Growth phase-dependent changes in the subcellular localization of pre-B-cell colony-enhancing factor[1]

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Abstract A cDNA encoding the homolog of the human pre-B-cell colony-enhancing factor (PBEF), a cytokine-like secreted protein, was isolated from a rat cDNA library. This protein existed in both the cytoplasm and nucleus of the cells, and the amount was higher in the cytoplasm than in the nucleus of proliferating PC-12 and Swiss 3T3 cells but higher in the nucleus than in the cytoplasm of the PC-12 cells treated with nerve growth factor and the 3T3 cells grown to a confluent state. Thus, the so-called PBEF is not a cytokine-like secreted protein but an intracellular protein associated with the cell cycle.

Key words: Pre-B-cell colony-enhancing factor; Cell cycle; Subcellular distribution; Cell nucleus

1. Introduction

A protein localized in the nucleus of PC-12 cells treated with nerve growth factor (NGF) was incidentally found to cross-react with an antibody prepared for another protein and the cDNA for the nuclear protein was cloned by screening with the antibody. PC-12 cells, derived from a rat pheochromocytoma [1], are known to respond to NGF by an arrest of cell division and an extension of neurites [1], and are widely used as a model system for neuronal differentiation. The amino acid sequence of the cloned protein showed 95% identity with that of the pre-B-cell colony-enhancing factor (PBEF) cloned from a human peripheral blood lymphocyte cDNA library [2], suggesting that this protein is a rat homolog of PBEF. The name of PBEF is based on the observation that it enhances the effect of stem cell factor and interleukin 7 (IL-7) on pre-B-cell colony formation [2] and it has been thought to be a cytokine-like secreted protein, although a signal sequence for secretion is not found in its amino acid sequence [2].

In this paper, we report the cloning and sequence analysis of the rat homolog of human PBEF. Immunochemical analysis showed that PBEF is ubiquitous in tissue distribution and exists in both the cytoplasm and nucleus of the cell in varying relative concentrations, depending upon the cell cycle phases.

2. Materials and methods

2.1. Cloning, sequencing, and expression

Since one of several antibodies raised against calmodulin-dependent protein kinase II (CaM-kinase II) obtained in this laboratory detected a nuclear protein in PC-12 cells treated with NGF, an oligo(dT)-primed cDNA library was constructed in λZAPII (Stratagene) using poly(A)$^+$ RNA, which was isolated from PC-12 cells by use of Fast-Track 2.0 mRNA isolation kit (Invitrogen) according to the manufacturer's manual, and approximately 7×10^5 plaques were screened with the antibody. Three positive clones of 2.0–2.3 kb were isolated, and the nucleotide sequences of the clones were determined by the dideoxynucleotide chain termination method [3], using a DNA sequencer model 4000L (LI-COR). A cDNA fragment containing the entire coding sequence was introduced into a baculovirus, AcNPV, using a Bac-To-Bac Baculovirus Expression System (Life Technologies). Sf9 cells infected with the recombinant baculovirus were grown at 27°C, as described previously [4]. The harvested cells were washed with phosphate-buffered saline (PBS), suspended in 10 volumes of 20 mM Tris–HCl buffer (pH 7.5 at 4°C) containing 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1% Tween 40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate the crude extract.

2.2. Northern blot analysis

Rat and human MTN membranes (Clontech) were hybridized with the ^{32}P-labeled probe for PBEF (nucleotides 1–1443) in ExpressHyb hybridization solution (Clontech) at 68°C for 1 h after prehybridization for 1.5 h at 68°C, and then washed with a solution consisting of 0.2×SSC and 0.1% sodium dodecyl sulfate (SDS) at 60°C.

2.3. Western blot analysis

A peptide, CVTKSYSFDEVRKNAQLNMEQDVAPH, consisting of the carboxyl-terminal 25 amino acids of rat PBEF (Fig. 1) with a cysteinyl residue added to the amino-terminus for coupling to a carrier protein, was conjugated to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent, the resultant conjugate was used to immunize Japanese white rabbits, and the antibody was purified by affinity chromatography on peptide-coupled Cellulofine, essentially as described previously [4]. Approximately 2.4 mg of the antibody was purified from 20 ml of the antiserum.

