Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 84-90

THE HIGH AFFINITY MURINE LAMININ RECEPTOR IS A MEMBER OF A MULTICOPY GENE FAMILY

María-Teresa Fernández*, Vincent Castronovo, C.N. Rao, and Mark E. Sobel+

Tumor Invasion and Metastasis Section, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892

Received December 21, 1990

The high affinity laminin receptor is differentially expressed in metastasis. We now report that there are multiple copies (6±1) of the laminin receptor gene in the murine genome of normal diploid cells as well as in cell lines derived from cancer cells. We have analyzed three distinct cDNA clones isolated from an Okayama-Berg cDNA library of transformed mouse fibroblasts that may represent transcripts of three different laminin receptor genes. Polymorphic changes include insertion of bases at the 5' terminus, a base substitution within the coding region resulting in an amino acid change from phenylalanine to leucine, a base substitution obliterating a polyadenylation signal, as well as changes in the length of the 3' untranslated domains. The discovery of multiple transcripts of laminin receptor genes suggests that there is a strong selective pressure to maintain laminin receptor expression in murine cells.

Interaction of cells with the extracellular matrix plays an important role in a variety of biological phenomena, including cell attachment, spreading, morphogenesis, differentiation, mitogenesis, neurite outgrowth, cell migration, and tumor cell metastasis (1,2). These cellular responses are mediated via specific cell surface receptors for extracellular attachment proteins such as fibronectin and laminin (2,3). Among the various laminin binding proteins that have been identified, the 67-kilodalton high affinity laminin receptor has been identified in a number of human and rodent normal and neoplastic cells and tissues (4-8) and is differentially expressed in metastasis (9,10).

cDNA clones of the 67-kilodalton laminin receptor have been isolated from both human and murine sources (6, 11-13). Some evidence has been presented to suggest that there are multiple laminin receptor genes in the human genome (14,15). In the present study, we report the existence of multiple copies of the laminin receptor gene in the mouse genome, and for the presence of polymorphic

*Present address: Departamento de Biología Funcional, Area de Bioquímica y Biología Molecular, Universidad de Oviedo, 33071 Oviedo, Spain.

+To whom correspondence and reprint requests should be addressed.

0006-291X/91 $1.50

laminin receptor mRNA transcripts in murine cells with size heterogeneity in the 3' untranslated domain.

MATERIALS AND METHODS

Cell lines. The metastatic M2 cell line and the low metastatic clone 19 of the K1735 murine melanoma have been described (16,17). Cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 u/ml penicillin and 100 ug/ml streptomycin, obtained from GIBCO (Grand Island, NY), at 37° C in a humidified atmosphere with 5% CO_2.

Hybridization probes. All hybridization probes were purified from acrylamide gels and nick translated as described (6). For initial cDNA library screening, the 650 bp HindIII-PstI human laminin receptor cDNA restriction fragment of pLR4-4 was used (6). In gene copy experiments, the 750 bp PstI-EcoRI murine laminin receptor cDNA restriction fragment of pMLR21 (13) or pMLR11 was used. In some experiments, sub-fragments of the murine cDNA were used, including a 200 bp BamHI-XhoI fragment representative of the 5' end of pMLR11, and a 550 bp XhoI-EcoRI fragment representative of the 3' end of pMLR11.

Isolation and Characterization of cDNA clones. An Okayama-Berg cDNA library of bovine papillomavirus type I transformed murine C127 fibroblasts (18) was kindly provided by Dr. C. Baker (National Cancer Institute, Bethesda, MD) and has been previously described (13). The library was transformed into Escherichia coli HB101. The library was then screened with ^{32}P nick translated cDNA insert of the human laminin receptor cDNA clone pLR4-4 by colony hybridization as described (13). Plasmid DNA was isolated and subjected to restriction endonuclease analysis and Southern hybridization as described (13).

DNA sequence analysis. The nucleotide sequence of overlapping restriction fragments of clones pMLR11 and pMLR29 was obtained by the chemical modification method of Gilbert and Maxam (19).

Isolation of genomic DNA. High molecular weight DNA was isolated from cultured cells after direct lysis in a buffer containing proteinase K and SDS as described (20). Normal diploid genomic DNA from adult Balb/c liver was obtained from Clontech Laboratories, Inc (San Francisco, CA).

