Possible involvement of the 440 kDa isoform of ankyrin$_B$ in neuritogenesis in human neuroblastoma NB-1 cells

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Abstract Two isoforms of brain ankyrin, 440 kDa and 220 kDa ankyrin$_B$, which are products of alternatively spliced pre-mRNA encoded by a single gene, are both expressed in human neuroblastoma NB-1 cells. Expression of the polypeptide and mRNA of the larger isoform increased upon induction of neurite outgrowth in NB-1 cells by dibutyryl cAMP, while those of the smaller isoform were unaffected. The expressed 440 kDa ankyrin$_B$ was concentrated at the tip of growing neurites and was partly co-localized with GAP-43. These results suggest that 440 kDa ankyrin$_B$ is one of the neuronal growth-associated proteins and provides an interesting example of gene regulation by alternative splicing in neuronal cells.

Key words: Ankyrin$_B$ (440 kDa); Neurite outgrowth; Growth-associated protein; Neuroblastoma cell; Human

1. Introduction

Ankyrins are a family of spectrin-binding proteins that link the spectrin/actin network to the cytoplasmic domain of integral proteins that include ion channels and cell adhesion molecules [1,2]. Ankyrins contain three domains; an N-terminal 89–95 kDa membrane- binding domain [3], a 62 kDa domain that binds spectrin [4] and a C-terminal domain that is the target of alternative splicing and is the most variable domain among different ankyrins.

Three different ankyrins are currently known to be expressed in brain tissue [1]: ankyrin$_R$, which is also expressed in erythrocytes; ankyrin$_B$, which is the major ankyrin in the brain; and ankyrin$_{node}$, which is localized in axonal initial segments and nodes of Ranvier of myelinated axons. Ankyrin$_B$ includes two isoforms of 220 kDa and 440 kDa which are products of alternatively spliced pre-mRNAs encoded by a single gene [5,6]. 440 kDa ankyrin$_B$ shares the same N-terminal and C-terminal domains as 220 kDa ankyrin$_B$ and contains, in addition, an inserted domain of 220 kDa located between the membrane/spectrin binding domains and the C-terminal domain [5–8]. The 440 kDa isoform is maximally expressed at an earlier stage of brain development where neurite outgrowth and synapse formation are actively conducted [9,10], and is possibly involved in these process. Several neuron-specific proteins such as GAP-43 [11] and SCG10 [12] have been shown to be intimately associated with neurite outgrowth and are designated neuronal growth-associated proteins.

The purpose of this study is to demonstrate the possible involvement of 440 kDa ankyrin$_B$ in neuritogenesis. Human neuroblastoma NB-1 cells, whose neurite extension can be stimulated by an external factor, dibutyryl cAMP [13], afford evidence that 440 kDa ankyrin$_B$ is also a neuronal growth-associated protein.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP and the multiprime DNA labeling system were from Amersham Corp. [^{125}I]Protein A was from ICN. Dibutyryl cAMP was from Sigma. All tissue culture media, sera and supplements were from Gibco.

2.2. Antibodies

Antibodies against human brain ankyrin were prepared as described [5] using recombinant proteins as antigens. An antibody raised against the recombinant protein corresponding to Region 2 in Fig. 1 recognizes both 440 kDa and 220 kDa isoforms and was used for immunoblot analysis. An antibody raised against Region 1 in Fig. 1 recognizes the 440 kDa isoform specifically and was used for immunocytochemical analysis. Anti-GAP-43 mouse monoclonal antibody was from Boehringer Mannheim and rabbit anti-mouse IgG, goat anti-rabbit IgG (TRITC-labeled) , goat anti-mouse IgG (FITC- labeled) and normal goat serum were from Sigma.

