Laminin-Induced Signaling in Tumor Cells: The Role of the \( M_6, 67,000 \) Laminin Receptor

Vered Givant-Horwitz, Ben Davidson, and Reuven Reich

1Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel, and 2Department of Pathology, The Norwegian Radium Hospital, Montebello, Oslo, Norway

ABSTRACT

The expression of the \( M_6, 67,000 \) laminin receptor, a nonintegrin laminin receptor, was found to be up-regulated in neoplastic cells and to directly correlate with invasion and metastatic potential. In the present study, we investigated the role of laminin receptor in mediating laminin effects and the involvement of the mitogen-activated protein kinases (MAPK) cascades and dual-specificity phosphatases in laminin signaling in human melanoma cells. Using stable transfection of A375SM melanoma cells, we established lines expressing reduced or elevated laminin receptor. The antisense-transfected cells demonstrated reduced attachment to laminin and reduced invasion through Matrigel-coated filters. In addition, both matrix metalloproteinase-2 (MMP-2) mRNA expression and activity were significantly reduced in the antisense-transfected cells. Antisense-transfected cells showed a reduction in mRNA level of the \( \alpha_6 \beta_1 \) integrin subunit isoform, whereas no change in the mRNA level of the \( \alpha_6 \alpha_1 \) isoform was observed. We found that exogenous laminin reduced the phosphorylated (active) form of extracellular signal-regulated kinase, c-Jun \( \text{NH}_2 \)-terminal protein kinase, and p38 in all of the cells, irrespective of the expression of the laminin receptor. Furthermore, the phosphorylation of extracellular signal-regulated kinase, c-Jun \( \text{NH}_2 \)-terminal protein kinase, and p38 was significantly higher in the cell lines expressing reduced laminin receptor, regardless of the exposure to exogenous laminin. This increase of MAPK phosphorylation was accompanied by a significant reduction in MKP-1 phosphatase mRNA level and a significant increase in PAC-1 phosphatase mRNA level. In conclusion, our results confirm the involvement of the laminin receptor in different mechanisms related to tumor dissemination and provide first evidence of the involvement of MAPK and dual-specificity phosphatases in its signal transduction pathway.

INTRODUCTION

Metastatic spread of cancer continues to be the greatest challenge to cancer cure. At the core of the process lies the changing adhesive preferences of the tumor cells, which determine their interactions with other cells and with the extracellular matrices, mainly in attachment and degradation processes (1–3).

Laminins are a family of extracellular matrix proteins that constitute the major noncollagenous glycoproteins found in the basement membrane and are involved in multiple important biological activities (3, 4), such as assembly of the basement membrane (3), cell attachment (3, 5), migration (3, 6), growth and differentiation (5, 7), neurite outgrowth (3, 8), and angiogenesis (5, 9). In addition, laminins promote the invasive phenotype of cancer. The interaction of cancer cells with laminin is a key event in tumor invasion and metastasis (2, 3, 10). One of the mechanisms by which laminin contributes to the metastatic spread is induction of proteolytic activity. It has been shown that in certain metastatic cells, but not in normal cells, laminin induces an increase in matrix metalloproteinase-2 (MMP-2) activity (11). MMP-2 is an extracellular matrix-degrading endopeptidase, which has a key role in invasion and metastasis and is frequently correlated with tumor progression (12, 13).

Laminin receptors are divided into two major groups: integrins and nonintegrin receptors. Insufficient data exist concerning the respective roles of integrins and nonintegrin receptors in mediating the effects of laminin (4, 14). The \( M_6, 67,000 \) laminin receptor is a nonintegrin receptor (2). A highly conserved multifunctional \( M_6, 37,000 \) protein (2, 15) is the precursor of the \( M_6, 67,000 \) laminin receptor but the exact manner by which it forms a mature \( M_6, 67,000 \) laminin receptor is not clear (16, 17). In addition to its physiological roles (18, 19), expression of the \( M_6, 67,000 \) laminin receptor has been shown to be up-regulated in neoplastic cells compared with their normal counterparts and to directly correlate with an enhanced invasive and metastatic potential in many malignancies (14, 20–22). The receptor has been implicated in laminin-induced tumor cell attachment (20, 23, 24) and migration (25), as well as in tumor angiogenesis (26), growth, invasion, and metastasis (2, 20, 24).

