Abstract  p67 (Munc-18), is a neuron-specific protein of 67 kDa, known for its ability to bind with syntaxin and also to copurify with neuronal cdc2-like kinase. Earlier, in situ hybridization and immunocytochemical analysis of rat trigeminal ganglion and hippocampal cells demonstrated the specific localization of p67 in nerve cells and its rich distribution in axons. In the present study, we have looked for p67 expression in normal human brain and various neuroectodermal tumors. Immunohistochemical and Western immunoblot analysis of normal human brain tissue using antibodies against the N- and C-termini of p67 demonstrated the specific localization of this protein in postmitotic neurons but not in glia. Among neuroectodermal tumors, expression of p67 was observed in 100% of the tumors of neuronal origin studied, especially in the mature neuronal cell population of these tumors. Western immunoblot analysis of non-neuronal neuroectodermal tumors failed to reveal the expression of this protein in majority of cases. However, in gliomas and meningiomas, mild cytoplasmic immunohistochemical staining of neoplastic cells was noted in 64.7% and 25% of cases, respectively. Observed mild immunohistochemical staining of these tumors could be due to immunoreactivity to low molecular weight degraded products of p67, as seen on Western blot. The findings suggest that p67, by virtue of its ability to be expressed in postmitotic neurons of adult human brain and in tumors of neuronal origin, may serve as a molecular tool to understand the growth and differentiation of the nervous system in general.

Key words  p67 (Munc-18) · Neuroectodermal tumors · Marker · Differentiation

Introduction

p67 (Munc-18), also referred to as RabSec 1, M-Sec 1 and N-Sec1, was independently identified by two different groups while studying what appears to be the two disparate functions of neurons, i.e., the regulation of cytoskeletal protein phosphorylation by neuronal cdc2-like kinase [21, 22] and the mechanism of synaptic vesicle docking and fusion to presynaptic membrane [9, 10, 11, 16]. Earlier findings based on in situ hybridization and immunocytochemical analysis of trigeminal ganglia and hippocampal neurons suggested the specificity of p67 to neurons and its rich distribution to axons [20, 21], implying a role in structure and function of axons. On the other hand, the reported 59% amino acid homology of this protein to Caenorhabditis elegans unc-18 [6] and also a limited identity to yeast Sec-1, both known to be the components of exocytotic secretory mechanism, implicated a role for p67 (Munc-18) in neurotransmitter release [11]. Since then, there have been several reports on the role of this protein, both as a positive and negative regulator of vesicular docking and release mechanism [3, 17, 20, 28]. Interestingly, the absence of p67 in adrenal medulla [21, 22], a neuroendocrine gland known for its ability to synthesize and release neurotransmitter, suggests that this protein may not be the universal regulator of vesicle docking and release mechanism. Further, the opulent localization of p67 (Munc-18) to axons [21, 22] and also the observation that this protein co-purifies with neuronal cdc 2-like kinase [21, 22, 25] and cytoskeletal protein (Shareef et al., submitted) implies that p67 (Munc-18) could also have functions other than the reported regulatory role in vesicular docking and release mechanism [9, 10, 11, 16]. The observation that p67 is constitutively expressed in tumor cell lines derived from neuronal cell lineage (IMR-32, HCN-1, NG-16, H4 and SK-NSH) but not from glial origin (HTB-169, C6, U 138MG, U 118MG, A 172, CRL...
Normal human brain tissue

Western immunoblot analysis of human brain tissue extract with antibodies against both N- and C-terminal regions of p67 fusion protein.

Materials and methods

Tissues

Human brain tissues, obtained from three adults who succumbed to road traffic accidents, were collected within 6 h postmortem. During hospitalization, these patients received only conventional anti-edema measures and were on assisted ventilation. After postmortem study, the brains were washed free of blood with cold phosphate-buffered saline (PBS). The different anatomical areas selected for analysis were frontal cortex, basal ganglia, hippocampus, cerebellum, medulla oblongata and cervical spinal cord. Thin blocks of tissue from these areas were kept frozen at –70 °C until use for Western immunoblot analysis. Thin slices from the immediate adjacent zones were fixed in 10% formalin for paraffin processing and immunohistochemistry.

