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Research Article

Expression and cellular distribution of perchloric acid-soluble protein is dependent on the cell-proliferating states of NRK-52E cells

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Abstract. To clarify the biological role of kidney perchloric acid-soluble protein 1 (K-PSP1), its expression and intracellular distribution were examined in normal rat kidney epithelial NRK-52E cells. K-PSP1 expression was low during the proliferating phase and high in the stationary phase, and shown to have a negative relationship with the protein-synthesizing activity of the cells. Immunocytochemical studies revealed that K-PSP1 is predominantly located in the cytosol, especially in endoplasmic reticulum and Golgi apparatus of proliferating cells. In the stationary phase, K-PSP1 was not detected immunologically even though protein and mRNA expression were high. This disappearance of reactivity with anti-serum seems to be due to a conformational change in K-PSP1 induced by unknown factors. These results suggest that the role of K-PSP1 is to regulate cell proliferation, and this may be related to a previously reported ability to inhibit protein synthesis.

Key words. Perchlolic acid-soluble protein; protein synthesis inhibitor; cell growth; immunoblotting; quantitative RT-PCR reaction; immunofluorescent cytochemistry.

Perchloric acid-soluble protein 1 (PSP1) is a protein originally isolated from rat liver. The manner by which it inhibits protein synthesis in the rabbit reticulocyte lysate differs from that of RNase A [1], and this inhibition is reportedly due to the ribonuclease activity of PSP1 [2]. Recently, PSP1-like 14-kDa protein synthesis inhibitor proteins have also been characterized in human monocytes [3] and mouse liver [4]. In addition, cDNA sequences of these proteins have been shown to have high homology with a new hypothetical family (YER057c/YJGF family) of small proteins, but their physiological role is still unknown. Since the sequence for these PSP1-like proteins is highly conserved in prokaryotes, cyanobacteria, fungi, and eukaryotes, they may play a vital role in the regulation of various cell functions. A 14-kDa trichloroacetic acid (TCA)-soluble translational inhibitor protein (p14.5) is upregulated during monocyte differentiation [3] and expression of kidney PSP1 (K-PSP1), a protein synthesis inhibitor isolated from rat kidney, is regulated in a differentia-

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tion-dependent manner. Immunohistochemical studies have shown that the expression of K-PSP1 increases from the 17th prenatal day to the 4th postnatal week in rat, whereafter expression levels remain constant until the 7th postnatal week [5]. These data suggest that PSP1 is involved in both cell proliferation and differentiation. Here, we examined the expression and intracellular distribution of K-PSP1 in normal rat kidney epithelial cells (NRK-52E cells).

Materials and methods

Cells and cell culture. NRK-52E cells were obtained from the American Type Tissue Culture Collection (Manassas, Va.). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, Md.) supplemented with nonessential amino acids (NEAA; ICN Biomedicals), 50 μg/ml streptomycin, 50 units/ml penicillin, and 5% fetal bovine serum (FBS; Whittaker Bioproducts, Walkersville, Md.). Cells were replated before reaching confluence using trypsin-EDTA (DIFCO) and the medium was changed every 3 days.

Protein synthesis. Protein synthesis was quantified by measuring [^{14}C]-leucine incorporation into the protein fraction. NRK-52E cells were cultured in 12-well plates for various periods, and 1 μCi/ml [^{14}C]-leucine was added to the culture medium during the last 8 h of the cultivation period. After removing the medium and washing the cells with phosphate-buffered saline (PBS, pH 7.4), proteins were fixed with ice-cold 5% TCA. To remove free [^{14}C]-leucine, the cells were washed twice with 5% TCA. Protein was solubilized with 0.5 ml 0.5 N NaOH-0.1% Triton X-100 and transferred to vials to which scintillation liquid was added. Radioactivity was counted using a liquid scintillation counter.

