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Secreted proteins of normal and *myc-ras* oncogene transformed rat embryo fibroblasts

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Abstract. Quiescent cultures of rat embryo fibroblasts synthesize and secrete several proteins in response to mitogenic stimulation. Two of these proteins have been characterized in this study and the effect of oncogenic transformation on these proteins was monitored. A serum induced 48,000 protein was shown to be related to plasminogen activator inhibitor while another serum-induced protein of M_r 45,000 was found to be an inhibitor of DNA synthesis. Transformation of rat embryo fibroblasts with oncogenes *myc* and *ras* resulted in drastic reduction in the level of these proteins. The reduced levels of protease inhibitor may be responsible for the loss of anchorage dependence of the transformed cells. The DNA synthesis inhibitor protein may act as a negative growth regulator and reduced levels of this protein in *myc-ras* transformed cells may accelerate the proliferation of these cells.

Keywords: Oncogenes; transformation; fibroblasts; secreted proteins.

1. Introduction

Neoplastic development is a multistep process involving sequential acquisitions of hermyable phenotypic changes such as immortality and anchorage independent growth. It is well known that cell transformation involves both activation of genes which enhance the proliferation of cells and inactivation of genes such as the tumour suppressor genes which negatively control the proliferation of cells. Transformation of cells by dominant oncogenes is accompanied by alteration in the programme of gene expression which ultimately lead to an escape from the genetic control and immune surveillance mechanisms. Several studies were directed to understand the molecular events of such evasion mechanisms and to determine the role of negative growth regulators which regulate cell proliferation (Holley *et al* 1980; Harel *et al* 1985; Keski-Oja and Moses 1987).

We have studied the expression of oncogenes and other early growth response proteins during transformation of normal rat embryo fibroblasts. Our results document the presence of exogenous genes in the cellular genome of transfected cells. Further, we have observed the loss of two early growth response proteins following transformation. One of these (the 48 kDa protein) is a protease inhibitor while the other (the 45 kDa protein) is a DNA synthesis inhibitor.

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Abbreviations used: REF, Rat embryo fibroblasts; PAI-1, plasminogen activators inhibitor-1; HOU, hydroxyurea.

2. Materials and methods

2.1 *Cells*

Primary rat embryo fibroblasts (REF) were isolated from 15–16 day old embryos of Fisher rats. Cells were grown in minimal essential medium (MEM) containing 10% fetal calf serum (Flow Labs) and gentamycin (50 μg/ml) at 37° C and passaged at regular intervals. Cells from fourth passage and above were used for the experiments. Cells were synchronized at quiescent phase and at Gl/S boundary as described by Nagashunmugam and Shanmugam (1987).

2.2 *Plasmids and antibodies*

PSV-c-*myc*-l and PEJ 6·6 (c-*Ha-ras)* (Land *et al* 1983) were obtained from Robert A Weinberg. pSV_2neo was provided by Michael D Cole. The antibodies against plasminogen activator inhibitor-1 (PAI-1) were obtained from D Luskutoff. The Plasmids were propagated by transformation of *Escherichia coli* HB 101 and isolated as described by Sambrook *et al* (1989). They were purified by centrifugation in CsCl gradients as described by Moore (1987).

2.3 *DNA transfections*

Plasmids (5 μg each) containing *myc* and *ras* oncogenes and 1 μg of pSV_2neo were introduced into REF according to the procedure of Graham and Van der Eb (1973) with minor modifications. The plasmid DNAs (total DNA concentration was made up to 20 μg with sheared calf thymus DNA, Boehringer, Mannheim) were co-precipitated with calcium phosphate and added to the cells. The cells were glycerol shocked for 90 s, washed with MEM without serum and then grown in 10% FCS containing medium for 48 h. When the cells reached confluence, they were split into 1:4 and then fed with 10 % FCS containing medium. Geneticin (G 418 400 μg/ml) was added to select the transformed cells. Cell death was evident within 4 days and G 418 resistant colonies appeared after 3–4 weeks. The colonies were pooled and propagated.

2.4 *Soft agar assay*

The transformed cells were assessed for their anchorage independence following growth in agar suspension as described by Crowe *et al* (1978). Cells were incubated at 37° C in a CO_2 incubator for two weeks with bi-weekly feedings. The colony formation was monitored after two weeks.

