LIGHT AND ELECTRON MICROSCOPE IMMUNOLABELLING AND FLOW CYTOMETRIC EVALUATION OF A 33 kDa STRESS PROTEIN DURING THE CELL CYCLE OF EUE CELLS GROWN IN HYPERTONIC MEDIUM

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Received for publication April 13, 1992 and in revised form July 17, 1992

The aim of this work was to look for the stress protein P₃₃, during long-term growth of human EUE cells in a hypertonic medium, with a polyclonal antibody against P₃₃ produced in rabbit, and detectable in cells by both light and electron microscopy, using fluorescein-, peroxidase-anti-peroxidase- and gold-indirect immunolabelling. P₃₃ was found to be mostly located in the cytoplasm, without apparent association with subcellular organelles; a slight, but significant, labelling was also observed in the nucleus. Dual parameter flow cytometric measurements of fluorescein-immunolabelled P₃₃ and DNA content (after propidium iodide staining) showed that the amount of P₃₃ increased progressively with increasing times of growth in a hypertonic medium in all phases of the cycle. Therefore there is no direct relationship between the increase of the P₃₃ and the exit of cells from the cycling compartment that occurs in a hypertonic environment.

Recently, we have reported that the synthesis of a 33 kDa protein (P₃₃) is enhanced when EUE (human embryonic epithelium) (13) cells were grown for long periods (4 days onwards) in a hypertonic (HT) medium. The level of this protein reverts to the controls when HT-adapted cells were transferred to the isotonic (IT) medium (7), suggesting that there is a direct relationship with resistance to the HT stress (7, 10).

Long term adaptation to an HT medium also entails other characteristics, such as reversible changes in the phenotype of EUE cells: cell volume enlarges (10); there is an increase and a restructuring of cytoskeletal intermediate filaments (4); sulpholipids, gangliosides (2), and enzyme activities linked to ion transport mechanisms (3) also increase. It is of special interest that the growth rate is slowed down under HT stress,

and that most cells are blocked in a resting state (12).

The aim of this work was first to localize, by both light and electron microscopy, the distribution of P₃₃ proteins in EUE cell populations grown in IT or HT medium. A second aim was to monitor the changes in P₃₃ expression at different times of growth in an HT medium, and to see whether the changes are related to the cell cycle changes.

To perform these analyses, a polyclonal antibody against P₃₃ was produced, to be used for fluorescence-, peroxidase-anti-peroxidase (PAP)-, and gold-indirect immunodetection. On suspensions of single cells, dual parameter cytometric measurements of DNA content and P₃₃ were also made, using propidium iodide staining and indirect immunofluorescent labelling.

MATERIALS AND METHODS

Cell cultures and growth conditions

EUE cells were grown in minimum essential

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Eagle's medium, supplemented with 10% newborn calf serum, 1% glutamine, 50 units/ml of streptomycin and penicillin. For the HT medium, the concentration of NaCl was raised from 0.136 M to 0.274 M in the Hank's salt solution used as a component of the culture medium (the final osmolarities were 305 mOsm and 495 mOsm).

Cells were grown in a 5% CO₂ atmosphere, in either IT or HT media during different time periods (1 to 20 d); in addition, 10-d HT-adapted cell samples were transferred to the IT medium, in which they were grown for 1 and 2 d (for the sake of simplicity, this culture condition will be defined as deadaptation, and the corresponding cells called de-adapted: DA).

Culture media were changed every two days, to ensure abundance of nutrients. Cell death was determined by the Trypan blue exclusion test for IT, HT, and DA cells, and proved to be comparable (less than 1%) for cells grown under all the culture conditions.

Anti-P₃₃ polyclonal antibody production

SDS-PAGE followed by western blotting with the P₃₃ antiserum

SDS-PAGE was carried out on cell extracts, following the procedure described by Laemmli and Favre (8), at T 10% and C 2.7%, in 10 × 8 gel slabs. All the samples were run in duplicate in two halves of the same gel (for rabbit immunization, see below).