Studies of laminin-induced signal transduction have focused on integrins (7, 27) and provided only limited data regarding the role of the \( M_6, 67,000 \) laminin receptor in signaling. It has been shown that a cell-laminin interaction via the \( M_6, 67,000 \) laminin receptor is an important step in signal transduction pathways (28) and that laminin and the \( M_6, 67,000 \) laminin receptor are probably involved in kinase-phosphatase cascades (8). Mitogen-activated protein kinase (MAPK) cascades transduce a diverse spectrum of extracellular and intracellular stimuli into alterations in gene expression and cellular function. The three major mammalian MAPK subgroups include extracellular signal-regulated kinases (ERKs), c-Jun \( \text{NH}_2 \)-terminal protein kinase (JNK)/stress-activated protein kinase, and p38 MAPK. The ERK pathway is mainly responsive to mitogens and growth factors and plays a key role in cell proliferation, survival, and differentiation. The JNK and p38 pathways are activated in response to chemical and environmental stress and to inflammatory cytokines. However, cross-links between the different MAPK cascades exist (29, 30). MAPKs are activated by phosphorylation on threonine and tyrosine residues located within the activation loop of the enzyme by dual-specificity MAPK kinases. Prolonged activation results in translocation of the activated MAPK into the nucleus (31). The MAPK level is a major point of regulation of the cascades, and MAPK activation level reflects a balance between the activities of the upstream kinases and protein phosphatases (29, 31). MAPK can be inactivated by serine/threonine phosphatases, tyrosine phosphatases, and dual-specificity MAPK phosphatases (DUSPs). The DUSPs show diverse distribution in different tissues and cell types, as well as differing substrate specificities, cellular location, and regulation (29, 31, 32).

In the present study, we investigated the role of the \( M_6, 67,000 \) laminin receptor in mediating the effects of laminin, its reciprocity with the \( \alpha_6 \) integrin subunit, and the involvement of the MAPK cascades and DUSPs in laminin signaling in human melanoma cell lines.
MATERIALS AND METHODS

Cell Lines and Plasmids. A super metastatic human melanoma cell line, A375SM (SM) was a generous gift from Prof. J. Fidler (M.D. Anderson Cancer Center, Houston, TX; Ref. 33). Cells were maintained in DMEM supplemented with 10% FCS, penicillin, streptomycin, amphotericin-1, -glutamine, sodium pyruvate, vitamins, and nonessential amino acids (Biological Industries, Beit Haemek, Israel). Cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

The plasmids containing the sense or antisense sequence of the MF, 37,000 laminin receptor precursor were a generous gift from Dr. H. Kleinman and Dr. Y. Yamada (NIH, Bethesda, MD). Laminin-1, collagen IV, and Matrigel were extracted from the Engelbreth-Holm Swarm tumor as described by Kleinman et al. (34).

Transfection. A375SM cells were transfected with the relevant plasmid using lipofection (Invitrogen, Life Technologies, Inc.) according to the manufacturer’s instructions. The culture medium was supplemented with 0.8 mg/ml G418 (Sigma, St. Louis, MO) 2 days after transfection and thereafter. G418 resistant clones: 40 antisense clones (SM-AS-101-140) and 40 sense clones (SM-S-1-40) were isolated. In addition to resistance to G418, the plasmid expression was verified by reverse transcription-PCR using the primers for the G418 resistance gene and by Western blot analysis using the anti-MF, 67,000 laminin receptor antibody, kindly provided by Dr. Kleinman (NIH).

