Acylated fibronectin: a new type of posttranslational modification of cellular fibronectin

Jutta Lange-Mutschler

Department of Transfusion Medicine, University of Ulm, Oberer Eselsberg 10, PO Box 15 64, D-7900 Ulm, FRG

Received 24 March 1986

Human fibroblasts were labelled with either [^{35}S]methionine or [^{3}H]palmitate and analyzed for the presence of ^{35}S- or ^{3}H-labelled fibronectin by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The majority of ^{35}S-labelled fibronectin was found in the extracellular matrix of cells which could be removed quantitatively by mild trypsin treatment. In contrast, ^{3}H-labelled fibronectin was found in a trypsin-resistant form. The ^{3}H label of fibronectin could be identified predominantly as [^{3}H]palmitate by HLPC analysis. The fatty acids remained stably associated with fibronectin during extraction and electrophoresis, indicating a covalent linkage. These results demonstrate that a subset of fibronectin different from extracellular matrix fibronectin is modified by acylation.

Fibronectin *Posttranslational modification* *Protein acylation* *Extracellular matrix*

1. INTRODUCTION

Fibronectin is a high-M_r glycoprotein present in closely related forms in the plasma of vertebrates, in the extracellular matrix, on cell surfaces and intracellular in a perinuclear distribution [1]. It has been implicated in a variety of biological functions, most of which involve cell-to-cell and cell-to-cell-matrix interactions, cell shape formation and cellular migration [2]. Some of these activities implicate interactions of cell surface associated fibronectin with intracellular cytoskeletal elements [2]. The molecular basis of a transmembrane link between fibronectin and the cytoskeleton is not understood. A direct insertion of fibronectin in plasma membranes seems to be unlikely since the cell attachment domain of fibronectin is rich in hydrophilic amino acids [3]. Alternatively, the interactions of fibronectin with the intracellular cytoskeleton might be mediated by membrane receptors for fibronectin. Although gangliosides [4], phospholipids [5], glycoproteins [6] and heparan sulfate proteoglycans [7] may play a role in the attachment of fibronectin with plasma membranes, a fibronectin receptor could not be identified as yet. During the past few years several proteins have been found to be posttranslationally modified by a covalent linkage to fatty acids [8]. The acylation of proteins is thought to anchor otherwise soluble proteins in membranes [9,10]. Therefore, I analyzed cellular fibronectin for the presence of covalent bound fatty acids.

Here, I describe that a subset of fibronectin is specifically modified by covalent attachment to palmitic acid and can be distinguished from extracellular matrix associated fibronectin on the basis of its resistance against trypsin treatment. These results may indicate that acylation of fibronectin mediates a tight attachment of fibronectin with plasma membranes.

2. MATERIALS AND METHODS

2.1. *Radiolabelling and extraction of cells*

2.5×10^6 Ge 38 cells (a human fibroblast cell line) grown in petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, were labelled for 4 h with either

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

100 μCi [^{35}S]methionine (NEN) in 10 ml methionine-free DMEM supplemented with 0.1% bovine serum albumin or with 5 mCi [^{3}H]palmitate (NEN) in 10 ml DMEM supplemented with 10% tryptose phosphate broth, 2.5% fetal calf serum and 5 mM sodium pyruvate. After washing 3 times with PBS the cells were incubated for 30 min at room temperature with extraction buffer [50 mM Tris (pH 7.0), 25 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EGTA, 1% Empigen BB (Albright and Wilson, Frankfurt), 1% NP40 and 10% Trasylol^{R} (Bayer, FRG)]. Cell extracts were sonified and clarified by centrifugation for 30 min at 106000 × g.

2.2. *Immunoprecipitation and gel electrophoresis*

Cell extracts were immunoprecipitated with antibodies against fibronectin [antibody 1, rabbit anti-human plasma fibronectin (Behring); antibody 2, rabbit anti-bovine plasma fibronectin (Calbiochem); antibody 3, PHM 13 (Serotoc), a monoclonal antibody specifically binding to cellular fibronectin] and analyzed by SDS-polyacrylamide gel electrophoresis as in [11]. Gels were stained with Coomassie brilliant blue R250 and the total amount of fibronectin determined by scanning the gel with a Quick Scan R&D densitometer (Helena Laboratories) using purified plasma fibronectin as standard. For the evaluation of ^{3}H or ^{35}S uptake in radiolabelled fibronectin, the fibronectin-containing areas of the gel were cut out of the gel, eluted and counted for radioactivity as in [11]. To determine the radioactive background, gel slices of equal size above and below the fibronectin-containing area were analyzed for radioactivity in parallel. The results are given as the ^{3}H cpm from the fibronectin area minus the ^{3}H cpm determined as background.

