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Dual Mechanism of Laminin Modulation of Ecto-5′-Nucleotidase Activity

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Abstract The myoblast cell surface activity of ecto-5′-nucleotidase was stimulated by a laminin substrate, whereas fibronectin and gelatin did not increase the AMPase activity of ecto-5′-nucleotidase. This increase was related to a higher expression of ecto-5′-nucleotidase on the surface of cells seeded on a laminin substrate, but without the mobilization of an intracellular pool of enzyme. Furthermore, laminin and its fragments E'_1 and E_8 modified the AMPase activity of the ecto-5′-nucleotidase purified from chicken striated muscle and reconstituted in liposomes. Over the range of concentrations used, intact laminin and its fragment E_8, consisting of the distal half of the long arm, stimulated the AMPase activity of ecto-5′-nucleotidase. By contrast, the large fragment derived from the short arms, designated E'_1, inhibited the AMPase activity. Furthermore, the monoclonal anti-ecto-5′-nucleotidase antibody, CG37, abolished the stimulatory effect of fragment E_8 on the AMPase activity of ecto-5′-nucleotidase but did not reverse the inhibitory effect of fragment E'_1. In conclusion, laminin stimulates the AMPase activity of ecto-5′-nucleotidase by two mechanisms: inducing the expression of ecto-5′-nucleotidase to the cell surface and direct modulation of the enzymatic activity.

Key words: ecto-5′-nucleotidase, laminin substrate, fibronectin, gelatin, AMPase activity

Laminin, a high-molecular-weight glycoprotein, is known to be involved in the attachment, proliferation, migration, and differentiation of cells [1]. Different domains of laminin have been shown to interact with various cell surface receptors [2] and to elicit a diversity of biological effects [3]. However, the molecular mechanism by which laminin influences cell function is still not fully understood.

The membrane-bound enzyme 5′-nucleotidase (EC 3.1.3.5) isolated from chicken gizzard smooth muscle was shown to specifically interact with the extracellular glycoproteins, laminin, and fibronectin [4]. Furthermore, laminin and fibronectin modulated the AMPase activity of purified chicken gizzard ecto-5′-nucleotidase [5]: laminin stimulated it, and fibronectin inhibited it. More recently, these effects of these two extracellular matrix glycoproteins on AMPase activity were also demonstrated on BCS-TC2 and Rugli cells (tested as intact cells and isolated plasma membranes) [6]. By its enzymatic activity, 5′-nucleotidase is considered to be the main producer of adenosine. This compound is known to be a potent effector molecule in a number of cellular events, for example, the modulation of blood flow resistance and neuronal activity (for a review, see [7]). Using monoclonal antibodies directed against the chicken gizzard ectoenzyme, we previously demonstrated the participation of 5′-nucleotidase in the spreading of chicken embryonic fibroblasts [8] and myoblasts [9] on a laminin substratum. Here, we report that laminin promote the expression of ecto-5′-nucleotidase on cultured myogenic cell membranes and directly stimulate the AMPase activity of the ecto-5′-nucleotidase purified from striated muscle. Using proteolysed laminin fragments and monoclonal antibodies directed against chicken ecto-5′-nucleotidase, we found that two fragments of laminin (proteolyzed fragments E'_1 and

Abbreviations used: phenylmethylsulfonyl fluoride (PMSF); α,β-methylene adenosine 5′-diphosphate (AMPCP); sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); bovine serum albumin (BSA); phosphate-buffered saline (PBS); N-ethyl maleimide (NEM).

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E_8) interacted with distinct ecto-5′-nucleotidase sites and could modulate the enzymatic activity with a dual effect: stimulation of the AMPase activity of ecto-5′-nucleotidase by fragment E_8 and inhibition by the fragment E'_1. These results constitute a model of dual regulation of the enzymatic activity of a laminin-binding protein by different domains of its ligand.

MATERIALS

The specificities of the poly- and monoclonal antibodies used in this study have been described previously [10–12]. Fibronectin (human plasma) was purchased from Institut Jacque-Boy (Reims, France). All reagents were of the highest quality available.