2.3. Cell culture

Human neuroblastoma NB-1 cells obtained from the Japanese Cancer Research Resources Bank were grown in 45% RPMI-1640 and 45% Eagle's minimum essential medium containing 10% fetal calf serum, 50 units/ml penicillin G and 50 μg/ml streptomycin sulfate and subcultured once a week at a split ratio of 1:6 [14,15]. For the stimulation of neurite outgrowth, they were cultured in the presence of 2 mM dibutyryl cAMP in poly-L-lysine coated plates for up to 5 days. The degree of neurite outgrowth, expressed as the total length of neurites in mm per cell in randomly chosen fields of phase contrast micrographs, was designated Neuritogenesis Index.

2.4. Immunoblot analysis

Cultured cells on plastic plates were washed once with Dulbecco's phosphate-buffered saline, mixed with SDS sample buffer, scraped from the plates and heated at 65°C for 20 min. Samples were electrophoresed on SDS-polyacrylamide gradient gels and were immunoblotted using [^{125}I]protein A to visualize antibodies as described by Davis and Bennett [16] after electrophoretic transfer to Immobilon P membrane (Millipore Corp.). In the case of the monoclonal antibody, rabbit anti-mouse IgG was used as a second antibody.

2.5. Northern blot analysis

Total RNA was extracted from cultured NB-1 cells using RNAzol methods (Cinna/Biotecx). Twenty mg of the isolated total RNA was fractionated by formaldehyde/1.2% agarose gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell, Inc.) as described [5]. Membranes were hybridized with ^{32}P-labeled ankyrin$_B$ cDNA corresponding to Region 2 and washed with 0.1 × SSC at 65°C before autoradiography.

2.6. Immunocytochemical procedures

Cells grown on coverglasses coated with poly-L-lysine were fixed with 4% formaldehyde for 15 min at room temperature and washed 3 times with PBS. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 10% normal goat serum and 1% bovine serum

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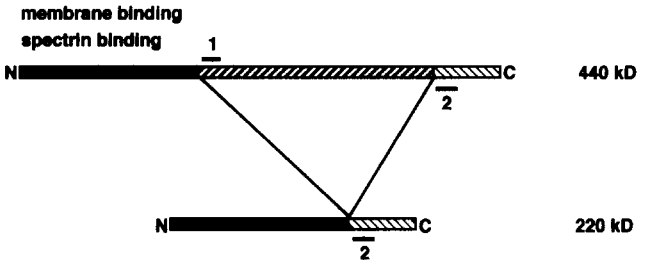

Fig. 1. Schematic organization of ankyrin$_B$ isoforms. Numbered bars mark the location of regions of the cDNA used to express proteins for preparation of antibodies and to prepare a probe for Northern blot analysis.

albumin in PBS for 30 min at room temperature. The coverglass was then incubated with 440 kDa ankyrin$_B$ antibody and GAP-43 antibody (4 μg/ml each), or with control IgG (4 μg/ml) in the presence of 0.05% Triton X-100 overnight at 4°C and washed five times with PBS containing 0.1% Tween 20. Ig molecules were visualized with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG.

3. Results

3.1. Induction of neurite outgrowth in NB-1 cells

While NB-1 cells extend neurites spontaneously, dibutyryl cAMP at 2 mM further stimulates neurite outgrowth [13] (Fig. 2). The Neuritogenesis Index was increased from 2.4 ± 0.4 μm/cell (mean ± S.D.) at day 1 to 30.7 ± 4.3 μm/cell at day 5 in the presence of dibutyryl cAMP at 2 mM, while it was also increased from 1.7 ± 1.1 μm/cell at day 1 to 11.5 ± 2.7 μm/cell at day 5 even in the absences of dibutyryl cAMP. Therefore, NB-1 cells cultured for 5 days in the presence of dibutyryl cAMP were compared with those cultured for 1 day with dibutyryl cAMP as a control to examine the effects of induction of neurite outgrowth on the expression and localization of ankyrin$_B$ isoforms.

