Cell Adhesion to Fibronectin Regulates Membrane Lipid Biosynthesis through 5'-AMP-activated Protein Kinase*

(Received for publication, May 29, 1997)

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We have shown that attachment to a fibronectin substrate stimulates two pathways of lipid biosynthesis in cultured human fibroblasts. Detachment of these cells (mechanically, with trypsin, or by RGDS peptides) caused a significant decrease in their 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and in their incorporation of [3H]acetate into fatty acids. This inhibition was substantially reversed by the reattachment of cells to fibronectin substrates, but not to poly-L-lysine substrates or to fibronectin in solution. Inhibiting phosphoprotein phosphatase activity with okadaic acid blocked the recovery of both biosynthetic activities.

Both 3-hydroxy-3-methylglutaryl-coenzyme A reductase and fatty acid biosynthesis are known to be inhibited by the action of 5'-AMP-activated protein kinase, which is activated by an increase in the level of AMP relative to ATP. For example, in our system, sodium azide and 2-deoxy-o-glucose increased the ratio of cellular AMP to ATP and caused a decrease in lipid biosynthesis. We then verified the prediction that detachment of cells from substrates also caused an increase in the AMP/ATP ratio. We therefore conclude that the attachment of cells to fibronectin promotes lipid biosynthesis, presumably in coordination with the cellular growth response evoked by attachment to the extracellular matrix.

The interaction of cells with the extracellular matrix leads to complex adaptive responses in the cytoplasm (1–3). In particular, the binding of fibronectin to plasma membrane integrins results in their clustering, promoting the colocalization of cytoskeletal proteins and the formation of focal adhesions. This leads to fibronectin-stimulated phosphorylation of tyrosine residues by focal adhesion kinase (1–3) as well as the phosphorylation of serine and threonine residues by protein kinase C (4, 5) and mitogen-activated protein kinases (6). Focal adhesion kinase also appears to stimulate cell growth through its interaction with pp60
src (4, 7, 8), induction of c-
murine sarcoma viral oncogene products
mRNAs (9), and activation of phosphatidylinositol 3-kinase (10). Cell adhesion to the extracellular matrix stimulates cell proliferation through a kinase cascade (2–4).

Membrane lipid biosynthesis is known to be coordinated with cell growth (11, 12) through the phosphorylation of key biosynthetic enzymes (13). The regulatory pathways are not fully understood. The impact of cell adhesion on the biosynthesis of the major membrane bilayer lipids has not been evaluated heretofore. Here we test the hypothesis that membrane lipid biosynthesis responds to the attachment of cells to the extracellular matrix.

EXPERIMENTAL PROCEDURES

Materials—[3H]Acetic acid (sodium salt, 100 mCi/mmol), [14C]acetate acid (sodium salt, 59 mCi/mmol), and 3-3H]mevalonolactone (60 Ci/mmol) were from National Institutes of Health. [3-14C]glutathione A (57.7 mCi/mmol) were from NEN Life Science Products. [5-3H]NADPH (60 Ci/mmol) was from American Radiolabeled Chemicals. [4-14C]cholesterol (53 mCi/mmol) and [N-methyl-3H]sphingomyelin (58 mCi/mmol) were from Amersham Corp. Tributylamine was from Fisher. Bovine plasma fibronectin and all other chemicals were from Sigma. Lipoprotein-deficient serum was prepared from fetal bovine serum as described (14). [14C]triglyceride and [14C]cholesterol esters, used as recovery standards, were purified by TLC from hepatoma cells that had been incubated for 4 h with [14C]acetate.

Cell Culture and Replating—Human foreskin fibroblasts were derived from primary explants and used between passages 4 and 15 (14). Some experiments were repeated with the F15AH rat hepatoma cell line (15). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. In studies using fibroblasts, cultures were preincubated overnight in medium containing 5% lipoprotein-deficient serum. Disconnection was by incubation with 0.5 mg/ml trypsin plus 0.5 mM EDTA for 2 min at 37 °C. Then 2.5 mg/ml egg white trypsin inhibitor was added, and the cells were chilled on ice for 5 min and washed with cold phosphate-buffered saline. The cells were suspended in buffer A (135 mM NaCl, 20 mM NaHEPES (pH 7.4), 3 mM KCl, 2 mM KH2PO4, 0.8 mM MgSO4, 1 mM CaCl2, and 1 mg/ml glucose). The suspended cells were either assayed immediately or, following a 10-min incubation at 37 °C, replated in buffer A in Petri dishes. The dishes had been precoated with 50 μg/ml poly-L-lysine or 5 μg/ml fibronectin overnight and then blocked with 1 mg/ml bovine serum albumin for 30 min and rinsed with phosphate-buffered saline prior to use. Reattachment was assessed by cholestrol mass measurement after 30 min. We found that 80–90% of the cells attached to poly-L-lysine and 70–80% of the cells attached to fibronectin.