METHODS

Enzyme Assay

Ecto-5′-nucleotidase activity was determined spectrophotometrically at 265 nm at 25°C as described in ref. [13]. The test solution (1 ml) contained 50 μM AMP, 0.1 M $CaCl_2$, 0.5 mM NaN_3, 4 μg/ml adenosine deaminase and 5 mM Hepes, pH 7.4. 1 U of enzyme corresponded to 0.132 μM AMP hydrolyzed × min^{-1}. Alternatively, AMPase activity was determined by a radioisotope assay using [^{14}C]-AMP as the substrate [14]. Briefly, in each assay, 500 μl containing 60 mM Tris · HCl, pH 7.4, 50 μM 5′-AMP, 0.01–0.015 μCi [^{14}C]-AMP (sp. act. 451 mCi/mmol, The Radiochemical Centre, Amersham, Buckinghamshire, UK), 0.1 mM $MgCl_2$ and 0.2 mM $CaCl_2$. The reaction was performed at 37°C for 15 min and stopped by the addition of 100 μl 0.15 M $ZnSO_4$ and 100 μl 0.3 N $Ba(OH)_2$ with mixing between each addition. Unreacted 5′-AMP was sedimented by centrifugation at 8,000 rpm for 10 min in a minifuge (Biofuge A, Heraeus, Sepatech). The radiation emitted by an aliquot of the supernatant was measured in a scintillation counter (Minaxi, B tricarb 4000 series, Packard). When required, 20 μM α,β-methylene adenosine 5′-diphosphate (AMPCP) was added to the test solution to specifically inhibit 5′-nucleotidase activity [14].

Cell Culture and Ecto-5′-Nucleotidase Activity Determination

Myoblasts were obtained from leg muscles of 11-day-old chick embryos as described previously [9]. Myoblasts were grown for 24 h on gelatin-coated flasks in DMEM Dulbecco's modified Eagle's medium (Flow Laboratories, Irvine, UK) containing 1% kanamycin, 1% streptomycin, 1% penicillin, 1% glutamine, and 1% fungizone and supplemented with 10% fetal bovine serum. Cells were washed twice with $CaCl_2$- and $MgCl_2$-free PBS, pH 7.4 (PBS^-) and harvested with 150 μg/ml of trypsin-TPCK diluted in PBS^-. The reaction was stopped by adding trypsin inhibitor to a final concentration of 10%. After centrifugation (500 *g* for 5 min), cells were resuspended in culture medium to 150,000 cells/ml (low density), and 1 ml was deposited into the substrate-coated wells (0.1% gelatin, laminin or fibronectin at 20 μg/ml each, respectively) of 24-well plates, as reported in [15]; under these conditions, 1 μg of fibronectin or laminin was deposited [15]. To obtain high-density cultures, cells were resuspended at 300,000 cells/ml.

For surface AMPase and ecto-5′-nucleotidase activity determinations, myoblasts were grown for 24 h. Cells were washed twice with 20 mM Hepes, pH 7.4, 0.15 M NaCl, 0.1 mM $MgCl_2$, and 0.2 mM $CaCl_2$ (buffer A) and incubated for 90 min at 37°C with 250 μl/well of buffer A containing 10 μM 5′-AMP and 0.006 μCi [^{14}C]-AMP. Assays were performed either in the presence or the absence of 40 μM AMPCP. At the end of the assay, incubation supernatants of 2 wells were pooled, collected and quickly mixed with 0.15 M $ZnSO_4$ and 0.3 N $Ba(OH)_2$. After centrifugation, the radioactivity in the supernatant was determined. Total cell AMPase and ecto-5′-nucleotidase activities were determined as follows: cells were washed with buffer A and lysed by the addition of 200 μl/well of 20 mM Hepes, pH 7.4, containing 2.5% (w/v) CHAPS and protease inhibitors (aprotinin, leupeptin, pepstatin A 10 μg/ml of each except for PMSF used at a final concentration of 1 mM). After lysis (15 min at room temperature), the same procedure was used to measure AMPase and ecto-5′-nucleotidase activities (see Table I). Intracellular AMPase activity was calculated as the difference between total and surface enzymatic activities. All measurements were made in duplicate and two independent experiments were run using different myoblast preparations. For each substrate, cell number was determined in triplicate using a hemocytometer. 5′-Nucleotidase activity was expressed as μmoles of AMP hydrolyzed per cell for 90 min. The rate of AMP hydrolysis was linear under these conditions. As a control, we checked the possibility of the binding of fetal

bovine serum 5′-nucleotidase to coated wells; no activity was found. In addition, the nonspecific hydrolysis (without cells) was routinely subtracted from each measurement. Furthermore, extracellular matrix protein preparations used in this study were devoid of AMPase activity. The cell uptake of adenosine was less than 3% and can be considered nonsignificant.

Binding of Anti-Ecto-5′-Nucleotidase IgG Polyclonal Antibodies to Cultured Myoblasts

Myoblasts were grown in 24-well plates coated with gelatin, laminin, or fibronectin. The presence of ecto-5′-nucleotidase at the cell surface was studied using a slightly modified version of the binding of IgG described in ref. [16]. Cells were washed twice with 18.2 mM Hepes, pH 7.2, 127 mM NaCl, 5.3 mM KCl (buffer B), and incubated with 5% BSA, 0.02% NaN_3 at room temperature for 45 min to saturate nonspecific sites. Cells were washed with buffer B and incubated with polyclonal antibody directed against ecto-5′-nucleotidase (IgG, 50 μg/well), 0.5% BSA, and 0.02% NaN_3 diluted in buffer B for 45 min. After extensive washing with buffer B containing 2% BSA and 0.1% NaN_3, cells were incubated with ^{125}I-labeled goat antirabbit IgG (2 μCi/well) diluted in buffer B with 0.5% BSA and 0.02% NaN_3 at room temperature for 45 min. Cells were washed twice with buffer B containing 2% BSA and 0.1% NaN_3 before solubilization with 1 N NaOH. Radioactivity in cell lysates was determined in a minigamma counter. For each substrate, the number of cells was determined in triplicate, using an hemocytometer.