Western blot analysis was performed essentially as described by Winston et al. [5]. Tissues obtained from 10-week-old female Wistar rats, and testis from male rats were homogenized with a Potter–Elvehjem homogenizer in 3 volumes of 20 mM HEPES–NaOH (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 0.1% Triton X-100 and 20 μg/ml each of the microbial protease inhibitors. After removal of the residues by centrifugation, the crude extracts were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide,

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[1] The nucleotide sequence of cDNA for rat PBEF has been submitted to the DDBJ/EMBL/GenBank under the accession number AB081730.

Abbreviations: PBEF, pre-B-cell colony-enhancing factor

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Rat:   MNAAAE-AEFN ILLATDSYKV THYKQYPPNT SKVYSYFECR EKKTENSKVR KVKYEETVFY GLQYILNKYL KGKVVTKEKI QEAKEVYREH FQDDVFNERG-100
Mouse: ******-**** ********** ********** ********** ********* ********** ********** ********** ********** **********
Human: **P***-**** ********** ********** ********** ********L* ********** ********** ********** ****D**K** ********K*
Carp:  *EQHTGA**** ********** ********** ********** ******PT*L* ****DK**** ******HR** **Q***R*** ********** ********K*

       WNYILEKYDG HLPIEVKAVP EGSVIPRGNV LFTVENTDPE CYWLTNWIET ILVQSWYPIT VATNSREQKK ILAKYLLETS GNLDGLEYKL HDFGYRGVSS -200
       ********** ********** ********** ********** ********** ********** *********R ********** ********** **S*******
       ********** *****I**** **F******** ********** ********** ********** ********** ********** ********** **********
       ********N* *****I**** ********** ****VS**** *******V** ****I***** ********** ******M*** *S*E****** **********

       QETAGIGASA HLVNFKGTDT VAGIALIKKY YGTKDPVPGY SVPAAEHSTI TAWGKDHEKD AFEHIVTQFS SVPVSVVSDS YDIYNACEKI WGEDLRHLIV -300
       ********** ****L***** ********** ********** ********** ********** ********** ********** ********** **********
       ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
       ********** ********** ****CV**** *********F ********** ********** *****IK**P *****I**** ********** ******G**E

       SRSTEAPLII RPDSGNPLDT VLKVLDILGK KFPVSENSKG YKLLPPYLRV IQGDGVDINT LQEIVEGMKQ KKWSIENVSF GSGGALLQKL TRDLLNCSFK -400
       ********** ********** ********** ****T***** ********** ********** ********** ********** ********** **********
       ****Q***** ********** *****E**** ****T***** ********** ********** ********** *M*****IA* ****G***** **********
       M**AD***VV ********** *****E**** ***LV***** **V****I** ********** *********D HR*****IA* ********** **********

       CSYVVTNGLG VNVFKDPVAD PNKRSKKGRL SLHRTPAGTF VTLEEGKGDL EEYGHDLLHT VFKNGKVTKS YSFDEVRKNA QLNMEQDVAP H
       ********** ********** ********** ********N* ********** ********** ********** ********** ***I****** *
       ********** I********* ********** ********N* ********** ****Q***** ********** *****I**** ***I*LEA*H *
       ********** ********** ********** ******S*D* ********** ****E***** **R***IV*T *T*****D** K*KESELEDL LL
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Fig. 1. Comparison of the deduced amino acid sequence of rat PBEF with those of mouse, human, and carp PBEFs. The deduced amino acid sequences of rat, mouse (GenBank/EMBL/DDBJ accession number AF234625), human (HSPBEF) [2], and carp (AB027712) PBEFs are aligned for maximal homology, matching amino acids being indicated by asterisks. Identities of rat PBEF with mouse, human, and carp PBEFs are 98, 95, and 88%, respectively.

