Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 9015–9019, December 1987
Cell Biology

Identification of a synthetic nonapeptide sequence that inhibits motility in culture of a melanoma subclone that possesses a high metastatic potential

(metastasis/thrombin/growth and differentiation factors)

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Communicated by Christian B. Anfinsen, August 19, 1987

ABSTRACT **A synthetic nonapeptide fragment of thrombin inhibits the cellular motility in culture of a human melanoma subclone that possesses a high metastatic potential in mice. Concomitant with the loss of ability to translocate in culture, these cells exhibit increases in the average length of actin cables and cellular surface area in contact with the substratum. The spreading activity is observed at a nonapeptide concentration of 1 nM within 1 hr of exposure at 37°C. Pretreatment of cells with this nonapeptide does not block signal transduction through plasma membrane receptors for the following growth or differentiation factors: α-melanotropin (α-melanocyte-stimulating hormone), nerve growth factor, and transforming growth factor type β. Results of the present study suggest an approach to cancer chemotherapy in which naturally occurring peptides from two functionally orthogonal classes may be used to perform two complementary functions: inhibition of metastasis and induction of differentiation.**

Melanoma is a malignancy of melanocytes, the pigment-producing cells of the skin. As in all cancers, the tumor cells exhibit behavior indicative of a preterminal-differentiation arrest. In addition to an incomplete repertoire of biologic functions, many transformed cells, particularly those of the melanocytic lineage, are highly metastatic. It is this latter property that most directly correlates with mortality (1–6). To gain insight into the molecular mechanisms of metastasis, *in vitro* assays such as time-lapse video microscopy coupled with morphometric analyses have been developed; there appears to be a high correlation between motility of a cell type in culture and its metastatic behavior *in vivo* (7–12).

In has been known for some time that thrombin is both a chemotactic agent and a mitogen for cells of the monocytic lineage. The latter activity has been localized to a 14-amino-acid sequence, residues 367–380 of the B chain of α-thrombin that includes loop "B" (residues 367–375) (13); the tetradecapeptide is designated LB-3-(1–14) herein. In the present work, the activity characteristics of this tetradecapeptide, which does not contain either thrombin's proteolytic activity or its mitogenic activity for fibroblasts, was assessed in cells belonging to a metastatic melanoma subclone (14); cell spreading was induced by a nanomolar peptide concentration.

The observed spreading activity was further localized by synthetic peptide chemistry, enabling identification of a synthetic nonapeptide fragment, LB-3-(6–14) comprising residues 372–380 of α-thrombin, as a factor capable of inducing spreading on glass and plastic of cells belonging to a human melanoma subclone that had been selected for its high metastatic potential (14). As a strong correlation between cell spreading and inhibition of motility was observed, complementarity between this factor and peptides that are known to affect growth and differentiation was investigated. The cell-spreading and inhibition-of-motility activities are shown here to be additive to the activities of α-melanocyte-stimulating hormone (α-MSH), nerve growth factor (NGF), and transforming growth factor type β (TGF-β).

MATERIALS AND METHODS

Materials. *Cells.* Human melanoma cells were a gift from I. J. Fidler of M. D. Anderson Hospital and Tumor Institute (14). Two clones were used in this study: one that has a low metastatic potential in athymic nude mice (the parental line), which will be referred to as A375-P, and one with a high metastatic potential in mice, a subclone of A375-P, which will be referred to as A375-M.

Cell growth supplies. Dulbecco's modified Eagle's (DME) medium (in powdered form), fetal calf serum, and glutamine were purchased from GIBCO. Trypsin/EGTA solution was from Meloy Laboratories (Springfield, VA). Bovine pancreatic insulin, transferrin, sodium selenite, progesterone, and putrescine were from Sigma. Plastic Petri dishes (35-mm diameter) and tissue culture flasks (25-cm^2 surface area) were from Falcon (3001 and 3012, respectively).

Peptides. Amino acids with *tert*-butyloxycarbonyl (Boc) groups on amino termini and appropriate protecting groups on side chains were purchased from Peninsula Laboratories (San Carlos, CA).

α-MSH and NGF were from Sigma. TGF-β, a gift from A. Komoriya (Rorer Biotech), was purified from outdated human platelets.

Cell-staining supplies. Rhodamine-labeled phalloidin was purchased from Molecular Probes (Junction City, OR), and Hoechst 33258 was from Calbiochem.

