Modulation of 5'-nucleotidase activity in plasma membranes and intact cells by the extracellular matrix proteins laminin and fibronectin

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Modulation of 5'-nucleotidase activity by the extracellular matrix proteins fibronectin, laminin and their fragments has been studied in plasma membrane preparations as well as in intact BCS-TC2 and Rugli cells. The ectoenzyme on plasma membranes is activated by laminin; fibronectin inhibits the AMPase activity on BCS-TC2 plasma membranes but no inhibitory effect is found in plasma membrane preparations from Rugli cells. These effects are dependent on the preincubation time and protein concentration. When the effect of the extracellular matrix proteins is studied on intact cells, both BCS-TC2 and Rugli cells show similar behaviour. A decrease in the enzyme activity is observed in the presence of fibronectin. The AMPase inhibitory activity is located on its 40 kDa fragment. No inhibitory activity is found in other fibronectin fragments, including the 140 kDa fragment which contains the RGDS cell-adhesion sequence. Laminin and its E1–4 and E8 fragments are able to activate the ecto-5'-nucleotidase activity of both BCS-TC2 and Rugli cells. The effect of the E1–4 fragment on intact cells is greater than that observed for the E8 fragment and uncleaved laminin. Our results suggest a bifunctional role for 5'-nucleotidase as ectoenzyme and cell receptor for extracellular matrix proteins.

INTRODUCTION

5'-Nucleotidase (EC 3.1.3.5) catalyses the hydrolysis of nucleoside 5'-monophosphates to nucleosides and inorganic phosphate. Nowadays, investigations of the ectoenzyme are mainly centred on structural aspects, such as its membrane topography, and on the role of the enzyme in the regulation of cellular functions and cell–extracellular matrix interactions. Several hypotheses concerning the membrane topography of the ectoenzyme have been proposed. It was initially described as a transmembrane protein with intracellular domains [1–3] or as a short-stalked integral membrane protein without a cytoplasmic domain [4]. More recently, it has been found that the ectoenzyme from several sources is released from the plasma membranes by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), suggesting that phosphatidylinositol (PI) is involved in the attachment of 5'-nucleotidase to the membrane [5–9]. Moreover, rat liver 5'-nucleotidase has been sequenced and shown to be linked to the plasma membrane through an inositol-containing glycolipid covalently attached to serine-523 [10].

Recently, two glycoproteins of the extracellular matrix, fibronectin and laminin, have been shown to interact directly and specifically with purified chicken gizzard 5'-nucleotidase [11,12] and to regulate the AMPase activity of this ectoenzyme [13]. Laminin and fibronectin are involved in a variety of biological processes, including cell adhesion and spreading, growth, migration and differentiation of a great number of cell types [14–19]. The interaction of these extracellular matrix glycoproteins with their cellular receptors triggers specific molecular mechanisms which induce changes in cell behaviour. However, these mechanisms are poorly understood. Taking into

account the above-mentioned modulation of 5'-nucleotidase activity by fibronectin and laminin, it is tempting to speculate that this ectoenzyme is involved in the regulation of cell behaviour by extracellular matrix components. Recently, the involvement of the ectoenzyme in chick embryo myoblast spreading on laminin has also been reported, monoclonal antibodies against 5'-nucleotidase being used [20].

The studies described above on the modulation of the AMPase activity of the ecto-5'-nucleotidase by fibronectin and laminin have been performed using plasma membrane preparations or the purified enzyme [11,13]. We compare in this paper the effect of laminin and fibronectin on the 5'-nucleotidase activity using intact cells as well as plasma membrane preparations to assess the biological significance of this modulation. In order to discuss the relationship between cell binding and AMPase-modulator domains in laminin and fibronectin, and the possible involvement of the ectoenzyme in the regulation of cell behaviour by both matrix proteins, the modulation of ecto-5'-nucleotidase by different laminin and fibronectin fragments has also been examined. These findings may have an impact on our understanding of the mechanism of interaction between cells and their external environment.

MATERIALS AND METHODS

Materials

[U-¹⁴C]AMP (50 mCi/mmol) was obtained from Amersham, and AMP and proteinase inhibitors were from Serva (Heidelberg, Germany). ZnSO₄ and Ba(OH)₂ were from Sigma (Munich, Germany). Sepharose–gelatin and Sepharose–heparin were from Pharmacia (Uppsala, Sweden). All other chemicals were of

Abbreviations used: PI-PLC, phosphatidylinositol-specific phospholipase C; PBS, phosphate-buffered saline; TEA buffer, triethanolamine hydrochloride buffer; PMSF, phenylmethanesulphonyl fluoride.

