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Rapid report

Identification of NAP-22 and GAP-43 (neuromodulin) as major protein components in a Triton insoluble low density fraction of rat brain

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Abstract

NAP-22 is a membrane-localized brain enriched acidic protein having a Ca^{2+}-dependent calmodulin binding activity. Further fractionation of the NAP-22 containing membrane showed the localization of NAP-22 in a Triton insoluble fraction of low density. Besides NAP-22, this fraction was found to contain GAP-43 (neuromodulin), trimeric G proteins, and some GPI-anchored proteins such as Thy-1 and N-CAM-120. Presence of some protein tyrosine kinases, such as src and fyn, was also shown.

Keywords: GAP-43; Neuromodulin; NAP-22; G protein; Triton insoluble; Membrane; (Rat brain)

NAP-22 is an acidic protein expressed predominantly in brain [1]. Physicochemical analysis of this protein showed its resemblance to two well known C-kinase substrates, GAP-43 (neuromodulin, F1, B-50, p46, p57) and MARCKS (myristoylated alanine rich C-kinase substrate, p80, p87), such as heat stability, solubility in 2.5% perchloric acid, low hydrophobicity, and having an acidic isoelectric point [1–3]. NAP-22 binds calmodulin in a Ca^{2+}-dependent manner and this binding is inhibited by phosphorylation with protein kinase C [4]. Biochemical extraction of NAP-22 from rat brain indicated its localization in the membrane fraction despite the very hydrophilic amino-acid sequence of the protein. Myristoylation of this protein in its N-terminal, shown using a vaculo virus expression vector system, partly explained its membrane localization [5]. An extraction experiment however also showed that most of the NAP-22 remains in the insoluble fraction even after a treatment with a solution containing Triton X-100, an unexplainable result from simple membrane localization through a myristoyl moiety [1]. To elucidate the localization mechanism of NAP-22, we first tried to isolate and characterize the components of the NAP-22 containing structure.

Rat brains were homogenized in a low ionic strength medium (TME: 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, at pH 7.8) containing 2% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. One half volume of 2.4 M sucrose solution was added to this fraction and the mixed sample was placed in centrifuge tubes. TME was overlaid and the

Abbreviations: TME, 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA (pH 7.8); TIF, Triton insoluble fraction of low density; GPI, glycosylphosphatidylinositol.

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sample was centrifuged at 70 000 × *g* for 6 h at 2°C using a swing rotor. A membrane fraction concentrated at the interface of TME and the sample solution was collected and recovered as a pellet after dilution with TME containing 1% Triton X-100. This fraction was suspended in TME containing 0.8 M NaCl and centrifuged again. The pellet fraction was suspended in TME containing 1 M sucrose and placed in the bottom of the centrifuge tube after the sucrose concentration was adjusted to 0.8 M. A continuous sucrose gradient from 0.8 M to 0.3 M was overlaid on this solution and the tube was centrifuged at 70 000 × *g* for 12 h. A white layer of membrane was observed at about 0.55 M sucrose concentration and this was collected. After dilution, this fraction was recovered as the Triton insoluble fraction of low density (TIF) by centrifugation. About 1.6% of the total protein was recovered in the TIF from 2–3 weeks old rat brain. Fig. 1 shows a preparation protocol of this fraction and Fig. 2a shows the protein components of this fraction analyzed with SDS-PAGE using 10% and 12% acrylamide gels. Four major protein bands were observed in the middle region of the 10% gel and, owing to an anomalous behavior of NAP-22 [1], one of the major bands seen in the 10% gel was split into two bands in the 12% gel. Immunoblotting analysis and a two dimensional gel electrophoresis showed the presence of tubulin, NAP-22, GAP-43 (neuromodulin), and actin in this fraction (Fig. 2). Two protein bands which did not react with these antibodies were excised from gels and partial amino-acid sequences were analyzed after protease digestion and HPLC separation of the digests. The obtained amino-acid sequences showed the identities of these proteins as the α-subunit of trimeric G protein Go, and the β-subunit of trimeric G proteins (Fig. 3). In case of epithelial or immunological cells, the Triton insoluble low density fraction was recently reported to contain many signal transducing proteins such as trimeric G proteins, src family protein tyrosine kinases, cytoskeletal proteins, and some glycosylphosphatidylinositol (GPI)-anchored proteins [6–9]. In addition to GAP-43, NAP-22, and the trimeric G proteins, the presence of src and fyn was also observed in the brain derived fraction (Fig. 4). The presence of some GPI-anchored proteins such as N-CAM and Thy-1 was also detected. Comparing the immunoreactivity of fraction 2 with those of fractions