3.2. Expression of ankyrin$_B$ isoforms in differentiating NB-1 cells

Two isoforms of brain specific ankyrin, 440 kDa and 220 kDa ankyrin$_B$, are both detected in NB-1 cells by immunoblot analysis. The amount of 440 kDa ankyrin$_B$ increased during a 5-day culture in the presence of 2 mM dibutyryl cAMP, while that of 220 kDa ankyrin$_B$ remained almost unchanged (Fig. 3A). Expression of GAP- 43 [11,17,18] was also increased upon induction of neurite outgrowth with dibutyryl cAMP. Messenger RNA (13 kb) encoding 440 kDa ankyrin$_B$ was increased in differentiated NB-1 cells, while mRNA (9 kb) encoding 220 kDa ankyrin$_B$ was nearly unaffected (Fig. 3B).

3.3. Localization of 440 kDa ankyrin$_B$ in differentiating NB-1 cells

Localization of 440 kDa ankyrin$_B$ in differentiating NB-1 cells was examined by indirect immunofluorescent microscopy using a 440 kDa ankyrin$_B$ specific antibody. In differentiated cells with long and complex neurites, 440 kDa ankyrin$_B$ is localized at the tip of neurites, along the neurite and plasma membrane of cell bodies (Fig. 4A,B). About 80% of the tips of growing neurites were strongly stained with the 440 kDa ankyrin$_B$ specific antibody. Small population of cells is still poorly differentiated even after the 5-day culture in the pres-

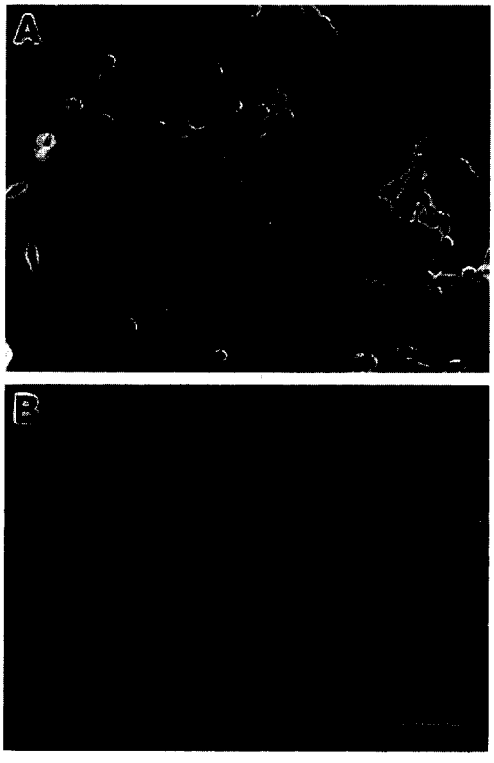

Fig. 2. Dibutyryl cAMP stimulates neurite outgrowth in NB-1 cells. NB-1 cells were cultured for 1 day (A) or 5 days (B) in the presence of 2 mM dibutyryl cAMP. Bar = 40 μm.

