Involvement of the YIGSR sequence of laminin in protein tyrosine phosphorylation

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Abstract We have examined the mechanism of signaling by the 67 kDa YIGSR binding protein of laminin and its properties in neuroblastoma cells. Ligand displacement analysis showed that the interaction with the C(YIGSR)$_3$-NH$_2$ peptide amide is of intermediate affinity (1.5×10^{-7} M). Cross-linking experiments with sulfo-MBS detected an additional protein with a molecular mass of 116 kDa that binds the YIGSR sequence. Incubation of neuroblastoma cells with C(YIGSR)$_3$-NH$_2$ peptide amide or antibody directed against the 67 kDa laminin binding protein induces tyrosine phosphorylation of proteins with a molecular mass ranging from 115 to 130 kDa and another heterogeneous protein group of 32 kDa.

Key words: Cell adhesion; Glycosyl phosphatidylinositol anchor; Laminin; Neuroblastoma; 67 kDa laminin binding protein; Signal transduction

1. Introduction

The glycoprotein laminin is a prominent constituent of the basement membrane and serves as an adhesion protein for a variety of cell types, especially epithelial and neuronal cells [1,2]. Laminin is also involved in cell proliferation, differentiation [3], tumor cell invasion and metastasis [4,5]. Various sites in the laminin molecule are responsible for distinct functions and have been identified using both proteolytic fragments and synthetic peptides [6]. For example the IKVAV sequence close to the carboxyl-end of the long arm in the α1 chain, the RGD containing sequence in the short arm of the α1 chain and the pentapeptide YIGSR sequence in the short arm of the β1 chain, all promote cell adhesion and migration [7–9]. Furthermore, hepatocyte attachment to laminin is inhibited by peptides containing these three sequences [10]. Besides serving as competitive inhibitors of cell adhesion and migration, synthetic peptides containing adhesive recognition sequences appear to induce several metabolic responses. Thus, induction of neurite outgrowth appears to be affected by the site containing the IKVAV sequence [9,11]. In addition, the pentapeptide, YIGSR, was shown to promote cell attachment and migration as well as to reduce the formation of lung colonies in mice injected with melanoma cells [12]. The biological effects of laminin are mediated by several laminin binding proteins (LBPs) that include the integrins [13,14] and several non-integrin LBPs [6]. One of the most studied non-integrin LBPs is a 67 kDa YIGSR binding protein that has been isolated from many cell types [15,16], including human neuroblastoma (NB) cells [17]. It now appears that cell-matrix interactions do not merely offer structural pegs to anchor cells in place but, at least in some cases, transmit signals that are integrated with other cellular activities [13,18]. For example, adhesion of human neuroblastoma cells to matrix proteins or aggregation by anti-integrin antibodies was found to promote tyrosine phosphorylation of p125FAK and several other proteins [19]. We here provide evidence that the C(YIGSR)$_3$-NH$_2$ peptide amide, containing a repeat of YIGSR binding motif of laminin, as well as antibodies directed against the 67 kDa LBP induce protein tyrosine phosphorylation.

2. Materials and methods

2.1. Reagents

The synthetic peptide amide, C(YIGSR)$_3$-NH$_2$, and the unrelated peptide, CYKNVRSKIGSTENLKHQPGGGKV, were synthesized on a 430A peptide synthesizer (Applied Biosystems) and further purified by HPLC. Before use, the peptides were dissolved in 10 mM HCl, and immediately added to the indicated buffers. NHS-LC-biotin was purchased from Pierce (Rockford, IL), and streptavidin conjugated to horseradish peroxidase was from Amersham International. PI-PLC was purchased from Oxford Glycosystems. Polystyrene latex beads were from Sigma. Polyclonal antibodies directed against the C(YIGSR)$_3$-NH$_2$ peptide amide were raised by multiple injections into rabbits. Antibody directed against the 67 kDa LBP was a gift from Dr. H. Kleinman. Monoclonal anti-P-Tyr antibody PY-20 was from Santa Cruz Biotechnology. Biotin-labeled peptide was prepared by incubating the C(YIGSR)$_3$-NH$_2$ peptide amide (5 mg/ml) in 100 mM sodium bicarbonate buffer, pH 8.0, with 1 mg/ml sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin), for 1 h at room temperature with shaking.

