Modulation of Fibronectin Gene Expression in Chondrocytes by Viral Transformation and Substrate Attachment

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Abstract. Chicken vertebral chondrocytes, which normally grow in suspension, synthesize large amounts of cartilage extracellular matrix proteins, but little fibronectin. We have analyzed the effects of both substrate attachment and transformation with a temperature-sensitive mutant of Rous sarcoma virus on fibronectin gene expression in these cells. Our experiments show that viral transformation increases fibronectin synthesis to a greater extent than substrate attachment. Furthermore, transformed chondrocytes have lost the ability to decrease fibronectin synthesis in response to suspension culture, suggesting that transformation alters the normal attachment-responsive control of fibronectin gene expression. Finally, infected substrate-attached chondrocytes shifted to the nonpermissive temperature for transformation use fibronectin RNA more efficiently in protein synthesis than cells grown under the other conditions, suggesting for the first time a role for translational control of fibronectin gene expression.

Fibronectin, a large glycoprotein that is a component of the surface and extracellular matrix of many types of cells, plays an important role in adhesion and maintenance of morphology. The functions of fibronectin and the control of its synthesis and accumulation have been examined most extensively in cultured fibroblasts, which are anchorage dependent, grow in monolayer, and synthesize large amounts of fibronectin (reviewed in references 23 and 46). Additional information has been obtained, however, by examination of cultured chondrocytes (cartilage-producing cells). These cells are anchorage independent and grow equally well in suspension as round, refractile, isolated cells and, attached to the substrate as polygonal cells. Under both conditions they synthesize large amounts of the cartilage extracellular matrix components, primarily type II collagen (reviewed in reference 41) and chondroitin sulfate proteoglycan (13, 31, 33). They differ, however, in that the substrate-attached cells synthesize more fibronectin (9, 12) and accumulate more fibronectin on the cell surface (42) than the cells grown in suspension.

With time in culture, substrate-attached chondrocytes often lose the ability to synthesize cartilage matrix proteins (reviewed in reference 41). Furthermore, transformation by Rous sarcoma virus, as well as treatment with the tumor promoter PMA, bromodeoxyuridine, retinoic acid, embryo extract and fibronectin (reviewed in reference 41), interferes with expression of the normal chondrocyte phenotype. These agents reportedly have vastly different effects on fibronectin synthesis; for example, synthesis is increased by viral transformation (1, 3, 20, 47) and PMA (18, 19), while retinoic acid has little effect (21, 22). However, interpretation of these experiments is complicated by the fact that in some laboratories normal (untreated) chondrocytes were grown in suspension (1, 3, 18, 19) and synthesized little or no fibronectin, while in others they were grown in monolayer (20–22) and synthesized a large amount. Furthermore, many of the agents which cause loss of the differentiated phenotype also cause substrate attachment of cells initially grown in suspension (18, 19, 35, 36; our unpublished observations). Thus in some cases comparisons have been made between normal suspended and treated attached chondrocytes; conclusions drawn from such comparisons may be misleading in the absence of controls for the effects of substrate attachment alone.

Thus the correlations among fibronectin synthesis, substrate attachment, and loss of the differentiated phenotype provoked by treatments such as viral transformation are complex and require more extensive analyses than those which have been performed previously. Further clarification of these issues will not only aid in understanding the mechanisms regulating fibronectin gene expression in chondrocytes, but will also have more general relevance for understanding the effects of fibronectin modulation on cell behavior. To explore these correlations further, we have performed a detailed analysis of fibronectin gene expression at both the protein and RNA levels. In these studies, we have analyzed the effects of viral transformation in both suspended and attached chondrocytes and compared them with the effects of substrate attachment alone. Our results show that the control of fibronectin gene expression in chondrocytes is unexpectedly complex and may involve both transcriptional and posttranscriptional regulatory mechanisms.
Materials and Methods