After the run, the gel was copied on nitrocellulose paper, following the procedure described by Towbin *et al.* (14). The nitrocellulose sheet was then cut into two halves. One was incubated for 2 hr at room temperature (r.t.) with a 1 : 500 dilution of the anti-P₃₃ antiserum and, after washing, for 1 hr with a 1 : 1,000 dilution of conjugated goat-anti rabbit IgG (DAKOPATTS, Copenhagen).

The immuno-positive bands were revealed by staining through an alpha-chloronaphthol-H₂O₂ solution (10 mg of alpha-chloronaphthol and 10 µl of 35% H₂O₂ in 100 ml PBS). The second half of the nitrocellulose sheet, also containing the molecular weight standard mixture, was stained with Coomassie-blue and compared with the other half.

Rabbit immunizations

The gel slice was fragmented prior to injection by repeated passages through a syringe and emulsified with Freund Adjuvant. Rabbits were immunized by injection into the hindfoot pads and intramuscularly. Each animal was given four doses at two weeks intervals. The first dose was administered in Freund Complete Adjuvant (Gibco Laboratories, New York, USA) while the others were administered in Freund Incomplete Adjuvant.

Blood samples were collected from the marginal

ear vein after the second booster and blood was allowed to clot for 60 min at 37°C. Rabbit antisera were tested by enzyme-linked immuno-absorbent assay on EUE extract-coated microtiter plates (see below). Rabbits were exsanguinated after the third booster.

Titration of rabbit antisera

Total EUE extracts (from both HT and IT cells) were coated (1 µg/well in 50 mM carbonate buffer pH 9) onto microtiter plates for 2 hr at 37°C. Plates were saturated with phosphate buffered saline (PBS) containing 3% newborn calf serum (Flow Laboratories, Irvine, U. K.) for 1 hr at 37°C. Serially diluted rabbit antisera, or pre-immune sera as controls, were added and incubated for 2 hr at 37°C. After extensive washing, horseradish peroxidase swine anti-rabbit antiserum (DAKO) was added for 1 hr at 37°C. Plates were extensively washed and a chromogenic substrate (o-phenylenediamine, SORIN Biomedica, Saluggia, Italy) was added. The enzymatic reaction was blocked after 10 min by addition of 2N H₂SO₄. The optical density (O. D.) at 492 nm was determined with an automated reader Titertek Multiscan (Flow Laboratories, Mac Lean).

Light microscopy immunocytochemistry

For light microscopy, cells were grown in monolayers on coverslips. After 5 days growth in either IT or HT medium, they were washed in PBS and fixed for 15 min at r.t. with 2% paraformaldehyde containing 0.2% glutaraldehyde in IT or HT PBS; they were then carefully washed in PBS. At this very low concentration of glutaraldehyde, no auto-fluorescence was observed in unstained samples, thus making a fluorescence quenching procedure unnecessary.

Indirect immunocytochemical detection was performed with FITC-conjugated secondary antibody and peroxidase-antiperoxidase labelling.

FITC-Immunofluorescence procedure: Cell cultures were treated for 10 min with PBS containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA). They were then incubated for 5 min in a 1 : 100 non-immune goat serum (Sigma Chemical Co., St. Louis MO, USA) before being treated for 30 min at r.t. with the anti-P₃₃ antibody diluted 1 : 100 in PBS containing 0.05% Tween 20 and 0.1% BSA. After thorough washing in PBS, the cells were incubated for 30 min at r.t. with the FITC-conjugated goat anti-rabbit antibody (DAKO, Glostrup, Denmark), diluted 1 : 30 in PBS containing 0.05% Tween 20 and 0.1% BSA. After washing in PBS, they were mounted with PBS-glycerol 1 : 1, and immediately observed and photographed with a Leitz Dialux.

