J. Biosci., Vol. 12, Number 3, September 1987, pp. 281–287. © Printed in India.

Secreted proteins of quiescent, serum-stimulated and over-confluent mouse embryo fibroblasts

M. SUBRAMANIAM and G. SHANMUGAM
Cancer Biology Division, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

MS received 9 January 1987; revised 15 July 1987

Abstracts. Quiescent and proliferating cultures of Swiss mouse embryo fibroblasts were pulse labelled with [^{14}C]-amino acids and the newly synthesized proteins that were secreted into the medium were resolved by electrophoresis on Polyacrylafde gradient gels. Conditioned media obtained from quiescent cultures that were stimulated to grow by the addition of 20% fetal calf serum showed the presence of two unique polypeptides of molecular weights 48000 and 26000. A polypeptide of molecular weight 45000 was present in increased amounts in serum-stimulated cells than in quiescent cells. This protein was also superinduced in quiescent cells by cycloheximide treatment. Mouse embryo fibroblasts grown under over-crowded conditions secreted two proteins of molecular weights 35000 and 11000. The 35 K polypeptide was shown to be related to the major excreted protein of transformed cells, since it was immunoprecipitated by an antiserum to major excreted protein. These results indicate that the 48 K and 26 K proteins may be proliferation specific proteins, while the 35 K protein present in the conditioned media of over-confluent cells may be a marker of morphological transformation.

Keywords. Mouse embryo fibroblasts; secreted proteins.

Introduction

Cultured cells release a number of enzymes and proteins into the surrounding growth medium. Some of the factors released by the cells condition the medium, enhancing the growth or attachment of the cells to the substratum (Nilsen-Hamilton *et al.,* 1980). Among the molecules identified as released into the medium by fibroblasts are plasminogen activator (Ossowski *et al.,* 1973), collagenase (Werb and Burleigh, 1974), fibronectin (Hynes *et al.,* 1977), collagen (Peterkovsky, 1972), a major excreted protein (Gottesman, 1978), a 58,000 dalton protein and a 58,000-62,000 dalton phosphoprotein (Senger *et al.,* 1979). Transformed cells release more plasminogen activator (Ossowski *et al.,* 1973), phosphoprotein (Senger *et al.,* 1979) and major excreted protein (MEP) (Gottesman, 1978) into the medium when compared wmyh their untransformed counterparts.

In order to understand the role of secreted proteins in the life of normal cells, we have studied the secreted proteins of secondary cultures of mouse embryo fibroblasts maintained under different growth conditions.

Materials and methods

Secondary cultures of mouse embryo fibroblasts were maintained in Eagle's basal medium containing 10% bovine serum. Cells were made quiescent by growing sub-

Abbreviations used: MEP, Major excreted protein; FCS, fetal calf serum; CH, cycloheximide; HBS, Hank's balanced saline; SDS, sodium dodecyl sulphate; M_r, molecular weight.

confluent cultures in 0·5% serum containing medium for 72 h (Subramaniam and Shanmugam 1986a, b). Quiescent cells were either treated with 20% fetal calf serum (FCS) or cycloheximide for various periods as indicated in the figure legends to analyse the FCS and cycloheximide (CH)-induced secreted proteins.

The labelling protocol for secreted proteins was the same as described earlier (Subramaniam and Shanmugam 1985). Briefly, monolayer (120 cm^2) cultures were washed thrice with Hank's balanced saline (HBS) and then incubated for 25–30 min with 10 ml of HBS containing [^{14}C] -amino acid mixture (50 μCi/flask). At the end of incubation, the labelling medium was removed and the cells were incubated for 30 min or 3 h in serum-free chase medium. At the end of incubation, the medium containing labelled secreted proteins were collected and centrifuged at 2000 *g* to remove the cell debri. The proteins in the labelled conditioned medium were precipitated with trichloroacetic acid and the secreted proteins were electrophoresed.

Immunoprecipation of secreted proteins of over-confluent cells were carried out following the procedure of Chackalaparampil *et al.* (1985). Approximately 10 ml of the labelled medium containing secreted proteins obtained from over-confluent cells were concentrated to 0·5 ml in a dialysis tubing using Aquacide. This 0·5 ml was made up to 1 ml in RIPA buffer [0·1% Triton X-100, 1·0% sodium deoxycholate, 0·1% sodium dodecyl sulphate (SDS), 0·15 M NaCl, 2·0mM phenylmethylsulphonyl fluoride and 0·05 M Tris-HCl pH 7·2] and centrifuged at 10000 *g* for 20 min. To the supernatant, 20 μl of anti-MEP serum was added and the mixture was first incubated at 37°C for 30 min and then kept at 4°C overnight. An excess of protein-A sepharose (30 μl of 50% slurry in RIPA buffer) was added and the mixture was further incubated at 4°C for 6–8 h. The absorbed complexes were washed once with RIPA buffer and then thrice with 50 mM Tris-HCl pH 7·4 containing 5% sucrose, 1% Nonidet p-40, 0·5 M NaCl and 5 mM EDTA. Distilled water was used for final washing. The immune complexes along with protein A sepharose were subsequently suspended in 50 μl of electrophoresis sample buffer and heated at 100°C for 3 min. The sepharose beads were pelleted by centrifugation and the proteins in the supernatent were used for electrophoresis.

