Modulation of fructose-2,6-bisphosphate metabolism by components of the extracellular matrix in cultured cells. Interaction with epidermal growth factor

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Abstract The use of NIH3T3 fibroblasts overexpressing different mutations of the EGF receptor shows that regulation of fructose-2,6-bisphosphate (Fru-2,6-P_2) metabolism by EGF is mediated by the kinase activity of the EGF receptor and suggests a PLCγ1-mediated mechanism. The effect of several extracellular matrix components on glucose metabolism was assessed by incubating A431 cells and NIH3T3 fibroblasts with heparin, laminin, fibronectin, collagen and PG-I and PG-II proteoglycans and measuring the levels of Fru-2,6-P_2. Laminin increased the levels of Fru-2,6-P_2 and heparin decreased the levels of the metabolite, whereas the other molecules did not have any effect. No effect of laminin or heparin in glucose uptake by the cell was observed. Laminin was able to modulate the effects of EGF on Fru-2,6-P_2 concentration, suggesting cross-talk between these agents.

Key words: Fructose-2,6-bisphosphate; Epidermal growth factor; Laminin; Heparin

1. Introduction

Many of the agents which regulate glucose metabolism act by altering the levels of fructose-2,6-bisphosphate (Fru-2,6-P_2), the main activator of the key enzyme in the glycolytic pathway, PFK-1. We have previously described that epidermal growth factor (EGF) can stimulate glucose metabolism in A431 cells [1]. Fru-2,6-P_2 levels are also increased in cells treated with other growth promoters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or transfected with several oncogenes [2,3], thus indicating that a mechanism exists that couples cell growth and ability to obtain energy from glucose.

Recently, several laboratories have focused their attention on the proliferative and morphological effects of the extracellular matrix (ECM) and its interactions with growth factor-triggered mechanisms, but its role on the control of energy metabolism has not been investigated. ECM components can affect cellular processes by its ability to bind growth factors [4] or by directly activating intracellular signaling pathways that modulate cell proliferation, differentiation or migration [5,6]. There is increasing evidence that ECM components modulate cellular activity through receptor-mediated mechanisms such as changes in protein phosphorylation [7–9], gene expression [10] and the generation of intracellular second messengers [11–13].

Among the ECM components and related molecules, laminin and heparin have been described as potent modulators of cell growth. Laminin is a large glycoprotein composed of many distinct domains [14] and is able to interact with several types of receptors on the cell surface [15]. Laminin has been described as a growth factor for many cell types and the regions involved in the mitogenic action have been localized in the EGF-like motifs from the short arms [16–18]. Heparin inhibits the proliferation of several cell types [19,20] and is able to modulate the expression of specific genes [21,22]. Recently, heparin has been shown to activate the FGF receptor 4 independently of the presence of the growth factor [23]. At the moment, though, the possibility that ECM molecules could exert an effect on the metabolic efficiency of the cell has not been investigated.

The aim of this work was twofold: first, to investigate the role of the EGF receptor on the control of Fru-2,6-P_2 levels by this factor and second, to determine whether glucose metabolism can be regulated by several ECM components.

2. Materials and methods

2.1. Materials

EGF from mouse submaxillary glands, laminin, collagen and fibronectin were from Boehringer Mannheim. Heparin (low molecular weight) was from Sigma or from Opocrin S.p.A. Biofarmaci (kindly given by Dr. S. Vannucchi, Istituto di Patologia Generale, University of Florence, Italy). PG-I and PG-II from bovine bone were kindly given by Dr. L.W. Fisher (NIH, Bethesda, MD, USA). Chondroitin sulfate was from Sigma. Reagents for cell culture were from Gibco. All other reagents were from Boehringer Mannheim or Sigma.

2.2. Cell culture

All cell lines were grown at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle medium containing 10% fetal calf serum with 100 IU penicillin/ml and 100 μg/ml streptomycin. A431 human epidermoid carcinoma cells were obtained from ATCC (CRL 1555). This cell line has been described to contain 2.6×10^6 EGF receptors/cell [24]. The NIH3T3 fibroblasts transfected with several mutations of the EGF receptor, pCO11 [25], F3 [26], Kin^- [27] and Dc214 [27] were kindly given by Dr. L. Beguinot (Laboratorio di Oncologia Molecolare, H.S. Raffaele, Istituto de Recovero a Cattere Scientifico, Milan, Italy).

