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CARBOXY-TRUNCATED INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-5 STIMULATES MITOGENESIS IN OSTEOBLAST-LIKE CELLS

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Summary: Recently we demonstrated that a 23 kDa form of IGFBP-5, derived from osteoblast-like cells, stimulates osteoblast mitogenesis and enhances IGF-I action. Because osteoblast-derived IGFBP-5 is smaller than recombinant intact IGFBP-5 (23 vs 30 kDa) and has decreased binding affinity for IGF-I, we proposed that the native 23 kDa form of IGFBP-5 was truncated at a carboxy-terminal position. We now show that a recombinant form of carboxy-truncated IGFBP-5 binds IGF-I with reduced affinity and stimulates mitogenesis in mouse osteoblasts. We also show that ^{125}I-truncated IGFBP-5 specifically binds to osteoblast monolayers with low binding affinity, similar to that seen with native 23 kDa IGFBP-5. These data indicate that carboxy-truncated IGFBP-5 stimulates osteoblast mitogenesis and suggest that reduced IGF-binding and cell-surface attachment are local mediators of this response.

Insulin-like growth factor (IGF) binding proteins (IGFBPs) are secreted by cells and regulate IGF-stimulated cell growth by autocrine or paracrine mechanisms. All six IGFBPs so far identified have been shown to inhibit IGF actions when added to cells in culture (1-6). In addition, IGFBP-1 (1), IGFBP-3 (3,4) and IGFBP-5 (7) can enhance IGF-I-stimulated

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Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BSA, bovine serum albumin.

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mitogenesis under varying incubation conditions. One proposed mechanism for this enhancing effect involves the association of the binding protein with the cell surface.

Recently we demonstrated that IGFBP-5, purified from media conditioned by human osteosarcoma cells, stimulated normal osteoblast mitogenesis without the need for exogenous IGF (7). Because our purified IGFBP-5 was smaller than the size predicted from the cDNA sequence, and had decreased binding affinity for IGF-I, we proposed that the 23 kD IGFBP-5 was truncated at the carboxy-terminus. We also suggested that its intrinsic mitogenic activity and its IGF-enhancing effect resulted from its ability to attach to the osteoblast surface (7).

The purpose of the present study was to prove that a carboxy-truncated form of IGFBP-5 has intrinsic mitogenic activity in osteoblasts, similar to native 23 kDa IGFBP-5.

MATERIALS AND METHODS

Construction and Expression of Recombinant Truncated IGFBP-5 - All procedures were identical to those previously described for the construction and expression of full-length IGFBP-5 (6) except for the substitution of a new 3' PCR primer (5'-AGATCTGTCGACCTACTGCTCAGACTCCTG-3') that was used in the PCR to generate the carboxy-truncated IGFBP-5 construct.

Purification of Truncated IGFBP-5 - Yeast cultures were harvested by centrifugation and the lysates were applied to an IGF-I affinity column. IGFBP-5 was eluted with 0.5 M acetic acid and concentrated by Speed Vac as previously described (7). Affinity purified IGFBP-5 was dialyzed twice against 4 liters of 0.1 M acetic acid and concentrated, and the reconstituted IGFBP-5 was applied to a C_{18} reversed-phased HPLC column and eluted using a 20-60% acetonitrile/0.05 % trifluoroacetic acid gradient. Fractions were collected in siliconized glass tubes and dried by Speed-Vac. Protein size and purity was determined by silver stain of SDS-PAGE and by N-terminal amino acid sequencing of IGFBP-5 transferred to polyvinylidine difluoride membranes as previously described (8).

IGFBP Binding Studies - Binding of ^{125}I-IGF-I was determined at different concentrations of intact and truncated IGFBP-5 as described (6). Bound and free IGF-tracer were separated using 2% charcoal suspension equilibrated with 0.1 M sodium phosphate buffer, 2% human serum albumin, pH 7.0. After incubation for 20 min and centrifugation, the supernatant was counted in a gamma counter.