The secreted proteins and the immune complexes were electrophoresed in high resolution Polyacrylamide gradient (5–18%) gels containing SDS (Laemmli and Favre, 1973). Following electrophoresis, the gels were stained, dried and flurographed (Bonner and Laskey, 1974).

Results

Figure 1 shows the secreted protein profiles of quiescent cells that were stimulated to grow by the addition of FCS for various periods. Two major mitogen induced proteins of molecular weights (M_r) 48000 and 26000 were found throughout the cell cycle. These proteins were not seen in the conditioned medium of quiescent cells. These results indicate that the 48 K and 26 K proteins may play a role in cell proliferation since they are proliferation specific proteins found only in cells passing through the proliferative stages. A 45000 dalton protein component seen in quiescent cells showed an increase in serum-stimulated cells. Af all the secreted proteins, the 45 K protein was super-inducible by cycloheximide treatment (Shanmugam *et al.,* 1987). Since the 45 K protein was the major secreted protein of CH-treated cells, the fate of

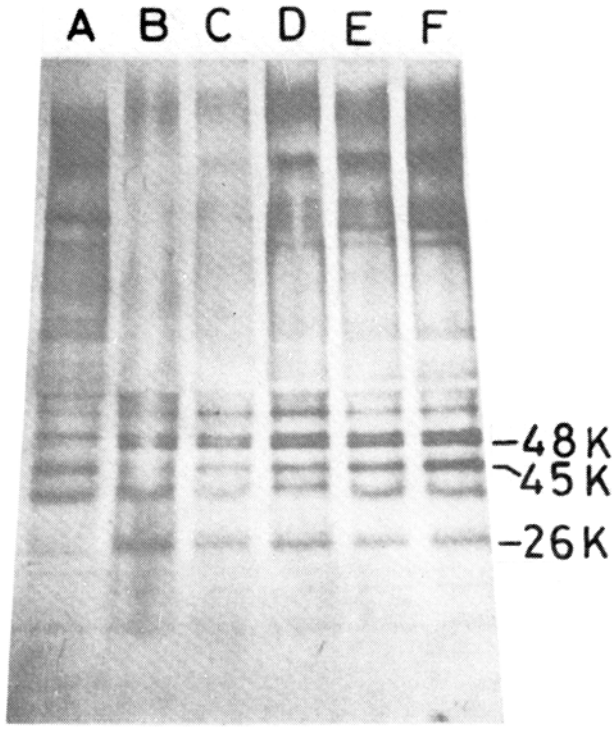


Figure 1. Secreted proteins of mouse embryo cells treated with 20% HCS. Quiescent mouse embryo fibroblasts were stimulated to grow with 20% HCS and the cells were labelled at different periods of stimulation. Hollowing 30 fn labelling and 30 fn chase, the labelled proteins in the conditioned medium were precipitated with TCA and electrophoresed in 5–18% Polyacrylafde gels and fluorographed. Each lane contained approximately 15,000 cpm. Lane A, secreted proteins of quiescent cells; lanes B, C, D, E and F show the profiles of secreted proteins of cells after 6, 12, 18, 24 and 30 h of stimulation, respectively.

the newly synthesized 45 K secreted protein was followed as described below. Quiescent mouse embryo fibroblasts were treated with CH for 6 h and immediately after this treatment the cells were washed and labelled for 30 min with [^{14}C]-amino acids and the labelled secreted proteins were chased for different periods (figure 2). Apart from the high M_r matrix proteins, the 45 K polypeptide was the first one to appear at the early period (30 min) of chase. At later chase periods, the accumulation of the 45 K protein progressively increased with concomitent increase in the level of other secreted proteins. Maximum amount of 45 K protein was observed in the medium 3 h after chase.

