Multidomain Synthetic Peptide B2A2 Synergistically Enhances BMP-2 In Vitro

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ABSTRACT: A multidomain, synthetic peptide designated B2A2 synergizes the activity of BMP-2. B2A2 interacts with BMP receptor isoforms, potentiating the action of BMP-2 in activating alkaline phosphatase and triggering Smad and MAPK signaling. B2A2's design permits its delivery as a local surface coating as well as a soluble co-factor, thus broadening potential bioengineering applications.

Introduction: BMP-2 induces osteogenic differentiation and accelerates bone repair. Although BMP-2 inhibitors have been discovered, no BMP-2 mimetics or enhancers that function in the physiological range have yet been found. Here we report that a synthetic peptide designated B2A2, consisting of (1) a BMP receptor-targeting sequence, (2) a hydrophobic spacer, and (3) a heparin-binding sequence, is a positive modulator of recombinant BMP-2.

Materials and Methods: Cultures of mesenchymal cell lines C2C12 and C3H10T1/2 were given B2A2, recombinant BMP-2, or both. Alkaline phosphatase (ALP) activity was assayed by conversion of paranitrophenol phosphate (PNPP). Signaling through Smad and MAP kinase pathways was monitored by Western blot. Receptor binding was assessed by incubating immobilized B2A2 with soluble recombinant receptor-Fc chimeras and detecting bound receptor by anti-Fc antibody ELISA. Surface coating of medical device materials was done by first dip-coating with silyl-heparin, followed by B2A2.

Results and Conclusions: Treatment of cells with B2A2 alone marginally increased ALP activity. However, B2A2 plus BMP-2 resulted in 5- to 40-fold augmentation of ALP compared with BMP-2 alone in C3H10T1/2 or C2C12 cells, respectively. This synergistic enhancement was observed over a broad concentration range (4–1000 ng/ml BMP-2). B2A2 interacted directly with BMP receptor isoforms (preferentially to BMPR-Ib and ActivinR-II). In cells, B2A2 + BMP-2 led to a repression of MAP kinase and an increase of Smad activation, consistent with known activation pathways of BMP-2. B2A2 was ineffective when paired with other cytokine/growth factors (basic fibroblast growth factor [FGF-2], TGF-β1, vascular endothelial growth factor [VEGF]). Simultaneous co-administration was not strictly required. Pulse-chase experiments revealed that temporal separations up to 1 h were still effective. B2A2 was also effective when delivered in a polystyrene- or stainless steel–coated surface through a heparin platform (silyl-heparin) while BMP-2 was added exogenously in solution. These results suggest that B2A2 might promote aggregation of receptor subunits, enabling BMP-2 to activate signaling pathways at effectively lower concentrations. Synthetic multidomain constructs like B2A2 may be useful to accelerate bone repair/deposition through augmentation of endogenous levels of BMP-2 or through local BMP-2 contained in artificial or engineered matrices.
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INTRODUCTION

BMPS ARE A group of proteins involved in the development of a wide range of organs and tissues from embryonic through adult stages.[1] BMPs also play important roles in tissue repair and remodeling processes after injuries.[2–6] In animal models, certain BMPs induce ectopic bone formation and enhance healing of critical-sized segmental bone defects. Clinical studies have shown that use of recombinant human BMPs (rhBMPs) is a safe and effective alternative to autologous bone grafting. rhBMP-2 and rhBMP-7 are approved for human use in spinal fusion and recalcitrant long-bone nonunions, respectively.[3–6]

At the cellular level, BMP-2 signaling involves two types of high affinity transmembrane serine/threonine kinase re-

