J. Biosci., Vol. 16, Number 4, December 1991, pp 175–186. © Printed in India.

Purification and characterization of a DNA synthesis inhibitor protein from mouse embryo fibroblasts

S SRINIVAS*, T NAGASHUNMUGAM** and G SHANMUGAM†
Cancer Biology Division, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India
Present address: *The Salk Institute, San Diego, California 92138, USA
**The Wistar Institute, Philadelphia, Pennsylvania 19104, USA

MS received 24 June 1991; revised 7 September 1991

Abstract. A DNA synthesis inhibitor protein was purified from the conditioned medium of cycloheximide treated mouse embryo fibroblasts. This protein has a molecular weight of 45,000 as determined by gel filtration and Polyacrylamide gel electrophoresis. The levels of the [^{35}S] methionine la belled 45 kDa protein in the medium and matrix were monitored across two cell cycles in synchronized cultures. The 45 kDa protein was present in higher levels in the medium of non-S-phase cells depicting a peak between the two S- phases. The DNA synthesis inhibitor protein was immunologically related to a chicken DNA-binding protein which showed similar cell cycle specific variations at the intracellular level. The purified 45 kDa protein inhibited DNA synthesis in murine and human cells. In mouse embryo fibroblasts, the DNA synthesis was inhibited to an extent of 86% by 0·25 μg/ml of the inhibitor, while higher amounts of the inhibitor were required to arrest DNA synthesis in human skin fibroblasts: in these cells, 4 μg/ml of the inhibitor inhibited DNA synthesis to an extent of 50%. The high levels of the 45 kDa protein in the medium of non-S phase cells and its DNA synthesis inhibitory potential suggest that this protein may be involved in the regulation of DNA synthesis during the cell cycle.

Keywords. DNA synthesis inhibitor; variations in cell cycle.

1. Introduction

The molecular mechanisms of regulation of cell growth by the endogenous proteins of the cell are of increasing interest. The role of autocrine growth factors in the stimulation of cell proliferation has been studied in detail (James and Bradshaw 1984; Heldin and Westermark 1984). However, much less is known about the role of endogenous growth inhibitor proteins on cell growth. A number of growth inhibitory proteins have been purified and characterized from various types of cells (Holley *et al* 1980; Harel *et al* 1985; Hsu and Wang 1986; Stein and Atkins 1986; Feltham *et al* 1987; Ervin *et al* 1989). Recently, transforming growth factor β was shown to act as a bifunctional modulator of cell growth (Roberts *et al* 1985). A secreted protein of molecular weight (M_r) 48,000 identified as plasminogen activator inhibitor-1 was suggested to be involved in the regulation of cell proliferation (Nagashunmugam *et al* 1989; Srinivas *et al* 1990).

We have recently shown that the conditioned media of non-S-phase fibroblast cultures contained large quantities of a 45 kDa protein, and this conditioned medium inhibited DNA synthesis (Nagashunmugam and Shanmugam 1987). In studies described here, the 45 kDa protein was purified from the conditioned medium of mouse embryo fibroblasts (MEF).The purified protein was shown to

†Corresponding author.
Abbreviations used: MEF, Mouse embryo fibroblasts; HOU, hydroxyurea; CH, cycloheximide.

inhibit DNA synthesis in murine and human cells. Studies on the levels of the 45 kDa protein across two cell cycles and its immunological relatedness to a chicken DNA binding protein (Matsuhashi *et al* 1987) that showed similar cell cycle specific variations are also described.

2. Materials and methods

2.1 *Synchronization of Cells*

Primary cultures of Swiss MEF were grown in Eagle's minimal essential medium (MEM) (Flow Labs., UK) containing 10% bovine serum (Flow Labs, UK) and gentamycin (50 μg/ml) at 37°C. Cells from the fourth passage onwards (secondary cultures) were used for experiments. Sub-confluent monolayers were synchronized by a double block method first at quiescence by growing for 48-72 h in 0·5% serum-containing medium and then at G1/S junction by treatment with 1 mM hydroxyurea (HOU) in MEM containing 10% bovine serum for 16 h. The human skin fibroblasts (LN 9 cells) were obtained from Dr M Digweed, and these cells were also synchronized as above. Cells were relieved of HOU arrest by washing the monolayers thrice with phosphate buffered saline followed by incubation in 10% serum-containing medium.

2.2 *Protein purification*

Quiescent monolayers in rollers were first treated with cycloheximide (3 μg/ml) for 15 h and then maintained for 1 h in serum-free MEM. One hundred ml of conditioned medium were concentrated to 0·5 ml by ultrafiltration using a 10 kDa cut-off Millipore membrane. The concentrated sample was fractionated in a LKB HPLC system using size-exclusion column (7·5 × 600 mm, TSK G-3000). The proteins were eluted with 20 mM Tris-HCl pH 7·2 and 1 mM EDTA at a flow rate of 0·2 ml/min and 0·5 ml fractions were collected. The molecular weights of the eluted proteins were estimated using proteins of known molecular weights. The peak fractions were pooled, lyophilized and dissolved in phosphate buffered saline (PBS). The protein solution was then filter-sterilized and assayed for DNA synthesis inhibitory activity. The protein concentration was determined according to Lowry *et al* (1951).

