Journal of Cellular Biochemistry 99:1524–1535 (2006)

Biological Activity of Rainbow Trout Ea4-Peptide of the Pro-Insulin-Like Growth Factor (Pro-IGF)-I on Promoting Attachment of Breast Cancer Cells (MDA-MB-231) via α2- and β1-Integrin

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Abstract E-peptide of pro-IGF-I was considered as biologically inactive. We have demonstrated that rainbow trout (rt) Ea4-peptide exerted biological activities in several established tumor cell lines [Chen et al., 2002; Kuo and Chen, 2002]. Here we report the activity of rtEa4-peptide in promoting attachment of human breast cancer cells (MDA-MB-231). While rtEa2-, rtEa3-, and rtEa4-peptides enhanced the attachment of MDA-MB-231 cells in a dose dependent manner, rtEa4-peptide possessed the highest activity. Antibodies specific to α2 and β1 integrins significantly inhibited the attachment of cells to rtEa4-peptide coated-plates by 40%. In addition, rtEa4-peptide induced the expression of fibronectin 1 and laminin receptor genes in MDA-MB-231 cells. Blocking new protein synthesis by cycloheximide significantly reduced the attachment of MDA-MB-231 cells to rtEa4-peptide coated wells by 50%. These results suggest that rtEa4-peptide may promote cell attachment by interacting with α2/β1 integrin receptors at the cell surface and by inducing the expression of fibronectin 1 and laminin receptor genes. Expression of fibronectin 1 gene induced by rtEa4-peptide in MDA-MB-231 cells was abolished by inhibitors of PI3K, PKC, Mek1/2, JNK1/2, and p38 MAPK signaling transduction molecules. These results suggested that induction of fibronectin 1 gene expression in MDA-MB-231 cells by rtEa4-peptide may be mediated via PI3K, PKC, Mek1/2, JNK1/2, and p38 MAPK signal transduction molecules. J. Cell. Biochem. 99: 1524–1535, 2006.

Key words: cancer cell attachment; rtEa4-peptide; pro-IGF-I; fibronectin 1 gene; laminin receptor gene

E-peptides are the carboxyl terminal domain of pro-insulin-like growth factors (IGFs), which are proteolytically cleaved from pro-IGFs during the post-translational processing to produce the mature IGFs [Duguay, 1999]. In human, three isoforms of pro-IGF-I mRNAs have been identified, encoding the same mature IGF-I but different E domain peptides (hEa-, hEb-, and hEc-peptides of 35, 77, and 40 amino acid residues) [Chew et al., 1995]. In rainbow trout (rt), four different isoforms of pro-IGF-I mRNAs have been identified [Shamblott and Chen, 1993]. These four different isoforms of pro-IGF-I mRNAs encode the same mature IGF-I peptide and four different lengths of E domain peptides designated as rtEa1-, rtEa2-, rtEa3-, and rtEa4-peptides due to their high amino acid sequence homology to the hEa peptide. Similar to the E-peptides of pro-hIGF-I, rtEa1-, rtEa2-, rtEa3-, and rtEa4-peptides possess a conserved motif of 15 amino acid residues at the N-terminus and a highly homologous region of 20 amino acid residues at the C-terminus of the hEa-peptide. An E-peptide with 97 amino acid residues was also identified in pro-IGF-II of rt, and it shared a high degree of amino acid sequence homology with E-peptide of human pro-IGF-II [Sussenbach et al., 1993].

Initially, E-peptide was assumed to function primarily in facilitating the proper folding and intermolecular disulfide bond formation of mature IGFs, and was thought not to possess

Grant sponsor: NSF; Grant number: IBN-0078067; Grant sponsor: USDA; Grant number: CONTR # 58-1930-0-009.

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Received 15 December 2005; Accepted 16 February 2006

DOI 10.1002/jcb.20914

any other biological activity [Duguay, 1999]. It is only recently that E-peptides had been shown to exert mitogenic activity in many cell lines. A synthetic human E-peptide of pro-IGF-II was shown to promote ^{3}H-thymidine incorporation in HIL8 hamster cells [Hylka et al., 1985] and a synthetic 22 amino acid residues of hEb-peptide promoted cell proliferation in normal and malignant bronchial epithelial cells [Siegfried et al., 1992]. Recently, our laboratory demonstrated that recombinant rtEa2-, rtEa3-, and rtEa4- but not rtEa1-peptide exerted mitogenic activity in a non-transformed mammalian cell line, NIH3T3 cells, and primary caprine mammary gland cells [Tian et al., 1999]. We have further shown that recombinant trout rtEa4-peptide induced morphological differentiation, inhibited anchorage independent growth, and reduced cancer cell invasion in many established human cancer cells such as MDA-MB-231, HepG2, HT-29, OVCAR, and SK-N-F1 [Chen et al., 2002; Kuo and Chen, 2002]. The broad spectra of biological activities of the rtEa4-peptide in a variety of tumor cells may have suggested the complexity in the action mechanism(s) of this peptide.

In studying the effect of recombinant rtEa4-peptide in human neuroblastoma cells (SK-N-F1), it was demonstrated that the effect of rtEa4-peptide in inducing morphological differentiation of neuroblastoma cells was mediated via the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated protein kinase (ERK1/2) signaling molecules of mitogen activated protein kinase (MAPK) pathway [Kuo and Chen, 2002]. However, the detailed molecular mechanism of the action of rtEa4-peptide remains further investigation. In this article, we report results of studies to determine the biological activity of the rtEa4-peptide in promoting the attachment of human breast cancer cells, MDA-MB-231, to substratum and the action mechanism of this biological activity.

MATERIALS AND METHODS

Cell Cultures

Human breast cancer cells, MDA-MB-231, were obtained from American Type Culture Collection (ATCC, Rockville, MD). Single-cell clones of MDA-MB-231, that have maintained their cancerous properties, were isolated from the ATCC stock and used throughout the studies. Cells were routinely maintained in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY) and were incubated in a 37°C incubator under a humidified atmosphere of 5% CO_2. For treatment with recombinant rtEa4-peptide, 5×10^5 cells were plated in a 35 mm × 10 mm culture dish in DMEM/F12 medium supplemented with desired concentrations of recombinant rtEa4-peptide or control proteins, and cells were maintained at 37°C in a 5% CO_2 incubator.