2.2. Cell cultures and induction of differentiation

Human neuroblastoma LA-N1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μg/ml gentamicin (Biological Industries, Kibbutz Beth Haemek, Israel). Mouse neuroblastoma×rat glioma hybrid NG108-15 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Both cell lines were incubated at 37°C, in a humidified atmosphere of 5% CO_2/95% air. The media were replaced every second day. For the induction of differentiation, the cells were replated at a concentration of 1×10^6 cells per 75 cm^2 Nunc tissue culture flasks in the same medium, containing 1 mM Bt_2cAMP for 3–5 days.

2.3. Labeling of cell surface receptors by biotinylation

Cells (5×10^7) were washed three times with PBS and incubated in 2 ml of PBS containing 1 mg/ml NHS-LC-biotin at 4°C for 1 h with shaking. Cells were washed three times with cold PBS containing 15 mM glycine to quench the reaction. The packed cells were suspended in a buffer containing: 20 mM Tris-HCl, pH 7.6; 130 mM NaCl; 1 mM $MnCl_2$; 1 mM $MgCl_2$; 1 mM $CaCl_2$ and a protease

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Abbreviations: LBP, laminin binding protein; GPI, glycosyl phosphatidylinositol; NB, neuroblastoma; PI-PLC, phosphatidylinositol-specific phospholipase C; Sulfo-MBS, *m*-maleimidobezoyl-*N*-hydroxysulfosuccinimide ester; C(YIGSR)$_3$-NH$_2$, C(YIGSR)$_3$-NH$_2$ peptide amide

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inhibitor mixture (10 μg/ml aprotinin; 5 μg/ml leupeptin; 10 μg/ml soybean trypsin inhibitor; 1 mM benzamidine; 5 μg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride). The cells were subjected to three cycles of freezing and thawing followed by homogenization in an all-glass homogenizer. The suspension was centrifuged at $12\,000\times g$ for 15 min at 4°C, and the precipitated particulate fraction was washed three times with the homogenization buffer. Membrane proteins were extracted from the particulate fraction with the homogenization buffer containing 1% Triton X-100 for 1 h at 4°C with shaking. Detergent-insoluble material was removed by centrifugation at $12\,000\times g$ for 15 min at 4°C. The supernatant fraction was either subjected immediately to affinity column chromatography or stored at −70°C [17].

2.4. ^{125}I-C(YIGSR)$_3$-NH$_2$ binding assay

C(YIGSR)$_3$-NH$_2$ was labeled with Na^{125}I (New England Nuclear) by the chloramine-T method [20], to a specific activity of $2.0–5.0\times10^9$ cpm/μmol. Bt$_2$cAMP-induced and uninduced LA-N1 cells (2×10^6 cells/well) were seeded in a 24-well plate, and then grown to a confluent state. The cells were washed twice with binding buffer (RPMI 1640; 2% FCS; 20 mM HEPES pH 7.4; 0.1% Na-azide) and incubated with the same buffer for 20 min at room temperature. The buffer was aspirated and the cells were further incubated for 30 min in 300 μl of binding buffer containing ^{125}I-C(YIGSR)$_3$-NH$_2$ ($0.7–360\times10^{-12}$ mol) at room temperature. Cells were then washed twice with binding buffer and lysed with 0.1% NaOH; radioactivity was counted in a gamma counter. Non-specific binding values were determined by adding unlabeled C(YIGSR)$_3$-NH$_2$ peptide amide in 100-fold excess to parallel samples and were subtracted from the total binding values obtained. Displacement assays were performed by the addition of unlabeled C(YIGSR)$_3$-NH$_2$ peptide amide as competitor at the indicated concentrations. Binding data were analyzed using the Cricket Graph and Ligand programs.

