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The calpain–calpastatin system is regulated differently during human neuroblastoma cell differentiation to Schwannian and neuronal cells

Yumiko Saito[a,*], Takaomi C. Saido[a], Kimihiko Sano[b], Seiichi Kawashima[a]

[a]*Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan*
[b]*Department of Pediatrics, Kobe University School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 550, Japan*

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Abstract Changes in expression of calpains and calpastatin during differentiation in GOTO cells were examined using antibodies specific to calpains and calpastatin. Neuronal differentiation induced by dibutyryl cyclic AMP elicited a remarkable decrease in m-calpain. No marked changes in the levels of calpains were found in bromodeoxyuridine-induced Schwannian differentiation. Calpastatin was detected as a single band of 110k in undifferentiated and in neuronally differentiated cells by Western blot analysis. However, the appearance of a 120k species was detected in Schwannian differentiation associated with morphological change. The data show that marked changes in m-calpain and calpastatin occur in a differentiation-specific manner.

Key words: Neuroblastoma; Differentiation; Calpain; Calpastatin

1. Introduction

Proteases have been implicated to be involved in various stages of neuronal development including glial proliferation, cell migration, and neurite outgrowth [1–3]. Many studies on calpains have been performed in the nervous system [4–10], but little is known about the roles of calpains in neuronal differentiation. In rat pheochromocytoma PC12 cells, cell-permeable calpain inhibitors cause initiation of neurite outgrowth without nerve growth factor (NGF) [11], and a relative decrease in calpain activity caused by an increase in calpastatin activity was observed during NGF-induced neurite outgrowth [12]. These results clearly suggest that the calpain–calpastatin system is associated with the neuronal differentiation process in the NGF-responsive PC12 cell line. It is not clear, however, whether quantitative and qualitative changes in calpains and calpastatin occur during neuronal differentiation of other cell lines and during other types of differentiation.

A human neuroblastoma cell line, GOTO, derived from adrenal gland tumors, has bipotential characteristics in differentiation; that is, treatment with bromodeoxyuridine (BrDU) causes the cells to differentiate into Schwannian cells [13,14], while withdrawal of serum from the culture medium induces neurite outgrowth and increases the level of neurofilament protein mRNA [15]. Thus, GOTO cells provide an attractive model system for studying the molecular details of Schwannian/neuronal cell differentiation. Since Schwannian/neuronal differentiation induced from precursor cells is accompanied by turnover of and profound changes in membrane proteins and cytoskeleton proteins which are the preferable substrates of calpain, we expected that the calpain–calpastatin system might be involved differently in each developmental pathway. To approach to this problem, we examined changes in the levels of m-calpain, μ-calpain, and calpastatin during Schwannian/neuronal differentiation in GOTO cells using specific antibodies to human calpains and calpastatin. The results show that there was a marked decrease in the level of m-calpain during neuronal differentiation, while a new species of calpastatin appeared during Schwannian differentiation.

2. Experimental

2.1. Materials

Antibodies specific to human m-calpain and μ-calpain 80k subunits were produced as described [16]. Monoclonal antibodies specific to human calpastatin were from Takara (Tokyo, Japan). RPMI 1640 medium was from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was from Cell Culture Laboratories (Cleveland, OH). Collagen type IV (0.3% solution in 1 mM HCl) derived from bovine placenta was obtained from Koken (Tokyo, Japan). Dibutyryl cyclic AMP (dbcAMP) was obtained from Yamasa (Chiba, Japan). Collagen type I-coated dishes were purchased from Corning. All unspecified chemicals were of analytical grade, and purchased either from Wako Pure Chemicals, Nacalai Tesque, or Sigma.

2.2. Cell culture

GOTO cells were cultured in RPMI medium supplemented with 10% FBS, 100 U/ml potassium penicillin G, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 on collagen type I-coated dishes.