Purification of Recombinant rtEa2-, rtEa3-, rtEa4-, and Control-Peptides

The coding sequences of trout Ea2-, Ea3-, and Ea4-cDNA were cloned into the expression vector pET15b [Tian et al., 1999]. The expression vector pET15b without the rtEa4-peptide cDNA insert was used for the preparation of control proteins. A single colony of *E. coli* strain BL21 (DE3) containing the specific expression construct was cultured at 37°C in 5 ml of LB broth for 4 h, then transferred into 500 ml LB broth, and allowed to grow until the OD_{600} reached approximately 0.6. The bacterial culture was then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and further incubated for 2 h. Cells were then spun down, resuspended in 10 ml of a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and sonicated. After the cell debris was removed by centrifugation at 35,000*g* for 20 min, the resulting supernatant was applied onto a His-Bind resin affinity chromatography column (Novagen, Madison, WI). The impurities were removed by washing the column initially with the binding buffer and then followed by the wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The recombinant protein bearing six histidine residues was eluted from the column using an elution buffer (1 M imidazole, 0.5 N NaCl, 20 mM Tris-HCl, pH 7.9). The protein was then dialyzed against 1× PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4) containing 10 mM EDTA, followed by 1× PBS containing a desired concentration of EDTA (20 μM EDTA/100 μg protein/ml) at 4°C. The protein was concentrated using 15–20 K PEG until the desired concentrations were obtained. The peptide concentration was determined by Lowry-based DC protein assay (Bio-Rad, Hercules, CA).

Cell Attachment Assay

Each well of the non-tissue-culture grade polysterene high binding 96-well microtiter plates (Corning, Corning, NY) was coated with various concentrations of the recombinant rtEa2-, rtEa3-, rtEa4-, control proteins, or laminin in 1× PBS at 4°C overnight. After the excess peptide was removed, the unoccupied sites on the wells were blocked with a solution containing 1 mg/ml of bovine serum albumin (BSA) at room temperature for 1.5 h. The coated wells were then washed once with PBS. Cells were released from culture flasks by digestion with 0.25% trypsin in 1× PBS + 0.02% EDTA, washed twice with DMEM/F12, and resuspended in DMEM/F12 at the concentration of 5×10^5 cells/ml. One hundred microliters of cell suspension was added into each coated well and cells were incubated for 1.5 h at 37°C with 5% CO_2 humidified atmosphere, wells were then rinsed twice with DMEM/F12 to remove non-attached cells. The relative number of attached cells was determined from the amount of DNA content of attached cells in each well following the Schirmer protocol [Schirmer et al., 1994]. Cells were lysed in 0.01% SDS, and DNA was stained with the Hoescht dye (H33258). The DNA content was determined using a CytoFlour plate reader with 360 nm excitation filter and 460 nm emission filter. The reading of DNA content in each condition was normalized with the reading of the control wells, which were coated with control proteins. The average value from six wells was used for each assay point and the assay was conducted at least twice in each experiment.

To test if α2 and β1 serve as receptors for binding of MDA-MB-231 cells to rtEa4-coated plates, mouse anti-human α2- and β1-integrin monoclonal antibodies (P1E6 and P5D2; Chemicon, Temecula, CA) were used. Cells were incubated with both antibodies for 2 h at 37°C before the cells were allowed to attach to rtEa4-coated plates for 1.5 h. Similarly, to test the effect of protein synthesis on the attachment of cells to rtEa4-coated plates, cells were incubated with 0.01–20 μM cycloheximide for 2 h before they were allowed to attach to rtEa4-peptide coated plates.

Isolation of Permanent Transformants

Two gene constructs, CMV-Ea4-cDNA-IRES-EGFP, and CMV-IRES-EGFP, cloned in the backbone of pIRES2-EGFP (Clontech, Palo Alto, CA) were used in the transfection studies. A signal peptide sequence of hIGF-I was fused to the N-terminus of the rtEa4-peptide cDNA to ensure that the synthesized E-peptide would be secreted out of the cell. The expression of these gene constructs was driven by from cytomegalovirus (CMV) promoter. Single-cell clones of MDA-MB-231 cells were cultured to 90% confluence in DMEM/F12 medium supplemented with 10% FBS. About 5×10^6 cells were harvested and resuspended in 1 ml of serum-free DMEM/F12 (1:1) medium containing 20 μg of constructs. The cells were electroporated in a BRL Cell-Porator (Life Technologies) using the following settings: low Ω, 1,180 micro Faraday (μF) capacitance, and two pulses at 200 V. Following electroporation, cells were resuspended in 12 ml of DMEM/F12 (1:1) medium supplemented with 10% FBS and seeded into two T-25 flasks to recover. Permanent transfectants expressing green fluorescence protein (GFP) were enriched in a medium containing neomycin (G418) at 1mg/ml for 10 days and followed with 500 μg/ml for continuous maintenance. Individual green cell clones of transfectants were isolated from the enriched population by the method of serial dilution [Chen et al., 2002].

Gene Expression and Comparative Real-Time RT-PCR

The relative levels of the expression of fibronectin 1 and laminin receptor in rtEa4-treated and non-treated cells were determined in an iCycler thermocycler (Bio-Rad), using 2-step real-time RT-PCR with SYBR Green I incorporation. Primers specific for fibronectin 1, laminin receptor, and β-actin mRNA were designed using a combination of oligo3 (Molecular Biology Insights, Cascade, CO) and vector NTI programs (Informax, Frederick, MD). The primer sequences were designed to be specific to the target genes using the Blastn search against the GeneBank database. The primer sequences and the expected amplicon sizes are indicated in Table I. Prior to conducting a real-time RT-PCR experiment, each primer pair was tested for its fidelity to amplify and yield a single, correct amplicon by analyzing the melting curve of the amplified product and by visualizing the PCR product on an agarose gel. At the completion of 50 cycles of amplification, the melting curve was

TABLE I. List of PCR Primers Used in This Study

Accession number	Gene	Primer sequences
BC014861	β-actin	Upper primer 5′TCGTGCGTGACATTAAGGAG3′ Lower primer 5′AGGAAGGAAGGCTGGAAGAG3′
XM_055254	Fibronectin 1	Upper primer 5′GCTTCCTGGCACTTCTGGTC3′ Lower primer 5′CTTCTTGTCCTACATTCGGCG3′
BC010418	Laminin receptor	Upper primer 5′TTGATGTGGTGGATGCTGG3′ Lower primer 5′TCCTTGGTCACTGCCTTCTC3′

obtained by changing the temperature from 95°C to 25°C at 0.5°C decrements per 10 s. If a single product was not obtained, the primers were re-designed and tested until effective primers were found. Optimum annealing temperature, $MgCl_2$ concentration, and SYBR Green I dilution were tested to give amplification efficiency greater than 95%.