Gene copy experiments. Gene copy numbers were determined by slot blot hybridization using a Minifold II slot-blotter (Schleicher & Schuell, Keene, NH). Standard curves of gene copy number were constructed using known amounts of pMLR11 cDNA, equivalent to 0, 6, 12, or 24 gene copies. The amounts of pMLR11 plasmid DNA used for the standard curves were calculated considering that the plasmid size was 4000 bp and that the mouse haploid genome contains 3×10^9 bp. Duplicates of pMLR11 plasmid DNA were applied into individual slots on nitrocellulose in parallel with duplicates of aliquots of murine genomic DNA, containing 0.5, 2.0 and 4.0 ug. Hybridization was carried out separately with three different murine laminin receptor cDNA restriction fragments as described above. Gene copy number was determined after scanning multiple-timed exposures of autoradiographs, using a 2202 Ultrascan Laser Densitometer (LKB). A standard curve (from pMLR11 DNA) was constructed for each of the probes and compared with the absorbance values obtained from the genomic DNA hybridized with the same probe. The known single copy gene of human osteonectin (21) was used to verify the validity and the accuracy of the gene copy number determination protocol. The data was analyzed by the method of least squares to generate standard curves.

RESULTS

Isolation and characterization of multiple laminin receptor cDNA clones. We previously reported the characterization of a murine laminin receptor clone (designated pMLR21) that was isolated by screening an Okayama-Berg cDNA library of transformed murine C127 cells using a human laminin receptor cDNA probe (13).

Shortly thereafter, we became aware of the report of a cDNA clone (designated here as pMak) for a major mouse mRNA isolated from an Okayama-Berg library of mouse L cells (12). The latter clone contained sequences that were remarkably similar to pMLR21, however there were three significant differences between the two reported sequences. One difference involved a base change (base 130 of pMLR21 and base 128 of pMak) from C to T, resulting in a change in amino acid 18 from leucine in pMLR21 to phenylalanine in pMak. It should be noted that in the human protein, the amino acid residue is phenylalanine (11). Another difference involved the 5' untranslated region. Primer extension experiments showed that pMLR21 probably contained a complete 5' sequence, which began with a C followed by a string of 6 Ts (13). The 5' sequence of pMak did not contain the initial 2 bases of pMLR21 (12). The most intriguing difference between pMLR21 and pMak involved a difference in the size of the 3' untranslated domain. Both clones, having been isolated from Okayama-Berg cDNA libraries, were obviously derived from polyadenylated mRNA species, however pMLR21 contained an additional 54 bases downstream between the end of pMak and its poly(A) tail. This placed the so-called polyadenylation signal AAUAAA of pMLR21 an unusually long distance (74 bases) upstream of the poly(A) tail.

The sequence variations between pMLR21 and pMak, coupled with some evidence for multiple human laminin receptor genes (14,15) led us to characterize additional murine laminin receptor cDNA clones that had been isolated from the same library as pMLR21. Two clones, pMLR11 and pMLR29, were selected for further analysis on the basis of size of cDNA insert, as determined by BamHI restriction endonuclease digestion and subsequent Southern blot hybridization as described (13). Restriction maps of pMLR11 and pMLR29 demonstrated that, like pMak, they contained all the sites previously described for pMLR21 with the exception of a PstI site located in the 3' untranslated domain of the latter clone (data not shown).

The complete nucleotide sequences of pMLR11 and pMLR29 were established and compared to that of pMLR21 (Figure 1). There are four differences between pMLR29 and pMLR21. First, inserted between bases 1 and 2 of pMLR21, pMLR29 contains two additional Ts. This results in an intial 5' sequence of $CT_{(8)}$ instead of the $CT_{(6)}$ of pMLR21. The second difference between pMLR29 and pMLR21 is that the former clone has the same base change at position 130 as does pMak; i.e., a T replaces a C with a resulting amino acid change from leucine to phenylalanine. The third difference between the two clones is that pMLR29 contains a base substitution at base 1015 from T to C. This results in the loss of a classic polyadenylation signal in pMLR29, even though it was synthesized from a polyadenylated mRNA. Finally, the poly(A) tail of pMLR29 begins 41 bases upstream of pMLR21; the 3' PstI site of pMLR21 is therefore missing from pMLR29.

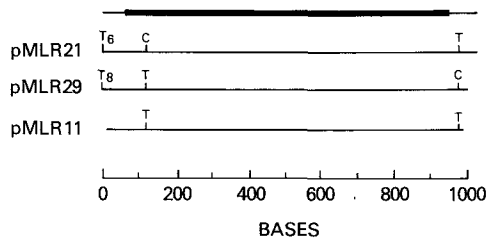

Figure 1. Comparison of murine laminin receptor cDNA clones

Shown on the top is a diagram of a full length cDNA clone for the murine laminin receptor, as determined by previous analysis of pMLR21 (13). Clones pMLR29 and pMLR11 are also represented. The latter two clones contain shorter 3′ untranslated regions compared to pMLR21. The initial 5′ 26 bases of pMLR21 are not present in pMLR11 and could not be compared. pMLR29 contains a string of 8 Ts after the inital C of its sequence, whereas pMLR21 contains a string of 6 Ts. At base 130 of pMLR21, clones pMLR11 and pMLR29 contain a T instead of a C, resulting in an amino acid change from leucine to phenylalanine. Finally, a mutation in the polyadenylation signal from AATAAA to AACAAA was found in pMLR29.