2.5. Cross-linking studies

Semi-confluent LA-N1 cells (4×10^7) were washed twice with PBS and collected by centrifugation for 5 min at 1000 rpm. The cell pellet was suspended in 2 ml of a buffer containing RPMI 1640; 20 mM HEPES, pH 7.4; 0.1% Na-azide and 250 μg/ml biotinylated C(YIGSR)$_3$-NH$_2$ peptide amide, and incubated at 4°C for 30 min with shaking. The cell suspension was divided into two 1 ml aliquots, the cells collected separately by centrifugation. One of the cell pellets was resuspended in a cross-linking buffer (50 mM HEPES buffer, pH 7.4; 150 mM NaCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$) containing 5 mM of freshly dissolved sulfo-MBS (Pierce). The other aliquot was suspended in cross-linking buffer alone. The two cell suspensions were incubated at 4°C for 2 h with shaking then washed three times with cross-linking buffer. The cells were then lysed with lysis buffer (20 mM Tris-HCl, pH 7.6; 130 mM NaCl; 1% Triton X-100; 1 mM $MnCl_2$; 1 mM $MgCl_2$; 1 mM $CaCl_2$ and a protease inhibitor mixture defined earlier). Cleared solubilized proteins (200 μg per lane) were resolved by 7.5% SDS-PAGE. The nitrocellulose membranes were quenched with 10% low fat milk in PBS at room temperature for 30 min. The membranes were incubated with streptavidin conjugated to horseradish peroxidase (1:750) in PBS containing 0.5% Tween-20 (PBST) for 30 min with shaking, and then washed four times for 5 min each with PBST. Detection of cross-linked proteins was carried out by the ECL method (Amersham).

2.6. Adhesion of peptides to cultured cells

Hybrid NG108-15 cells were grown to the confluent state in 10 cm plastic plates. The cells were washed twice with RPMI 1640 devoid of fetal calf serum. To each plate 5 ml of this medium containing 100 μg/ml of the indicated peptides was added. The cells were incubated at 37°C for the indicated time intervals. At the end of the incubation cells were washed twice with ice cold PBS and immediately frozen in liquid nitrogen. To each plate 100 μl of sample buffer was added, and the lysed cell suspension was collected with the aid of a rubber policeman followed by boiling for 5 min. Proteins were resolved by means of 7.5% SDS-PAGE and analyzed by immunoblotting with monoclonal anti-P-Tyr antibodies followed by the ECL method.

2.7. Selective cell adhesion to coated polystyrene latex beads

Beads (0.05 ml) were suspended in 0.45 ml of PBS (without Ca^{2+} or Mg^{2+} cations) containing 200 μg of C(YIGSR)$_3$-NH$_2$ peptide amide or 200 μg of anti-67 kDa LBP antibodies. The mixture was incubated for 30 min at room temperature with gentle shaking. The beads were then collected by centrifugation at 2000 rpm for 10 min and resuspended in 0.5 ml PBS at room temperature. NG108-15 cells were serum-starved for 16 h, washed twice and detached with PBS. The cell suspension (0.2 ml, 4×10^6 cells) was mixed with 0.1 ml (1.8×10^8) latex beads coated with C(YIGSR)$_3$-NH$_2$ or with anti-67 kDa antibody. The suspension was incubated at room temperature for 30 min with gentle shaking and then harvested by centrifugation for 10 min at 2000 rpm. Cells were solubilized in a buffer containing 50 mM HEPES buffer, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1 mM sodium orthovanadate; 50 mM sodium pyrophosphate; 50 mM NaF; 1 mM EDTA; 1 mM EGTA and a protease inhibitor mixture. The extracted proteins were resolved by means of 12% SDS-PAGE followed by immunoblotting with monoclonal anti-P-Tyr antibody followed by the ECL method (Amersham).