2.3. *HPLC analysis of lipids*

Lipids from [^{3}H]palmitate-labelled fibronectin purified by immunoprecipitation were extracted with 2 N HCl in 82% methanol for 5 h at 70°C and dried in vacuo, followed by treatment with 0.5 KOH in methanol for 1 h at 40°C. After extraction of lipid in acetone, a mixture of appropriate lipid standards was added, and the fatty acids derivatized with 4-Br-phenacyl bromide according to [12]. Samples were subjected to a 27 × 4 cm HPLC column on Shandon ODS hypersil (5 μm) using methanol as eluant. Fractions were collected every 10 s and the absorbance profile of the phenacyl esters identified spectrometrically. Radioactively labelled phenacyl esters of fatty acids were detected by scintillation counting.

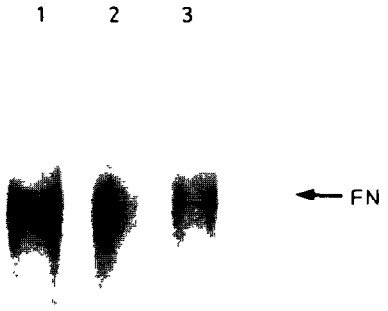
3. RESULTS AND DISCUSSION

Fibronectin in extracts of [^{3}H]palmitate-labelled human Ge 38 cells was immunoprecipitated with different polyclonal anti-fibronectin antibodies (antibodies 1 and 2) and a monoclonal anti-fibronectin antibody (antibody 3) and analyzed on 5% SDS-polyacrylamide gels as shown in fig.1. Fibronectin immunoprecipitated by all of the three antibodies used was ^{3}H-labelled.

Since it is known that up to 50% of the ^{3}H label in [^{3}H]palmitate-labelled cells were metabolically converted into amino acids which subsequently were incorporated into the polypeptide backbone of proteins [13], the ^{3}H label in fibronectin was analyzed by HPLC as described in section 2.3. As shown in fig.2, approximately one half of the ^{3}H label in fibronectin could be recovered as the phenazyl esters of [^{3}H]palmitic acid, whereas the other half of the label purified in the front peak of HPLC. Short fatty acids should evaporate under our conditions (drying of samples in vacuo), whereas hydrophobic amino acids are soluble in the organic phase. Therefore, we suppose that the front peak in our HPLC experiments comprises radioactively labelled hydrophobic amino acids rather than short fatty acids. These results clearly demonstrate that fibronectin is in fact bound to palmitic acid. Several criteria indicate a covalent linkage between fibronectin and fatty acids:

(i) Fatty acids remained associated with fibronectin during extraction with ionic and nonionic detergents (Empigen BB, NP40, see section 2.1).
(ii) The fatty acids could not be removed from fibronectin by boiling in 3% SDS for 5 min.
(iii) The fatty acids were still bound to fibronectin after SDS-polyacrylamide gel electrophoresis.
(iv) Fatty acids could only be quantitatively cleaved from fibronectin by acid hydrolysis (see section 2.3).

Most of the acylated proteins, especially those bound to palmitic acid, are glycosylated intrinsic



FN precipitated from ^{3}H-palmitic acid labelled cells	Antibody number 1	2	3
^{3}H-c.p.m.	1052	677	472
μg FN	29	25	13

Fig.1. Detection of [^{3}H]palmitate-labelled fibronectin in human fibroblasts. [^{3}H]Palmitate-labelled Ge 38 fibroblasts were lysed and immunoprecipitated with antibodies to human (antibody 1) or bovine plasma fibronectin (antibody 2) or a monoclonal antibody (antibody 3). Immunoprecipitates were run on 5% SDS-polyacrylamide slab gels and stained with Coomassie blue (left). As a standard, purified human fibronectin (5, 10 and 20 μg) was run on the same gel. Fibronectin was quantitated densitometrically, eluted and counted for incorporated ^{3}H label (right). The results represent the specific label of fibronectin (see section 2.2).