2.3. Differentiation

Schwannian differentiation. Cells were plated at 1×10^4 cells/cm^2 on collagen type-I coated dishes. After 24 h incubation, cells were maintained in the medium with or without 5 μg/ml BrDU for 16 days with a change of medium every 72 h.

Neuronal differentiation. 0.1% solution of collagen type IV solution in 1 mM HCl derived from bovine placenta was spread as a film over the dish and allowed to dry at room temperature under ultraviolet irradiation for 1 h. Cells were plated at 0.25×10^4 cells/cm^2 on collagen type-IV coated dishes. After 24 h incubation, the medium was changed to fresh N2 medium and dibutyryl cyclic AMP (dbcAMP) was added at a concentration of 1 mM. N2 medium consists of the DMEM/Ham's F12 (1:1) base medium supplemented with 25 mM HEPES, 5 μg/ml insulin, 50 μg/ml transferrin, 5 μg/ml putrescine, 5 μg/ml progesterone, and 52 μg/ml sodium selenite. The medium was changed every other day.

2.4. Preparation of cell homogenates and subcellular fractions

All experimental operations were performed on ice. Cells were collected by centrifugation in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and washed twice with PBS. Cells were disrupted in a tightly fitting Dounce homogenizer in homogenizing buffer (100 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 100 μM phenylmethanesulfonyl fluoride, 100 μM diisopropyl fluorophosphate, 50 μM leupeptin, pH 7.2) until the nuclei became micro-

*Corresponding author. Fax: (81) (3) 5685 6609.

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scopically free. The homogenate was then ultracentrifuged at 100,000 × g for 60 min. The supernatants and the pelleted debris were termed the cytoplasmic fraction and membrane fraction, respectively. Nuclei were contained in the membrane fraction.

2.5. Immunoblot analysis

Cell homogenates prepared as described above were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nylon membranes. The membranes were treated with polyclonal antibodies specific

