Journal of Biochemistry and Molecular Biology, Vol. 33, No. 5, September 2000, pp. 370-378

JOURNAL OF
Biochemistry
and
Molecular Biology

Laminin-1 Phosphorylation by Protein Kinase A: Effect on self assembly and heparin binding

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Received 26 April 2000, Accepted 30 June 2000

Incubation of purified laminin1-nidogen1 complexes with [γ-^{32}P]-ATP in the presence of the catalytic subunit of the protein kinase A (cAMP-dependent protein kinase) resulted in the phosphorylation of the alpha chain of laminin-1 and of the nidogen-1 molecule. Aminoacid electrophoresis indicated that phosphate was incorporated on serine residues. The phosphorylation effect of laminin-1 on the process of self assembly was studied by turbidometry. In these experiments, the phosphorylated laminin-1 showed a reduced maximal aggregation capacity in comparison to the non-phosphorylated molecule. Examination of the laminin-1 network under the electron microscope showed that the phosphorylated sample formed mainly linear extended oligomers, in contrast to controls that formed large and dense multimeric aggregates. Heparin binding on phosphorylated laminin-1 in comparison to controls was also tested using solid-phase binding assays. The results indicated an enhanced heparin binding to the phosphorylated protein. The results of this study indicate that laminin1-nidogen1 is a substrate for protein kinase A *in vitro*. This phosphorylation had an obvious influence on the laminin1-nidogen1 network formation and the heparin binding capacity of this molecule. However, further studies are needed to investigate whether or not this phenomenon could play a role in the formation of the structure of basement membranes *in vivo*.

Keywords: Basement membrane, Extracellular phosphorylation, Heparin, Laminin-1, Protein Kinase A.

Introduction

Laminins, the major cell binding molecules in basement membranes, are cross shaped glycoproteins that consist of three distinct disulfide linked polypeptide chains (α, β, and γ). The prototype laminin, named laminin-1, according to the proposed nomenclature (Burgeson *et al.*, 1994), was isolated from the matrix of the Engelbreth-Holm-Swarm (EHS) tumor (Timpl *et al.*, 1979). Laminin-1 molecules have a characteristic ability to self assemble into laminin networks that interact with other basement membrane molecules as well as with cell surface associated macromolecules. Laminin-1 is copurified with nidogen-1 (entactin) (Paulsson *et al.*, 1987), a 158 kDa protein (Mann *et al.*, 1989). Nidogen-1 acts as a molecular ligand between laminin-1 and other basement membrane structural components (Dziadek, 1995). Via nidogen-1, laminin-1 binds to type IV collagen, as well as to the core protein of perlecan (Dziadek, 1995). Laminin-1 also has the capacity to interact with heparin, a negatively charged variant of heparan sulphate, a glycosaminoglycan that is present in basement membrane and cell surface proteoglycan side chains (Yurchenco and Schittny, 1990, Yurchenco *et al.*, 1985).

The formation of laminin networks is considered to be a major event in the supramolecular organization of basement membranes *in situ* (Yurchenco *et al.*, 1985; Yurchenco *et al.*,1990; Yurchenco and Schittny, 1990). Self assembly of laminin-1 has been attributed to the globular domains at the N-terminal ends of the short arms (Yurchenco and Schittny, 1990) and possibly to the E3 fragment from the globular domain of the long arm of the molecule (G domain) (Charonis *et al.*, 1986). This association is calcium dependent and influenced by the presence of heparin (Yurchenco *et al.*, 1990).

The interaction of laminin-1 with heparin/heparan sulfate proteoglycans is considered to be important for the structure of basement membranes (Yurchenco and Schittny, 1990). The

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major heparin binding site has been also localized in the above mentioned G domain of the molecule (Yurchenco *et al.*, 1990), which is divided into five subdomains G1-G5 (Sung *et al.*, 1997). An active heparin binding site is located in the G4 and proximal G5 subdomains as well as a cryptic heparin binding site (Sung *et al.*, 1993) with a higher affinity to heparin that is localized at the G1 domain. Interestingly, the G domain of laminin-1 contains consensus sequences that could be phosphorylated by protein kinase A (cAMP-dependent protein kinase) (Cohen,1988; Sasaki *et al.*, 1988).

In fact an extracellular role for protein kinase A has been suggested (Shaltiel *et al.*, 1993). This kinase is specifically released together with its co-substrates ATP and Mg^{+2} after physiological stimulation of blood platelets, resulting in the phosphorylation of vitronectin (Shaltiel *et al.*, 1993).

In addition to vitronectin, several extracellular proteins (Mc Guire *et al.*, 1988; Nel *et al.*, 1988; Feige *et al.*, 1989) are substrates of various protein kinases. Furthermore, several studies have shown that the extracellular environment contains various ecto- and exo-protein kinases (Ehrlich and Kornecki, 1987; Ehrlich, 1996). These protein kinases that operate at the cell surface utilize extracellular ATP, which are present in the extracellular milieu of a variety of tissues (Ehrlich *et al.*, 1990; Ehrlich *et al.*, 1998). The laminin1-nidogen1 complex, an extracellular key regulatory structure, may serve as a substrate of such an enzyme and therefore undergo important post-translational modifications, such as phosphorylation-dephosphorylation.