RNA was extracted using Trizol reagent following the recommended protocol from the manufacturer (Invitrogen, Carlsbad, CA). The RNA quality was assessed by agarose gel electrophoresis. In addition, to confirm that the purified RNA samples were free of DNA contamination, the RNA samples were used as templates for direct amplification of β-actin sequence without prior reverse transcription. In each RT reaction, 1 μg of total RNA was reverse transcribed in a 20 μl reaction containing 100 ng oligo dT, 10 mM dNTP, 200 mM DTT, 1× first strand buffer, 1 reaction unit of Powerscript reverse transcriptase (Clontech) at 42°C for 1.5 h. The cDNA reaction was then used as the template in PCR reactions, which was carried out in a 96-well plate. One tenth of the cDNA reaction was used in a PCR reaction of 100 μl containing 0.2 mM dNTP, 3 mM $MgCl_2$, 0.4 μM of each primer, 1 μM fluorescein, 1:20,000 dilution of 10,000× SYBR, and 1× PCR buffer. The reaction mixture of 20 μl was used per well and the average data of four wells were collected to represent each sample. At least two independent experiments were done to ensure the reproducibility of the results. The thermal profile was 1 cycle of 95°C for 3 min, and 50 cycles of 95°C for 15 s, appropriate annealing temperature for 15 s, and 72°C for 30 s. The cycle threshold, C_T, was determined from the fluorescence value, which was 10 times the mean standard deviation of fluorescence of the base line cycles. The relative gene expression was determined using the arithmetic formula: $2^{-[(S\Delta CT-C\Delta CT)]}$, where ΔCT is the difference in C_T value between the gene of interest and the housekeeping gene (β-actin), and SΔCT−CΔCT is the difference between the sample ΔCT and control ΔCT.

Gene Regulation

The regulation of fibronectin 1 gene expression in response to rtEa4-peptide treatment via common signaling molecules of the focal adhesion kinase (FAK)/mitogen-activated protein kinase (MAPK) pathway was tested using the following inhibitors; PD98095, SB202190, SP600125, Tamoxifen, and Wortmannin. One milliliter of MDA-MB-231 cells (1×10^6 cells/ml) was treated with 0–10 μM inhibitors (below their cytotoxic concentrations), and incubated in a 37°C incubator under 5% CO_2. After 2 h of incubation, cells were treated with 2.56 μM of recombinant rtEa4-peptide or control proteins for additional 1.5 h. RNA was extracted from the cells using Trizol reagent as described before. The expression of fibronectin 1 gene in each sample was determined using a 2-step real time RT-PCR as described earlier. At least two independent experiments were conducted to confirm the reproducibility of the results.

RESULTS

Trout Ea-Peptides Promote Breast Cancer Cell Attachment

Results of preliminary studies showed that treating MDA-MB-231 cells with 1.4 μM recombinant rtEa4-peptide for less than 1 h resulted in the appearance of a flatten, spread-out cell morphology and an increase in the numbers of cells attached to the substratum over time (data not shown). This observation led us to raise the question whether rtEa4-peptide may promote the attachment of MDA-MB-231 cells to substratum. To answer this question, a cell attachment assay was conducted on MDA-MB-231 cells. MDA-MB-231cells were allowed to attach on rtEa4-peptide-, laminin-, or control protein-coated wells for 1.5 h, and the non-attached cells

were removed by washing with PBS. Most cells remain attached to the rtEa4-peptide- and laminin-coated wells, whereas very few cells remain attached to control protein-coated wells (Fig. 1), suggesting that rtEa4-peptide enhanced the attachment of cells to substratum. It is interesting to note that the morphology of the attached cells on rtEa4-peptide-coated wells was different from those attached to laminin-coated wells. In rtEa4-coated wells, few cells showed protruding cell membrane (spindle-like) morphology (Fig. 1), and most cells were rounded with ruffled edges, similar to the preliminary result of treating cells with rtEa4-peptide. The morphology of cells in laminin-coated wells showed spindle morphology. While a dose-dependent increase in cell attachment was observed with different concentrations of rtEa4-peptide or laminin, the control proteins did not show any activity in promoting cell attachment (Fig. 2A), suggesting that the attachment activity of rtEa4-peptide was not contributed by the His-Tag sequence associated with the recombinant rtEa4-peptide. Different subtypes of trout Ea-peptides (rtEa2-, rtEa3-, and rtEa4-peptides) were assayed for their activities in promoting cell attachment, and the

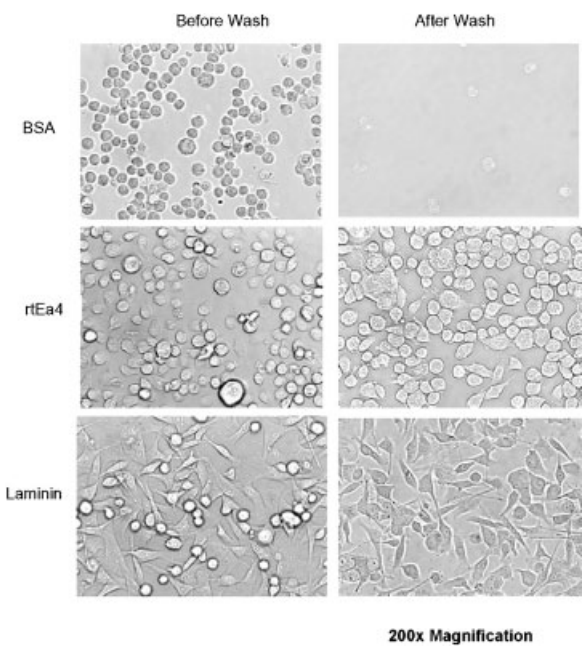

Fig. 1. Attachment of MDA-MB-231 cells to rtEa4-peptide-, laminin-, and BSA-coated wells. Recombinant rtEa4-peptide (10 μg/ml), laminin (10 μg/ml), or control protein (10 μg/ml) was coated on a non-tissue-culture, polysterene, high binding, 96 microtiter wells in 1× PBS over night at 4°C. After the excess peptides were removed, the unoccupied sites on the wells were blocked with a solution containing 1 mg/ml of BSA at room temperature for 1.5 h. MDA-MB-231 cells (5×10^5) were allowed to adhere on rtEa4-peptide-, laminin-, and control protein-coated wells for 1.5 h in an incubator at 37°C with a humidified atmosphere with 5% CO_2. Non-attached cells were washed-off twice with 1× PBS. Images of cells in the middle of each well of both before and after washing, were taken at 200× magnification in an inverted microscope (Olympus IX50) equipped with phase contrast objectives. After washed with 1× PBS solution, most cells remain attached on the rtEa4-peptide- and laminin-coated wells, whereas very few cells remain attached on the control protein-coated wells.