Peroxidase-anti-peroxidase procedure: Cells were

treated with PBS containing 0.05% Tween 20, and the endogenous peroxidases were blocked with 3% H₂O₂ in 10% methanol for 7 min at r.t.. Cells were incubated for 30 min in a 1:100 non-immune goat serum (Sigma) in PBS containing 0.05% Tween 20 and 0.1% BSA, and were reacted for 30 min at r.t. with a 1:100 anti-P₃₃ antibody in PBS containing 0.05% Tween 20 and 0.1% BSA.

After washing in PBS for 30 min, cells were incubated with a 1:50 goat-anti-rabbit antibody (Sigma) in PBS for 2 hr at r.t.. The successive incubation with a 1:100 dilution in PBS of rabbit peroxidase-anti-peroxidase antibody (Miles Laboratories Inc., Elkhart In, USA) was for 1 hr at r.t., and was followed by incubation with 0.05% diaminobenzidine in 0.05% Tris-HCl buffer pH 8 containing 0.01% H₂O₂ in darkness for 20 min. To enhance the final product of reaction, cells were treated for 5 min with 0.5% OsO₄ in 0.1 M phosphate buffer pH 7.4. Finally, they were dehydrated in a graded ethanol series, and slides were mounted in Eukitt.

The two procedures were also applied to semithin sections from Epon embedded pellets, prepared for electron microscopy. Some modifications were introduced: 1) the semithin sections were treated with Na ethanolate for 15 min at r.t. to solubilize the epoxin resin; 2) incubation in the anti-P₃₃ antibody was done overnight in a moist chamber at 4°C.

To control specificity, anti-P₃₃ antibody was replaced by non-immune rabbit serum (Sigma) in both labelling procedures.

Electron microscopy immunocytochemistry

IT and HT cells grown in Falcon flasks were collected by mild trypsinization and fixed in suspension for 1 hr at 4°C in 2% p-formaldehyde containing 0.2% glutaraldehyde in either IT or HT PBS. After dehydration through a graded ethanol series, the pellets were embedded in Epon 812.

Thin sections (600–700 Å), mounted on nickel grids, were used.

After etching the resin with 10% H₂O₂ for 15 min at r.t., the thin sections were incubated for 3 min with non-immune goat serum 1:100 in PBS, then in the anti-P₃₃ antibody 1:100 in PBS containing 0.05% Tween 20 and 0.1% BSA.

As controls of specificity of the anti-P₃₃ antibody binding, thin sections were incubated in non-immune rabbit serum 1:100 instead of the primary antibody.

After 5 min washing in PBS containing 0.05% Tween 20 and for 15 min in PBS alone, all the sections were incubated for 30 min at r.t. with a secondary goat-anti-rabbit antibody conjugated with 15 nm colloidal gold particles (Aurion, Wageningen, The

Netherlands), using a 1:6 dilution in PBS containing 0.05% BSA-C (an acetylated and partially linearized bovine serum albumin, Aurion).

The grids were air dried, then stained with 8% aqueous uranyl acetate: acetone (1:1, v:v), and photographed under a Philips EM 300 electron microscope.

The labelling density over the nucleus and cytoplasm (i.e. number of gold particles per unit area) was evaluated by a graphic tablet from photographs taken at the same magnification.

Flow cytometry

Immunofluorescent detection of P₃₃

For flow cytometry, cells collected by mild trypsinization, were washed with PBS, fixed with 4% formaldehyde in PBS for 10 min at 4°C, and finally washed with PBS. For P₃₃ immunolabelling, 10⁶ cells were resuspended for 1 hr at r.t. in a 1:100 dilution of antiserum in PBS containing 0.05% Tween 20 and 0.1% BSA. Cells were then washed in PBS and resuspended in a 1:20 dilution of FITC-conjugated goat anti-rabbit antibody (DAKO). Cells were finally washed in PBS before being reacted with propidium iodide (PI) for DNA.

The level of background fluorescence due to non-specific binding of the FITC-conjugated antibody was established from control cell specimens processed as previously described, but either without exposure to the primary antibodies or with exposure to a rabbit non-immune serum. Cells above the corresponding value of green fluorescence were considered as labelled. For all the culture conditions and periods, at least two experiments were performed.