2.3. Treatment of the cells with ECM components, EGF and other effectors

Cells were plated in triplicate and the experiments were performed 48 h after confluence. Under these conditions, cells are quiescent and the glycolytic flux is low. Twenty hours before the end of the incubation, the medium was removed and a low glucose, glutamine-free medium containing 6 mM glucose, 126 mM NaCl, 14 mM $NaHCO_3$, 38 mM KCl, 0.9 mM Na_2HPO_4, 0.6 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.3 mM $CaCl_2$, 20 mM HEPES pH 7.2 (incubation medium) was

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Abbreviations: ECM, extracellular matrix; Fru-2,6-P_2, fructose-2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; EGF, epidermal growth factor; PLC, phospholipase C; PK-C, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA-responsive element

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added to the plates with different concentrations of the indicated ECM components. After the incubation, the medium was rapidly removed and the monolayers immediately frozen in liquid nitrogen and stored at −80°C. The triplicate plates were treated, processed and analyzed separately.

In the case of treatment with EGF, the incubation medium was renewed 90 min before adding the factor to eliminate possible secreted molecules and restore the initial glucose concentration. 100 ng/ml EGF was added to the cells for the indicated times. Controls received 100 μM acetic acid, as vehicle.

Down-regulation of protein kinase C (PK-C) was achieved by culturing the monolayers in the presence of 500 nM TPA during the 20 h of preincubation in incubation medium [1].

2.4. Analytical procedures

Fru-2,6-P_2 was measured in alkaline extracts prepared by adding 1 ml 50 mM NaOH to 35 mm plates and heating the samples at 80°C for 10 min. After centrifugation at 10000×*g* for 10 min, Fru-2,6-P_2 was measured in duplicate in the supernatant by the method described by Van Schaftingen et al. [28].

The uptake of 2-deoxyglucose was determined as described previously using 2-deoxy-D-[U-^{14}C]glucose [29].

Protein concentration was determined using bovine serum albumin as standard.

3. Results

3.1. The effect of EGF on Fru-2,6-P_2 levels depends on the kinase activity of the EGF receptor

EGF increased the levels of Fru-2,6-P_2 in pCO11 fibroblasts transfected with the wild-type EGF receptor in a similar manner as previously described in our laboratory for A431 cells (Fig. 1, [1]). The time course showed a maximal increase in Fru-2,6-P_2 concentration of up to twofold and this value did not decrease significantly for at least 4 h (data not shown). The EC_{50} determined in pCO11 fibroblasts was 0.3 ng/ml vs.

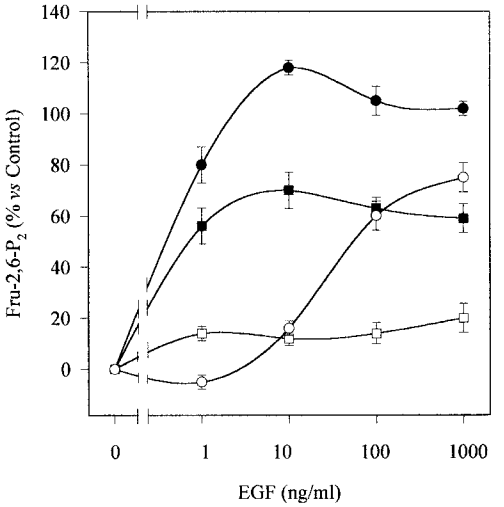

Fig. 1. Effect of EGF on Fru-2,6-P_2 levels in NIH3T3 fibroblasts expressing different mutations of the EGF-receptor. pCO11 (●), F3 (■), Dc214 (○) and Kin$^-$ (□) cells were grown on 24 well plates and after 2 days of confluence, they were maintained for 20 h in incubation medium. Two hours before the end of the incubation, different concentrations of EGF or 0.1 mM acetic acid were added to the cells. Fru-2,6-P_2 was determined in alkaline extracts as described in Section 2. The percentage increase from the basal level of Fru-2,6-P_2 is represented (basal values: pCO11: 25.4; F3: 16.9; Dc214: 76.9; Kin$^-$: 3.9 pmol/mg protein). The number of receptors per cell is: pCO11: 150000; F3: 250000; Dc214: 70000–150000 and Kin$^-$: 50000–70000. The average of five different experiments is represented.