Western immunoblotting. NRK-52E cells (1×10^4 cells/10-cm dish) were cultured for various periods and then trypsinized with 0.02% trypsin. The cells were homogenized in a 0.25 M sucrose buffer using a Potter–Elvehjem-type homogenizer, and the protein concentration was determined with a BCA protein assay kit (Pierce). Immunoblotting was performed according to the method described by Oka et al. [1]. Briefly, 40 μg protein homogenate was electrophoresed on a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) and immunoblotted with anti-PSP1 serum (1:3000).

RNA extraction and semiquantitative RT-PCR. Total RNA was prepared using TRIzol (GIBCO-BRL) from the cells cultured for various periods. Relative quantitation of PSP mRNA was performed using RT-PCR [6]. Briefly, cDNA was synthesized from 0.2 μg total RNA using a commercial kit (Amersham), according to the manufacturer's instructions. cDNAs (10 ng) were amplified by PCR using specific PSP primers (sense primer: 5′-GCC ATT GGT GCC TAC AGC CA-3′, anti-sense primer: 5′-AAA GGC CCC TGG ACA G-3′), or glyceraldehyde-3-phosphate dehydrognase (GAPDH) primers (sense primer: 5′-GAT GCT GGT GCT GAG TAT GTC G-3′; anti-sense primer: 5′-GTG GTG CAG GAT GCA TTG CTG A-3′). The total reaction volume (10 μl) included 1 μM of each primer, 200 μM deoxy-NTP mixtures, 1.5 mM MgCl, 10 × buffer, and 1 unit of Taq DNA polymerase (Fermentas). The PCR was executed using the GeneAmp PCR system 2400 (Perkin Elmer-Cetus) under the following conditions: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. PCR products were electrophoresed on a 2% agarose gel containing 5 μg/ml ethidium bromide, and the DNA bands were visualized under ultraviolet light. The molecular sizes of the amplified K-PSP1 and GAPDH were determined by comparison with the molecular size markers. Gel bands were quantified using a computer program obtained from the NIH.

Immunofluorescent cytochemistry. Immunofluorescent cytochemistry was executed following the method described by Musil et al. [7]. Briefly, cells grown on uncoated glass coverslips were washed with PBS and fixed for 20 min at room temperature with 4% paraformaldehyde dissolved in PBS. The fixed cells were treated with PBS containing 1% bovine serum albumin (BSA) for 30 min, and incubated overnight at 4° C with serum (1:3000) prepared from nonimmunized or PSP-immunized rabbits. After washing three times with PBS for 15 min each, the cells were incubated for 2 h at room temperature with Cy-2-conjugated goat anti-rabbit IgG (1:3000) (Amersham) dissolved in 0.1% BSA-PBS. The cells were then incubated with 50 μg/ml propidium iodide at room temperature for 5 min. Finally, the coverslips were washed thoroughly, mounted onto glass slides with 50% glycerol, and the cells were photographed using a TCS NT Leica (Germany) confocal laser scanning microscope.

Results

Time course of [^{14}C]-leucine incorporation into cells in various stages of proliferation. As shown in figure 1, [^{14}C]-leucine incorporation in NRK-52E cells was highest on day 2, at which time the cell density was low (around 2.5×10^4 cells/dish). [^{14}C]-leucine incorporation then reduced as the cell number increased until settling at a low constant value when the cells reached the stationary phase at around 7×10^5 cells/dish.

Change in K-PSP1 expression during proliferation. Expression of K-PSP1 in NRK-52E cells during cell proliferation was analyzed by immunoblotting. In this experiment, cells exhibited a logarithmic prolifera-