Cell Culture

The isolation, culture, and viral infection of primary vertebral chondrocytes (from 12-d-old chicken embryos; SPAFAS, Inc., Norwich, CT) were performed using minor modifications of procedures described previously (1, 11, 34). Suspended cells collected after 5-7 d (hence, free of contaminating adherent fibroblasts) were the basis for all subsequent manipulations in culture conditions, which are outlined in Fig. 1. Primary chondrocytes were treated for 30 min with 200 U/ml of hyaluronidase to promote cell attachment (34), then suspended in complete medium and plated on tissue culture dishes (Falcon Labware, Oxnard, CA) at 10^6 cells per dish. Half the plates were infected with tsLA24A (ts-RSV), a conditional mutant of Rous sarcoma virus. At 36°C the permissive temperature for transformation; 41°C is the nonpermissive temperature for transformation.

Protein Synthesis

Duplicate dishes were labeled for 5 or 15 min at the appropriate temperature (36 or 41°C) in 1.5 ml of prewarmed Hank's balanced salt solution containing 100 μCi/ml of [35S]methionine. Cells were then solubilized by boiling in 100 μl of 10 mM sodium phosphate, pH 6.8, containing 2% SDS, and protease inhibitors were added to the following concentrations: 5 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 0.5% aprotinin, and 5 mM benzamidine. Identical labeling efficiencies were observed in cells grown at 36°C. Suspended and substrate-attached cells often displayed differences in labeling efficiency. However, the magnitude and direction of the differences varied from one experiment to another; thus we assume that the differences were due to experimental variation, rather than to intrinsic differences in properties of the cells.

RNA Analysis

Total cellular RNAs were isolated by differential precipitations (2) from 20-30 plates of cells grown under all the conditions described above. For hybridization analysis, RNAs were denatured by glyoxalation (30) and subjected to electrophoresis at 70 V through 1% agarose gels. The amount of fibronectin RNA in transformed, substrate-attached chondrocytes was assigned a value of 100; the amounts in cells grown under other conditions were then expressed relative to that value. Each value shown in the histogram is the average of three to five independent experiments using cells from different batches of embryos; vertical bars show the standard deviation. The values for uninfected cells represent averages of values for cells grown at 36 and 41°C since there were no detectable differences between cells grown at the two temperatures.

Statistical Analysis

Differences in the calculated values for protein synthetic rates and steady-state RNA levels were assessed by means of a two-tailed t test.

Results

Relative rates of fibronectin synthesis were determined by pulse-labeling cells for 15 min with [35S]methionine. Typical autoradiographs are shown in Fig. 2, and the results of several experiments are depicted graphically in Fig. 3. Fibronectin synthesis was nearly undetectable in primary vertebral chondrocytes grown in suspension (Figs. 2 and 3, lanes 1), even when they were maintained in culture for a month (not shown). The effects of substrate attachment were examined in uninfected chondrocytes that had been attached for 1-14 d and maintained at 36°C or shifted to 41°C.
attached for 9-10 d and put back into suspension at the third subculture. Fibronectin synthesis was decreased in these resuspended cells (Figs. 2 and 3, lanes 2) to a level comparable to that found in the primary cells. Note that the effects of substrate attachment and resuspension were similar whether the cells were maintained at 36°C or shifted to 41°C 3 d before harvest. Thus the values shown in Fig. 3 (lanes 2 and 3) represent an average of cells grown at 36 and 41°C.

It is important to note that, in terms of the overall pattern of protein synthesis, the substrate-attached chondrocytes are similar to the primary cells maintained in suspension, but they are not identical. The substrate-attached cells display slightly decreased synthesis of type II collagen and significantly decreased synthesis of the chondroitin sulfate proteoglycan core protein. Furthermore, in addition to increased fibronectin synthesis, the attached cells display moderately increased synthesis of vimentin, suggesting that the expression of genes concerned with attachment and maintenance of cell shape may be increased coordinately by substrate attachment. Thus, in a quantitative sense, substrate-attached chondrocytes are not phenotypically normal. They can, however, be fully restored to the normal phenotype by returning them to suspension culture.

