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GROWTH HORMONE-DEPENDENT INSULIN-LIKE GROWTH FACTOR (IGF) BINDING PROTEIN BOTH INHIBITS AND POTENTIATES IGF-I-STIMULATED DNA SYNTHESIS IN HUMAN SKIN FIBROBLASTS

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SUMMARY: This study investigates the effects of BP-53, the acid-stable IGF-binding subunit of the circulating 150 kDa IGF-binding protein complex, on IGF-I-stimulated thymidine incorporation by neonatal human skin fibroblasts. When cells were incubated for 24 h with IGF-I in serum-free medium, and thymidine incorporation studied over the final 4-h period, maximal stimulation (4- to 7-fold) was seen with 30 ng/ml IGF-I, with a half-maximal effect at 6.8±1.2 ng/ml (SD, n=4). Co-incubation of IGF-I with increasing concentrations of pure BP-53 caused dose-dependent inhibition of IGF-I-stimulated thymidine incorporation, which was complete when IGF-I and BP-53 were equimolar. In contrast, preincubation of cells with BP-53 for 8-48 h before adding IGF-I resulted in a potentiation of the subsequent IGF-I effect. The potentiation was maximal (2-fold) at a BP-53 concentration of 150 ng/ml, and appeared to act by increasing the maximal rate of thymidine incorporation rather than the sensitivity of this process to IGF-I. Since neonatal fibroblasts produce a protein which is identical to BP-53 in size and immunoreactivity, these results suggest an autocrine role for BP-53 in regulating fibroblast responsiveness to IGF-I.

The existence of binding proteins (BP) for peptides of the insulin-like growth factor (IGF) family has been recognized for over a decade. Several such proteins have been isolated and characterized in recent years, from both human (1-6) and non-primate (5, 7-10) sources. BPs for which an N-terminal amino acid sequence is known appear to fall into two structurally related classes : proteins of approximately 30 kDa, such as those from human amniotic fluid and rat BRL-3A cells (1,3,7,8), and proteins of approximately 50 kDa isolated from adult human and rat serum (5,6). Many other BP species, identified by binding studies in a wide variety of biological fluids, have not yet been classified in this way.

In human serum the major BP species exists as part of a GH-dependent complex of approximately 150 kDa (11-13), which appears to contain, in addition to BP and IGF-I or IGF-II, a non-IGF-binding, acid-labile protein of approximately 100 kDa (13,14). We have previously isolated the binding subunit of this complex, of which the predominant form is a 53 kDa acid-stable glycoprotein (termed BP-53), with high affinity (K_a= 2-3 x 10^{10} L/mol) for both IGF-I and IGF-II (6). A minor BP form of 47 kDa, with apparently identical properties, always co-purifies with BP-53 (6).

Abbreviations: BP, binding protein; IGF, insulin-like growth factor.

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Impure BP preparations have been found to inhibit various IGF actions *in vitro*, including stimulation of glucose transport by adipocytes, sulfate incorporation by chondrocytes, and thymidine incorporation in fibroblasts (15-18). Pure preparations of a 34 kDa BP isolated from amniotic fluid or human decidua, and the homologous protein from rat BRL-3A cells, have also been shown to inhibit IGF actions in several tissues (19-21) but, interestingly, in one recent study the amniotic fluid protein had a marked potentiating action on IGF-stimulated DNA synthesis in porcine aortic smooth muscle cells and human, mouse and chick embryo fibroblasts (22). Because comparable studies have not previously been performed using a well-characterized preparation of the major BP species from adult human serum, we have now tested the effects of pure BP-53 on DNA synthesis in human fibroblasts. In this report we describe the inhibitory effect of this protein when it is co-incubated with IGF-I, and contrast this with the potentiating effect seen when cells are preincubated with BP-53 prior to the addition of IGF-I.

Materials and Methods

Natural human IGF-I and IGF-II were purified from Cohn Fraction IV of human plasma, as previously described (23). Recombinant DNA-derived human IGF-I (24) was generously donated by Dr. B.D. Burleigh, International Minerals and Chemical Corp., Terre Haute, IN. BP-53 was isolated from Cohn Fraction IV by affinity chromatography and reverse-phase high performance liquid chromatography (6).