Attachment Assay. 96-well plates covered with various concentrations of laminin-1 and blocked by 2% BSA were used for the attachment assays. Cells were harvested by brief exposure to 1 mM EDTA, washed with serum-free medium, and added to the laminin-coated wells (50,000 cells). After 1 h of incubation, nonadherent cells were removed by two gentle washes with PBS, and the attached cells were stained using 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma). Cells were lysed with DMSO, and the color intensity of the solution was read at 540 nm. All values are expressed in terms of the absorbance of A375SM cells normalized to 100%. The results are expressed as the mean ± SE of the relative absorbance in three independent experiments.

Invasion Assay. Boyden chamber chamber invasion assays were performed as described previously by Reich et al. (35). Matrigel (reconstituted basement membrane; 25 µg) was flooded on a polycarbonated filter (PVDF; Nuclepore; Whatman, Maidstone, United Kingdom). Fibroblast conditioned medium (obtained from confluent NIH-3T3 cells cultured in serum-free DMEM) was used as the chemoattractant. Cells were harvested by brief exposure to 1 mM EDTA, washed with serum-free medium, and added to the laminin-coated wells (50,000 cells). After 1 h of incubation, nonadherent cells were removed by two gentle washes with PBS, and the attached cells were stained using 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma). Cells were lysed with DMSO, and the color intensity of the solution was read at 540 nm. All values are expressed in terms of the absorbance of A375SM cells attached to 10 µg of laminin normalized to 100%. The results are expressed as the mean ± SE of the relative absorbance in three independent experiments.

Analysis of MMP Activity (Zymography). Cells (200,000) were incubated for 24 h in serum-free medium, and the resultant supernatant was extracted from the Engelbreth-Holm Swarm tumor as described by Kleinman et al. (34).

Western Blot Assay. Cells (150,000) were plated on a 6-well plate. Twenty-four h later, the culture medium was changed to a serum-free medium containing different concentrations of laminin-1. After various incubation periods, the cells were washed in cold PBS and lysed in 1% NP40, 0.5 mM EDTA, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM sodium orthovanadate, and 0.1% SDS (Sigma). Fifteen µg of protein from each sample, under reducing conditions, were loaded on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to Immobilon transfer membranes (Millipore, Bedford, MA). Membranes were blocked in Tris Buffered Saline with Tween 20 (TBST; 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) containing 5% BSA (Sigma) for 2 h at room temperature and then incubated overnight in 4°C in 5% BSA in TBST containing anti-ERK, anti-phospho JNK, or anti-phospho p38 (Biosource, Camarillo, CA). Membranes were then washed three times for 10 min with TBST followed by 1 h incubation with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) in TBST containing 5% BSA. After being washed four times for 10 min with TBST, membranes were developed by enhanced chemiluminescence (ECL; Pierce, Rockford, IL), according to the manufacturer’s specifications. Membranes were stripped, blocked, and then incubated overnight at 4°C in 5% BSA in TBST containing anti-ERK, anti-JNK (Biosource), or antiphospho-p38 (StressGen Victoria, British Columbia, Canada). All values are expressed in terms of A375SM cells normalized to 100%. Results are expressed as the mean ± SE of three independent experiments.

Reverse Transcription-PCR Procedure. Cells (750,000) were incubated in serum-free medium for 24 h. Total RNA was extracted using a commercial kit (Tri-Reagent; Sigma). Total RNA (0.5 µg) were reverse transcribed using M-MLY Reverse Transcriptase (Promega, Madison, WI), incubated for 2 h in 37°C, followed by 5 min in 95°C, and diluted 1:5 with RNase-free water. The cDNA sample was further processed by PCR using the primer pairs listed in Table 1 (36–41). Using a DNA thermal cycler (PTC-100; MJ Research Inc.). The optimal number of amplification cycles was evaluated for each primer pair, and the specificity of each primer pair was verified by sequencing. Taq DNA Polymerase (Promega) was used for the PAC-1, MKP-4, G418 resistance gene, MMP-2, integrin α6 subunit, and 28S PCR tests, Ampliclone DNA polymerase (Perkin-Elmer, Roche, NJ) was used for MKP-1 and MKP-5 PCR tests. Cycle parameters were: MKP-1 — denaturation at 92°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s, for 32 cycles; PAC-1 — denaturation at 94°C for 60 s, annealing at 64°C for 60 s, and extension at 72°C for 30 s.