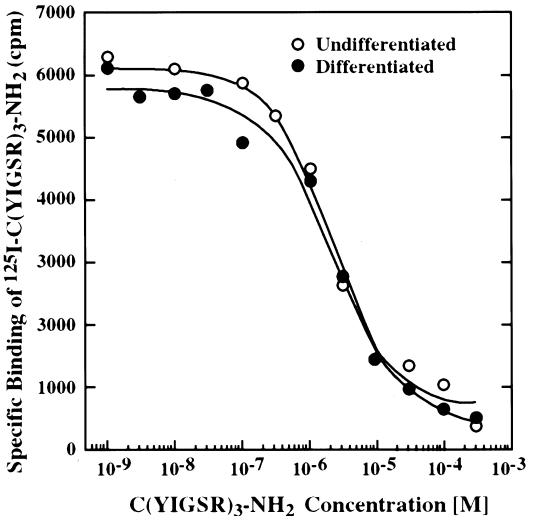

Fig. 1. Inhibition of specific ^{125}I-C(YIGSR)$_3$-NH$_2$ binding to neuroblastoma LA-N1 cells. Bt$_2$cAMP-differentiated and undifferentiated LA-N1 cells (0.15×10^6/well) were seeded in 24-well plates and grown to confluent state. Cells were then washed twice with binding buffer followed by 20 min incubation at room temperature with the same medium. The medium was then replaced with binding buffer (300 μl/well) containing ^{125}I-labeled C(YIGSR)$_3$-NH$_2$ (1×10^5 cpm/well) and unlabeled C(YIGSR)$_3$-NH$_2$ that was added as a competitor at the concentrations indicated. The cells were incubated for 45 min at room temperature, washed twice with binding buffer and lysed with 0.1% NaOH prior to determination of radioactivity.

3. Results

3.1. Binding studies

We have previously shown that detergent-solubilized 67 kDa LBP binds to immobilized C(YIGSR)$_3$-NH$_2$ [17]. It was important to show that native 67 kDa LBP expressed on intact LA-N1 cells will bind to the YIGSR binding site of laminin. Binding studies were performed using intact human neuroblastoma LA-N1 cells and ^{125}I-labeled C(YIGSR)$_3$-NH$_2$. Values of dissociation constant were estimated by Scatchard analysis [21]. ^{125}I-labeled C(YIGSR)$_3$-NH$_2$ was added, together with unlabeled C(YIGSR)$_3$-NH$_2$, or with an unlabeled and unrelated peptide, at the indicated concentrations. Equilibrium binding was attained after 30 min at room temperature. A K_d value of 1.3×10^{-7} M for the C(YIGSR)$_3$-NH$_2$ peptide amide was obtained. Displacement experiments yielded a very similar K_d value of 1.5×10^{-7} M (Fig. 1). These experiments also show that there was no significant difference

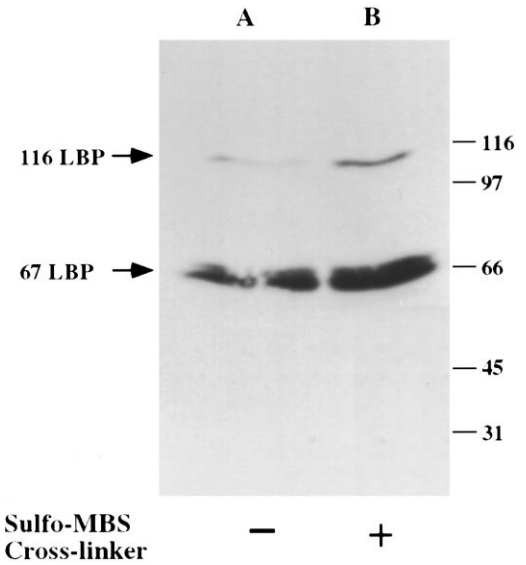

Fig. 2. Cross-linking of biotinylated C(YIGSR)$_3$-NH$_2$ to intact LA-N1 cells. LA-N1 cells were mixed with biotinylated C(YIGSR)$_3$-NH$_2$ and divided into two aliquots. In one aliquot, cells were treated with the heterobifunctional cross-linker sulfo-MBS (B). The other aliquot was not treated with the cross-linker and served as a control (A). Cells were washed, lysed and proteins were resolved by means of 7.5% SDS-PAGE followed by Western blot analysis with streptavidin conjugated to horseradish peroxidase. Detection of the cross-linked proteins was carried out by the ECL method. Each lane represents a lysate from 2×10^7 cells, containing about 200 μg protein.

in the affinity between differentiated and undifferentiated LA-N1 cells. The results suggest that the interaction between the 67 kDa LBP and the C(YIGSR)$_3$-NH$_2$ peptide amide is of intermediate affinity.

