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# LOCALIZATION OF PHOSPHOPROTEIN PP 105 IN CELL LINES OF VARIOUS SPECIES

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Summary: The cellular location of phosphoprotein pp 105 was determined in various mouse cell lines with rabbit anti mouse pp 105 serum. Immunofluorescence was predominantly observed in the nucleoli in addition to a diffuse but weaker fluorescence of the whole nucleus. Cell surface fluorescence was obtained only with cells grown in suspension cultures. The presence of pp 105 in normal mouse tissue was demonstrated with tissue extracts by immunobinding assays. Cross-reacting phosphoproteins with the same molecular weight were detected in hamster and human cell lines as well as in chicken cartilage cells and Drosophila embryonic cells. Endogenous phosphorylation of pp 105 studied with purified mouse nucleoli showed optimal activity at isotonicity, pH 8.7, in the presence of 10 mM magnesium.

In previous studies we observed that a phosphoprotein with molecular weight 105 000 Da (pp 105) is one of the major proteins which become accessible to endogenous phosphorylation on the surface of intact cells after growth transition from monolayer to suspension cultures (1). The surface pp 105 was found to be identical to a phosphoprotein present in the cytosolic fraction after cell disruption at low magnesium concentration in the absence of calcium ions (2). Isolation of the cytosolic pp 105 at high purity allowed the preparation of specific antisera. In this paper, we present data on the cellular location of pp 105 using cell lines of various species and immunofluorescence techniques. Data on the presence of pp 105 in various normal mouse tissues were established by the immunobinding assay.

## MATERIALS AND METHODS

Cells: The following cell lines were investigated: STU 51A-232B mouse fibroblasts, a subline of SV 40-transformed embryonic cells of STU mice; STU D1756

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mouse fibroblasts, a subline derived from transformation of STU mouse embryonic cells with Rous sarcoma virus, supplied by Dr. Kurth, Frankfurt, FRG; K 42-A2 hamster fibroblasts, a subline derived from an SV 40-induced hamster tumor; HT29 human colon carcinoma cells (3); Drosophila embryonic Kc cells(4) obtained from Dr. Saumweber, Tübingen, FRG. Chicken cartilage cells were a primary culture derived from 10-day-old chicken embryos. Mouse embryonic cells were prepared from 18-day-old embryos. L 1210 mouse leukemia cells, 3T3, RAJI and HeLa cells were obtained from Flow Laboratories. Suspension cultures were established in MEM (spinner modification without Ca) (1).

Tissue extracts, nuclei and nucleoli: Tissue extracts were obtained by homogenization of minced tissue in PBS containing 0.5 mM PMSF, followed by two 5 sec cycles of ultrasonication (Branson sonifier, 75 W) and centrifugation at 2000 xg for 5 min. Nuclei and nucleoli were prepared from suspension cells following the method of Muramatsu and Onishi (6) but using higher concentrations of magnesium (5 mM) in the first step.

Sera: anti pp 105 serum was obtained by immunizing a rabbit with 1 mg highly purified native pp 105 (2) suspended in complete Freund's adjuvant (1:1), and boosting 2 months later with 1 mg pp 105 suspended in incomplete Freund's adjuvant (1:1). Peroxidase-conjugated and FITC-conjugated goat anti rabbit IgG was purchased from Miles Laboratories.

Immunobinding assay (Western blot): The method of Towbin et al.(5) was modified. After electrophoresis and transfer of samples with 80 µg total protein of cell and tissue extracts, the nitrocellulose strips were incubated in saturation buffer (10 mM Tris-HCl pH 7.0 containing 0.15 M NaCl, 0.1% sodium azide, 10% fetal calf serum, 10% goat preimmune serum, 3%bovine serum albumin). After 3-6 hr incubation at room temperature, rabbit anti pp 105 serum (100 µl/20 ml saturation buffer) was added and incubated overnight. The strips were then washed 5 times in 10 mM Tris-HCl/0.15 M NaCl pH 7.0 and incubated for 12 hr with peroxidase-conjugated goat anti rabbit IgG diluted 1:1000 with wahing buffer containing 10% fetal calf serum, 3% bovine serum albumin and 0.02% merthiolate. After 5 washes with washing buffer, the peroxidase reaction was developed with a mixture of 20 ml 25 mM Tris-HCl pH 7.5, 4 ml 4-chloro-1-naphthol (3 mg/methanol) and 8 µl hydrogen peroxide (30%).