Neonatal human fibroblasts were grown from foreskin explants, and maintained at 37°C in 25 cm^2 Corning flasks (Corning, NY) in Ham's F10 medium (Flow Laboratories, Herts, UK) supplemented with 28 mM Na Hepes, 60 mg/liter penicillin, 100 mg/liter streptomycin and 10% fetal bovine serum (Cytosystems, Sydney, Australia). Cells, used between generations 5 and 10, were trypsinized and plated in 4.5 cm^2 12-place multiwells (Flow) at a subconfluent density of 3.0 x 10^4 cells/ml in 2 ml supplemented F10 medium containing 10% serum. After 24 h the medium was replaced with a 1:1 mixture of F10 medium and RPMI 1640 medium (Flow), serum-free and supplemented with Hepes, penicillin and streptomycin as above, plus 1 g/liter bovine albumin (RIA Grade, Sigma, St. Louis, MO). The thymidine concentration of this medium is 1.5 μM. Cells were incubated 48 h under these conditions unless the effect of preincubation with BP-53 was being tested, when the medium was replaced (normally after 24 h) with the same medium containing various concentrations of BP-53, and the incubation continued to a total of 48 h. The medium was again replaced by the same medium without BP-53, containing IGF-I or other substances being tested in the thymidine incorporation assay, and after 20 h [^{3}H]thymidine (Amersham, UK, 40-50 Ci/mmol) was added at 1 μCi/ml. After 4 h of [^{3}H]thymidine incorporation, the medium was removed and the cells were washed in succession with 0.15 M NaCl, 100 g/liter trichloroacetic acid, and methanol, then dissolved in 0.75 ml 0.5% sodium dodecyl sulfate. Aliquots of 0.6 ml were mixed with 8 ml Pico-Fluor 30 scintillant (Packard, Downers Grove, IL) and counted in a Packard Tricarb 300 liquid scintillation counter. DNA concentrations were measured on duplicate 75 μl aliquots of solubilized cells by the method of Cesarone et al. (25). Since correction for cell DNA content did not alter the results, all data are presented uncorrected for DNA content.

Results

Figure 1(a) shows the stimulation of [^{3}H]thymidine incorporation into DNA by increasing concentrations of IGF-I. In 4 similar experiments, maximal stimulation (4- to 7-fold above basal incorporation) was seen at 30 ng/ml IGF-I, with a half-maximal effect at 6.8±1.2 ng/ml IGF-I (mean±SD). These figures are similar to the maximal 3.5-fold stimulation by IGF-I, with a half-maximal effect at 9 ng/ml IGF-I, reported by Conover et al. (26) for adult human fibroblasts. Recombinant DNA-derived IGF-I was indistinguishable from the natural peptide in its stimulatory effect (not shown). IGF-II was approximately half as potent as IGF-I in stimulating

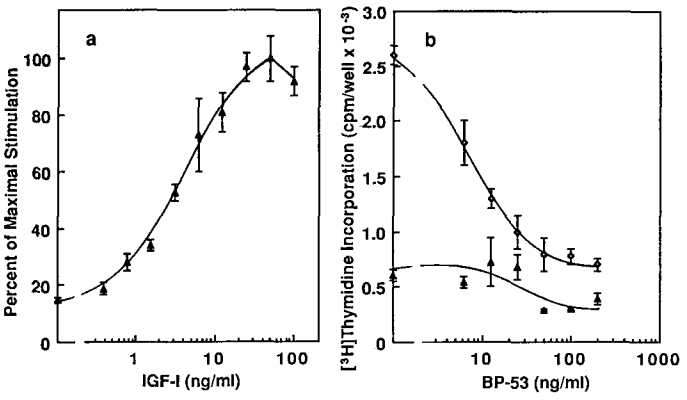

Figure 1. (a) Stimulation of [^{3}H]thymidine incorporation in human skin fibroblasts by increasing concentrations of IGF-I. (b) Inhibition of [^{3}H]thymidine incorporation in the presence of 7.5 ng/ml IGF-I (*open symbols*) or no IGF-I (*solid symbols*) by co-incubation with increasing concentrations of pure BP-53. Data are means ± SEM for triplicate incubations.

[^{3}H]thymidine incorporation (not shown), but was not tested further in this study as it is likely that IGF-I and IGF-II act through the same (type I) receptor (26).

