Regulation of type VI collagen synthesis in transformed mesenchymal cells

Thomas SCHREIER,* Robert R. FRIIS,† Kaspar H. WINTERHALTER* and Beat TRÜEB*

*Laboratorium für Biochemie I, ETH Zentrum, CH-8092 Zürich, and †Ludwig Institut für Krebsforschung, Inselspital, CH-3010 Bern, Switzerland

We have analysed the effects of oncogenic transformation on the expression of type VI collagen in mesenchymal cells. Synthesis of type VI collagen was almost completely inhibited in fibroblasts transformed by DNA or RNA tumour viruses or in cells derived from spontaneous mesenchymal tumours. Inhibition of type VI collagen synthesis appears, therefore, to be a common phenomenon of transformed mesenchymal cells. When introduced into normal cells by viral vectors, the 'nuclear' oncogene *v-myc* had an inhibitory effect similar to that of the 'cytoplasmic' oncogene *v-src*. Fibroblasts infected with a temperature-sensitive strain of Rous sarcoma virus (NY68) produced type VI collagen at the restrictive, but not at the permissive temperature. If such cells were shifted from the permissive to the restrictive temperature, synthesis of the individual subunits of type VI collagen was co-ordinately induced. These results demonstrate that the activity of a single oncogene product is sufficient to inhibit type VI collagen expression.

INTRODUCTION

Transformation of fibroblasts by oncogenic viruses causes a number of changes, including alterations in cellular growth control, changes in cellular morphology and alterations in the synthesis of a discrete group of proteins. While the rate of synthesis is increased for some proteins, it is decreased for others (Hynes, 1976; Dubbelman & Yamada, 1982). This change appears to be specific, since the production of most cellular proteins is not affected. Two well-characterized proteins whose synthesis is markedly reduced upon transformation are fibronectin and type I collagen. A third protein that is reduced in simian virus 40 (SV40)-transformed fibroblasts to an extent similar to or even higher than that of fibronectin and type I collagen, has recently been identified as type VI collagen (Carter, 1982*b*; Heller-Harrison & Carter, 1984).

Type VI collagen is a dumb-bell-shaped molecule with two large globular domains linked by a short collagenous triple helix (see Engel *et al.*, 1985, for a review). The protein is composed of three different, disulphide-linked polypeptide chains. Two of these chains exhibit very similar molecular masses (140 kDa), whereas the third chain is considerably larger (200–260 kDa) (Trüeb & Winterhalter, 1986; Schreier *et al.*, 1987). Type VI collagen is synthesized and secreted by fibroblasts and other mesenchymal cells; on cell surfaces and in the extracellular matrix it forms extended filaments with a characteristic periodicity of 100 nm (Bruns *et al.*, 1986).

Carter has demonstrated that SV40-transformed fibroblasts produce drastically lower amounts of type VI collagen than normal cells (Carter, 1982*b*). Biosynthesis of the molecule appeared to be blocked at the transcriptional level, for SV40-transformed fibroblasts did not contain any translatable mRNA for type VI collagen (Trüeb *et al.*, 1985). A reduction in the synthesis of type

VI collagen may have profound effects on the adhesive properties of transformed cells as this collagen is known to promote cell attachment (Carter, 1982*a*). Furthermore, low levels of type VI collagen may disturb the integrity of the extracellular matrix as this protein is normally associated with fibronectin and other matrix components. Thus, it is conceivable that a reduction of type VI collagen synthesis may contribute to the unrestricted growth of transformed cells.

In the present study we have analysed the effects of transformation by three tumour viruses containing different oncogenes on the biosynthesis of type VI collagen in fibroblasts. We show that inhibition of type VI collagen synthesis is a common phenomenon of transformed cells and that the activity of a single oncogene is sufficient for inhibition.