2.3 *Inhibitor assay*

DNA synthesis inhibitory activity was assayed using MEF and LN9 cells grown in 24 well plates. Each well contained 2×10^4 cells in 0·5 ml of growth medium. Cells were synchronized by HOU treatment as mentioned earlier. The HOU arrested MEF and LN9 cells were washed thrice with PBS and maintained in fresh growth medium in the presence or absence of purified DNA synthesis inhibitor protein. The DNA synthesis was monitored by labelling the cells with 5 μCi/ml [^{3}H] thymidine for 30 min after 2 h of release from HOU arrest. At the end of labelling, the cells in the wells were washed thrice with cold PBS and acid precipitable radioactivity was determined (Russell *et al* 1984).

2.4 *Gel electrophoresis*

The HOU arrested and stimulated monolayers (25 cm^2) were washed twice with PBS and labelled with 25 μCi of [^{35}S] methionine for 30 min in 2 ml of Hanks balanced salt solution. The radioactivity was later chased for 30 min in serum-free medium. The secreted and the extracellular matrix proteins were isolated as described earlier (Nagashunmugam *et al* 1989), electrophoresed in 5–18% Polyacrylamide gradient gels containing sodium dodecyl sulphate (SDS) and fluorographed (Nagashunmugam *et al* 1989). The relative amounts of the 45 kDa protein was deduced by laser densitometry of the fluorograms.

3. Results

In our previous studies, we have identified a group of early growth response proteins in MEF (Subramaniam and Shanmugam 1985, 1986a, 1987, 1988). One of these, the 45 kDa protein was present in large amounts in the growth medium of non-S-phase cells. Of all the secreted proteins of MEF, the 45,000 dalton protein was super-induced by cycloheximide treatment (figure 1). For these studies, quiescent cells were treated with cycloheximide (CH) (3 μg/ml) for 15 h after which the CH containing medium was removed and the cells were labelled with [^{35}S] methionine for 30 min. The radioactivity was then chased for different periods in serum-free chase-medium. Figure 1 shows the fate of the labelled 45 kDa protein at different periods of chase. The 45 kDa protein was the major secreted protein in the medium of CH treated cells in the first 1 h of chase. Other proteins started to accumulate in the medium from 2 h onwards.

3.1 *45 kDa protein levels in synchronized cells*

When the DNA synthesis of synchronized cells that were released from HOU-arrest was monitored by labelling with [^{3}H] thymidine, two peaks of DNA synthesis (one at 3 h and the other at 24 h after release) were observed (figure 2). The levels of the 45 kDa protein was quantitated at various periods after release from HOU-arrest by labelling the cells with [^{35}S] methionine and analysing the labelled secreted proteins present in the chase-medium. In our previous studies we observed a decline in the level of the 45 kDa secreted protein as the cells entered into peak DNA synthesis (Nagashunmugam and Shanmugam 1987). Here, we report a second decrease in the level of the 45 kDa protein at the beginning of the second S-phase; this decline continued up to the peak of DNA synthesis of the second S-phase (figure 2). When the levels of the 45 kDa protein were quantitated by laser densitometry, a 3-4-fold decrease in its level was observed at the first and second S-phase peaks, in comparison to its level in the culture media of non-S-phase cells 12–18 h after release from HOU arrest.

3.1a *45 kDa protein is a component of extracellular matrix*: Some of the secreted proteins like fibronectin and vitronectin are extracellular matrix components. These proteins were shown to have important roles in cell adhesion and cell-cell communication. Therefore, we looked for the presence of the 45 kDa protein in the