In an attempt to investigate the possible effects of phosphorylation on laminin-1 function, self association and heparin binding were tested after in vitro phosphorylation of the complex by protein kinase A.

Experimental Procedures

Preparation of Laminin1-Nidogen1 The laminin1-nidogen1 complex (kindly provided by Dr. A.S. Charonis, University of Minnesota, Minneapolis MN) was isolated from the mouse Engelbreth-Holm-Swarm tumor, using a modification of the method of Timpl *et al.*, 1979, as described by Palm and Furcht, 1983. The lyophilized preparation was reconstituted in 50 mM Tris pH 7.4, 500 mM NaCl, 10 mM EDTA at a final concentration of about 1 mg/ml. The solution was then dialyzed overnight against 1 M $CaCl_2$, in 50 mM Tris HCl pH 7.4, 500 mM NaCl, and subsequently overnight against 50 mM Tris HCl pH 7.4, 500 mM NaCl. The solution was then stored in aliquots under liquid nitrogen until use. A weight extinction coefficient of A 1% 1 cm 280 = 8,3 was used to calculate the laminin-1 concentrations (Mc Carthy *et al.*, 1983). Molecular weight determinations, and verification of the integrity and purity of the isolated laminin-1, were tested by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described below.

Phosphorylation by Protein Kinase A The laminin1-nidogen1 preparation was phosphorylated with [γ-^{32}P]-ATP (110 gBq/mmol) (Amersham International plc). In 125 µl [γ-^{32}P]-ATP (0,27 mM) 175 µl of a 25.7mM magnesium acetate water solution was added. For non-radioactive ATP experiments a similar solution was utilized (Sigma-Aldrich Chemie GmbH).

Protein kinase A, catalytic subunit, was purchased from Sigma. The lyophilized powder was reconstituted to a concentration of approximately 50 µl/ml in distilled water containing 6 mg/ml dithioerythrytol.

The phosphorylation mixture contained a 250 µl 0.05 M citrate phosphate buffer pH 5.8, 250 µl laminin1-nidogen1 solution (1 mg/ml), 100 µl ATP solution (0,27 mM ATP, 15 mM magnesium acetate) and a 20 µl protein kinase solution (50 µl/ml protein in 6 mg/ml dithioerythrytol). The final pH of the mixture was 6.5. Phosphorylation was performed by incubation at room temperature for 30 minutes and the reaction was stopped either by addition of Deoxycholate-Trichloroacetic acid, as described below, or by chilling in ice. As positive controls, we used partially dephosphorylated casein and histone (Sigma-Aldrich Chemie GmbH). Samples in which the enzyme was omitted from the phosphorylation mixture served as negative controls. As an additional negative control we used samples where the 0.1 mg/ml of type III protein kinase inhibitor (Sigma-Aldrich Chemie GmbH) was added.

The phosphorylated protein was precipitated by Deoxycholate-Trichloroacetic acid. For this reason 100 µl 0.17% of sodium deoxycholate was mixed with each sample. After incubation at room temperature for 10 minutes 100 µl of a 100% Trichloroacetic acid solution was added to each sample. The samples were then mixed and centrifuged at 3000 g for 20 minutes. The pellet was washed with ice cold acetone and resuspended in 20 µl NaOH 0.1 N.

Five µl of each sample was used for dot blot autoradiography on a 0.45 µm cellulose nitrate membrane (Whatman). Additionally, the radioactivity of another five microliters of each sample was measured in a liquid scintillation counter (1209 Rackbeta-LKB). For the estimation of the stoichiometry of the phosphorylation reaction the radioactivity of 5 µl aliquots of 1 : 1000 and 1 : 10000 dilutions of the initial γ-^{32}P ATP solution in 0.1 N NaOH was also measured in the liquid scintillation counter. The molecular ratio of phosphate incorporate to laminin-1 was then calculated according to the specific activity of [γ-^{32}P]-ATP, the amount of laminin-1 and radioactivitity measured in each sample.

SDS Electrophoresis and Autoradiography Phosphorylated samples were also analyzed by SDS polyacrylamide gel electrophoresis according to the method of Laemmli (Laemmli,1970) on a 6% slab gel with a 2% stacking gel. Before electrophoresis, the samples were incubated at 100°C for 3 minutes in 10 µl of the sample buffer (3% SDS, 0.08 M Tris HCl pH 6.8, 15% glycerol, 0.01% bromophenol blue and

5% v/v β-mercaptoethanol). After electrophoresis the gels were developed with coomassie blue staining and dried on a gel dryer apparatus. The radioactivity of the protein bands was then detected using a Molecular Dynamics Phosphorimager apparatus.

Phosphoamino Acids Analysis In order to detect phosphorylated aminoacids, after phosphorylation and precipitation the pellet was resuspended and hydrolyzed under a vacuum in 50 μl of 6 N HCl for 1 h at 100°C. The sample was then dried in a speed vac apparatus (Savant) and dissolved in 10 μl of a pH: 3.5 buffer (10 : 100 : 1890 pyridine : acetic acid : H_2O) containing 0.1 mg unlabell phosphoserine and phosphothreonine (Sigma-Aldrich Chemie GmbH). The sample was then subjected to electrophoresis on Whatman paper for 50 minutes at 480 V. Phosphoamino acids were visualized with ninhydrin and radioactivity was detected by autoradiography.

