Influence of hyperthermia on the phosphorylation of ribosomal protein S6 from human skin fibroblasts and meningioma cells

W.W. Richter, K.D. Zang and O.-G. Issinger*

Institut für Humangenetik, Universität des Saarlandes, 6650 Homburg-3, FRG

Received 25 January 1983

Skin fibroblasts and meningioma cells, derived from primary cultures of the same patients have been used to study the influence of hyperthermia on (i) cell morphology and (ii) phosphorylation pattern of ribosomal and ribosome-associated proteins. Incubation of tumour cells and fibroblasts up to 7 h at 42°C did not significantly change the cell morphology as compared to control cells kept at 37°C. At 42°C ribosomal protein S6 is shifted cathodically indicating a loss of negative charge, however no quantitative dephosphorylation of S6 was observed. Meningioma cells and fibroblasts did not differ significantly with respect to S6 phosphorylation.

Human *Tumour cell* *Hyperthermia* *Phosphorylation* *Translation* *Ribosome*

1. INTRODUCTION

Hyperthermal treatment of tissue cultures from different sources initially leads to a drastic reduction in the biosynthesis of almost all cellular proteins, subsequently giving rise to a complete new set of so-called heat shock proteins (HSP) [1–7]. These effects are accompanied by a rapid dephosphorylation of an M_r 36000 protein tentatively assigned as ribosomal protein S6 [8,9]. If cells are maintained at 42°C for several hours the rate of protein synthesis gradually increases again. The increase in protein synthesis and re-formation of large polysomes at 42°C does not occur when RNA synthesis is inhibited by actinomycin D while recovery of protein synthesis following return to 37°C occurs even in the presence of the drug. These results indicate that hyperthermia causes a specific lesion in protein synthesis at the level of polypeptide chain initiation which can be reversed either by restoration of normal temperatures or by RNA synthesis at 42°C [3].

Ribosomal protein S6 becomes rapidly and quantitatively dephosphorylated in *Drosophila* and plant cells after applying heat shock conditions [8,9]. Since the rise in temperature for these kinds of cells can easily be as high as 12–15°C we wanted to find out whether human fibroblasts and tumour cells would behave similarly, albeit at 42°C, since this is a clinically acceptable hyperthermia. Furthermore, we also looked for changes in the phosphorylation pattern of ribosome-associated proteins which consist mostly of initiation and elongation factors of protein synthesis, the former possibly involved in the above described reversibility of hyperthermal effects on initiation of protein synthesis.

2. MATERIALS AND METHODS

2.1. *Tissue culture and labelling conditions*

Material from human meningiomas and skin biopsies of the same patients were cut into small pieces and explanted as particle cultures in medium TC 199 with the addition of 10% fetal calf serum (FCS) [10,11]. After forming a dense monolayer the cells were trypsinised and subcultured under the same conditions. Prior to radioactive labelling, cells were starved for 12 h in a phosphate-deficient TC 199 medium in the presence of dialysed FCS

* To whom correspondence should be addressed

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

(10%). Cells were propagated at 0.2–0.5 × 10^5 cells/2 ml medium in 25 cm^2 Falcon flasks. In vivo labelling was done with 1 mCi [^{32}P]orthophosphate/Falcon flask for the times indicated in the legends to figures.

2.2. *Isolation and 2D-PAGE characterization of ribosomal proteins*

After labelling, cells were washed and lysed by the addition of a non-ionic detergent buffer [12]. Nuclei and mitochondria were removed by centrifugation. The total ribosomal fraction was isolated from the postmitochondrial supernatant by centrifugation in the presence of 130 KTM-3 buffer (130 mM KCl, 3 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4) as in [13]. Ribosomal proteins were extracted with acetic acid, dialysed against 7.5% propionic acid and then lyophilised. Two-dimensional PAGE was carried out in the first dimension in the presence of 8 M urea, 10 mM dithioerythreitol, 10 mM bis-Tris (pH 4.5) [13]. Under these conditions most proteins become protonated and move to the cathode. The second dimension electrophoresis was carried out in 12.5% polyacrylamide gels in the presence of SDS. Gel slabs were treated for fluorography [14] exposed on Fuji RX (NIF) X-ray films for the times indicated.

3. RESULTS AND DISCUSSION

Incubation of fibroblasts and tumour cells at 37°C and 42°C up to 10 h did not lead to any significant change in cell morphology. What one can observe is a tendency of the epithelioid tumour cells to form a more extended cell shape with knob-like structures at the peripheral endings in contrast to tumour cells which have been kept at 37°C. Fibroblasts do not show any such change in cell morphology under identical growth conditions (not shown).