HMG-CoA Reductase Activity—Cells were extracted in 50 mM KH2PO4, 5 mM dithiothreitol, and 5 mM EDTA containing 1% KYO EOB (Procter and Gamble Co.) and 50 mM NaF (16). To extract attached cells, this buffer was added directly to the flask, and the cell residue was scraped into tubes on ice. To extract suspended cells, trypsin inhibitor was added, cells were pelleted and then washed with phosphate-buffered saline, and the final pellet was resuspended in the extraction buffer. The assay used [3H]hydroxy-3-methylglutaryl coenzyme A as a substrate, with [3H]mevalonolactone as a recovery standard (16). Sterol and Fatty Acid Biosynthesis—Cells on plates or in suspension were incubated at 37 °C with 20 μCi/ml [3H]acetate for 45 min (for fatty acids) or 60 min (for sterols). Plates were released with trypsin; cells attached to poly-L-lysine or fibronectin were gently scraped from the dish. The cells were washed, resuspended, and extracted with 5 volumes of chloroform/methanol (2:1, v/v). [14C]Triglycerides, [14C]cholesterol esters, and/or [14C]sphingomyelin were used as recovery standards.

1 The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HPLC, high performance liquid chromatography.

2 This work was supported by National Institutes of Health Grant HL 28448. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I

| Cells                        | HMG-CoA reductase activity | Relative activity | Mean ± S.D. | (n = 3) |
|------------------------------|----------------------------|-------------------|-------------|---------|
| Exp. 1                       |                            |                   |             |         |
| Plated                       | 5771                       | 1.0               | 1.0         |         |
| Suspended                    | 2924                       | 0.5               | 0.58 ± 0.09 |         |
| Replaced on poly-L-lysine    | 2428                       | 0.4               | 0.42 ± 0.03 |         |
| Replaced on fibronectin      | 5378                       | 0.9               | 0.98 ± 0.15 |         |
| Exp. 2                       |                            |                   |             |         |
| Replaced on poly-L-lysine    | 239                        | 0.2               | 0.43 ± 0.22 |         |
| Replaced on fibronectin      | 1020                       | 1.0               | 1.0         |         |
| Released by RGDS peptide    | 543                        | 0.5               | 0.48 ± 0.03 |         |

In Experiment 1, cells were dissociated with trypsin and either incubated in suspension in buffer A or replated onto poly-L-lysine- or fibronectin-coated dishes. The medium of parallel flasks of cells was replaced with buffer A. The cells were incubated for 1 h at 37 °C, and then HMG-CoA reductase activity was assayed as described under “Experimental Procedures.” In Experiment 2, cells in buffer A were allowed to attach to fibronectin- or poly-L-lysine-coated plastic for 30 min. Fresh buffer with or without 0.75 mg/ml RGDS peptide was added, and the dishes were incubated for 45 min at 37 °C. The dissociated cells were removed and pelleted by centrifugation. HMG-CoA reductase activity was measured as described under “Experimental Procedures.” More than 85% of the fibronectin-attached cells were released by the RGDS peptide; the release of cells attached to poly-L-lysine was negligible. The second and third columns show the results of a representative experiment; the fourth column gives the mean results of three experiments.

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Effect of cell attachment on HMG-CoA reductase activity—

Dissociation of confluent monolayers of fibroblasts led to a 42% decrease in the activity of HMG-CoA reductase, a rate-limiting enzyme in sterol biosynthesis (Table I, Experiment 1). Similar results were obtained by releasing cells from the flask with trypsin plus EDTA or by mechanical scraping. The decrease in enzyme activity occurred immediately upon detachment and persisted for at least 2 h of incubation. Comparable results were obtained with rat hepatoma cells.

Dissociated cells were replated on tissue culture plastic coated with either poly-L-lysine (for nonspecific attachment) or fibronectin (for specific attachment) (20). Attachment to poly-L-lysine had little effect on enzyme activity. On the other hand, reattachment to fibronectin led to full recovery of HMG-CoA reductase activity within 1 h (Table I, Experiment 1). Plating cells on untreated tissue culture plastic in the presence of 10% serum or 5% lipoprotein-deficient serum led to recovery of HMG-CoA reductase activity similar to that observed when cells were replated on fibronectin (data not shown); this effect was most likely mediated by the fibronectin or other matrix components present in the serum.

