Phosphorylation of fibronectin in quiescent and growing cell cultures

Iqbal Unnisa Ali*

Meloy Laboratories, 2501 Research Boulevard, Rockville, MD 20850, USA

Received 8 November, 1982

Phosphorylation of fibronectin was studied in quiescent *vs* growing cells from several species. Fibronectin secreted by actively growing cells exhibits a significantly higher level of phosphorylation than does the fibronectin secreted by quiescent cells of the same species.

Fibronectin *Phosphorylation* *Growing* vs. *quiescent cells*

1. INTRODUCTION

Fibronectin, a high M_r glycoprotein present on the surfaces of various cell types and also in blood plasma, is important for several biological functions [1–4]. A role for fibronectin has been suggested in a wide variety of cellular activities such as cell-to-cell and cell-to-substratum adhesion [5–7], cell shape formation and cellular migration [8,9], organization of intracellular cytoskeletal elements and extracellular matrix [10,11], reticuloendothelial clearance of foreign particles [12] and embryogenesis and differentiation [13–15].

The amount of cell surface fibronectin is usually very much decreased on cultured tumor cells and in cells transformed in vitro with oncogenic viruses or by chemical carcinogens [1–4]. Compared to nonproliferating quiescent cells fibronectin is present in reduced amounts on the surfaces of rapidly growing and mitotic cells [16,17]. This may not be surprising in view of the fact that rapidly growing and mitotic cells exhibit several biochemical changes that are typical of transformed cells [18,19].

Fibronectin from transformed cells is phosphorylated to a much higher extent than that from normal cells [20]. Since the functions of a variety of enzymes and other proteins are regulated by phosphorylation, it was of interest to study phosphorylation of fibronectin produced by quiescent and growing cells.

2. MATERIALS AND METHODS

2.1. *Cells*

Human skin fibroblasts, GM0077, obtained from the Institute For Medical Research (Camden, NJ) and a mouse fibroblast line, NIH/3T3, were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS). The hamster cell line, NIL-8, was grown in DMEM + 5% FCS.

2.2. *Radioactive labeling*

Monolayers of either quiescent or rapidly growing cells, which were 50% confluent, were labeled with [^{32}P] orthophosphate (500 μCi/ml; carrier-free; New England Nuclear) and [^{3}H]leucine (100 μCi/ml; 100 Ci/ml; New England Nuclear) in medium lacking phosphate and leucine, plus 5% dialyzed FCS.

2.3. *Isolation of fibronectin*

Fibronectin was isolated from the labeled conditioned medium of NIL 8 cells and NIH/3T3 fibroblasts by 2 cycles of immunoprecipitation using antihamster fibronectin serum as in [20]. Mono-

* Present address: HEM Research, Inc., 12220 Wilkins Avenue, Rockville, MD 20852, USA

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

clonal antibodies designated as 3E3 [21] were used to precipitate the fibronectin from the conditioned medium of GM0077 cells.

SDS-polyacrylamide gel was run as in [22]. ^{32}P and ^{3}H radioactivity in the immune complex of fibronectin was determined by scintillation counting.

3. RESULTS AND DISCUSSION

Fig. 1 shows a representative experiment of the kinetics of phosphorylation of fibronectin in relation to [^{3}H]leucine incorporation in quiescent *vs* growing NIL 8 cells. At all time points the ratio of ^{32}P:^{3}H in fibronectin was higher in growing, than in quiescent cells. Especially at earlier time points, i.e., 2 h and 4 h, the ^{32}P:^{3}H ratio in this experiment was 8-fold and 5-fold higher in growing cells than in quiescent cells. With increasing length of labeling period (8 h and 24 h) fibronectin from growing cells showed about 1.5–3-fold increase in ^{32}P:^{3}H ratio.

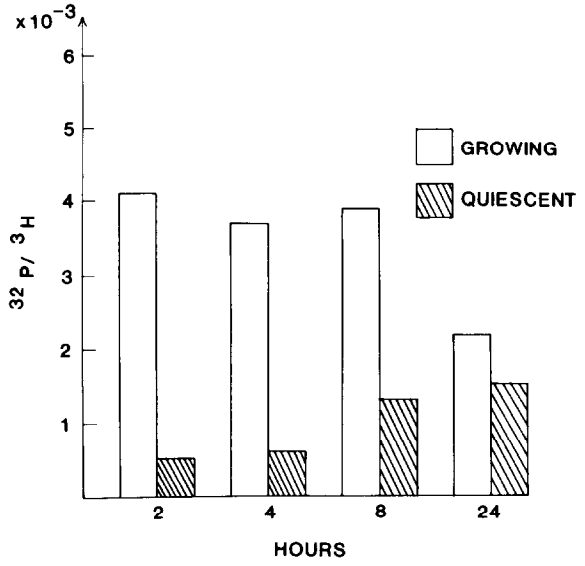

Fig. 1. Kinetics of phosphorylation of fibronectin in relation to [^{3}H]leucine incorporation: 2×10^{5} and 2×10^{4} NIL 8 cells were seeded in 60 mm Petri dishes in DMEM with FCS. On day 4 cells were labeled with [^{32}P]orthophosphate and [^{3}H]leucine for indicated time periods (see section 2 for details of labeling conditions and isolation of fibronectin).