Propidium iodide DNA staining and flow cytometric analyses

After processing for immunofluorescence, cell samples were stained for 30 min at r.t. with 5 µg/ml PI in water containing 100 Kunitz units/ml bovine pancreas RNase type A (Sigma).

Dual parameter measurements of green fluorescence (identifying P₃₃-positive cells) versus red fluorescence (PI-DNA content) were made with a Becton Dickinson (Sunnyvale, CA, USA) FACStar flow cytometer, under the following conditions: laser excitation power of 200 mW at 488 nm, 510–540 nm pass band filter for the green fluorescence detector, and 610 nm long-pass filter for the red fluorescence detector. At least 20,000 cells per sample were measured. Data were collected in list-mode with a Consort 30 software program running on a dedicated Hewlett Packard computer, and presented as either histograms of single parameters (for DNA) or plots for correlated green-to-red fluorescence signals. This approach makes it possible to study P₃₃ in single cells and to

relate it directly to specific cycle phases. Evaluation of cytometric data on dual-parameter plots was done by box analysis. FITC-immunolabelled cells were those with green fluorescence values exceeding the background threshold as reported above. The significance of the differences in mean green-fluorescence values was determined by the Student *t* test. The ranges for G_{0/1}, S and G₂+M phase cells were established on the basis of the corresponding DNA content histograms.

RESULTS

Titration of rabbit antisera

Rabbit sera were titrated by ELISA on both, HT and IT EUE extract-coated microtiter plates, after the second booster and after the final bleeding. Pre-immune sera were unreactive with both extracts. O. D. of immune sera (1 : 1,600) on HT extracts was 0.718 after the second booster and rose to 1.1 in the final bleeding. O. D. of the final bleeding on IT extracts at the same dilution was 0.129.

Immunoblotting of HT and IT extracts (Fig. 1) confirmed the specificity of our antiserum, which gave positive reactions for the P₃₃ protein band on both samples, with the expected quantitative differences: the HT extracts were stained from 10 to 20 times more intensely than IT extracts, depending also on the thoroughness of transfer from the gel to the nitrocellulose, which is never complete, but usually 30 to 50%. Some minor slightly positive bands, migrating in lower molecular weight regions, appeared when larger quantities of samples were applied to the gel (more than 20 µg on the 10×8 gel slabs).

Immunolabelling of P₃₃ at light and electron microscopy

In the monolayer cell cultures reacted with the secondary FITC-conjugated antibody, positive cells were found in both HT and IT cultures (Fig 2, 3). Nevertheless, there was a much stronger fluorescence in the HT cells. Cell immunolabelling was mostly cytoplasmic and evenly distributed throughout the inner cytoplasmic areas surrounding the nucleus, and between the lipid droplets, which are especially

FIG. 1. Immunoblotting of HT and IT EUE cell extracts with the anti-P₃₃ antiserum.

Lane 1: standard molecular weight mixture stained with Coomassie blue (from top to bottom: bovine serum albumin 66.2 kDa; ovalbumin 42.7 kDa; carbonic anhydrase 32 kDa; trypsin inhibitor 21.5 kDa; lysozyme 14.4 kDa).

Lane 2: HT EUE cell extract stained with Coomassie blue.

Lane 3: immunoblotting of HT EUE cell extract with the anti-P₃₃ antiserum.

Lane 4: immunoblotting of IT EUE cells extract with the anti-P₃₃ antiserum.

numerous in HT cells (where they appeared as dark spots). At the cell periphery, where the cytoplasm is extremely flattened and thin, fluorescence was comparatively weaker. Lesser fluorescence, which could be due to overlying cytoplasm, was observed on the nucleus. The immunolabelling patterns obtained with the peroxidase-anti-peroxidase procedure (Fig. 4, 5) were similar to the immunofluorescence features described above.