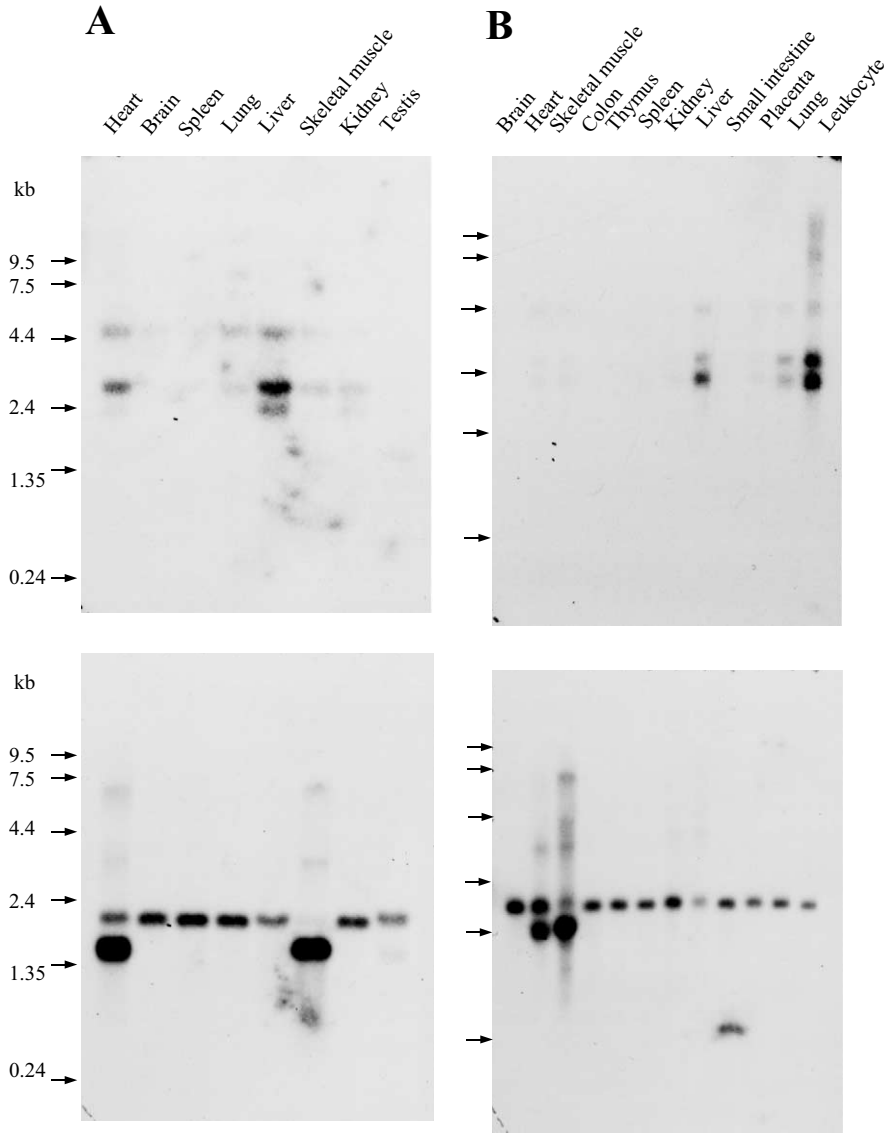

Fig. 2. Tissue distribution of PBEF on Northern blot analysis. Rat (A) and human (B) MTN membranes (Clontech), on which 2 and 1 μg each of poly(A)$^+$ RNA, respectively, from the indicated tissues had been blotted, were hybridized with a ^{32}P-labeled probe for rat PBEF (nucleotides 1–1443) (upper panel) or ^{32}P-labeled human β-actin cDNA (Clontech) (lower panel). RNA sizes in kb are given on the left.

and then the protein bands were transferred to a polyvinylidene difluoride membrane (Fluorotrans, Pall Bio Support). The membrane was blocked with 5% non-fat milk in PBS for 30 min at 24°C, and then incubated with antibodies to rat PBEF in the blocking buffer at 4°C overnight, followed by incubation with 30 μg/ml goat anti-rabbit immunoglobulins (IgA+IgG+IgM) conjugated with peroxidase (Organon Teknika) at 24°C for 1 h. Positive bands were detected with diaminobenzidine tetrahydrochloride and H_2O_2 in the presence of $CoCl_2$.