We also measured cholesterol biosynthesis in intact cells from the incorporation of $^3$H]acetate into sterols (Fig. 1). Biosynthetic activity decreased in suspended cells compared with growing cells and recovered upon reattachment to fibronectin, but not to poly-L-lysine. The HMG-CoA reductase activity in detached cells was about half of the control (Table I), whereas incorporation of $^3$H]acetate into sterols dropped to about one-third (Fig. 1). This difference might reflect the fact that the activity of squalene epoxidase also appeared to be inhibited upon cell suspension.

Effect of Cell Detachment on Fatty Acid Biosynthesis—The incorporation of $^3$H]acetate into fatty acids in dissociated cells was ~15% of that in attached cells. Reattachment of the cells to fibronectin led to substantial recovery, whereas reattachment to poly-L-lysine had no effect (Fig. 1).

Dissociation by RGDS Peptide—To test whether fibroblast attachment to fibronectin was mediated by integrins, we used the RGDS peptide as a soluble competitor for integrin binding sites (20). Incubation with 0.75 mg/ml RGDS peptide for 45 min led to the release of >85% of the cells plated on fibronectin. There was no detectable release following addition of the RGDS peptide to cells plated on poly-L-lysine. Detaching cells from fibronectin by the RGDS peptide reduced their HMG-CoA reductase activity to half that of attached cells (Table I, Experiment 2). Addition of serum fibronectin (10 μg/ml) to cells in

Thin-layer Chromatography—To resolve cholesterol esters and triglycerides, we used solvent A: petroleum ether/ethyl acetate/acetic acid (90:10:1, v/v/v). To isolate free fatty acids, we used petroleum ether/ethyl acetate/acetic acid (80:20:1). To isolate sterols, we used isopropyl ether/ethanol containing 50 mM acetic acid plus 0.01 mM EDTA. For acids.

Desorption of cholesterol mass and the radioactivity incorporated into total lipids was most likely mediated by the fibronectin or other matrix protein in the aqueous phase was separated and re-extracted using a dummy aqueous phase.

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2 K. Page and Y. Lange, unpublished observations.
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**Effect of cell attachment on sterol and fatty acid biosynthesis.** Fibroblasts were dissociated with trypsin and either incubated in suspension in buffer A (Sus) or replated onto poly-L-lysine-coated (Ply) or fibronectin-coated (Fn) dishes. 

**FIG. 1.**

![Graph showing the effect of cell attachment on sterol and fatty acid biosynthesis.](image)

**Effect of Detachment of Cells on AMP and ATP Levels—**Both acetyl-coenzyme A carboxylase and HMG-CoA reductase are inhibited by phosphorylation mediated by 5'-AMP-activated protein kinase (reviewed in Refs. 13, 22, and 23). This kinase is activated by a high ratio of 5'-AMP to ATP (24, 25). We therefore measured AMP, ADP, and ATP levels in plated and suspended cells.

The ratio of AMP to ATP increased 2.5-fold upon cell suspension both in fibroblasts (Fig. 2A) and in hepatoma cells (data not shown). This moderate increase in the AMP/ATP ratio is associated with a ~7-fold decrease in the incorporation of \(^{3}\text{H}\)acetate into fatty acids (Fig. 1). A nonlinear relationship between these variables was also reported in studies of freshly isolated hepatocytes (24, 26). Several factors could account for this acute response. (a) Some ATP and/or ADP could be broken down to AMP during cell extraction so that the AMP/ATP ratio was actually lower in vitro. On the other hand, no such hydrolysis was found when exogenous nucleotide standards were included in the extraction. (b) The pathway leading from the nucleotide ratio to the activity of the biosynthetic enzymes is complex (26). For example, a small activation of the kinase could lead to a large amount of phosphorylation, hence inhibition, of the biosynthetic enzymes. (c) Furthermore, 5'-AMP activates the kinase both directly (27) and through stimulation of an upstream kinase (26). This compound action could lead to a variable nonlinear response to the AMP/ATP ratio.

We further tested the validity of the hypothesis that the observed changes in the AMP/ATP ratio could regulate the lipid biosynthetic enzymes by manipulating this parameter in other ways. As expected (28), we found that the addition of the energy poison sodium azide to plated cells evoked a 1.8-fold increase in the AMP/ATP (Fig. 2B) and a 2-fold decrease in the incorporation of \(^{3}\text{H}\)acetate into lipids (Fig. 2C). Similarly, incubation with 50 mM 2-deoxy-D-glucose increased the AMP/ATP ratio in the cells 2.6-fold (see also Ref. 29) and decreased lipid biosynthesis ~3-fold (data not shown). The fact that these in vitro manipulations of the AMP/ATP ratio elicited a smaller response in lipid biosynthesis than did detachment raises the possibility that other, parallel pathways could be involved in the physiologic response.