The presence of increasing concentrations of pure BP-53 during the 24-h incubation with IGF-I resulted in a dose-dependent inhibition of the IGF-I effect. As illustrated in Figure 1(b), stimulation of [^{3}H]thymidine incorporation by 7.5 ng/ml IGF-I (i.e. 1 nM) was fully reversed by 50 ng/ml BP-53, an equimolar concentration. Basal [^{3}H]thymidine incorporation was also inhibited slightly by high concentrations of BP-53, suggesting that the basal rate might be due in part to endogenous IGF-I.

In contrast to the inhibitory effect of BP-53 when co-incubated with IGF-I, preincubation of fibroblasts with BP-53 prior to the addition of IGF-I resulted in a potentiation of the stimulatory effect of subsequently added IGF-I on [^{3}H]thymidine incorporation. In three similar experiments, a preincubation period with BP-53 of at least 8 h was required, with 24-h or 48-h preincubations having no greater effect, and no effect after only 4 h (Figure 2). The [^{3}H]thymidine incorporation rate in the absence of IGF-I was not significantly altered by preincubation for any length of time with BP-53.

Figure 3(a) shows a dose-response curve for the potentiation of IGF-stimulated incorporation of [^{3}H]thymidine by preincubation with increasing concentrations of BP-53. Up to 2-fold potentiation was seen when cells were preincubated with 150 ng/ml BP-53, with a half-maximal potentiation at 20-30 ng/ml. As seen in Figure 3(b), IGF-I at a concentration of 5 ng/ml stimulated [^{3}H]thymidine incorporation half-maximally regardless of the concentration of BP-53 present during the preincubation period, indicating that the potentiating effect of BP-53 was on the maximal rate of [^{3}H]thymidine incorporation obtained, rather than on the sensitivity of the cells towards IGF-I.

Discussion

This study demonstrates that a purified preparation of BP-53, the IGF-binding subunit of the circulating 150 kDa IGF-BP complex, can either inhibit or potentiate IGF-stimulated

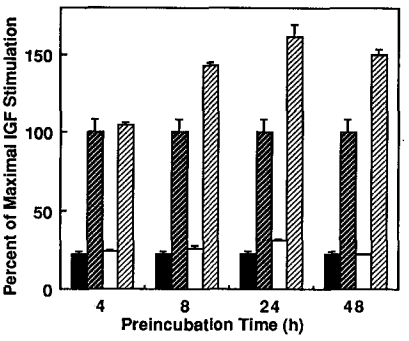

Figure 2. Potentiation of IGF-I-stimulated [^{3}H]thymidine incorporation in human fibroblasts by preincubation for various times with pure BP-53 before the addition of IGF-I. For each time point, the four bars indicate : *black,* no BP in the preincubation (period 1), and no IGF-I present during the final 24-h period (period 2); *dark hatched,* no BP in period 1, 30 ng/ml IGF-I in period 2; *white,* 50 ng/ml BP-53 in period 1, no IGF-I in period 2; and *light hatched,* 50 ng/ml BP-53 in period 1, 30 ng/ml IGF-I in period 2. Data are means ± SEM for triplicate incubations, expressed in terms of the maximal IGF-I stimulated incorporation without BP-53 preincubation. By analysis of variance and Duncan's multiple-range test, IGF-I-stimulated incorporation was significantly higher after 8, 24 or 48 h preincubation with BP-53 than after 4 h ($P<0.001$).

[^{3}H]thymidine incorporation in neonatal skin fibroblasts, depending upon the incubation conditions used. The possibility that these effects might represent physiological mechanisms is strongly suggested by the observation that these cells secrete an IGF-binding protein which is indistinguishable from plasma BP-53, both in its size on sodium dodecyl sulfate polyacrylamide gel electrophoresis and by its immunoreactivity towards an antiserum raised against plasma BP-53 (27). Serum-free medium conditioned by fibroblasts contains over 50 ng/ml immunoreactive BP-53, increasing to over 300 ng/ml in the presence of 10% fetal calf serum (27). Thus the concentrations of BP-53 required to achieve maximal inhibition or potentiation of IGF-stimulated