Western Blotting Analysis using Enhanced Chemiluminescence (ECL) A non-radioactive phosphorylated sample was used for the direct detection of phosphoserine using a specific anti-phosphoserine monoclonal antibody (Sigma) and ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Five μl from the phosphorylation mixture were placed on the membrane, which was then blocked for 2 hours with 5% Bovine Serum Albumin (BSA) that was dissolved in a Phosphate Buffered Saline (PBS/ 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl) pH 7.5. The primary antibody was applied in a 1 : 500 dilution in PBS and incubated overnight. The membrane was then washed with PBS-Tween20 and the horseradish peroxidase-labelled secondary antibody was then added in a 1 : 3000 dilution and incubated for one hour. ECL detection reagents were then added to the membrane and incubated for one minute. The film used to detect light emitting from the peroxidase reaction was exposed for 15 seconds. Histone was used as a positive control (Sigma-Aldrich Chemie GmbH). Samples from which the enzyme was omitted from the phosphorylation mixture served as negative controls.

Turbidity Measurements For turbidity formation estimation, samples of phosphorylated laminin1-nidogen1, as well as negative controls, dialyzed overnight at 4°C against PBS at 4°C. Laminin-1 concentration was then estimated by the measurement of absorbance at 280 nm, as described previously. Aliquots in PBS pH 7.5, containing 140 μl/ml phosphorylated laminin-1 or controls, with an equal concentration of laminin-1, were incubated for 60 min in quartz cuvettes in a water bath at 37°C. The change in absorbance at 360 nm was monitored in a Shimadzu UV1020 spectrophotometer every 10 minutes.

Electron Microscopy The pattern of the polymerization of laminin-1 was examined under the electron microscope using a negative staining technique (Hayat, 1986). For this reason, after the development of turbidity, as described in the previous section, one small drop of each sample was placed on a formvar-carbon coated 200 mesh copper grid (Ted Pella Inc Redding CA). The excess amount was removed by the edge of a filter paper leaving a thin monolayer. A drop of a 2% uranylacetate aqueous solution was placed onto the grid for 60 seconds. The excess amount of the liquid was removed by a filter paper and the grid was allowed to dry at room temperature. The grids were examined with a JEOL transmission electron microscope operating at 60 KV. We examined 47 optic fields from each case at a final magnification of 66000. The amount of linear extended oligomers were evaluated by counting each network formation with a diameter of less than 82 nm (in our prints <0.57 cm). This maximum diameter was calculated by taking into account the three arms interaction model of the laminin network (Yurchenco, 1994) and a length for each short arm of about 34 and 48 nm (Bruch *et al.*, 1989). A total area of 326 μm^2 (forty seven optic fields) from each case was examined by two independent researchers.

Solid Phase Heparin Binding Assay The binding of [^{3}H] heparin (American Radiolabelled Chemicals inc.) to the phosphorylated laminin-1 in comparison to controls was estimated by direct binding assays performed in 96 well polystyrene plates (Skubitz *et al.*, 1988). Phosphorylated laminin1-nidogen1 or negative controls, were diluted in 50 mM Tris HCl pH 7.4 to a final concentration of 40 μg/ml. Fifty μl of these samples were placed in each well of a 96 well polystyrene plate (Kartel SPA). The plate was left uncovered until dry, usually overnight, at room temperature under vacuum in a desiccator containing silica gel.

In pilot experiments, the wells with the bottoms covered with laminin1-nidogen1 were washed three times with 200 μl of 10 mM Tris-HCl-HEPES buffer pH 6.8 containing 0.5% Triton X-100 and then twice with distilled water. Proteins attached to the plastic well were solubilized with 20 μl of 0.5 M NaOH 1% SDS and incubated at 60°C for 30 min. Protein concentration was then estimated using the enhanced protocol of the Bicinchonic Acid method (Peirce) (Sorensen and Brodbeck, 1986).

For heparin binding estimation, 96 well polystyrene plates (Kartel SPA) were covered with phosphorylated laminin1-nidogen1 or control samples as described above. Dry coated wells were filled with 200 μl of 2 mg/ml bovine serum albumin in 10 mM Tris-HCl-HEPES, 0.01% $CaCl_2$ buffer pH 6.8 and incubated for 2 hours at 37°C. After this treatment, the buffer was removed from each well and 50 μl of various [^{3}H]-Heparin concentrations in the same buffer, in the presence or absence of 100 fold-excess of unlabelled heparin (Sigma-Aldrich Chemie GmbH) were added to the wells. After incubation at 37°C for three hours the unbound heparin was removed by washing three times with 200 μl of 10 mM Tris-

HCl-HEPES buffer pH 6.8 containing 0.5% Triton X-100. Attached tritiated heparin was solubilized by incubation with 100 µl of prewarmed 0.05 N NaOH and 1% SDS for 30 min at 60°C. The radioactivity of the solution was then counted in the liquid scintillation counter. Specific heparin binding was defined as the amount of tritiated heparin bound in the absence of unlabelled heparin minus the amount of tritiated heparin bound in the presence of 100-fold excess non-radiolabelled heparin. In all cases the non-specific binding was less than 150 cpm.