2.5 *Southern blotting*

The cellular DNA from transfected cells was extracted and approximately 10 μg of DNA was digested with HindIII and BamHI restriction endonucleases at 37° C

overnight. The digested fragments were separated by agarose (0·7%) electrophoresis. Lambda DNA digested with HindIII was used as molecular weight marker in adjacent lanes. The digested cellular DNA was transferred onto a nylon (Hybond) membrane by overnight blotting (Southern 1975). After blotting was completed, the DNA on the membrane was immobilized by exposing to UV illumination for 3 min.

2.6 *Slot blotting and hybridization*

RNA was extracted from transfected cells and quantitated spectrophotometrically (Srinivas *et al* 1991b). Serial dilutions of RNA were denatured by heating at 65° C for 10 min followed by rapid cooling in ice and blotted on a nylon (Hybond) membrane using a BRL slot-blot apparatus. The RNA on the nylon membrane was immobilized by exposure to UV irradiation for 3 min. Hybridizations were carried out using plasmid DNA probes which were labelled with [α-^{32}P] dCTP using a oligolabeling kit (Pharmacia). The hybridizations and washings were carried out in a Bachofer hybridization oven as described by Srinivas *et al* (1991b).

2.7 *Pulse-chase labelling of proteins and SDS-PAGE*

The secreted proteins of normal and transfected REF were labeled with [^{35}S] methionine and electrophoresed in 5–18% Polyacrylamide gels containing SDS (Srinivas *et al* 1991a).

2.8 *Purification and assay of 45 kDa protein*

To purify the 45 kDa protein from the conditioned medium of REF cultures, quiescent monolayers of REF grown in roller bottles were treated with cycloheximide (3 μg/ml) for 16 h. The cells were then rinsed thrice with PBS and incubated in serum free medium for 1 h. The conditioned medium from these cells was concentrated by ultrafiltration using a 10 kDa cut-of Millipore membrane and the 45 kDa protein was purified by HPLC using a 7·5 × 600 mm LKB-TSK 3000 size-exclusion column (Srinivas *et al* 1991a). The DNA synthesis inhibitor assay using purified 45 kDa protein was carried out as described by Srinivas *et al* (1991a). For the inhibitor assay, normal and *myc-ras* transformed REF were grown in 24 well plates. Each well contained 2 × 10^6 cells in 0·5 ml growth medium. Cells were synchronized at Gl/S by treatment with 1 mM hydroxyurea (HOU) in MEM containing 10% serum for 16 h. The HOU arrested 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 labeling the cells with 5 μCi/ml of [$_3$H] thymidine (specific activity 18 Ci/m mol) in the last 30 min of 3 h incubation of cells with the inhibitor. At the end of labelling, the cells in the wells were washed thrice with cold PBS and TCA precipitable radioactivity was determined by collecting the precipitate from each well on a Millipore nitrocellulose membrane and liquid scintillation counting. The assays were repeated thrice and the experimental variations were in the range of 5 to 8%.

3. Results

3.1 *Transformation of REF with oncogenes*

Normal REF were cotransfected with *myc* and *ras* oncogenes along with pSV_2neo as a selection marker. The G 418 resistant colonies were pooled and propagated Figure la and b respectively show normal REF and a bifringent transformed colony. The transfected cells formed colonies in soft agar (figure 1c) indicating anchorage independent growth fulfilling one of the characteristics of transformed cells.