Notably, although the 3′ untranslated domain of pMLR29 is shorter than that of pMLR21, it is longer than that of pMak.

The sequence of pMLR11 varies from both pMLR29 and pMLR21. It does not contain a complete 5′ untranslated region, making it impossible to determine if it contains a CT_6 or a CT_8 sequence at its 5′ end. It contains the same base change at position 130 as pMLR29 and pMak. It contains an intact polyadenylation signal. Its 3′ untranslated domain is the smallest of the clones, identical in size (and sequence) to that of pMak.

Analysis of murine genomic DNA for laminin receptor sequences. The particular combination of base changes and varying 3′ untranslated regions demonstrated for pMLR21, pMLR29 and pMLR11, and the repetitive pattern of those polymorphisms, led us to predict that they may represent transcripts of multiple, distinct laminin receptor genes. If this were the case, we would expect to see multiple bands of hybridization on mouse genomic DNA blots. Genomic DNA from diploid adult Balb/c mouse liver was digested with restriction endonucleases EcoRI, HindIII, BamHI, PstI, and BglI and hybridized stringently with an internal cDNA insert of pMLR21 (Figure 2). Multiple bands of hybridization were observed with all the enzymes tested, consistent with the possibility of multiple laminin receptor genes. We cannot rule out the possibility, however, that there are multiple sites for each of the restriction enzymes tested within the introns of the laminin receptor gene that would explain the complex pattern of Southern blot hybridization. An identical pattern of hybridization was obtained using genomic DNA isolated from murine melanoma cell lines (data not shown).

To confirm the presence of multiple laminin receptor genes, the gene copy number was directly determined using a slot blot hybridization method. A representative experiment is shown in Figure 3. Using known amounts of plasmid