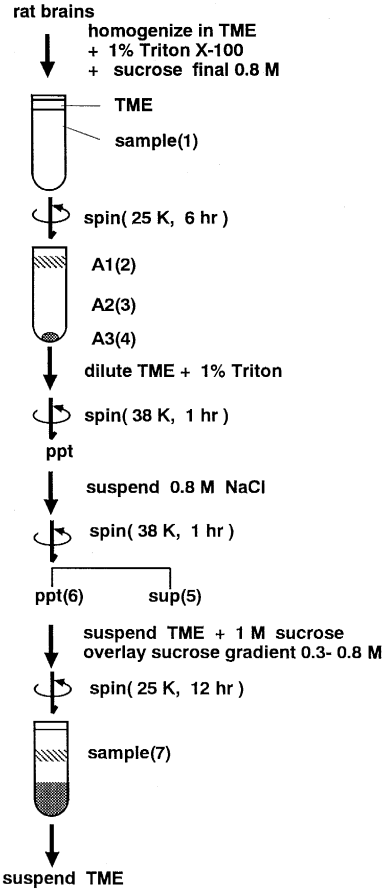

Fig. 1. A flow chart of the preparation of the Triton-insoluble low density fraction (TIF) from rat brain. Fractions obtained during preparation are numbered in parentheses. First sucrose density gradient centrifugation was done to separate the homogenate (1) to three fractions: Triton insoluble low density fraction, A1 (2); Triton soluble fraction, A2 (3); and Triton insoluble high density fraction, A3 (4). Soluble proteins were also recovered in the Triton soluble fraction, A2 (3). Fraction A1 was collected and recovered as a pellet after dilution with TME containing 1% Triton X-100. The pellet was homogenized with a solution containing 0.8 M NaCl and centrifuged to separate the supernatant (5) and pellet (6). The NaCl insoluble fraction was applied to a continuous sucrose centrifugation and a membranous fraction recovered at a low density region was collected as TIF (7).

3 and 4, localization of NAP-22 and Thy-1 in TIF was evident (Fig. 4). In contrast to some enrichment of fyn and the α-subunit of trimeric G protein Gi, little enrichment of src and N-CAM in TIF was observed. Phosphatidylinositol-specific phospholipase C (PI-PLC) is known to solubilize GPI-anchored proteins specifically [6,9]. Application of this enzyme to this fraction showed specific solubilization of a

couple of proteins, and immunoblotting with specific antibodies identified two of them as N-CAM and Thy-1 (data not shown).

Many signal transducing proteins were reported to be localized in a low density Triton insoluble fraction [6–9] and a recent study using mouse cerebellum showed the localization of some GPI-anchored proteins such as F3, N-CAM-120, and Thy-1 in a Triton insoluble low density fraction with an immunoblotting technique [8]. In this study, we showed that GAP-43, NAP-22, and Go protein are also present in this fraction prepared from whole rat brain. The accumulation of GAP-43 and Go protein in the growth cone membrane fraction is known and their biochemical interaction is well studied [10]. Our study described here shows that their possible interaction may not be restricted to the growth cone region. Localization of GAP-43 in the Triton insoluble fraction was reported previously and this localization was ascribed to the interaction of this protein with cytoskeletal protein such as actin filaments since the GAP-43 enriched fraction contains also actin and its regulatory proteins [11]. Our study showed the localization of GAP-43 in the Triton insoluble fraction of low density. Presence of actin was also shown in this fraction but the amount of actin was relatively low compared to that of GAP-43 (Fig. 2b) and electron microscopic observation showed little filamentous structures in this fraction (data not shown). Dialysis of this fraction to low ionic solution, a condition to depolymerize actin filaments, caused little effect in the solubility of GAP-43 (data not shown). These results suggest that the interaction of GAP-43 to the