Secreted proteins of over-confluent cells

When secondary cultures of mouse embryo fibroblasts were maintained under crowded condition for more than a month, the cells in the monolayers became round in morphology and formed foci. These cells showed the morphology of transformed cells (figure 3). In order to know whether the over-confluent cells secrete proteins specific for their altered morphology, the secreted proteins of these cells were

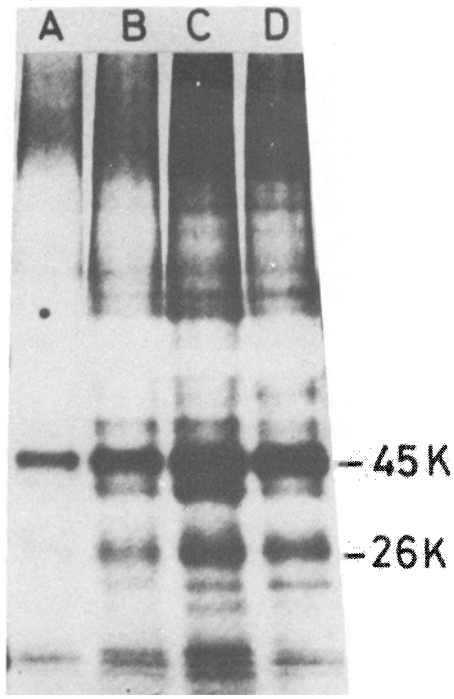


Figure 2. Fate of pulse labelled 45 K secreted protein. Quiescent mouse embryo fibroblasts were incubated with CH (3 μg/ml) for 6 h. After incubation, the cells were washed quickly with PBS and pulse labelled with [^{14}C]-afno acids for 30 fn. Hollowing the labelling, the cells were washed and incubated in serum-free chase medium for various time points. At the end of each time point the labelled proteins in the conditioned medium were precipitated with TCA and electrophoresed in 5-18% Polyacrylafde gels and fluorographed. Lane A, shows the protein profile after 30 min of chase. Lanes B, C and D depicts the protein profiles at 1,2 and 3 h of chase, respectively.

analysed. Secondary cultures of mouse embryo fibroblasts of confluent, over-confluent and serum-stimulated cells were labelled and the proteins obtained from the conditioned medium after 3 h of chase were electrophoresed in polyacrylamide gradient gels. Figure 4 shows the secreted protein profiles of these cells. Lanes A and B show the secreted protein profile of sub-confluent and confluent cells, respectively. Lane C shows the protein pattern of over-confluent cells in which two proteins of M_r 35 K and 11 K were unique to these cells. It is interesting to note the presence of the proliferation-specific 48 K protein in the proliferating sub-confluent (lane A) and over-confluent (lane C) cells. The confluent cells specifically show the presence of a 21 K secreted protein (lane B). The 35 K protein resembled the MEP by its M_r MEP was first identified by Gottesman (1978) and this protein was found to be synthesized in larger amounts by various transformed cells. Another protein with the M_r of 11 K which was seen only in over-confluent cells may be transforming growth factor -β because the M_r of the latter comes in the range of 11 K (Lawrence, 1985).

Since the 35 K secreted protein of over-confluent cells and MEP had the same M_r, immunoprecipitation experiments were carried out using anti-MEP serum. The labelled secreted proteins of over-confluent cells were immuno-precipiated with anti-MEP serum. The lane E in figure 4 shows the immunoprecipitation profile of

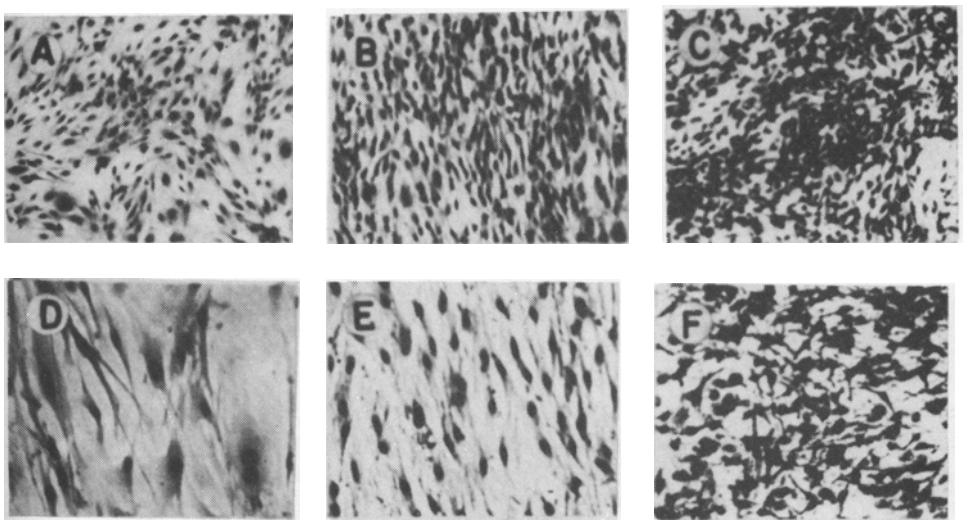
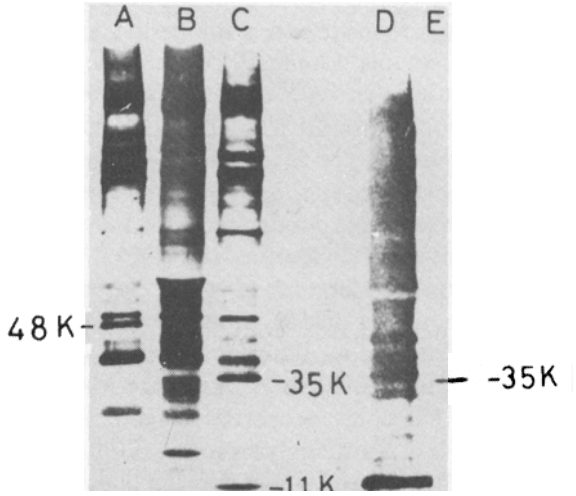