Ecto-5′-Nucleotidase Purification and Preparation of Proteoliposomes

In order to obtain enough material to carry out proteoliposome reconstitution experiments, ecto-5′-nucleotidase was isolated from chicken striated muscle. It should be note that the molecular and the enzymatic properties of 5′-nucleotidase obtained from myoblast culture or from striated muscles are similar [17]. Plasma membranes were purified essentially as reported in [18], and ectoenzyme 5′-nucleotidase was purified by affinity chromatography using polyclonal anti-5′-nucleotidase IgGs coupled to Sepharose 4B by their Fc fragment, as recommended by the manufacturer. The enzyme was eluted as described in [19]. Briefly, plasma membrane proteins were solubilized in 20 mM Tris · HCl buffer, pH 7.4, 0.1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM PMSF (buffer C) containing 150 mM NaCl and 60 mM octylglucoside and other protease inhibitors (10 μM Foy; 5 μg/ml each of aprotinin, leupeptin, and pepstatin) for 30 min at 4°C. Insoluble material was pelleted, and the supernatant was mixed gently for 1 h at 4°C with Sepharose 4B equilibrated in buffer C containing 150 mM NaCl and 0.1% Triton X-100. After this preabsorption step, unbound material was incubated with immobilized anti-ecto-5′-nucleotidase IgG for 2 h at 4°C and poured onto a column. The column was washed with 15 ml of buffer C containing 0.5 M NaCl and 0.1% Triton X-100 and then with 15 ml of buffer C containing 0.2 M NaCl and 0.1% Triton X-100. The enzyme adsorbed onto the column was eluted with 50 mM triethylamine (pH 11.5) containing 0.1% Triton X-100. Fractions of 1 ml were collected in tubes containing 200 μl of 1 M Tris · HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM PMSF.

Purified ecto-5′-nucleotidase was incorporated into phospholipid vesicles by mixing for 10 min at 4°C with buffer C containing 150 mM NaCl, 34 mM octylglucoside, and 25 mg of pretreated phosphatidylcholine (Sigma Chemical Co., Munich, Germany). The mixture was dialyzed for 48 h at 4°C against buffer C to remove detergent. Proteoliposomes were pelleted by centrifugation at 100,000*g* for 1 h, resuspended in buffer C, and conserved in a nitrogen atmosphere at 4°C.

Other Procedures

Protein concentrations were determined using Bradford's method [20]. Laminin–nidogen complexes were prepared from EHS mouse tumor as described by Paulsson et al. [21], modified as follows. Briefly, the tumor (60 g) was homogenized in 50 mM Tris · HCl, 150 mM NEM, and 1 mM PMSF (buffer A), and then diluted to 1,200 ml with buffer A. The extract was centrifuged at 4°C for 20 min at 8,000 rpm (GSA rotor, Sorvall RC-SB). The insoluble material was homogenized and washed two times with buffer A. Laminin–nidogen was extracted under agitation in 300 ml of buffer A containing 10 mM EDTA, at 4°C for 1 h. Then laminin-nidogen was centrifuged for 20 min at 4°C at 800 rpm (SS 34 rotor).

This procedure was performed twice and the pooled supernatants were dialyzed at 4°C against 20 mM Tris · HCl, pH 9.6, 2 mM EDTA. Di-

alyzed extracts were chromatographed on a QA-Tris-acryl S column (Pharmacia, Uppsala, Sweden, 4.5 × 20 cm) equilibrated with 20 mM Tris · HCl, pH 9.6, without EDTA (buffer A). The elution was performed with buffer A containing 250 mM NaCl. Next, the laminin–nidogen complexes were precipitated by ammonium sulfate (60% saturation) overnight at 4°C under agitation. The laminin-nidogen complexes were pelleted by centrifugation at 4°C for 30 min at 10,000 rpm (GSA rotor). The precipitate was resuspended in 50 ml of 50 mM Tris · HCl, pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 0.5 mM NEM, 1 mM PMSF, and dialyzed overnight. The laminin-nidogen complexes were purified by chromatography on Agarose-Biogel A5 M (BioRad, 1.5 × 135 cm) and eluted with the same buffer, the complexes (500 μg/ml) were used immediately or stored at −80°C.

