Communication

Signaling Site of Laminin with Mitogenic Activity*

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Laminin, a basement membrane glycoprotein, has diverse biological activities including cell adhesion, growth, and differentiation. However, little is known concerning the signal transduction and active site involved in cell growth. In this study, we have shown that laminin and a 19-mer peptide (PA22-2) from the carboxyl-terminal end of the long arm of the laminin A chain, which was previously shown to promote cell adhesion and neurite outgrowth, stimulate thymidine incorporation and cell growth of PC12 cells. Laminin and PA22-2 (PA) were also found to induce a rapid and transient mRNA expression of c-fos and c-Jun protooncogenes in PC12 cells. Further, both laminin and PA stimulated the DNA binding activity of c-Fos and c-Jun protein complex to the AP-1 site. We have also found that there is a correlation between cell growth, c-fos expression, and the ability of cell attachment to laminin or to PA in different cell types. These results suggest that the PA sequence is a potent site in laminin for both signal transduction and cell growth.

The most well characterized form of laminin consists of three chains: A, B1, and B2 (1). Several active sites of laminin have been identified (see Ref. 2 for review). These include a pentapeptide, YIGSR, a peptide F9 from the B1 chain, and a RGD-containing site of the A chain, all of which promote cell attachment (3-5). In addition, an IKVAV-containing synthetic peptide (PA) was shown to promote cell adhesion, spreading, and migration (6, 7). PA also stimulated neurite outgrowth of PC12 cells after priming with nerve growth factor (NGF) (6). Without NGF priming, neither laminin nor PA increased neurite outgrowth of PC12 cells.

The effects of laminin on cell growth were observed when fibroblasts (8), Schwann cells (9), myoblasts (10), and fibrosarcoma cells (11) were attached and grown on laminin-coated plates. Recent reports have demonstrated that laminin and its proteolytic fragments (1-4) containing the inner rod-like segments from its short arms, which consist of cysteine-rich motifs, stimulate thymidine incorporation in cultured cells possessing EGF receptors (12). However, the active site of laminin and signal transduction involved in laminin-induced cell growth was not identified. Here we find that another site on laminin containing the IKVAV sequence is active for cell growth and for inducing c-fos and c-jun expression and DNA binding activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell culture medium was obtained from GIBCO. Rat pheochromocytoma PC12 cells (from Dr. G. Guroff, National Institutes of Health) were grown in Dulbecco's modified Eagle's medium containing 7.5% fetal bovine serum and 7.5% horse serum. Mouse fibroblast NIH 3T3 cells (from Dr. S. A. Aaronson, National Institutes of Health) were grown in Dulbecco's modified Eagle's medium containing 10% calf serum. Mouse neuroblastoma Neuro-2a cells were grown in RPMI 1640 medium containing 15% fetal bovine serum. U-937, HL60, and KatoIII cells were obtained from American Type Culture Collection.

Synthetic Peptides and Laminin—Peptides were synthesized using an automated model 430A synthesizer (Applied Biosystems). Peptides were further purified using preparative high performance liquid chromatography. Peptides used were as follows: PA (CSARKQAASIKVAVSADR, A chain residues 2080-2108), S7 (CSARKQAASGVASADR, A chain residues 2091-2108), S13 (SRNLSEIKLASSRARKQAASIKVAVSADR, A chain residues 2080-2108), a YIGSR-containing peptide (CDPGYIGSR, B1 chain residues 926-933), and D11 (DELKNLTGPKESVDNI, A chain residues 1954-1970). Cysteine in PA, S7, and the YIGSR peptide does not occur in the native chains. Laminin prepared from Engelbreth-Holm-Swarm tumor, as described previously (6), was kindly provided by Dr. H. K. Kleinman (National Institutes of Health).

Cell Attachment—Cell attachment assay to laminin (20 µg) or PA (20 µg) was performed in a 96-well tissue culture plates as described (6). The data were expressed as % compared with PC12 cell attachment to laminin (100%) from the average of two experiments in duplicate (mean ± S.D.).