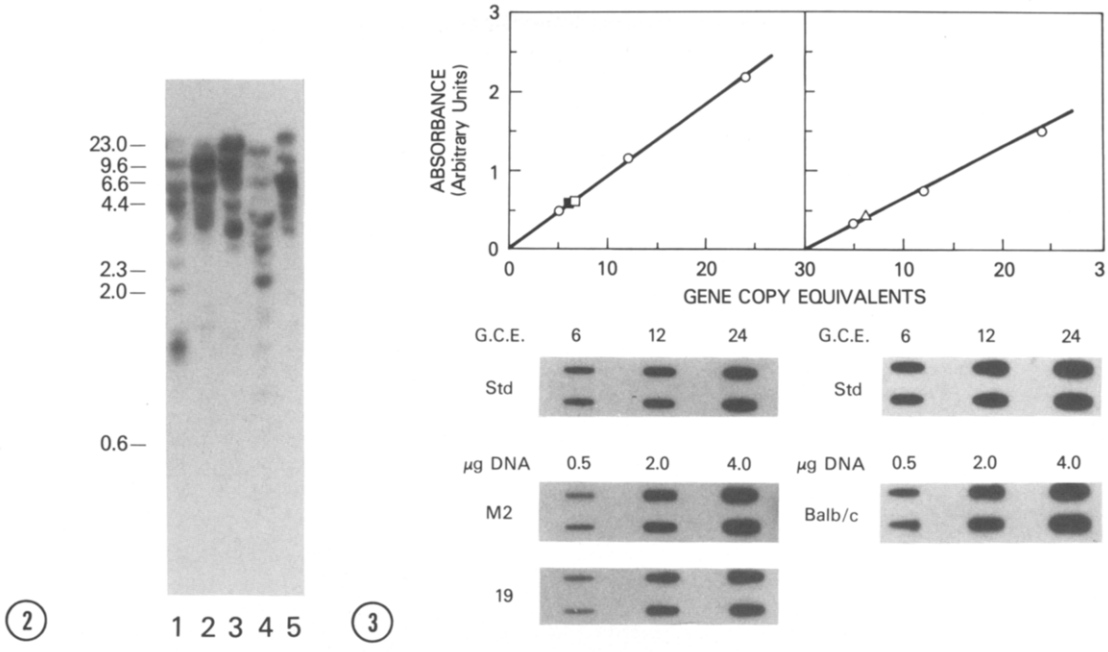

Figure 2. Identification of murine laminin receptor genomic sequences

Normal diploid DNA from Balb/c adult mouse liver (20 µg per sample) was digested with restriction endonucleases EcoRI (lane 1), HindIII (lane 2), BamHI (lane 3), PstI (lane 4), and BglI (lane 5) and fractionated by size on a 0.8% agarose gel. After transfer to nitrocellulose, hybridzation was performed under stringent conditions with a nick translated 750 bp cDNA insert of pMLR21 as described in Materials and Methods. Filters were washed down to 0.2 x SSC (3 mM Na citrate, 30 mM NaCl) in the presence of 0.2% SDS at 65°C. Molecular size standards were generated by the HindIII digestion of bacteriophage lambda DNA.

Figure 3. Murine laminin receptor gene copy number

DNA slot hybridization analysis was carried out as described in Materials and Methods. In this representative experiment, the 550 bp XhoI-EcoRI fragment of pMLR11 was used as a probe. [TOP] Autoradiographic images of the slot hybridzations shown below were scanned as described in Materials and Methods. The absorbance of the slots is displayed in arbitrary units versus the number of gene copy equivalents. Open circles represent the values of the standard curves of pMLR11 DNA. On the left, the closed and open squares represent the average of two values of genomic DNA from M2 and clone 19 cells of the K1735 melanoma, respectively. On the right, the open triangle represents the average of two values of normal diploid mouse liver DNA. [BOTTOM] Duplicate samples containing increasing amounts of pMLR11 and mouse genomic DNAs were applied to nitrocellulose and hybridized as described in Materials and Methods. Representative slot blots are shown for pMLR11 DNA (Std), M2, clone 19, and normal diploid Balb/c liver DNA. G.C.E. designates gene copy equivalents.

DNA as a standard curve, the copy number of the laminin receptor gene per haploid murine genome was found to be 6 ± 1. The same results were obtained for diploid Balb/c adult mouse liver and for the DNA of two different murine melanoma-derived cell lines (Figure 3). Furthermore, identical data were obtained using cDNA probes of varying lengths (representative of different regions of the gene, see Materials and Methods). To verify the validity of our technique, we determined that the human osteonectin gene, known to be a single copy gene (21), is represented once in the human genome.

DISCUSSION

In the present study, we report that there are multiple copies of the laminin receptor gene in the murine genome. This is the case for diploid DNA found in adult mouse liver, as well as in DNA found in tissue culture lines derived from murine melanomas. In addition to direct gene copy number experiments, the pattern of hybridizable laminin receptor DNA fragments in murine genomic DNA restricted with a variety of different endonucleases is consistent with the presence of multiple laminin receptor genes. Furthermore, we have isolated multiple cDNA clones which contain base variations as well as heterogeneity in the length of the 3' untranslated domain. The three cDNA clones characterized in the present study, therefore appear to represent the mRNA transcripts of 3 distinct laminin receptor genes and are probably not the result of cloning artifacts.

The three cDNA clones described in this report were isolated from an Okayama-Berg cDNA library made from polyadenylated mRNA (18). Nonetheless, pMLR29 did not contain a classic polyadenylation signal. Although rare, others have reported that the hexanucleotide AAUAAA is not required for polyadenylation of some mRNAs, such as those for the dihydrofolate reductase gene (22). In contrast to the case for dihydrofolate reductase, in which the multiple-sized mRNA transcripts are derived from a single gene, the multiple laminin receptor cDNAs described in this report appear to represent transcripts from three distinct genes.

As was found for the dihydrofolate reductase gene (22,23) the length of the 3' untranslated domain appears to differ amongst the various laminin receptor mRNAs. It is not clear if this results in a functional change in the ability to be translated, however it is interesting to note that the shorter transcript cloned by Makrides et al (12) from mouse L cells appears to be under translational control. Nonetheless, our laboratory has recently demonstrated that there is a direct correlation between varying amounts of laminin receptor mRNA and expressed laminin receptor protein present in a variety of human breast carcinoma-derived cell lines (unpublished data). The regulation of laminin receptor expression appears to be complex, involving transcriptional, translational, and post-translational mechanisms (12,13). In this report, a further possible complexity has been elucidated; i.e., the presence of multiple laminin receptor genes that may be differentially expressed. In any case, the evidence presented in this report for multiple copies as well as transcripts of the laminin receptor gene suggests that there is considerable selective pressure to maintain laminin receptor mRNA levels in murine cells.