After pre-incubation of fibroblasts and tumour cells in a phosphate-deficient medium for 12 h, before applying hyperthermal conditions, cells were either labelled with [^{32}P]orthophosphate or were first incubated at 42°C then followed by the addition of label. Fig.1 shows a time-dependent incorporation experiment where the uptake of radioactive phosphorus was measured over an extended period of time. These experiments were carried out

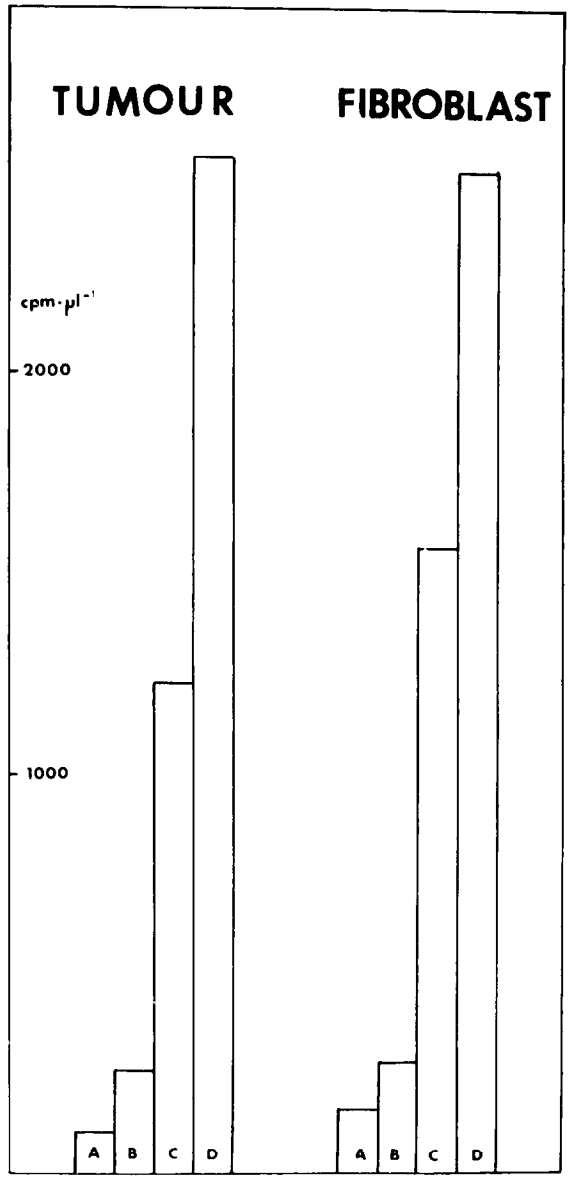

Fig.1. Incorporation of [^{32}P]orthophosphate into the ribosomal protein fraction after various times of incubation of fibroblasts and meningioma cells at 37°C. Incubation of the cells was carried out in the presence of [^{32}P]orthophosphate (500 µCi/ml, A,B,D). Incubation was for: (A) 15 min; (B) 30 min; (D) 10 h. (C) Incubation was carried out for 7 h in the presence of 250 µCi radioactive phosphorus, then followed by the addition of another 250 µCi for further 3 h. Aliquots (1 µl) were removed from extracted ribosomal material prior to two-dimensional PAGE and the trichloroacetic acid-precipitable material was determined in a liquid scintillation counter.

in order to exclude that any lack of protein phosphorylation was due to an exhaustion of the phosphate source in the medium. As one can see, even after 10 h incubation there is still a continuous uptake of label.

The ribosomal fraction was isolated by differential centrifugation in the presence of 130 mM KCl and thus contained all factors commonly found to be associated with ribosomes during the course of the translation process. The use of ribosomes which had not been subject to high salt treatment (500 mM KCl) prior to two-dimensional PAGE analysis should allow detection of phosphoproteins from both, ribosomal and ribosome-associated proteins. We had shown that both protein fractions can be analysed on the same gel system and can be distinguished by their migration behaviour in both dimensions so that unambiguous identification was ensured [15].

Phosphorylation of ribosomal protein S6 has been reported for several mammalian species and tissues ([16,17] and references therein). Although ribosomal protein S6 can exist in a multiply-phosphorylated state, in most mammalian tissues it is normally phosphorylated to much lower extent. Increased phosphorylation of S6 from tissue cultures has been reported when serum is restored [18,19] thus indicating a possible correlation with cellular proliferation.

So far no studies with human fibroblasts and meningiomal cells have been done with respect to the influence of elevated temperatures on the phosphorylation/dephosphorylation of protein S6. Therefore a comparative study between fibroblasts and meningioma cells was conducted in order to look for tumor-specific differences after applying hyperthermal conditions.