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analytical grade and were purchased from Merck (Darmstadt, Germany). Media, sera, antibiotics and trypsin/EDTA solutions for cell culture were from Flow Laboratories (Rockville, MD, U.S.A.); multiwell tissue culture plates and plastic culture flasks were from Costar (Cambridge, MA, U.S.A.).

Cells and tissue culture

Two established cell lines were used throughout these studies: Rugli and BCS-TC2. Rugli cells, derived from a rat glioblastoma (a generous gift from Professor B. Odermatt, University Hospital, Zurich, Switzerland) were cultured in Dulbecco's modification of Eagle's medium. BCS-TC2 cells, obtained in our laboratory from a human colon adenocarcinoma [21], were grown in RPMI-1640 medium. Standard media were supplemented with 10% heat-inactivated foetal calf serum, penicillin (50 IU/ml), streptomycin (50 µg/ml) and L-glutamine (300 µg/ml). Cells were grown to confluence in 75 cm² Falcon plastic flasks and incubated at 37 °C in a CO₂/air (1:19, v/v) humidified atmosphere. The culture medium was routinely changed every 2 days; confluent cultures were detached and propagated by treatment with trypsin (0.05% trypsin/0.02% EDTA). Cell harvesting for AMPase assays was also performed by trypsin treatment of nearly confluent cultures. After centrifugation, cells were allowed to recover for at least 1 h in supplemented medium, washed with phosphate-buffered saline (PBS; 50 mM-sodium phosphate/0.15 M-NaCl, pH 7.5) and resuspended in assay buffer at the required concentration.

Preparation of plasma membranes

Cell plasma membranes were obtained by a modification of the method described by Lesot *et al.* [22]. Cell cultures were washed twice with PBS, incubated for 30 min in 10 mM-triethanolamine hydrochloride buffer, pH 7.4 (TEA buffer) containing 8.5% (w/w) sucrose and proteinase inhibitors [1 mM-phenylmethanesulphonyl fluoride (PMSF) and 1 mM-N-ethylmaleimide], and homogenized in a Dounce homogenizer. After centrifugation at 1200 g for 15 min, the supernatants were pelleted at 20000 g for 1 h, resuspended in 8.5% (w/w) sucrose in TEA buffer and layered on a discontinuous sucrose density gradient composed of 10 ml each of 40% and 17% (w/w) sucrose in TEA buffer. Plasma membranes were collected, after centrifugation at 130000 g for 3 h on a Beckman SW27 rotor, from the 17/40% sucrose interface and pelleted at 20000 g for 1.5 h. Plasma membrane preparations were stored at -20 °C in AMPase assay incubation buffer (see below).

Protein preparations

Laminin was isolated from EHS mouse tumours as described by Timpl *et al.* [23] and stored at 4 °C in 50 mM-Tris (pH 7.4)/0.4 M-NaCl, in the presence of PMSF and *p*-chloromercuribenzoate, each 10 µg/ml. Elastase digestion (elastase from porcine pancreas, EC 3.4.21.36) was performed according to Ott *et al.* [24] and Goodman *et al.* [14] in 0.2 M-bicarbonate for 16 h at 37 °C using an enzyme/substrate protein ratio of 1:100 (w/w), and was stopped by addition of PMSF. Digestion products were separated on agarose 1.5M. E3 fragment was used without further purification. After dialysis against 50 mM-sodium bicarbonate, the E1-4 and E8 fragments were further purified by chromatography on Sepharose-heparin. E8 binds, whereas E1-4 flows through; E8 was eluted with 0.5 M-NaCl. Laminin fragments were stored in the same conditions as described for the intact molecule.

Fibronectin was purified from human plasma by Sepharose-gelatin affinity chromatography and further chromatography on DEAE-cellulose, as described [25], and it

was stored at 20 °C in 50 mM-Tris (pH 7.4)/0.4 M-NaCl/1 mM-EDTA. Fibronectin fragments were obtained by successive digestion with cathepsin D (bovine spleen, EC 3.4.23.5; enzyme/substrate protein 1:250, w/w) and plasmin (human plasma, EC 3.4.21.7; enzyme/substrate protein 1:1000, w/w). Separation was performed by chromatography on Sepharose-gelatin and Sepharose-heparin (both from Pharmacia) as described [25,26]. All fragments were further purified, after dialysis against 50 mM-Tris/0.1 M-NaCl/1 mM-EDTA, by chromatography on Ultrogel AcA 44. Fragments were concentrated by ultrafiltration and stored at -20 °C in the same buffer.