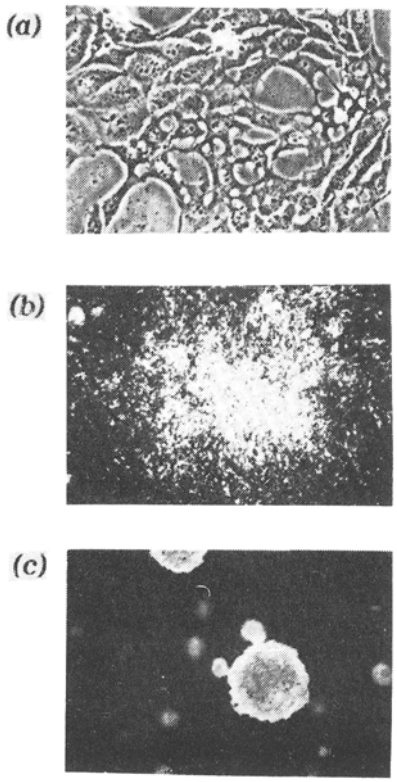


Figure 1. Transformation of REF with oncogenes *myc* and *ras.* **(a),** Normal REF; **(b),** bifringent colony of *myc* and *ras* transformed cells; **(c),** the *myc-ras* transformed colony in soft agar culture. Secondary rat embryo fibroblasts were transfected with *myc* and *ras* oncogenes by calcium phosphate co-precipitation as described in §2. The cells were allowed to proliferate and transformed foci were observed 21 days after transfection. Soft agar assay was performed as described in § 2.

To check the integrity and expression of the transfected oncogenes in REF, high molecular weight DNA and cytoplasmic RNAs were extracted from transfected cells. The DNA was subjected to restriction enzyme digestion and Southern blotting. The blots were hybridized with ^{32}P-labelled probes. The hybridization of a 9·5 kb fragment with *myc* probe indicates that the transfected *myc* oncogene is

intact in the recipient cells; similarly the presence of a 6·6 kb fragment that hybridizes with *ras* probe suggests that the transfected *ras* oncogene also is intact in transfected cells (figure 2).

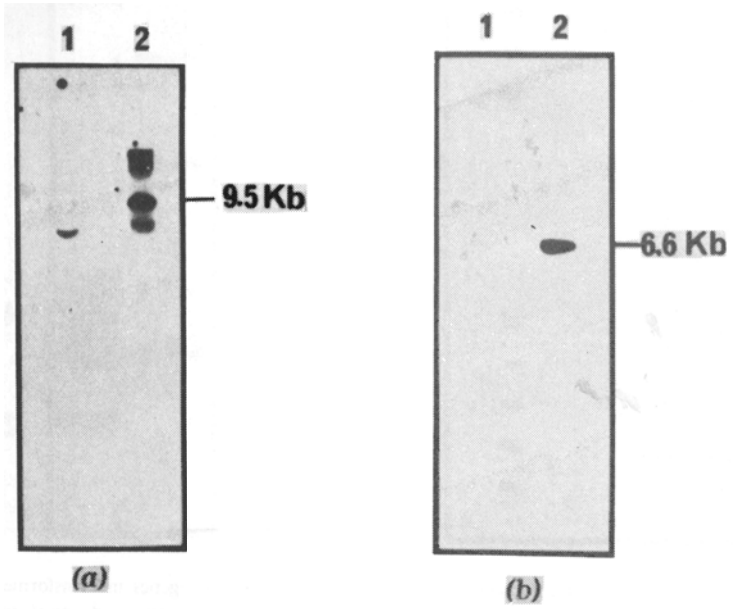
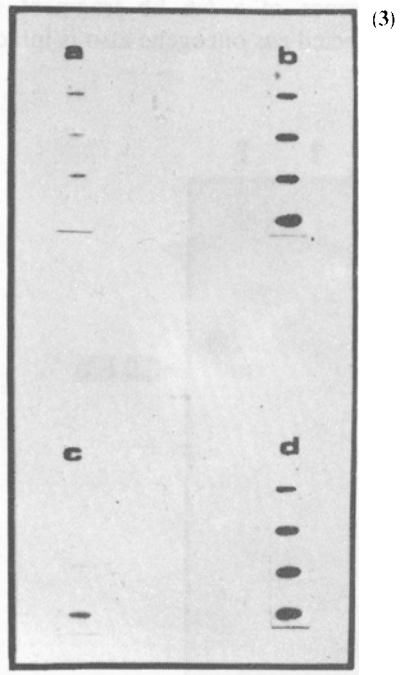