2.4. Immunocytochemical analysis

PC-12 cells and Swiss 3T3 cells were cultured on collagen type I-coated coverslips (Iwaki, Japan) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 10% horse serum and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, respectively, in a humidified incubator at 37°C under an atmosphere of 5% CO_2. The cells grown on coverslips were rinsed with PBS, fixed with 3.7% formaldehyde in PBS for 30 min, washed three times in PBS, and then permeabilized with 0.3% Triton X-100 in PBS for 5 min. After washing in PBS, the cells were incubated with 3% normal goat serum (Cappel) and 0.1% Triton X-100 in PBS for 1 h, incubated overnight at 4°C with 0.44 μg/ml antibodies against rat PBEF, washed three times in 0.1% Triton X-100 in PBS, and then incubated with 1 μg/ml Alexa Fluor 488 goat anti-rabbit IgG (H+L) (highly cross-adsorbed) (Molecular Probes) in 3% normal goat serum and 0.1% Triton X-100 in PBS for 2 h. After washing three times in 0.1% Triton X-100 in PBS, cell nuclei were visualized by staining with 0.2 μg/ml Hoechst 33258 (Polysciences) for 5 min, followed by washing in three changes of PBS. The cells were mounted in Vectashield (Vector Laboratories), and then examined by fluorescence microscopy under a Zeiss Axiovert microscope (Carl Zeiss) equipped with a 4,6-diamino-2-phenylindole/fluorescein isothiocyanate/tetrarhodamine isothiocyanate (DAPI/FITC/TRITC) filter set (Chroma Technology) using a Zeiss 40× Plan-Neofluor lens (Carl Zeiss). Microscopic data were analyzed by CELLscan system with CELLview image visualization and EPR image restoration systems (Scanalytics).

2.5. Other analytical procedures

SDS–PAGE was carried out according to the method of Laemmli [6]. Proteins were determined by the method of Lowry et al. [7] as modified by Peterson [8] with bovine serum albumin as a standard.

3. Results and discussion

3.1. Cloning and amino acid sequence of rat PBEF

Since a nuclear protein in PC-12 cells treated with NGF was incidentally detected by an antibody raised against CaM-kinase II, a λZAPII cDNA library constructed from PC-12 cells was screened by plaque hybridization with the antibody. When the nucleotide sequence of the cDNA clone isolated was determined, the deduced amino acid sequence was found to be very similar to that of human PBEF (2), as shown in Fig. 1. The coded protein consisted of 491 amino acids with a molecular weight of 55 437 Da. The overall identities of this protein with mouse, human, and carp PBEFs are 98, 95, and 88%, respectively. Its very high sequence homologies with mouse, human, and carp PBEF indicate that this protein is a rat homolog of PBEF, and that the overall amino acid sequence of PBEF is highly conserved, suggesting that this protein plays a physiologically important role.

Northern blot analysis of rat tissues detected three bands, corresponding to molecular sizes of 2.3 kb, 2.6 kb, and 4.5 kb, as shown in Fig. 2A, in agreement with the result of Samal et al. [2] that the mRNA for human PBEF is represented in species of three different molecular sizes of about 2.0 kb, 2.4 kb, and 4.0 kb.

3.2. Tissue distribution of PBEF

The tissue distribution of PBEF was examined by Northern blot analysis (Fig. 2) and Western blot analysis (Fig. 3). As shown in Fig. 2A, mRNA for PBEF was expressed in various tissues, at the highest level in liver and at the second highest in heart. In human tissues, the maximum amount of PBEF mRNA was found in peripheral blood leukocyte, the second highest in liver, and the next in lung (Fig. 2B). The amount of PBEF mRNA was higher in heart than in lung in rats, but