Laminin and fibronectin preparations and their proteolytic digestion fragments were routinely checked by SDS/PAGE for the absence of degradation products. Nidogen from mouse EHS tumours was kindly provided by Dr. R. Timpl (Max-Planck Institute für Biochemie, Munich, Germany).

Enzyme assay

5'-Nucleotidase activity was measured in plasma membranes, freshly harvested cells (suspension assay) or cell monolayers (monolayer assay), as previously described [27]. A modification of the method described by Gentry & Olsson [28] was followed. In brief, the AMPase activity of the ectoenzyme was determined by a radioisotope assay; the incubation mixture contained 100 µM-AMP and 20 mM-glycerophosphate in incubation buffer (50 mM-Tris/HCl, pH 7.4, containing 5 mM-MgCl₂, 5 mM-MnCl₂ and 0.14 M-NaCl). AMPase activity was expressed as nmol of AMP hydrolysed in 15 min at 37 °C unless otherwise stated. In general, 13 µg of plasma membrane protein was used in each assay; the number of cells is indicated in each experiment.

Suspension assays were performed with cells obtained by trypsin treatment, pelleted by centrifugation, and incubated for at least 1 h in supplemented culture medium for recovery from potential membrane damage. After centrifugation, cells were resuspended in an appropriate volume of the assay buffer, and 5'-nucleotidase activity, cell number and protein concentration were determined. Monolayer assays were performed 2 or 3 days after the appropriate number of cells had been seeded in 24-well culture dishes. Culture medium was then removed and the monolayers were washed twice with PBS; the wells were used to either evaluate the actual cell number or measure 5'-nucleotidase activity (adding 500 µl of the assay mixture to the wells).

Matrix protein stock solutions were dialysed and diluted in the assay buffer in order to obtain the required concentrations; BSA, diluted in the incubation buffer at the same concentration, was used as control. Cells and plasma membrane preparations, prepared as described above, were preincubated with matrix proteins or BSA at different temperatures and time-intervals, and 5'-nucleotidase activity was determined afterwards. Controls without cells or plasma membrane proteins were also routinely performed in order to verify the absence of endogenous or contaminant AMPase activity in the different protein and fragment preparations.

Other assays

Release of 5'-nucleotidase with PI-PLC from *Bacillus thuringiensis* (kindly provided by Dr. M. G. Low, Columbia University, New York, NY, U.S.A.) was performed on cells growing in monolayer. The cells were incubated with PI-PLC (0.75 unit/ml) in culture medium for 60 min at 37 °C. AMPase activity was measured both in the cell layer and culture medium in the presence or absence of β-glycerophosphate, as described [27].

Protein concentration was determined by the method of Bradford [29] with BSA as standard. SDS/PAGE was performed as described by Laemmli [30].

Table 1. Characteristics of the ecto-5'-nucleotidase from BCS-TC2 and Rugli cells

Ecto-5'-nucleotidase activity was determined at 37 °C as described in the Materials and methods section and is expressed as nmol of AMP hydrolysed/mg of protein in 15 min for plasma membranes and nmol of AMP hydrolysed/cell in 15 min for cells in suspension or monolayer. Apparent K_m values were obtained from the plots of v_0 versus AMP concentration by non-linear regression analysis. The percentage of ecto-5'-nucleotidase activity released after PI-PLC treatment of intact cells was calculated as 100 minus the percentage of remaining AMPase activity present on the cell monolayer referred to the total cell activity before the PI-PLC treatment.

Ecto-5'-nucleotidase	BCS-TC2	Rugli
Activity		
Plasma membranes	475 ± 14	4245 ± 460
Cells in suspension (1 × 10 ⁵)	8.7 ± 5.7	38.7 ± 9.6
Cells in monolayer (1 × 10 ⁵)	36.7 ± 10.0	115.3 ± 24.0
Release after PI-PLC treatment (%)	20	50
$K_{m\text{ app.}}$ (μM)	19.9 ± 2.1	40.3 ± 5.3

Fig. 1. SDS/PAGE of laminin, fibronectin and their fragments

Purity of the different preparations was determined by SDS/PAGE. (a) Lane 1, laminin (5% linear gel, reducing); lanes 2 and 3, E1-4 and E8 laminin fragments respectively (3-5% gradient gel, non-reducing). (b) Lanes 4-10 were on a 10% linear gel. Lane 4, molecular-mass markers; lanes 5-10, fibronectin and its fragments (30, 40, 140, 60 and 31 kDa respectively).

RESULTS

The 5'-nucleotidase activity values for plasma membrane preparations and intact BCS-TC2 and Rugli cells are shown in Table 1. The hydrolysis of AMP followed Michaelis-Menten kinetics. Apparent K_m values as well as the release of the ectoenzyme by treatment with PI-PLC of these cells are also indicated.