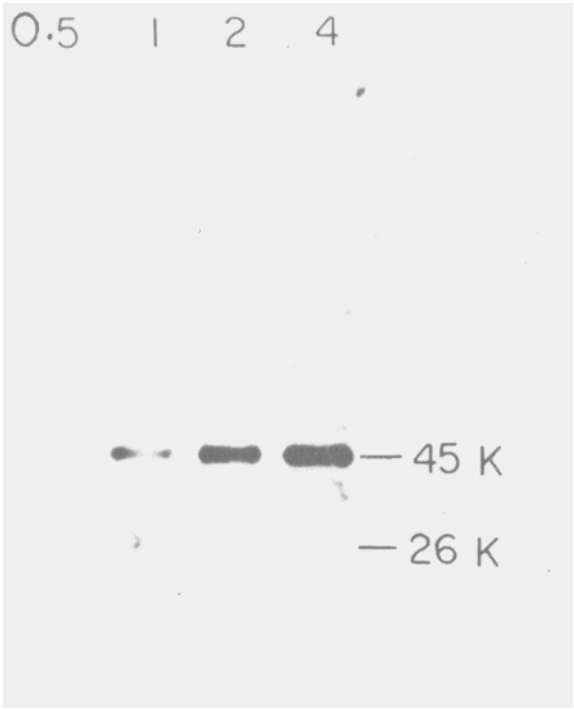

Figure 1. Secreted proteins of cycloheximide treated cells. Quiescent MEF were incubated with CH (3 μg/ml) containing medium for 15 h. At the end of incubation. CH-containing medium was removed, cells were washed and then labelled with [^{35}S] methionine for 30 min. After this pulse-labelling, the radioactive medium was removed, cells were washed and then incubated for different periods in serum free chase-medium. The labelled proteins secreted into the medium were analysed by electrophoresis in 5 to 18% Polyacrylamide gradient gels containing SDS. The protein bands were visualized by fluorography. The numbers at the top of the lanes indicate hours of incubation of labelled cells in the chase-medium.

extracellular matrix and the results are shown in figure 3. Maximum levels of the 45 kDa protein were observed in both the matrix and medium at 12 h after release from HOU arrest; at this time, the DNA synthesis declined to background levels. When the DNA synthesis went up in the second S-phase (20 h onwards), the amount of the matrix-associated 45 kDa protein also declined. The major component of the extracellular matrix of MEF is a 48 kDa protein identified as plasminogen activator inhibitor (Nagashunmugam *et al* 1989), this protein did not show any spectacular variation across the cell cycle as that exhibited by the 45 kDa protein. However, a slight decrease in the level of the 48 kDa protein at 12 h after release of cells from HOU arrest was observed (figure 4); at this time (12 h after HOU release), the cells were expected to be at mitosis and the cells had a rounded shape. The decreased level of the protease inhibitor al the time of mitosis may correlate with increased pericellular proteolytic activity which results in the disruption of anchorage of these cells enabling them to become round and loosely attached to the substratum.

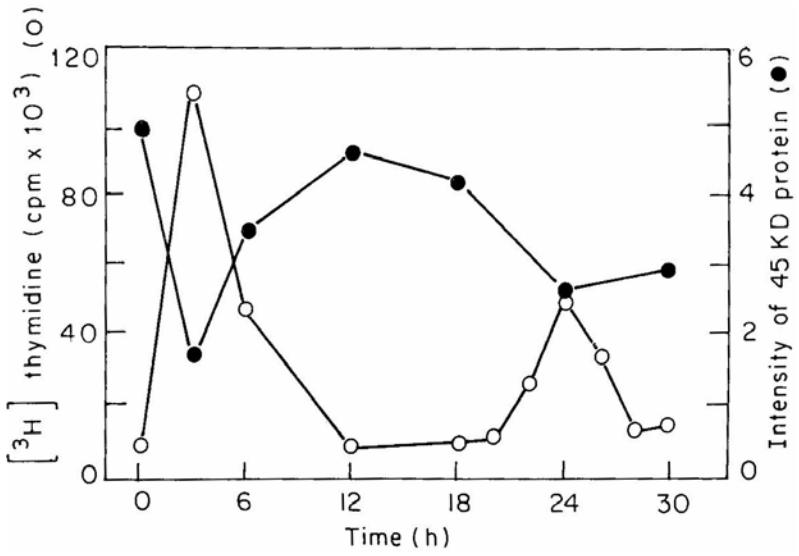

Figure 2. Levels of 45 kDa protein in the growth medium across two S-phases.
One set of MEF that were released from HOU-arrest were pulse-labelled for 30 min with [^{35}S] methionine at the indicated periods after release. Another set was labelled for 30 min with [^{3}H] thymidine under the same conditions. [^{3}H] thymidine incorporation into DNA was estimated from TCA-insoluble counts. [^{35}S] methionine-labelled secreted proteins were isolated and electrophoresed in Polyacrylamide gradient gels as described under § 2·4. [^{35}S] methionine incorporation into the secreted 45 kDa protein is represented as the intensity of the radioactive 45 kDa protein. The intensity was quantitated by laser densitometry of the 45 kDa protein bands of the fluorograms of gels in which the proteins were electrophoresed.

3.2 *45 kDa protein is related to P28 DBP*

Matsuhashi *et al* (1987) have shown that a 45 kDa protein present in serum-stimulated NIH 3T3 cells reacted with a monoclonal antibody raised against a chicken DNA binding protein called P28. The P28 protein was shown to be synthesized in the cells during late G1 and the synthesis of this protein ceased at the S-phase (Matsuhashi *et al* 1987). To establish the relatedness of these proteins, the 45 kDa DNA synthesis inhibitor protein secreted by MEF was reacted with monoclonal antibody against P28 antigen. Figure 4 shows the electrophoretic profile of immunoprecipitated proteins of medium and matrix. The electrophoretic pattern shows a 45 kDa protein as the sole labelled protein of the immunoprecipitate, indicating that the 45 kDa protein is related to the P28 antigen.