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ceptors, namely type IA (BMPR-IA) and type IB (BMPR-IB), and a lower affinity type II receptor (BMPR-II).[1,7,8] Whereas BMPs have been reported to bind to other members of the TGF-β receptor superfamily such as the Activin receptors,[9–11] for BMP-2 induction of osteogenic differentiation, an active ligand/receptor complex is understood to consist of BMP-2, BMPR-I, and BMPR-II in a 2:2:2 ratio.[12] It has been shown that multiple BMP receptors are present as oligomers at the cell surface before ligand binding[13] and that BMP-2 signaling results from binding to preaggregated receptor complexes rather than free receptors in the membrane.[8] After ligand binding, the type II receptor phosphorylates the type I receptor. The activated type I receptor then phosphorylates members of the Smad family of intracellular proteins—Smad1, Smad5, and Smad8—which in turn assemble into heteromeric complexes with Smad4 that translocate into the nucleus to regulate transcription of target genes.[11,14,15] In addition, the activated receptor complexes can trigger the p38 MAP kinase pathway independent of the Smad pathway.[9,16]

In vivo, BMP signaling is highly regulated by restricted temporal and spatial expression of BMPs and their cell-surface receptors (BMPRs),[17,18] and the interplay of these endogenous regulatory factors can vary under normal, developmental, or injury states. However, for therapy, exogenously applied rhBMP-2 is required at very high doses. For example, levels around 1 mg/ml of rhBMP-2 are used in spinal fusion cages (up to 8 mg/cage), an amount that is three to four orders of magnitude higher than what is typically found endogenously.[5] Administration of such a high dose of recombinant protein is not only costly, but might also be associated with other adverse effects such as bony overgrowth and immunological reactions. Therefore, the development of positive modulators of BMP-2 to enhance BMP activities may be of practical significance. Clinically, for example, they could be used in bone fillers to augment endogenous BMP-2 at bone margins, thereby increasing the effectiveness of the fill material. Alternatively, they could be used with demineralized bone matrix or BMP-2 containing implants to increase the effectiveness and/or reduce the dose of the exogenous rhBMP-2.

Few modulators of BMP signaling with therapeutic potential, however, have been described. Negative regulators that antagonize BMP have been reported, such as the secreted inhibitory proteins noggin, cerberus, gremlin, and chordin.[12,18] These proteins interact directly with the BMP molecules. Noggin, for example, binds to BMP at epitopes that prevent it from docking with type I and type II receptors, thereby sequestering BMP into an inactive complex.[18] A positive regulator, on the other hand, might be exemplified by the Xenopus Twisted gastrulation (xTsg) protein. This acts as a permissive signal in embryos by dislodging latent BMPs sequestered by Chordin, thus allowing BMP signaling to proceed.[19] In a different mechanism, sonic hedgehog (Shh) enhances BMP-2 effects in C3H10T1/2 and ST2 cells (although not C2C12 nor MC3T3-E1 cells) by increasing the percentage of cells in a population that are competent to respond to BMP-2.[20] Furthermore, basic fibroblast growth factor (bFGF or FGF-2), retinoic acid, prostaglandin E_1 (PGE_1), and ascorbate have been reported to act synergistically with BMP in some cell culture systems,[21–26] and an anti-inflammatory drug and an inhibitor of MAP kinase have also been reported to act synergistically with BMP-2.[27,28]

We hypothesized that a synthetic multidomain peptide construct designed to promote the preformation of receptor complexes could mimic BMP-2 or at least positively modulate the activity of exogenous BMP-2. In this study, we show that a novel synthetic peptide, designated B2A2, could synergistically enhance the bioactivity of BMP-2. The data suggest a mechanism of action that is distinct from previously described agents. The unique ability of B2A2 to enhance the activity of BMP-2 suggests a new set of strategies for the clinical treatment of bone lesions may be possible.