The distribution patterns of P₃₃ could be resolved better on semithin section, reacted with both the FITC- and PAP procedures. The immunolabelling

FIG. 2. EUE cells grown 5 d in HT medium. The FITC-immunolabelling is mostly in the cytoplasm, between the dark lipid vacuoles. There is lesser fluorescence over the nucleus. ×1,200

The inset shows that there is still low fluorescence over the nucleus in a semithin (1 µm) section in which there is no overlying cytoplasm. ×2,000

FIG. 3. In EUE cells, grown in IT medium, the FITC-immunolabelling is of very low intensity. Fig. 3a. Negative control IT sample, in which anti-P₃₃ antibody was replaced by non-immune rabbit serum. ×1,200

FIG. 4. The peroxidase-anti-peroxidase immunolabelling in HT cells is also strongly positive in the cytoplasm (the lipid vacuoles appear light). ×1,200

FIG. 5. The peroxidase-anti-peroxidase immunoreaction is weakly positive in IT cells. Fig. 5a. Negative control IT sample, in which anti-P₃₃ antibody was replaced by non-immune rabbit serum. ×1,200

FIGS. 6, 7. Thin sections of EUE cells grown 5 days in HT medium. Immunogold labelling and uranyl acetate staining. The 15 nm colloidal gold particles are scattered throughout the cytoplasm, without any significant association with cell organelles or cytoskeletal filaments (asterisks in Fig. 7). There are also gold particles on the nucleus (arrows).

Fig. 6. $\times 26,000$ Fig. 7. $\times 30,750$

FIG. 8. EUE cells grown in IT medium have very slight immuno-gold labelling, on both cytoplasm and nucleus. $\times 26,000$

Fig. 8a. Negative control IT section, in which anti- P_{33} antibody was replaced by non-immune rabbit serum. $\times 26,000$

TABLE 1. *Density of gold particles in the cytoplasm and nucleus of EUE cells grown in IT or in HT medium*

Culture Medium		Cytoplasm	Nucleus
Isotonic	P < .002	0.84 ± 0.13 (0.23 ± 0.02)	0.48 ± 0.08 (0.27 ± 0.04)
Hypertonic		2.14 ± 0.44 (0.18 ± 0.02)	1.41 ± 0.46 (0.20 ± 0.07)
		P < .03	
		P < .02	
			P < .001

Data are indicated as the means $\pm$ standard deviations, the background values are reported in parentheses.

appeared to be evenly distributed throughout the cytoplasm. The nucleus, no longer covered by a cytoplasmic layer, was still weakly positive (Fig. 2, inset).

In all cases, the immunolabelling was specific,

since a negative reaction was obtained when the primary antibody was replaced by non-immune rabbit serum.

The distribution patterns of P₃₃ were also analyzed by electron microscopy. In the HT cells, gold par-

FIGS. 9a-c. Upper row: DNA histograms, after PI staining, of IT (a), 10 d HT (b) and DA (c) EUE cells. In HT medium, there are lesser S-phase cells having intermediate values between the G_{0/1} and the G₂+M peaks.

Lower row: Dual parameter cytograms of PI-DNA content versus FITC labelling of IT (a), 10 d HT (b) and DA (c) EUE cells. In the contour-graph drawings, dots represent single events measured, while the lines (from outside to inside) connect points for which the frequency of events was 10, 50, 100 or 200.

FIG. 10. In the graph, the mean amount of green fluorescence of FITC-labelled EUE cells following immunolabelling of P₃₃ was correlated with the time of growth in HT medium. Each point represents the mean value of at least three experiments.

ticles were evenly distributed throughout the cytoplasm, including at the cell periphery. There were no apparent associations of these granules with subcellular structures (such as plasma membrane, cell organelles or cytoskeletal filaments: Figs. 6, 7). The density of the gold particles in the cytoplasm was significantly higher (more than twice) in HT than in IT cells (Fig. 8, Table 1).