Hybridization analysis of RNAs from uninfected cells grown under all the conditions described above (Fig. 4, panels 1–3) indicated that fibronectin RNA levels precisely paralleled the protein synthesis data (for a quantitative comparison of fibronectin synthesis and steady-state RNA levels in these cells, see Fig. 3, lanes 1–3). Thus, the effects of substrate attachment and resuspension on fibronectin synthesis are determined primarily by modulating the steady-state amount of fibronectin RNA.

The combined effects of viral transformation and substrate attachment on fibronectin synthesis were examined in chondrocytes that were infected with ts-RSV and grown at 36°C, fore harvesting. These cells displayed a fivefold increase in fibronectin synthesis (Figs. 2 and 3, lanes 2). The reversibility of this increase was examined in cells that had been attacched for 9-10 d and put back into suspension at the third subculture. Fibronectin synthesis was decreased in these resuspended cells (Figs. 2 and 3, lanes 2) to a level comparable to that found in the primary cells. Note that the effects of substrate attachment and resuspension were similar whether the cells were maintained at 36°C or shifted to 41°C 3 d before harvest. Thus the values shown in Fig. 3 (lanes 2 and 3) represent an average of cells grown at 36 and 41°C.

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The combined effects of viral transformation and substrate attachment on fibronectin synthesis were examined in chondrocytes that were infected with ts-RSV and grown at 36°C,
the permissive temperature for transformation. At the time of harvest they had been attached to the substrate for 12-14 d. The amount of fibronectin synthesis in these cells (Figs. 2 and 3, lanes 4) was approximately twice as high as in uninfected substrate-attached cells (Figs. 2 and 3, lanes 2), and 10 times as high as in primary chondrocytes (Figs. 2 and 3, lanes 1). When the substrate-attached transformed cells were returned to suspension culture (Figs. 2 and 3, lanes 5), fibronectin synthesis remained at the same high level, indicating that transformed chondrocytes have lost the ability to modulate fibronectin synthesis in response to suspension culture.

To determine whether the transformation-induced increase in fibronectin synthesis was reversible, the substrate-attached transformed cells were shifted to 41°C, the nonpermissive temperature for transformation (Figs. 2 and 3, lanes 6). Surprisingly, although these infected cells reacquired the polygonal morphology characteristic of normal substrate-attached chondrocytes, fibronectin synthesis remained at the same high level as in the transformed cells (Figs. 2 and 3, lanes 4). However, when the infected cells were not only shifted to 41°C but also returned to suspension culture, fibronectin synthesis was reduced to 31% of that observed in transformed cells (Figs. 2 and 3, lanes 7). This was similar to the amount in uninfected cells that were attached and resuspended (Figs. 2 and 3, lanes 3). These results indicate that infected chondrocytes shifted to the nonpermissive temperature for transformation have regained the ability to respond to suspension culture by an appropriate reduction in fibronectin synthesis.

To ensure that the fibronectin synthesis we observed in the experiments described above reflected relative synthetic rates, and not the net effect of synthetic rates and preferential degradation (for example, in the transformed cells), or preferential stabilization (for example, in the infected substrate-attached cells at 41°C), cells were pulse-labeled for 5 min instead of for 15 min. The relative amounts of fibronectin synthesis were very similar to those observed in the 15-min labeling experiments (not shown), suggesting that preferential stabilization or degradation is not responsible for the unexpectedly high level of fibronectin synthesis in infected substrate-attached cells shifted to the nonpermissive temperature for transformation.

It is important to note that the infected substrate-attached cells shifted to 41°C are nearly identical, in terms of synthesis of most proteins, to the uninfected substrate-attached cells (for example, compare synthesis of type II collagen in Fig. 2, lanes 2 and 6). This indicates that the continued high synthesis of fibronectin in these cells is not due to incomplete inactivation of src, the transforming gene of ts-RSV, by shifting the cells to the nonpermissive temperature for transformation.