Protein and fragment purifications

Laminin fragments were obtained from nidogen-free laminin preparations by limited elastase digestion as described in the Materials and methods section. After chromatography on agarose 1.5M, fragment E3 was essentially homogeneous as observed by the elution profile and SDS/PAGE. Fragment E8 required a further purification step on Sepharose-heparin in order to eliminate contaminating E1-4 fragment. Fig. 1(a) shows the electrophoretic pattern of laminin, E8 and E1-4 fragments under reducing conditions. A molecular weight of 400 kDa was estimated for E1-4 fragment. In fragment E8, the truncated laminin A and B chains are not covalently linked under SDS/PAGE denaturing conditions giving two bands of approximately 140 kDa and 75 kDa as also described previously [14].

E1-4 and E8 fragments showed adhesion- and spreading-promoting activities when laminin fragments were screened on BCS-TC2 cells. These results are similar to those reported previously for Rugli cells [14]. E8 fragment promoted rapid cell adhesion and spreading of BCS-TC2 cells, similar to or greater than that observed for laminin. E1-4 fragment induced a similar cell-adhesion process but a lower extent of spreading, whereas cell to cell aggregation was promoted (results not shown).

Preparations of fibronectin and its proteinase digestion fragments purified from human plasma appeared, from SDS/PAGE analysis, to be homogeneous (Fig. 1b).

Influence of extracellular matrix proteins on AMPase activity in plasma membranes

The effect of extracellular matrix proteins on the 5'-nucleotidase activity of plasma membrane preparations from cultured cells has been studied. Preincubation with fibronectin and laminin resulted in a modification of the AMPase activity present in these preparations. Different times and temperatures of preincubation were assayed for the enzyme present in plasma membrane

Table 2. Effect of laminin and fibronectin on 5'-nucleotidase activity of BCS-TC2 and Rugli cells at different preincubation times

Monolayers or cells in suspension were preincubated at 37 °C for different time-intervals with laminin and fibronectin. Afterwards, 5'-nucleotidase activity was measured as described in the Materials and methods section. Parallel assays were performed under identical conditions but in the absence of both matrix proteins in order to obtain 100% activity values (100% activity without preincubation: for BCS-TC2 cells = 12.5×10^{-5} and 26.2×10^{-5} nmol of AMP hydrolysed/cell in 15 min in suspension and monolayer respectively; Rugli cells = 38.2×10^{-5} and 113.8×10^{-5} nmol of AMP hydrolysed/cell in 15 min in suspension and monolayer respectively). Data are means of at least three independent experiments with triplicate determinations (± s.d.).

		Nucleotidase activity (%)									
		BCS-TC2 cells					Rugli cells				
Matrix protein	Time (h)...	0	1	2	3	4	0	1	2	3	4
Laminin (60 nM)											
Suspension		100 ± 5	108 ± 5	136 ± 7	140 ± 7	155 ± 7	102 ± 9	112 ± 5	132 ± 6	140 ± 14	145 ± 12
Monolayer		105 ± 9	135 ± 9	174 ± 8	184 ± 12	204 ± 13	100 ± 9	139 ± 10	144 ± 12	155 ± 11	184 ± 11
Fibronectin (30 nM)											
Suspension		98 ± 8	85 ± 5	55 ± 4	50 ± 3	22 ± 1	96 ± 9	77 ± 3	53 ± 3	20 ± 1	23 ± 2
Monolayer		96 ± 4	88 ± 7	77 ± 5	70 ± 4	21 ± 2	100 ± 7	83 ± 8	43 ± 4	12 ± 1	10 ± 1

Fig. 2. Effect of laminin and fibronectin on the AMPase activity of plasma membrane preparations of BCS-TC2 cells

Plasma membrane proteins (13 μ g) were assayed for AMPase activity in the presence of different concentrations of laminin (●) and fibronectin (■), with (—) or without (---) preincubation at 37 °C for 3 h. Activity is expressed (left) as nmol of AMP hydrolysed/mg of protein in 15 min and (right) as a percentage of the control in the absence of matrix proteins (100% activity = 480 nmol of AMP hydrolysed/mg of protein in 15 min). Data are means of three independent experiments with triplicate determinations.

preparations of both cell lines. A 3 h preincubation period at 37 °C was found to be necessary in order to achieve maximal 5'-nucleotidase activation by laminin and inhibition by fibronectin.