In addition, for testing laminin's specific affinity for heparin we repeated the above described assay using a constant amount of [^{3}H]heparin that was mixed with chondroitin/dermatan sulfate (Sigma-Aldrich Chemie GmbH). We used this sulfrated polysaccharide as a possible competitor of heparin.

Results

***In vitro* phosphorylation of laminin1-nidogen1 complex by Protein Kinase A** In order to test if laminin-1 is a substrate of protein kinase A, purified laminin1-nidogen1 complex from a EHS mouse tumor was incubated for 30 minutes at room temperature in the presence of the catalytic subunit of protein kinase A and [γ-^{32}P]-ATP. A similarly treated laminin-1 mixture, where the enzyme was omitted, served as a negative control and dephosphorylated casein as a positive control. As an additional control, samples, where a protein kinase A inhibitor (Walsh *et al.*, 1971) was added, were used. The radioactivity of the samples was detected by dot blot autoradiography (Figure 1A) and by beta counter measurements (data not shown). The results indicated that the phosphate was incorporated into both substrates (laminin1-nidogen1 and casein) in the samples where the enzyme was present and no inhibitor was added.

According to the specific activity of [γ-^{32}P]-ATP, the amount of laminin-1 and the radioactivitity measured in each sample of the stoichiometry of the reaction was estimated to be 2.9 ± 0.3 mol of phosphate per mol of laminin-1.

The samples were also analyzed on SDS polyacrylamide gels under reducing conditions. Coomassie blue staining of the gels showed two main band. One was at about 400 kDa, corresponding to the alpha chain of laminin-1 and one was at about 200 kDa, corresponding to the beta and gamma chains of laminin-1. A faint band at about 150 kDa was also present, corresponding to the known molecular weight of nidogen-1 (Mann *et al.*, 1989) (Figure 1B). Polyacrylamide gels were dried and exposed on a phosphor imager apparatus. Development of the phosphor imager screen picture showed clearly phosphorylation on a band at about 150 kDa, corresponding to the nidogen-1 chain. The alpha chain of laminin-1 at 400 kDa was also phosphorylated. A weak phosphorylation signal was also observed at the position of

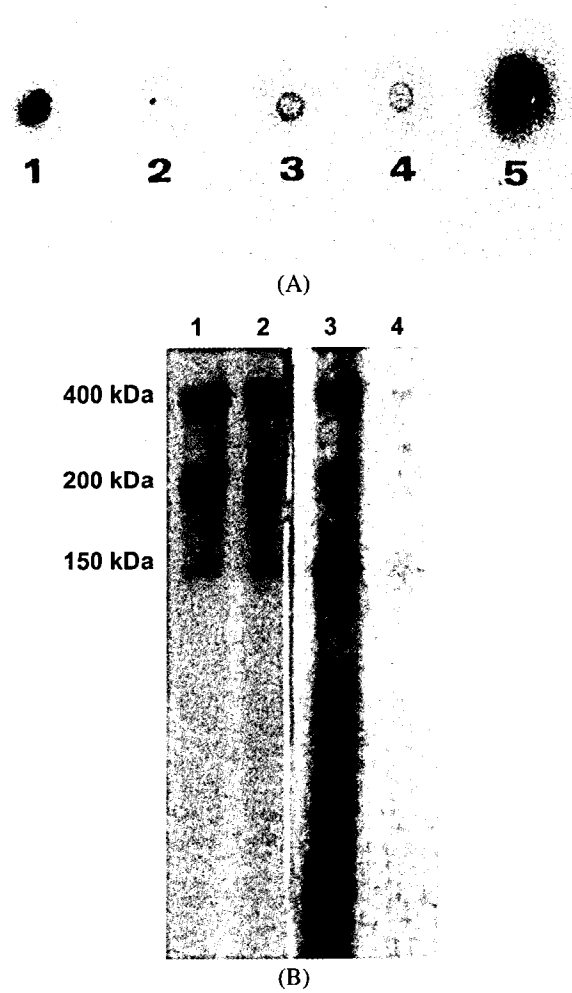

Fig. 1. (A) Dot blot autoradiography after phosphorylation. Purified Laminin-nidogen was incubated with protein kinase A and [γ^{32}P]-ATP. Dephosphorylated casein served as a positive control. Samples where protein kinase A was omitted served as negative controls. As an additional negative control, samples where protein kinase was inhibited by a specific inhibitor were also used. After phosphorylation the protein was precipitated and resuspended in 20 µl 0,1 N NaOH as described under "Experimental Procedures". Five microliter of each sample were used in each dot blot position. The numbers given stands for the following mixtures: (1) phosphorylated laminin-nidogen complex, (2) no enzyme in laminin's phosphorylation mixture, (3) the inhibitor was added in laminin's phosphorylation mixture, (4) casein phosphorylation mixture where the inhibitor was added (5) phosphorylated casein. (B) SDS electrophoresis and autoradiography of the phosphorylated samples. Phosphorylated samples and controls were analyzed by SDS electrophoresis on 6% polyacrylamide gel. After Coomassie blue staining phosphorylation was detected by autoradiography on a phosphorimager apparatus. Lanes 1-2: coomassie blue stained SDS gel. Lanes 3-4: Autoradiography. Lanes 1 and 3: Phosphorylated Laminin-Nidogen. Lanes 2 and 4: Negative control where protein Kinase A was omitted.