Table 1 Sequences of sense and antisense primers used for reverse transcription-PCR amplification

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<th>Primers pairs</th>
<th>Product size (bp)</th>
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for 120 s, for 33 cycles; MKP-4—denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 60 s, for 30 cycles; MKP-5, G418 resistance, MMP-2, and integrin α6 subunit—denaturation at 94°C for 30 s, annealing at 63.5°C, 60°C, 55°C, and 55°C, respectively, for 60 s, and extension at 72°C for 90 s, for 30, 32, 29, and 30 cycles respectively; and 28S—denaturation at 94°C for 35 s, annealing at 63°C for 50 s, and extension at 72°C for 30 s, for 19 cycles. PCR products were separated on 1.5% agarose gel containing 0.05 μg/ml ethidium bromide, visualized under UV light, and photographed. The picture was scanned (UMAX Astra 1200S), and the intensity of the individual bands was quantified with NIH image 1.62. All values were divided by the value of the corresponding 28S and expressed in terms of A375SM cells normalized to 100%. Results of the G418 resistance gene are expressed in terms of the transfected plasmid that was used as positive control, normalized to 100%. Results are expressed as the mean ± SE of three independent experiments.

Statistics. Using nonparametric statistical analysis, P < 0.05 was considered statistically significant.

RESULTS

Establishing Melanoma Cell Lines Expressing Reduced or Increased Mr 67,000 Laminin Receptor. Forty stable G418-resistant antisense and G418-resistant sense clones were selected. Plasmid expression was verified by reverse transcription-PCR for G418 resistance gene, showing the presence of this mRNA only in the transfected cells (SM-AS-109, SM-AS-112, SM-S-19, and SM-S-23 in Fig. 1A) and not in the wild-type cells (A375SM; Fig. 1A). Western blot analysis showed that the expression of Mr 67,000 laminin receptor was significantly reduced in the antisense-expressing cell lines (SM-AS-109 and SM-AS-112 in Fig. 1B) compared with the wild-type A375SM cell line, and was significantly increased in the sense-expressing cell lines (SM-S-19 and SM-S-23 in Fig. 1B). Two antisense-expressing cell lines (SM-AS-109 and SM-AS-112) and two sense-expressing cell lines (SM-S-19 and SM-S-23) were chosen for additional analysis. The antibody recognized also a Mr 37,000 protein. Expression of this protein was also reduced in the antisense-expressing cells (data not shown).

Mr 67,000 Laminin Receptor Mediates Cell Attachment to Laminin. The ability of the cells expressing different levels of the Mr 67,000 laminin receptor to attach to laminin was evaluated by attachment to varying laminin doses. The antisense-transfected cell lines, SM-AS-109 and SM-AS-112, showed significantly reduced ability to attach to various amounts of bound laminin compared with the parental A375SM cells, whereas the sense-transfected cell lines, SM-S-19 and SM-S-23, showed significantly increased attachment to laminin compared with the wild-type cells (Fig. 2).

Mr 67,000 Laminin Receptor Is Involved in the Invasion of Melanoma Cells. The invasion ability of the cell lines expressing different levels of the Mr 67,000 laminin receptor was evaluated by the Matrigel invasion assay. Results show that the antisense-transfected cells (SM-AS-109 and SM-AS-112) have reduced ability to traverse Matrigel-covered filters compared with wild-type cells and sense-transfected cells (Fig. 3).