Fig. 2 shows the dependence of the 5'-nucleotidase activity on fibronectin and laminin concentration with and without preincubation at 37 °C for 3 h. AMPase activity of 13 μ g (26 μ g/ml) of BCS-TC2 plasma membrane proteins is about 180% of the control when preincubated in the presence of 40 nM-laminin (18 μ g/assay). The activity is decreased to 30% after preincubation with 40 nM-fibronectin (9 μ g/assay).

No modification of this activity is observed when the plasma membrane preparations are assayed in the presence of type I or type IV collagens, or nidogen. Identical results are obtained after preincubation in the presence of these proteins. BSA has no effect on AMPase activity. Moreover, the effects induced by either laminin or fibronectin are almost identical in the presence of 0.5% BSA, which confirms the specificity of the interactions between these proteins and 5'-nucleotidase.

Plasma membrane preparations from Rugli cells show a slightly different pattern of modification of 5'-nucleotidase activity by extracellular matrix proteins. Laminin induces an activation of the enzyme, but always lower than that obtained for BCS-TC2 plasma membranes; a 16 h preincubation with 60 nM-laminin resulted in activity values of 140% of the controls. Fibronectin did not inhibit the 5'-nucleotidase activity in Rugli plasma membrane preparations (results not shown).

Quantitative differences were detected among different laminin and fibronectin preparations in the modulation of 5'-nucleotidase activity. 5'-Nucleotidase activation by different laminin stocks ranges from 120 to 190% of AMPase activity at 60 nM-laminin. A wider variation is detected among fibronectin preparations; 60 nM-fibronectin induces 5'-nucleotidase activity inactivation which ranges from 80 to 20% of remaining AMPase activity. Both laminin and fibronectin lose their ability to modulate 5'-nucleotidase activity after thermal denaturation at 70 °C for 60 min. All these data strongly suggest an important role for the protein conformation in these effects of laminin and fibronectin.

Fig. 3. Effect of laminin and fibronectin on the AMPase activity on intact (a) BCS-TC2 and (b) Rugli cells

AMPase activity determinations were performed with intact cells in suspension (—) or in monolayer (---). Cells were preincubated at 37 °C for 3 h with different concentrations of laminin (●), fibronectin (■) or BSA (□). Activity values are expressed as percentages of the control activity measured without matrix proteins. Data are means of three independent experiments with triplicate determinations. (a) BCS-TC2 cells (1×10^5 cells per assay); 100% activity = 11.9×10^{-5} and 34.2×10^{-5} nmol of AMP hydrolysed/cell in 15 min, in suspension and monolayer assays respectively. (b) Rugli cells (2.5×10^4 cells per assay); 100% activity = 36.4×10^{-5} and 99.8×10^{-5} nmol of AMP hydrolysed/cell in 15 min, in suspension and monolayer assays respectively.

Modulation of AMPase activity on intact cells by laminin and fibronectin

To gain further support for the physiological relevance of the observed effect, 5'-nucleotidase modulation by extracellular matrix proteins required to be verified on intact cells.

The effect of laminin and fibronectin was time-dependent and required preincubation of cells with the matrix proteins. The results obtained with BCS-TC2 cells are similar to those obtained with their plasma membrane preparations. An increase in the enzyme activity was observed in the presence of laminin while fibronectin exerted the opposite effect. The results obtained are summarized in Table 2. These effects were highly dependent on the preincubation time and were observed when the cells were assayed for AMPase activity in either suspension or monolayer.

Similar results were obtained with Rugli cells (Table 2). However, in contrast with the experiment using Rugli plasma membranes where fibronectin did not inhibit the nucleotidase activity, fibronectin showed an inhibitory effect on intact Rugli cells.

Fig. 4. Effect of laminin and its elastase digestion fragments E1-4 and E8 on the AMPase activity of BCS-TC2 plasma membrane preparations (a) and intact cells (b)

Plasma membrane proteins (13 μ g) (a) or 1×10^5 cells (b) were preincubated at 37 °C for 3 h in the presence of different concentrations of laminin (●) or its fragments (▲, E1-4; ■, E8). Activity is expressed (left) as nmol of AMP hydrolysed/mg of protein in 15 min in plasma membrane preparations (a) and nmol of AMP hydrolysed/cell in 15 min ($\times 10^5$) on intact cells (b). On the right, the activity is expressed as the percentage of the respective controls. Data are the means of three independent experiments; each experimental point was determined at least in triplicate.

Activation by laminin and inhibition by fibronectin increased with preincubation time up to 3-4 h, levelling off thereafter. Therefore a preincubation time of 3 h was selected for further experiments with intact cells in order to exclude a possible loss of cell viability during the preincubation period.