Mitogenic Assays - Cell proliferation was estimated by ^{3}H-thymidine incorporation as previously described (6,7). Mitogenic assays were performed under serum-free conditions in primary cultures of osteoblast-like cells released from neonatal (3 day) mouse calvaria (7) and in the human osteosarcoma cell line, SaOS B-10 (6). IGF-I used in these studies was provided by Ciba Geigy AG and Chiron Corp., Emeryville, CA and the antiserum to intact recombinant IGFBP-5 was generated in rabbits (BAbCo, Berkeley, CA) using KLH-conjugated recombinant IGFBP-5. Values are the mean $\pm$ SE of 6 replicate wells. All experiments were performed at least twice with similar results.

Cell Binding of Truncated IGFBP-5 - Binding of truncated IGFBP-5 to monolayer cultures of mouse osteoblast-like cells was performed using radioiodinated and unlabeled truncated IGFBP-5 as previously described (7). The specific activity of ^{125}I-IGFBP-5 was 120-150 μCi/μg.

RESULTS AND DISCUSSION

The cDNA for IGFBP-5 was modified by PCR (9) to encode for amino acids 1-169 and to allow subcloning into the yeast expression vector pBS24Ub (10,11) at the carboxy-terminus of the ubiquitin-encoded gene (6). The truncated IGFBP-5 cDNA was sequenced to verify that no mutations had been introduced by PCR. Yeast cells were transformed with the truncated IGFBP-5 construct (BP5 2.2) and induced for expression by growth and concomitant depletion of glucose from the culture medium.

HPLC purified truncated IGFBP-5 migrated in SDS-polyacrylamide gels (nonreduced) as two major bands of 24 and 18 kDa and a minor band at 22 kDa (by silver stain). Approximately equal amounts of the 24 and 18 kDa forms were observed (data not shown). Amino acid sequencing of each band from polyvinylidine difluoride membranes revealed that all 3 forms had the same N-terminal amino acid sequence, LGSFVH, identifying it with IGFBP-5 (12,13). The 24 kDa form is similar in size to the native 23 kDa IGFBP-5 (7) and the 18 kDa form is likely a carboxy-truncated fragment of the 23 kDa form.

Truncated IGFBP-5 demonstrated reduced binding affinity to IGF-I compared to intact IGFBP-5 (Figure 1). Specific ^{125}I-IGF-I binding to intact IGFBP-5 reached a maximum of 28% at a binding protein concentration of 25 ng/ml; half-maximal binding occurred at a concentration of 5 ng/ml. Specific ^{125}I-IGF-I binding to truncated IGFBP-5 was 7.7% at a concentration of 2500 ng/ml IGFBP-5. The apparent low affinity of recombinant truncated IGFBP-5 for IGF-I compares favorably with the native 23 kDa truncated IGFBP-5 (7). Low affinity IGF-binding is consistent with truncation of a portion of the carboxy-terminus of IGFBP-5 as described for other IGFBPs (14, 15).

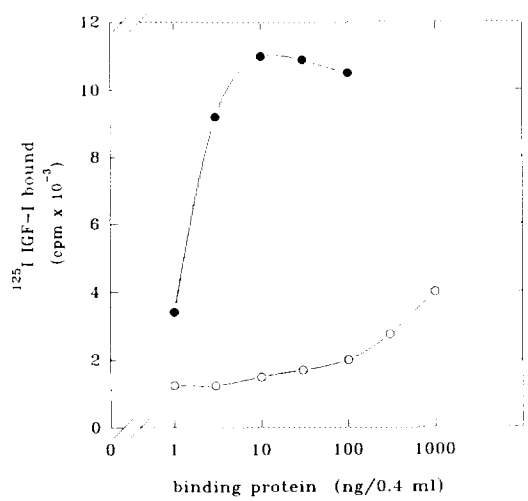

Figure 1. ^{125}I-IGF-I binding to intact and truncated IGFBP-5. Binding of ^{125}I-IGF-I to increasing concentrations of IGFBP-5. Closed circles represent intact IGFBP-5 and open circles represent truncated IGFBP-5.