Hybridization analysis of RNAs indicated that in transformed chondrocytes (Fig. 4, panels 4 and 5) fibronectin RNA levels precisely paralleled the relative rates of fibronectin synthesis, whether the cells were attached or suspended (for a quantitative comparison, see Fig. 3, lanes 4 and 5). Surprisingly, however, in infected substrate-attached cells shifted to 41°C, fibronectin RNA was reduced to 43% of the amount in transformed cells (Fig. 4, panel 6). This decrease is the response one would expect after a shift to nonpermissive temperature, since the amount is similar to that observed in uninfected substrate-attached cells (cf. lanes 2 and 6 in Fig. 3). Recall, however, that fibronectin synthesis was not decreased at all in those cells (Figs. 2 and 3, lanes 6); the difference between the high synthetic rate of fibronectin and the much lower steady-state amount of fibronectin RNA was found to be statistically significant at the 0.01 level. These results suggest that fibronectin RNA is used more efficiently in substrate-attached chondrocytes that have been rescued from transformation than in both uninfected cells and transformed cells.

To determine whether the apparent increase in translational efficiency was due to alteration in an intrinsic property of the fibronectin RNA itself, RNAs from infected cells were translated in vitro in a rabbit reticulocyte lysate (not shown). Translatability of the RNAs precisely paralleled steady-state RNA levels determined by hybridization analysis (for a quantitative comparison, see Fig. 3). Thus the unexpectedly high rate of fibronectin synthesis in infected substrate-attached cells shifted to 41°C may reflect an altered property of the cells, rather than an altered property of the fibronectin RNA in those cells.

When infected cells were restored to suspension culture at 41°C, the amount of fibronectin RNA was reduced further,
to 16% of that in transformed cells (Fig. 4, panel 7). This amount correlates well with the rate of fibronectin synthesis, (Fig. 3, lane 7) and is almost identical to the amount observed in the uninfected cells which were substrate-attached and then returned to suspension culture (cf. lanes 2 and 7 in Fig. 3). These results indicate that the effects of viral transformation on fibronectin synthesis are fully reversible only when the cells are restored to suspension culture at the non-permissive temperature for transformation (41°C).

Discussion

In the experiments reported here, we have defined in detail the effects of viral transformation on fibronectin gene expression in both attached and suspended vertebral chondrocytes, and have compared them with the effects of substrate attachment alone. These studies have led us to some unexpected findings which suggest that the regulation of fibronectin gene expression in chondrocytes is more complex than previously suspected. Specifically, we have shown that fibronectin synthesis is increased fivefold by substrate attachment, while viral transformation in conjunction with substrate attachment results in an additional twofold increase. Furthermore, whereas the induction of fibronectin synthesis due to substrate attachment is fully reversible in control cells, it is not reversible in transformed cells. These results indicate that transformed chondrocytes have lost the normal attachment-responsive control of fibronectin gene expression, and suggest that the effects of transformation are dominant over the effects of substrate attachment.

The effects of viral transformation on fibronectin gene expression have been studied most extensively in chicken embryo fibroblasts, which normally display high levels of fibronectin synthesis. Transformation of these cells by Rous sarcoma virus results in reduced fibronectin synthesis (reviewed in references 23 and 46) and steady-state RNA levels (2, 15), reflecting decreased transcription of the fibronectin gene (39). As with the effects on fibroblasts, we anticipate that the increased steady-state RNA levels in transformed chondrocytes will be due to increased transcriptional activity of the fibronectin gene in these cells.