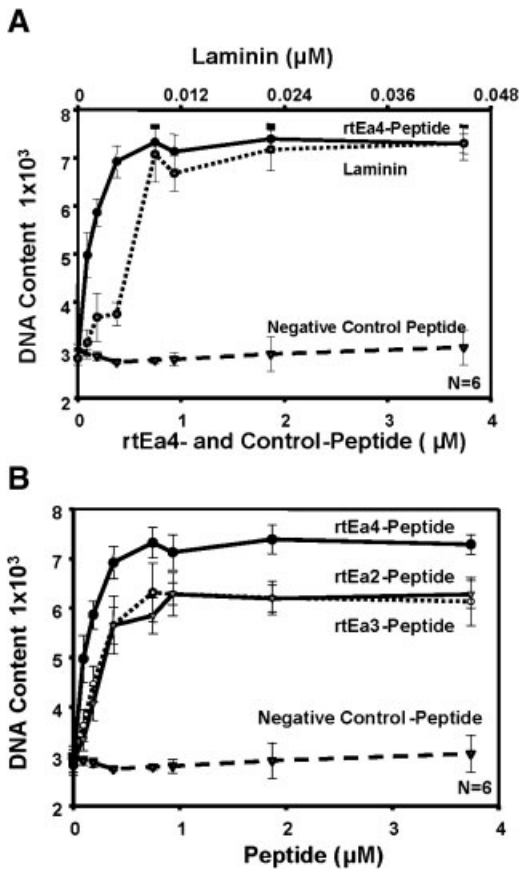

Fig. 2. Rainbow trout Ea4-peptides promotes attachment of MDA-MB-231 cells. Zero to 3.7 μM of rtEa2-, rtEa3-, rtEa4-, or control proteins were used to coat wells of 96-well plates as described previously. In addition, 0–0.046 μM of laminin was also used to coat wells of he 96-well plates as positive controls. MDA-MB-231 cells (5×10^4) were allowed to attach to the peptide coated wells for 1.5 h. After non-attached cells were removed, the relative numbers of attached cells on each well were determined using H33258 DNA staining dye. The fluorescent readings of attached cells on the peptide-coated wells were normalized with the fluorescent readings of non-specific binding cells on the BSA-coated wells. At least two independent experiments were conducted in order to ensure the reproducibility. **A**: the effect of rtEa4-peptide on cell attachment; (**B**) the effects of rtEa2- and rtEa3-peptides on cell attachment.

results showed that, although rtEa2- and rtEa3-peptides promoted attachment of MDA-MB-231 cells to the substratum in a dose-dependent manner, rtEa4-peptide exhibited a significantly higher activity (Fig. 2B).

rt Ea4-Peptide Promotes Cell Attachment via Integrin Receptors

Since one of the major groups of receptors involved in cell attachment is integrin receptors, we wondered if the enhanced cell attachment activity of rtEa4-peptide may involve integrin receptors. To test this possibility, monoclonal antibodies specific for integrin receptors were used to block the attachment activity of the rtEa4-peptide. Since both α2 and β1 integrins have been reported to be highly expressed in MDA-MB-231 cells [Doerr and Jones, 1996], we focused our studies on these two receptors. As shown in Figure 3A,B, attachment of MDA-MB-231 cells to rtEa4-coated substratum was reduced by approximately 40% when cells were pre-incubated with anti-α2 integrin, suggesting that α2 integrin receptors might be involved in the attachment of cells on rtEa4-peptide coated substratum. In addition, the attachment of MDA-MB-231 cells to rtEa4-peptide coated plates was also significantly decreased by pre-treating cells with anti-β1 integrin (Fig. 3B), suggesting that β1 integrin receptor might also be involved in the attachment of MDA-MB-231cells on rtEa4-peptide coated substratum. The attachment of MDA-MB-231 cells to laminin was known to involve β1-integrin receptor but not α2-integrin receptor [Maemura et al., 1995; Sonohara et al., 1998], antibodies against β1- and α2-integrin receptors were, therefore, used as positive and negative controls for the attachment of MDA-MB-231 cells to laminin-coated plates. As expected, the binding of MDA-MB-231 cells to laminin-coated wells was reduced by prior treatment of cells with anti-β1 integrin receptor but not with anti-α2 integrin receptor (Fig. 4A,B).

rt Ea4-Peptide Induced the Expression of Fibronectin 1 and Laminin Receptor Genes

Since treatment of MDA-MB-231 cells with monoclonal antibodies specific to integrin receptors only resulted in 40% of inhibition of cell attachment, it suggests that other molecules may also contribute to the binding of MDA-MB-231 cells to substratum. Preliminary results of microarray analysis conducted in our laboratory (Chen et al., unpublished results) showed that transfection of an rtEa4-peptide gene (containing the signal peptide sequence of human IGF-I) into MDA-MB-231 cells resulted in upregulation of fibronectin 1 and laminin receptor genes. Since both genes are known to function in promoting the attachment of cells to substratum, we hypothesized that rtEa4-peptide may also promote cell attachment by inducing the expression of fibronectin 1 and laminin receptor genes. To test this hypothesis, we first determined the expression levels of fironectin 1 and lamin receptor genes in MDA-MB-231 cells transfected with an rtEa4-peptide gene construct and the control-construct (same backbone as rtEa4-peptide construct minus rtEa4-peptide sequence) by the comparative real-time RT-PCR analysis. The expression levels of fibronectin 1 and laminin receptor genes were induced approximately 2.2 and 1.4 folds (after normalized with the expression of a house keeping gene, β-actin), respectively, in rtEa4-peptide gene transfected cells compared to that in the control cells (Fig. 4A). These results supported the preliminary results of the microarray analysis. In addition, we also determined the expression levels of fibronectin 1 and laminin receptor genes in MBA-MB-231 cells treated with the recombinant rtEa4-peptide. As reported in the previous section, induction of cell attachment to rtEa4-coated substratum was observed within 1.5 h, therefore, the same incubation period was also used to determine the levels of expression of fibronectin 1 and laminin receptor genes in response to treatment with recombinant rtEa4-peptide. As shown in Figure 4B,C, rtEa4-peptide induced the elevation of fibronectin 1 and laminin receptor mRNA in 1.5 h following the addition of rtEa4-peptide. These results led to a hypothesis that, in response to rtEa4-peptide, MDA-MB-231 cells increased the expression levels of both fibronectin 1 and laminin receptor genes, which in turn result in promoting cell attachment. To prove this hypothesis, the attachment of MDA-MB-231 cells to the rtEa4-coated substratum was determined when new protein synthesis was inhibited by cyclohexamide. MDA-MB-231 cells treated with cycloheximide for 2 h prior to exposure to rtEa4-peptide coated wells resulted in an approximately 40% reduction in cell attachment (Fig. 5). This observation supported