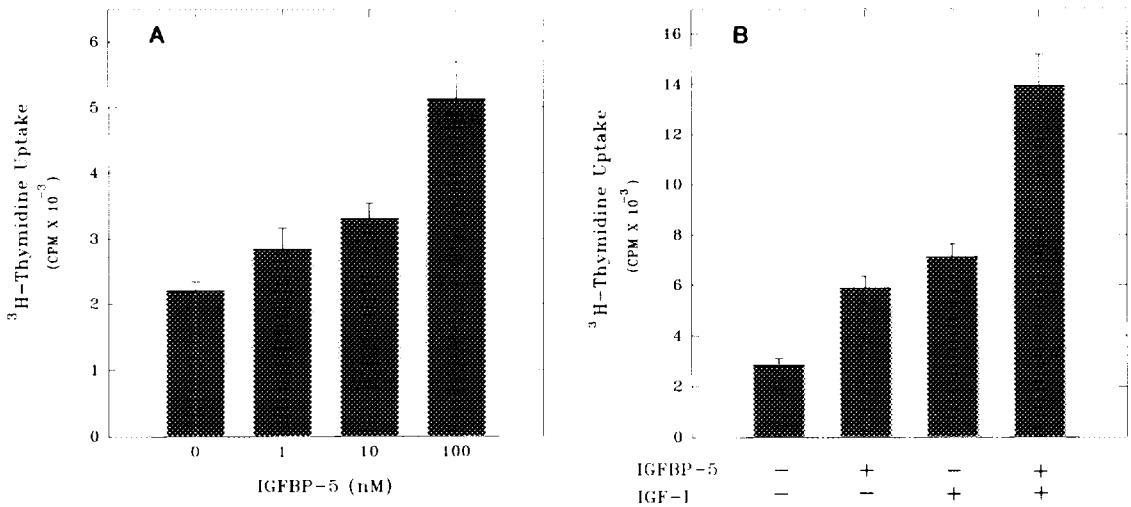

Figure 2. Stimulation of mouse osteoblast mitogenesis by truncated IGFBP-5. A) Cultures maintained in serum-free media containing 0.1% BSA were incubated overnight with varying concentrations of truncated IGFBP-5. B) Cultures maintained in serum-free media containing 0.1% BSA were incubated overnight without and with 65 nM IGFBP-5 in the absence or presence of 10 nM IGF-I.

Recombinant truncated IGFBP-5 stimulated mitogensis in cultured mouse osteoblasts under serum-free conditions (Figure 2A). The dose response effect shows that 10 nM IGFBP-5 stimulated mitogenesis by 54% and 100 nM IGFBP-5 increased mitogenesis by 138% (2.4 fold) above controls ($p<0.01$). Truncated IGFBP-5 also enhanced IGF-I-stimulated osteoblasts mitogenesis (Figure 2B); the mitogenic response to 65 nM IGFBP-5 was similar to the response of 10 nM IGF-I and the combination of both was additive. In B-10 human osteosarcoma cells, truncated IGFBP-5 caused a significant enhancement of IGF-I stimulated mitogenesis ($p<0.05$) and showed no intrinsic activity at concentrations up to 30 nM IGFBP-5 (data not shown). Co-incubation of 30 nM IGFBP-5 with 0.1-10 nM IGF-I enhanced B-10 mitogenesis 8-19% when compared to IGF-I alone. This weak enhancing effect of truncated IGFBP-5 in osteosarcoma B-10 cells contrasts with the inhibitory effect of intact IGFBP-5 on IGF-I-stimulated B-10 cell mitogenesis (6). The reason for the different mitogenic responses of truncated IGFBP-5 in B-10 and mouse calvarial cells is unclear but may be related to species differences and/or to the basal level of mitogenesis (transformed vs. normal).