3.2. Resistance of the 67 kDa LBP to treatment with phosphatidylinositol-specific phospholipase C

The putative transmembrane domain of the 67 kDa LBP predicted by the cDNA sequence suggests a very short transmembrane domain of only 16 amino acid residues [15,22]. It is reminiscent of certain other cell surface proteins that lack intracellular domains. Examples of such proteins include Thy-1, CEA, the variant surface glycoproteins (VSG) of *Trypanosoma brucei*, N-CAM, the scrapie prion [23] and the receptor for ciliary neurotrophic factor [24]. These proteins contain a membrane spanning domain that is cleaved off and the proteins are subsequently anchored to the cell surface by a glycosyl phosphatidylinositol (GPI) linkage. In general, the GPI linkage is sensitive to treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). We therefore examined whether the 67 kDa LBP is anchored through a GPI linkage by determining whether PI-PLC could release the 67 kDa LBP from LA-N1 cells. The cells were first labeled with NHS-LC-biotin, this technique specifically labels surface membrane proteins including the 67 kDa LBP [17]. The biotin-labeled cells were then treated with PI-PLC. Particulate fractions from untreated and PI-PLC treated cells were extracted with detergent and subjected to C(YIGSR)$_3$-NH$_2$ affinity column chromatography. The purified 67 kDa LBP was subjected to SDS-PAGE followed by Western blot analysis with streptavidin conjugated to horseradish peroxidase [17]. It was found that PI-PLC treatment did not reduce the amount of biotin-labeled 67 kDa LBP in the treated cells (not shown), suggesting that the 67 kDa LBP is not anchored to the cell surface through a GPI linkage, or by association with another protein that is anchored through a GPI linkage.

3.3. Cross-linking studies

The above results raised the possibility that in NB cells the 67 kDa LBP binds to the C(YIGSR)$_3$-NH$_2$ in a cooperative manner through association with another protein. For this reason we attempted to chemically cross-link biotin-labeled C(YIGSR)$_3$-NH$_2$ peptide amide with intact LA-N1 cells, using the heterobifunctional cross-linker sulfo-MBS. Cross-linking studies were carried out with human neuroblastoma LA-N1 cells, as well as with hybrid NG108-15 cells. As shown in Fig. 2, the labeled peptide was extensively cross-linked to the 67 kDa LBP and to an additional protein species with molecular mass of ca. 116 kDa. It thus appears that both the 67 kDa LBP and some other binding proteins may function in concert to promote the adhesion of the cell to the YIGSR site of laminin. To ascertain that the associated protein participates in the binding of C(YIGSR)$_3$-NH$_2$, the chemiluminescence of the same blot was allowed to decay completely and then reacted with polyclonal antibodies directed against C(YIGSR)$_3$-NH$_2$. Control experiments showed that any remaining streptavidin conjugated to horseradish peroxidase was inactive after 4 days under these conditions. Fig. 3 shows that proteins with the same molecular mass were also detected by the antibodies, thus establishing the presence of cross-linked C(YIGSR)$_3$-NH$_2$ in these proteins.

3.4. Signaling via the 67 kDa LBP

It is likely that the adhesion of cells to the YIGSR sequence of laminin through the 67 kDa LBP may involve specific signaling events. We therefore analyzed the response of the hybrid NG108-15 cell line to the presence of the C(YIGSR)$_3$-NH$_2$ as compared to an unrelated peptide. These cells have been reported to contain high levels of the 67 kDa LBP [16], which might facilitate the detection of such a specific mechanism. To examine the role of tyrosine phosphorylation in