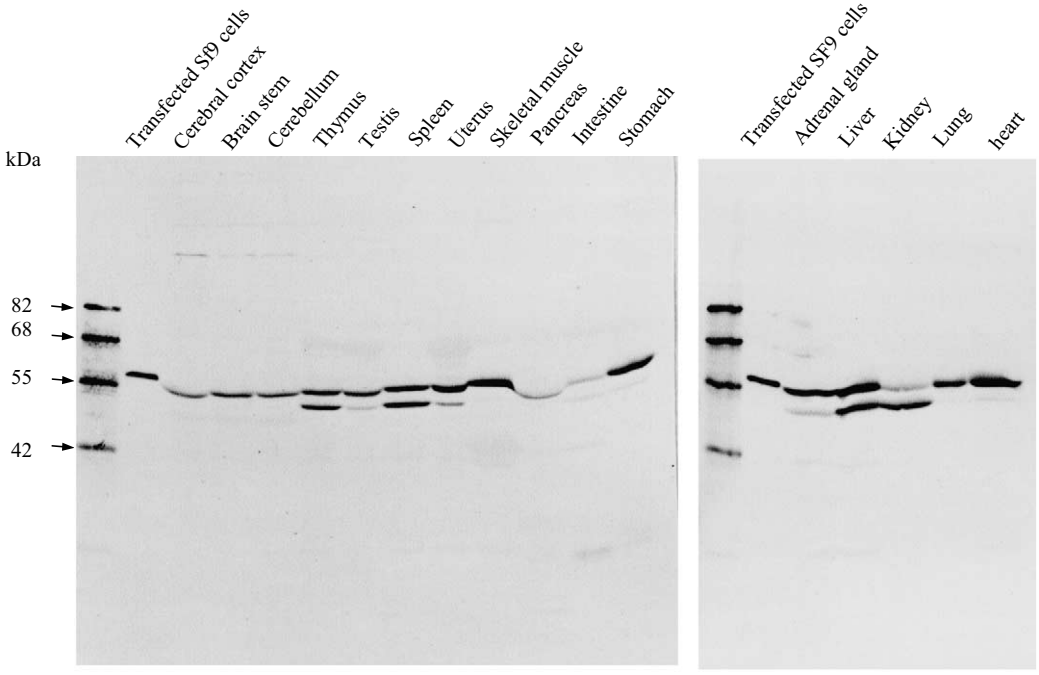

Fig. 3. Tissue distribution of PBEF on Western blot analysis. Approximately 0.2 μg protein of the crude extract of Sf9 cells transfected with baculovirus carrying a cDNA encoding rat PBEF (transfected Sf9 cells) and 40 μg protein of the crude extracts of the indicated rat tissues were subjected to Western blot analysis with approximately 0.5 μg/ml antibodies against rat PBEF, as described in Section 2. Dr. Western (Oriental Yeast) was used as protein size markers.

