Effect of Dominant-Negative Expression of L1 Cytoplasmic Domain on the Localization of Brain Ankyrins in Human Neuroblastoma NB-1 Cells

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Two isoforms of brain ankyrin, 440 kD and 220 kD ankyrin$_B$, and neuronal cell adhesion molecule L1, which can bind to brain ankyrin, are both expressed in human neuroblastoma NB-1 cells. To examine the potential importance of the interaction between brain ankyrins and L1 in neuronal cell functions, an expression vector encoding a cytoplasmic domain of L1 has been constructed and transfected in NB-1 cells. The results obtained show that the expressed L1 cytoplasmic domain is ineffective in perturbing the interaction between brain ankyrins and L1, possibly because it exists in a monomeric form.

Key words —— brain ankyrin, cell adhesion molecule L1, neuroblastoma cell

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] Ankyrin$_B$, which is the major ankyrin in the brain, includes two isoforms of 220 kD and 440 kD which are products of alternatively spliced pre-mRNAs encoded by a single gene.[3,4] The 440 kD isoform is maximally expressed at an earlier stage of brain development[3] when neurite outgrowth and synapse formation actively occurs, and has been suggested to be involved in these processes[5,6] like neuronal growth-associated protein (GAP)-43.[7] Human neuroblastoma NB-1 cells, in which neurite extension can be stimulated by an external factor, dibutyryl cAMP,[8] afford evidence that 440 kD ankyrin$_B$ is also a neuronal growth-associated protein.[9–11]

The membrane-binding domain of brain ankyrin can bind to the cytoplasmic domains of the neurofascin/L1/NrCAM family of cell adhesion molecules in the nervous system.[12,13] Recently, we reported that the localization of L1was similar to that of 440 kD ankyrin$_B$ in the brain tissue, while it was similar to that of 220 kD ankyrin$_B$ in cultured neurons, suggesting that the interaction of L1 with brain ankyrins in neurons is affected by their environment.[14]

The purpose of this study is to examine the potential importance of the interaction between brain ankyrins and L1 in neuritogenesis and cell morphology. For that purpose, an expression vector encoding a cytoplasmic domain of L1 has been constructed to inhibit competitively the binding of brain ankyrins to L1 in NB-1 cells. The results obtained show that the expressed L1 cytoplasmic domain is ineffective in perturbing the interaction between brain ankyrins and L1, possibly because it exists in a monomeric form.

MATERIALS AND METHODS

Materials —— An antibody against human brain ankyrin was prepared as described[3] using recombinant protein as an antigen. This antibody recognizes both the 440 kD and the 220 kD isoforms. Anti-HA and anti-Myc mouse monoclonal antibodies were 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. All tissue culture media, fetal bovine sera, and supplements were from GIBCO BRL (MO, U.S.A.).

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 contain-

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ing 10% fetal bovine serum, 50 units/ml of penicillin G, and 50 μg/ml of streptomycin sulfate and subcultured once a week at a split ratio of 1 : 6.[9,15] For transfection of expression vectors, NB-1 cells were cultured in poly-L-Lysine-coated chamber slides (Lab-Tek [8 well], Corning-Costar).

Construction and Transfection of a Vector Expressing the L1 Cytoplasmic Domain —— Complementary DNA corresponding to the human L1 cytoplasmic domain was obtained through reverse transcriptase (RT)-PCR using total RNA from NB-1 cells as a template. Primers for RT-PCR were designed so that the cDNA contains the HA-tag sequence at the 5′-end in the frame. Obtained cDNA was sequenced to confirm the absence of mutation and then subcloned into a multiple cloning site of an expression vector (pcDNA 3.1, Stratagene). For transient expression of the L1 cytoplasmic domain bearing the HA-tag, NB-1 cells precultured in the chamber slides for 2 days were treated with OPTI-MEM containing plasmid DNA at 0.1 μg/ml and Lipofectin (GIBCO BRL) for 6 hr. The cells were then cultured in the presence of 10% fetal bovine serum for up to 72 hr.