Indirect immunofluorescence: Suspension cells, isolated nuclei and nucleoli were absorbed to poly-lysine-coated glass slides and incubated with fetal calf serum for 10 min to block free poly-lysine. Adherent cells were used directly. For fixation, the samples were washed with PBS, incubated with 1%(surface staining) or 4% formaldehyde in PBS (intracellular staining) for 10 min at room temperature. Thereafter, the samples were washed twice with PBS for 5 min, incubated in a 1:80 dilution of anti pp 105 serum for 40 min at 37°C, washed 3 times with PBS and then incubated with a 1:80 dilution of goat anti

rabbit IgG conjugated with FITC for 45 min. After washing, the samples were mounted in glycerol/PBS (9:1). Fluorescence was observed with a Zeiss II R 5 microscope and photographed on Kodak-Tri X-Pan film.

Standard phosphorylation procedures: Aliquots of isolated nucleoli, cellular sonicates and tissue extracts were brought to 37°C and phosphorylated in a buffer with final concentrations of 50 mM sodium phosphate pH 7.8, 10 mM magnesium chloride and 10 mM ß-mercaptoethanol by adding [$\gamma$-$^{32}$P] ATP (30 µCi/100 µl sample volume; specific activity 40 Ci/mmol). After 2 min the reaction was stopped by adjusting the samples to 10 mM EDTA (pH 7.0) and 1% SDS, followed by 5 min heating at 100°C. When samples were used for immunoprecipitation (7), phosphorylation was stopped by 5 mM p-chloro-mercuriphenylsulfonic acid (PCMPS; Sigma Chem.). $^{32}$P-incorporation into pp 105 was determined after SDS polyacrylamide gel electrophoresis and autoradiography of the dried gels. Thereafter, the pp 105 bands were cut out, soaked in water and solubilized with Soluene (Packard Instr.) followed by determination of radioactivity. In vivo phosphorylation was established with logarithmically growing STU 51A-232B suspension cells ($10^9$ cells/500 ml) by a 12 hr pulse with 1 mCi $^{32}$P-orthophosphate in phosphate-free medium.

## RESULTS

Cellular localization of pp 105: A rabbit antiserum against electrophoretically pure pp 105 was used to characterize the location of pp 105 in STU 51A-232B suspension cells from which pp 105 was isolated. The specificity of the antiserum was tested by immunoprecipitation (7) and the immunobinding assay (5) using endogenously phosphorylated cellular sonicates. Most samples showed only pp 105 as reacting protein. In a few cases, some additional cross-reacting components with 98, 92, 87, 83 and 60 KDa were identified, containing up to 45% of total $^{32}$P incorporated into the immunoprecipitated proteins. The intracellular location could be demonstrated by strong immunofluorescence of nucleoli, predominantly in the periphery, and a diffuse but weaker fluorescence of the whole nucleus (Fig. 1a). The background fluorescence of the nucleus is absent when isolated nuclei were studied (Fig. 1b), but fluorescence is still strong in nucleoli even after isolation (Fig. 1c). All other mouse cell lines tested, including mouse embryonic cells (Fig. 1e), yielded similar results. Cell surface fluorescence was studied with non-permeabilized cells. Only suspension cells showed surface fluorescence, partly with a capping phe-