Elastase digestion of laminin-nidogen complexes and fractionation of laminin fragments were conducted according to Paulsson et al. [22]. The E'_1 and E_8 fragments were further purified [23] after dialysis against 50 mM sodium bicarbonate by passage over heparin-Sepharose (Pharmacia) pre-equilibrated in the same buffer: E_8 was bound while E'_1 flowed through the column. E_8 was eluted with 0.3 M sodium chloride. E_3 and E_4 fragments were used without further purification. The purity of laminin fragments was analyzed by SDS-PAGE (Fig. 1) as described by Laemmli [24]. Laminin was coupled to cyanogen bromide-actived Sepharose 4B according to the manufacturer's instructions (Pharmacia). The integrity of 5′-nucleotidase was analysed by SDS-PAGE and silver nitrate staining as reported in [25]. Proteoliposomes containing ecto-5′-nucleotidase (20 μl, 3.5 μg) were mixed for 16 h at 4°C with laminin or its fragments at different concentrations as indicated in the results.

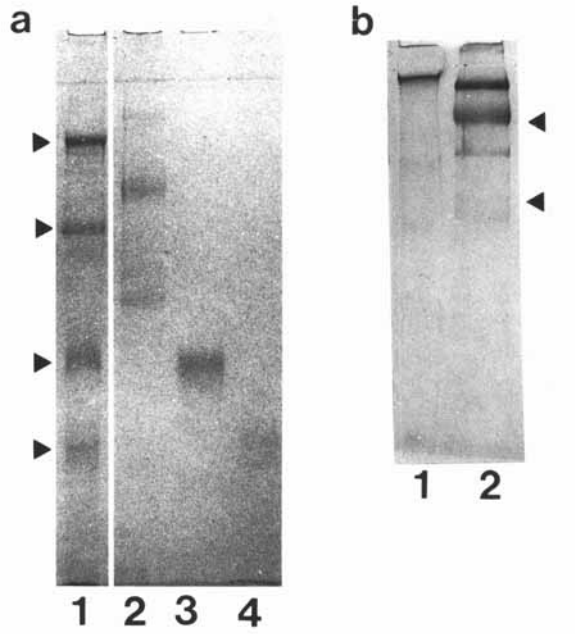

Fig. 1. SDS-PAGE of laminin–nidogen complexes and laminin fragments Electrophoresis was performed in 7% **a:** Gel electrophoresis under nonreducing conditions of E_8, E_4, and E_3 fragments E_8 fragment was obtained by purification on heparin–Sepharose and eluted with 50 mM sodium bicarbonate, lane 2 E_4 and E_3 fragments were used without further purification as indicated in Materials and Methods, lanes 3 and 4, respectively Lane 1 molecular weights of the prestained standards (BioRad) myosin, 205,000, phosphorylase B, 106,000, bovin serum albumin, 80,000, ovalbumin, 49,500 **b:** Lane 1 E_1 fragment under nonreducing conditions E_1 fragment corresponded to the nonretained fraction after purification on heparin–Sepharose Lane 2 laminin–nidogen complex under reducing conditions Proteins were detected by staining with Coomassie brilliant blue R

RESULTS AND DISCUSSION

Effect of Extracellular Matrix on the Activity of Ecto-5′-Nucleotidase From Cultured Myoblasts

In a first series of experiments, we investigated the effect of the composition of extracellular matrix on the myoblast AMPase activity. The experimental conditions used, e.g., concentration of extracellular glycoprotein, were the same as those previously determined for a myoblast adhesion assay [9]. When the cell surface AMPase activity was set at 100% for cells cultured on gelatin, we observed that the AMPase activity on the surface of myoblasts cultured on laminin or fibronectin was 106% ± 2 or 90% ± 2, respectively. To discriminate between ecto-5′-nucleotidase and the activity of non specific phosphatases, the same assay was performed in the presence of AMPCP, a specific and potent inhibitor of ecto-5′-nucleotidase activity. Under these conditions, a specific increase of cell surface ecto-5′-nucleotidase activity was detected when myoblasts were cultured on a laminin substrate (Table I), whereas no such an increase was observed when cells were seeded on a fibronectin or gelatin substrate. This indicates a specific effect of laminin, and not an effect on cell growth as demonstrated by Turnay et al. [26], since identical activation of chick myoblast growth was obtained in the presence of laminin and fibronectin (data not shown). In addition, the specific ecto-5′-nucleotidase activity was in the same range, whether measured on intact cells

TABLE I. Ecto-5′-Nucleotidase (Ecto-5′-N) Activity on Myoblasts Cultured on Laminin, Fibronectin, or Gelatin Substrates*

Matrix	Cell surface ecto-5′-N activity[a]	% of cell surface AMPase activity	Total cell ecto-5′-N activity[a]	% of total AMPase activity
Gelatin	0.37 ± 0.15	19 ± 8	0.57 ± 0.15	16 ± 4
Fibronectin	0.37 ± 0.07	21 ± 4	0.40 ± 0.12	13 ± 4
Laminin	1 ± 0.05	47 ± 3	1.11 ± 0.12	30 ± 3

*Ecto-5′-nucleotidase activity was calculated from total AMPase activity by means of specific inhibition with 40 μM AMPCP. The nonspecific hydrolysis (without cells) was subtracted from each measurement. Laminin and fibronectin were used at 20 μg/ml each and gelatin at 0.1%.