EXPERIMENTAL

Cells and viruses

Chicken fibroblasts were prepared from 10-day-old chicken embryos of specific pathogen-free White Leghorn eggs (Lohmann-Tierzucht G.m.b.H., Cuxhaven, F.R.G.). Primary cultures were grown under an atmosphere of 5% (v/v) CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 10% (v/v) tryptose phosphate broth, 50 µg of ascorbate/ml, 100 µg of streptomycin/ml and 100 units of penicillin/ml. The fibroblasts were infected with the Schmidt-Ruppin strain of Rous sarcoma virus (RSV; subgroup A) or with the corresponding temperature-sensitive mutant NY68. Quail embryo fibroblasts transformed by the replication-defective myelocytomatosis virus MC29 (Q8 cells; Bister *et al.*, 1977) and normal quail fibroblasts were provided by Dr. E. Nigg (ETH, Zürich). Normal human fibroblasts (WI38, Malme-3), SV40-transformed fibroblasts (WI38 VA13),

and cells derived from a fibrosarcoma (HT-1080) and from a rhabdomyosarcoma (A-204), were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). All of these cells were grown at 37 °C in DMEM supplemented with 10 % (v/v) fetal calf serum, 50 µg of ascorbate/ml, 100 µg of streptomycin/ml and 100 units of penicillin/ml. In one experiment with WI38 cells, sodium orthovanadate (10–20 µM, Sigma) or phorbol 12-myristate 13-acetate (0.1–1 µM, Sigma) was added to the culture medium.

Preparation of cell culture proteins

Confluent cell layers were rinsed with phosphate-buffered saline and dissolved in hot SDS sample buffer containing 4 % (v/v) mercaptoethanol. Proteins secreted into the culture medium were concentrated by precipitation with ammonium sulphate at 33 % saturation. The precipitates were dissolved in SDS sample buffer. Alternatively, secreted proteins were labelled metabolically with [³⁵S]methionine (New England Nuclear, Boston, MA, USA, 100 µCi/ml) and immunoprecipitated as described (Trüb & Winterhalter, 1986). Cell numbers were determined by assessing the DNA content of cultures grown in parallel under strictly identical conditions (Labarca & Paigen, 1980).

Gel electrophoresis and immunoblotting

Proteins were resolved under reducing conditions on 5–10 % (w/v) gradient polyacrylamide gels as described by Laemmli (1970). Special care was taken to ensure that the amount of protein in samples from normal and transformed cells corresponded to exactly the same cell

numbers. The gels were stained with Coomassie Blue or processed for fluorography (Bonner & Laskey, 1974). For immunoblots, proteins were transferred to nitrocellulose and reacted with affinity-purified antibodies directed against the α1(VI) and α2(VI) subunits of human or chicken type VI collagen (Zimmermann *et al.*, 1986; Schreier *et al.*, 1987) or against chicken fibronectin (a gift of Dr. L. Vaughan, ETH, Zürich). Bound antibodies were visualized by ¹²⁵I-labelled protein A and autoradiography (Trüb & Bornstein, 1984). For quantification the autoradiographs were scanned with a CD50 densitometer (Desaga, Heidelberg, F.R.G.).

RESULTS

Human fibroblasts transformed by the DNA tumour virus SV40 grew in culture with the typical morphology of neoplastic cells and did not release infectious virus particles (Girardi *et al.*, 1966). Proteins were extracted from the cell layer of such fibroblasts with SDS sample buffer and resolved on gradient polyacrylamide gels (Fig. 1). When the resulting polypeptide pattern was compared with the pattern obtained in a similar way from normal embryonic or adult fibroblasts, no major differences were observed. On immunoblots, however, a striking difference was detected using antibodies against human α1(VI) and α2(VI) collagen subunits. These antibodies reacted strongly with a band of 140 kDa originating from the cell layer of normal fibroblasts, but did not react with any of the bands originating from the cell layer of transformed fibroblasts. A similar observation was made with the conditioned media. Antibodies against

Fig. 1. Influence of transformation by a DNA tumour virus on the synthesis of type VI collagen in human fibroblasts

Normal embryonic fibroblasts (WI38, lane 1), normal adult fibroblasts (Malme-3, lane 2) and SV40-transformed fibroblasts (WI38 VA13, lane 3) were grown for 6 days at 37 °C. Proteins from the cell layers (L) were extracted with SDS sample buffer and resolved on gradient polyacrylamide gels. The gels were stained with Coomassie Blue or processed for immunoblotting with antibodies against human α1(VI) and α2(VI) (140 kDa) subunits. Proteins in the media (M) of cultures labelled with [³⁵S]methionine were immunoprecipitated with the same antibodies, resolved on gradient gels and visualized by fluorography. The amount of protein loaded on to each slot corresponded to equal cell numbers.