Mr 67,000 Laminin Receptor Is Involved in Regulation of MMP-2 Activity. The involvement of the Mr 67,000 laminin receptor and MMP-2 in tumor progression, invasiveness and metastasis is well established (2, 12, 14). However, no association between the two molecules has been shown, except for the report of laminin-induced MMP-2 activity in cancer cells (11). The expression and the activity of MMP-2 were analyzed by reverse transcription-PCR and by zymography in the cell lines expressing different levels of the Mr 67,000 laminin receptor. The antisense-transfected cells, SM-AS-109 and SM-AS-112, showed significant decrease in MMP-2 mRNA level compared with wild-type cells and sense-transfected cells, as shown using reverse transcription-PCR (Fig. 4A). Furthermore, zymography showed that the activity of MMP-2 was significantly reduced in the antisense-transfected cells (SM-AS-109 and SM-AS-112) compared with wild-type cells and sense-transfected cells (Fig. 4B).
a6B Integrin Subunit mRNA Level Is Associated with the Expression of M6 67,000 Laminin Receptor. Studies showed co-
regulation of the a6 integrin subunit and the M6 67,000 laminin receptor (23, 42, 43). One study demonstrated that a reduction of a6 integrin subunit expression was accompanied by a decrease in the cell surface expression of the M6 67,000 laminin receptor (43). In the present study, we analyzed the inverse effect, i.e., the consequence of decreased expression of the M6 67,000 laminin receptor on integrin a6 subunit mRNA level. The primers that were used resulted in two bands, representing the alternatively spliced isoforms a6A and a6B (44, 45). a6A, 392 bp in length, corresponds to nucleotides 3033–3425 in the mRNA of human a6 integrin subunit, whereas a6B, 262 bp in length, corresponds to nucleotides 3033–3260 and 3390–3425 of a6 integrin subunit mRNA. A significant decrease in the level of a6B isoform mRNA was seen in the antisense-transfected cells compared with sense-transfected and wild-type cells, whereas no change in a6A isoform mRNA level was seen between the different cell lines (Fig. 5).

The M6 67,000 Laminin Receptor Signal Transduction Induces Dephosphorylation of ERK, JNK, and p38 MAPK. It has been shown that laminin induces dephosphorylation in neuronal cells (8) and that the signal transduction pathways of laminin-binding integrins involve MAPK cascades (7, 46, 47). However, there is no data regarding the involvement of MAPK cascades in M6 67,000 laminin receptor signal transduction. The expression and the activity (phosphorylation) of the kinases ERK, JNK, and p38 were analyzed in cell lines expressing various levels of the M6 67,000 laminin receptor. Results clearly show that there is a significant increase in the activity (phosphorylation) of ERK, JNK, and p38 in the antisense-transfected cells compared with wild-type cells (an increase of 24%, 42%, and 16% was seen in the phosphorylation of ERK, JNK, and p38, respectively, in SM-AS-109 and an increase of 29%, 80%, and 19% was seen in the phosphorylation of ERK, JNK, and p38, respectively, in SM-AS-112; Fig. 6). In the sense-transfected cells (SM-S-23), a decrease in the phosphorylation of ERK (16%) and p38 (22%) and a nonsignificant increase in the phosphorylation of JNK compared with the nontransfected cells were seen (Fig. 6). No change was seen in the expression level of the kinases. This pattern was seen at all incubation times (data not shown), indicating that the elevated phosphorylation level in the antisense-transfected cells is inherent and not temporary. Therefore, the results suggest that as part of its signal transduction pathway, the M6 67,000 laminin receptor induces dephosphorylation, thus decreased activity, of the three MAPK cascades, rather than decreased expression of the enzymes.