3.3 *DNA synthesis inhibition by purified 45 kDa protein*

For the purification of the 45 kDa protein, cells were grown in roller bottles and treated with cycloheximide for 15 h; the first hour conditioned media of these cultures were collected, clarified and concentrated by ultrafiltration. The concentrated medium was chromatographed in TSK 3000 HPLC column. Figure 5 shows the elution profile of the 45 kDa protein which coincides with the ovalbumin

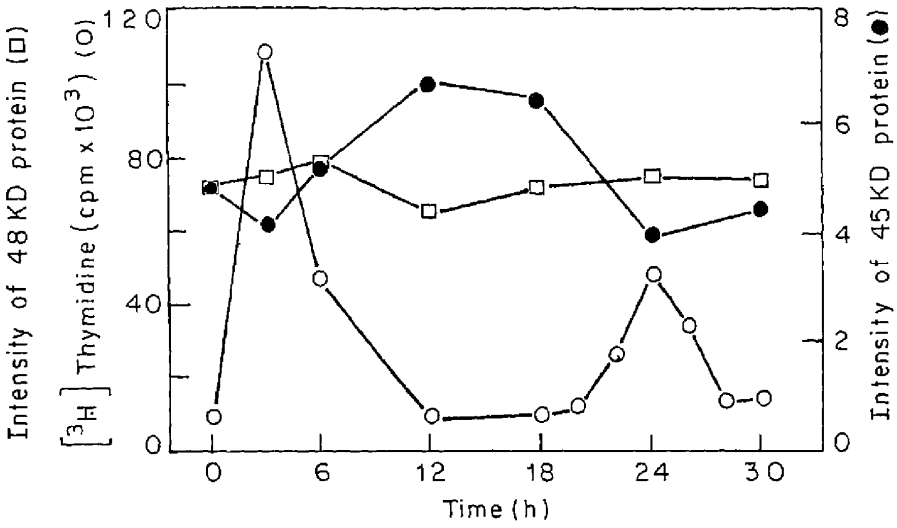

Figure 3. Levels of 45 kDa protein in the extracellular matrix across two S-phases.

One set of MEF that were released from HOU-arrest were pulse labelled for 30 min with [^{35}S] methionine. Labelled cells were removed at the indicated periods after release and the extracellular matrix proteins were isolated as described in §2·2. The labelled proteins were resolved by 5-18% PAGE and fluorographed. A 48 kDa protein identified previously as plasminogen activator inhibitor and the 45 kDa protein were the predominant components of the extracellular matrix. The intensities of the labelled 48 and 45 kDa protein bands were quantitated by laser densitometry. Another set of MEF were labelled for 30 min with [^{3}H] thymidine under the same conditions, and the thymidine incorporation into DNA was determined from TCA-insoluble radioactivity.

retention time. The 45 kDa protein fractions were pooled, lyophilized and dissolved in PBS. After filter sterilization, the protein was used in DNA synthesis inhibition assays.

Cells synchronized in G1/S border by the double block method were released by the addition of medium containing 20% foetal calf serum, in the presence or absence of column fractions. No inhibition of DNA synthesis was observed with fractions lacking the 45 kDa protein. Figure 6a shows the inhibition of DNA synthesis in MEF in the presence of various concentrations of the 45 kDa protein. Maximum DNA synthesis inhibition was observed at and above 0·25 μg/ml of inhibitor in MEF (figure 6a). The DNA synthesis inhibitory activity was found to be dose-dependent, and the half maximal inhibitory activity, calculated from figure 6a, was at 0·15 μg/mml of the 45 kDa protein. No DNA synthesis inhibitory activity was observed at this concentration in LN9 human fibroblasts. However, at higher concentration of the inhibitor, the DNA synthesis in these cells was also inhibited. In human fibroblasts, maximum inhibition of DNA synthesis (53%) was achieved with 4 μg/ml of the inhibitor protein and half maximum inhibition was observed with 1 μg/ml of the inhibitior (figure 6b).

Maximum inhibition of DNA synthesis was observed at 2 h after addition of the inhibitor in cells released from HOU arrest (figure 7). DNA synthesis was inhibited to an extent of 45% at 1 h and 84% after 2h of treatment of MEF with the inhibitor. Later, a gradual increase in [^{3}H] thymidine incorporation in inhibitor

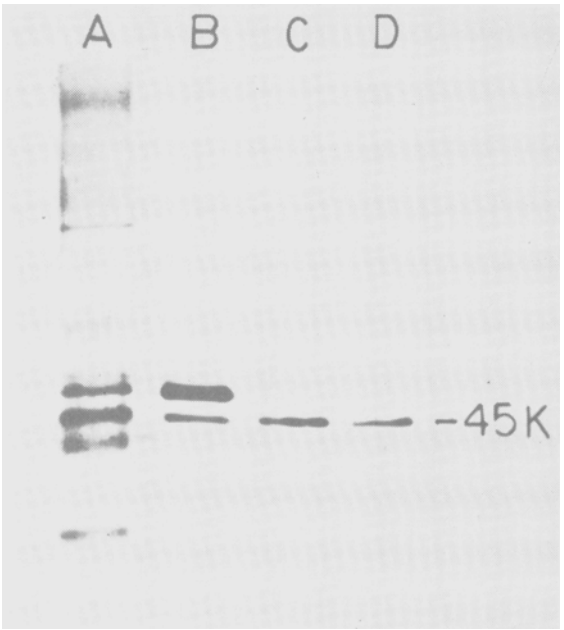

Figure 4. Immunoprecipitation of 45 kDa protein.