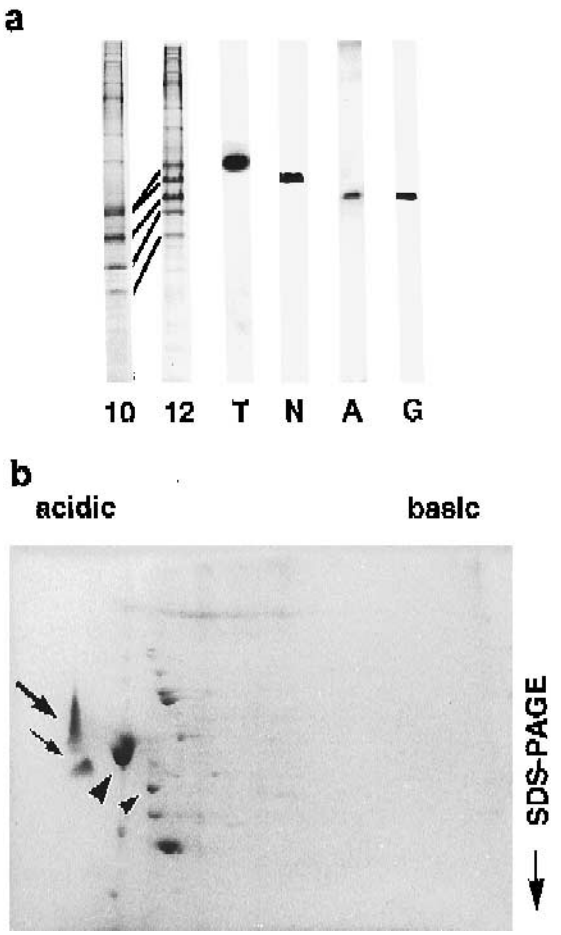

Fig. 2. Protein components of the TIF. (a) Each 5 μg protein was analyzed with SDS-PAGE using 10 or 12% acrylamide gels (lane 10 and 12, respectively) and stained with CBB. Corresponding protein bands are indicated with bars. Proteins separated using a 12% gel were transferred to a nylon sheet and blotted using anti-tubulin (T, Amersham), anti-NAP-22 (N, monoclonal antibody described in [1]), anti-actin (A, Amersham), and anti-GAP-43 (G, Transduction Lab) antibodies. Immunoblotting was done using ECL system (Amersham). (b) Proteins in TIF (120 μg) were analyzed with the two-dimensional gel electrophoresis using a 10% acrylamide gel for SDS-PAGE and stained with CBB as described previously [1]. NAP-22 (large arrow), GAP-43 (small arrow), tubulin (large arrowhead), and actin (small arrowhead) are shown.