Fig. 3 shows the modification of 5'-nucleotidase activity of BCS-TC2 (Fig. 3a) and Rugli (Fig. 3b) cells as a function of fibronectin and laminin concentrations. Assays were performed, either in suspension or monolayer, with an appropriate cell number in order to suppress cell density effects [27]. No modification of the enzyme activity was detected when the intact cells were incubated with different concentrations of BSA.

In both cell types the laminin effect was stabilized at about 150 % AMPase activity of the control when assayed in suspension. In monolayer, the laminin effect was more pronounced and did not stabilize even at higher laminin concentrations, reaching more than 200 % AMPase activity (Fig. 3). Fibronectin inhibited the AMPase activity of intact cells in a concentration-dependent manner (Fig. 3). In suspension culture at 250 nM-fibronectin the activity was totally lost. Inhibition was higher in Rugli cells, where 80 % inhibition was achieved at 30 nM-fibronectin (Fig. 3b). A similar inhibition was reached in monolayer culture of BCS-TC2 cells at 60 nM and in suspension at 180 nM (Fig. 3a).

5'-Nucleotidase activation by laminin fragments in plasma membrane preparations and intact BCS-TC2 cells

Laminin fragments E1-4 and E8 are able to induce activation of the AMPase activity present in plasma membrane preparations of BCS-TC2 cells. E3 laminin fragments do not promote any effect on the AMPase activity. The modification of 5'-nucleotidase activity of BCS-TC2 plasma membrane preparations and intact cells with respect to the concentration of laminin and E1-4 and E8 fragments has been considered. Fig. 4(a) shows the activation of the AMPase activity present in 13 μ g of BCS-TC2 plasma membrane proteins. E1-4 and E8 fragments stimulated 5'-nucleotidase activity to a similar extent, but to a greater extent than that observed for intact laminin. However, at very low concentrations, E1-4 is the most effective activating factor. A 300 % activity value is reached at 100 nM of both fragments while only 180 % activity is obtained with a similar concentration of laminin.

5'-Nucleotidase activation was also observed when intact BCS-TC2 cells in suspension were preincubated in the presence of laminin and its E1-4 and E8 fragments (Fig. 4b). Several differences were found in comparison with the experiments performed with plasma membrane preparations. At saturating concentrations, E1-4 fragment enhances the ecto-AMPase activity more than do the E8 fragment or the intact laminin

Table 3. Effect of different fibronectin fragments on the 5'-nucleotidase activity of BCS-TC2 and Rugli cells

Cells were preincubated at 37 °C for 3 h in the presence of fibronectin and its different proteolytic fragments, each at 200 nM. Assays were performed with 1×10^5 and 2.5×10^4 cells in suspension for BCS-TC2 and Rugli cells respectively. The inhibition is referred to the control value in the absence of fibronectin or its fragments (100 % activity = 12.1×10^{-5} and 32.0×10^{-5} nmol of AMP hydrolysed/cell in 15 min for BCS-TC2 and Rugli cells respectively). Blanks without cells but in the presence of fibronectin or its fragments were always performed. Data are the average ($\pm$ S.D.) of at least three independent experiments with triplicate samples.

Fibronectin fragment	Domain	Binding activity	AMPase inhibition (%)	
			BCS-TC2	Rugli
30 kDa	N-Terminus (N ₁)	Fibrin, heparin	< 5	< 5
40 kDa	Adjacent to N ₁	Gelatin, collagen	76 $\pm$ 4	74 $\pm$ 5
140 kDa	Central region	Cell (RGDS), DNA	< 5	< 5
60 kDa	C-Terminus (-S-S-)	Heparin, fibrin	< 5	< 5
31 kDa	C-Terminus	Fibrin	< 5	< 5
40 kDa + gelatin			77 $\pm$ 4	75 $\pm$ 3
Intact fibronectin			80 $\pm$ 6	75 $\pm$ 5
Denatured fibronectin			< 5	< 5

Fig. 5. Effect of fibronectin and its fragments on the AMPase activity of BCS-TC2 cells

Concentration-dependence of the AMPase activity inhibition by fibronectin (■), its 40 kDa (●) and 140 kDa (▲) fragments and BSA (□). Activity is expressed as the percentage of the control (100% activity = 12.1×10^{-5} nmol of AMP hydrolysed/cell in 15 min). Data are means of four independent experiments; each point was determined at least in triplicate.

molecules, while E1-4 and E8 fragments show similar behaviours at low concentrations. A 100 nM-E1-4 concentration yields 170% AMPase activity while only 140% is obtained with a similar concentration of E8 and laminin. Moreover, saturation is achieved at lower protein concentrations with both laminin fragments, around 40 nM, than with intact laminin, 80 nM.