[a]AMPase activity is expressed as micromoles of AMP hydrolyzed X 10^5 per cell in 90 min and correspond to the mean of three separate experiments with triplicate determinations (±SD). The hydrolysis of 5′-AMP was found to be linear for 90 min at 37°C. Surface ecto-5′-nucleotidase was determined on intact nonconfluent cells. Total cell ecto-5′-nucleotidase activity was determined in cell homogenates.

(cell surface activity) or in a cell homogenate (total activity) (Table I). Thus from these data, we deduced that the increased cell surface ecto-5′-nucleotidase activity was not due to laminin-induced mobilization of an intracellular pool of ecto-5′-nucleotidase. Two nonexclusive hypotheses can be advanced from these results to explain the specific increase of cell-surface ecto-5′-nucleotidase activity when myoblasts were cultured on laminin: (1) the higher expression of ecto-5′-nucleotidase on the cell surface; (2) the positive modulation of the enzymatic activity by laminin.

Laminin Induced the Cell-Surface Expression of Ecto-5′-Nucleotidase

To follow the cell-surface expression of ecto-5′-nucleotidase, we evaluated the specific binding of a polyclonal antibody directed against chicken gizzard ecto-5′-nucleotidase. This antibody specifically recognizes the ecto-5′-nucleotidase from chicken striated muscle [10]. The binding of the anti-5′-nucleotidase was monitored as described in [16] (see Materials and Methods).

Regardless of the experiment considered, a higher fixation of antibodies to cells cultured on laminin substrate was observed. All the experiments (1.5×10^5 cells/ml) reported above and in this section were performed on cells seeded at a low density. When confluent cells were used, the effect of laminin was no longer detectable (data not shown). This influence of cell density on the modulation of ecto-5′-nucleotidase activity by the extracellular matrix has also been reported for other cell lines [6]. Interestingly, a similar observation was made for an other ecto-enzyme, galactosyltransferase, which can serve as a laminin-binding protein [16]. However, laminin-induced mobilization of an intracellular pool seemed to be necessary for cell surface expression of galactosyltransferase [16]. Our results indicate that this is not the case for ecto-5′-nucleotidase. This postulate is in line with a previous study that reported a very low intracellular pool of ecto-5′-nucleotidase [27]. Even if laminin triggers the expression of ecto-5′-nucleotidase on myoblasts, we cannot exclude direct stimulation of the AMPase activity by the extracellular matrix. One approach to investigate this possibility was to isolate ecto-5′-nucleotidase from striated muscle and to follow the effect of laminin on its AMPase activity.

Purification of Ecto-5′-Nucleotidase From Striated Muscle and Modulation of Its Enzymatic Activity by Laminin

In order to simulate in vivo conditions for a membrane-bound enzyme, ecto-5′-nucleotidase was reconstituted in phospholipid vesicles. These conditions were previously shown to optimize the modulating effect of extracellular glycoproteins on the AMPase activity of ecto-5′-nucleotidase isolated from chicken gizzard smooth muscle [5]. After detergent extraction, ecto-5′-nucleotidase was purified from chicken skeletal muscle by immunoaffinity chromatography. The activity of the eluted enzyme was completely inhibited by 20 μM AMPCP, a specific inhibitor of the membrane-bound form of 5′-nucleotidase [28] (Fig. 2). A homogeneous enzyme preparation, at least 95% pure (specific activity 15 U/mg protein as determinated spectrophotometrically) was obtained which exhibited an apparent M_r of 79,000 as determinated by SDS-PAGE (Fig. 2, insert). Our preliminary experiments showed that ecto-5′-nucleotidase from chicken striated muscle can be incorporated into liposomes. Like the chicken gizzard enzyme [5], when mixed vesicles were passed through a laminin-Sepharose column, only liposomes containing ecto-5′-nucleotidase in an outside-out orientation were retained, whereas the inside-out oriented liposomes passed unimpeded through the column (as verified by measurement of their