Table 1
Fru-2,6-P_2 levels in A431 cells and EGF receptor-transfected NIH3T3 fibroblasts pretreated with laminin and incubated with or without EGF

Cell line	EGF	Fru-2,6-P_2 (pmol/mg)	
		Control	Laminin (%)
A431	−	45.6±2.2	61.9±3.7 (136)
	+	73.7±3.9	76.4±2.6
pCO11	−	25.4±0.9	39.6±1.3 (156)
	+	43.4±2.5	42.1±2.2
F3	−	16.4±0.9	24.4±0.8 (149)
	+	25.8±1.0	27.1±1.6
Dc214	−	76.5±5.2	115.1±6.4 (150)
	+	130.2±7.5	132.3±7.8
Kin$^-$	−	3.9±0.1	6.4±0.2 (164)
	+	4.2±0.2	7.2±0.2

Cells were grown on 24 well plates and after 2 days of confluence, they were maintained for 20 h in incubation medium and with or without 100 μg/ml laminin. Two hours before the end of the incubation, 100 ng/ml EGF or 0.1 mM acetic acid were added to the cells. Fru-2,6-P_2 was determined in alkaline extracts as described in Section 2 (n=5). The percentage increase caused by laminin vs. control cells is indicated in parentheses.

30 ng/ml in A431 cells, indicating that fibroblasts were more sensitive to EGF, probably because A431 cells have a very low proportion of high affinity receptors [30].

Several fibroblast cell lines expressing different mutations of the EGF receptor were analyzed (Fig. 1 and Table 1). Kin$^-$ fibroblasts, which express a receptor lacking the tyrosine kinase activity, did not respond to EGF. The Dc214 mutants have a deletion of 214 carboxy-terminal amino acids, including the five putative autophosphorylated tyrosines [27]. In these cells, the sensitivity to EGF is lower than in pCO11 cells in terms of EC_{50} (30 ng/ml vs. 0.3 ng/ml) and maximal response achieved (70% vs. 120%). The F3 receptor mutants have the main autophosphorylated tyrosines (Y1173, Y1148 and Y1068) substituted with phenylalanine [26]. In these cells, the EC_{50} coincided with that from pCO11 cells, but the maximal response was lower. The numbers of receptors per cell expressed in each cell line and the Fru-2,6-P_2 basal levels are indicated in the legend of Fig. 1.

3.2. Fru-2,6-P_2 levels can also be regulated by several ECM components

To analyze whether the glycolytic response can be modified by the ECM, A431 cells were preincubated for 20 h in the presence of several ECM components and the level of Fru-2,6-P_2 was determined. As can be observed in Fig. 2, laminin increased and heparin decreased the concentration of Fru-2,6-P_2. Other ECM components such as the proteoglycans PG-I and PG-II, collagen and fibronectin or chondroitin sul-

Table 2
2-Deoxyglucose uptake in A431 cells and pCO11 fibroblasts pretreated with laminin or heparin

	2-Deoxyglucose uptake (nmol/min×mg)		
	Control	Laminin	Heparin
A431	0.26±0.01	0.24±0.01	0.22±0.01
pCO11	0.74±0.01	0.77±0.01	0.73±0.01

Cells were grown on 24 well plates and after 2 days of confluence, they were maintained for 20 h in incubation medium and with or without 100 μg/ml laminin or 200 μg/ml heparin. 2-Deoxyglucose uptake was determined as described in Section 2 (n=4).

Table 3
Effect of heparin on Fru-2,6-P_2 levels in A431 cells and pCO11 fibroblasts depleted of PK-C

Cell line	Non-depleted (%)	PK-C-depleted (%)
A431	60.1 ± 3.1	64.5 ± 3.5
pCO11	77.2 ± 1.9	78.0 ± 2.1

Cells were grown on 24 well plates and after 2 days of confluence, they were maintained for 20 h in incubation medium in the absence (non-depleted) or presence of 300 nM TPA (PK-C-depleted) and with or without 200 μg/ml heparin. Fru-2,6-P_2 was determined in alkaline extracts as described in Section 2. PK-C depletion was assessed by the lack of effect of TPA on Fru-2,6-P_2 levels in these cells. 100% is the Fru-2,6-P_2 level in non-depleted cells in the absence of heparin. The average of three different experiments is represented.

fate did not show any effect. The effect of laminin and heparin was dependent on their concentration. The same effects were observed in pCO11 fibroblasts. Glucose transport inside the cells were determined by measuring the uptake of 2-deoxyglucose in the presence of laminin and heparin. No significant differences were observed under our experimental conditions (Table 2).