In another experiment phosphorylation of fibronectin from 3 cell lines (GM0077, NIH/3T3, and NIL 8) was studied in quiescent *vs* rapidly growing cells after 4 h of labeling with [^{32}P]orthophosphate and [^{3}H]leucine. Table 1 shows that growing cells of all 3 cell lines had ~3–7-fold higher ^{32}P:^{3}H ratio than quiescent cells. This increase in phosphorylation of fibronectin may in part, but could not entirely be due to increased uptake of [^{32}P]orthophosphate by growing cells as the ^{32}P:^{3}H ratio in the cell-associated and secreted proteins after 4 h of labeling was only 1.3–2.4-fold higher in growing, than in quiescent cells.

Fig. 2. Electroporetic mobilities of fibronectin from quiescent, and growing NIL 8 cells. [^{32}P]Orthophosphate labeled fibronectin isolated from the conditioned medium of cells labeled for 4 h was run on 7% SDS–polyacrylamide gel: [a] quiescent cells; [b] growing cells.

Table 1

Ratio of [^{32}P]orthophosphate to [^{3}H]leucine incorporation in fibronectin

Cells	Fibronectin		Cell-associated proteins[a]		Secreted proteins[b]	
	^{32}P:^{3}H ratio	Ratio of growing: quiescent cells	^{32}P:^{3}H ratio	Ratio of growing: quiescent cells	^{32}P:^{3}H ratio	Ratio of growing: quiescent cells
GM0077 growing	0.823	4.84	6.94	1.38	2.26	1.52
GM0077 quiescent	0.170		5.01		1.48	
NIH 3T3 growing	0.092	3.06	6.65	2.13	2.40	2.40
NIH 3T3 quiescent	0.030		3.12		1.00	
NIL 8 growing	0.061	6.7	7.49	2.01	1.83	2.23
NIL 8 quiescent	0.009		3.72		0.82	

[a] Total trichloroacetic acid – insoluble counts of cell lysate
[b] Total trichloroacetic acid – insoluble counts of the conditioned medium

Cell-associated and secreted proteins: 2×10^5 and 2×10^4 NIL 8 cells, and 3×10^5 and 3×10^4 NIH 3T3 cells were grown in DMEM + FCS for 4 days in 60 mm Petri dishes. For sparse GM0077 cells, 24 h before the labeling a confluent and quiescent monolayer in 60 mm dish was split at a 1:2 ratio. Labeling with [^{32}P]-orthophosphate and [^{3}H]leucine was for 4 h (see section 2 for details of labeling conditions and isolation of fibronectin)

Phosphorylation of polypeptides has been shown to alter their electrophoretic and chromatographic mobilities [23–26]. Fibronectin from transformed hamster cells, that had an increased level of phosphorylation compared to the protein from normal cells, also showed a slower electrophoretic mobility in SDS gels [20]. The same is also true for fibronectin from growing cells. Fig. 2 shows a slower migration of fibronectin from growing NIL 8 cells than that from quiescent cells after a 4 h labeling period. In this particular experiment fibronectin from growing cells had an 8-fold increase in ^{32}P:^{3}H ratio compared to the fibronectin from quiescent cells. In other experiments using NIL 8 or other cell lines and different lengths of labeling periods where the increase in phosphorylation was only 3–4-fold or less, the difference in electrophoretic mobility was not very obvious.

It is clear from the results presented above that like the protein from transformed hamster cells, fibronectin from growing hamster cells is phosphorylated to a higher extent than fibronectin from quiescent cells. In several experiments after a 4-h labeling period the ^{32}P:^{3}H ratio of fibronectin in actively growing cells was 5–10-fold higher than the ratio in fibronectin from quiescent cells. An 8–12-fold increase in ^{32}P:^{3}H ratio of fibronectin was reported for transformed NIL 8 hamster cells [20]. Furthermore, preliminary experiments showed that the ^{32}P:^{3}H ratio of fibronectin from the transformed hamster cell line NIL 8-HSV, was not significantly affected by its state of growth. The significance of these observations remain to be elucidated.

In light of the importance of reversible phosphorylation reactions of proteins as an index of their functional and regulatory activities [27,28] several laboratories have studied cell cycle associated differences in activities of protein kinases. Differences have been found in the activities of

both cAMP-dependent and cAMP-independent nuclear and cytoplasmic protein kinases which vary with the state of phosphorylation of proteins during different growth stages [29–34]. Phosphorylation of fibronectin is a highly specific and conserved phenomenon [20] occurring only on serine residue(s) [20,35]. Information on the kinase(s) responsible for phosphorylation of fibronectin and on the involvement of the phosphorylated region, if any, in various functional aspects of fibronectin will contribute to a better understanding of how this important protein functions at the cell surface and in blood plasma.

ACKNOWLEDGEMENTS

I am grateful to Dr Michael Pierschbacher and Dr Erkki Ruoslahti for the monoclonal antibody to human plasma fibronectin, 3E3, used in this study. I thank Dr Robert Bassin for his support and encouragement during the course of this study, Harry Rieck and Kathleen Noer for technical assistance, and Pam Shanahan for typing the manuscript. This research was supported by the National Cancer Institute under contract N01-CP-01013 with Meloy Laboratories.

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