Fig. 2. Influence of transformation by RNA tumour viruses on the synthesis of type VI collagen in avian cells

Normal quail fibroblasts (lane 1), quail fibroblasts transformed by MC29 containing the oncogene *myc* (lane 2), normal chicken fibroblasts (lane 3) and chicken fibroblasts transformed by RSV containing the oncogene *src* (lane 4), were grown for 5 days at 37 °C. Proteins from the cell layers (L) were extracted with SDS sample buffer; proteins in the media (M) were precipitated with ammonium sulphate. The proteins were resolved on gradient polyacrylamide gels and the gels were processed for immunoblotting with antibodies against chicken $\alpha 1(\text{VI})$ (140 kDa) and $\alpha 2(\text{VI})$ (130 kDa). The amount of protein loaded on to each slot corresponded to equal cell numbers.

$\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ subunits precipitated polypeptides of 140 kDa and 260 kDa from the radiolabelled media of normal cells, but did not precipitate any polypeptides from those of transformed cells. The 260 kDa component represents the $\alpha 3(\text{VI})$ subunit of type VI collagen, which is co-precipitated with the 140 kDa component because the components are linked by disulphide bonds (Trüb & Winterhalter, 1986). These experiments demonstrate that the synthesis of type VI collagen is completely blocked after transformation of fibroblasts by a DNA tumour virus.

The effects of RNA tumour viruses carrying well-defined oncogenes were studied in the following experiments. Quail fibroblasts transformed by the retrovirus MC29 divided rapidly in culture and exhibited an epithelial-like morphology. Proteins were harvested from the cell layer and the medium of such fibroblasts and analysed by the immunoblotting technique (Fig. 2, left-hand side). No traces of type VI collagen could be identified in these protein preparations with antibodies against chicken $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ subunits. Large amounts of type VI collagen, however, were detected by the same method in protein preparations from the cell layer and medium of normal quail fibroblasts used as a control. Since MC29-transformed cells do not produce virus particles (Bister *et al.*, 1977), inhibition of type VI collagen synthesis cannot be a side-effect of viral infection, but must be caused by the transforming activity of the viral oncogene *myc*. *myc* is known to encode a DNA-binding protein located in the cell nucleus.

An oncogene coding for a plasma-membrane-bound tyrosine kinase is *src*, the transforming gene of RSV.

When chicken fibroblasts infected with RSV were grown to confluence, only minimal amounts of type VI collagen were deposited in the cell layer or secreted into the medium as demonstrated by immunoblotting (Fig. 2, right-hand side). In contrast, uninfected control fibroblasts contained 7–10 times more type VI collagen in their cell layer and their medium. Since it is possible with the chicken protein to separate $\alpha 1(\text{VI})$ from $\alpha 2(\text{VI})$ on gradient polyacrylamide gels (Schreier *et al.*, 1987), the two subunits can be analysed separately. Densitometric scanning of the individual lanes shown in Fig. 2 revealed that the two subunits are reduced to the same extent after transformation by RSV.

To study the effect of the oncogene *src* on the synthesis of type VI collagen in more detail, we used a temperature-sensitive mutant of RSV (NY68). Cells infected with this mutant produce virus particles at both the permissive (35 °C) and the restrictive (41 °C) temperature. The transformed phenotype, however, is expressed only at the permissive temperature owing to the thermolability of the *src* gene product encoded by the NY68 mutant. Fibroblasts infected with NY68 were propagated at both the restrictive and the permissive temperature. Proteins were harvested from the cell layers and the conditioned media of these fibroblasts and analysed on polyacrylamide gels (Fig. 3). When the resulting protein patterns were compared, some differences in the quantity of three cell layer proteins (molecular mass 200–250 kDa) could be observed, but no such differences were noted with the proteins obtained from the conditioned media. On immunoblots, antibodies raised against the $\alpha 1(\text{VI})$ subunit of chicken type VI collagen detected a

Fig. 3. Inhibition of type VI collagen synthesis by the *src* gene product

Chicken fibroblasts infected with a temperature-sensitive mutant of RSV (NY68) were grown for 4 days at the restrictive (41 °C, lane 1) or the permissive (35 °C, lane 2) temperature. Proteins were harvested from the cell layers (L) and the media (M) and resolved on gradient polyacrylamide gels. Gels were stained with Coomassie Blue or processed for immunoblotting with antisera against $\alpha 1(\text{VI})$ or $\alpha 2(\text{VI})$ subunits. The amount of protein loaded on to each slot corresponded to equal cell numbers. Lane S shows a standard of the subunits $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$.