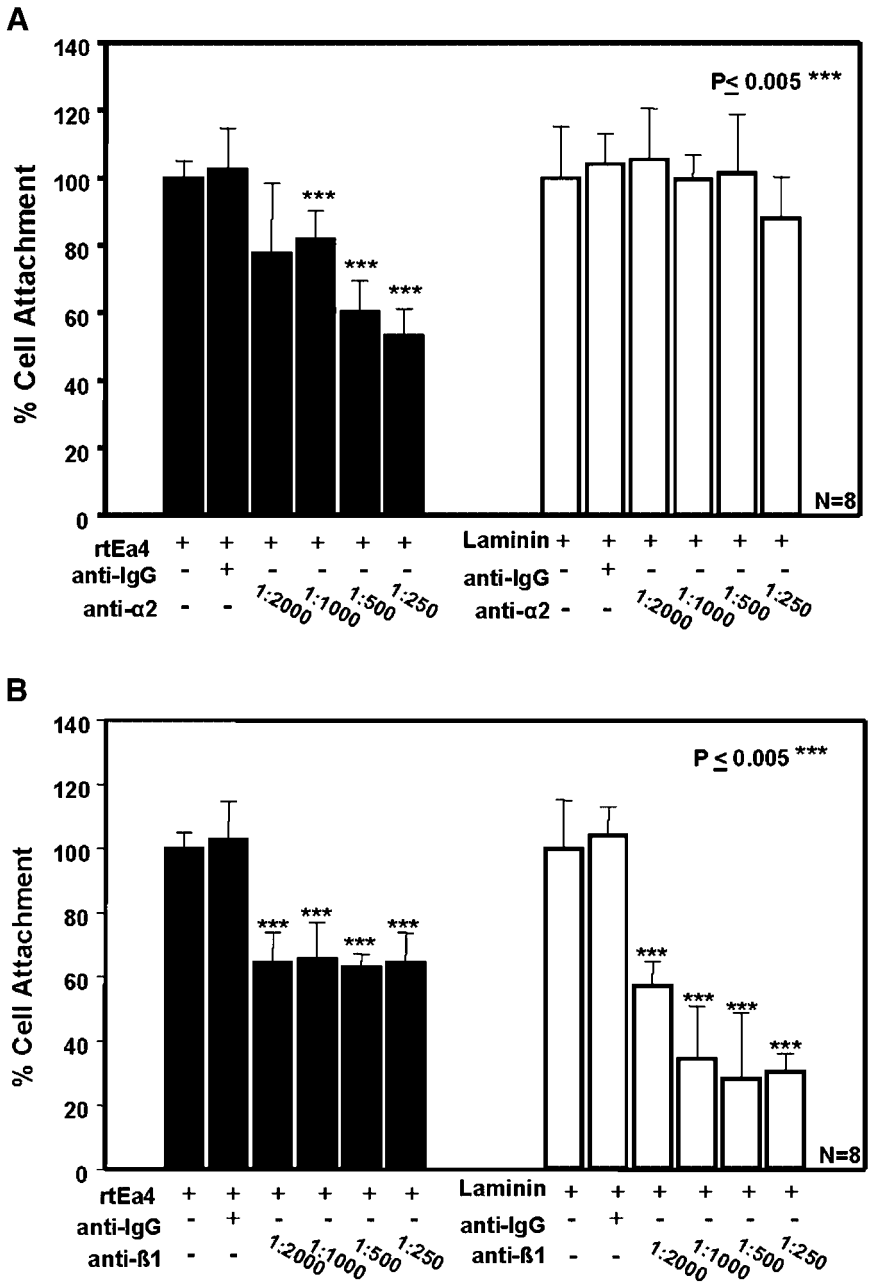

Fig. 3. Effect of α2- and β1-integrin receptor on the attachment of MDA-MB-231 cells on the EIa4-coated plate. Recombinant rtEa4-peptide, laminin, or control protein was coated on a 96 well plate as described previously. MDA-MB-231cells (5×10^4) in DMEM/F12 medium were treated with various dilutions of antibodies against α2- and β1-integrin receptors for 2 h at 37°C. Treated cells were then allowed to attach to the peptide-coated plates for 1.5 h at 37°C. Non-attached cells were removed by washing twice with 1× PBS. The relative numbers of attached cells were determined by staining with H33258 DNA dye as described previously. Each data point is the average from eight wells. At least three independent experiments were conducted to confirm the reproducibility of the experiment. **A**: Effect of anti-α2 integrin on the attachment of MDA-MB-231 cells on rtEa4- and laminin-coated wells; (**B**) effect of antibody against β1 integrin receptor on the attachment of MDA-MB-231cells on rtEa4- and laminin-coated wells.

our hypothesis that the effect of rtEa4-peptide in promoting the attachment of MDA-MB-231 cells to substratum could in part be mediated through the synthesis of new fibronectin 1 and laminin receptor induced by rtEa4-peptide.

rt Ea4-Peptide Mediates Gene Expression via Signal Transduction Pathways

Kuo and Chen [Chen et al., 2002] reported earlier that induction of morphological