Assays of Growth Promoting Activity—Subconfluent cells in a Costar 24 well plate were washed twice with phosphate-buffered saline, incubated in serum free medium containing 1 µM selenium and 5 µg/ml transferrin for 48-72 h, and then stimulated by adding laminin (1-100 µg/ml), PA (1-100 µg/ml), S7 (100 µg/ml), S13 (100 µg/ml), D11 (100 µg/ml), YIGSR (100 µg/ml), EGF (100 ng/ml), and NGF (100 ng/ml) for 18 h. EGF and NGF were purchased from Collaborative Research. [3H]Thymidine (Amersham Corp.) (2 µCi/ml) was added and further incubated for 5 h. The incorporation of radioactivity was measured as described (13). The data from the average of two experiments in duplicate were expressed as fold increase compared to control (no addition to the culture) (mean ± S.D.). Cell number was determined by a Coulter counter. Quiescent cells (4 x 10⁴) were stimulated by adding laminin (100 µg/ml) or PA (100 µg/ml), and after 2 days the cell number was counted. The data were expressed as fold increase compared with control from the average of two experiments in triplicate (mean ± S.D.).

Northern Blot Analysis and Protein Analysis—The quiescent cells were incubated with laminin (50 µg/ml) or PA (50 µg/ml) at various times. Total RNA preparation, Northern blot analysis of 10 µg of each RNA, hybridization with c-jun (from Dr. D. Nathans, Johns Hopkins University) or c-fos probes (Lofstrad Labs Limited) was performed as described previously (14). For protein analysis, labeling with 150 µCi of L-[³⁵S]methionine (Amersham), preparation of cell lysates, immunoprecipitation with Fos antibody (Ab) (Oncogene Science), and sodium dodecyl sulfate electrophoresis were performed as described (15). Equivalent aliquots (equal cpm) were loaded on each lane.

Gel Mobility Shift Assay—Nuclear extracts were prepared as de-
FIG. 1. The effect of laminin and PA on thymidine incorporation in PC12 cells. [3H]Thymidine incorporation was measured as described under "Experimental Procedures." The data is expressed as fold increase compared to control.

scribed (16) from PC12 cells untreated or treated for 15 min with laminin (50 µg/ml) or PA (50 µg/ml). A gel shift assay was carried out according to the manufacturer's methods (Stratagene gel shift kit) using 3 µg of each nuclear extract. Quantitation was made by densitometric scanning of the gels using Microtek Scanner. Jun Ab was obtained from Oncogene Science.

RESULTS AND DISCUSSION

PA, as well as laminin, stimulated thymidine incorporation in PC12 cells without NGF priming. As shown in Fig. 1, PC 12 cells responded to the presence of 1–100 µg/ml PA or laminin in a dose-dependent manner. A longer synthetic peptide (S13), which contains the PA sequence concentrations at 100 µg/ml, also stimulated thymidine incorporation to a similar degree. The effect of PA on thymidine incorporation was specific, since S7, which has a substitution of isoleucine by a glycine, has little thymidine incorporation activity compared to PA. A YIGSR-containing peptide from the B1 chain and D11 located near the PA site of the A chain also failed to stimulate thymidine incorporation comparable to PA (Fig. 1).

Protooncogenes such as c-fos and c-jun are thought to have regulatory roles in cell proliferation (see Refs. 18 and 19 for review). Expression of c-fos and c-jun is induced rapidly and transiently following a variety of mitogens (18, 19). Signal transduction involved in laminin-induced cellular changes is

FIG. 2. Induction of c-fos and c-jun mRNA and protein expression by laminin and PA. The quiescent PC12 cells were incubated with laminin (50 µg/ml) or PA (50 µg/ml) at various times. Total RNA preparation, Northern blot analysis of 10 µg of each RNA, and hybridization with a c-jun or c-fos probes were performed as described under "Experimental Procedures." a, time course of c-fos mRNA expression. b, time course of c-jun mRNA expression. c, superinduction of c-jun mRNA expression in the presence of cycloheximide. d, time course study of c-fos and c-jun protein expression. C, control; LM, laminin; PA, PA22-2; CHX, cycloheximide.
Jun mRNA was reached the maximum levels at 30 min after addition of c-fos and c-jun is unknown. We have found that laminin and PA rapidly and transiently induce c-fos and c-jun mRNA expression in PC12 cells. Fig. 2a shows the induction of c-fos mRNA by Northern blot analysis, following the addition of laminin (50 μg/ml) or PA (50 μg/ml) to quiescent PC12 cells. Both laminin and PA increased the c-fos mRNA, with the maximum level at 15 min, and thereafter the mRNA levels rapidly declined (lanes 1–7). In the presence of cycloheximide (10 μg/ml), the expression of c-fos mRNA was superinducible (lanes 8–11). Similarly, c-jun mRNA was reached the maximum levels at 30 min after the addition of laminin (50 μg/ml) or PA (50 μg/ml) (Fig. 2b). In the presence of cycloheximide (10 μg/ml) c-jun mRNA was also superinducible (Fig. 2c). Cycloheximide (10 μg/ml) alone induced c-fos and c-jun mRNA expression similar to laminin-mediated c-fos and c-jun mRNA expression occurs in the presence of cycloheximide (10 μg/ml) c-jun mRNA was also superinducible (lanes 12–15).