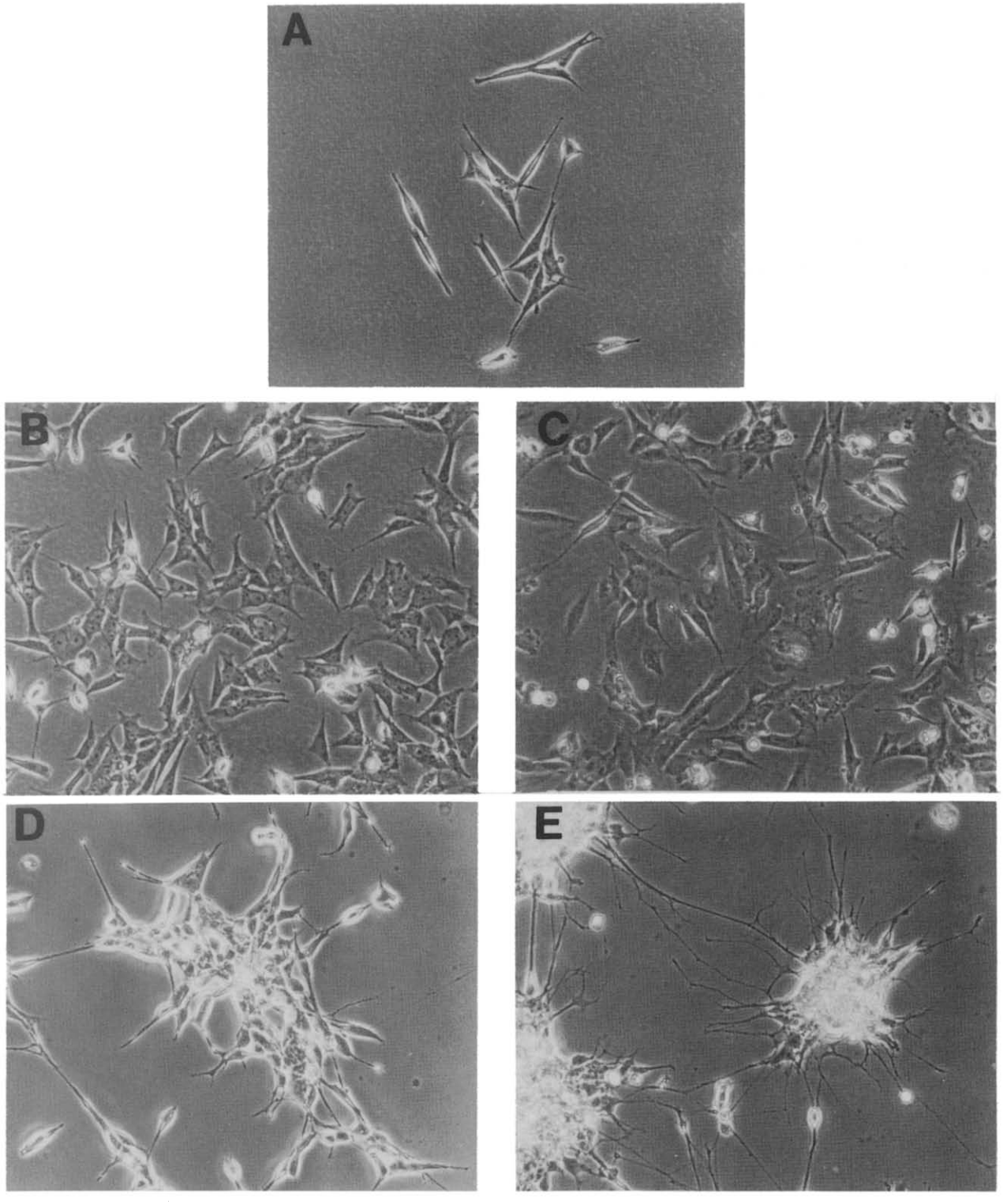

Fig. 1. Morphological appearance of the human neuroblastoma cell line GOTO. GOTO cells were cultured in the presence of (A) serum-containing growth medium, (B) 5 μg/ml BrDU for 2 days, (C) 5 μg/ml BrDU for 5 days, (D) 1 mM dbcAMP/N2 medium for 3 days, or (E) 1 mM dbcAMP/N2 medium for 6 days. Cells were photographed using an invert, phase-contrast microscope. × 200.

for the 80k subunit of human m-calpain or μ-calpain or a monoclonal antibody to human calpastatin domain III for 3 h at 37°C, and subjected to alkaline phosphatase staining. In some experiments, monoclonal antibodies to human calpastatin domains I and IV were used. The bands on the membranes were quantitated with a densitometer (ACI Japan. Digitizer 256).

3. Results

3.1. Morphological changes in GOTO cells

GOTO cells were small and spindle-shaped cells in serum-containing medium (Fig. 1A). Two days after treatment with BrDU, the cells began to enlarge and flatten, and possesed polygonal cytoplasm that adhered firmly to the substrate (Fig. 1B,C). GOTO cells grew slowly from 2 days and reached only 15% of control cells 7 days after treatment with BrDU. The viability of the BrDU-treated GOTO cells was 87.5 ± 3.4% (S.D.) during the period. It has been reported that BrDU induces an increase in the amount of S100 protein [13,14] and in the activity of cyclic nucleotide phosphodiesterase (CNPase) [13] in GOTO cells, suggesting that BrDU-treated GOTO cells have Schwannian characteristics. On the other hand, using a substratum coated with purified collagen type IV, N2 medium with dbcAMP induced the formation of large cellular aggregates (pseudoganglia) until 3 days (Fig. 1D). After the formation of cellular aggregates, the cells began to initiate neurite outgrowth, and finally the aggregates were interconnected by long neurites over 6 days (Fig. 1E).