membrane proteins [8,13]. In contrast, cellular fibronectin is localized predominantly in the extracellular matrix, at the plasma membrane and in small amounts in the cytoplasm as the newly synthesized precursor of cellular fibronectin [2,14]. Extracellular matrix associated fibronectin can be quantitatively removed by mild trypsin treatment, whereas plasma membrane fibronectin and the intracellular precursor of cellular fibronectin are protected [14]. In accordance with the data of Yamada [14] I could quantitatively remove extracellular matrix associated fibronectin by treating living monolayer cells with 5000 units trypsin/1 $\times$ 10^{6} Ge 38 cells for 10 min at room temperature, as judged by immunofluorescence microscopy (not shown). For the determination of the localization of acylated fibronectin Ge 38 cells were labelled either with [^{35}S]methionine or [^{3}H]palmitate in parallel. Half of the cells were treated in situ with trypsin as described above in order to remove extracellular fibronectin. Fibronectin from trypsin-treated and untreated cells was purified by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The label incorporated in fibronectin was quantitatively determined as described for fig.1. The majority (up to 70%) of total cellular fibronectin as well as [^{35}S]methionine-labelled fibronectin could be digested by trypsin treatment, whereas [^{3}H]palmitate-labelled fibronectin was resistant to trypsin treatment under our labelling conditions (fig.3). Together with the immunofluorescence data one can conclude that approx. 70% of the total cellular fibronectin as well as fibronectin syn-

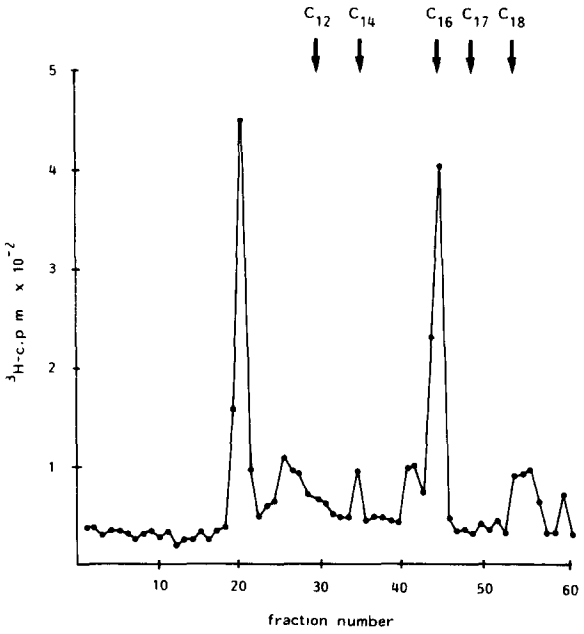


Fig.2. HPLC analysis of [^{3}H]palmitate-labelled fibronectin. Ge 38 cells were labelled with [^{3}H]palmitate, extracted and immunoprecipitated using antibody 1. Fatty acids bound to immunoprecipitates of fibronectin were released by acid hydrolysis and subjected to HPLC analysis as described in section 2.3. The positions of the non-labelled internal lipid standards are indicated.

thesized during the 4 h labelling period is localized in the extracellular matrix. In contrast, acylated fibronectin could not be digested by trypsin treatment, indicating that this subset of cellular fibronectin is predominantly attached with the plasma membrane as described for nearly all palmitoylated proteins [13].

Although the role of posttranslational modifications of proteins with long-chain fatty acids remains to be determined, studies of transforming