Methods. *Cell growth conditions.* A375 cells were carried in DME medium containing 10% fetal calf serum. For experiments, plating was at a density of 1×10^4 cells per ml with 1 ml added per 35-mm Petri dish [into which a square (22 mm on a side) glass coverslip had been placed] and 3 ml added per tissue culture flask. Twenty-four hours after plating, the medium was changed to DME medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (30 nM), progesterone (10 nM), putrescine (0.1 mM), and glutamine (2 mM). Peptides were added 24 hr after cells had been in this defined medium.

Peptide synthesis and purification. Sequences of peptides synthesized are listed in Fig. 1. The parent, LB-3-(1–14), was

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Abbreviations: LB-3, parent tetradecapeptide; LB-3-(x-y), LB-3 peptides with x = amino terminus and y = carboxyl terminus; α-MSH, α-melanotropin (α-melanocyte-stimulating hormone); NGF, nerve growth factor; TGF-β, transforming growth factor type β.

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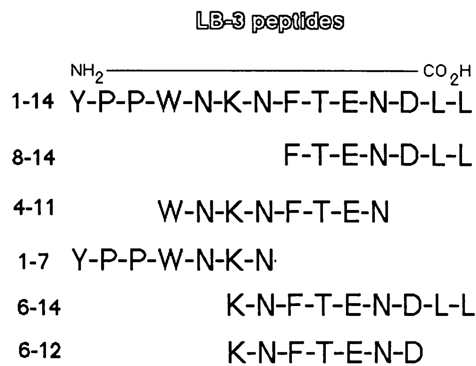

FIG. 1. Sequences of LB-3 peptides synthesized.

synthesized with a peptide synthesizer (Applied Biosystems, Foster City, CA). All other peptides were synthesized by a modification of the Merrifield method (15) with the following changes: diisopropylcarbodiimide was used in place of dicyclohexylcarbodiimide as the coupling agent, 1-hydroxybenzotriazole was added during the asparagine and glutamine couplings, and *N,N*-dimethylformamide was substituted for methylene chloride as the solvent in several of the couplings in the first group of peptides synthesized, with eventual conversion to dimethyl formamide in the final group. Phenylacetamidomethyl-linked amino acids (Boc protection) were used as the starting materials.

After HF deprotection and cleavage of peptides from phenylacetamidomethyl-conjugated resins, the crude peptides were purified by reverse-phase high-pressure liquid chromatography using C_{18} columns and gradients of water and acetonitrile in the presence of 0.1% trifluoroacetic acid. Purity was then assessed to be homogeneous by rechromatography of the peptides. Amino acid analyses, using the Pico-Tag method (Waters Division of Millipore), were consistent with the compositions of peptides listed in Fig. 1.

Incubations of cells with peptides. Thrombin fragments—i.e., LB-3 and LB-3 fragments in Fig. 1—were added at a concentration of 1 nM to cells, and incubations were carried out at 37°C in the presence of 5% CO_2/95% air. Fragments that showed suboptimal activity were added up to 100 nM.

α-MSH, NGF, and TGF-β were found to induce the phenomena described herein at 10 nM, 50 ng/ml, and 0.1 nM, respectively.

Cell staining. Cells on coverslips were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 15 min at ambient temperature. After removal of the formaldehyde solution and three washes with PBS, coverslips were removed from Petri dishes and placed in mini-Coplin jars to which cold (−20°C) acetone was added. The jars were left at −20°C for 5 min, after which the acetone was removed and the coverslips were permitted to dry. Coverslips were incubated with 33 nM rhodamine-labeled phalloidin in PBS for 15 min in the dark. After five PBS washes, incubation with PBS containing 1 μM Hoechst 33258 was carried out for 3 min. After five more PBS washings, coverslips were mounted in PBS/glycerol, 1:1 (vol/vol), on microscope slides and sealed with nail polish.

Video recording and area quantitation of cells. To study the perturbations on motility and to quantitate the surface changes induced by the peptide factors, video images of cells in tissue culture flasks with tightened caps were recorded from a Zeiss phase microscope using a Panasonic time-lapse video tape recorder (NV-8030). Data files were transferred from 8-inch (20.3 cm) reel film to VHS cassettes for storage and later analysis. Cellular images were displayed in the freeze-frame mode by a Panasonic videocassette recorder (model PV 1563) onto a Mitsubishi television monitor. Areas were traced from 23 control cells and 29 cells exposed to LB-3-(6–14) onto transparency film, the outlined images were cut out, and the plastic figures were weighed. Standardization was accomplished by filming an electron microscopic grid with two known periodicities and converting these distances into magnified lengths—i.e., the two periodic distances on the grid, 6.25 and 25 μm, were magnified to 3.75 and 15 cm, respectively. A series of nine squares ranging from 5.0 cm up to 15 cm on a side were then cut out from the transparency material and weighed.