3.2. Changes in calpain levels in GOTO cells during differentiation

Changes in the levels of m- and μ-calpains during Schwannian and neuronal differentiation detected by Western blot analysis are shown in Fig. 2. Schwannian differentiation induced by BrDU in GOTO cells caused a slight increase in the level of μ-calpain at 5 days (Fig. 2A), which returned to the original level after 16 days (data not shown). In contrast, neuronal differentiation induced by treatment with N2 medium/dbcAMP for 6 days caused a remarkable decrease in m-calpain immunoreactivity (Fig. 2A). Fig. 2B shows the time course for the change in the level of m-calpain using Western blot analysis. The bands on the membranes were quantitated by evaluating the stained area with a densitometer and expressed as the relative intensities to 0 day (100%). Areas under peaks were 101% after 2 days, 77% after 3 days, 67% after 4 days, 53% after 5 days, and 30% after 6 days. The change shows that the decrease in m-calpain is not associated with cellular aggregation occurring up to 2 days, but rather with the beginning of neurite formation starting at 4 days. The cells seeded on collagen type IV-coated dishes under serum-containing conditions showed no change in immunoreactivity to either anti-m-calpain or anti-μ-calpain over a period of 6 days.

3.3. Changes in calpastatin levels in GOTO cells during differentiation

To examine whether the decrease in m-calpain is accompanied by a change in calpastatin, calpastatin levels were analyzed by Western blot using an antibody specific to human calpastatin domain III. In non-treated GOTO cells, a single band of 110k calpastatin was detected. During neuronal differentiation, there was no substantial quantitative or qualitative change for 6 days in calpastatin after treatment with serum-free N2 medium/dbcAMP (Fig. 3A). Interestingly, however, Schwannian differentiation induced by BrDU for 5 days caused the appearance of a new form, 120k calpastatin (Fig. 3A). We next exam-

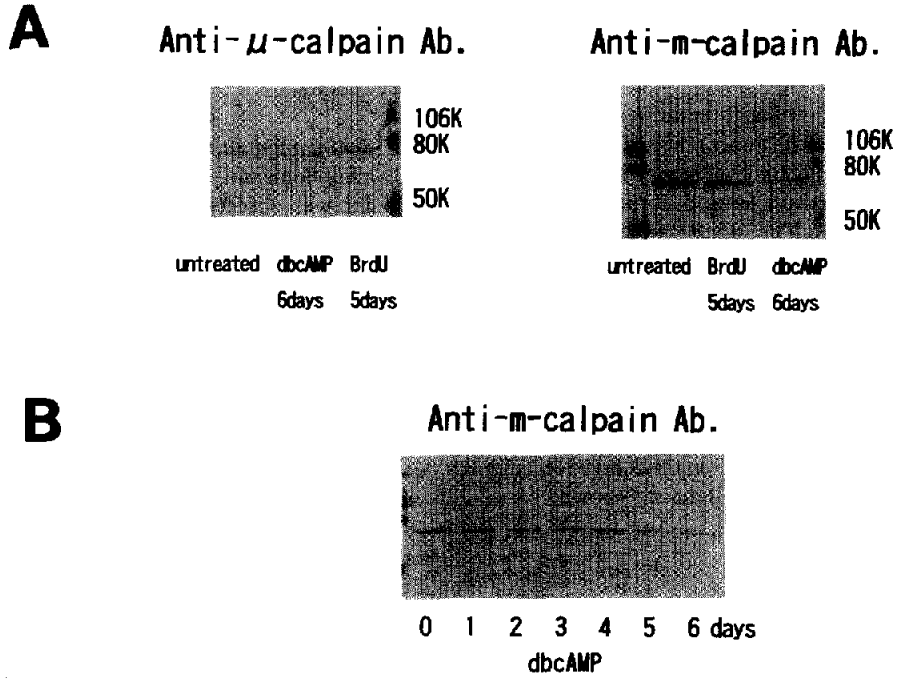

Fig. 2. Effect of BrDU or dbcAMP/N2 on the level of the calpains in GOTO cells as determined by immunoblotting. (A) GOTO cells were treated with 5 μg/ml BrDU for 5 days or 1mM dbcAMP/N2 medium for 6 days. The proteins (30 μg) from whole cells were separated on 10% SDS-polyacrylamide gels and transferred electrophoretically to nylon membranes. The membranes were treated with polyclonal antibodies specific to the 80k subunit of human m-, or μ-calpain and subjected to alkaline phosphatase staining. (B) GOTO cells were treated with 1 mM dbcAMP/N2 medium for the indicated number of days. Thirty micrograms of protein from whole cells on each day were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The nylon membranes were treated with m-calpain-specific antibody.