Laminin-1 Induces Dephosphorylation of ERK, JNK, and p38 MAPK. The direct effect of exogenous laminin-1 on MAPK cascades was evaluated by Western blot analysis with A375SM cells. Various concentrations of laminin-1 (0–15 μg/ml) were added to A375SM cell culture. After various incubation times (5, 10, 30, and 60 min), the cells were harvested and Western blot analysis was performed. Results show that in A375SM cells, exogenous soluble laminin-1 induced temporary dephosphorylation of ERK, JNK, and p38 MAPK in a dose-dependent manner after 30 min of incubation (data not shown). Furthermore, cells expressing various levels of M6 67,000 laminin receptor were exposed to 15 μg/ml soluble laminin-1 for 5, 10, 30, and 60 min, followed by Western blot analysis. This shows that the exogenous soluble laminin-1 induced a significant reduction in the phosphorylation of ERK, JNK, and p38 in all of the cell lines, only after 30 min of incubation. The reduction in ERK phosphorylation was to 44, 48, 58, and 57% of basal activities in SM-AS-109, SM-AS-112, A375SM, and SM-S-23 cell lines, respectively. The reduction in JNK phosphorylation was to 45, 65, 77, and 52% of the basal levels and the reduction in p38 phosphorylation was to 32, 35, 47, and 52% of basal levels in SM-AS-109, SM-AS-112, A375SM, and SM-S-23 cell lines, respectively (Fig. 6). The reduction of phosphorylation induced by laminin-1 was seen in all cell lines, independently of the expression level of the M6 67,000 laminin receptor. However, the elevated phosphorylation level of all MAPK in cells expressing reduced M6 67,000 laminin receptor (SM-AS-109 and SM-AS-112) was still seen during the exogenous laminin-1-induced dephosphorylation (Fig. 6).

MKP-1 and PAC-1 Are Inversely Involved in the M6 67,000 Laminin Receptor Signal Transduction. To further investigate the role of the M6 67,000 laminin receptor on MAPK cascades, the
expression of several MAPK phosphatases, which are involved in the regulation of ERK, JNK, and p38 (31, 32), was studied in the cells expressing different levels of the Mr 67,000 laminin receptor. Four phosphatases of the DUSP family, MKP-1, PAC-1, MKP-4, and MKP-5 were found to be expressed in the cells, of which two phosphatases, MKP-1 and PAC-1, were found to be affected by the expression level of Mr 67,000 laminin receptor. We observed that mRNA level of MKP-1 was significantly lower in the antisense-transfected cells (SM-AS-109 and SM-AS-112) compared with sense-transfected cells (SM-S-19 and SM-S-23; +, P < 0.05). MKP-1 mRNA levels in SM-AS-109 and SM-AS-112 cell lines were reduced to 29% and 23% of the nontransfected cell values, respectively, and a slight increase was seen in the sense-transfected cells (Fig. 7). Interestingly, in contrast to MKP-1 mRNA expression pattern, the antisense-transfected cells had a significantly increased level of PAC-1 mRNA compared with the other cell lines (an increase of 61% in SM-AS-109 and of 66% in SM-AS-112 compared with the A375SM cells, with no significant changes in sense-transfected cell lines; Fig. 7). Thus, decreased expression of Mr 67,000 laminin receptor is associated with decreased level of MKP-1 mRNA and increased level of PAC-1 mRNA.

**DISCUSSION**

The aim of the present study was to broaden the understanding of the role of the Mr 67,000 laminin receptor in mediating the effects of laminin, its reciprocity with the α6 integrin subunit, and the involvement of the MAPK cascades and DUSPs in laminin signaling in human melanoma cell lines. This was achieved through comparison of melanoma cell lines expressing various Mr 67,000 laminin receptor levels, which were established by stable transfection.