**Effect of Okadaic Acid on HMG-CoA Reductase Activity—**The inactivation of HMG-CoA reductase by phosphorylation is reversed by a specific phosphatase (30), which can be inhibited by okadaic acid (26). We therefore plated cells onto poly-L-lysine or

**FIG. 2.**

![Graph showing AMP and ATP levels in plated, suspended, and sodium azide-treated fibroblasts.](image)
Fibroblasts were dissociated, washed, and resuspended in buffer A containing 100 nM okadaic acid or dimethyl sulfoxide as solvent control (<1%). Aliquots of the cells were replated on plastic coated with fibronectin or poly-L-lysine. After a 30-min incubation at 37 °C, 4 μCi/ml [3H]acetate was added, and the cells were incubated for a further 60 min at 37 °C. The incorporation of [3H] into total lipids was assayed as described under “Experimental Procedures.” Assays were performed in duplicate and agreed to within 20%. The third and fourth columns give the results of a typical experiment; the fifth column gives the mean results of three experiments.

| Substrate       | Addition            | ³H incorporation | Relative | Mean ± S.D. (n = 3) |
|-----------------|---------------------|------------------|----------|---------------------|
| Poly-L-lysine   | None                | 259 dpm/μg cholesterol | 1.0       | 1.0                 |
|                 | Okadaic acid        | 239 dpm/μg cholesterol | 0.9       | 1.0 ± 0.07          |
| Fibronectin     | None                | 470 dpm/μg cholesterol | 1.8       | 1.8 ± 0.32          |
|                 | Okadaic acid        | 225 dpm/μg cholesterol | 0.9       | 0.9 ± 0.02          |

Acknowledgment—We thank T. L. Steck for critical reading of the manuscript.

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Fibronectin Binding Regulates Protein Kinase

The dissociation of cells from a fibronectin substrate leads to a reversible increase in the ratio of AMP to ATP in the cytoplasm (22). That the dissociation of cells from a fibronectin substrate leads to a small shift in “energy-charge ratio” (22) could then activate cytoplasmic 5’-AMP-dependent kinase (13), leading to the phosphorylation and consequent inhibition of acetyl-coenzyme A carboxylase and HMG-CoA reductase (13, 22, 23, 31). The predicted outcome is the inhibition of the biosynthesis of fatty acids, cholesterol, and other isoprenoid derivatives (24, 25). This action of the 5’-AMP kinase could serve multiple homeostatic purposes, as follows.

(a) Activation of 5’-AMP-dependent kinase by a decreased energy charge would conserve energy reserves under conditions of cellular stress (24), simulated here by detachment from the substrate. Similar responses may be why insulin (32) and heat shock (24) also raise the AMP/ATP ratio and activate the 5’-AMP-dependent kinase.

(b) Cell growth is coordinated with membrane synthesis (33). It is therefore appropriate that detachment from the extracellular matrix sends a signal to decrease membrane lipid biosynthesis (12, 34). (c) A reduction in the synthesis of mevalonic acid by HMG-CoA reductase could lead to the inhibition of dolichol synthesis, affecting the provision of dolichol sugars for the biosynthesis of membrane and matrix glycoproteins and glycolipids (12, 34). (d) Decreased isoprenoid biosynthesis could similarly lower the prenylation of G proteins, compromising their ability to up-regulate cell growth and drive the cell cycle (34).

Membrane phosphoinositide metabolism is also sensitive to the binding of cells to the extracellular matrix (35). However, this response presumably reflects the role of these lipids in intracellular signaling, rather than in membrane biogenesis for cell growth.

Finally, the mechanistic link between fibronectin binding and the regulation of the AMP/ATP ratio remains to be determined. Fibronectins not only interact with integrins through RGD sites (21), but they also bind to heparan moieties on syndecans, signal-transducing integral plasma membrane proteoglycans (36). However, since the effects on lipid biosynthesis were reversed by RGDS peptides, it is parsimonious to postulate the involvement of integrins rather than syndecans. Conceivably, fibronectin binding affects the activity of adenylate kinase, the enzyme that catalyzes the equilibration of AMP, ADP, and ATP. Modulation of adenylate kinase activity may serve as an intracellular signaling mechanism (37), and its role in the coordination between cell-surface adhesion and lipid biosynthetic activity is worth exploring.