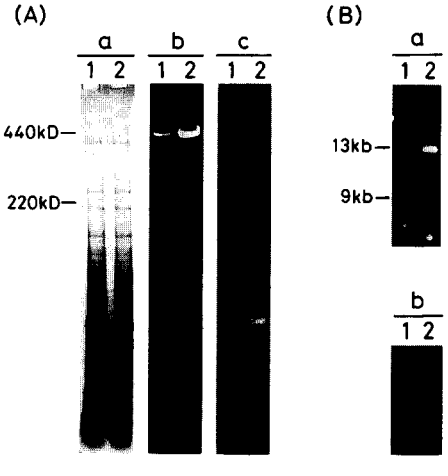

Fig. 3. An up-regulation of 440 kDa ankyrin$_B$ associated with neurite outgrowth in NB-1 cells . (A) Total proteins of NB-1 cells cultured for 1 day (lane 1) or 5 days (lane 2) in the presence of 2 mM dibutyryl cAMP were separated on SDS-polyacrylamide gel, transferred to Immobilon-P membrane and immunoblotted using ankyrin$_B$ antibody raised against Region 2 in Fig. 1, which recognizes both 440 kDa and 220 kDa isoforms (b) and monoclonal antibody against GAP-43 (c). Coomasie blue stained gel is also shown (a). (B) Twenty micrograms each of total RNA isolated from NB-1 cells cultured for 1 day (lane 1) or 5 days (lane 2) in the presence of 2 mM dibutyryl cAMP were separated on formaldehyde/1.2% agarose gel and transferred to nitrocellulose membrane. The blot was hybridized with ^{32}P-labeled cDNA probe derived from Region 2 in Fig. 1 (a). Ethidium bromide stained bands of 18S RNA are also shown (b).

