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Cellular fibronectin is induced by epidermal growth factor, but not by dexamethasone or cyclic AMP in rat liver epithelial cells

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Epidermal growth factor (EGF) induces fibronectin (FN) and FN mRNA in rat liver epithelial cells, under conditions where the factor also induces the cells to migrate. Newly synthesized protein is secreted into the medium and deposited as substratum-bound extracellular matrix. The levels of mRNA and the amount of protein synthesized are not influenced by cyclic AMP or dexamethasone, factors that have been found to modulate FN expression in other cells. However, the cells are sensitive to the factors, suggesting a cell-specific regulation. The EGF-induced RNA contains the sequences EIIIA and EIIIB characteristic of cellular fibronectin.

Epidermal growth factor; Cell migration; Gene expression; mRNA splicing variant; Extracellular matrix; Cellular fibronectin; (Rat liver epithelial cell)

1. INTRODUCTION

The dimeric, high M_r glycoprotein fibronectin (FN) is a component of normal plasma and of the extracellular matrix (ECM) of many cells, and fulfills essential functions in homeostasis, cell growth and differentiation [1]. FN is also required for migration during development (e.g., neural crest cells [2]) and is involved in liver tissue repair [3] and liver cell migration [4]. From the single FN gene, up to 12 (rat [5]) or 20 (human [18]) variants can be derived by differential splicing of the primary transcript. The mRNA for plasma FN is devoid of the ED I (EIIIA) and ED II (EIIIB) sequences, while cellular fibronectin contains both sequences, in variable combinations with a third domain of differential splicing [5,6]. It has also been shown that cells transformed with retroviruses carrying temperature-sensitive oncogenes can modulate the splicing pattern with changes in temperature [7]. Overall, however, the mechanisms that determine the changes in the splicing pattern, and the role of the different proteins that result from it, remain to be determined.

For some cell types it has been established that the amount of fibronectin they produce can be modulated by glucocorticoids [8], cyclic AMP [9,10] and neoplastic transformation [1], but these data do not yet allow conclusions on the mechanisms that control the cell specific and developmentally regulated expression of the fibronectin gene.

We have recently reported that rat liver epithelial cells induced to migrate by EGF deposit ECM migration tracks that contain FN as one of its components, and also synthesize increased amounts of the protein [4]. We now show that the induction of FN by EGF occurs shortly after exposure of the cells to the growth factor and is associated with an increase in the amount of mRNA for the cellular type of FN. The induction of FN in mouse fibroblastic cells released from serum starvation has been reported recently by others [11].

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2. MATERIALS AND METHODS

2.1. *Cell culture and analysis of labeled proteins*

The cells were cultured in serum-free medium supplemented with insulin (2 μg/ml), insulin plus EGF (10 ng/ml) as previously described [4], and in addition with dexamethasone (Sigma; 5×10^{-8} M) or cyclic AMP (dibutyryl cyclic AMP, Sigma; 5×10^{-4} M). Labeling was achieved with 100 μCi/ml [3.7 MBq/ml] of [^{35}S]methionine for 2 h [4], and as indicated in the figure legend. The labeled medium proteins were separated on 5 to 15% polyacrylamide gels and analyzed by fluorography as previously described [4]. Substratum-bound ECM proteins were prepared as described [12], solubilized in sample buffer, separated on gradient gels and processed as the medium proteins. The 240 kDa band of these gels corresponds only to fibronectin, as previously determined by immunoprecipitation, immunoblotting and by 2D gel analysis.