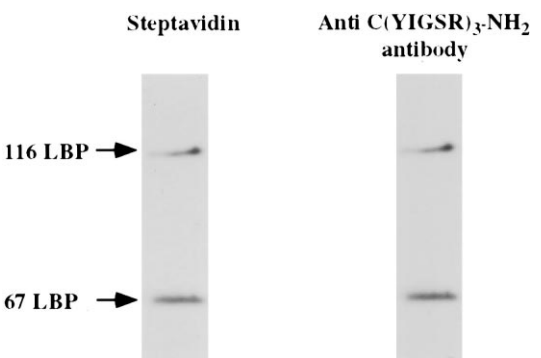

Fig. 3. Detection of cross-linked biotinylated C(YIGSR)$_3$-NH$_2$ with anti-C(YIGSR)$_3$-NH$_2$ antibodies. LA-N1 cells were mixed with biotinylated C(YIGSR)$_3$-NH$_2$ peptide and treated with the heterobifunctional cross-linker sulfo-MBS. Cells were washed, lysed and proteins were resolved by means of 7.5% SDS-PAGE followed by Western blot analysis with streptavidin conjugated to horseradish peroxidase. Detection of the cross-linked proteins was carried out by the ECL method. Chemiluminescence of the proteins were then allowed to decay completely, and the blot was incubated with polyclonal antibodies directed against C(YIGSR)$_3$-NH$_2$. Immunolabeled proteins were also detected by the ECL method.

signal transduction by the 67 kDa LBP, we asked whether attachment of the C(YIGSR)$_3$-NH$_2$ to cultured cells results in tyrosine phosphorylation of intracellular proteins. To each plate of confluent hybrid NG108-15 cells, 5 ml of medium containing 100 μg/ml of the indicated peptides was added. Fig. 4 (lanes B and C) demonstrates that exposure of cultured cells to C(YIGSR)$_3$-NH$_2$ peptide amide induced tyrosine phosphorylation of several proteins with a molecular mass of 115–130 kDa. The induction of tyrosine phosphorylation was rapid and transient, reaching a maximum level within 1 min. In contrast, when cells were exposed to an unrelated polypeptide (CYKNVRSKIGSTENLKHQPGGGKV) of similar length, and the same molar concentration tyrosine phosphorylation of these proteins was hardly detected (Fig. 4, lane A). To obtain selective ligation of the 67 kDa LBP to the C(YIGSR)$_3$-NH$_2$ without concomitant stimulation by growth factors or cell shape changes, serum-starved NG108-15 cells were detached and allowed to adhere in suspension to polystyrene latex beads coated with C(YIGSR)$_3$-NH$_2$ for 30 min at 37°C. To detect a more complete repertoire of phosphorylated proteins a higher percentage (12%) of acrylamide gel was used for SDS-PAGE. As shown in Fig. 5 (lane B), the same high molecular weight proteins of 115–130 kDa underwent phosphorylation on tyrosine residues as observed above (Fig. 4). In addition, YIGSR-dependent tyrosine phosphorylation of a heterogeneous group of proteins with a molecular weight of about 32 kDa was detected in these cells. By contrast, in the presence of uncoated beads, tyrosine phosphorylation was hardly detected (Fig. 5, lane A). We have further investigated whether clustering of the 67 kDa LBP caused by antibody directed against it, is sufficient to induce tyrosine phosphorylation of these proteins. This antibody was raised against a bacterial fusion protein encoded by the β-galactosidase gene plus 0.9 kbp from the carboxyl end of the 1.1 kbp cDNA encoding a 32 kDa protein. It has been found that this antibody recognizes 32, 42 and 67 kDa proteins, and has been designated as anti-67 kDa antibody [10]. Serum-starved NG108-15 cells were detached and allowed to adhere in suspension to polystyrene latex beads coated with anti-67 kDa antibody and incubated for 30 min at 37°C. Fig. 5 illustrates that clustering of the 67 kDa LBP by the corresponding antibody resulted in activation of protein tyrosine kinases capable of phosphorylating the same group of proteins that were phosphorylated in response to C(YIGSR)$_3$-NH$_2$ coated beads (Fig. 5, lane C).