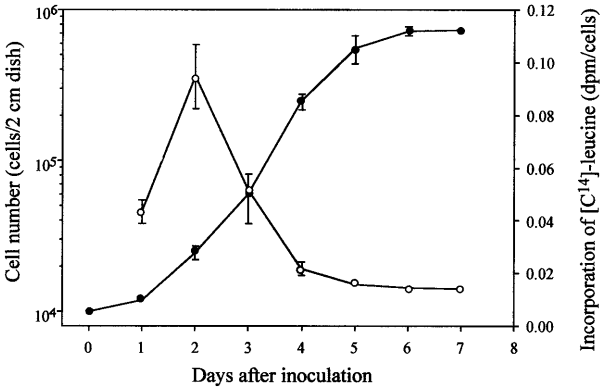

Figure 1. [^{14}C]-leucine incorporation during cell proliferation of NRK-52E cells. NRK-52E cells were cultured for various time periods, cells were counted (filled circles) and assayed for protein-synthesizing activity (open circles). Isotope incorporation was carried out by replacing the medium with fresh medium containing 2 μCi [^{14}C]-leucine, following which, cells were cultured for 8 h before measurements were taken. Results are expressed as the mean ± SE (n = 3).

tion pattern from 24 to 144 h and entered stationary phase 216 h after inoculation (fig. 2A). K-PSP1 expression was very low between 48 and 96 h, and then rose with the increase in cell number (fig. 2B) in three independent experiments.

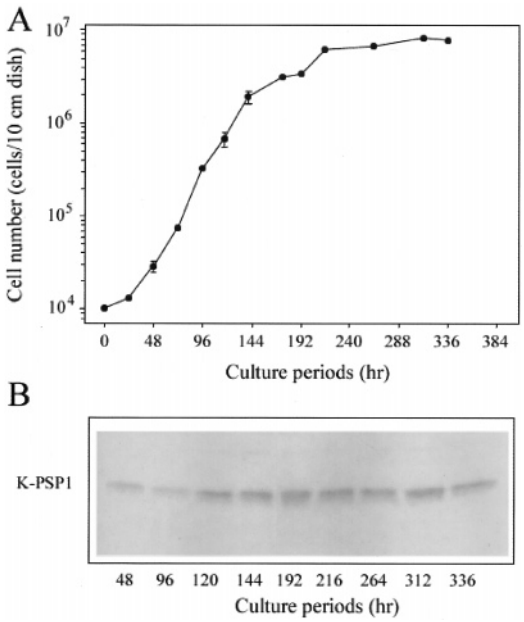

Figure 2. Expression of K-PSP1 during cell proliferation. (*A*) NRK-52E cells were seeded at an initial density of 1×10^4 cells/10-cm dish. They were detached from the dish using trypsin and viable cells were counted using a hemocytometer in the presence of trypan blue. (*B*) Total protein (40 μg) from NRK-52E cells was electrophoresed on a 15% SDS-PAGE gel and immunoblotted using anti-PSP antiserum.

mRNA expression of K-PSP1 was determined by RT-PCR. The extracted total RNA was first reverse transcribed and the resulting templates were used for PCR. Cycle time for PCR semiquantification was examined from 16 to 40 cycles. Twenty-four cycles for K-PSP1 and 29 cycles for GAPDH were shown to be the lowest consistently detectable cycle numbers, and these were therefore employed for further analysis of K-PSP1 and GAPDH (fig. 3A). As shown in figure 3B, GAPDH mRNA expression was almost constant although the cells were proliferating logarithmically. On the other hand, expression of K-PSP1 mRNA was very low during the first 24–96 h after inoculation, and gradually increased from 120 to 168 h after inoculation. The cells maintained this high expression level in the stationary phase.

Intracellular distribution of K-PSP1. The intracellular distribution of K-PSP1 in NRK-52E cells was examined by immunofluorescent cytochemistry. In figure 4, K-PSP1 is represented as green spots when stained with Cy-2 dye and nuclei are seen as red spots when stained with propidium iodide. Green fluorescence was not observed when the cells were treated with nonimmunized serum as the primary antibody (data not shown). When the cells were cultured for 24, 48, 72 and 96 h, staining was mainly seen in the cytoplasm. We show K-PSP1 to be generally localized throughout the whole cytosol, possibly concentrated in the endoplasmic reticulum (ER) and Golgi. A similar distribution has been reported for the ER and Golgi 30-kDa protein (ERG30) [8], α-mannosidase II (ManII) [9], and the ER/Golgi soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNAR) [10]. K-PSP1 was not observed in cells in the stationary phase after 240 h culture (fig. 4F).