RESULTS

Morphologic Observations. Within 1 hr of exposure to the tetradecapeptide fragment from α-thrombin designated LB-3-(1–14) at the nanomolar level, human melanoma cells from a subclone (A375-M) that exhibits a high metastatic potential when injected i.v. into athymic nude mice (14) resulted in: (*i*) an increase in surface area per cell in contact with the substratum by >2-fold (Fig. 2; also see Fig. 4), (*ii*) a decrease in the average density of f-actin as indicated by rhodamine-phalloidin staining (Fig. 3), and (*iii*) the loss of ability for a single cell to translocate its entire surface. The latter is in sharp contrast to the high motility exhibited by untreated cells. The morphologic phenotype in A375-M induced by

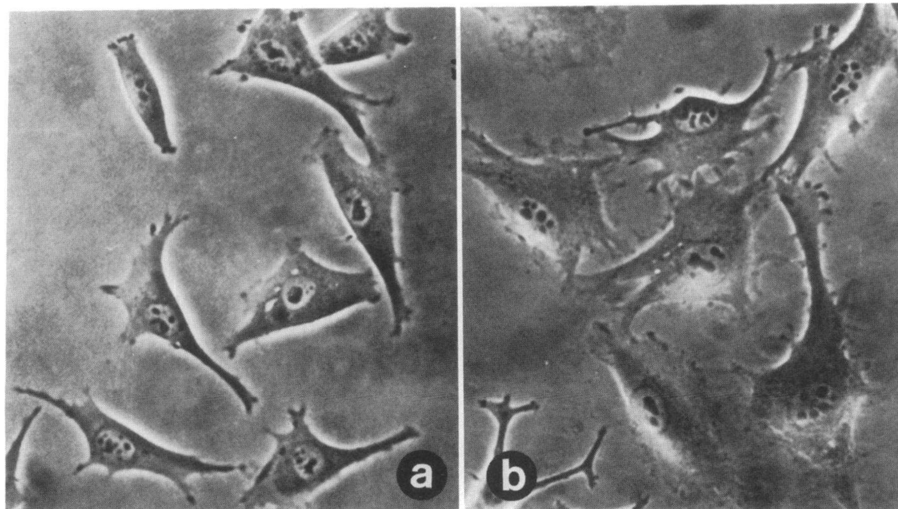

FIG. 2. Phase-contrast micrographs of cells of the A375-M melanoma subclone. (*a*) Control. (*B*) LB-3-(1–14)-treated (1 nM for 1 hr).

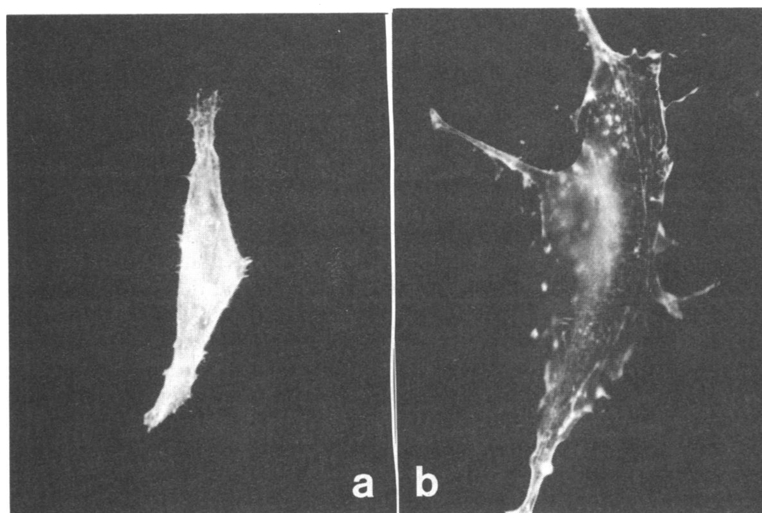

FIG. 3. Fluorescence micrographs with rhodamine filters of A375-M cells. (*a*) Control. (*b*) LB-3-(1–14)-treated.