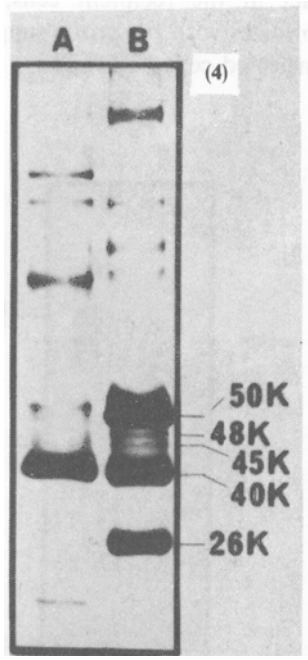
Figure 2. Southern hybridization of *myc* and *ras* oncogene transformed cell DNAs. DNAs from transfected and control cells were digested with BamHI. The digested fragments were electrophoresed on 0·7% agarose gels, blotted on a nylon membranes and hybridized either with [α-^{32}P] labelled pSV-c-myc linearized with BamHI or with BamHI fragment of pEJ c-Ha- . **(a, b)** Hybridization pattern with *myc* **(a)** and *ras* **(b)** probe. Lane 1, normal REF, lane 2, *myc-ras* transfected REF.

In another set of experiments RNA from transfected cells was blotted on a nylon membrane and hybridized with the labelled oncogene probes. The analysis of expression of *myc* and *ras* oncogenes at the transcriptional level is shown in figure 3. The expression of transfected *myc* and *ras* oncogenes is 3–4-fold higher in transformed cells in comparison to their level in normal cells. These results and the Southern hybridization data show that both the transfected genes were integrated as well as expressed in the recipient cells.

3.2 *Secreted proteins of normal REF*

Figure 4 shows the pattern of secreted proteins of quiescent and serum stimulated rat embryo fibroblasts. Secreted proteins from quiescent and serum-stimulated cells were labelled with [^{35}S] methionine, analysed by SDS-PAGE and visualized by autoradiography. Serum stimulation of quiescent cells resulted in the induction of proteins of molecular weights 50,000, 48,000, 45,000 and 26,000. The 48 kDa protein appeared in the medium in the first few hours after the addition of serum.





Figures 3 and 4. **(3)** Expression of transfected *myc* and *ras* oncogenes in transformed cells. Cytoplasmic RNAs were isolated from oncogene transfected cells and serial two-fold dilutions of RNA were blotted on a nylon membrane and hybridized with labelled probes (*myc* and *ras)* as described in §2. **(a)** Control cell RNA hybridized with *myc* probe; **(b)** transfected cell RNA hybridized with *myc* probe; **(c)** control cell RNA hybridized with *ras* probe; **(d)** transfected cell RNA hybridized with *ras* probe. **(4)** Effect of serum stimulation on the levels of secreted proteins. **(A),** Quiescent cells; **(B)** serum-stimulated cells. Sub-confluent monolayers of REF were synchronized at quiescent phase by serum starvation. The cells were stimulated with 20% FCS containing medium for 3 h and labelled with [^{35}S] methionine for 30 min and the radioactivity was chased for 30 min in serum-free medium. The proteins from the chase medium were analysed by SDS-PAGE and fluorographed.

The inducibility of 48 kDa secreted protein by serum or growth factors and also its molecular weight suggested that it could be related to plasminogen activator inhibitor (PAI). In order to establish the identity of this protein, immunoprecipitation was carried out using antiserum to endothelial cell type plasminogen activator inhibitor. Results shown in figure 5 indicate that the 48 kDa protein is indeed related to PAI-1.

The 45 kDa protein of REF showed properties similar to that of a murine 45 kDa protein such as presence in large amounts in the growth medium of HOU arrested cells and negligible amounts in the conditioned medium of cells that were e e ed from H O U arrest which synthesized DNA at peak levels (Nagashunmuga and Shanmugam 1987). Therefore, this protein was purified and its ability to inhibit DNA synthesis was assessed. Normal REF that were synchronized at Gl/S border by double block method were released from arrest by the addition of 20% FCS containing medium in the presence or absence of purified 45 kDa protein. Figure 6 shows the inhibition of DNA synthesis in these cells in the presence of