Immunocytochemical Procedures ——Cells grown on cover glass coated with poly-L-Lysine were fixed with 4% formaldehyde for 15 min at room temperature and washed 3 times with phosphate-buffered saline (PBS). Fixed cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 10% normal goat serum and 1% bovine serum albumin in PBS for 30 min at room temperature. The cover glass was then incubated with ankyrin$_B$ antibody and HA or Myc antibody (4 μg/ml each) in the presence of 0.05% Triton X-100 overnight at 4°C and washed five times with PBS containing 0.1% Tween 20. IgG molecules were visualized with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG and observed under a confocal laser microscope (TCS4D, Leica).

RESULTS AND DISCUSSION

Two isoforms of brain ankyrin, 440 kD and 220 kD ankyrin$_B$, and neuronal cell adhesion molecule L1, which can bind to brain ankyrin, are both expressed in human neuroblastoma NB-1 cells.[9] To examine the potential importance of the interaction between brain ankyrins and L1 in neuronal cell functions, an expression vector encoding a cytoplasmic domain of L1 was constructed (Fig. 1) and transfected to NB-1 cells. L1 cytoplasmic domain bearing HA-tag was successfully expressed in NB-1 cells. Immunocytochemical staining of the transfected cells revealed intense staining of L1 in the cytosol, which completely overlapped with the staining of HA (Fig. 2A and 2B). The expression of the L1 cytoplasmic domain was evident as early as 24 hr after transfection and lasted for 72 hr at least after transfection (data not shown).

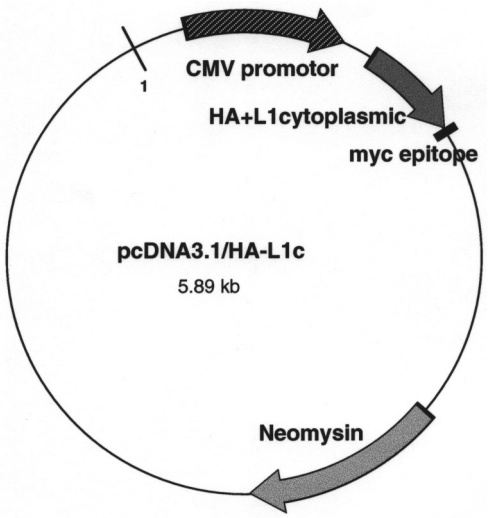

Fig. 1. Organization of the Expression Vector Encoding the L1 Cytoplasmic Domain, pcDNA3.1/HA-L1c

Complementary DNA corresponding to the L1 cytoplasmic domain bearing the HA-tag sequence at the 5′ end was inserted into the multiple cloning site of pcDNA 3.1.

Next, the effects of dominant negative expression of the L1 cytoplasmic domain on the cell morphology and localization of brain ankyrins in NB-1 cells were examined. Transfected cells were stained with double-label immunofluorescence using anti-HA and anti-ankyrin$_B$ antibodies. As a transfection control, pcDNA 3.1 was used and detected using anti-Myc antibody. The morphology and ankyrin$_B$ staining of the cells with intense staining of anti-HA did not show any significant difference from those with intense staining of anti-Myc (Fig. 2C and 2E, respectively). There were some rounded cells with poor neurites among the cells with anti-HA staining but the incidence was not significantly different from that among cells with anti-Myc staining (data not shown).

These results indicate that the dominant negative expression of the L1 cytoplasmic domain does not affect either the localization of brain ankyrins or the neurite extension in NB-1 cells significantly. It has been shown that the interaction of L1 with brain ankyrin requires dimerization of L1.[16] In our experi-