In the presence of cycloheximide (10 μg/ml) c-jun mRNA was also superinducible (Fig. 2c). Cycloheximide (10 μg/ml) alone induced c-fos and c-jun mRNA expression similar to laminin-treated cells (data not shown). These results suggest that laminin-mediated c-fos and c-jun mRNA expression occurs through different mechanism of the cycloheximide induction and is independent of protein synthesis. The cycloheximide effect is consistent with the previous report using a different growth factor (20).

It has been previously reported that when Fos is immunoprecipitated, the level of c-jun protein (Jun) (p39) increases in parallel with the level of c-fos protein (Fos) (15, 21, 22). Fig. 2d shows a time course study by immunoprecipitation analysis using anti-Fos monoclonal antibody (Fos Ab) with cell lysates from PC12 cells stimulated with laminin (lanes 3, 6, 9, and 12) or PA (lanes 4, 7, 10, and 13). The synthesis of Fos and Jun was increased about 2–3-fold 15 min after treatment with laminin or PA, and Fos and Jun appeared to be stable for 100 min. We also detected 190–200-kDa Fos-related proteins which appeared to be inducible.

Fos and Jun are transcriptional factors which play a pivotal role in the signal transduction process (see Refs. 19 and 23 for review). Fos and Jun form a heterodimer through a leucine zipper domain, and this heterodimer has high affinity binding to the AP-1 site (consensus sequence TGA(G/C)TCA) (24, 25). To see whether laminin and PA activate DNA binding activity of these proteins, a gel mobility shift assay was employed, with 2 ng of a 32P-labeled double-stranded 22-mer oligonucleotide probe containing the AP-1 consensus sequence (TGA(T/C)A) (AP-1), and 3 μg of nuclear extracts from PC12 cells (Fig. 3). Although untreated PC12 cells contained readily detectable levels of binding activity of nuclear factors to the AP1 site (Fig. 3a, lane 1), there was significant increase (about 1.5–1.8-fold) 15 min after addition by laminin (Fig. 3a, lane 2) or PA (lane 3). The significant increase was seen until 90 min after addition of laminin or PA (data not shown). This binding was specific as formation of the retarded complex was competitively inhibited by a 10-fold excess of unlabeled AP-1 (lane 4), but not by a 100-fold excess of unlabeled oligonucleotide without the AP-1 consensus sequence (lane 6). To examine whether the retarded band contained Fos and Jun, Fos Ab and Jun Ab were employed. When either Fos or Jun Ab were preincubated with the nuclear extracts for 1 h at room temperature, the gel retardation of the AP-1 site was blocked (Fig. 3b, lanes 3–6), but not by preincubation with non-immune IgG (lane 8). These results confirmed that Fos and Jun were present in the retarded complex. Thus, both laminin and PA stimulated DNA binding activity of the Fos-Jun complex to the AP-1 site.

The growth of many cells is thought to be dependent on cell adhesion to extracellular matrix components (26, 27). Recent study has shown that adhesion of suspension-arrested cells rapidly induces growth-associated genes (c-fos, c-myc, and actin) expression (28). A correlation between laminin- or PA-mediated adhesion, cell growth and gene expression is unknown. Therefore, we next studied whether there was a correlation between cell attachment to PA, or laminin, c-fos expression, and cell growth (thymidine incorporation and cell number) in different cell types. Table I summarizes the results. We find a correlation between cell attachment to PA or laminin, c-fos expression (Northern blot, not shown), and cell growth. PC12 cells, NeuroIIa, and NIH 3T3 cells attached very well to laminin and PA. In these cells, thymidine incor-