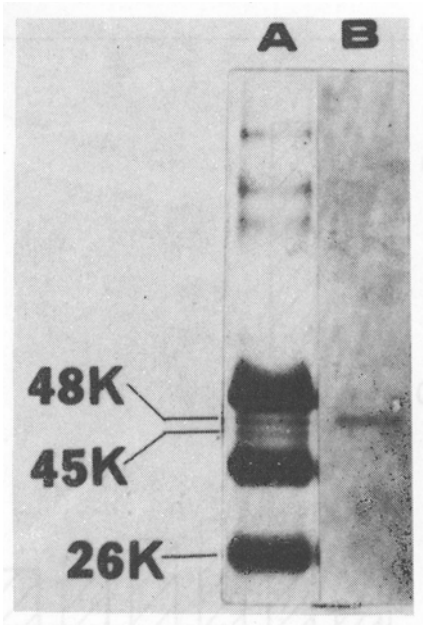


Figure 5. Immunoprecipitation of 48 kDa secreted protein with antibodies to plasminogen activator inhibitor-1 (PAI-1). Quiescent monolayers were stimulated with 20% FCS for 3 h and then labelled with [^{35}S] methionine for 30 min and chased for 30 min. The secreted proteins in the chase medium were concentrated and immunoprecipitated as described in § 2. **(A)** Labelled proteins of conditioned medium of serum-stimulated cells. **(B)** Conditioned medium proteins of serum-stimulated cells immunoprecipitated with PAI-1 antibodies. The proteins were analysed by SDS-PAGE and fluorographed.

various concentrations of purified 45 kDa protein. Maximum DNA synthesis inhibition was observed with 0·5 μg/ml of 45 kDa protein.

3.3 *Loss of 48 kDa and 45 kDa protein in transformed cells*

Cell transformation is known to manifest in a myriad of changes. To follow the pattern of secreted proteins of normal and transformed cells, they were labelled with [^{35}S] methionine and the secreted proteins were isolated and analysed by SDS-PAGE. Figure 7 shows the protein profile of normal and *myc-ras* transformed cells. A drastic reduction in the levels of both the 48 kDa and the 45 kDa protein was evident in transformed cells. Fibronectin (the top most band) also disappears upon transformation as shown by others (Olden and Yamada 1977; Vaheri and Mosher 1978).

3.4 *Effect of purified 45 kDa protein on DNA synthesis in myc-ras transformed cells*

Since the transformed cells showed reduced levels of 45 kDa protein, an attempt was made to check the DNA synthesis inhibitory activity of the 45 kDa protein in *myc-ras* transformed cells. For this purpose, cells that were synchronized at Gl/S

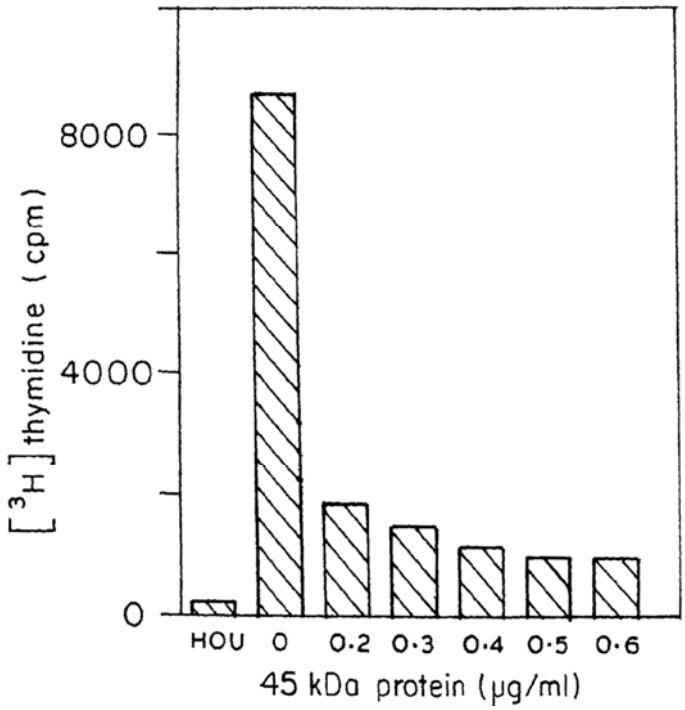


Figure 6. Effect of purified 45 kDa protein on DNA synthesis in REF. DNA synthesis was monitored in cells that were released from HOU arrest by incubation with 10% FCS in the presence or absence of different concentrations of purified 45 kDa protein. The cells were labelled with [^{3}H] thymidine in the last 30 min of 3 h incubation and the DNA synthesis was quantitated by the estimation of TCA precipitable radioactivity as described in §2.

border were released from growth arrest by the addition of 20% FCS containing medium in the presence or absence of various concentrations of the 45 kDa protein. Figure 8 shows the inhibition of DNA synthesis in transformed cells following addition of 45 kDa protein. Low concentrations of 45 kDa protein (0·5 μg/ml) inhibited DNA synthesis to an extent of 63% and maximum inhibition (75%) was observed in the presence of 8 μg/ml of this protein.