Ribosomal protein S6 from human fibroblasts

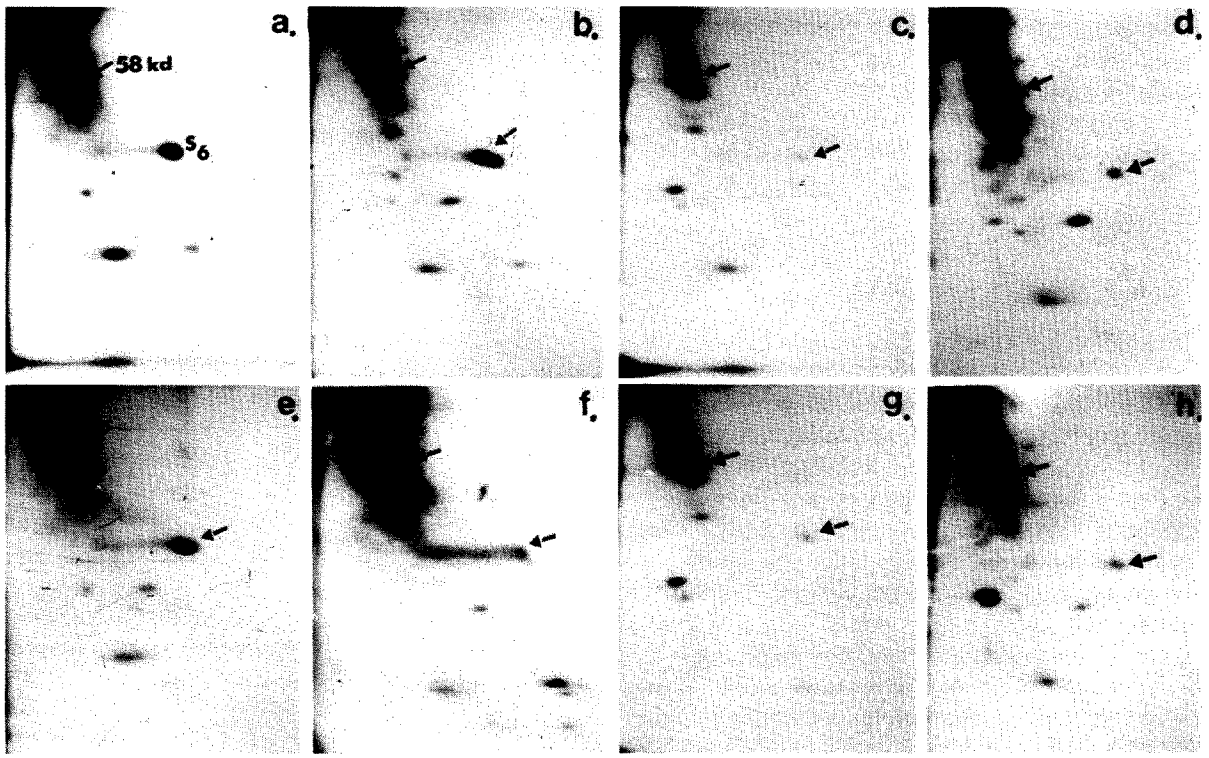

Fig.2. Fluorographs from [^{32}P]orthophosphate-labelled ribosomal and ribosome-associated proteins. Electrophoresis in the first dimension was from the anode to the cathode. Second-dimensional electrophoresis was in a 12.5% gel in the presence of SDS. Phosphorylated ribosomal proteins from human fibroblasts (a–d) and meningioma cells (e,h). Times of incubation were: 60 min, 37°C (a,e); 5 h, 37°C (b,f); 5 h, 42°C (d,h). Fibroblasts (c) and meningioma cells (g) after incubation for 3 h at 37°C followed by a shift to 42°C for 2 h.