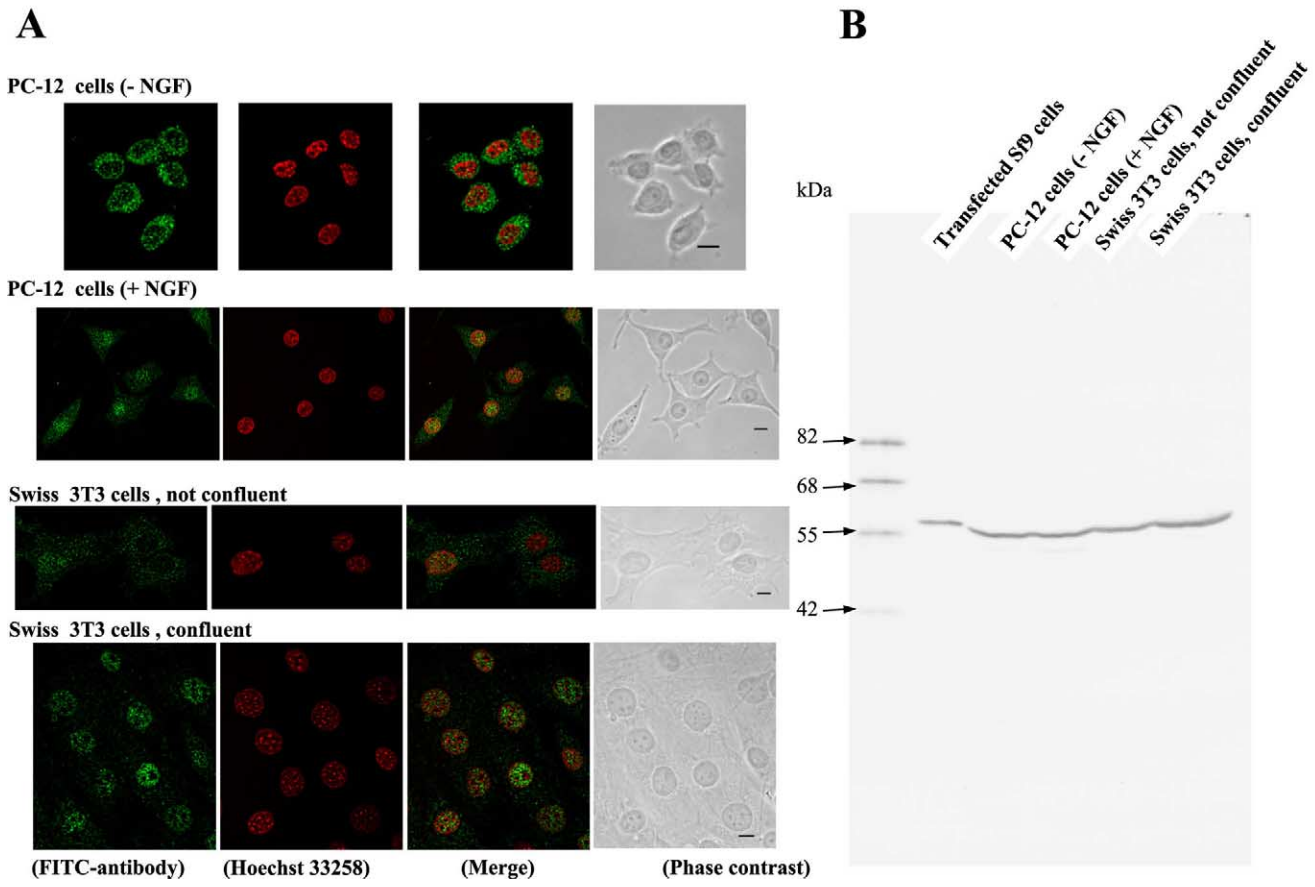

Fig. 4. Subcellular distribution of PBEF. A: PC-12 cells cultured in the absence of NGF (−NGF) or cultured for 6 days in the presence of NGF (+NGF), where the culture medium containing no NGF or 100 ng/ml NGF (2.5S NGF, mouse) (Promega) was changed every 3 days, and Swiss 3T3 cells cultured before (not confluent) or after reaching confluency (confluent) were stained by means of indirect immunofluorescence with antibodies against PBEF, as described in Section 2. The nuclei of the cells were stained with Hoechst 33258. A proper plane of the images was selected and the images of Alexa Fluor 488 (the left row of panels) and Hoechst 33258 (the next row) were merged together (the third row) using MetaMorph Imaging System (Universal Imaging Corporation). Phase-contrast images of the cells are shown in the right row of panels. Bar = 10 μm. B: Approximately 0.2 μg protein of the crude extract of Sf9 cells transfected with baculovirus carrying a cDNA encoding rat PBEF (transfected Sf9 cells), and 40 μg protein of the crude extracts of PC-12 cells cultured in the absence of NGF (PC-12 cells (−NGF)) or cultured for 6 days in the presence of NGF (PC-12 cells (+NGF)), and Swiss 3T3 cells before (Swiss 3T3 cells, not confluent) or after reaching confluency (Swiss 3T3 cells, confluent) were subjected to Western blot analysis with approximately 3 μg/ml antibodies against rat PBEF.

higher in lung than in heart in humans. The hybridization results with β-actin cDNA control probe (Fig. 2, lower panels) indicate that our Northern blot analysis was achieved successfully. As shown in Fig. 3, Western blot analysis revealed that PBEF occurred in all tissues examined, the content varying depending upon the tissue. Western blot analysis also revealed the existence of two species of PBEF, and the larger one coincided in position with recombinant PBEF expressed in Sf9 cells. The larger species was major in brain, testis, uterus, skeletal muscle, pancreas, intestine, stomach, adrenal gland, lung, and heart, and the smaller species was major in kidney. Both species existed in an equivalent amount in thymus, spleen, and liver. It is not yet clear whether the smaller species of PBEF is a degradation product of the larger species, an alternatively spliced variant, or an isozyme of PBEF.