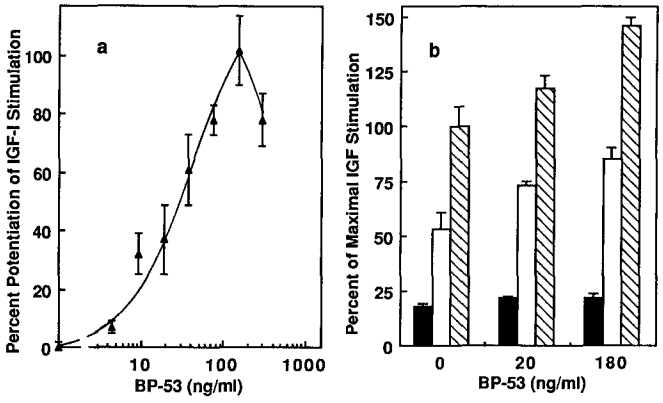

Figure 3. The effect of preincubation with increasing concentrations of pure BP-53 on IGF-I-stimulated [^{3}H]thymidine incorporation. Preincubations were for 24 h, after which cells were changed to fresh medium containing (a) 5 ng/ml IGF-I or (b) no IGF-I (*black bars*), 5 ng/ml IGF-I (*white bars*) or 30 ng/ml IGF-I (*hatched bars*), without BP-53. Data are means ± SEM for triplicate incubations, expressed in terms of the maximal IGF-I stimulated incorporation without BP-53 preincubation.

[^{3}H]thymidine incorporation in neonatal fibroblasts are within the range actually found in culture medium conditioned by these cells.

Since co-incubation of IGF-I and BP-53 resulted in inhibition of the stimulatory effect of IGF-I, it appears that the BP-IGF complex is inactive in this system. This conclusion is in accord with many other reports that various IGF BPs are able to inhibit a range of IGF actions (15-19, 21), but contrasts with the observation by Elgin et al. (22) that a preparation of amniotic fluid BP was able to potentiate IGF-stimulated mitogenesis in several cell types. Other studies also suggest that some IGF-BP complexes may be biologically active. Cornell et al. (28) found that serum subjected to anion-exchange chromatography at pH 8.1 fractionated into a BP-IGF complex rich in IGF-I, with very little activity in a rat adipocyte assay for insulin-like activity, and an IGF-II-rich complex which had strong insulin-like activity. Another recent preliminary report suggests that co-incubation of a plasma BP preparation with IGF-I could potentiate the stimulatory effect of IGF-I on DNA synthesis in hamster kidney fibroblasts (29).

Whereas co-incubation of IGF-I with BP-53 was clearly inhibitory in our study, fibroblasts that had been exposed to BP-53 prior to the addition of IGF-I showed an increased responsiveness to IGF-I action. This potentiation required between 8 and 24 h to reach its maximal effect, a considerably longer time than would be required for BP-53 to bind any free IGFs in the culture medium. Nevertheless, it is possible that the potentiating effect results from the binding of free IGFs by the BP. For example, if endogenous IGFs normally act to desensitize fibroblasts to IGF stimulation, either by receptor down-regulation or by some post-receptor mechanism, then the effective removal of free IGFs for an extended period by complexing them to BP-53 might result in a heightened sensitivity of the cells towards IGFs.

Alternatively, it is possible that BP-53 acts directly on fibroblasts, sensitizing the cells towards IGFs. This might occur independently of the IGF-binding function of the BP, or might be due to a modulation of IGF binding to its receptors on the fibroblast cell surface, resulting from the association of BP-53 with the cells. The latter hypothesis is supported by studies indicating that a 35 kDa IGF BP released by fibroblasts can associate with the cell surface and affect IGF-I binding (30,31). This protein is the same size as an IGF BP which is secreted by neonatal fibroblasts together with the 53 kDa BP and cross-reacts with an antibody raised against plasma BP-53 (27). Whether this protein is a processed form of the 53 kDa protein, or a separate gene product with an epitope in common with BP-53, is not known.

Whichever explanation is correct, this study provides new evidence that the action of IGF-I on fibroblast DNA synthesis can be modified in both inhibitory and stimulatory ways by a 53 kDa IGF BP apparently identical to one released by these cells. This observation therefore strongly suggests an autocrine role for BP-53 in regulating fibroblast responsiveness to IGF-I.

Acknowledgement

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