MEF 12 h after release from HOU arrest were labelled with [^{35}S] methionine for 30 min. Following a 30 min chase incubation, the labelled medium and extracellular matrix proteins were isolated as described in §2.4. For immunoprecipitation, antibodies to P28 protein provided by Dr K Hori was used and the method described by Srinivas *et al* (1990) was followed. The immunoprecipitates were analysed by SDS-PAGE and fluorographed. (A), secreted proteins of conditioned medium; (B), extracellular matrix proteins; (C), immunoprecipitate of proteins from conditioned medium; (D), immunoprecipitate of proteins from extracellular matrix. In each case proteins derived from one 75 cm^2 monolayer was used.

treated cells was observed which reached a maximum at 7 h after release from HOU arrest. During this period, only a basal level of DNA synthesis was observed in control cells that were released from HOU arrest in the absence of inhibitor protein. The growth inhibition after 7 h of release from HOU arrest was 56% of the maximum incorporation observed in control cells 3 h after release from HOU arrest. The decrease in the inhibitory activity of the protein after its presence for longer periods in the medium may be due to the inactivation of the protein. The DNA synthesis inhibitory activity was destroyed by heating the inhibitor protein at 60°C for 1 h.

4. Discussion

The results presented here show that secondary cultures of MEF secrete a growth inhibitory protein whose secretion is regulated during the cell cycle. The amount of the 45 kDa protein in the conditioned medium of S-phase cells was about one-fourth of that observed at G2 and other phases, suggesting an inverse correlation between DNA synthesis and the levels of the 45 kDa secreted protein.

DNA synthesis inhibitory proteins have been identified from the membranes of

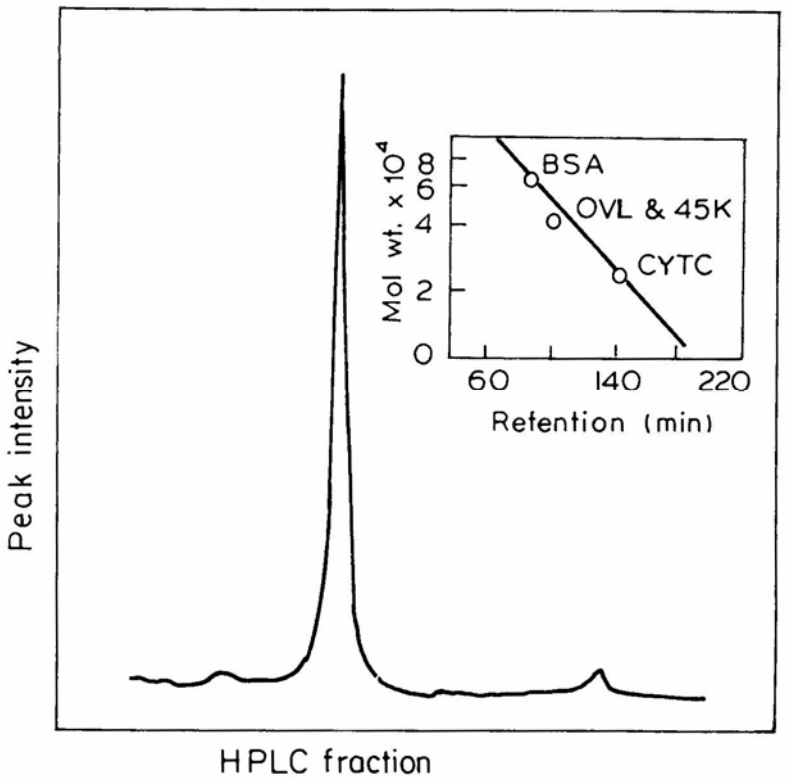

Figure 5. Purification of 45 kDa protein.

Conditioned media from cycloheximide treated MEF were concentrated by ultrafiltration (see §2·2) and chromatographed in a size exclusion (TSK 3000) HPLC column. The main figure shows the elution profile of the 45 kDa protein while the inset dipicts molecular weight calibration in which the retention times of standard marker proteins and the 45 kDa protein were ploted against log molecular weight.

quiescent and senescent human fibroblasts (Feltham *et al* 1987) and from the 3T3 cell plasma membranes (Whittenberger and Glaser 1977). Low molecular weight polypeptide inhibitors of DNA synthesis have been identified in the medium of mouse fibroblasts (Wells and Mallucci 1983). A 45,000 dalton protein called inhibitory diffusible factor (IDF) has been purified from the conditioned medium of density arrested NIH 3T3 cells and the N-terminal amino acid sequence of this protein has been determined (Blat *et al* 1989). Bohmer *et al* (1987) have shown that antibodies raised against bovine mammary derived growth inhibitor react with mouse fibroblast growth inhibitor.