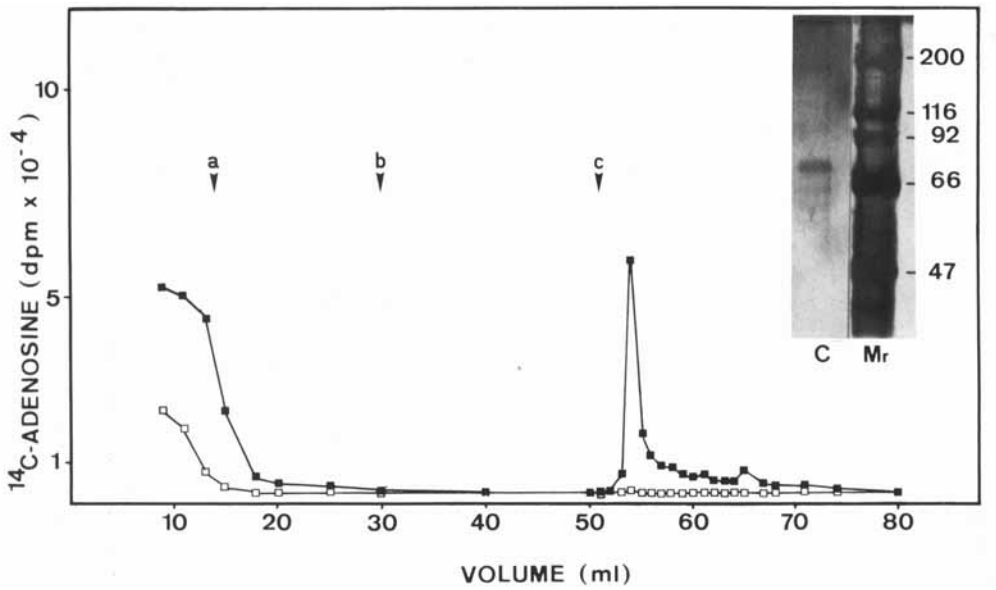

Fig. 2. Immunoaffinity purification of ecto-5′-nucleotidase from chicken striated muscle on immobilized anti-5′-nucleotidase. The column was equilibrated with 20 mM Tris · HCl, pH 7.4, 0.2 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1% Triton X-100, 1 mM PMSF (buffer C) containing 0.15 M NaCl, and sequentially eluted with the buffer C supplemented with 0.5 M NaCl (a); 0.2 M NaCl (b) and finally with 50 mM triethylamine, pH 11.5 (c). Total AMPase activity (■), AMPase activity in the presence of 20 μM AMPCP (□). Insert: SDS-PAGE and silver nitrate staining of the purified ecto-5′-nucleotidase recovered in the triethylamine-eluted fraction (C), specific activity 15 U/mg protein, determined spectrometically at 265 nm, M_r indicates the migration of the standards used.

AMPase activity after addition of detergent) (data not shown). The adsorbed phospholipid vesicles could not be eluted by the addition of up to 1 M NaCl; the AMPase activity could only be eluted from the column after inclusion of 1% Triton X-100. No retention of reconstituted ecto-5′-nucleotidase by immobilized bovine serum albumin (BSA) was found.

We also found that chicken striated muscle ecto-5′-nucleotidase reconstituted in a phospholipid environment interacted more strongly with laminin than its detergent-solubilized counterpart which was eluted from the same column at 0.1 M NaCl (data not shown). A similar binding behavior had been observed for chicken gizzard ecto-5′-nucleotidase before and after its reconstitution in proteoliposomes [4]. A marked stimulatory effect of intact laminin on reconstituted ecto-5′-nucleotidase was noted and was concentration-dependent. A 1.5–2-fold stimulation of the activity was reached when 50–80 nM of laminin were used (Fig. 3). This stimulation was optimized by preincubation for 16 h at 4°C, as described for the chicken gizzard enzyme [5]. Because no AMPase activity was detected in our laminin preparations, we excluded the possibility that this stimulation by laminin was due to trace contamination by AMP-hydrolyzing enzymes. In addition, the effect of laminin on the AMPase activity of ecto-5′-nucleotidase could be suppressed by the addition of polyclonal antibodies directed against the laminin-nidogen complex (data not shown). BSA did not modify the AMPase activity, indicating that the mere increase in protein concentration did not interfere with the ecto-5′-nucleotidase activity determination. In order to locate the laminin domain(s) responsible for the stimulation of the AMPase, we prepared proteolysed fragments E'_1, E_8, E_4, and E_3 of laminin and their effects on the AMPase activity were examined.

Dual Effect of Laminin Fragments on the AMPase Activity of Ecto-5′-Nucleotidase

The assay was run with fragments obtained from the same preparation of laminin that had been used in the experiments illustrated in Figure 3. The results obtained indicate a differential effect of these fragments on the AMPase activity (Fig. 3). Fragment E_8, which is derived from the long arm of laminin, stimulated the AMPase activity in a concentration-dependent manner, reaching a maximal increase of 50–100%. However to obtain this percentage of stimulation, 200 nM of fragment E_8 were required versus 30 nM of laminin. This difference