Fresh samples of tumor tissue (n = 72) were collected from resected neurosurgical specimens. The tissues were sliced into two, one half frozen for Western immunoblot analysis and the other half fixed in buffered formalin for paraffin processing. The various tumors analyzed, following the standard classification of brain tumors [12], included neuronal/glioneuronal and embryonal tumors (n = 14), neoplastic and choroid plexus tumors (n = 34), meningothelial and benign nerve sheath tumors (n = 24).

Antibodies

Polyclonal antibodies (1256) raised in rabbit, specific to N terminus [20, 22] and C terminus (Momin et al., submitted) of p67 were used. The specificity of N-terminal anti-p67 antibody was checked by immunodepleting the antibody with purified C-terminal truncated p67 fusion protein [22].

Tissue extraction

Tissues from different regions of human brains (n = 3) were collected at postmortem. Both the normal and tumor specimens (n = 72) were homogenized (1:10 w/v) in ice-cold 50 mM TRIS-HCl pH 7.4, 145 mM NaCl (TBS) containing protease inhibitors (1 mM PMSF, 0.2 mM AEBSF, 0.2 mM benzamidine and 1 μg/ml leupeptin). Homogenates were cleared by centrifugation at 30,000 g for 30 min at 4 °C and the supernatants were estimated for protein contents [1].

SDS-PAGE and Western immunoblot analysis

Protein samples (20 μg) were separated by SDS-PAGE on 12% gels [14] and blotted onto polyvinylidene difluoride (PVDF) membranes [24] followed by blocking of the membrane(s) for 30 min with TBS containing 0.25% Tween 20 (TBST). Membranes were incubated with rabbit anti-p67 antibody (N terminus; 1:5000) or anti-p67 (C terminus; 1:2000) diluted in TBST containing 0.25% bovine serum albumin (BSA) at room temperature for 90 min. After four wash cycles of 15 min each with TBST, the blots were incubated for 60 min with alkaline phosphatase conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, USA), diluted in TBST containing 0.25% BSA, followed by wash cycles of 15 min with TBST. Immunoblots were developed with BCIP and NBT (Sigma) chromogen system.

Immunohistochemical staining procedure

Paraffin sections (5 μm thick) mounted on poly-L-lysine-coated glass slides, were taken for the study. The sections were dewaxed in xylene, rehydrated by passing through a series of graded alcohols and rinsed in phosphate buffer (0.01 M, pH 7.2). Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide (Merck, India) in methanol for 30 min. Labeling was performed using polyclonal primary antibodies against the N- and C-termini of p67 (1:100) and monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:50) for 1 h at room temperature. The secondary antibodies (anti-rabbit or anti-mouse) conjugated with peroxidase (1:100) were used. The enzyme reactions were visualized using a substrate solution of 3,3′diaminobenzidine (DAB) and hydrogen peroxide. The sections were lightly counterstained with Harris’s hematoxylin, dehydrated, cleared in xylene and mounted with DPX (Fluka Chemika, Switzerland). Omission of the primary antibody served as the negative controls.

Analysis of immunohistochemical results

p67 expression was analyzed by immunohistochemistry independently by two observers (V.S. and S.K.S.) in a blinded fashion. There was general agreement between both the observers in the parameters studied. In the few discrepancies, a second evaluation course was done to arrive at an agreement. The specimens were scored visually as negative (–) if p67 was completely lacking or if less than 5% of tumor cells were labeled; mild (+) if immunoreactivity was found in small clusters of tumor cells or in diffusely scattered single cells amounting up to 25% of cells labeled; moderate (+++) if more than 5% but less than 25% of cells were positive; and intense (+++) if more than 50% of cells were positive. Statistical analysis in the groups of neuronal/embryonal, glial and meningothelial/schwannian tumors was carried out using the Chi-square test.

Results

Fig. 1 A, B Western blot analysis of p67 expression in different regions of the normal human brain tissue. A Immunoblot using N-terminal-specific anti-p67 antibody (1:5000). B Immunoblot using C-terminal-specific anti-p67 antibody (1:2000). Both the antibodies recognize the same protein band corresponding to 67 kDa (arrow) in all the regions.