Modulation of 5'-nucleotidase activity by fibronectin fragments on BCS-TC2 intact cells

The effect of different fibronectin fragments on ecto-5'-nucleotidase activity is shown in Table 3. After preincubation of BCS-TC2 intact cells with 200 nM-fibronectin, or with its different fragments, only the intact molecule and the 40 kDa fragment showed AMPase activity inhibition.

The concentration-dependence of the inhibition is almost identical for fibronectin and its 40 kDa fragment (Fig. 5). The incubation of the 40 kDa fragment with gelatin had no effect on the inhibitory activity of this fragment (Table 3). A stabilization around 80% inhibition is reached at 120 nM. Preincubation of BCS-TC2 cells with other fibronectin fragments, including the 140 kDa fragment which contains the RGDS sequence, had no effect on the ecto-5'-nucleotidase activity (Fig. 5 and Table 3).

Effect of laminin and fibronectin fragments on 5'-nucleotidase activity of Rugli cells

Similar experiments to those described for BCS-TC2 cells were performed with Rugli cells. The effects are similar to those obtained with BCS-TC2 cells; they are summarized in Fig. 6. Both laminin fragments (E1-4 and E8) induce ecto-5'-nucleotidase activation, the effect being greater for E1-4 (190% activity at 120 nM) than for E8 or the intact laminin molecule (150% activity at 120 nM).

The ecto-5'-nucleotidase inactivation induced by fibronectin was also achieved with the 40 kDa fragment. Moreover, as observed in BCS-TC2 cells, both fibronectin and its 40 kDa fragment exert an almost identical concentration-dependent effect. The incubation of the 40 kDa fragment with gelatin had

Fig. 6. Effects of laminin, fibronectin and their fragments on the AMPase activity of Rugli cells

Cells (2.5×10^4) were preincubated at 37 °C for 3 h in the presence of different concentrations of each effector. ▲, E1-4; ■, E8; ●, laminin; △, 140 kDa; □, fibronectin; ○, 40 kDa. The activity is expressed as percentage of control values (100% activity = 32.0×10^{-5} nmol of AMP hydrolysed/cell in 15 min). Data are means of three independent experiments; each point was obtained at least in triplicate.

no effect on the inhibitory activity of this fragment. No effect was detected with the other fibronectin fragments assayed (Table 3).

DISCUSSION

Extracellular matrix proteins affect cell functions such as adhesion, spreading, migration, proliferation and differentiation. Among these proteins, laminin and fibronectin seem to be responsible for a major proportion of these cell-matrix interactions [14,16-18,31,32]. However, the mechanisms through which these proteins exert such functions are not yet fully understood. The interaction of these molecules with their respective receptors in the cell membrane is known to induce alterations of cell morphology, possibly through a reorganization of the intracellular cytoskeleton [7,33,34]. The interaction of the ectoenzyme 5'-nucleotidase with fibronectin and laminin *in vitro* [11-13] and the possible existence of different isoforms of the ectoenzyme [5,9], linked to the cell membrane through a PI anchor [5,10] or through hydrophobic domains [1-4,8], suggest a role for the ectoenzyme as a cell receptor for the extracellular matrix [12,35] acting as a modulator of the adhesive properties and locomotion of cells [5].

The modulation of the AMPase activity of purified 5'-nucleo-

tidase by laminin and fibronectin when the enzyme was solubilized with detergents or incorporated into liposomes has been described [11,13]. However, the understanding of the importance of this modulation *in vivo* requires its demonstration on intact cultured cells. Two cell lines with different 5'-nucleotidase activities and characteristics (Table 1) were used as experimental models to study this regulation. Our results clearly show that the ectoenzyme present in BCS-TC2 plasma membrane preparations and intact cells is stimulated by laminin and inhibited by fibronectin (Figs. 2 and 3a). However, in Rugli plasma membrane preparations the 5'-nucleotidase activity is not affected by fibronectin, and the activation of the ectoenzyme by laminin is lower than with BCS-TC2 plasma membranes. However, when the effect of both matrix proteins is assayed on intact Rugli cells, similar results to those obtained for BCS-TC2 cells are observed. This suggests that the 5'-nucleotidase activity and its modulation by extracellular matrix proteins is strongly dependent on its microenvironment, as has also been suggested by other authors [13,36].

Laminin stimulation of the AMPase activity is greater in cells assayed in monolayer than in the same cells assayed in suspension. We have observed that preincubation with laminin induced cell aggregation in suspension. This aggregation could increase the cell density effects previously detected in both types of cell [27] and thus be responsible for the decrease in the AMPase activity.