To test whether there is an interaction between laminin and the EGF receptor, all the fibroblast cell lines carrying mutations of the EGF receptor were incubated with laminin (Table 1). In all cases, laminin induced an increase in Fru-2,6-P_2 levels of up to 150% on average. This effect can be seen even in the kinase-defective mutants. When cells were pretreated for 20 h with laminin before the treatment with EGF, it can be observed that the effects of EGF and laminin on Fru-2,6-P_2 were not additive in any of the cell lines (Table 1).

To study whether PK-C and heparin interact at some point of their mechanism, A431 cells and pCO11 fibroblasts were depleted of PK-C by long-term treatment with TPA [1] and treated with heparin in the same conditions as above. PK-C depletion was assessed by the lack of effect of TPA on Fru-2,6-P_2 levels in these cells. Heparin was able to diminish the level of Fru-2,6-P_2 in PK-C-depleted cells similarly to control cells, indicating that PK-C is not directly involved in the mechanism of heparin action (Table 3). The acute treatment with TPA is able to produce an increase in the level of Fru-2,6-P_2 in pCO11 fibroblasts as well as in A431 cells (Fig. 3, [1]). Nevertheless, the effect of TPA is lower in heparin-pretreated cells, especially in A431 cells where it is completely abolished. This fact indicates that heparin is able to modulate the PK-C-mediated increase of Fru-2,6-P_2 (Fig. 3).

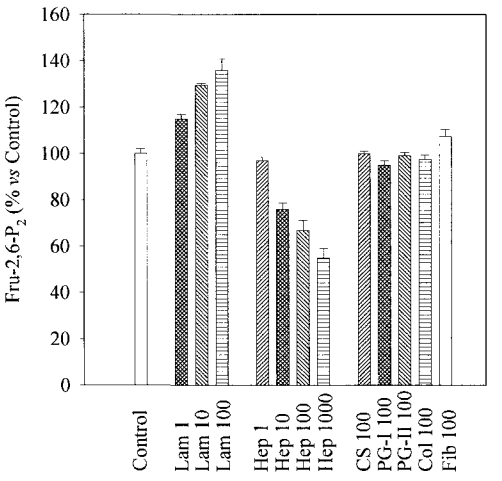

Fig. 2. Fru-2,6-P_2 levels in A431 cells incubated in the presence of several components of the extracellular matrix. Cells were grown on 24 well plates and after 2 days of confluence, they were maintained for 20 h in incubation medium in the presence of several components of the ECM at the indicated concentrations (μg/ml). Fru-2,6-P_2 was determined in alkaline extracts as described in Section 2. Hep: heparin; Lam: laminin; CS: chondroitin sulfate; PG-I: biglycan; PG-II: decorin; Col: collagen; Fib: fibronectin. The average of five different experiments is represented.

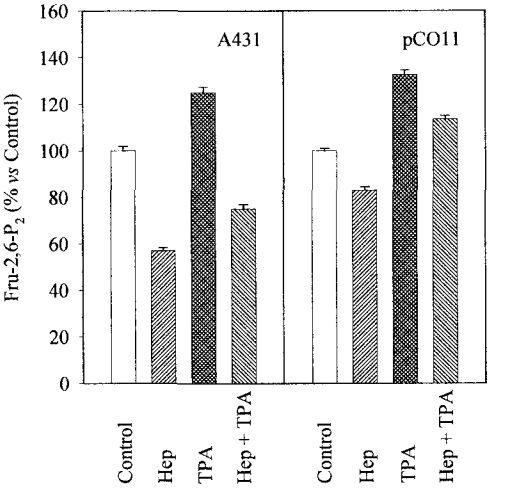

Fig. 3. Effect of TPA on Fru-2,6-P_2 levels on NIH3T3 fibroblasts and A431 cells pretreated with heparin. Cells were grown on 24 well plates and after 2 days of confluence, they were maintained for 20 h in incubation medium and with or without 200 μg/ml heparin. Two hours before the end of the incubation, 150 nM TPA or 0.1% DMSO were added to the cells. Fru-2,6-P_2 was determined in alkaline extracts as described in Section 2. The average of three different experiments is represented.