4. Discussion

The results presented here show that secondary cultures of REF secrete several early growth response proteins such as the 48 kDa, 45 kDa and 26 kDa proteins in addition to some high molecular weight proteins upon exposure to serum mitogens. The levels of the 48 kDa and 45 kDa proteins are reduced drastically upon transformation by oncogenes *myc* plus *ras.*

It is well established that many malignant tumours and transformed cell lines produce and secrete increased amounts of several proteases including collagenases (Liotta *et al* 1986) and plasminogen activators (PA) (Dano *et al* 1985). The

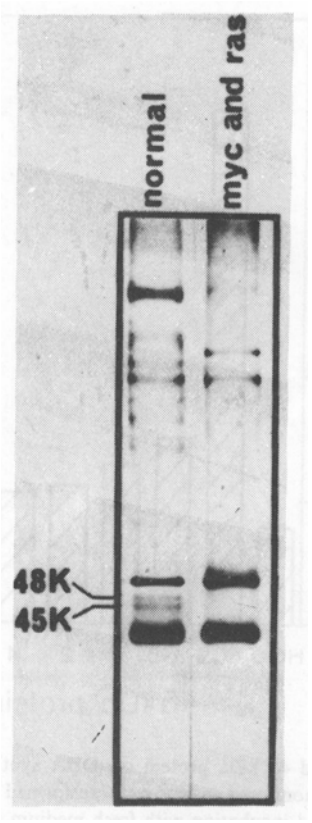


Figure 7. Secreted proteins of normal and oncogene transformed cells. Normal and oncogene (*myc* and *ras*) transformed REF were labelled with [^{35}S] methionine for 30 min and chased for 30 min. The proteins in the chase medium were resolved by electrophoresis in SDS-PAGE and fluorographed.

association of plasminogen activator expression with the transformed phenotype suggested the possibility that plasminogen activators or plasminogens may be involved in tumour growth, tissue degradatation, tumour invasion and metastasis causing connective tissue destruction. The disappearance of secreted PAI-1 in *myc-ras* transformed cells may lead to an increase in the activity of extracellular proteases which destroy the anchorage thus enabling the transformed cells to lose adhesion. Since malignant cell growth can lead to active matrix degradation both *in vivo* and *in vitro*, it is associated with low levels of PAI-1 in the transformed cells. Recent experiments with HT 1080 fibrosarcoma cell line suggest an inverse relationship between PAI-1 expression and transformation (Laiho *et al* 1987).

A large body of studies have accumulated describing the role of growth factors in the stimulation of cell growth; however, much less is known about the role of endogenous proteins that are inhibitors of cell growth. Harel *et al* (1983) have purified a growth inhibitor called IDF 45 from dense cultures of 3T3 cells. This protein was found to act in the Gl phase of the cell-cycle and shown to inhibit the growth and DNA synthesis in chick embryo fibroblasts (Blat *et al* 1987).