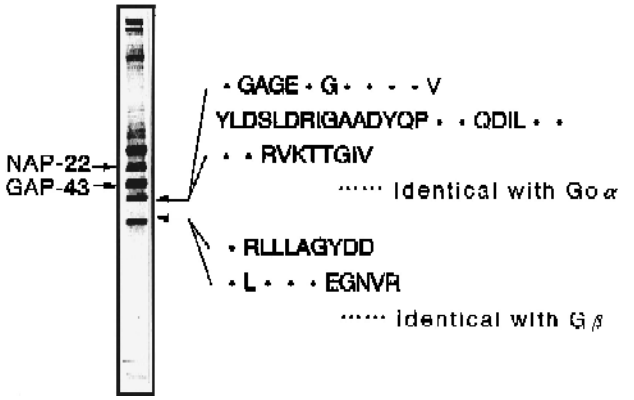

Fig. 3. Partial amino-acid analysis of two major bands in TIF. Protein bands were electrophoretically eluted from SDS-gels and digested with trypsin or lysyl endopeptidase (Wako, Japan). After HPLC fractionation, some peptides were sequenced with a peptide sequencer (ABI, 476A). These partial sequences were found in the reported sequences of Go α-subunit (accession No. 232135, and 120976) and β-subunit of G proteins (U34958, and U34959) of rat. A CBB-stained 12% acrylamide gel and partial amino-acid sequences derived from two of these bands are shown.

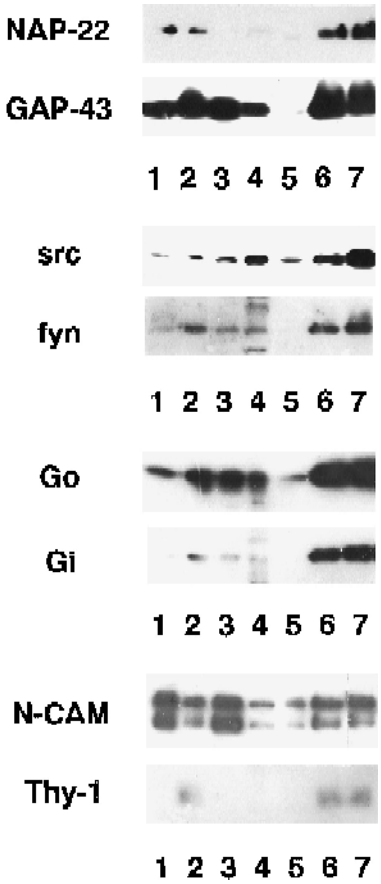

Fig. 4. Protein components in the TIF. Fractionation of NAP-22, GAP-43, and other proteins during the preparation of TIF is shown with immunoblotting using antibodies against NAP-22 [1], GAP-43, src and fyn (UBI), α-subunits of Gi and Go (kindly supplied from Dr. K. Saitoh, Suntory Institute for Bioorganic Research, Japan), N-CAM (Sigma), and Thy-1 (Cedarlane). Lanes 1–7 correspond to the fraction numbers described in Fig. 1 and each 2 μg protein was electrophoresed using 12% acrylamide gels. Each antibody except for anti- N-CAM recognized one band having a predicted mobility in SDS-PAGE. Anti-N-CAM reacted with two bands and these were judged as N-CAM-140 (upper) and N-CAM-120 (lower) from their mobilities [8]. Only appropriate regions of the sheets are shown.

membrane components other than actin filaments may cause the localization of GAP-43 in this fraction, although the molecular mechanism is unclear at present. The TIF prepared from cultured neuronal cells showed similar SDS-PAGE pattern to that of TIF from whole brain, suggesting that these proteins are present in neuron (data not shown). Myelin membrane fraction and growth cone fraction, two well known brain-derived low density fractions [12,13], were prepared and further treated to obtain the TIF as described in Fig. 1. The TIF from myelin membrane fraction showed characteristic protein components but these proteins were not the major components in the brain derived TIF. In contrast, protein components in the growth cone TIF were basically the same as those of brain, suggesting further the neuronal origin of the brain derived TIF.

In epithelial and immunological cell membranes, the presence of Triton insoluble region is well recognized, although the precise molecular mechanisms of Triton insolubility and protein interactions in this fraction are unclear at present [6,7,9]. Presence of some cytoskeletal proteins in this fraction suggests the existence of a specific translocation system of this region within the cell. The co-existence of cell surface molecules such as GPI-anchored proteins and signal transducing molecules such as trimeric G proteins and protein tyrosine kinases suggests the possibility of the presence of a putative signal transducing region in neuronal cells, which region is proposed to exist in the membranes of epithelial and immunological cells. Further characterization of the components in this fraction will help to elucidate the signal transducing mechanisms of neuronal cells.

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