4. Discussion

The experiments performed in transfected fibroblasts showed that the tyrosine kinase activity of the EGF receptor is essential for the effect on Fru-2,6-P_2 levels, like other actions of the factor, including those related to the metabolic activity of the cell such as glucose and amino acid uptake [31]. The replacement of the three main autophosphorylated tyrosines from the carboxy-terminal tail of the receptor with phenylalanine (F3 mutants) reduces its kinase activity and internalization [32] and decreases the phosphatidylinositol turnover [26]. Our results show that F3 cells also have a reduced response to EGF, indicating that receptor autophosphorylation is essential to maintain the complete response to EGF. We also have analyzed the EGF response in fibroblasts with a deletion of the 214 carboxy-terminal residues of the receptor, including the five putative autophosphorylated tyrosines. This Dc214 mutant has a decreased ability to interact with phospholipase Cγl (PLCγl) [33], whereas the phosphorylation of *ras*GAP is similar to that obtained with the wild-type receptor [34]. Interestingly, in this cell line the glycolytic response to EGF is reduced. These results suggest that the signaling mechanism of EGF is mediated by PLCγl rather than *ras*GAP proteins. Thus, PLCγl would be the link between the EGF receptor and the calcium-mediated stimulation

of glycolysis by EGF previously described in our laboratory [1].

Our results show that, of all the ECM components tested, only laminin and heparin were able to modify the basal levels of Fru-2,6-P_2. Since one of the parameters regulating the level of Fru-2,6-P_2 is the availability of glucose, glucose transport inside the cell was determined, but no effect was seen in heparin- or laminin-treated cells. Laminin increases the level of Fru-2,6-P_2 up to 136–164% whereas heparin decreases the amount of the metabolite to 55–65%. These effects were observed in confluent cultures and were caused by soluble effectors in the absence of serum. This argues for a direct effect of both molecules rather than for indirect effects caused by changes of cell attachment or cell shape [16].

A controversy exists on the possibility that the mitogenic activity of laminin is contained within the EGF-like repeats of the molecule and mediated by the EGF receptor [16,17]. To study whether the EGF receptor is involved in the action of laminin, we analyzed the effect of this molecule in the transfectants carrying different mutations of the receptor. Laminin was able to increase the level of Fru-2,6-P_2 in all the cell lines, including the kinase-defective mutants. Furthermore, the stimulatory effects of EGF and laminin are not additive. This fact indicates that, although independent of the EGF receptor, there is cross-talk between the mechanisms induced by laminin and by EGF. A possible explanation would be that the pretreatment of the cells with laminin renders difficult the interaction of EGF with its receptor, but it has been shown that the binding of EGF occurs even in the presence of a 50-fold excess of laminin [16]. Whether the effect of laminin occurs through the activation of a laminin receptor such as the β1 integrins, as in melanoma cells [35], is unknown, although in this case it could provide a link between intracellular signaling mechanisms mediating cell growth and metabolism.

Finally, heparin is a complex glycosaminoglycan with a potent antiproliferative activity. Heparin seems to exert its inhibitory action by inhibiting the binding of the AP-1 transcription factor to the TPA-responsive element (TRE) regions in the promoter of specific genes [36,37], thus blocking one or more steps in PK-C-dependent pathways [38,39]. PFK-2 can be one of these genes, since it is activated by TPA in A431 cells and fibroblasts [1–3]. Our results indicate that heparin blocks the glycolytic response induced by TPA, suggesting that heparin may act at the transcriptional level according to the observations mentioned above. Nevertheless, since it is not known whether the PFK-2 isozyme from fibroblasts contains a TRE element in its promoter [40], other possibilities cannot be ruled out. In any case, the decrease of Fru-2,6-P_2 caused by heparin is also visible in PK-C-depleted cells, indicating that this enzyme is not a step in its mechanism. The effect is not related to the charge density since other negatively charged molecules, such as chondroitin sulfate, do not alter Fru-2,6-P_2 levels. One possibility is that heparin modifies the activity of a growth factor receptor, as has been described for the FGF receptor 4, whose tyrosine kinase activity is directly activated by heparin [23].

In conclusion, these results indicate that, similarly to soluble growth factors such as EGF or oncogenes such as *src*, specific components of the ECM, such as laminin or heparin, can modulate the levels of Fru-2,6-P_2 and, consequently, the glycolytic activity of the cell. Since glycolytic activation is a short-term response and proliferation takes place after several hours, a mechanism that ensures that energy is obtained from glucose prior to the start of cell proliferation should exist, independently of the molecule that initiates this process.

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