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Laminin-1 induces endocytosis of 67KDa laminin receptor and protects Neuroscreen-1 cells against death induced by serum withdrawal

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ABSTRACT

Although the function of laminin in the basement membrane is known, the function of soluble "neuronal" laminin is unknown. Since laminin is neuroprotective, we determined whether the soluble laminin-1 induces signaling for neuroprotection via its 67KDa laminin-1 receptor (67LR). Treatment of Neuroscreen-1 (NS-1) cells with laminin-1 or YIGSR peptide, which corresponds to a sequence in laminin-1 β1 chain that binds to 67LR, induced a decrease in the cell-surface expression of 67LR and caused its internalization. Furthermore, intracellular cAMP-elevating agents, dibutyryl-cAMP, forskolin, and rolipram, also induced this internalization. Both soluble laminin-1 and YIGSR induced a sustained elevation of intracellular cAMP under defined conditions, suggesting a causal role of cAMP in the endocytosis of 67LR. This endocytosis was not observed in cells deficient in protein kinase A (PKA) nor in cells treated with either SQ 22536, an inhibitor for adenylyl cyclase, or ESI-09, an inhibitor for the exchange protein directly activated by cAMP (Epac). In addition, when internalization occurred in NS-1 cells, 67LR and adenylyl cyclase were localized in early endosomes. Under conditions in which endocytosis had occurred, both laminin-1 and YIGSR protected NS-1 cells from cell death induced by serum withdrawal. However, under conditions in which endocytosis did not occur, neither laminin-1 nor YIGSR protected these cells. Conceivably, the binding of laminin-1 to 67LR causes initial signaling through PKA and Epac, which causes the internalization of 67LR, along with signaling enzymes, such as adenylyl cyclase, into early endosomes. This causes sustained signaling for protection against cell death induced by serum withdrawal.

1. Introduction

Laminins are heterotrimers of α, β, and γ chains that are present as major constituents of the basement membrane [1]. In the central nervous system, laminin is localized to the basement membranes of blood vessels and in reactive astrocytes [2]. Although laminin functions by providing structural support for the basement membrane, it also exhibits several biological activities in the nervous system, aiding in the stimulation of neurite outgrowth, cell attachment, cell migration, and neuronal survival [3]. Various isoforms of laminin that are found in the neuronal cell bodies and axons are referred to as 'neuronal' laminins [4–8]. Either neurons produce these laminins by themselves, or they take up those produced by astroglial cells [5]. The functions of neuronal laminins are unknown.

Neurons in the central nervous system have a variety of laminin receptors, such as integrins, dystroglycan, and 67KDa laminin receptor (67LR) [9,10]. The 67LR-binding site has been identified to the YIGSR pentapeptide sequence in the β1 chain of laminin [1]. 67LR is a highly conserved protein and is produced as a 37kDa protein that subsequently undergoes dimerization to form the 67kDa protein that is expressed on the cell surface [11,12]. It internalizes prion proteins and various viruses and bacteria [11]. Besides this cargo-carrying function, the signaling mechanisms associated with 67LR are not clearly known. Recently, the early

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endosomes resulting from the internalization of receptors have been shown to serve as signaling platforms and may generate signals for a sustained period [13,14]. It is not known whether the early endosomes resulting from the internalization of 67LR can exhibit such cell signaling.

Intracellular messengers, like cAMP, play crucial roles in influencing various neuronal functions [15]. It is not known whether the signaling or internalization of 67LR can produce an increase in cAMP. Unlike the adenylyl cyclase associated with the plasma membrane which causes a transient elevation of cAMP, the same enzyme when associated with early endosomes may contribute to a sustained elevation of cAMP [14]. cAMP elicits its actions through at least two effectors in nervous system: protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac) [16]. It is not known whether these effectors play a role in soluble laminin-mediated signaling.

Apoptotic cell death plays an important role in the development of a normal nervous system [17]. Neurons deprived of trophic support die [17]. Currently, therapeutic potential of neurotrophins for treating various neurodegenerative diseases is being explored [18]. Serum deprivation of PC12 and other cell lines is utilized as a model for studying the mechanisms of neuronal death, in order to identify neuroprotective agents and elucidate their mechanisms [19].

Here we show for the first time that laminin-1 and its peptide, YIGSR, induce internalization of 67LR along with signaling enzymes, such as adenylyl cyclase, into early endosomes. This causes a sustained generation of cell signaling that increases the survival of Neuroscreen-1 (NS-1) cells from death induced by withdrawal of serum.