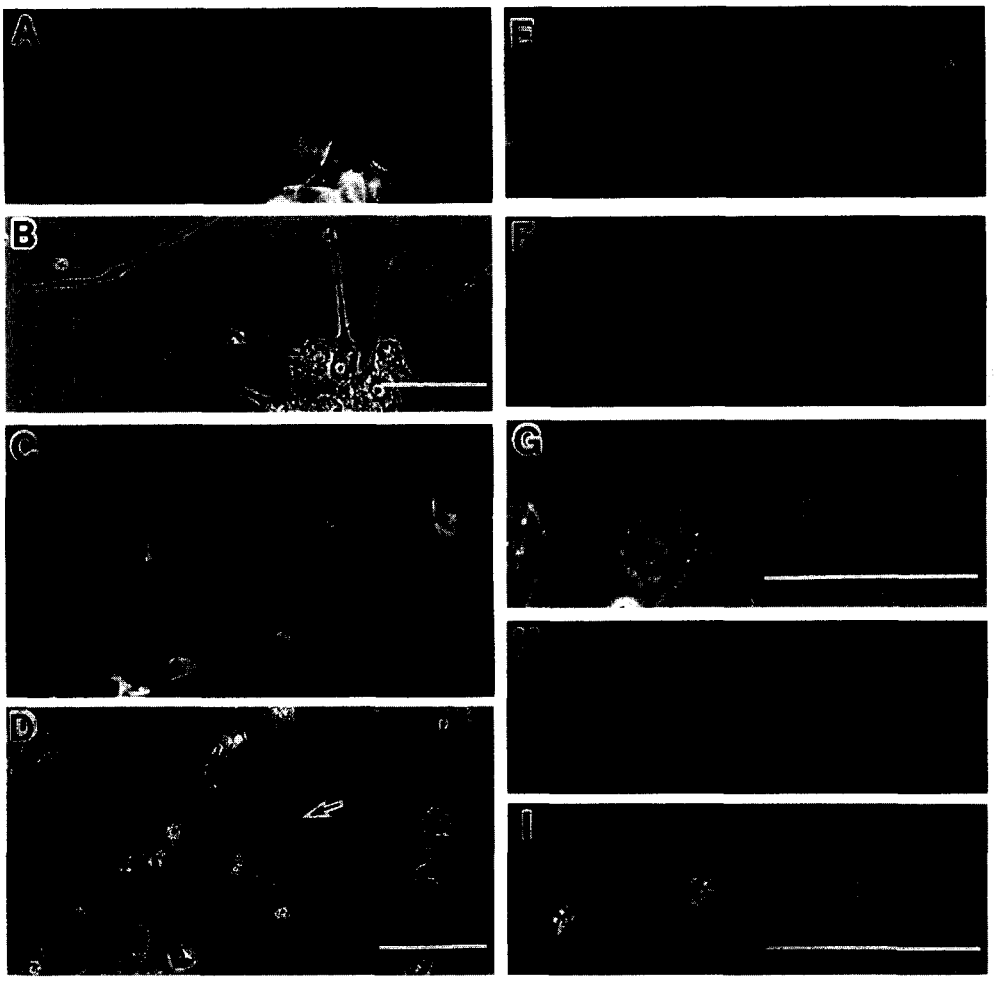

Fig. 4. Localization of 440 kDa ankyrin$_B$ and GAP-43 in differentiating NB-1 cells. NB-1 cells cultured for 5 days in the presence of 2 mM dibutyryl cAMP were labeled by indirect immunofluorescence with 440 kDa ankyrin$_B$ specific antibody (A,C,E), monoclonal antibody against GAP-43 (F) or control IgG (H) and photographed under fluorescent microscopy. Corresponding phase-contrast micrographs (B,D,G,I) are also shown. Arrows (C,D) indicate the cells which differentiated poorly and were immunostained weakly. Bars = 40 μm.

ence of dibutyryl cAMP and shows much weaker staining with the 440 kDa ankyrin$_B$ antibody than the differentiated cells (Fig. 4C,D, indicated by arrows). Intense staining of GAP-43, a well-established marker for the axonal growth cone, was observed at about 41% of the tips of growing neurites, essentially all of which were also strongly stained with the 440 kDa ankyrin$_B$ specific antibody (Fig. 4E–G).

4. Discussion

Expression of brain-specific isoforms of ankyrin, 440 kDa and 220 kDa ankyrin$_B$, is differentially regulated in the developing brain [5]. 220 kDa ankyrin$_B$ is the major isoform in adult brain, while 440 kDa ankyrin$_B$ is maximally expressed in developing neonatal rat brain, with a peak at postnatal day 10, and decreases to about 30% of the maximal level in adult brain [5]. Expression of neuronal growth-associated proteins like GAP-43 and SCG10, which are shown to be intimately associated with neurite outgrowth in neuronal cells both in vivo and in vitro [11,12,17,18], is also regulated in the manner similar to that of 440 kDa ankyrin$_B$ during the brain development [12,19].

Human neuroblastoma NB-1 cells express both 440 kDa and 220 kDa ankyrin$_B$. Upon induction of neurite outgrowth in the cells, expression of 440 kDa ankyrin$_B$ and GAP-43 was selectively increased and part of them were colocalized at the growth-cone like structure. Furthermore, NB-1 cells with few growing neurites showed little staining with the 440 kDa ankyrin$_B$ specific antibody (Fig. 4,B,C). These results clearly indicate that 440 kDa ankyrin$_B$ is one of the neuronal growth-associated proteins.

440 kDa ankyrin$_B$ is a component of the membrane skeleton and its membrane binding domain can interact with a neuronal cell adhesion molecule related to neurofascin and L1 [2], some of which are shown to exist at the growth cone [20]. It is therefore likely that 440 kDa ankyrin$_B$ is providing a specialized linkage between membrane skeleton and integral membrane proteins including cell adhesion molecules at the growth cone.

During the up-regulation of 440 kDa ankyrin$_B$ associated with neurite outgrowth in NB-1 cells, polypeptide and mRNA of the 220 kDa isoform remained almost unchanged (Fig. 3). Two mRNAs of 13 kb and 9 kb, encoding 440 kDa and 220 kDa ankyrin$_B$, respectively, are generated from the same pre-mRNA by alternative splicing [5,6]. Therefore, the selective

increase of 13 kb mRNA could be brought about either by selective stabilization of the 13 kb mRNA, as reported in the case of NGF and GAP-43 mRNA [21], or by preferential splicing to produce the mRNA [22], in addition to transcriptional up-regulation. Although nothing is known about genomic sequences of ANK2 (the gene encoding ankyrin$_B$), including the promoter region and intron– exon arrangement, the above results suggest that the gene may have DNA elements responsive to cAMP and may provide a unique example of alternative splicing in neuronal cells.

In conclusion, the expression of polypeptide and mRNA of 440 kDa ankyrin$_B$ in human neuroblastoma NB-1 cells is intimately associated with the outgrowth of neurites. 440 kDa ankyrin$_B$ thus can be regarded as a member of the neuronal growth-associated proteins and may provide an interesting example of gene regulation by alternative splicing in neuronal cells.

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