In order to know the subunit structure of this protein, the crude extract of Sf9 cells transfected with baculovirus carrying PBEF cDNA was subjected to a column of Sephacryl S-200 (high resolution) (Pharmacia/LKB Biotechnology). The elution position of PBEF was monitored by immunoblotting, and its molecular weight was estimated from its elution position to be about 65 000 Da (data not shown), suggesting that PBEF is a monomeric protein.

3.3. Subcellular distribution of PBEF

The subcellular distribution of PBEF in cultured cells was immunocytochemically examined using antibodies raised against a peptide corresponding to the carboxyl-terminal 25 amino acids of rat PBEF. As shown in Fig. 4A, proliferating PC-12 cells (the uppermost panels) which were cultured in the absence of NGF were much more strongly immunostained in the cytoplasm than in the nucleus, but non-proliferating PC-12 cells (the second panels from the top), which had been cultured for 6 days with NGF to differentiate into neuronal cells, were more strongly immunostained in the nucleus than in the cytoplasm. Swiss 3T3 cells grown in monolayer (the lowest panels) were strongly immunostained in the nuclei and only weakly stained in the cytoplasm. In contrast, two dividing Swiss 3T3 cells appearing at the right in the second panels from the bottom were strongly stained in the cytoplasm. A non-dividing cell appearing at the left in the same panels was rather strongly stained in the nucleus. Thus, PBEF

appears to exist more abundantly in the cellular nuclei than in the cytoplasm in non-proliferating cells, but more abundantly in the cytoplasm than in the nuclei in proliferating cells, suggesting that PBEF is a cell cycle-associated intracellular protein. The question of whether nuclear and cytoplasmic PBEFs are the same or not was examined by Western blot analysis of the crude extracts of PC-12 cells grown in the presence and absence of NGF and also Swiss 3T3 cells before and after reaching confluency, as shown in Fig. 4B. No differences in the mobilities and intensities of the positive bands of the two crude extracts of PC-12 cells and Swiss 3T3 cells were observed, and their mobilities coincided with that of recombinant PBEF expressed in Sf9 cells, indicating that PBEF molecules did not undergo modification such as proteolysis in the cells.

The nuclear localization of PBEF raised some doubt as to whether PBEF is a cytokine-like secreted protein, as suggested before [2]. Our attempts to detect PBEF in the culture media, in which PC-12, Swiss 3T3, NG 108-15, P3-X63, and L6 cells had been heavily cultured, by antibodies against rat PBEF were all unsuccessful (data not shown). These results, taken together with the fact that PBEF does not have a signal sequence for secretion in its amino acid sequence [2] and any homology with other cytokines in any currently available database [9], indicate that PBEF is not a cytokine-like secreted protein from these cells under the experimental conditions tested. However, in contrast to our contention, it has recently been reported that treatment of human amnion-derived epithelial (WISH) cells with 10 ng/ml recombinant human PBEF increases the expression of mRNAs for IL-6 and IL-8 [10], supporting the concept that PBEF is a cytokine-like protein [2].

In the present study, PBEF is shown to be ubiquitous in its tissue distribution and to exist in both the cytoplasm and nucleus. The ratio of the content in the nucleus to that in the cytoplasm was low in proliferating cells, but high in non-proliferating cells whose cell division was inhibited by differentiation or contact inhibition. PBEF in the nucleus could not be distinguished from that in the cytoplasm by SDS–PAGE, indicating that proteolytic cleavage is not involved in the translocation of PBEF from the cytoplasm to the nucleus. In order to understand the biological significance of PBEF in cell division, the detailed relationship between the nuclear translocation and cell cycle is under investigation in this laboratory.

It has recently been reported that PBEF is a nicotinamide phosphoribosyltransferase, an enzyme involved in nicotinamide adenine dinucleotide (NAD) biosynthesis [11].

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