Discussion

Among PSP1-like proteins, p14.5 is expressed in mononuclear phagocytes in a differentiation-dependent manner, undergoing upregulation during monocyte differentiation [3]. In previous studies, K-PSP1 expression appeared to be related to development of the kidney, being little expressed in immature kidney, but highly expressed in mature kidney [5]. These results suggest that PSP1-like proteins are related to cellular differentiation. In addition, our results suggest that K-PSP1 is also related to cell proliferation: the expression of K-PSP1 mRNA and protein was lower in cells in the proliferating phase than in the stationary phase, and the expression pattern for K-PSP1 in proliferating cells is negatively correlated with the protein synthesis pattern. When K-PSP1 expression is low, protein synthesis activ-

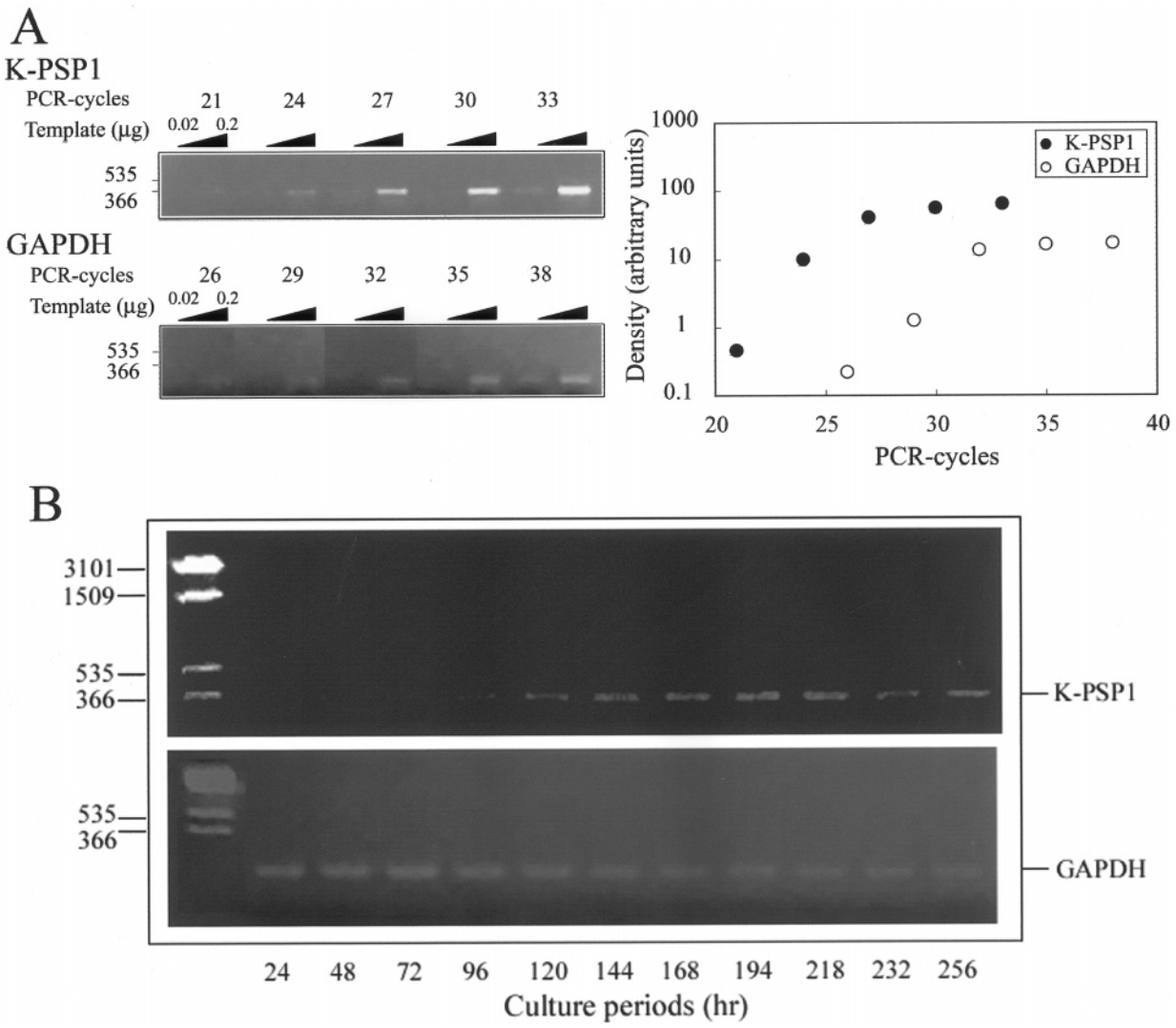

Figure 3. Expression of K-PSP1 mRNA during cell proliferation. K-PSP1 was semiquantified by RT-PCR. (*A*) Optimization of PCR cycles. Aliquots (0.02 or 0.2 μg) of total RNA isolated from NRK-52E cells at 256 h were reverse transcribed and PCR-amplified for various PCR cycles. The products were separated on a 2% agarose gel containing ethidium bromide, visualized on an ultraviolet transilluminator, and analyzed using the NIH image program. (*B*) Expression of K-PSP1 mRNA in NRK-52E cells cultured for various periods. Semiquantitive RT-PCR was performed by reverse transcribing 0.2 μg total RNA followed by PCR amplification for 24 cycles with K-PSP1 primers and 29 cycles with GAPDH primers.

ity is high, and vice versa. These results suggest that K-PSP1 is involved in protein synthesis when the cell is in the proliferating phase. The mechanism of this protein synthesis inhibition has recently been described, and attributed to the ribonuclease activity of PSP1 [2]. The p14.5 promoter contains binding sites for the ETS family transcription factor PU.1, a candidate transcription factor for mediating macrophage-specific gene expression. The p14.5 promoter has other binding sites for c-Myc and the α-palindrome-binding protein, both of which are related to cell growth and differentiation [11–14]. Although the PSP1 promoter has not yet been identified, its regulation may be similar to that of the p14.5 promoter, being controlled by transcription factor(s) involved in cell proliferation.

Previous immunohistochemical data demonstrated that PSP1 and p14.5 are distributed mainly in the cytosol and only slightly in the nucleus of rat liver and kidney cells [1, 3]. In our immunofluorescent cytochemical study of NRK-52E cells, K-PSP1 was located in the cytosol of cells only in the proliferating phase, but was not seen in cells in the stationary phase. This inability to immunostain the protein despite its high expression in stationary phase cells suggests that antibody binding may be inhibited by conformational changes due, e.g., to phosphorylation, isoprenylation, or interaction with