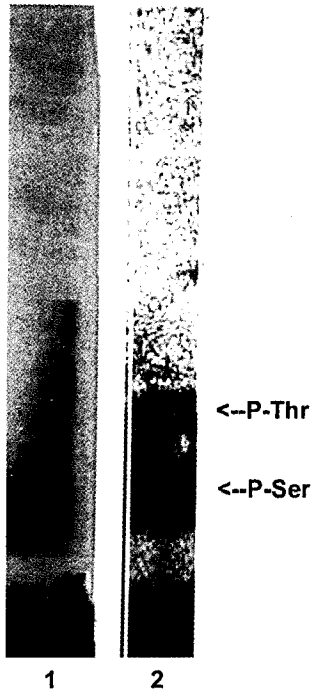

Fig. 2. Phosphoamino acids analysis. Purified Laminin-nidogen was incubated with protein kinase A and [γ^{32}P]-ATP and the protein was precipitated and resuspended as described under "Experimental Procedures". The pellet was then hydrolyzed in 50 µl of 6 N HCl for 1h at 100°C. The sample was dried in a speed vac apparatus, dissolved in 10 µl of electrophoresis buffer and then subjected to electrophoresis on a Whatman paper. Phosphoserine was detected by autoradiography and standards were ninhydrin stained. Lane 1: Autoradiogram of lane 2. Lane 2: Ninhydrin stain of ^{32}P-labelled laminin mixed with phosphoamino acid standards.

beta and gamma chains of laminin-1 (200 KDa). Thus, the phosphorylation of these chains can not be excluded as well. No signal was observed on the lane of the negative control where protein kinase was omitted. (Figure 1B).

In order to exclude the possibility of non-covalent ATP or phosphate binding on laminin1-nidogen1, the phosphorylated sample was hydrolyzed and analyzed by electrophoresis on Whatman paper (Figure 2). Phosphorylated serine was detected with this analysis, providing evidence that protein kinase A phosphorylates laminin1-nidogen1 mainly on serine residues.

In order to investigate if phosphorylation influences laminin-1 functional properties, larger amounts of the protein were phosphorylated using non radioactive ATP (Sigma) according to the above described conditions. Serine phosphorylation was verified in these samples by Western blotting analysis on dot blots using a specific antiphosphoserine monoclonal antibody (Figure 3).

Effect of phosphorylation on laminin-1 self association

The alpha chain of laminin-1 is involved in the process of laminin-1 self association (Yurchenco *et al.*, 1985). Since, as the above experiments indicated, the alpha chain of laminin-1 can be phosphorylated by protein kinase A, the effect of this event on laminin-1 self association was tested.

Phosphorylated laminin-1, as well as the controls, were

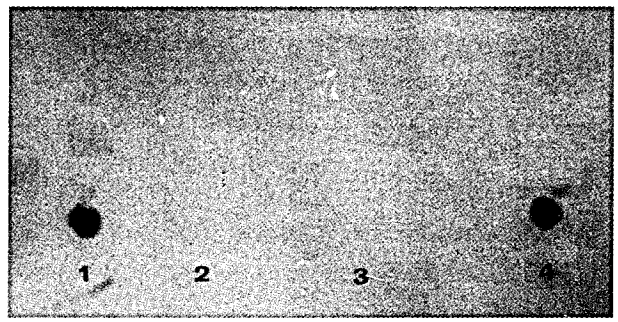

Fig. 3. Detection of phosphoserine by a monoclonal phosphoserine antibody. Laminin was phosphorylated using non-radioactive ATP. Non-phosphorylated laminin treated the same way, except that protein kinase A was omitted from the phosphorylation mixture served as a negative control whereas histone served as a positive control. Five µl of each sample were placed on nitocellulose membrane and incubated with a specific antiphosphoserine monoclonal antibody. Horse raddish peroxidase-labelled secondary antibody was then added. Light ommited from the substrate oxidation reaction was detected using a photographic film. (1) Phosphorylated laminin, (2) laminin's mixture where protein kinase A was ommited, (3) histone's mixture where protein kinase A was ommited and (4) phosphorylated histone.

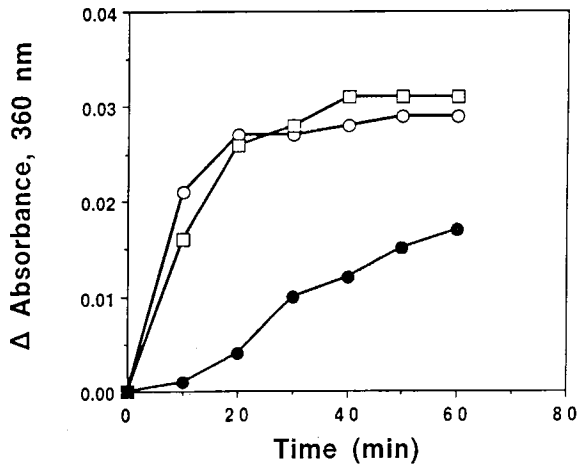

Fig. 4. Turbidity formation of phosphorylated laminin and control. After phosphorylation with non radioactive ATP the samples were dialyzed against phosphate buffered saline (PBS) (4°C) and then incubated at 37°C. The change in absorbance at 360 nm was recorded every 10 minutes and plotted against time. All the samples contained the same protein concentration (140 µl/ml). Phosphorylated laminin polymerized less rapidly and produced significantly lower turbidity than the control. ●-●: Phosphorylated laminin, ○-○: control without ATP, □-□: control without protein kinase A.