LB-3-(1–14) approximates that of the untreated parent clone (A375-P), which has a low metastatic potential (14).

When followed by time-lapse video microscopy, the LB-3-(1–14)-treated A375-M cells remained in contact with the same area of substratum throughout the observation time (usually 3–6 hr). Although the cells remained stationary with respect to translocatory movement, rippling motions were evident through the plasma membranes over entire cellular surfaces, with particularly strong oscillations and undulations at the cellular peripheries.

Examination of the actin network using the fibrillar actin (f-actin)-specific molecule phalloidin to which rhodamine had been covalently bound (Fig. 3) indicated the presence of long thick cables with accumulations of fibrillar material in focal contacts at the periphery in the LB-3-(1–14)-treated cells (Fig. 3*b*). This is in contrast to the higher density of f-actin observed in the control cells, where the fluorescent pattern was composed of shorter cables more uniformly distributed than in the treated cells and few if any observable focal contacts at the periphery (Fig. 3*a*).

Peptides. To localize the spreading activity in the LB-3-(1–14) sequence, the following series of peptides was synthesized: the amino-terminal heptapeptide, LB-3-(1–7), the carboxyl-terminal heptapeptide, LB-3-(8–14), and the middle octapeptide, LB-3-(4–11) (Fig. 1). When evaluated for their cell-spreading activity, LB-3-(1–7) showed virtually no activity, even when tested up to the 100 nM level. LB-3-(8–14) at 1 nM exhibited finite but not complete activity; LB-3-(4–11) was significantly less active than LB-3-(8–14). As LB-3-(1–14) contains a lysine as residue 6 and LB-3-(8–14) exhibited significant but not full activity, this heptapeptide was extended to the sequence of LB-3-(6–14) as shown in Fig. 1. The resulting nonapeptide inhibited cellular motility concomitantly with the appearance of oscillatory activity over the entire melanoma cell surface; furthermore, the induction of cell spreading was as effective and at least as efficient as with the parent sequence LB-3-(1–14). LB-3-(6–12) was then synthesized and tested to determine if the two carboxyl-terminal leucines are essential for activity; however, LB-3-(6–12) showed virtually no activity above that of controls.

The results of quantitation of the cell spreading and shape changes induced by LB-3-(6–14) are shown in Fig. 4; they indicate that the average surface area in contact with the substratum for control cells is 205 ± 50 μm^2 and for LB-3-(6–14)-treated cells is 432 ± 91 μm^2. Additionally, the question of overall shape was addressed by taking the ratio of the major axis, defined as the longest straight path in a cell, and the minor axis, defined as the sum of the single longest paths perpendicular to the major axis in both directions. The major-to-minor axis ratio was calculated to be 2.03 ± 0.78 for control cells and 1.61 ± 0.32 for LB-3-(6–14)-treated cells.

The question of whether cells that have undergone physical perturbations such as those induced by the active LB-3 peptides can still transduce signals for functions such as differentiation and growth was addressed by pretreatment of A375-M cells for 1 hr at 37°C with 1 nM LB-3-(6–14) and subsequent exposure to α-MSH, NGF, or TGF-β. Effects of the latter three factors were not apparent until at least 48 hr for α-MSH and NGF and 24 hr for TGF-β. In each case the morphologic phenotypes observed reflected a sum of the activities from the individual peptides—i.e., the total surface