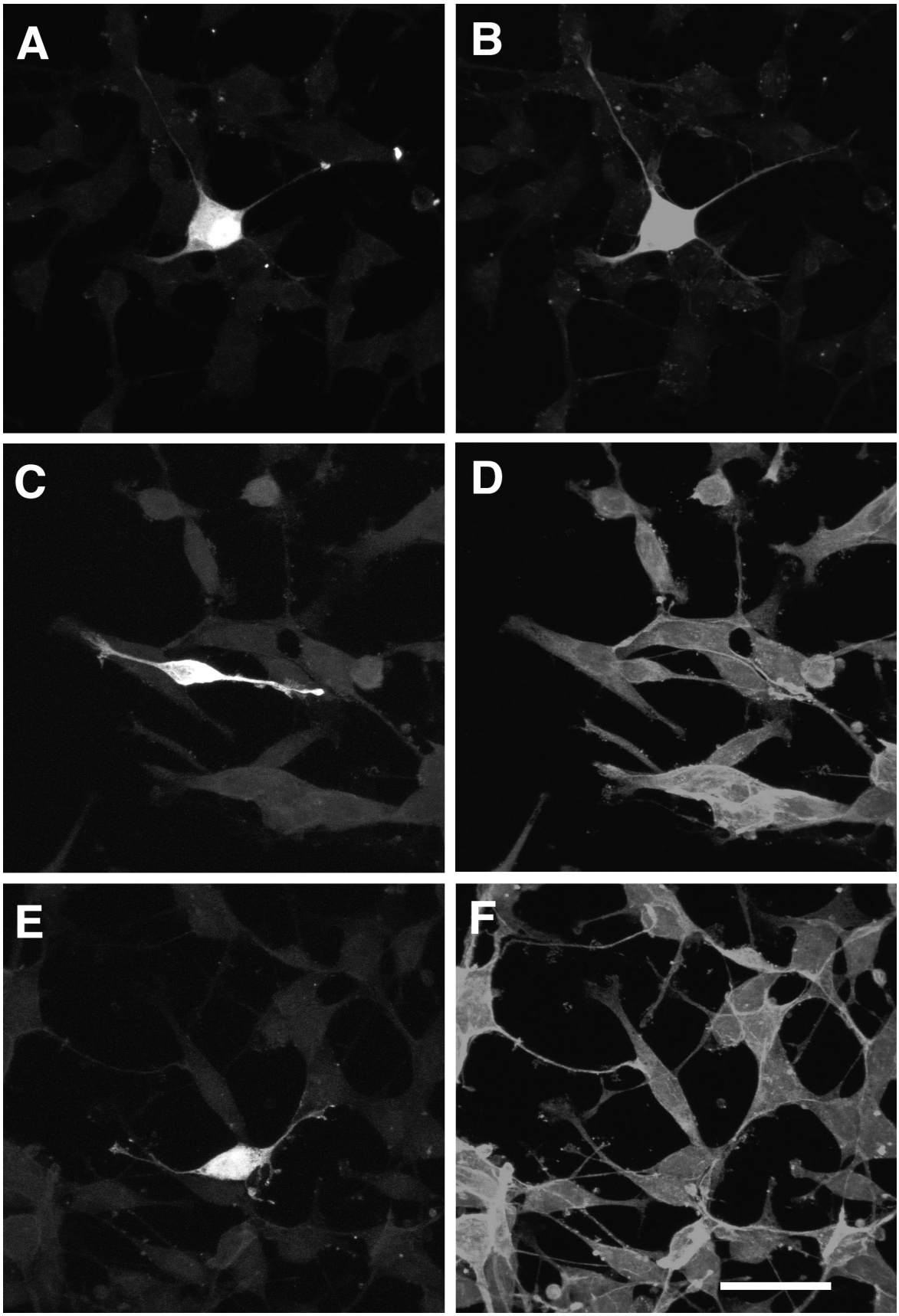

Fig. 2. Immunocytochemical Localization of Ankyrin$_B$ Isoforms and L1 in Human neuroblastoma NB-1 Cells

NB-1 cells cultured for 2 days after transfection with pcDNA3.1/HA-L1c or control vector (pcDNA3.1) were stained with double-label immunofluorescence using anti-HA (A, C) or anti-Myc antibody (E), and anti-L1 (B) or anti-brain ankyrin antibody (D, F), simultaneously. Scale bar, 40 μm.

mental system, the expressed the L1 cytoplasmic domains are supposed to exist as a monomer, which may be the reason why the dominant negative expression of the L1 cytoplasmic domain does not affect either the localization of brain ankyrins or morphology of NB-1 cells. To confirm these results, a sophisticated strategy to dimerize the L1 cytoplasmic domains in NB-1 cells by microinjecting anti-HA antibody or other dimerizing agents is essential.

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