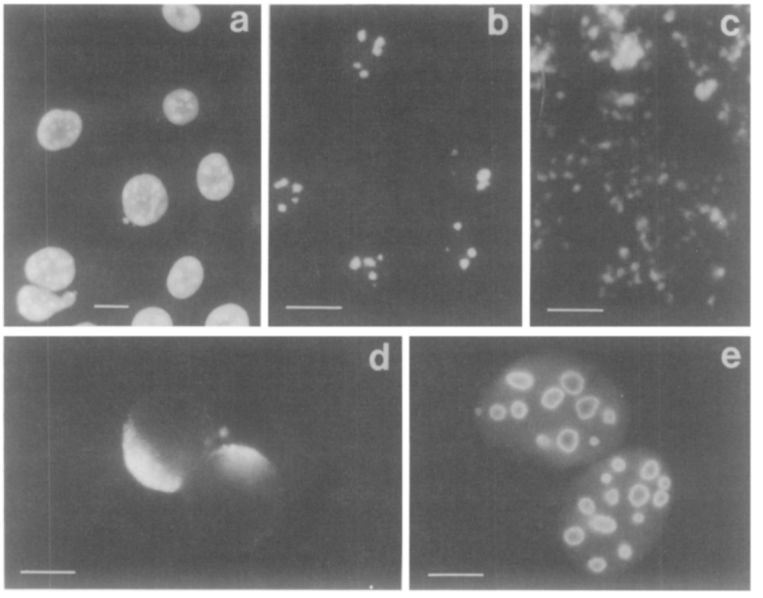


Figure 1: Immunofluorescent microscopy using rabbit anti pp 105 serum and goat anti rabbit IgG conjugated with FITC as second antibody: The specimen was fixed with 4% formaldehyde and permeabilized with methanol. (a) STU 51A-232B suspension cells; (b) isolated nuclei; (c) isolated nucleoli; (e) mouse embryonic cells; (d) STU 51A-232B suspension cell fixed only with 1% formaldehyde without permeabilization.

nomenon (Fig. 1a). Immunobinding studies with tissue extracts from normal mouse thymus, spleen, lymph nodes, liver, kidney, brain, placenta and epididymal fat pads, showed significant amounts of pp 105 in all tissues. Interspecies cross-reactity of anti mouse pp 105 serum was studied with cellular sonicates of hamster and human cell lines, chicken cartilage cells and Drosophila embryonic cells using the immunobinding assay. All cells exhibited a cross-reacting main protein in the range of 100-105 KDa (Fig. 2a-e).

Endogenous phosphorylation of isolated nucleoli: The electrophoretic pattern of phosphoproteins resulting from endogenous phosphorylation of nucleoli (Fig. 2f) shows pp 105 as a main nucleolar component. The time course of nucleolar phosphorylation of pp 105 is given in Fig.3a. Optimal reaction was observed at pH 8.7 (Fig. 3b) and isotonicity (Fig. 3c) in the presence of 10 mM magnesium (Fig. 3d). In the latter case, phosphorylation was performed in 0.1 M Tris-HCl buffer pH 7.5. At zero magnesium concentration, i.e. in the presence of 20 mM EDTA, pp 105 was not phosphorylated. The isoelectric point of pp