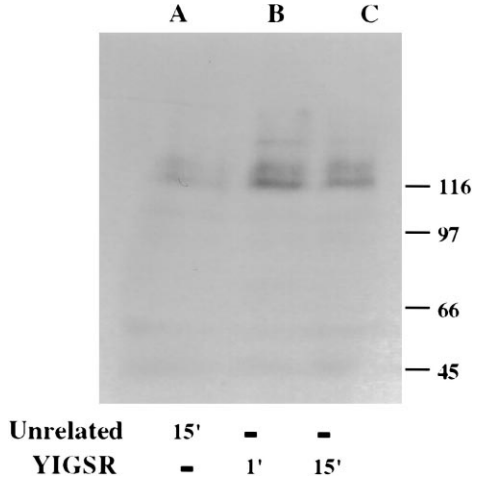

Fig. 4. Tyrosine phosphorylation following adhesion of soluble C(YIGSR)$_3$-NH$_2$ to hybrid NG108-15 cells. Confluent NG108-15 cells were washed twice with RPMI 1640. 5 ml of RPMI 1640 medium containing 100 μg/ml of unrelated peptides (A) and 100 μg/ml C(YIGSR)$_3$-NH$_2$ (lanes B and C) was added to each plate. Following incubation for the indicated time intervals cells were washed with ice-cold PBS and frozen in liquid nitrogen. Cells were then lysed with sample buffer and subjected to 7.5% SDS-PAGE. The blotted membranes were incubated with anti-P-Tyr monoclonal antibodies.

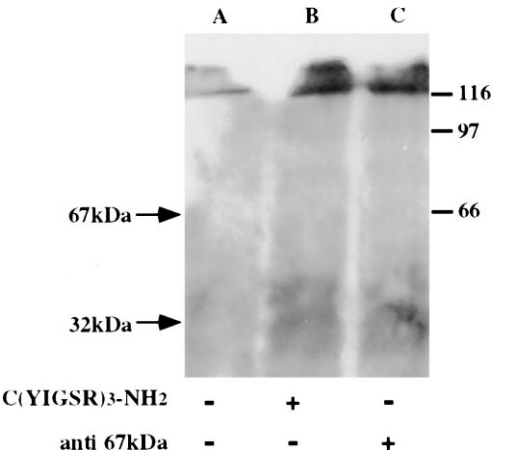

Fig. 5. Tyrosine phosphorylation following adhesion of cells to beads coated with C(YIGSR)$_3$-NH$_2$ or to beads coated with antibody directed against the 67 kDa LBP. Serum-starved confluent NG108-15 cells were incubated with uncoated polystyrene latex beads (A), with polystyrene latex beads coated with C(YIGSR)$_3$-NH$_2$ (B), or with polystyrene latex beads coated with antibodies directed against the 67 kDa LBP (C). Following incubation, cells were collected and lysed. Proteins were subjected to 12% SDS-PAGE and blotted. The blotted membranes were incubated with P-Tyr monoclonal antibodies. Each lane represents a lysate from 1×10^6 cells.

4. Discussion

The 67 kDa LBP represents a family of receptors found in neurons, muscle cells, chondroblasts and many other cell types that are involved in cell attachment to the extracellular matrix (ECM) [8]. Although the phenomenology of laminin interactions is well known, the biochemical and molecular basis for the binding of the YIGSR sequence to the 67 kDa LBP has not been examined in detail. The 67 kDa LBP was isolated in our laboratory from human NB LA-N1 and from the hybrid NG108-15 cells [17], and served as the basis for our studies. Binding experiments revealed that the interaction of the C(YIGSR)$_3$-NH$_2$ with the 67 kDa LBP appears to be of an intermediate-affinity (K_d 1.3×10^{-7} M). Displacement experiments yielded roughly the same K_d value (1.5×10^{-7} M). These data also demonstrate that the ^{125}I-C(YIGSR)$_3$-NH$_2$ interacts with a single class of binding site in a dose-dependent, saturable and reversible manner. The affinity for YIGSR found in these experiments is lower than that reported previously for the binding of [^{125}I]laminin to NG108-15 (K_d 2×10^{-9} M); chick spinal cord (K_d 2.5×10^{-9} M) and PC-12 cells (K_d 1.1×10^{-9} M) [16]. One possible explanation could be related to the difference in size and conformation between the relatively short C(YIGSR)$_3$-NH$_2$ peptide and the whole laminin molecule that was used in the other studies. The results

may also point to the importance of the adjacent amino acid sequences found in the intact laminin molecule.