Laminin is a substrate for invading tumor cells, and there is a correlation between the attachment ability of a cell and its metastatic potential (3, 48). The ability of tumor cells to transverse Matrigel-coated filters correlates with their ability to form metastases in animals (49). In the present study, cell lines expressing reduced Mr 67,000 laminin receptor demonstrated a significantly reduced ability to attach to laminin and to traverse Matrigel-coated filters compared with parental cells and sense-transfected cells (SM-S-19 and SM-S-23) and wild-type cells (A375SM). MKP-1 mRNA levels in SM-AS-109 and SM-AS-112 cell lines were reduced to 29% and 23% of the nontransfected cell values, respectively, and a slight increase was seen in the sense-transfected cells (Fig. 7). Interestingly, in contrast to MKP-1 mRNA expression pattern, the antisense-transfected cells had a significantly increased level of PAC-1 mRNA compared with the other cell lines (an increase of 61% in SM-AS-109 and of 66% in SM-AS-112 compared with the A375SM cells, with no significant changes in sense-transfected cell lines; Fig. 7). Thus, decreased expression of Mr 67,000 laminin receptor is associated with decreased level of MKP-1 mRNA and increased level of PAC-1 mRNA.
SM-AS-109 cells (at various time points. The antisense-transfected SM-S-23 the sense-transfected cells (and SM cant increment in the phosphorylation level of all with specific antibodies directed against the total enzymes (active and nonactive), pan ERK, pan JNK, and pan p38, with or without exogenous laminin-1, at various time points. The antisense-transfected cells (SM-AS-109 and (SM-AS-112) show significant increment in the phosphorylation level of all MAPK compared with the parental cells (SM) and the sense-transfected cells (SM-S-23; **, P < 0.05). Exogenous soluble laminin-1 induced significant dephosphorylation of all MAPK in all cell lines after 30 min of incubation (+, P < 0.05). A. phosphorylated ERK after 30 min of incubation with exogenous laminin-1. B. phosphorylated JNK after 30 min of incubation with exogenous laminin-1. C. phosphorylated p38 after 30 min of incubation with exogenous laminin-1.

The interaction of cancer cells with laminin has been established as key event in tumor invasion and metastasis (3, 48), apparently in part via laminin-induced proteolytic activity of MMP-2 in metastatic cells (11). In the present study, the involvement of the $M_r 67,000$ laminin receptor in the induction of MMP-2 activity was demonstrated by significantly reduced MMP-2 mRNA level and activity in the cells expressing reduced $M_r 67,000$ laminin receptor. These findings implicate the $M_r 67,000$ laminin receptor, which is overexpressed in many cancer types (2, 14), in laminin-induced tumor dissemination and provide a possible mechanism, through induction of collagen IV degradation, for the previously seen correlations between expression and provide a possible mechanism, through induction of collagen IV degradation, for the previously seen correlations between expression and protein dephosphorylation in neural cells during process formation (8). In the present study, it was clearly demonstrated that the addition of exogenous soluble laminin-1 results in a significant decrease in the phosphorylation (activation) of ERK, JNK, and p38 after 30 min of incubation. This soluble laminin-induced dephosphorylation was independent of the $M_r 67,000$ laminin receptor level, because it was seen in all cell lines irrespective of the expression level of the receptor. These findings suggest that additional laminin-related signal transduction pathways exist. The possible involvement of different laminin receptors in the cellular response is supported by our findings regarding the effect of the $M_r 67,000$ laminin receptor expression level on MAPK phosphorylation. The basal phosphorylation extent of ERK, JNK, and p38 was significantly higher in cell lines expressing reduced $M_r 67,000$ laminin receptor, compared with parental cells and sense-transfected cells, regardless of the exposure to exogenous laminin-1. Thus, these findings support the idea that additional exogenous soluble laminin-1 increases additional protein dephosphorylation, apparently not via the $M_r 67,000$ laminin receptor.

Studies of signal transduction related to integrins have implicated the laminin-binding integrins $6B4$ and $6B6$ in activation of ERK/ JNK and ERK cascades, respectively. In these studies, surface-bound laminin was used, and adhesion plaque elements were involved (7, 46, 47). On the other hand, it was shown that bound laminin induces protein dephosphorylation in neural cells during process formation (8). In the present study, it was clearly demonstrated that the addition of exogenous soluble laminin-1 results in a significant decrease in the phosphorylation (activation) of ERK, JNK, and p38 after 30 min of incubation. This soluble laminin-induced dephosphorylation was independent of the $M_r 67,000$ laminin receptor level, because it was seen in all cell lines irrespective of the expression level of the receptor. These findings suggest that additional laminin-related signal transduction pathways exist. The possible involvement of different laminin receptors in the cellular response is supported by our findings regarding the effect of the $M_r 67,000$ laminin receptor expression level on MAPK phosphorylation. The basal phosphorylation extent of ERK, JNK, and p38 was significantly higher in cell lines expressing reduced $M_r 67,000$ laminin receptor, compared with parental cells and sense-transfected cells, regardless of the exposure to exogenous laminin-1. Thus, these findings suggest that the $M_r 67,000$ laminin receptor induces prolonged dephosphorylation of ERK, JNK, and p38 and that additional exogenous soluble laminin-1 induces additional temporary dephosphorylation, apparently not via the $M_r 67,000$ laminin receptor.

