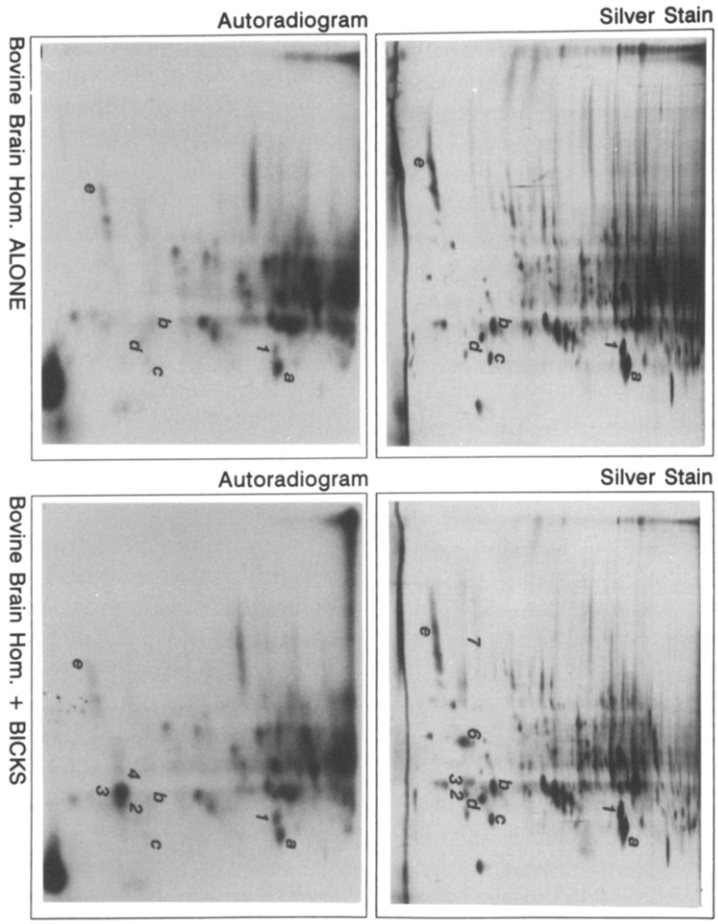

Fig. 3. Two-dimensional electrophoresis of ADP-ribosylated bovine brain homogenate in the presence and absence of BICKS (NG). The relevant spots on the 2D gels (pH 3.5–10.0, 15% SDS-PAGE) and autoradiograms (12 h, −80°C) are labelled as per the key in Fig. 2. ADP-ribosylated BICKS is clearly evident as an intense radioactive spot (spot 3, pI 4.6) in the autoradiogram derived from bovine brain homogenate incubated in the presence of purified BICKS.

isation of which appears to be entirely cytosolic [6]. Of course, the difference in the subcellular localisation of NG and B-50 may be due, at least in part, to a different level of ADP-ribosylation in each protein. It has been reported that the amino-terminus of B-50 binds to an α-subunit of the G-protein, G_0, and thereby regulates aspects of G-protein-mediated signal transduction [19–21]. This activity may extend to NG, and amino-terminal ADP-ribosylation of both proteins could affect this proven interaction of B-50 and hypothetical interaction of NG. Moreover, independent of either NG or B-50, G-protein-mediated signal transduction is thought to involve activation of ADP-ribosyltransferases [17].

The interaction CaM and NG, or B-50, can be attributed to essentially identical CaM binding domains in each protein, and the likely ability of this domain to adopt a basic amphiphilic α-helical secondary structure [22]. It is noteworthy that ADP-ribosylation of four out of six of the putative acceptor sites immediately carboxyl-terminal to the phosphorylation site in this domain would introduce a net negative charge into the region of greatest amphiphilicity. In a situation analogous to the phosphorylation of NG and B-50, ADP-ribosylation within the CaM binding domain may prevent binding, although this has yet to be established. Clearly, multi-site ADP-ribosylation distributed between the amino terminus and the CaM binding region is also possible, and results of preliminary studies suggest that this may be the case as α-chymotryptic digestion of [^{32}P]ADP-ribosylated B-50 gives rise to radiolabelled amino and carboxyl-terminal fragments following cleavage at a single aromatic residue (Zwiers et al., unpublished).

The generation of large quantities of radiolabelled ADP-ribosylated NG and B-50 for peptide mapping purposes has so far been unsuccessful, for a combination of technical reasons. Primarily, the bovine brain homogenate is a source of NADase, deribosylating, and protease activity, in addition to ADP-ribosyltransferase activity. Unfortunately, not all of the competing enzyme activities can be adequately inhibited and what appear to be lower affinity reactions are stimulated by an increase in the concentration of added NG or B-50. We are currently attempting to purify NG and B-50 ADP-ribosyltransferase activity from bovine brain. In contrast, bacterial toxins [23–25] are readily available but

neither cholera not pertussis toxin have shown significant NG or ADP-ribosylating activity in our hands (Zwiers et al., unpublished). This may be due to a lack of specificity for these two substrates, or it could be due to the lack of either a relevant ADP-ribosylation factor, an essential co-factor for certain transferases [25], or the lack of some less sophisticated, and yet essential, ingredient in the assay buffer.

The interaction of B-50 with G-protein subunits [20–22], the inhibition of polyphosphoinositide metabolism by p-B-50 [10], and the binding of dp-B-50 to CaM [4], have all contributed to the suggestion that the role of B-50-like proteins in the nervous system is to attenuate, if not regulate, neuronal signal transduction. The identification of a further post-translational modification (ADP-ribosylation) that applies to both NG and B-50 could be interpreted as supporting this view. Strictly speaking the regulatory hypothesis so far applies to B-50 alone, and it is precisely the identification of NG in concentrations equivalent to those of B-50 [6], but with a cytosolic localisation, that mitigates against suggestions that include, for example, the process of concentrating CaM at the plasma membrane by B-50 [11,22], since it is equally likely that cytosolic NG has the exact opposite effect. Perhaps the significance of post-translational modifications of NG and B-50 can be better appreciated within the context of proteins that undergo similar modification and are closely linked with neuronal architecture and routine metabolism, for example, tubulin and actin. ADP-ribosylation of tubulin leads to inhibition of microtubule assembly and depolymerization of assembled microtubules [26]. Similarly, ADP-ribosylation of non-muscle actin, the major component of the actin-rich submembraneous cortex [27], inhibits its ability to polymerize [28]. The functional consequence of this kind of post-translational modification on these proteins is difficult to define if the explanation is limited solely to molecular changes at the protein level, since each tubulin or actin molecule is part of a larger entity that is invested with the function – the microtubule or membrane skeleton. Therefore, the functional consequences tend to be descriptions made at a gross cellular level; as an increase in neurite outgrowth, for example, which does little to explain the precise function of either tubulin or actin as essentially structural proteins with responsibility for transportation of essential neuronal materials, or the maintenance of neuronal shape. Similarly, it may not be possible to assign the function of NG or B-50 within the context of their susceptibility to a single post-translational modification. Therefore, the significance of B-50-like proteins in different subcellular compartments, being substrates for a wide variety of enzyme systems, and interacting with other proteins like CaM, may relate to the functional idea of molecular adaptability. In short, by virtue of being supremely regulatable, the B-50-like proteins may provide the molecular flexibility by which a simple repertoire of structural proteins and major signal transduction systems, that are otherwise common to most cell types, respond to a variety of stimuli that are characteristic of the neuronal environment. Consequently, we predict that more B-50-like proteins, with common susceptibilities to post-translational modification, will emerge in the future.

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