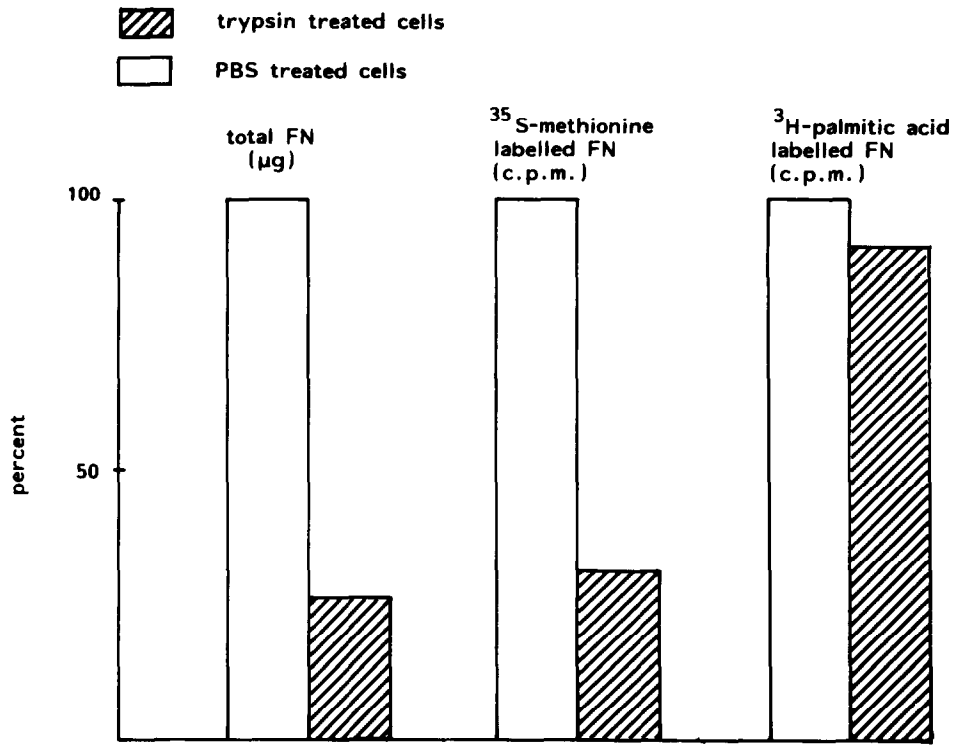


Fig.3. Comparison of the trypsin sensitivity of total fibronectin, [^{35}S]methionine-labelled fibronectin and [^{3}H]palmitate-labelled fibronectin. Ge 38 cells were labelled with either [^{3}H]palmitate or [^{35}S]methionine and treated with trypsin (5000 units/1 × 10^6 cells for 10 min at room temperature). Cells were extracted and immunoprecipitated with antibody 2. Fibronectin from trypsin-treated cells or cells treated with PBS as control was purified on SDS-polyacrylamide slab gels. The determination of total fibronectin (μg) or radioactively labelled fibronectin ^{3}H cpm or ^{35}S cpm was carried out as described in fig.1.

proteins of viruses [9,10,15] as well as of cellular proteins which are involved in the regulation of cell growth [16,17] indicate that the binding of proteins to palmitic acid may stabilize the attachment of proteins with the plasma membrane. Since fibronectin performs most of its functions by interactions with cell surfaces, the study of acylated fibronectin and its possible interactions with plasma membranes and/or components of the cytoskeleton might help in understanding the multiple functions of this major cellular protein on the regulation of cell shape, cell growth and cell mobility at the molecular level.

ACKNOWLEDGEMENTS

I gratefully acknowledge the help of Ms Claudia Herre in performing the experiments. I also thank Dr Ingo Melzner who performed the HPLC analysis of fatty acids, and Ms Angela Erkert for help in the preparation of the manuscript. This investigation was supported by grant La 551/1-1 from the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Pearlstein, E., Gold, L.I. and Garcia Pardo, A. (1980) Mol. Cell. Biochem. 29, 103–127.
[2] Ruoslathi, E., Engvall, E. and Hayman, E.G. (1980) Coll. Res. 1, 95–128.
[3] Pierschbacher, M.D. and Ruoslathi, E. (1984) Nature 309, 30–33.
[4] Kleinman, H.K., Martin, G.R. and Fishman, P.H. (1979) Proc. Natl. Acad. Sci. USA 76, 3367–3371.
[5] Perkins, R.M., Kellie, S., Patel, B. and Critchley, B.R. (1982) Exp. Cell Res. 141, 231–243.
[6] Tarone, G., Galetto, G., Prat, M. and Camoglio, P.M. (1982) J. Cell Biol. 94, 179–186.
[7] Laterra, J., Silbert, J.E. and Culp, L.A. (1983) J. Cell Biol. 96, 112–123.
[8] Magee, A.I. and Schlesinger, M.J. (1982) Biochim. Biophys. Acta 694, 279–289.
[9] Sefton, B.M., Trowbridge, I.S. and Cooper, J.A. (1982) Cell 31, 465–474.
[10] Lange-Mutschler, J. and Henning, R. (1984) Virology 136, 404–413.
[11] Lange-Mutschler, J. and Henning, R. (1982) Virology 117, 173–185.
[12] Borch, R.F. (1975) Anal. Chem. 47, 2437–2439.
[13] Olson, E.N., Touler, D.A. and Glaser, L. (1985) J. Biol. Chem. 260, 3784–3790.
[14] Yamada, K.M. (1978) J. Cell Biol. 78, 520–541.
[15] Williamson, B., Christensen, A., Hubbert, N.L., Papageorge, A.G. and Lowry, D.R. (1984) Nature 310, 583–586.
[16] Omary, M.B. and Trowbridge, I.S. (1981) J. Biol. Chem. 256, 12888–12892.
[17] Voronova, A.F., Buss, J.E., Patschinsky, T., Hunter, T. and Sefton, B.M. (1984) Mol. Cell. Biol. 4, 2705–2713.