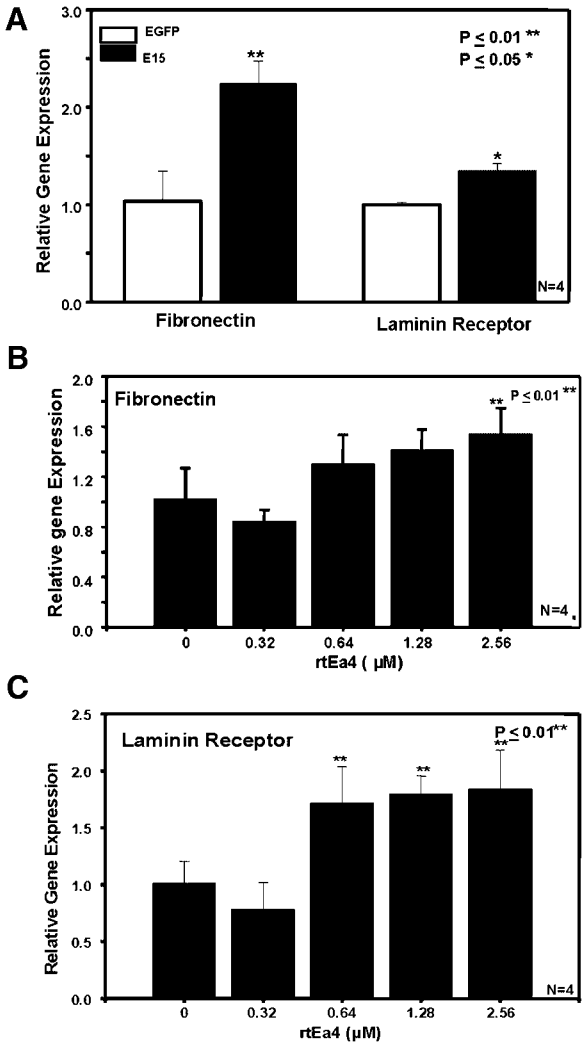

Fig. 4. Expression of fibronectin 1 and laminin receptor genes on MDA-MB-231 cells in response to rtEa4-peptide treatment. The expression of fibronectin 1 and laminin receptor genes in MDA-MB-231 cells transfected with rtEa4-peptide gene or treated with recombinant rtEa4-peptide was determined by the comparative real-time RT-PCR analysis. Total RNA was isolated from MDA-MB-231 cells using Trizol reagent (Invitrogen). First-strand cDNAs were reverse transcribed and were subsequently used as templates in the PCR amplification using gene specific primers for either fibronectin 1 or laminin receptor gene. The relative gene expression was normalized to the expression of the house keeping gene (β-actin) and was determined using an arithmetic formula: $2^{-[(S\Delta CT-C\Delta CT)]}$ as described in Materials and Methods. Each data point is the average of the last four samples, and the results were plotted in the bar histograms. At least two independent experiments were conducted with similar results obtained. **A**: Expression of fibronectin1 and laminin receptor genes in cells transfected with rtEa4- or control-protein genes; (**B**) expression of fibronectin 1 gene; and (**C**) expression of laminin receptor gene in cells treated with recombinant rtEa4-peptide.

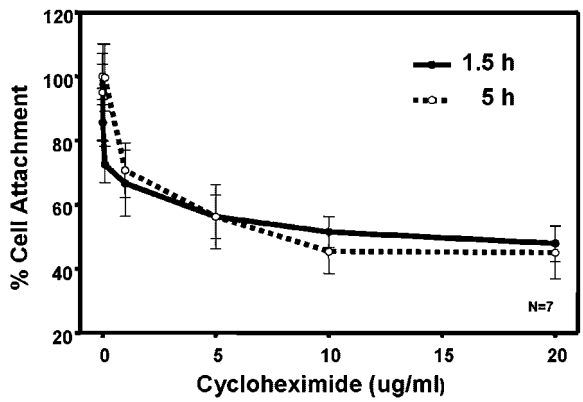

Fig. 5. Effect of cycloheximide on the attachment of MDA-MB-231 cells on the rtEa4-coated wells. Recombinant rtEa4-peptide (1 μM) was used to coat a 96 well plate as described previously. MDA-MB-231 cells in DMEM/F12 medium were incubated with 0–20 μg/ml of cycloheximide for 2 h at 37°C. Cycloheximide treated cells (5×10^4) were allowed to attach on rtEa4-peptide-coated wells for an additional 1.5 or 5 h. After non-attached cells were removed by washing with 1× PBS, the amount of cells attached to the peptide-coated wells were determined staining with H33258 DNA dye as describe earlier. After the results of cell attachment on rtEa4-peptide coated wells were normalized with the result of cell attachment on BSA-coated wells, the average percentage of cells attached to rtEa4-peptide-coated wells were calculated from eight wells, and the results plotted against the concentration of cycloheximide. At least two independent experiments were conducted and similar results were obtained. No toxicity was observed under the concentration of cycloheximide used in the study.

differentiation in SK-N-F1 cells by rtEa4-peptide was mediated via ERK1/2 and PI3K of MAPK signal transduction pathways. Since we have shown that α2/β1 integrin receptors might be involved in rtEa4-induced cell attachment to substratum, we hypothesized that rtEa4-peptide induced expression of fibronectin 1 and laminin receptor genes in MDA-MB-231 cells may be mediated via signaling molecules of FAK/MAPK pathways, which are the primary signal transduction pathways of integrin receptors. To test this hypothesis, the expression of fibronectin 1 gene in MDA-MB-231 cells in response to rtEa4-peptide treatment was monitored in the presence of inhibitors of FAK/MAPK pathways. Cells were treated with various concentrations of the inhibitors in non-cytotoxic doses as recommended by manufacturers and the expression of fibronectin 1 gene was measured in cells treated with rtEa4-peptides or control proteins by comparative real-time RT-PCR. As shown in Figure 6, inhibitors of JNK1/2, Mek1/2, p38 MAPK, PI3K, and PKC clearly suppressed the induction of