Figure 3. Photomicrographs of cells grown under different growth conditions. Secondary cultures of mouse embryo fibroblasts were grown on cover slips inside petri dishes. Cells at different growth conditions were fixed and stained with Giemsa stain and photographed: **A and D.**.Sub-confluent cells. **B and E.**. Confluent cells; **C and F.**. Over-confluent cells (A, B and C, X40; D, E and F X100).



Figured 4. Analysis of proteins of sub-confluent, confluent and over-confluent cells. Secondary cultures of mouse embryo fibroblasts were maintained under crowded conditions for over a month. After a prolonged maintenance at this stage, some cells in the monolayer became round in morphology and formed foci, similar to that of transformed cells. Cells were labelled with [^{14}C]-amino acids for 30 fn. Hollowing labelling, the cells were incubated in serum-free chase medium for 3 h. After this chase period, the labelled proteins in the conditioned medium were concentrated and immunoprecipitated with anti-MEP serum as described in methods. The secreted proteins and the immunoprecipitate were electrophoresed in 5-18% Polyacrylafde gel and fluorographed. Lane A, secreted proteins of sub-confluent cells; lane B, secreted proteins of confluent cells; lanes C and D, secreted proteins of over-confluent cells; lane E, immunoprecipitate obtained using anti-MEP serum of Secreted proteins of over-confluent cells.

over-confluent cells. It is interesting to note that the 35 K polypeptide was the only protein precipitated by anti-MEP serum indicating that the 35 K protein reported here is related to the MEP of transformed cells. This implies that the over-confluent cells mimic the transformed cells in secreting some of the transformation specific proteins.

Discussion

In this investigation, it has been shown that quiescent mouse embryo fibroblasts stimulated with serum mitogens released a set of proteins into the medium. Notable among these are proteins with the M_r of 48 K, 45 K and 26 K The 48 K and 26 K proteins were the major serum-induced proteins which were synthesized within a few hours of serum addition (figure 1). These proteins also persisted for a longer periods after serum stimulation. Since the 48 K and 26 K proteins are seen only in the medium of proliferating cells, these two proteins are designated as proliferation specific proteins. Proteins of similar M_r have been shown to be induced in Balb-C 3T3 cells by growth factors (Nilsen-Hamilton *et al.,* 1982). Our recent studies indicate the possible involvement of the 45 K secreted protein in the inhibition of DNA synthesis at the end of S-phase (Nagashanmugam and Shanmugam, 1987).

When secondary cultures of mouse embryo fibroblasts are maintained under crowded conditions for a long period (30 days), the cells overcome the density-dependent inhibition of growth and form colonies (figure 3). These over-confluent cells secrete novel polypeptides which were not found in confluent and serum-stimulated cells (see figure 4). Notable among these were the 35 K and 11 K secreted proteins which were absent in other types of cells. The 35 K secreted protein of over-confluent cells was immunoprecipitated with anti-MEP serum (see figure 4, lane E). MEP is a transformation related secreted protein (Gottesman, 1978). Increased MEP secretion was first observed in Kirstein sarcoma virus-transformed NIH 3T3 fibroblasts. The secretion of this protein is increased 50–100 fold in transformed cells, *versus* the NIH 3T3 parent cell line (Gottesman, 1978). Various agents induce an increase in the synthesis and/or secretion of MEP: Harvey and Moloney sarcoma viruses and SV40, phorbol ester tumor promoters and platelet derived growth factor (Gottesman, 1978; Cabral *et al.,* 1982; Gottesman and Sobel, 1980; Scher *et al.,* 1982). Although the function for MEP is not known (Gal *et al.,* 1985), recent work has suggested that MEP has some properties in common with the well-studied lysosomal enzymes. MEP is a phosphoglycoprotein (Gottesman and Cabral, 1981). Sahagian and Gottesman (1982) have shown that the phosphate associated with this protein is present as mannose-6-phosphate. These residues allow binding of MEP to phosphomanosyl receptors (Sahagian and Gottesman, 1982), which is required for targeting of lysosomal enzymes in cultured fibroblasts (Gonzalez-Noriega *et al.* 1980).