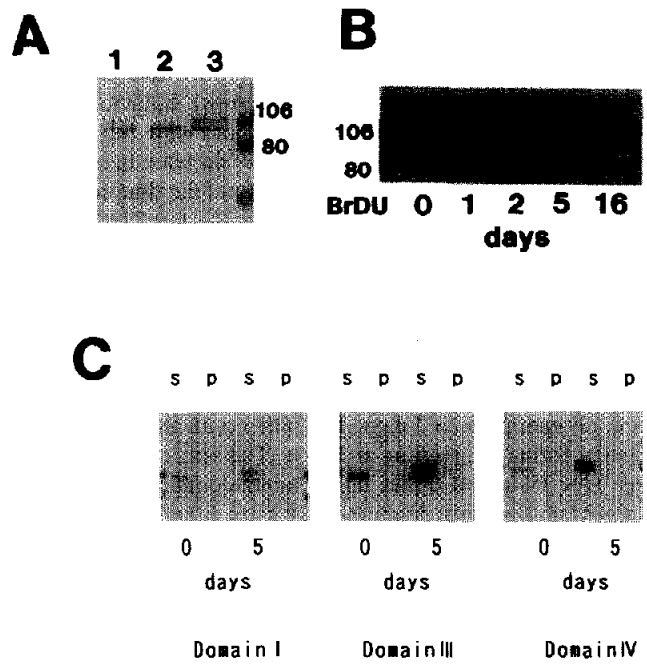

Fig. 3. Western blot analysis of calpastatin in GOTO cells after treatment with BrDU or dbcAMP/N2 medium. (A) GOTO cells were cultured in serum-containing growth medium for 6 days (lane 1), with 1 mM dbcAMP/N2 medium for 6 days (lane 2), or with 5 μg/ml BrDU for 5 days (lane 3). Thirty micrograms of protein from whole cells were subjected to 7.5% SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The nylone membranes were treated with monoclonal antibody to human calpastatin domain III. (B) GOTO cells were cultured in the presence of 5 μg/ml BrDU for the indicated number of days. Proteins from whole cells were analyzed as described in (A). (C) GOTO cells were collected and separated into fractions of cytoplasm (s) and membranes (p) after treatment with 5 μg/ml BrDU for 5 days. Each fraction was subjected to immunoblotting using antibodies to human calpastatin domains I, III and IV.

ined time-dependent changes in calpastatin during Schwannian differentiation in GOTO cells. The appearance of 120k calpastatin was noted faintly at 1 day. Its level reached a peak after 2 days, and this level remained unchanged during 16 days of treatment with BrDU (Fig. 3B). The subcellular localization of calpastatin is shown in Fig. 3C. Nearly all 110k and 120k calpastatins were located in the cytosol, and these calpastatins were recognized not only with an antibody against domain III but also with antibodies against domain I and IV (Fig. 3C). Immunocytochemical studies of GOTO cells in serum-containing medium showed that these calpastatins were distributed diffusely in the cytoplasm, but not in membranes nor nuclei; a more intense but similar staining pattern was observed in cells after treatment with BrDU for 5 days (data not shown).

4. Discussion

Using the combination of a bipotent differentiating model, GOTO, and antibodies specific to human calpains and calpastatin, we have demonstrated that the dynamic changes in the calpain–calpastatin system occur in a differentiation-specific mannner.