2.2. *RNA extraction and hybridizations*

Total RNA was extracted by the standard guanidine isothiocyanate method [13] with modifications [14]. Typically, 10 μg of RNA were obtained from 2×10^5 cells. 20 μg of total RNA were separated on formaldehyde-containing gels, transferred to nitrocellulose membranes (Schleicher and Schuell) and hybridized [15] for 24 h at 43°C with probes pFH 154 [16] to detect total FNmRNA, M13ED1 (ED1 domain [17]) or pFN 4H 0.95 (ED II domain [18]), that had been nick-translated to a specific activity of 1.5×10^8 dpm/μg. The concentration of labeled probes during the hybridization was 7.5×10^5 dpm/ml. These cDNA probes of human FN are more than 80% homologous to rat FN DNA and share large segments of complete homology [16]. Filter blocking and hybridization was performed overnight at 43°C in rotating 50 ml cell culture polypropylene tubes (Falcon; Nunc), using 3 × SSPE, 50% formamide, 10% (w/v) PEG 6000, 0.1% SDS, 50 μg/ml denatured calf thymus DNA and 500 μg/ml yeast tRNA. Posthybridization washes were performed in 1 × SSC, 50% formamide, 0.1% SDS at 43°C.

3. RESULTS AND DISCUSSION

The time course of synthesis of soluble fibronectin after addition of EGF to the cultures is shown in fig.1. The appearance of the newly synthesized protein in the medium is prevented by inhibitors of Asn-linked glycosylation (unpublished). The amount of newly synthesized FN deposited onto the substratum is also increased by EGF, as revealed by radioactivity counting of the FN bands of fluorographed gels of labeled matrix preparations from two and four days after plating the cells. The amount of radioactive matrix fibronectin of cultures maintained in insulin plus EGF was increased 3- and 4.3-fold, respectively (2nd and 4th day), with respect to the cultures maintained in insulin alone. In agreement with previous results [4], no increase in cell number by EGF was observed in these cultures. It remains to be determined, if the insoluble FN of the matrix and migration tracks belongs to a different splicing variant than the protein secreted into the medium.

EGF also induces FN mRNA (fig.2). This RNA contains the EIIIA/EDI and EIIIB/EDII segments (fig.3) characteristic of the cellular type of fibronectin [5,6] indicating that most, if not all, FN RNA codes for the cellular form of the protein. The relative proportions of both domains of alternative splicing however, have not been determined quantitatively, but the degree or kinetics of induction of the message containing these domains is not significantly different (fig.3).

The induction of FN messenger is suggested to result mostly from increased transcription, but a stabilization of the RNA cannot be excluded at the present time. However, in the few cases in which EGF has been identified unambiguously as an inducer of gene expression, the inducibility was found to be dependent on specific enhancer sequences [19,20]. Similar sequences occur at −260 of the 5′-region of the rat [22] and at −268 of the human [10] fibronectin genes.

Earlier reports had indicated that glucocorticoids [8,21] and cAMP [9] can modulate the synthesis of the protein [8], or the formation of

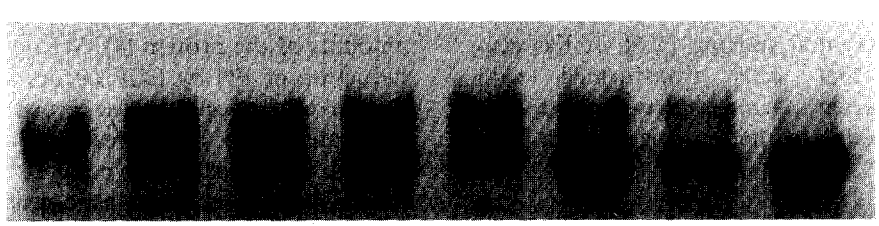

Fig.1. Time course of fibronectin induction by EGF. Secreted fibronectin from cells labeled for 2 h, collected after addition of EGF at the times (h) indicated on each gel slot. Cultures supplemented only with insulin (In), and with insulin plus EGF (IE).