Subramaniam and Shanmugam (1985, 1987, 1988) have shown that quiescent MEF secrete several proteins upon serum-stimulation. The results presented here show that among the secreted proteins, the 45 kDa protein alone is superinduced by cycloheximide treatment. The superinduction of the 45 kDa protein by CH suggests that the synthesis of this protein may be under the control of a labile repressor protein. Similar conclusion was also arrived at for the other CH-inducible proteins (Kruijer *et al* 1984; Makino *et al* 1984; Subramaniam and Shanmugam 1986b). The inhibition of extracellular levels of the 45 kDa protein by actinomycin-D (Subramaniam and Shanmugam 1988) shows that the synthesis and secretion of

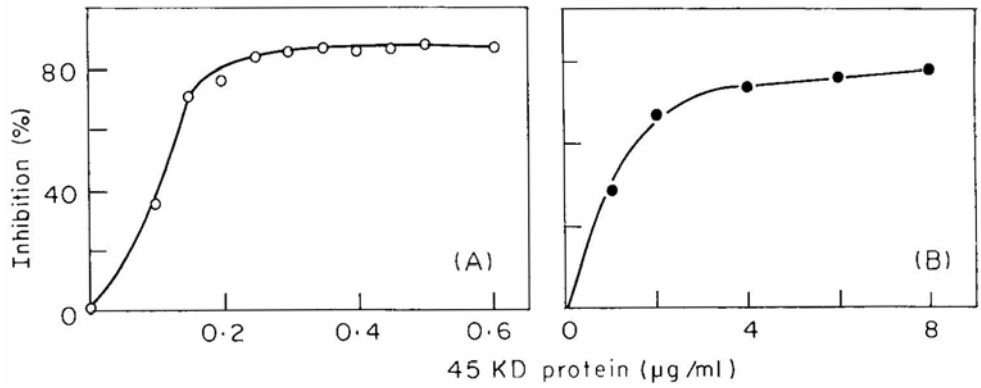

Figure 6. **(A)** Effect of purified 45 kDa protein on DNA synthesis in MEF.
The growth of MEF were arrested at G1/S junction by HOU treatment for 15 h. At the end of this period, HOU was removed, cell monolayers were washed and incubated for 2·5 h in fresh growth medium containing the indicated amounts of 45 kDa protein after which they were incubated another 30 min with [3H] thymidine. HOU released control monolayers were similarly incubated for 2·5 h in fresh medium followed by 30 more min in medium containing [3H] thymidine. Per cent inhibition of DNA synthesis was determined from the TCA insoluble radioactivities of control and 45 kDa protein treated monolayers. **(B)** Effect of purified 45 kDa protein on DNA synthesis in LN9 human fibroblasts. The protocol followed for this study is identical to that described above for MEF.

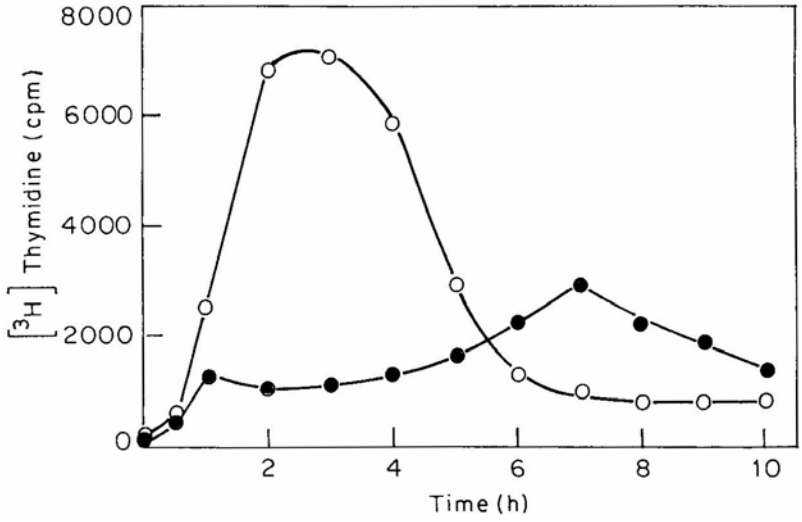

Figure 7. Time course of inhibition of DNA synthesis by the 45 kDa protein.
Monolayers of MEF were labelled for 30 min with [^{3}H] thymidine (5 μCi/ml) at the indicated periods after release from HOU arrest in the presence or absence of the 45 kDa protein (0·3 μg/ml). DNA synthesis was monitored by determining TCA insoluble radioactivity. The X-axis indicates time (in hours) after release of cells from HOU arrest. In the case of control, the cells were released from HOU arrest by using fresh medium containing foetal calf serum. To monitor DNA synthesis in the presence of the inhibitor, the cells were released from HOU arrest using fresh medium containing foetal calf serum and the 45kDa protein; these cultures were later labelled with [^{3}H] thymidine. (O), DNA synthesis in control cells; (●), DNA synthesis in 45 kDa protein-treated cells.

this protein involve newly made mRNA species. Santaren and Bravo (1987) observed a similar superinduction of a 45 kDa protein by CH in 3T3 cells and the synthesis of this protein was also inhibited by actinomycin-D.