prominent band of 140 kDa in the preparations derived from the cell layer and medium of fibroblasts grown at the restrictive temperature. No immunoreactive polypeptide, however, was present in the preparation from the cell layer of fibroblasts grown at the permissive temperature and only traces were detectable in the preparation from the respective medium. Analogous results were obtained with antibodies against the $\alpha 2(\text{VI})$ subunit. The results indicate that the *src* gene product is responsible for the simultaneous reduction of the two type VI collagen subunits. Control experiments with uninfected fibroblasts proved that the reduction was not caused by a direct effect of the temperature change. In fact, normal cells grown at 35 °C produced 1.5-fold more type VI collagen than cells grown at 41 °C. When NY68-infected cells were grown to semi-confluence at the permissive temperature and then shifted to the restrictive temperature, induction of the two type VI collagen subunits became apparent after 4 h (Fig. 4). During the following 70 h, the amount of the subunits increased in a co-ordinate, complex way. A similar complex induction behaviour was observed with other batches of NY68-infected cells. In contrast, a monophasic induction curve was obtained when the synthesis of fibronectin was analysed in an analogous way (Fig. 4). Though the shape of the induction curves is difficult to interpret, these experiments demonstrate that the subunits of type VI collagen are co-ordinately controlled in transformed cells.

A number of oncogenes are known to exert their transforming capacity by encoding protein kinases.

Therefore, we investigated the effects of substances causing changes in the level of protein phosphorylation on type VI collagen synthesis. Addition of sodium orthovanadate to the culture medium of fibroblasts was shown to result in a 40-fold increase in the level of phosphotyrosine as this drug effectively inhibits phosphotyrosyl phosphatases (Klarlund, 1985). Tumour-promoting phorbol esters such as phorbol myristate acetate are known to activate directly protein kinase C, since they can substitute for diacylglycerol (Castagna *et al.*, 1982). When added to the culture medium of human fibroblasts in non-toxic doses, both substances induced dramatic changes in the morphology of the cells. Neither of the drugs, however, had any effect on the biosynthesis of type VI collagen. Down-regulation of type VI collagen synthesis by oncogene-encoded kinases such as pp60^{src} must therefore be accomplished by other (more subtle) changes than those induced by vanadate or phorbol esters.

Type VI collagen is normally synthesized by mesenchymal cells such as fibroblasts and muscle cells. Consequently, we checked cell lines derived from spontaneous mesenchymal tumours for the production of type VI collagen. When analysed by the immunoblotting technique, neither cells from a fibrosarcoma (HT 1080), nor cells from a rhabdomyosarcoma (A 204), synthesized any material which reacted with our antibodies. Inhibition of type VI collagen synthesis is therefore not restricted to cells transformed by viruses, but represents a common phenomenon of malignant mesenchymal cells.

Fig. 4. Effect of a temperature shift on the synthesis of type VI collagen and fibronectin in NY68-infected cells

Chicken fibroblasts infected with NY68 were grown at the permissive temperature (35 °C) to semi-confluence and then shifted to the restrictive temperature (41 °C). Proteins from the cell layers were harvested at the time points indicated and analysed by the immunoblotting technique with antibodies against $\alpha 1(\text{VI})$, $\alpha 2(\text{VI})$ and fibronectin (FN) (continuous line). Results obtained with cells kept at the permissive temperature throughout the experiment are given by a dashed line.

DISCUSSION

In the present study, we have analysed the effects of viral transformation on the biosynthesis of type VI collagen in fibroblasts. Four major conclusions can be drawn from our results. (1) Transformation of fibroblasts by DNA or RNA tumour viruses results in a reduction of type VI collagen synthesis. This reduction appears to be a common phenomenon of malignant cells as it is also observed with cells derived from spontaneous mes-