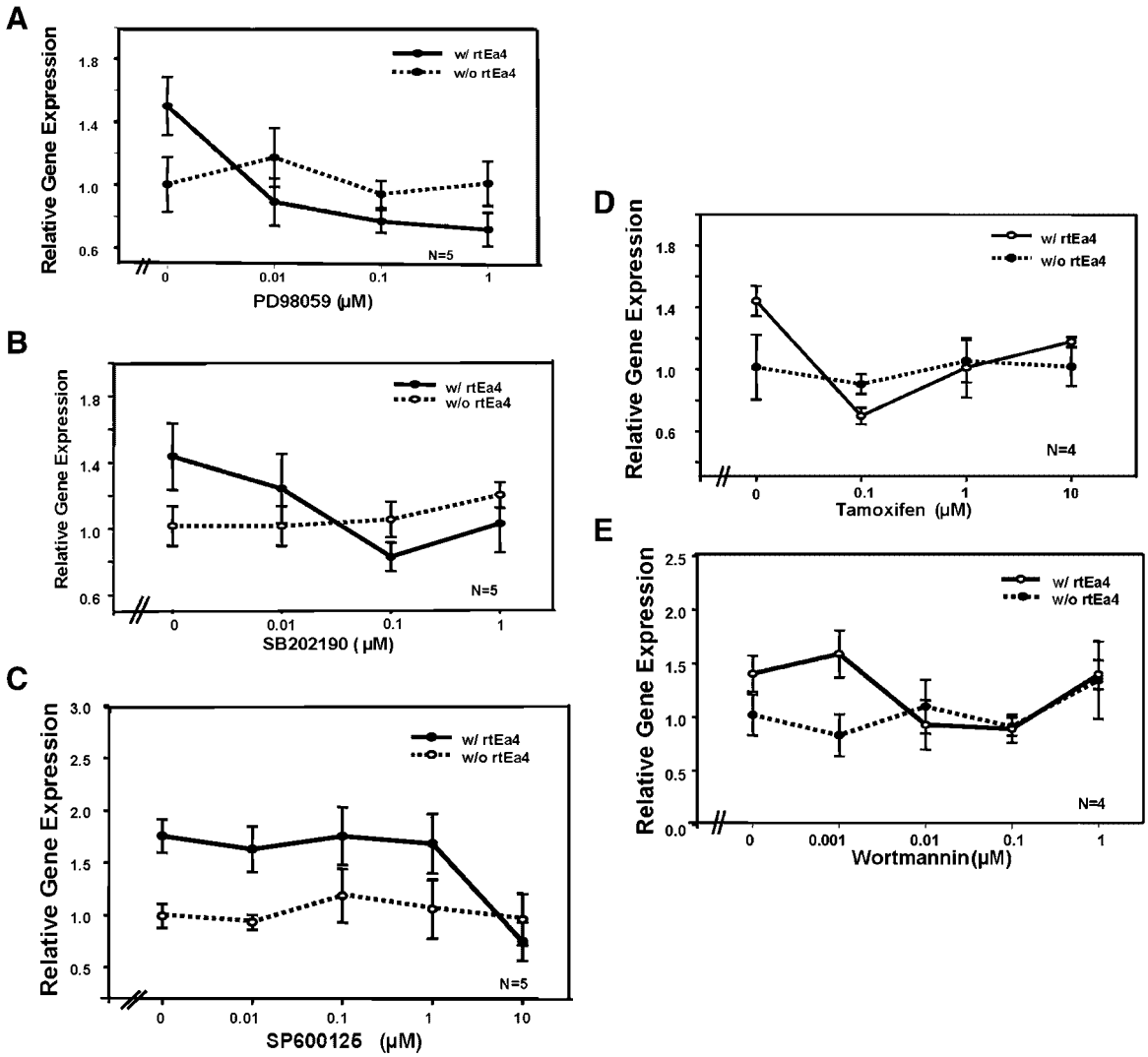

Fig. 6. Effect of FAK/MAPK signaling pathway inhibitors on the expression of fibronectin 1 gene in MDA-MB-231 cells treated with rtEa4-peptide. MDA-MB-231 cells were treated with 0–10 μM of inhibitors for the signaling molecules of the FAK/MAPK pathways for 2 h prior to treatment with 2.56 μM of rtEa4-peptide or control proteins for an additional 1.5 h. Total RNA was extracted from the treated cells and used for determination of levels of fironectin 1 mRNA by real-time RT-PCR analysis following the method described in Materials and Methods. PD98059, SB202190, SP600125, Tamoxifen, and Wortmannin (**panels A–E**) were the inhibitors specific to Mek1/2, p38 MAPK, JNK, PKC, and PI3K, respectively. Each data point was four repeat determinations with the same RNA preparation, and at least two independent experiments were performed to ensure the reproducibility.

fibronectin 1 gene expression in MDA-MB-231 cells treated with rtEa4-peptide. The results suggested that rtEa4-peptide regulated the expression of fibronectin 1 gene via JNK1/2, Mek1/2, p38 MAPK, PI3K, and PKC signaling molecules.

DISCUSSION

In this article, we demonstrated that rt Ea2-, Ea3-, and Ea4-peptides exhibited an activity in promoting the attachment of MDA-MB-231 cells to the substratum. These results are in good agreement with our earlier observation [Chen et al., 2002] that MDA-MB-231 cells remained attaching on the culture plate for 7 days in the presence of rtEa4-peptide in a serum free medium. We have further observed that the attachment of MDA-MB-231cells on rtEa4-peptide coated substratum happened as early as 1.5 h of incubation. All three forms of rtEa-peptides, namely rtEa2-, rtEa3-, and rtEa4-peptides, promoted cell attachment in a dose dependent manner; however, rtEa4-peptide

exhibited a significantly higher cell attachment activity. Since the sequences of rtEa2- and rtEa3-peptides are different from the sequence of rtEa4-peptide only by the absence of domain 2 or 3, respectively [Shamblott and Chen, 1993], the active domain(s) of the peptide responsible for the cell attachment activity must be residing in these domains. This could account for the observation that the activity of rtEa4-peptide on promoting cell attachment was much higher than that of rtEa2- or rtEa3-peptide. Although, the amino acid sequences in domains 2 and 3 of rtEa-peptides share no overall similarity to any known motifs of any adhesion molecules, interestingly, a DRG sequence motif was present in domain 2. This DRG sequence is similar to the well characterized conserved RGD motif of many adhesion molecules such as fibronectin, fibrinogen, glycoprotein gB, and Triflavin, which is recognized by integrin receptor [Sheu et al., 1996; Bennett, 2001; Danen and Yamada, 2001; Wang et al., 2003]. However, whether the DRG sequence may play a role as a recognition sequence for integrin receptor remains to be investigated.