The cDNA-deduced amino acid sequence of the 67 kDa LBP suggests a very short transmembrane domain [15,22]. We therefore examined whether this protein is anchored through a GPI linkage; our experiments did not support this assumption. It is also possible that the cellular functions of the 67 kDa LBP are facilitated by an associated protein. The results of the cross-linking studies support the notion that at least one more protein subunit is involved in the adhesion of the 67 kDa LBP to the YIGSR sequence of laminin. Two independent lines of evidence agree with this conclusion. First, the $C(YIGSR)_3$-NH_2 is chemically cross-linked in intact cells to an additional protein species with molecular mass of ca. 116 kDa. Alternatively this protein band may represent a complex of the 67 kDa LBP and an additional protein of molecular mass of about 50 kDa (Fig. 2). Second, both the 67 kDa and the 116 kDa proteins were found to be cross-linked to the $C(YIGSR)_3$-NH_2 peptide, as revealed by the anti-YIGSR antibodies (Fig. 3). It thus appears that adhesion to the YIGSR peptide sequence of laminin requires the participation of at least one more protein molecule. It was previously shown that differentiated LA-N1 cells show increased adhesion to laminin as compared to undifferentiated cells, although the expression of the 67 kDa LBP in these cells is down-regulated [17]. In addition, competition experiments showed the same order of affinity between the 67 kDa LBP and the YIGSR sequence in differentiated and undifferentiated LA-N1 cells (1.5×10^{-7} M). It is, therefore, possible that the presence of the 67 kDa associated protein might facilitate the enhanced adhesion of differentiated LA-N1 cells to laminin.

Tyrosine phosphorylation is known to regulate many physiological events including cell-cell and cell-matrix interactions [25]. In the present studies two classes of proteins were found to be phosphorylated in response to the interaction of the $C(YIGSR)_3$-NH_2 sequence with the 67 kDa LBP. One group of proteins had a molecular mass of 115–130 kDa (Fig. 4), which may be analogous to cytoplasmic focal adhesion kinase (p^{125FAK}). Indeed, immunoblotting of proteins derived from peptide-treated cells, with anti-p^{125FAK} antibody showed that the p^{125FAK} co-migrated on SDS-PAGE to the same position as the above phosphorylated proteins (not shown). This finding suggests that 67 kDa LBP may mediate signaling events involving tyrosine phosphorylation of cytosolic p^{125FAK}, as was found for integrin interaction with laminin in NB cells [19]. Another heterogeneous group of tyrosine phosphorylated proteins was found to have a molecular mass of around 32 kDa (Fig. 5), and their identity remains to be determined. It is possible that this heterogeneous group of phosphorylated proteins is related to the 33 kDa precursor protein of the 67 kDa LBP [15,22]. In the light of these results we suggest two mechanisms of signaling to account for phosphorylation of tyrosine residues in this molecule. The above proteins may undergo autophosphorylation on a tyrosine residue, which is generally indicative that it encodes a phosphotyrosine kinase (PTK) as a result of activation by the cell adhesion to the YIGSR sequence. Alternatively, the 32 kDa group of proteins may be phosphorylated by another unknown PTK. As tyrosine phosphorylation of these proteins can also be triggered by an antibody directed against the 67 kDa LBP, it is likely that dimerization or oligomerization of the 67 kDa LBP is required for activating an associated protein tyrosine kinase (Fig. 5, lane C). It therefore appears that in neuroblastoma cells tyrosine phosphorylation occurs in response to $C(YIGSR)_3$-NH_2 binding to the 67 kDa LBP, or to antibody-induced clustering of the 67 kDa LBP.

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