**TABLE I**

| Cell line      | Laminin Attachment | Mitogenic thymidine | Cell no. | PA Attachment | Mitogenic thymidine | Cell no. | c-fos* |
|----------------|--------------------|--------------------|---------|---------------|--------------------|---------|-------|
|                | %                  | -fold              | -fold   | %             | -fold              | -fold   |       |
| PC12           | 100                | 4.5 ± 1.3          | 1.68 ± 0.03 | 100           | 3.7 ± 0.4          | 1.60 ± 0.03 | ++    |
| NeuroIIa       | 100                | 4.3 ± 1.2          | 1.60 ± 0.05 | 100           | 3.3 ± 0.2          | 1.60 ± 0.04 | ++    |
| NIH 3T3        | 77.0 ± 6.0         | 3.7 ± 0.9          | 1.57 ± 0.06 | 68.0 ± 3.0    | 2.9 ± 0.3          | 1.66 ± 0.11 | ++    |
| U937           | 38.8 ± 6.3         | 2.1 ± 0.8          | 1.37 ± 0.13 | 43.5 ± 1.0    | 1.5 ± 0.6          | 1.39 ± 0.16 | +     |
| KataII         | 40.5 ± 2.5         | 2.1 ± 0.3          | 1.30 ± 0.10 | 44.0 ± 7.0    | 1.6 ± 0.2          | 1.30 ± 0.10 | +     |
| HL00           | 18.5 ± 1.5         | 1.0 ± 0.3          | 1.00 ± 0.06 | 10.0 ± 1.0    | 1.0 ± 0.2          | 1.12 ± 0.04 | -     |

* c-fos expression was performed by Northern blot analysis as described under “Experimental Procedures.” The expression 15 min after PA (100 μg/ml) treatment was compared with c-fos expression of untreated cells. ++, strong induction; +, induction; –, no induction. Cell attachment assay, and mitogenic assay (thymidine incorporation and cell number) were performed as described under “Experimental Procedures.”
poration and cell numbers were increased by about 2.9-4.5-fold and 60-70%, respectively, in the presence of laminin or PA, and c-fos mRNA expression were strongly induced. U937 and KatoII cells which attached moderately to laminin and PA showed about 1.5-2.1-fold increase of thymidine incorporation, and 30-40% increase in cell number. c-fos mRNA expression was induced in these cells, but was less prominent compared with that in PC12 cells. In contrast to these cells, HL60 cells which did not attach to laminin and PA well did not show any increase of cell growth and thymidine incorporation. c-fos mRNA was not induced at all in these cells. The result is in contrast to the previous report that mitogenic activity of P1 on Swiss 3T3 cells, a proteolytic fragment containing a portion of the short arms of the three laminin chains, was not associated with cell attachment (12). Although the reason for the difference is unclear, it is conceivable that several different sites of laminin may promote cell growth as has been found for cell attachment.

Since a number of different laminin receptors including integrin receptors and non-integrin receptors have been identified (29-31), P1 could function to promote cell growth through interacting with distinct receptors and through different signal transduction pathways. The authors also suggest that EGF receptor (EGFR) may be involved in laminin-induced mitogenesis, because laminin simulated cell growth of Swiss 3T3 cells, but did not stimulate that of a mutant cell line of Swiss 3T3 cells (NR6) which is deficient of EGFR (12). As shown in Table I, wild type NIH 3T3 cells which have EGFR responded well to laminin. The magnitude was comparable to EGF (data not shown). To investigate whether EGFR is involved in laminin-induced mitogenesis, we used mutant NIH 3T3 cells (clone 2.2) devoid of endogenous EGFR, and its isogenic NIH 3T3 cells, which were transfected with EGFR cDNA and expressed EGFR in amounts similar to wild type NIH 3T3 cells (32). Quiescent clone 2.2 cells and transfected NIH 3T3 cells were stimulated by adding laminin (100 µg/ml), and cell number was counted after 2 days. Laminin stimulated clone 2.2 and transfected cell number 1.85 ± 0.04-fold and 1.70 ± 0.08-fold, as compared with the control, respectively. The results suggest that EGFR is not directly involved in laminin-induced mitogenesis in NIH 3T3 cells. Although the involvement of EGFR in NR6 cells cannot be ruled out, it is reasonable to assume that the failure to respond to laminin is due to the absence or unavailability of a receptor for laminin. The fact that laminin did not compete with EGF for EGFR (12) and that EGF had little effect on cell growth of PC12 cells further supports arguments against the direct involvement of EGFR in laminin-mediated cell growth.

Although the molecular mechanism of laminin-mediated induction of c-fos and c-jun is not clear, the increased DNA binding activity of the Fos and Jun complex appears to be due to both transcriptional and post-translational activation. It has been reported that dephosphorylation events are involved in laminin-mediated neurite outgrowth in neuronal cells (33). More recent work has shown that activation of protein kinase C decreases phosphorylation of Jun at sites that negatively regulate its DNA binding activity to the AP-1 site (34). Identification of laminin- or PA-specific receptor and elucidation of signal transduction pathway will facilitate the understanding of laminin-mediated cell growth.

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