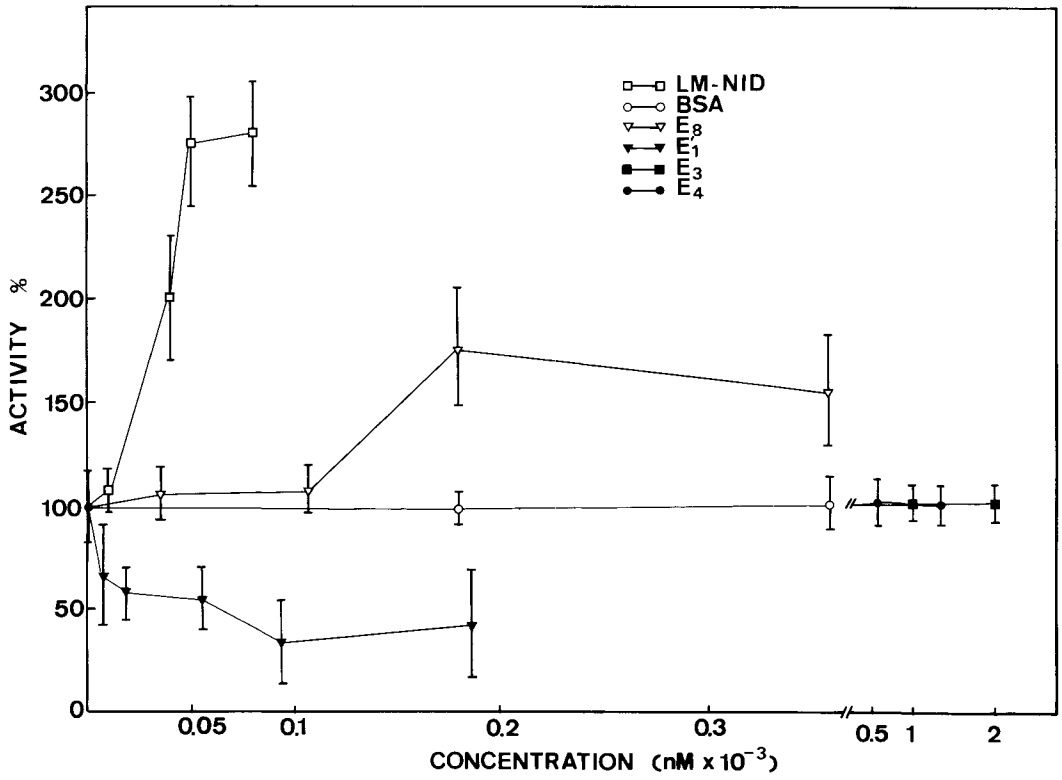

Fig. 3. Influence of laminin and its proteolyzed fragments on the AMPase activity of reconstituted ecto-5'-nucleotidase Ecto-5'-nucleotidase activity was measured using an optical assay system, 100% value of activity (0 03 U) corresponded to 3 5 μg of ecto-5'-nucleotidase incorporated into liposomes The abscissa gives the concentration of proteins or laminin fragments in the test cuvette The results are expressed in nM taking into account the following M_r 1,220,000 (LM–NID laminin–nidogen), 530,000 (E'_1) 280,000 (E_8), 75,000 (E_4), 50,000 (E_3), 68,000 (BSA) Different preparations of laminin and fragments (3 independent batches) were used, and the results presented are the more commonly observed

could be due to (1) a partial denaturation of fragment E_8 during its isolation procedure; (2) modifications of the conformational state of fragment E_8 when included in the laminin molecule or after its isolation by proteolysis; and (3) the participation of other parts of the laminin molecule in the stimulatory effect. These possibilities were consistent with our results, since the stimulatory effect of this fragment depended upon the preparation used. Nevertheless, the results shown in Figure 3 were the more commonly observed. By contrast, fragment E'_1, which consists of three segments originating from the center of the short arms of laminin devoid of its long arm and of the globular domain of chain B1, inhibited the AMPase activity in a concentration-dependent manner, decreasing it by 70% at 100 nM of E'_1. On the other hand, E_3 (which corresponds to the C-terminal of chain A) and E_4 (the globular domain of the short arm of chain B1), used at concentrations up to 2,000 and 1,300 nM, respectively, did not modify the AMPase activity of ecto-5'-nucleotidase. Interestingly, it was recently reported [6] that laminin and fragment E_8 stimulated the AMPase activity of two cell lines regardless of whether assays were performed on intact cells or isolated plasma membranes. However, fragment E_{1-4} also stimulated AMPase activity under the experimental conditions used. This discrepancy between our results and theirs could be due to (1) differences in the tissue origin of the cell used (brain and colon vs. muscle); (2) species, i.e., human vs. chicken; (3) physiological state: tumor vs. normal cells; (4) methodological approach: plasma membrane vs. purified ecto-5'-nucleotidase reconstituted in phospholipid vesicles; and (5) slight differences in the fragment used: E_{1-4} vs. E'_1. Further studies will be needed to elucided the reasons for these differences in the effects of short arm-derived fragments of laminin in the modulation of ecto-5'-nucleotidase activity. How-