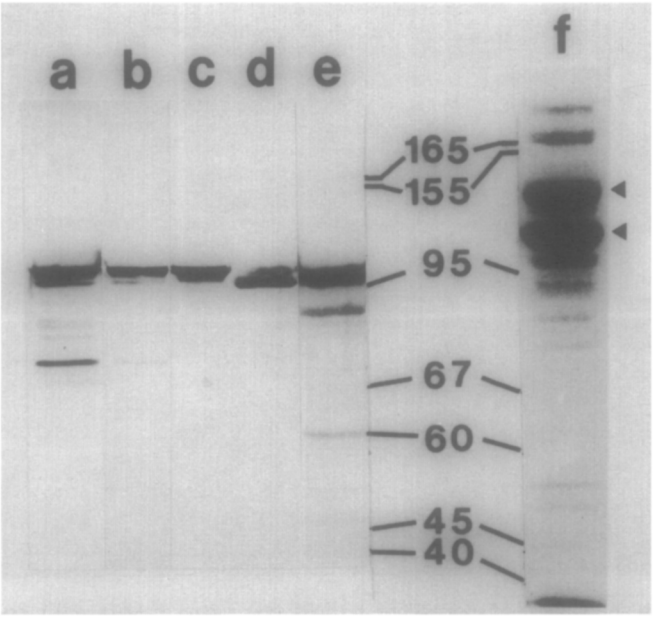


Figure 2: Western blot of cellular sonicates using rabbit anti mouse pp 105 serum: (a) mouse STU 51A-232B cells; (b) hamster K 42-A 2 cells; (c) human HT 29 cells; (d) Drosophila embryonic $K_c$ cells; (e) chicken cartilage cells; (f) autoradiography of the phosphoprotein pattern derived from endogenous phosphorylation of purified nucleoli (arrows indicate pp 135 and pp 105). Cross-reacting proteins have molecular weights of 98 KDa (a-e); 87 KDa (e); 83 KDa (a), 60 KDa (a).

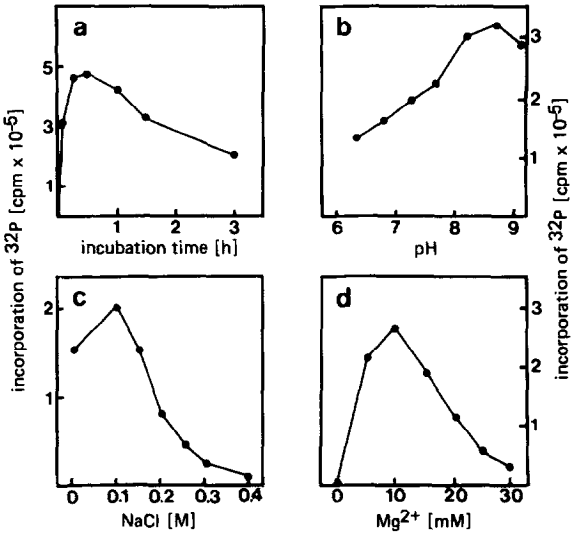


Figure 3: Endogenous phosphorylation of isolated nucleoli from STU 51A-232B suspension cells: (a) time course; (b) dependence on pH; (c) dependence on NaCl concentration; (d) dependence on magnesium concentration. Each sample representing one dot contained 20µg nucleolar protein.

105 varied between pI 5.9 and 6.2. Furthermore, the pattern of phosphoamino-acids of pp 105 resulting from endogenous phosphorylation of isolated nucleoli was compared to in vivo phosphorylation of logarithmically growing suspension cells during a 12 hr pulse with $^{32}$P-orthophosphate. The ratios phosphoserine: phosphothreonine determined by thin-layer electrophoresis (8) after 4 hr hyrolysis in 6 N HCl at 110°C, were 65:1 after in vitro and 10:1 after in vivo phosphorylation; phosphotyrosine could not be detected.

## D I S C U S S I O N

Phosphoprotein pp 105, orginally detected on the cell surface of mouse suspension cells by endogenous phosphorylation of intact cells (1) could now be identified as a major nucleolar protein using immunofluorescence techniques. Its location is not restricted to nucleoli, a weaker pp 105-specific fluorescence was observed in the whole nucleus. The maintenance of its nucleolar location during disruption of cells required the presence of calcium. Cell disruption in the absence of calcium at low magnesium concentration (0.2 mM) rendered most pp 105 into a soluble form which could be isolated from the cytosolic fraction (2). Phosphoprotein pp 105 is most likely identical with the major nucleolar protein C 23 from rat Novikoff hepatoma cells having a molecular weight 103-110 000, pI 5.2-5.5 and a phosphoserine: phosphothreonine ratio 35:1 (9-11). Analogy to C 23 protein was also assumed for a 110 000 Da acid-soluble nuclear phosphoprotein from mouse ascites sarcoma cells (12) and for a 100 000 Da nucleolar and ribosomal phosphoprotein from hamster ovary cells (13). In only case (9), intracellular location was studied by immunofluorescence yielding a C 23 location different from that observed by us. This discrepancy is most likely due to an experimental artifact, since the authors did not prefix cells with 4% formaldehyde before permeabilization with acetone which, in our experience, can lead to elution and secondary absorption. To date, there have been no reports of pp 105 surface fluorescence of suspension cells analogous to our observations. Investigations of the nature of cell surface pp 105 are the subject of further studies.

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