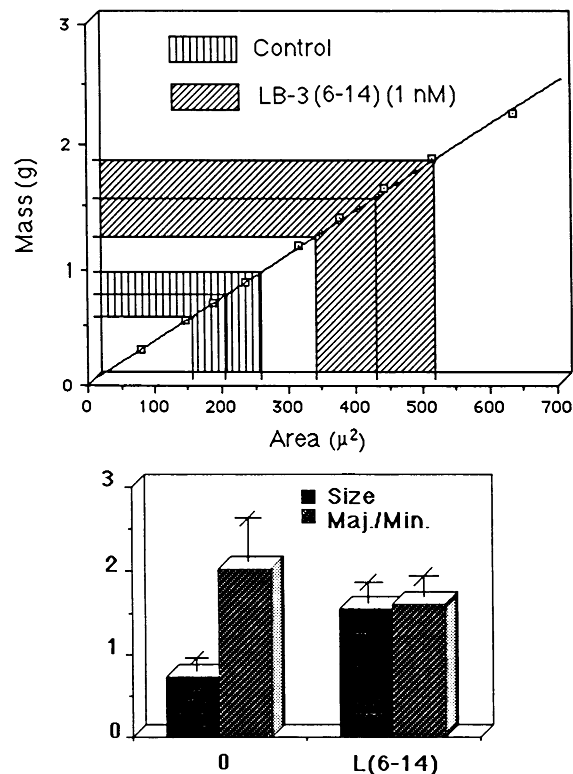

FIG. 4. Determination of area occupied and relative dimensions of A375-M cells. (*Upper*) Masses of outlined cells on plastic transparencies as a function of area per cell in real space. (*Lower*) Bar diagrams with error bars of size (solid bars) and major-to-minor axes (hatched bars) as a function of treatment.

area in contact with the substratum of the LB-3-(6–14)-treated cells was increased in addition to the appearance of cytoplasmic granules in cells treated with α-MSH, neurites on cells treated with NGF, and an increased density of f-actin in cells treated with TGF-β. With respect to motility, all cells treated with LB-3-(6–14) exhibited the characteristic LB-3-(1–14) decreased translocatory activity concomitantly with surface oscillations and peripheral undulations. Neither α-MSH nor NGF *per se* affected the motility of the cells; however, TGF-β was *ca.* 70% as effective as the most active fragments, LB-3-(1–14) and LB-3-(6–14), with respect to inhibition of motility.

DISCUSSION

In this work the effects of a synthetic fragment of thrombin, named LB-3-(1–14), on cells from a subclone of a human melanoma line are examined. The subclone, A375-M, was previously selected for the property of high metastatic potential upon intravenous injection into athymic nude mice (14). Upon exposure to culture medium containing 1 nM LB-3-(1–14), a tetradecapeptide that possesses no esterolytic activity and has previously been shown to be mitogenic for three monocytic leukemic cell lines (13), the surface area of the A375-M cells in contact with the substratum increases by a factor of >2 (Fig. 4). Examination of these treated cultures by time-lapse video microscopy indicates that the spread cells are immotile relative to both untreated A375-M cells and the parent clone, A375-P. The latter exhibits a much lower metastatic potential (14).

The minimal sequence necessary to effect such behavior was localized by synthesizing the overlapping oligopeptides listed in Fig. 1. Full activity was found in a nonapeptide sequence, LB-3-(6–14), that includes one hydrophobic terminus—i.e., two leucines—and a terminus with the charged amino acid lysine. Thus, the presence of hydrophobicity at the carboxyl terminus and an amino acid with a side chain possessing a charge at the amino terminus is suggested as being significant for induction of cell spreading and motility inhibition in A375-M cells.

The treated cells exhibit not only an increased surface area in contact with the substratum but also a less polarized overall morphology as indicated by the relative ratios of major to minor axes shown in Fig. 4; however, the magnitude of the error bars for the control-cell values suggests that the relatively immotile LB-3-(6–14)-treated cells should be considered as a morphologic subset of the highly motile control cells. As the control cells change direction and shape at a finite rate, the images measured for this group in a single frame may represent transitional shapes; therefore, a range of polarized morphologies would be expected to be a part of the cells' motility spectrum.

Since complete mitoses were recorded in many cells from all cultures assayed, including those that exhibit the surface changes characteristic of the above-described spreading effect, none of the peptides listed in Fig. 1 can be considered to be cytotoxic. Therefore, the physiologically compatible nature of LB-3-(6–14) and its potential value as a prototype of a new class of antimetastatic agents raises the question of whether cells treated with this peptide can still transduce other signals such as those for differentiation and growth through receptors in their plasma membranes. To answer this question, cells that had been pretreated for 1 hr at 37°C with 1 nM LB-3-(6–14) were exposed to α-MSH, NGF, or TGF-β. (NGF was chosen for use in this study because the embryologic origin of melanoma cells is the neural crest.) LB-3-(6–14), which was present throughout the incubations, did not affect the signal transduction of any of these peptides. In general the only differences between cultures that had been pretreated with LB-3-(6–14) and those that received only the inducing agents are that the former exhibited the cell shape characteristics induced by LB-3-(6–14) and reduced motility; these differences are in addition to the structures induced by the second set of peptides. Thus, melanin appears in cytoplasmic granules of cells treated with α-MSH (16), neurites are present on cells treated with NGF (17), and an increased density of f-actin with cables of greater lengths are seen in TGF-β-treated cells. It should be noted that, of the three signal-transducing agents *per se*, only TGF-β inhibits motility at all; this is consistent with the suggestion that the increased tightness and decreased number of colonies in agar that are induced by TGF-β in normal rat kidney cells might be an indication for the factor's possible role in modulating metastatic capability and invasiveness (18). Significantly, transduction of differentiation by α-MSH and NGF can still be mediated by glycoproteins in plasma membranes that have undergone a global perturbation. Thus, with peptides from two functionally orthogonal classes—i.e., one class that induces a physical perturbation such as cell spreading and a second class that induces signal transduction by directly binding to membrane receptors—the two complementary functions of inhibition of motility and induction of differentiation can be effected in culture.