The different effect exerted on the 5'-nucleotidase activity by both extracellular matrix proteins also indicates that the interaction of laminin and fibronectin with 5'-nucleotidase possibly takes place at different sites of the ectoenzyme. Interaction of laminin and fibronectin with 5'-nucleotidase on intact cells suggests a possible role for the ectoenzyme as a membrane receptor for both proteins. The recognition of and binding to different adhesive ligands, such as laminin and fibronectin, have been previously described for other cellular receptors such as integrins [33,34,37]. From this point of view, we have identified domains of the extracellular matrix proteins, laminin and fibronectin, that are responsible for the 5'-nucleotidase modulation described here. The laminin fragments that can modulate 5'-nucleotidase activity are E1-4 and E8, the same ones as are responsible for the interaction of laminin with cell surface receptors [13,18,19,38,39]. In contrast, the inactivation of the ectoenzyme by fibronectin is not dependent on the 140 kDa fragment which is its cell-binding fragment [17]; only the 40 kDa fragment shows AMPase activity inhibition. This fragment binds gelatin and collagen and is involved in the fibrous polymerization of fibronectin [17,35].

Preparations of the 40 kDa fragment incubated with gelatin retained inhibitory activity. Thus we propose that binding of fibronectin to cells is through this domain of the molecule, independently of the gelatin-binding sequences. The role of the 40 kDa domain as an additional cell-recognition site has previously been proposed. De Petro *et al.* [40] described a transformation-enhancing activity of the gelatin-binding fragment of human plasma fibronectin.

It cannot be excluded that the inhibition of the AMPase activity by fibronectin or its 40 kDa fragment occurs indirectly through modifications of the microenvironment of the ectoenzyme. However, a direct interaction between both proteins on the cell surface is more probable in view of the direct interaction found between fibronectin and purified chicken gizzard 5'-nucleotidase, either solubilized with detergents or reconstituted into liposomes [8]. Stochaj *et al.* [12] have also found that the binding process between 5'-nucleotidase incorporated into liposomes and fibronectin is saturable, specific and independent of the RGDS sequence of fibronectin. However, further studies are required in order to assess the possible

function of the 40 kDa fragment as a co-operative site for the fibronectin-cell interaction.

E1-4 and E8 laminin fragments are involved in laminin recognition by different types of laminin receptors [14,18]. Our results show that these two fragments are also involved in the regulation of 5'-nucleotidase activity. Laminin, E1-4 and E8 fragments probably enhance 5'-nucleotidase activity by inducing conformational changes in the ectoenzyme. These changes can be induced either by direct interaction or indirectly as a result of the laminin interaction with other cellular receptors. Recently, it has been reported that cell spreading on laminin substrate involves concanavalin A-binding proteins, among them the ecto-5'-nucleotidase [20,41,42]. These pieces of evidence and our own results strongly support the hypothesis of a direct interaction between laminin and 5'-nucleotidase which could take place between the E1-4 and E8 fragments and 5'-nucleotidase, inducing the activity enhancement reported here.

The increase in the AMPase activity on intact cells produced by fragment E1-4 is significantly greater (170 % compared with 145 % AMPase activity) than that obtained with the E8 fragment (Figs. 4b and 6). However, with plasma membrane preparations, the same AMPase activity enhancement is found with both fragments (Fig. 4a). These differences must be explained by cellular mechanisms of rearrangement of the enzyme in the cell surface of intact cells which could be different from those in plasma membrane preparations. On intact cells, the direct interaction of the E1-4 fragment with the 5'-nucleotidase could induce surface patching and capping of this ectoenzyme, resulting in a stimulation of the enzyme activity.

Our results point to a possible function of the ecto-5'-nucleotidase as a cell receptor for extracellular matrix proteins. A similar bifunctional role as ectoenzyme and cell-adhesion molecule has been described for the rat liver ecto-ATPase [43]. Nevertheless, more studies are required to elucidate this bifunctional role as well as the mechanisms of 5'-nucleotidase modulation by laminin and fibronectin. It would be of great interest to study the effect of these laminin fragments on purified ecto-5'-nucleotidase and to study the AMPase modulation on cell lines that express specific laminin receptors towards one of the two mentioned fragments.

We thank Professor Angel Martín-Municio and Professor José G. Gavilanes for critically reading, valuable discussion of and greatly improving the manuscript. This work was supported by grant no. PB88-0129 from the Comisión Asesora de Investigación Científica y Técnica (Spain).

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Received 1 May 1991/26 July 1991; accepted 30 July 1991