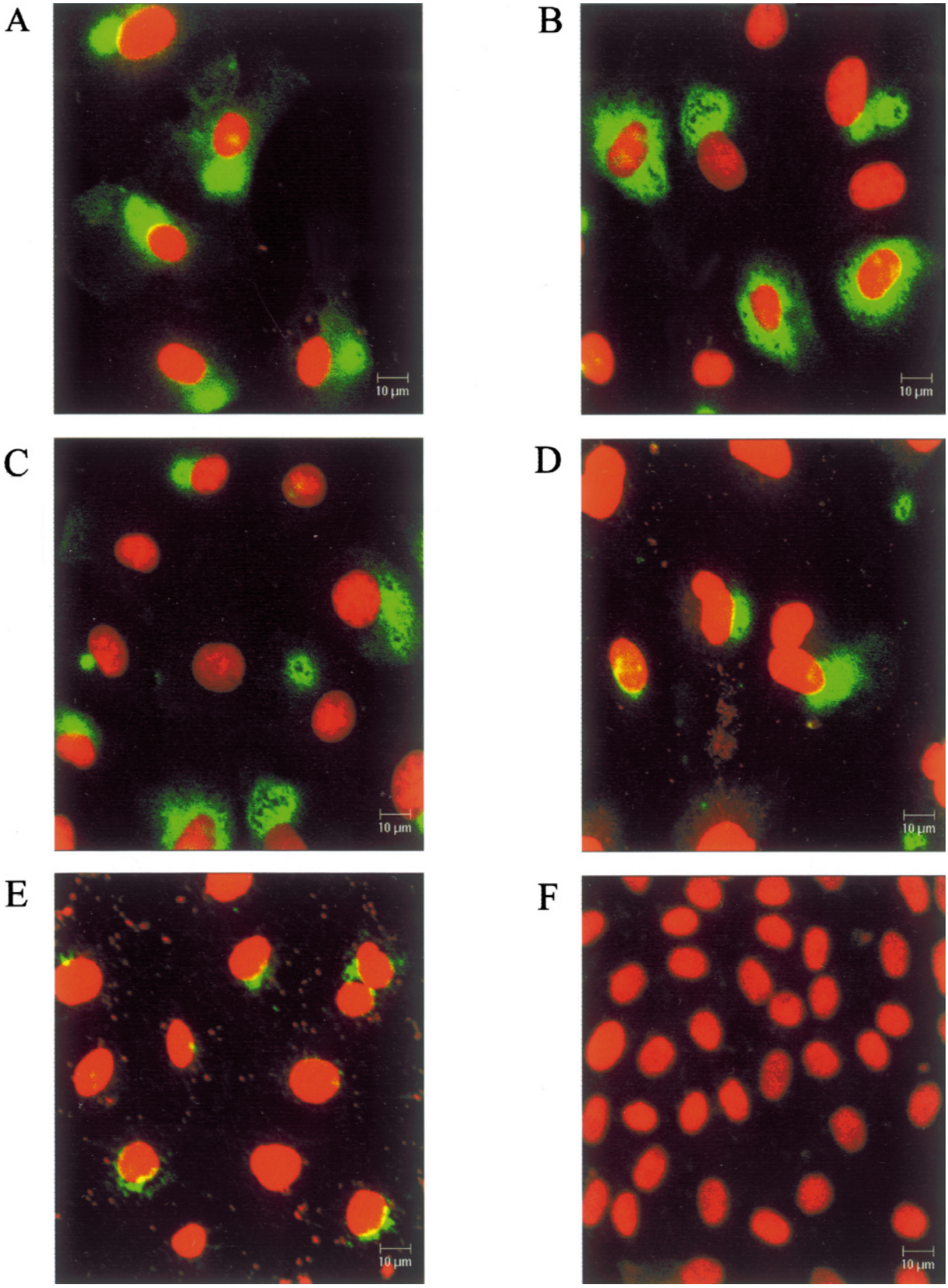

Figure 4. Immunofluorescent localization of PSP in NRK-52E cells. NRK-52E cells were cultured on a cover glass and then fixed for analysis. PSP was visualized by indirect immunofluorescence with anti-PSP antiserum using the Cy2-conjugate, and the nuclei were stained with propidium iodide. Cells were shown to be in the growth phase at 24 h (*A*), 48 h (*B*,*C*), 72 h (*D*) and 96 h (*E*) and in the stationary phase at 240 h (*F*).

another protein. Further studies are currently underway to elucidate this problem. In addition, this immunohistochemical study suggested that K-PSP1 is located in the ER or Golgi apparatus. An ER location is reasonable, since this site plays an important role in protein and lipid synthesis [15, 16]. Since PSP1 has been reported to exert fatty acid-binding activity [17, 18], it may be related to the transport or processing of fatty acids in the ER as well as to protein synthesis.

PSP1-like proteins have been found in various cell types of both prokaryotes and eukaryotes. Furthermore, expression of PSP1-like proteins has been reported to be tissue specific. Although PSP1 is predominantly located in liver and kidney cells, there are other proteins with similar activity. For example, PSP2, purified using the same method as for PSP1, is a heart-type fatty acid-binding protein [18, 19]. PSP1-like proteins are apparently expressed in a variety of tissues and may play an important role in the regulation of cell proliferation and differentiation in a tissue-specific manner.

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