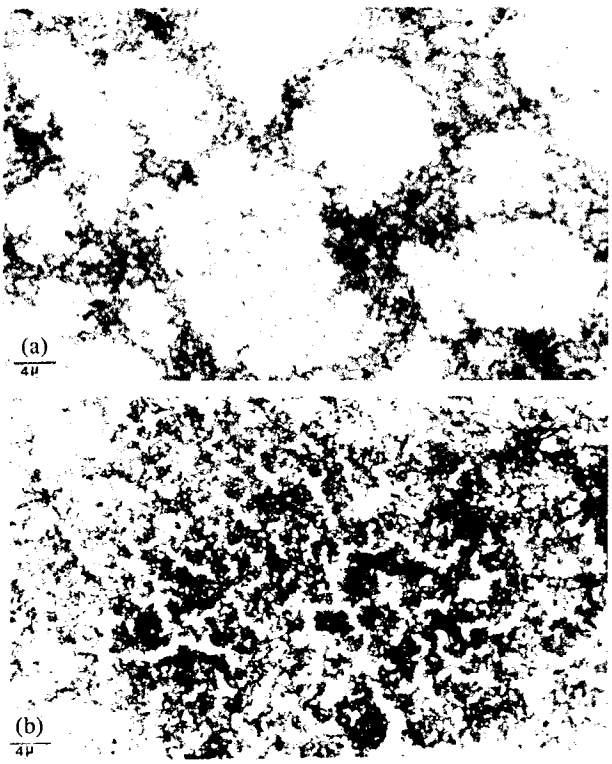

Fig. 5. Network appearance of Phosphorylated laminin and control under Electron Microscope. After turbidity experiments (figure 5) phosphorylated laminin and controls were examined under the electron microscope using a negative staining technique as described under "Experimental Procedures". Forty seven optic fields (a total area of 326 μ^2) from each case was examined. (a) Phosphorylated laminin. (b) Non-phosphorylated laminin

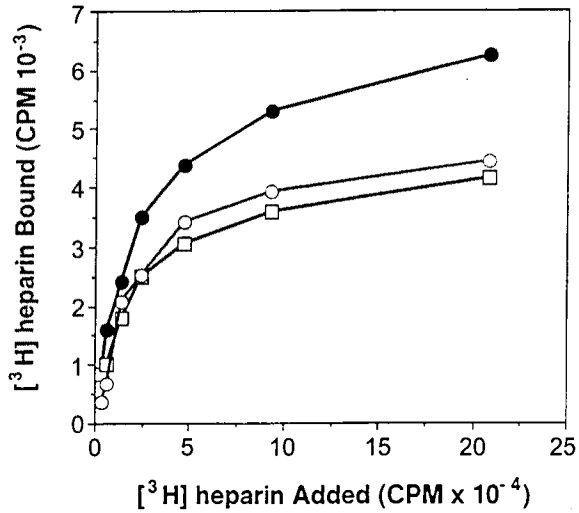

Fig. 6. Binding of [^{3}H] heparin to phosphorylated or control laminin coated into 96-well plates. 96 well plates were coated either with phosphorylated laminin or with controls (about 1.8 μg/well). Increasing concentrations (0.5-33.3 μg/ml) of [^{3}H] Heparin (specific activity about 290 cpm/ng) were added to triplicate wells and tritiated heparin was allowed to bind for 3h by incubating at 37°C. At the end of the incubation time, unbound [^{3}H] heparin was removed by washing. The non-specific binding was less than 150 cpm. Total cpm of bound tritiated heparin were plotted against total cpm of added [^{3}H] heparin (highest added concentration was 33.3 μg/ml). ●-●: Phosphorylated laminin, ○-○: control without ATP, □-□: control without protein kinase A.

incubated at 37°C and absorbance at 360 nm was monitored every 10 minutes. The change in absorbance was plotted against time and compared with the change in absorbance of similarly treated samples where ATP or protein kinase A were omitted from the phosphorylation mixture (controls), as described under "Experimental Procedures". Phosphorylated laminin-1 polymerized less rapidly and reached a significantly lower plateau of absorbance in comparison to controls (Figure 4).

In order to visualize the pattern of polymerization after turbidity formation, small drops of each sample were examined under the electron microscope using a negative staining technique, as described under "Experimental Procedures". A total area of 326 μ^2 from each case was evaluated. A qualitative difference in the polymerization pattern of the phosphorylated laminin-1, in comparison to the control, was obvious. (Figure 5). Non-phosphorylated laminin-1 was polymerized in large multimeric aggregates in which linear extended oligomers were rare (b). On the other hand, phosphorylated laminin-1 formed more linear extended oligomers with few multimeric aggregates (a). In an attempt to quantitate this morphological difference, the amount of obvious oligomers were counted by two independent researchers. A total of 12593 linear extended oligomers were counted on the prints taken from phosphorylated laminin-1 and only 2931 on the prints taken from controls.