Specificity of the mitogenic effect of truncated IGFBP-5 is demonstrated in Figure 3. As shown, pre-immune serum and IGFBP-5 antiserum (1:200 dilution) showed similar mitogenic responses and 100 nM IGFBP-5 co-incubated with pre-immune serum (1:200) stimulated mitogenesis by 50% above control values ($p<0.05$). When IGFBP-5 antiserum (1:200) was co-incubated with 100 nM IGFBP-5, the mitogenic effect was completely neutralized.

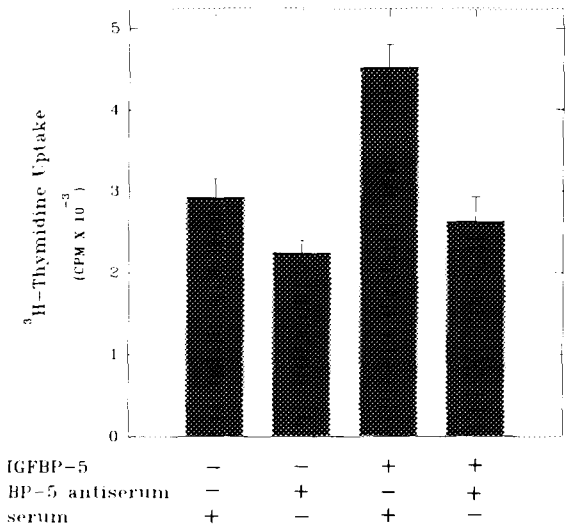

Figure 3. Neutralization of truncated IGFBP-5-stimulated mitogenesis by IGFBP-5 antiserum. Osteoblast cultures maintained in serum-free media containing 0.1% BSA were incubated overnight with either pre-immune rabbit serum (1:200 dilution) or IGFBP-5 antiserum (1:200 dilution) in the absence or presence of 100 nM truncated IGFBP-5.

Binding of ^{125}I-truncated IGFBP-5 to monolayers of mouse osteoblasts is shown in Figure 4. Specific binding was 3-4% of the total counts added and half-maximal displacement occurred with 1000 ng/ml of unlabeled truncated IGFBP-5. While this cellular binding affinity is slightly higher than that found with the native 23 kD IGFBP-5, where half-maximal

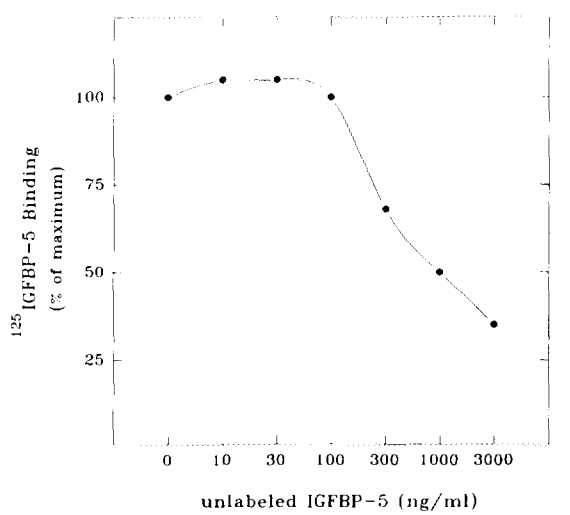

Figure 4. Binding of ^{125}I-truncated IGFBP-5 to mouse osteoblast monolayers. Confluent monolayers maintained in serum-free media were exposed to ^{125}I-IGFBP-5 with increasing concentrations of unlabeled IGFBP-5 for 2 hours at 4°C. The cells were washed, solubilized and cell-associated radioactivity was determined. Values are the mean ± SE of 4 wells.

displacement approximated 4000 ng/ml (7), both demonstrate relatively low binding affinity for the osteoblast surface.

In summary, we have shown that a carboxy-truncated form of IGFBP-5 can be produced in a yeast expression system employing the ubiquitin fusion method. The truncated IGFBP-5 product stimulated mitogenesis in mouse osteoblast-like cells and enhanced IGF-I stimulation, similar to native 23 kDa IGFBP-5. We suggest that the mitogenic and IGF-enhancing actions of truncated IGFBP-5 are mediated by low affinity binding to the osteoblast surface.

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