The extent of protein phosphorylation is balanced by antagonism of kinase and phosphatase activities, and the DUSP family plays a pivotal role in regulating the activity of MAPK (29, 31, 32). To further
investigate $M_{67}$, 67,000 laminin receptor-induced MAPK dephosphorylation, the expression of several DUSPs, MKP-1, PAC-1, MKP-4, and MKP-5, which were found to be expressed in the A375SM melanoma cell line, was studied in cells expressing different levels of the receptor. Results show that the increase in MAPK phosphorylation in cells expressing reduced $M_{67}$, 67,000 laminin receptor is accompanied by a significant reduction in MKP-1 mRNA level and a significant increase in PAC-1 mRNA level, with no change in MKP-4 and MKP-5 mRNA levels. Interestingly, it is well established that prolonged activation of MAPK results in translocation of the activated kinases into the nucleus (29, 31) and that MKP-1 and PAC-1 are nuclear enzymes, which are regulated on the transcriptional level and are encoded by early response genes that are either growth factors or stress inducible (29, 31, 53, 54). Overexpression of the ubiquitous enzyme MKP-1, which dephosphorylates ERK, JNK, and p38 (32), has been found in several malignancies (53, 55) and has been reported to be inversely related to apoptosis (36, 56). Our findings are in agreement with these reports, as evidenced by a decrease in MKP-1 mRNA levels. Interestingly, it is well established that pro-apoptotic mechanisms, because it has been shown that an increase in ERK phosphorylation results in transcription and activity of PAC-1, which in turn dephosphorylates and inactivates ERK (54, 57).

In conclusion, our findings provide novel insight regarding the understanding of various aspects related to the role of the $M_{67}$, 67,000 laminin receptor in tumor progression and dissemination. Cells expressing reduced $M_{67}$, 67,000 laminin receptor demonstrate a less aggressive phenotype, as reflected by their reduced invasiveness through Matrigel, diminished attachment to laminin, and decreased MMP-2 expression and activity. In addition, reduction in MKP-1 mRNA transcription, which has been reported to be inversely correlated with tumor malignancy (55, 56), was seen in cells with reduced $M_{67}$, 67,000 laminin receptor expression. This reduction could mediate both the increase in MAPK phosphorylation and in PAC-1 mRNA level that was seen in cells characterized by decreased expression of $M_{67}$, 67,000 laminin receptor. Interestingly, the most aggressive cellular phenotype was characterized by a higher level of the $M_{67}$, 67,000 laminin receptor and a reduced activity of MAPK. Satoh et al. (20) showed that reduced expression of the $M_{67}$, 67,000 laminin receptor in murine lung cancer cell line results in a reduction in tumor formation in mice. In our study, exogenous laminin-1, which increases the metastatic potential of tumor cells (2, 48), induced dephosphorylation of the MAPK. We can therefore speculate that reduced activity of MAPK is associated with increased malignancy of tumor cells. Supporting this hypothesis is the finding that increased level and activity of certain MAPK in ovarian carcinoma cells in effusions is associated with clinical parameters of improved outcome and significantly longer overall survival (58). In summary, the $M_{67}$, 67,000 laminin receptor affects fundamental signal transduction pathways and enzyme activities that are involved in cancer. Elucidation of additional roles of this receptor may extend our understanding of the mechanisms underlying tumor dissemination.

REFERENCES


Laminin-Induced Signaling in Tumor Cells: The Role of the Mr 67,000 Laminin Receptor

Vered Givant-Horwitz, Ben Davidson and Reuven Reich

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