2. Materials and methods

2.1. Materials

Laminin-1 isolated from Engelbreth-Holm-Swarm sarcoma, dibutyryl-cAMP, forskolin, rolipram, and SQ 22536 were obtained from Sigma-Aldrich. YIGSR-NH_2 and GRGDS peptides were from CPC Scientific. ESI-09 was from Calbiochem. Anti-67LR-(MluC5) mouse monoclonal antibody, mouse monoclonal antibody that recognizes all isoenzymes of adenylyl cyclase, and mouse IgM were from Santa Cruz Biotechnology. Rabbit antibody against early endosomal antigen1 (EEA1) was from GeneTex.

2.2. Cell culture and treatments

PKA-deficient PC12 cells (A132.7) originally cloned by Dr. John Wagner and parent PC12 cells were kind gifts from Dr. Louis Hersh (University of Kentucky, Lexington). Stocks of NS-1, PKA-deficient PC12, and parent PC12 cells were grown on flasks coated with poly-L-lysine in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum, 50 units/ml penicillin, and 0.05 mg/ml streptomycin.

2.3. Indirect immunofluorescence and colocalization

Cells were grown on poly-D-lysine-coated 12-mm glass coverslips in a 24-well culture plate to 50% confluency and treated with the indicated agents for 2 h. Cells were then fixed with 4% paraformaldehyde. Cells that were not permeabilized were used for detecting the cell-surface 67LR protein; cells that were permeabilized with 0.25% Triton-X-100 were used to stain intracellular proteins. These cells were blocked with 5% goat serum. Then, cells were incubated with mouse anti-67LR antibody (1:300)- either alone or in combination with rabbit anti-EEA1 (1:300) antibody for 24 h at 4 °C. They were then incubated with Alexa Fluor 488-conjugated antimouse goat secondary antibody, either alone or in combination with Alexa Fluor 594-conjugated antirabbit goat secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature. Nuclei were stained with 4′6′-diamidino-2-phenylindole (DAPI). Images were taken using an LSM 800 Zeiss confocal microscope. For the colocalization study, all images were collected with 63 × 1.4NA oil objective. 12 images were collected for stack. The z-step size was 0.4 μm. Colocalization was quantified by Pearson's correlation coefficient and Manders' colocalization coefficient using the JaCop plug-in of ImageJ software.

2.4. Biotinylation assay for endocytosis

67LR internalization was quantitated by biotinylation using the previously published procedure [20]. Briefly, NS-1 cells were labeled with sulfo-NHS-SS-biotin (0.8 mg/ml) for 30 min at 4 °C. Samples were transferred to 37 °C and incubated with laminin-1, YIGSR peptide, and dibutyryl-cAMP at 37 °C for 15 min to induce internalization. Then, samples were treated with glutathione to cleave the biotin associated S-S bond to selectively strip it from the cell surface. Cells were homogenized and the internalized biotinylated proteins were isolated with streptavidin-agarose beads. After eluting the protein from the beads, samples were subjected to Western immunoblotting to detect 67LR.

2.5. Quantitation of cAMP

The direct cAMP enzyme-immunosorbent assay kit (Enzo Life Sciences) was used for quantitation of cAMP. Cells (4000-7000) were seeded in a 96-well culture plate, treated with various agents, and lysed in 0.1 M HCl. cAMP was quantitated in the supernatants. Both standards and test samples were acetylated to increase the sensitivity of the assay. Protein was measured using 660nm assay reagent (Pierce) after lysing cells in a buffer containing detergent. The cAMP levels were expressed per mg cellular protein.

2.6. Withdrawal of serum support

Subconfluent cells (25% confluence) were grown in 96-well plates and preconditioned with laminin-1, YIGSR, or dibutyryl-cAMP, forskolin, or rolipram in phenol red-free RPMI medium supplemented with 0.2% heat-inactivated horse serum and 0.1% fetal calf serum for 24 h. Then, cells were washed twice with a medium devoid of both serum and phenol red and then kept in the same medium and treated again with the abovementioned agents. After 24 h, the cell viability was determined by thiazolyl blue tetrazolium bromide (MTT) assay [21].

2.7. Statistical analysis

All values are expressed as means ± SE. Statistical significance was determined by the Student's t-test. In some cases where indicated, data was analyzed using one-way analysis of variance, followed by post hoc Scheffe's test. $p < 0.05$ was considered statistically significant. Statistical analyses were performed with StatView software.

3. Results

3.1. Internalization of 67LR by laminin-1, YIGSR and cAMP

Unless otherwise mentioned, we used only the soluble laminin-1 in all experiments. In non-permeabilized cells, the surface expression of 67LR was substantially decreased by a treatment with

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