4.1. Neuronal differentiation in GOTO cells

During neuronal differentiation, the amounts of m-calpain markedly decreased. Since the change in the level in calpastatin was insignificant during neuronal differentiation, simple calculation would result in a decrease in net calpain activity. The decrease in the m-calpain level is not associated with cellular aggregation but rather with neurite outgrowth. Oshima et al. presented evidence that NGF causes a decrease in the activity of calpain in morphologically differentiating PC12 cells [12]. In our preliminary experiment, a functional calpastatin peptide caused an increase in the number and length of neurites when added together with dbcAMP/N2 medium in GOTO cells. These results suggest that the decrease in calpain activity may be directly related to neurite outgrowth, possibly by controlling available soluble pools of substrates such as cytoskeleton [12,17]. However, in PC12 cells, the decrease in calpain activity is not due to a decrease in calpain itself, but to an increase in the activity of calpastatin. Differences in the mechanisms for the relative decrease in calpain activity might reflect differences in neuronal cell types or in the pathway for neurite outgrowth; dbcAMP/N2 induces the formation of pseudoganglia and finally the aggregates are interconnected by long neurites in GOTO cells, while NGF induces two or three neurites from a single cell body leading finally to a complex branching pattern in PC12 cells.

The decrease in the amount of m-calpain leads neuronally-differentiated GOTO cells to be richer in μ-calpain than in m-calpain. This result obtained in cultured cells is consistent with many immunohistochemical studies in nervous tissue; that is, μ-calpain is found predominantly in neuronal perikarya rather than in glia [5–7]. Developmental switching of m-calpain in GOTO cells also corresponds well with the immunohistochemical observation reported by Hamakubo et al. [5] that m-calpain disappears in neuronal cells during rat brain development. Differences in the developmental pathways suggest differences in physiological function between calpain species, and it seems likely that μ-calpain plays a crucial role in neuronal cell function.

4.2. Schwannian differentiation in GOTO cells

During Schwannian differentiation, no dramatic change in calpains was observed, however, heterogeneity in calpastatin (110k and 120k) was detected by immunoblotting. Of particular interest is our observation that the appearance of the 120k form is characteristic for Schwannian differentiation and not detected in neuronal differentiation of GOTO cells and SH-SY5Y cells (data not shown). We confirmed that an increase in the immunoreactivity of S100αβ protein was not noted until 2 days of BrDU treatment, and intense staining was detected after 5 days (data not shown). These results show that the change in calpastatin is associated with cell flattening (Fig. 1B) but not with S100αβ protein expression.

The molecular diversity of calpastatin has been reported in other tissues and cell lines [18–22], but the present study is the first in which calpastatin heterogeneity is observed during differentiation at the cellular level. The brain has been found to contain two calpain-specific inhibitory fractions, CS-0.1, identified as an ordinary calpastatin, and CS-0.2, which has been only partially purified [18]. Interestingly, these fractions show developmental variations in postnatal mouse brain [23]. Since CS-0.2 contains proteins smaller than 110k and does not crossreact with polyclonal and monoclonal anti-calpastatin antibodies, neither 110k nor 120k calpastatin in GOTO cells corresponds

to CS-0.2. The diversity of calpastatin is generated whether at the posttranscriptional stage by exon skipping in domains L and I [20,22] or at the posttranslational stage, including proteolytic processing for erythrocyte calpastatin [20] and phosphorylation [21]. The microheterogeneity of calpastatin (116k and 118k), as labeled metabolically with ^{32}P in hematopoietic cell lines, was reported by Adachi et al. [21]. They showed that more than 30% of phosphorylated calpastatins were distributed in the membrane fraction. Since both the 110k and 120k species in GOTO cells are localized in the cytosol fraction, it seems unlikely that the diversity is mainly caused by phosphorylation. Experiments are now underway in our labolatory to elucidate the molecular basis for 110k and 120k heterogeneity. Whatever the nature of 110k and 120k, it is important to note that 120k calapstatin is closely associated with the acquisition of the Schwannian phenotype in neuroblastoma cells.

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