Effect of laminin-1 phosphorylation on heparin binding

Heparin binding on laminin-1 was closely related with laminin-1 self association (Yurchenco and Schittny, 1990). Therefore, the following experiments examined the effect of phosphorylation on heparin binding to laminin-1. Heparin binding to laminin-1 was tested using solid phase binding assays. In these assays a constant amount of phosphorylated laminin-1 or controls was placed in the wells of polystyrene plates. In order to exclude if the differences between the phosphorylated laminin-1 and controls could be attributed to a different amount of protein bound to the polystyrene plate, protein binding to the plastic was tested. Phosphorylated laminin-1 or controls were dried onto the polystyrene plates and after washing the plates in a similar manner as in assays, the amount of bound protein was estimated using a sensitive assay (Bicinchonic Acid method), as described under "Experimental Procedures". The amount of protein bound to the plastic did not differ between phosphorylated laminin-1 and the control and was estimated to be 1.8 ± 0.3 μg per well.

In order to test heparin binding to phosphorylated laminin-1

in comparison to the controls, various concentrations of [^{3}H]-heparin were added to the polystyrene wells, the coated with a constant amount (1.8 ± 0.3 μg) of phosphorylated laminin-1 or controls. After incubation at 37°C for three hours the amount of bound [^{3}H]-heparin was measured as described under "Experimental Procedures". In these solid phase experiments [^{3}H]-heparin bound to the phosphorylated laminin-1 and as well the controls. However, a significantly higher amount of [^{3}H]-heparin bound, under similar conditions, to the phosphorylated laminin-1 in comparison to the controls (Figure 6). This binding was specific since it was saturable and could be displaced by an excess of heparin, but not chondroitin sulfate (data not shown).

Discussion

Laminin-1 is involved in two general types of interactions; besides its ability to bind to the other basement membrane macromolecules, it interacts with various cell surface molecules, including integrins, the 67 kD metastasis associated receptor and the cell surface sulfated glycolipids (Paulsson, 1992). These interactions with cell surface molecules influence the morphology, growth, adhesion and migration of various cell types. Laminin-1 also has the ability to bind with heparin (Yurchenco *et al.*, 1990). Since heparin is a variant of heparan sulphate, like side chains that are present in basement membrane and on cell surface, this interaction is considered to be rather significant (Mecham, 1991).

The results of this study indicate that laminin-1 and nidogen1 are in vitro substrates of protein kinase A. This enzyme transfers phosphate groups from ATP molecules to several proteins at serine or threonine residues (Cohen, 1988). Physiological substrates for protein kinase A contain two or more adjacent basic aminoacids N terminally to the phosphorylatable residue (Cohen, 1988). According to the known sequence (Sasaki et al., 1988), the alpha chain of laminin-1 contains the sequences RRS (329-331), RKIRS (1832-1836), RKGVS (2430-2434) that could be phosphorylated by protein kinase A. The RKGVS sequence belongs to the carboxyterminal domain (Domain G: residues 2110-3060). The sequence RKIRS belongs to the helical domain of laminin-1 (domains I and II: residues 1538-2109) and the sequence RRS belongs to the cysteine rich domain V (residues 252-495). Additionally, several other sequences containing serine or threonine adjacent to aminoterminally basic aminoacids, such as KKS (2241-2243), KMRTS (2621-2625), KGRT (2766-2769) and KRKAFMT (2791-2797), should not be excluded, as previous studies have shown, since a variety of sequences can serve as a phosphorylation substrate of protein kinase A (Cohen, 1988, Walsh and Van Patten, 1994). Known substrates with sequences different than consensus around the target serine also interact with the protein kinase A catalytic subunit (Walsh and Van Patten, 1994).

As mentioned previously, a weak phosphorylation signal was also observed at the possition representing the beta and gamma chain of laminin-1 (200 KDa). Thus phosphorylation of these cannot be excluded. Indeed, their known sequence (Pikkarainen *et al.*, 1987; Pikkarainen *et al.*, 1988; Hunder *et al.*, 1989; Ikoren *et al.*, 1989; Kallunki *et al.*, 1991) reveals some possible phosphorylation sites on both chains. Specifically, the following sequences of beta chain may serve as phosphorylation substrates of protein kinase A: RKVS (1225-1228), KRAS (1279-1282) as well as KRLVT (482-486), KKT (1687-1689) and KKT (1707-1709). As for the gamma chain, the following sequences represent possible protein kinase A phosphorylation sites.: RKVS (1556-1559) as well as RKT (164-166), RRDT (585-588), RRET (709-712), KGKT (1327-1330) and RKT (1593-1595).