The results on the secreted proteins of over-confluent cells in combination with the data available in the literature (see above) suggest that over-confluent cells mimic transformed cells in secreting the 35 K protein and imply that they may be in a preneoplastic condition. Further biochemical work on these cells would throw light on how the normal cells grown under different growth conditions get converted into the preneoplastic and transformed cells. The secreted proteins reported in this study

may play important roles in cellular communication of mitogen-stimulated signal in proliferating cells.

Acknowledgements

This study was supported by a grant from the Department of Science and Technology, New Delhi. We thank Dr. Michael Gottesman for providing the anti-MEP serum.

References

Bonner, W. M. and Laskey, R. A. (1974) *Eur. J. Biochem.,* **46,** 83.
Cabral, F., Gottesman, M. M. and Yuspa, S. H. (1981) *Cancer Res.,* **41,** 2025.
Chackalaparampil, I., Banerjee, D., Poirier, Y. and Mukherjee, B. B. (1985) *J. Virol.,* **53,** 841.
Gal, S., Willingham, M. C. and Gottesman, M. M. (1985) *J. Cell Biol.,* **100,** 535.
Gonzalez-Noriega, A., Grubb, J. H., Talkad, V. and Sly, W. S. (1980) *J. Cell Biol.,* **85,** 839.
Gottesman, M. M. (1978) *Proc. Natl. Acad. Sci. USA,* **75,** 2767.
Gottesman, M. M. and Sobel, M. E. (1980) *Cell,* **19,** 449.
Gottesman, M. M. and Cabral F. (1981) *Biochemistry,* **20,** 1659.
Hynes, R. O., Destree, A., Mautner, V. M. and Ali, I. U. (1977) *J. Superamol. Struct.,* **7,** 397.
Laemmli, U. K. and Favre, M. (1973) *J. Mol. Biol.,* **80,** 575.
Lawrence, D. A. (1985) *Biol. Cell,* **53,** 93.
Nagashanmugam, T. and Shanmugam, G. (1987) *Cell Biol. Int. Rep.,* **11,** 147.
Nilsen-Hamilton, M., Hamilton, R. T. and Adams, G. (1982) *Biochem. Biophys. Res. Commun.,* **108,** 158.
Nilsen-Hamilton, M., Shapiro, J. M., Massoglia, S. L. and Hamilton, R. T. (1980) *Cell,* **20,** 19.
Ossowski, L., Unkeless, J. C, Tobia, A., Quigley. J. P., Rifkin, D. B. and Reich, E. J. (1973) *J. Exp. Med.,* **137,**112.
Peterkovsky, B. (1972) *Arch. Biochem. Biophys.,* **152,** 318.
Sahagian, G. G. and Gottesman, M. M. (1982) *J. Biol. Chem.,* **257,** 11145.
Scher, C. D., Handrickson, S. L., Whipple, A. P., Gottesman, M. M. and Pledger, W. J. (1982) *Cold Spring Harbor Conf. Cell Prolif.,* **9,**289.
Senger, D. R., Wirth, D. F. and Hynes, R. O. (1979) *Cell,* **16,** 885.
Shanmugam, G., Subramaniam, M. and Nagashunmugam, T. (1987) *in Biomembranes: Structure, Biogeniesis and Transport* (eds C. Rajamanickam and L. Packer) (New Delhi: Today and Tomorrow's Printers and Publishers) (in press).
Subramaniam, M. and Shanmugam, G. (1985) *Cell Biol. Int. Rep.,* **9,** 51.
Subramaniam, M. and Shanmugam, G. (1986a) *J. Cell. Physiol.,* **126,** 47.
Subramaniam, M. and Shanmugam, G. (1986b) *Cell Biol Int. Rep.,* **10,** 323.
Werb, Z. and Burleigh M. C. (1974) *Biochem. J.,* **137,** 373.