Gold immunolabelling was also present over the nucleus (Fig. 6), although at a significantly lower frequency than in the cytoplasm, as shown in table I. This is in agreement with the patterns observed by light microscopy of semithin sections. These labelling patterns were also specific, since the background, evaluated after incubation with non-immune rabbit serum, was very low in both HT and IT cells.

Flow cytometric determination of P₃₃ expression versus DNA content in single cells

After dual-parameter cytometric measurements of P₃₃ and DNA content, labelled cells were found in all cycle phases (Figs. 9a, b, lower row). FITC green fluorescence intensity was taken as a rough estimate of the amount of P₃₃. Despite rather wide variability (the CV values ranged from 35 to 50%), the mean values of the whole cell populations were significantly different in IT and HT cells at all the incubation times ($P < 0.001$), and increased progressively with increasing time of growth in HT medium (Fig. 10).

The frequencies of cells with P₃₃ contents above the upper limit of green fluorescence intensity of IT

cells also increased with the time of growth in HT medium (after 20 days, more than 90% of the cells had higher green fluorescence values).

According to their nuclear DNA content after PI staining (Fig. 9, upper row), cells can be divided into three main classes: corresponding to G_{0/1}, G₂+M (with twice the amount of DNA of the previous class), and S phase cells (with intermediate DNA contents). In all culture conditions, P₃₃ content increased from G_{0/1} to the G₂+M phase. Dual parameter cytograms (Fig. 9) show that a given amount of P₃₃ must be present before a cell can proceed into the S phase and onwards. In fact, all the cells with relatively lower green fluorescence values have DNA contents in the G_{0/1} range.

EUE cells, de-adapted for at least 1 d in IT medium, had amounts of P₃₃ comparable to those of IT controls (Fig. 9c).

DISCUSSION

Electrophoretic data (7) showed that the relative amount of P₃₃, compared to other protein bands, reached a plateau after 4 d of adaptation. The results of our cytometric study are consistent with the electrophoretic data, since the progressive increase of P₃₃ content per cell (up to 20 d of growth in HT medium) parallels the enlargement of cell volume as observed in a previous work (10).

Immunolabelling of P₃₃ showed, by both, light

and electron microscopy, that this protein is mostly located in the cytoplasm, and does not bind to cytoplasmic organelles. These findings are in agreement with the biochemical indication that P₃₃ is, for the most part, a soluble protein; in fact, more than 80% of this protein was extracted from cell lysates after exposure to low ionic strength solutions in the absence of detergents. However, a small amount is apparently organelle-bound, being extracted after SDS treatment (7), and this is consistent with our evidence of nuclear immuno-labelling. It is, however, unlikely that P₃₃ plays a similar role in the nucleus to the role hypothesized for the cytoplasm. At the present state of the research, the hypothesis of a regulatory role of nuclear P₃₃ on cell cycle progression cannot be ruled out.

The rapid increase of P₃₃, after exposure to the HT medium and its reversion to IT values upon de-adaptation, points to a direct involvement of this protein in the mechanism of resistance to HT stress.

The chemical nature of P₃₃ is not completely established. However, preliminary data on the amino acid sequence of this protein (6) suggests a homology of P₃₃ with aldose reductase (5). It has recently been reported that this enzyme activity is high in HT cultured cells from renal medulla. The role of aldose reductase should be to increase the endocellular osmotic pressure in order to cope with the higher tonicity of the extracellular milieu (1). A similar role could be envisaged for cytoplasmic P₃₃ of EUE cells which are of epithelial origin, as are renal medullary cells. It will be of interest to see whether P₃₃ is increased in other cell types grown in an HT medium, and, especially, if this mechanism for resisting HT stress is also exploited by non-epithelial cells.

ACKNOWLEDGEMENTS

This work was supported by the Italian MURST (60% grants). Dual parameter cytometric evaluations were performed with the FACStar cytometer of the Centro Grandi Strumenti (Pavia University). We also wish to thank Mrs. Marinella Volonte', Mrs. Paola Veneroni and Mr. Sergio Veneroni for their technical assistance.

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