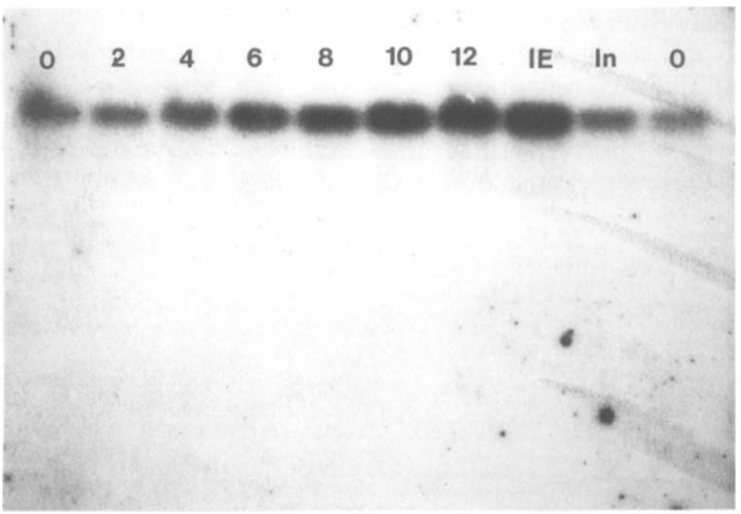

Fig.2. Time course of FN mRNA induction by EGF. RNA extracted from cultures after addition of EGF at the times (h) indicated on each gel slot, and hybridized with pFH 154 DNA to demonstrate the presence of FN mRNA. RNA from cultures supplemented only with insulin (In), and with insulin plus EGF (IE).

immunohistologically detectable FN networks around cells [9,21]. The recent cloning of the 5′-genomic segments of the FN gene has revealed regulatory sequences for glucocorticoid- and cAMP-mediated regulation both in human and rat DNA [10,22]. With the cells of the present experiments, however, neither dexamethasone nor cyclic AMP modulate the induction of FN, either in the presence of insulin alone or in association with EGF (fig.4 and unpublished results). However, both factors have been found to effectively modulate the expression of several other genes in these cells (Bade and Feindler, in preparation). Therefore, if the specific control sequences have not been deleted or otherwise mutated, the lack of response is probably due to mechanisms that cell-specifically prevent a transcriptional activation of the FN gene.

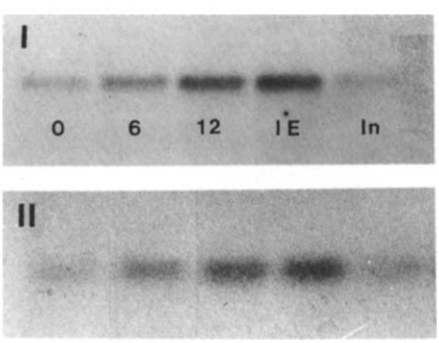

Fig.3. Sequences of alternative splicing in FN mRNA, demonstrated by hybridization with probes for EDI (I) and EDII (II) sequences, respectively. Further details as for fig.2.

It has recently been reported [11] that serum and EGF induce the expression of the fibronectin gene in fibroblastic mouse cells. In that study, however, the induced synthesis of the protein was associated directly with the growth response occurring after release from serum starvation. In contrast, with the epithelial cells of our experiments, the induction of the protein is associated with the induction of migration, in the absence of a growth stimulation by the factor ([4] and above). In this context it is interesting to note that neither dexamethasone nor cyclic AMP affect the induction of migration by EGF (unpublished).

The system is being further used for testing the interesting possibility that EGF might, in addition

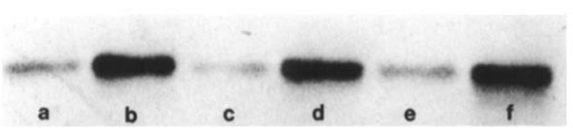

Fig.4. Fibronectin mRNA from cultures supplemented only with insulin (a), insulin plus EGF (b), insulin plus dexamethasone (c), insulin, EGF and dexamethasone (d), insulin plus cAMP (e), and insulin, EGF and cAMP (f), respectively, demonstrated by hybridization with pFH154.

to its (cell specific) regulation of the amount of FN produced by the cells, also modulate its splicing pattern in relation to the migratory behaviour it induces. These studies will require RNase protection assays that include the use of probes specific for the third domain (V/IIICS) of differential splicing.

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