TABLE II. Anti-Ecto-5′-Nucleotidase Binding to the Myoblast Cell Surface

	^{125}I-labeled anti-rabbit IgG binding (cpm)	
Matrix	I	II
Gelatin	1,300 ± 50	—
Fibronectin	1,540 ± 55	911 ± 45
Laminin	4,050 ± 20	1,200 ± 135

I and II Two separate experiments using different myoblasts preparations Data are the means of triplicate determinations (±SD) Binding of second antibody ^{125}I-labeled IgG anti-rabbit was reported for 10,000 cells as described under Materials and Methods Laminin and fibronectin were used at 20 μg/ml each and gelatin at 0 1%

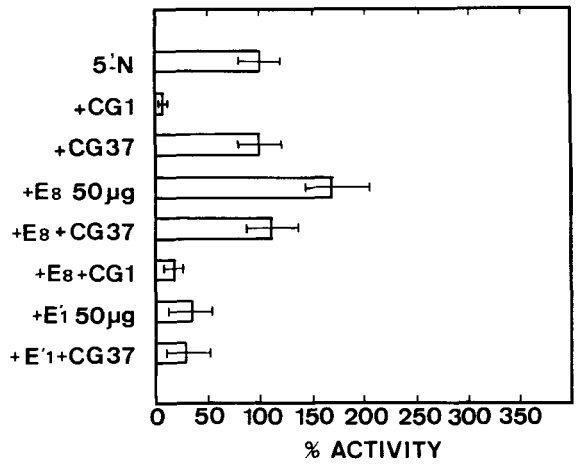

Fig. 4. Effects of fragments E_8 and E'_1, and monoclonal antibodies CG1 and CG37 on the AMPase activity of reconstituted ecto-5′-nucleotidase 100% value of activity (0 03 U) correspond to 3 5 μg of ecto-5′-nucleotidase incorporated into liposomes 5′-N AMPase activity in the absence of effectors Monoclonal antibodies CG1 and CG37 were added at the concentration of 10 μg/ml and 100 μg/ml, respectively

ever, from all these data, it is clear that laminin and its fragments are able to modulate the activity of ecto-5′-nucleotidase in various cell types. To obtain more information on the mechanism by which the laminin fragment modulates AMPase activity, we used characterized monoclonal antibodies directed against chicken gizzard ecto-5′-nucleotidase in a competitive assay.

Effect of Monoclonal Antibodies on the Modulation of Ecto-5′-Nucleotidase Activity by Laminin Fragments

Several monoclonal antibodies directed against the ecto-5′-nucleotidase from chicken gizzard smooth muscle have been produced [11]. Some of these antibodies, such as CG1, strongly inhibit the AMPase activity, while CG37 does not modify this activity, and this differential effect was also seen on the AMPase activity of ecto-5′-nucleotidase from striated muscle (Fig. 4). The inhibitory effect of CG1 cannot be reversed by the addition of 180 nM of fragment E_8. Interestingly, CG37, which is known to displace the ecto-5′-nucleotidase–laminin interaction [8,9], has different actions on the modulating effects of E'_1 and E_8: it reversed the stimulatory effect of E_8, but it was unable to abolish the inhibitory effect of E'_1 (Fig. 4). This result could suggest that E_8 and E'_1 interact with different domains of the ecto-5′-nucleotidase molecule. Hence, a rough estimate can be made of their affinities to skeletal muscle ecto-5′-nucleotidase from the 50% values of their modulating effects: 10 nM E'_1 and 150 nM E_8. Interestingly, the dissociation constant of the intact laminin–nidogen complex from reconstituted chicken gizzard ecto-5′-nucleotidase was recently determined to be 30 nM [29]. The two active proteolyzed laminin fragments (E'_1 and E_8) used in this study were previously shown to elicit different biological effects, including cell adhesion [3], cell differentiation [30], and cell migration [31]. To the best of our knowledge, this paper reports for the first time differential effects of proteolyzed fragments of laminin on the enzymatic activity of an ectoenzyme. These results might also indicate that ecto-5′-nucleotidase can bind to two structurally different domains of laminin. In basement membranes, only the long arm of laminin and not the central cross is thought to be exposed to cells [32]. Therefore, under physiological conditions, laminin would stimulate AMPase by its interaction with the distal end of its long arm. Only in situations such as tissue remodelling, wound healing or metastasis would laminin's central cross be exposed. Indeed, the direct binding of purified chicken gizzard ecto-5′-nucleotidase to the central cross and E_8 under certain conditions has been demonstrated [33]. Therefore, we can hypothesize that the arrangement and composition of the extracellular matrix would modulate the production of adenosine by ecto-5′-nucleotidase. The relevance of such a regulation remains to be explored in different physiological states.

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