and meningiomal cells became increasingly phosphorylated after 5 h incubation at 37°C resulting in an anodic shift of the radioactive spot (fig.2b,f). There is only one other protein which became phosphorylated to almost the same extent as ribosomal protein S6 and which according to its migration position in the gel is a non-ribosomal protein (M_r ~58000) possibly one of the subunits of initiation factor 2 (eIF-2) which had been shown earlier to become phosphorylated in vitro and in vivo [20,21]. We wanted to find out whether there is a specific cellular mechanism with respect to phosphorylation/dephosphorylation of ribosomal protein S6 and non-ribosomal proteins when unphysiological conditions are prevailing. Therefore we followed the phosphorylation of translational proteins during hyperthermal stress, since it also had been shown that high temperature treatment of tissue cultures from *Drosophila* and plant cells led to a quantitative dephosphorylation of protein S6 [8,9]. When fibroblasts and meningioma cells were incubated for 3 h at 37°C and then shifted to 42°C (fig.2c,g) S6 moved back to its former position (cathodic shift) and was also less phosphorylated than usually at 37°C. The same holds true when cells were pre-incubated for 2 h at 42°C and were then labelled for 5 h (fig.2d,h) or even when incubated for 2 h at 44°C (not shown). However, protein S6 always retained radioactive label. No significant difference concerning the phosphorylation behaviour of protein S6 was found between fibroblasts and meningioma cells. The lack of complete dephosphorylation of S6 could be due to the relative small increase in temperature; i.e., 5°C, as compared to 12°C and 15°C used for the heat shock of *Drosophila* and plant cells [8,9]. Although hyperthermal conditions influence the phosphorylation status of protein S6, the overall incorporation of radioactive phosphorus into trichloroacetic acid-precipitable material was not reduced. In contrast the high-M_r protein is unaltered despite hyperthermal conditions and seems to follow a different phosphorylation/dephosphorylation mechanism as protein S6 (fig.2b,f;d,h). Assuming a regulatory role for the initiation of protein synthesis through phosphorylation of translational proteins (e.g., initiation factors, ribosomal proteins and aminoacyl-tRNA synthetases as shown in [22] it would be reasonable to postulate a system which:

(i) Guarantees a continuous and selective initiation process (e.g., at the level of initiation factor phosphorylation); and

(ii) Allows for the regulation of mRNA translation velocity and quantity in response to unphysiological influences by scaling up or down protein synthesis after various stimuli via phosphorylation/dephosphorylation of protein S6.

ACKNOWLEDGEMENTS

We wish to thank Mrs V. Klein, H. Schulz and M. Thönnes for excellent technical assistance. This work was supported by DFG grant Is. 19/7.

REFERENCES

[1] McCormick, W. and Penman, S. (1969) J. Mol. Biol. 39, 315–320.
[2] Walsh, C. (1980) J. Biol. Chem. 255, 2629–2635.
[3] Hickey, E.D. and Weber, L.A. (1982) Biochemistry 21, 1513–1521.
[4] Laundry, J., Bernier, D., Chrétien, P., Nicole, L.M., Tanguay, R.M. and Marceau, N. (1982) Cancer Res. 42, 2457–2461.
[5] Schlesinger, M.J., Ashburner, M. and Tissières, A. (eds) Heat Shock: From Bacteria to Man, Cold Spring Harbor Laboratory, New York.
[6] DeDomenico, B.J., Bugaisky, G.E. and Lindquist, S. (1982) Proc. Natl. Acad. Sci. USA 79, 6181–6185.
[7] Cato, A.C.B., Sillar, G.M., Kioussis, J. and Burdon, R.H. (1982) FEBS Lett. 145, 57–61.
[8] Glover, C.V.C. (1982) Proc. Natl. Acad. Sci. USA 79, 1781–1785.
[9] Scharf, K.D. and Nover, L. (1982) Cell 30, 427–437.
[10] Handen, D.G. and Brunton, S. (1965) in: Human Chromosome Methodology (Yunis, J.J. ed) pp.57–73, Academic Press, London, New York.
[11] Paul, J. (1970) in: Cell and Tissue Culture (4th edn) Livingstone, Edinburgh, London.
[12] Nilsen-Hamilton, M., Allen, W.R. and Hamilton, R.T. (1981) Anal. Biochem. 115, 438–449.
[13] Issinger, O.-G. and Beier, H. (1978) Mol. Gen. Genet. 160, 323–339.
[14] Chamberlain, J.P. (1979) Anal. Biochem. 97, 169–183.
[15] Reichert, G. and Issinger, O.-G. (1981) Biochim. Biophys. Acta 654, 268–278.

[16] Leader, D. (1980) in: Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation (Cohen, P. ed) Molec. Asp. Cell. Reg. vol.1, pp.203–233, Elsevier Biomedical, Amsterdam, New York.
[17] Bielka, H. (1982) in: The Eukaryotic Ribosome, pp.76–78, Springer, Berlin, New York.
[18] Lastick, S.M., Nielsen, P.J. and McConkey, E.H. (1977) Mol. Gen. Genet. 152, 223–230.
[19] Haselbacher, G.K., Humbel, R.E. and Thomas, G. (1979) FEBS Lett. 100, 185–199.
[20] Issinger, O.-G., Benne, R., Hershey, J.W.B. and Traut, R.R. (1976) J. Biol. Chem. 251, 6471–6474.
[21] Benne, R., Edman, J., Traut, R.R. and Hershey, J.W.B. (1978) Proc. Natl. Acad. Sci. USA 75, 108–112.
[22] Damuni, Z., Claudwell, F.B. and Cohen, P. (1982) Eur. J. Biochem. 129, 57–65.