Transcriptional activation, however, appears not to be the sole mechanism controlling fibronectin gene expression in chondrocytes. We have also shown that in infected substrate-attached chondrocytes that have been rescued from transformation, fibronectin RNA is translated more efficiently than in the cells grown under other conditions. To our knowledge, these experiments provide the first demonstration of translational control of fibronectin gene expression. This apparent increase in translational efficiency could theoretically arise either from an alteration in the primary structure of the RNA itself or from an alteration in the translational machinery of the cell. Since the primary transcription products of the fibronectin gene can be spliced in several different ways (32, 37), such alternative RNA processing might give rise to RNAs with different intrinsic translational efficiencies. Although we have not ruled out the existence of alternate splicing of fibronectin RNAs in these cells, the results of the in vitro translation experiment mitigate the possibility that such a mechanism causes the increased translational efficiency of fibronectin RNA in infected substrate-attached chondrocytes grown at 41°C. If the primary structure of the RNA were altered in such a way, one would expect to find RNA function altered in vitro as well as in the intact cells. However, the RNA from these cells does not display increased translational efficiency in vitro. Thus the simplest explanation for these data is that the increased translational efficiency is mediated by an alteration in the translational machinery of the cell.

There is at least one other protein, in addition to fibronectin, which is overproduced in infected substrate-attached chondrocytes shifted to the nonpermissive temperature for transformation. In experiments to be reported elsewhere, we have shown that vimentin synthesis is induced dramatically by transformation with ts-RSV. Furthermore, when the substrate-attached cells are shifted to 41°C, vimentin synthesis remains elevated while steady-state RNA levels decrease. Thus vimentin RNA also displays increased translational efficiency in these cells. Note that both of these proteins have been implicated in attachment, cell spreading, and maintenance of normal cell morphology. Furthermore, vimentin gene expression is modulated by manipulations in adhesion and cell spreading (5, 7). Thus it seems possible that the apparent translational control described here for fibronectin may be a common regulatory mechanism for RNAs encoding proteins involved in cell shape and behavior, for example, other components of the cytoskeleton and extracellular matrix. A likely candidate for such control is heparan sulfate proteoglycan, which has also been implicated in substrate attachment and which is overproduced in the infected substrate-attached chondrocytes at 41°C (38).

Translational control due to alterations in cell shape and substrate attachment has been described previously (4, 16, 17). Suspension of anchorage-dependent cells results in a slow decline in translation of most cellular messenger RNAs, apparently due to modification of the mRNAs themselves. Transformed cells (which are not anchorage dependent) do not display such translational inhibition (44). The results we have described above differ from those earlier experiments in several ways. First, there is no difference between suspended and attached chondrocytes in the overall rate of protein synthesis. This result suggests that the lack of translational inhibition in transformed cells (44) is not due to the transformed phenotype per se, but to the absence of anchorage dependence, which is also a property of some non-transformed cells (such as chondrocytes). Furthermore, infected chondrocytes that are substrate attached and grown at the nonpermissive temperature for transformation display the selectively increased translational efficiency of only a few RNAs. This selective increase is apparently not due to modification of these RNAs, since the in vitro translation of these RNAs precisely reflects the steady-state RNA levels determined by hybridization analysis.

The previously described association of actively translating polyribosomes with the cytoskeleton (6, 10, 27, 28, 40) may suggest an explanation for the apparent translational control we have observed in chondrocytes. Marchisio et al. (29) have examined the cytoskeleton in ts-RSV infected chondrocytes at permissive and nonpermissive temperatures. They found that transformed chondrocytes lose the stress fibers characteristic of substrate-attached cells (as do transformed fibroblastic and epithelial cells; reviewed in reference 24). In infected substrate-attached chondrocytes cultured at the nonpermissive temperature, stress fibers reap-
peer; however, the distribution of microfilaments is not fully restored to the pattern characteristic of uninfected cells. Thus, our data suggest a possible correlation between the altered distribution of microfilaments (or some other less well-characterized component of the cytoskeleton) and the apparent increase in translatable activity of a subset of cellular RNAs.

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