ACKNOWLEDGMENTS

M.-T. Fernandez was a fellow of the FICYT, Principado de Asturias, Spain, during the initial stages of this work. The authors are grateful to Dr. Carl

Baker (National Cancer Institute, Bethesda, MD) for the Okayama-Berg library, and also thank Dr. Marian Young (National Institute of Dental Research, Bethesda, MD) for the osteonectin cDNA clone, and M. Christine Schmitt for help in preparing genomic DNA from murine melanoma cell lines.

REFERENCES

1. Liotta, L.A., Rao, C.N. & Wewer, U.M. (1986) Annu. Rev. Biochem., 55, 1037-1057.
2. Ruoslahti, E. (1988) Annu. Rev. Biochem., 57, 375-413.
3. Liotta, L.A., Wewer, U.M., Rao, C.N., Schiffmann, E., Stracke, M., Giurguis, R., Thorgeirsson, U., Muschel, R. & Sobel, M. (1987) Anti-Cancer Drug Design, 2, 195-202.
4. Malinoff, H.L. & Wicha, M.S. (1983) J. Cell. Biol., 96, 1475- 1479.
5. Lesot, H., Kuhl, U. & von der Mark, K. (1983) EMBO J., 2, 861- 865.
6. Wewer, U.M., Liotta, L.A., Jaye, M., Ricca, G.A., Drohan, W.N., Claysmith, A.P., Rao, C.N., Wirth, P., Coligan, J.E., Albrechtsen, R., Mudryj, M. & Sobel, M.E. (1986) Proc. Natl. Acad. Sci. USA, 83, 7137-7141.
7. Rao, C.N., Barsky, S.H., Terranova, V.P. & Liotta, L.A. (1983) Biochem. Biophys. Res. Commun., 111, 804-808.
8. Sugrue, S. (1988) Differentiation, 38, 169-176.
9. Castronovo, V., Colin, C., Claysmith, A.P., Chen, P., Lifrange, E., Lambotte, R., Liotta, L.A., & Sobel, M.E. (1990) Amer. J. Pathol., 137, 1373-1381.
10. Castronovo, V., Taraboletti, G., Liotta, L.A., & Sobel, M.E. (1989) J. Natl. Canc. Inst., 81, 781-786.
11. Yow, H., Wong, J.M., Chen, H.S., Lee, C., Steele, G.D. & Chen, L.B. (1988) Proc. Natl. Acad. Sci. USA, 85, 6394-6398.
12. Makrides, S., Chitpatima, S.T., Bandyopadhyay, R., & Brawerman, G. (1988) Nucl. Acids Res., 16, 2349.
13. Rao, C.N., Castronovo, V., Schmitt, M.C., Wewer, U.M., Claysmith, A.P., Liotta, L.A. & Sobel, M.E. (1989) Biochemistry, 28, 7476-7486.
14. Van den Ouweland, A.M.W., Van Duijnhoven, H.L.P., Deichmann, K.A., Van Croningen, J.J.M., de Leij, L., & Van de Ven, W.J.M. (1989) Nucl. Acids Res., 17, 3829-3843.
15. Segui-Real, B., Rhodes, C., & Yamada, Y. (1989) Nucl. Acid. Res., 17, 1257.
16. Fidler, I.J. (1984) Cancer Inv. and Metastasis: Biologic and Therapeutic Aspects (Nicholson, G.C. & Milas, C. eds.) pp. 5-26, Raven Press, New York.
17. Kalebic, T., Williams, Talmadge, J.E., Kao-Shan, C-S., Kravitz, B., Locklear, K., Siegal, G.P., Liotta, L.A., Sobel, M.E. & Steeg, P. (1988) Clin. Expl. Metastasis, 6, 301-318.
18. Yang, Y.C., Okayama, H., & Howley, P.M. (1985) Proc. Natl. Acad. Sci. USA, 82, 1030-1034.
19. Gilbert, W., & Maxam, A. (1973) Proc. Natl. Acad. Sci. USA, 70, 3581-3584
20. Davis, L.G., Dibner, M.B., & Battery, J.F. (1986) Basic Methods in Molecular Biology. pp. 44-50. Elsevier Science Publishing Co., Inc. New York.
21. Young, M.F., Day, A., Dominguez, P., McQuillan, C., Fisher, L., & Termine, J.D. (1990) Connect. Tisuue Res., 24, 17-28.
22. Setzer, D.R., McGrogan, M., & Schimke, R.T. (1982) J. Biol. Chem., 257, 5143-5147.
23. Setzer, D.R., McGrogan, M., Nunberg, J.H. & Schimke, R.T. (1980) Cell, 22, 361-370.