The 45 kDa protein was the first protein that appeared in the medium within 30 min chase incubation of pulse-labelled cells. Since high extracellular levels of the 45 kDa protein were observed in the conditioned medium within an hour of CH treatment, this antibiotic was used to enrich this protein selectively for purification purposes.

Extracellular matrix proteins like fibronectin, vitronectin, collagen and laminin play important roles in cell substrate adhesion and cell-cell interaction by binding against specific cell surface receptors (Kleinman *et al* 1981; Hasegawa *et al* 1985; Yamada *et al* 1985; Laptin 1986; Hynes 1987). One of the 45 kDa secreted proteins of 3T3 cells was a glycoprotein associated with extracellular matrix (Santaren and Bravo 1987). In our studies, the matrix associated 45 kDa protein showed variations across cell cycle, while the major matrix protein (PAI-1) showed little variation (figure 4).

Matsuhashi *et al* (1987) identified a 45 kDa protein in NIH 3T3 cells whose intracellular level was regulated during cell cycle; the synthesis of this protein ceased during S-phase. A monoclonal antibody (P28) raised against a chicken DNA binding protein reacted with the intracellular 45 kDa protein of NIH 3T3 cells (Matsuhashi *et al* 1987). Here, we show that the secreted and the matrix associated 45 kDa proteins of MEF, are immunologically related to the 45 kDa protein of NIH 3T3 cells. Also the 45 kDa DNA synthesis inhibitory factor described here is similar in molecular weight and mode of action to the IDF 45 described by Harel *et al* (1985). Further characterization of this protein is necessary to confirm its identity with IDF 45. Experiments are underway for sequencing this protein.

Several mechanisms may be postulated for the inhibitory action of 45 kDa protein on DNA synthesis. The protein may inhibit the transport of [^{3}H] thymidine or deplete the intracellular nucleotide pool. Also it is not known whether the 45 kDa protein is internalized by specific receptors or by other mechanisms. The ongoing studies in our laboratory are aimed at unravelling the mechanism of action of this protein on the inhibition of DNA synthesis.

The 45 kDa DNA synthesis inhibitor protein might be one among the negative growth regulators that control the proliferation of cells triggered by positive growth regulators such as mitogens (Stocker 1973; Stocker and Piggolt 1974). Future directions of these studies include raising of antibodies and DNA clones to the 45 kDa protein and its gene to understand the role of this protein in cell proliferation. Preliminary studies showed reduced levels of this protein in rapidly proliferating oncogene (*myc*-ras) transformed rat cells (M V V S Varaprasad and G Shanmugam, unpublished results). Higher amounts of the 45 kDa protein in normal cells may exert a negative control in these cells and regulate the rate of cell division.

Acknowledgements

We thank Dr K Hori for providing the monoclonal antibody against P28 chicken DNA binding protein and Dr M Digweed of Institute of Human Genetics, Berlin for providing the LN 9 normal human diploid fibroblasts. SS and TNS were supported by senior research fellowships from the Council of Scientific and

Industrial Research, New Delhi. This study was supported by the Department of Science and Technology, New Delhi.

References

Bohmer F D, Sun Q, Pepperle M, Muller T, Eriksson U, Wang J L and Grosse R 1987 Identification of a polypeptide growth inhibitor from bovine mammary gland; *Biochem. Biophys. Res. Commun.* **148** 1425-1431

Blat C, Bohlen P, Villaudy J, Chatelain G, Golde A and Harel L 1989 Isolation and amino-terminal sequence of a novel cellular growth inhibitor (Inhibitory Diffusible Factor 45) secreted by 3T3 fibroblasts; *J. Biol Chem.* **264** 6021-6024

Ervin P R, Kaminski M S Jr, Cody R L and Wicha M S 1989 Production of mammastatin, a tissue-specific growth inhibitor by normal human mammary cells; *Science* **244** 1585–1587

Feltham N, Fahey D and Knight E Jr 1987 A Growth inhibitory protein secreted by human diploid fibroblasts; *J. Biol. Chem.* **262** 2176-2179

Harel L, Blat C and Chatelain G 1985 Regulation of cell proliferation. Inhibitory and stimulatory factors diffused by 3T3 cultured cells; *J. Cell Physiol.* **123** 139-143

Hasegawa T, Hasegawa E, Chen W-T and Yamada K M 1985 Characterization of a membrane-associated glycoprotein complex implicated in cell adhesion to fibronectin; *J. Cell Biochem.* **28** 307-318

Heldin C H and Westermark B 1984 Growth factors: mechanism of action and relation to oncogenes; *Cell* **37** 9-20