The mechanism of inhibition of DNA synthesis by the 45 kDa protein is not clear

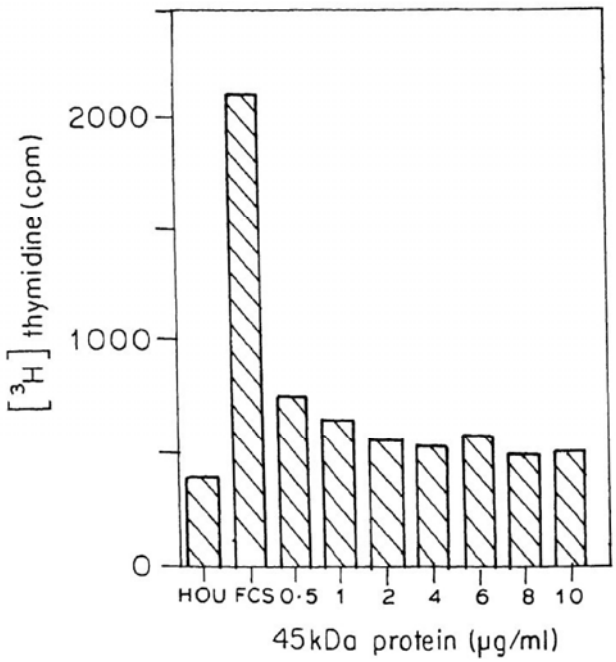


Figure 8. Effect of purified 45 kDa protein on DNA synthesis in *myc-ras* transformed cells. DNA synthesis was monitored in *myc-ras* transformed cells that were released from HOU-arrest by washing and incubation with fresh medium in the presence or absence of various concentrations of 45 kDa protein. DNA synthesis was quantitated by labelling the cells with [^{3}H] thymidine in the last 30 min of 3 h incubation of cells released from HOU arrest (Srinivas *et al* 1991a).

but it is possible that this protein may bind to a cell surface specific receptor(s) to generate a negative regulatory signal to the cell. Further studies are necessary to check this possibility. Alternately this protein may inhibit the transport of [^{3}H] thymidine or may deplete the intracellular nucleotide pool.

The phenomenon of cell transformation leads to multitude of changes in the cellular and biochemical processes. Oncogene mediated cell transformation may result in the perturbation of negative regulatory circuits which control the rate of cell proliferation upon transformation. It appears from the studies presented here that the 45 kDa protein may function as a negative growth regulatory protein inhibiting DNA synthesis and subsequently cell growth. The observation that *myc-ras* transformed REF exhibit drastic reduction in the level of the 45 kDa secreted protein and the finding that this protein inhibits DNA synthesis suggest that the 45 kDa protein may interfere with the rapid proliferation potential of transformed cells.

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References

Blat C, Villaudy J, Rouillard D, Golde A and Harel L 1987 Modulation by the *src* oncogene of the effect of inhibitory diffusible factor IDF 45; *J. Cell. Physiol.* **130** 416–419

Crowe R, Ozer H and Rifkin D 1978 Anchorage requirements; in *Experiments with normal and transformed cells* (New York; Cold Spring Harbor Laboratory) pp 18–27

Dano K., Andreasen P A, Grondahl-Hansen J, Kristensen P, Nielsen L S and Skriver L 1985 Plasminogen activators, tissue degradation and cancer; *Adv. Cancer Res.* **44** 139–266

Graham F L and Van der Eb A J 1973 A new technique for the assay of infectivity of human adenovirus 5 DNA; *Virology* **52** 456–463

Harel L, Blat C and Chalelain G 1983 Density dependent inhibition of growth. Fractionation of inhibitory diffusible factor released by dense cultures of 3T3 cells; *Biol. Cell.* **48** 11–16

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

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

Keski-Oja J and Moses H L 1987 Growth inhibitory polypeptides in the regulation of cell proliferation; *Med. Biol* **65** 13–20

Land H, Parada L F and Weinberg R A 1983 Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes; *Nature* (*London*) **304** 596–602

Laiho M, Saksela O and Keski-Oja J 1987 Transforming Growth-Factor β induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase; *J. Biol Chem.* **262** 17467–17474

Liotta L A, Rao C N and Weaver U M 1986 Biochemical interactions of tumor cells with the basement membrane; *Annu. Rev. Biochem.* **55** 1037–1057

Moore D 1987 *Current Protocols in Molecular Biology* (New York: Greene Publishing Associates and Wiley-Interscience)

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

Olden K and Yamada K M 1977 Mechanisms of the decrease in the major cell surface protein of chick embryo fibroblasts after transformation; *Cell* **11** 957–969

Sambrook J, Fritsch E F and Maniatis T 1989 *Molecular Cloning—A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press) P.l.38–1.39

Southern E M 1975 Detection of sequences among DNA fragments separated by gel electrophoresis; *J. Mol Biol* **38** 503–517

Srinivas S, Nagashunmugam T and Shanmugam G 1991a Purification and characterization of a DNA synthesis inhibitor protein from mouse embryo fibroblasts; *J. Biosci.* **16** 175–186

Srinivas S, Sironmani T A and Shanmugam G 1991b Dimethyl sulfoxide inhibits the expression of early growth response genes and arrests fibroblasts at quiescence; *Exp. Cell Res.* **196** 279–286

Vaheri A and Mosher D F 1978 High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation; *Biochim. Biophys. Acta* **516** 1–25