Laminin-1 is a large molecule with few possible phosphorylation sites on it. Thus, a weak phosphorylation was expected. For this reason radioactivity was detected either on a larger amount of protein after precipitation, or by longer exposure times. However, this fact does not diminish the importance of the phosphorylation of laminin-1 since several proteins undergo significant modifications of their properties due to phosphorylation of as few as one serine residue per molecule (Walsh and Van Patten, 1994; Brown and Cori, 1961). The possibility that the signal observed in autoradiography experiments is due to non-covalent ATP binding on laminin-1 can be excluded, since phosphate was incorporated to laminin-1 only in the presence of protein kinase and not in samples where the enzyme was omitted, or in samples where the specific inhibitor of PKA was added. Furthermore, these data were verified by aminoacid electrophoresis and western blotting analysis with the use of a specific antiphosphoserine monoclonal antibody.

As far as the properties of the molecule are concerned, phosphorylation promoted heparin binding to laminin-1 and partially inhibited laminin-1 polymerization.

The major heparin binding site on laminin-1 was localized in the globular domain of the long arm of the molecule (G domain) (Yurchenco *et al.*, 1990). This domain is divided into five subdomains (Sung *et al.*, 1993) and contains an active heparin binding site that is located in the G4 and proximal G5 subdomain and a cryptic heparin binding site (Sung *et al.*, 1993) with a higher affinity to heparin that is localized at the G1 domain. Interestingly, the G domain of laminin-1 contains sequences that could be phosphorylated by protein kinase A (Titani *et al.*, 1975; Cohen, 1988; Sasaki *et al.*, 1988). It may be suggested that phosphorylation of the laminin-1-nidogen1 complex leads to conformation changes of the laminin-1 molecule that expose the above mentioned cryptic heparin binding site. A similar mechanism has been proposed for vitronectin (Chain *et al.*, 1990). Vitronectin, an extracellular heparin and cell binding protein, can be phosphorylated by protein kinase A that is released from platelets along with ATP and Mg^{++} during platelet stimulation (Bruch *et al.*, 1984; Mc Guire *et al.*, 1988). Phosphorylation alters the function of vitronectin since it reduces significantly its ability to bind to

the inhibitor 1 of the plasminogen activator (Shaltiel *et al.*, 1993). Interestingly, the phosphorylation site of vitronectin is located near its heparin binding domain (Mc Guire *et al.*, 1988) and heparin enhances vitronectin phosphorylation by exposing its phosphorylation site (Chain *et al.*, 1991). A basic fibroblast growth factor provides another example of an important extracellular molecule that is a substrate for protein phosphorylation. This phosphorylation enhances the basic fibroblast growth factor's affinity with its receptor (Feige and Baird, 1989). It is interesting to note that the phosphorylation of the basic FGF can also be modulated by heparin (Feige and Baird, 1989). It is also important to mention that collagen IV, which is a major component of the basement membrane, is also phosphorylated by the protein kinase A (Revert *et al.*, 1995). As shown, the human a3 chain of collagen IV contains a five residue motif (KRGDS) at the amino-terminal region that serves as the substrate of cAMP-dependent protein kinase *in vitro*. As indicated by the above researchers this phosphorylation may play an important role in the process of cell attachment on basement membrane.

In addition to the alpha chain of the laminin-1, the nidogen-1 molecule was also phosphorylated by the protein kinase A. A possible phosphorylation site on this molecule, according to the known sequence of the molecule [database: Swiss-Prot Updates (33.0+, 4/19/96)] and protein kinase A specificity (Cohen,1988, Titani *et al.*,1975), is KKDES (residues 208-212) that belongs to the larger amino-terminal globular domain, a region where collagen IV and perlecan bind to the nidogen-1 molecule (Aumailley *et al.*, 1989; Mann *et al.*, 1989; Dziadek, 1995). In addition to the above mentioned sequence, another two may be considered as possible phosphorylation sites: RTRS (347-350) and KKT (963-965).

A main characteristic of the structure of basement membranes is the formation of a backbone that is constituted by two molecular networks. The type IV collagen network and the laminin-1 network. These two networks are connected mainly by the nidogen-1 molecules (Dziadek, 1995). The influence of the phosphorylation of nidogen-1 or/and laminin-1 and collagen type IV on the interactions between these molecules and basement membrane formation will be the scope of further investigations.

Another finding of the present report is the influence of phosphorylation on laminin-1 self association. Self assembly of laminin-1 has been attributed to globular domains at the N-terminal ends of the short arms (Yurchenco and Schittny, 1990) and possibly to the E3 fragment from the globular domain of the long arm of the molecule (G domain) (Charonis *et al.*, 1986). Phosphorylation at the sequence RRS, belonging to the cysteine rich domain V (residues 252-495), adjacent to the amino terminal globule (domain VI) of laminin-1, may play a role in this phenomenon. Previous work indicated a regulatory effect of heparin on laminin-1 polymerization *in vitro* (Kouzi-Koliakos *et al.*, 1989; Yurchenco *et al.*, 1990). This effect, combined with the possibility of protein phosphorylation, may endow laminin-1 with increased structural flexibility.

A modification of several functions of laminin-1 after *in vitro* phosphorylation by the protein kinase A has been reported in this paper. However, further research is required in order to reveal the exact phosphorylation site/s on laminin's molecule, as well as the biological significance of the phenomenon.

Acknowledgments The authors are indebted to Professor Aris Charonis for providing EHS tumor laminin and for his helpful discussions and comments during the course of the study. The authors would also like to thank Ms Katerina Paschalidou for excellent technical assistance.

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