Holley R W, Bohlen P, Fava R, Baldwin J H, Kleeman G and Armour R 1980 Purification of kidney epithelial cell growth inhibitors; *Proc. Natl. Acad. Sci. USA* **77** 5989-5992

Hsu Y M and Wang J L 1986 Growth control in cultured 3T3 fibroblasts. V. Purification of an M_r 13000 polypeptide responsible for growth inhibitory activity; *J. Cell Biol.* **102** 362-369

Hynes R O 1987 Integrins: a family of cell surface receptors; *Cell* **48** 549-554

James R and Bradshaw R A 1984 Polypeptide growth factors; *Annu. Rev. Biochem.* **53** 259–292

Kleinman H J, Klebe R J and Martin G R 1981 Role of collagenous matrices in the adhesion and growth of cells; *J. Cell Biol.* **88** 473-485

Kruijer W, Cooper J A, Hunter T and Verma I M 1984 Platelet-derived growth factor induces rapid but transient expression of c-*fos* gene and protein; *Nature* (*London*) **312** 711-716

Leptin M 1986 The fibronectin receptor family; *Nature* (*London*) **321** 728-730

Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951 Protein measurement with the Folin phenol reagent; *J. Biol. Chem.* **193** 265-275

Makino R, Hayashi K and Sugimura T 1984 c-*myc* transcript is induced in rat liver at very early stage of regeneration or by cycloheximide treatment; *Nature* (*London*) **310** 697-698

Matsuhashi S, Watanabe T and Hori K 1987 An antigen expressed in proliferating cells at late G1-S Phase; *Exp. Cell Res.* **170** 351-362

Nagashunmugam T and Shanmugam G 1987 S-phase mouse embryo fibroblasts secrete varying amounts of a 45000 dalton protein; *Cell Biol. Int. Rep.* **11** 147-155

Nagashunmugam T, Srinivas S and Shanmugam G 1989 Effect of dimethyl sulfoxide on mouse embryo fibroblasts: inhibition of plasminogen activator deposition and interference with early events of serum-stimulated growth; *Biol. Cell* **66** 307-315

Roberts A B, Anzano M A, Wakfield L M, Roche N S, Stern D F and Sporn M B 1985 Type B transforming growth factor: A bifunctional regulator of cellular growth; *Proc. Natl. Acad. Sci. USA* **82** 119-123

Russell W E, Van Wyk J J and Pledger W J 1984 Inhibition of the mitogenic effects of plasma by a monoclonal antibody to somatomedin C; *Proc. Natl. Acad. Sci. USA* **81** 2389-2392

Santaren J- F and Bravo R 1987 Immediate induction of a 45 K secreted glycoprotein by serum and growth factors in quiescent mouse 3T3 cells; *Exp. Cell Res.* **168** 494-506

Srinivas S, Nagashunmugam T and Shanmugam G 1990 Translocation of plasminogen activator inhibitor-1 during serum stimulated growth of mouse embryo fibroblasts; *J. Biosci.* **15** 351-359

Stein G H and Atkins L 1986 Membrane-associated inhibitor of DNA synthesis in senescent human diploid fibroblasts: Characterization and comparison to quiescent cell inhibitor; *Proc. Natl. Acad. Sci. USA* **83** 9030-9034

Stocker M G P 1973 Role of diffusion boundary layer in contact inhibition of growth; *Nature* (*London*) **246** 200-203

Stocker M G P and Piggolt D 1974 Shaking 3T3 cells: further studies on diffusion boundary effects; *Cell* **3** 207-215

Subramaniam M and Shanmugam G 1985 Intracellular and secreted proteins of density-inhibited serum-arrested and proliferating mouse embryo cells; *Cell Biol. Int. Rep.* **9** 51-60

Subramaniam M and Shanmugam G 1986a Effect of cycloheximide and growth factors on gene expression in quiescent mouse embryo fibroblasts; *J. Cell. Physiol.* **126** 47-52

Subramaniam M and Shanmugam G 1986b A serum-induced 29 kD protein of mouse embryo fibroblasts is tightly bound to the chromatin; *Cell Biol. Int. Rep.* **10** 323-329

Subramaniam M and Shanmugam G 1987 Secreted proteins of quiescent serum-stimulated and over-confluent mouse embryo fibroblasts; *J. Biosci.* **12** 281–287

Subramaniam M and Shanmugam G 1988 Effects of serum cycloheximide and actinomycin D on protein secretion by quiescent mouse embryo fibroblasts; *Mol. Biol. Rep.* **13** 133-138

Wells V and Mallucci L 1983 Properties of a cell growth inhibitor produced by mouse embryo fibroblasts; *J. Cell. Physiol.* **117** 148-154

Whittenberger B and Glaser L 1977 Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes; *Proc. Natl. Acad. Sci. USA* **74** 2251-2255

Yamada K M, Akiyama S K, Hasegawa T, Hasegawa E, Humphries M J, Kennedy D W, Nagata K, Urushihara H, Olden K and Chen W-T 1985 Recent advances in research on fibronectin and other cell attachment proteins; *J. Cell. Biochem.* **28** 79-97