enchymal tumours. (2) The extent to which type VI collagen is reduced in transformed fibroblasts exceeds that observed earlier with fibronectin or type I collagen. The syntheses of the latter two proteins were reported to be reduced 2–10-fold in various transformed cell lines when compared to normal cells (Olden & Yamada, 1977; Sandmeyer *et al.*, 1981a). The synthesis of type VI collagen, however, was found to be completely blocked in SV40- or MC29-transformed cells. In RSV-transformed cells, the synthesis was at least 7–10-fold reduced. In this case, the actual reduction might even be greater, since it is possible that a small proportion of the cells used for our experiments were not susceptible to viral infection and consequently continued to synthesize type VI collagen at a normal rate. In contrast, each individual cell in preparations of SV40- or MC29-infected fibroblasts must be transformed as these cells were obtained by clonal selection. (3) The expression of the individual subunits of type VI collagen is controlled in a co-ordinate way. In our experiments, the two subunits $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ have been analysed; the third subunit has not been included because antibodies of high titre are not available against the $\alpha 3(\text{VI})$ subunit of human or chicken origin. It is generally assumed that the subunits are encoded by different genes which may be located on different chromosomes. Thus, a common mechanism must exist for the co-ordinate repression of the $\alpha 1(\text{VI})$ and the $\alpha 2(\text{VI})$ subunit. (4) The activity of a single oncogene is sufficient for inhibition of type VI collagen synthesis. Different oncogenes exert similar inhibitory effects. Recently, it has been established that oncogenes can be grouped into two different classes on the basis of the function and location of their protein products (Weinberg, 1985). The gene products of the first class are located at the plasma membrane or in the cytoplasm, while those of the second class are located in the cell nucleus. Oncogenes of the first class (e.g. *src*) can co-operate with oncogenes of the second class (e.g. *myc*) to confer a fully tumorigenic phenotype on a normal cell. In our experiments, *myc* as well as *src* caused a reduction of type VI collagen synthesis when introduced into normal cells by viral vectors. This reduction could not have been caused by a side-effect of viral infection, because MC29-transformed cells do not produce infectious virus particles: the transforming agent present in preparations of MC29 is defective for replication (Bister *et al.*, 1977). Furthermore, a special strain of RSV was used in one of our experiments which contains a temperature-sensitive mutation in the transforming gene *src*. This mutation does not affect production of the virus particles. Cells infected with the mutant viruses had a transformed phenotype at the permissive and a normal phenotype at the restrictive temperature. Synthesis of type VI collagen was reduced only at the permissive, but not at the restrictive temperature, clearly proving that the activity of the *src* gene product was solely responsible for this reduction.

The exact mechanism by which the synthesis of type VI collagen is blocked in transformed cells remains to be elucidated. In a recent publication we presented evidence that inhibition might occur at the transcriptional level (Trüeb *et al.*, 1985). There we demonstrated by a translation assay *in vitro* that SV40-transformed fibroblasts do not contain any active mRNA for type VI collagen. The molecular mechanisms by which the syntheses of fibronectin and type I collagen are regulated

in transformed cells have been studied in some detail and may serve here as model cases. In both examples, it was proven with complementary DNA probes and nuclear run-off transcription experiments, that inhibition occurred at the transcriptional level (Tyagi *et al.*, 1983; Sandmeyer *et al.*, 1981*b*). The mRNA levels of type I collagen were found to be reduced in *src*-, *mos*-, *H-ras*- and *Ki-ras*-transformed cell lines (Liau *et al.*, 1985). To gain further insight into the molecular mechanism, the promoter region of the $\alpha 2(I)$ collagen gene was fused to an unrelated marker gene (Schmidt *et al.*, 1985). When fibroblasts containing this hybrid transcription unit were transformed by *v-mos*, the expression of the marker gene was inhibited to a similar degree to the expression of the endogenous collagen gene. Thus, the regulatory elements affected upon transformation must be located within the promoter region of this gene. Transcription factors that may interact with such regulatory sequences are now being investigated (Setoyama *et al.*, 1985; Hatamochi *et al.*, 1986). It is conceivable that protein factors exist which specifically regulate the activities of the genes coding for fibronectin, type I and type VI collagen. Upon transformation, these factors might become neutralized or inactivated and consequently the transcription of the respective genes would cease. If identical or closely related factors were responsible for the activities of an entire battery of genes coding for different extracellular matrix proteins, the levels of all these proteins would drop in concert. In fact, co-ordinate regulation has been observed for different subunits of type I collagen (Sandmeyer *et al.*, 1981*b*; Sobel *et al.*, 1981) and type VI collagen (this study). To examine further this possibility, specific DNA probes are required for each of the proteins involved. While probes for type I collagen and fibronectin have been available for some time, cloning of DNA probes for type VI collagen has just been reported (Chu *et al.*, 1987).

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