In this article we have shown that treatment of MDA-MB-231 cells with antibodies raised against $\alpha 2$ and $\beta 1$ integrins resulted in a significant reduction of cells attached to rtEa4-peptide coated wells. These results suggested that rtEa4-pepitde might serve as an adhesion molecule to promote the attachment of MDA-MB-231 cells to the substratum. It is known that both $\alpha 2$ and $\beta 1$ integrins are the major receptors for collagen, and these molecules could also serve as the receptors for laminin in human melanoma cells [Elices and Hemler, 1989]. In MDA-MB-231 cells, it has been reported that antibodies against $\alpha 2$- or $\beta 1$-receptor reduced the attachment of cells to collagen I, III, and IV [Lichtner et al., 1998]. However, antibodies against $\alpha 2$ integrin receptor did not affect the attachment of MDA-MB-231 cells to laminin-coated substratum, which were in a good agreement with results reported by others [Maemura et al., 1995; Lichtner et al., 1998; Sonohara et al., 1998]. The inhibition of MDA-MB-231 cell attachment to rtEa4-coated plates by treatment with antibodies against $\alpha 2$- or $\beta 1$-integrins, suggested that rtEa4-peptide itself might also serve as an adhesion molecule and promote cell adhesion via the $\alpha 2$- or $\beta 1$-integrin receptor. However, the reduction of cell attachment to rtEa4-peptide coated plates by treatment with integrin antibodies was approximately 40%. This result does not rule out the possibility that rtEa4-peptide could bind to other integrin receptors or other receptors to mediate cell attachment. Alternatively, rtEa4-peptide might mediate cell attachment by inducing other adhesion molecules or cell adhesion receptors. The latter hypothesis was supported by the following two lines of evidence. Inhibition of new protein synthesis by cycloheximide resulted in the reduction of attachment of MDA-MB-231 cells to rtEa4-coated culture plates. Furthermore, levels of fibronectin 1 and laminin receptor mRNAs in MDA-MB-231 cells were elevated in response to rtEa4-peptide treatment and the expression of both genes were shown to correlate with the enhanced cell attachment [Grinnell and Feld, 1979; Castronovo et al., 1991]. These findings provide evidence supporting the notion that induction of adhesion molecules or cell adhesion receptors in MDA-MB-231 cells by rtEa4-peptide may assist in the attachment of these cells to the substratum. While the induction of fibronectin 1 gene in MDA-MB-231 cells by rtEa4-peptide lasted for a period of 12 h, the induction of laminin receptor gene was observed only in the first 1.5 h (data not shown). The differences in the duration of gene induction could reflect the differences of the threshold level of rtEa4-peptide required for the induction of both genes. Since rtEa4-peptide was added at the beginning of the experiment without subsequent supplementation of the protein, the longer induction period of fibronectin 1 mRNA suggested that the concentration of rtEa4-peptide required for the induction of fibronectin 1 gene is much lower than that of laminin receptor gene.

In an attempt to determine how rtEa4-peptide may be involved in the induction of fibronectin 1 gene expression, we have observed that treatment of MDA-MB-231 cells with inhibitors of JNK1/2, Mek1/2, p38MAPK, PI3K, and PKC in FAK/MAPK pathways resulted in blocking the induction of fibronectin 1 mRNA by rtEa4-peptide. These results suggest that rtEa4-peptide may induce the expression of fibronectin 1 and laminin receptor genes a series of signal transduction cascades. In searching for receptor molecules specific for rtEa4-peptide, Kuo and Chen [Kuo and Chen, 2003] have recently detected the presence of membrane binding components specific for trout rtEa4-peptide in human neuroblastoma

(SK-N-F1) cells. Together these results led us to propose the following mechanism to account for the action of the rtEa4-peptide in promoting cell attachment. In addition to rtEa4-peptide serving directly as the adhesion molecule by binding to integrin receptors, it may also bind to specific cell membrane receptor molecules to activate FAK/MAPK signal transduction cascades. The binding of rtEa4-peptide to the receptor(s) results in the upregulation of fibronectin 1, laminin receptor, and other adhesion molecules or adhesion cell receptor genes, which can also facilitate the attachment of cells to the substratum. Integrin receptors contain a ligand binding site with the affinity of approximately 1×10^{-6} M [Ginsberg et al., 1990], which is similar to one of the identified binding sites of rtEa4-peptide on the cell membrane of SK-N-F1 cells [Kuo and Chen, 2003]. It is conceivable that rtEa4-peptide may bind to $\alpha 2/\beta 1$ integrin receptors and this binding might result in the upregulation of expression of fibronectin 1 and laminin receptor genes via the activation of FAK/MARK signal transduction pathways. Although the rtEa4-peptide binding sites detected on the membrane of human neuroblastoma cells have not been shown to be present in MDA-MB-231 cells [Kuo and Chen, 2003], similar binding sites like these might serve as the receptors for rtEa4-peptide action to upregulate fibronectin 1 and laminin receptor genes in MDA-MB-231 cells in response to treatment with rtEa4-peptide.

Over the past two decades, the roles of IGF-I in regulating numerous developmental processes and cellular activities have been have been intensively investigated. However, the biological functions of E-peptides that are part of the pro-IGF-I have never been addressed until recently. It was shown earlier that Ea4-peptide of trout pro-IGF-I possessed mitogenic activity in non-transformed cells [Tian et al., 1999]. More recently we have further shown that the same peptide exerted inhibitory activities on anchorage-independent growth and invasion in established human cancer cells in vitro [Chen et al., 2002; Kuo and Chen, 2002]. In the present study we have shown that rtEa4-peptide enhanced the attachment of breast cancer cells (MDA-MB-231) by inducing the expression of fibronectin 1 and laminin receptor genes. Furthermore, Siri et al. [2006] showed that rtEa4-peptide inhibited invasion of the invasive breast cancer cells (MDA-MB-231) by suppressing the expression of *uPA*, *tPA* and *PA11* genes. It is intriguing to find that an E-peptide of the pro-IGF-I not only is biological active, but also elicits functions that are entirely different from its mature hormone under certain conditions. This is similar to the C-peptide of pro-insulin that elicits functions different from the mature insulin [Johansson et al., 1992; Ido et al., 1997; Wahren et al., 2000]. Although the physiological relevance of E-peptide activities remains unclear, Kuo and Chen [2002, 2003] have demonstrated the presence of a high affinity binding component specific for rtEa4-peptide on the membrane of human neuroblastoma cells (SK-N-F1), and the E-peptide induced morphological differentiation and inhibition of anchorage-independent growth of SK-N-F1 cells is reversed by signal transduction inhibitors involving MAP kinase/PI3-kinase cascades. Overall, these new findings highlight the presence of additional levels of control over the functionality of the IGF system. Further studies on the newly emerged E-peptide functions might prove that it plays important roles in regulating normal and abnormal growth and development as part of the IGF system. Together with the recent reports on the function of pro-insulin C-peptide, these findings could potentially revolutionize our perspectives on peptide hormone precursors. Although more studies are required to address the biological roles of the rtEa4-peptide, our novel discoveries point to the importance of questioning the previously established concepts of IGF actions.

ACKNOWLEDGMENTS

This research was supported by grants from NSF (IBN-0078067) and USDA (CONTR # 58-1930-0-009) to T.T.C.

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