When the sequence of LB-3-(6–14) was compared with those in the protein sequence data bank, a nonapeptide sequence that has identity in four of nine residues and two additional conservative replacements was found in the F-1 glycoprotein of respiratory syncytial virus (Fig. 5) (19, 20). The similar amino acid sequence contains the putative significant residues of LB-3-(6–14), namely a lysine and two leucines an equal distance apart. Since the glycoprotein promotes formation of syncytia only when glycosylated, it is

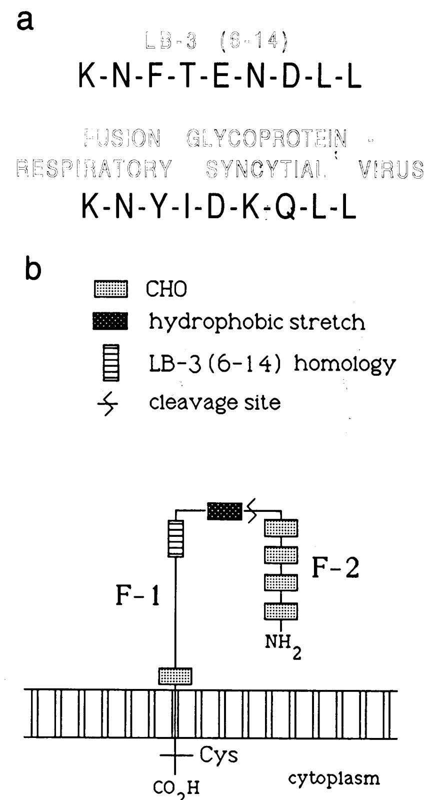

FIG. 5. LB-3-(6–14) and F-1 fusion glycoprotein of respiratory syncytial virus similarity. (*a*) Amino acid sequences of LB-3-(6–14) and F-1. (*b*) Putative preprocessed structure of F-1 with approximate position of sequence homology indicated by box enclosing horizontal lines. In the mature processed form, F-1 and F-2 are held together by disulfide bonds, and the cysteine proximal to the plasma membrane on the cytoplasmic side of F-1 is a palmitate derivative.

conceivable that the similar sequence in F-1 is important for cell spreading, a process that is a precursor step to syncytial formation. This class of activity has not been reported previously for thrombin (21).

In conclusion, a nonapeptide sequence that is found in thrombin and shows similarity with a stretch of the F-1 glycoprotein from respiratory syncytial virus has been shown to induce cell spreading and reduce motility in culture of a human melanoma cell line that exhibits metastatic behavior *in vivo*. Of potential importance are the peptide's noncytotoxicity and the complementary behavior it exhibits with differentiation-inducing agents. It should be of interest to determine whether these effects produce a decrease in the metastatic behavior of treated cells *in vivo*. As the surface morphology of A375-M cells treated with LB-3-(6–14) is quite perturbed, it is conceivable that the accessibility of membrane proteins and glycoproteins is altered so that the immune surveillance network is notified.

The following individuals are acknowledged for their interest throughout this work in addition to the specific contributions listed below: Dr. A. Komoriya for the gift of TGF-β and constructive criticisms of this manuscript, Dr. K. A. O'Connell for the O.B.R. System and fine manuscript editing, Dr. S. Lin for the use of the video recording equipment, Dr. C. B. Anfinsen for pedagogy in the art of manual peptide synthesis and use of his laboratory for chemical procedures, and Dr. M. Edidin for support from National Institutes of Health Grant AI14584. B.S.P. was a fellow of the Cancer Research Institute.

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