MATERIALS AND METHODS

Materials

C2C12 cells and C3H10T1/2 cells were purchased from American Type Culture Collection (Manassas, VA, USA). *E. coli*– or Chinese hamster ovary (CHO) cell–derived recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN, USA). Soluble BMP-2 receptors (BMPR-I and -II, and Activin receptor-II) were also obtained from R&D Systems in the form of recombinant receptor ectodomains fused to immunoglobin Fc. Endostatin-Fc, FGF-2, and vascular endothelial growth factor (VEGF) were supplied by the Biological Resources Branch Developmental Therapeutics Program, National Cancer Institute. TGF-β1, BSA, anti-phosphorylated MAP kinase antibody, and anti-human Fc antibody conjugated to horseradish peroxidase were from Sigma Aldrich (St Louis, MO, USA). Anti-phosphorylated-Smad1/5/8 antibodies were from Cell Signaling Technology (Beverly, MA, USA). FBS, DMEM/F12 medium, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). B2A2 was synthesized by the W. M. Keck Biotechnology Resource Center, a core facility at Yale University School of Medicine, by the solid phase Fmoc method. B2A2 consists of a receptor targeting domain, a hydrophobic spacer domain, and a heparin-binding domain; this design is detailed elsewhere.[29] The spacer and heparin-binding domains are contained within a linear backbone chain, KK-[NH(CH$_2$)$_5$CO]$_3$-RKRKLERIAR. The heparin-binding sequence is derived from c-*jun*/jun D (RKRKLERIAR) that conforms to the canonical XBBXXBX heparin-binding motif.[30] The receptor targeting domain containing a pair of identical sequences (AISMLYLDENEKVVL, amino acid residues 91–105 of mature human BMP-2) is grown off the backbone at the N-terminal lysines.[31] A similar peptide, B2A2Rev, was synthesized in an identical manner except that the amino acid sequence of the receptor targeting domain was reversed. Silyl-heparin is benzyl-tetra(dimethylsilylmethyl)oxycarbamoyl-heparin; its synthesis is detailed elsewhere.[32] In brief, silyl-heparin is made by reacting the hydrophobic group benzyl-tetra(dimethylsilylmethyl)-oxycarbamoyl-succinimide with heparin, thereby resulting in an amphipathic heparin derivative that can be adsorbed

onto hydrophobic surfaces. For coating purposes, silyl-heparin was used as a 0.25% solution in 60% isopropanol.

Alkaline phosphatase activity assay

C2C12 cells and C3H10T1/2 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, with DMEM/F12 medium supplemented with 10% serum and antibiotics. For the BMP-2–induced alkaline phosphatase (ALP) assay, cells were plated in 96-well (1×10^4/well) dishes in regular growth medium. Twenty-four hours later, when the cells formed a confluent monolayer, medium was replaced with DMEM/F12, supplemented with 2% serum and containing BMP-2 and/or B2A2 at concentrations described in the Results section. At 4–5 days after induction, ALP activity was determined as described by Akiyama et al.[33] with modifications. Briefly, cells were washed once with PBS and lysed with 0.1% Triton X 100 in 10 mM Tris HCl, pH 9.0. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Then, ALP activity was measured by adding ALP buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 1 mg/ml PNPP, pH 9.0), incubating in 37°C, and absorbance (405 nm) was read at 15, 30, and 60 minutes using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The activity was generally expressed as optical density (OD) per milligram protein per hour. Statistical significance was determined by paired two-tailed Student's *t*-tests.

Receptor binding assay

B2A2 or negative controls (insulin or unrelated peptide) were adsorbed onto wells of 96-well plates in 10 mM bicarbonate buffer for 1 h at 37°C (50 µg/ml). After rinsing 10 times with H_2O, wells were blocked for 1 h in 1% BSA/PBS at 37°C. Plates were rinsed, aliquots of soluble receptor-Fc chimeras (5 µg/ml in 3% BSA/PBS) were added, and plates incubated for 1 h at 37°C. After rinsing, horseradish peroxidase (HRP)-conjugated goat anti-human Fc antibody (1: 1000 in 3% BSA/PBS) was added, and the plates were incubated for 45–60 minutes at 37°C. After rinsing 15 times with H_2O, the chromogenic substrate ABTS was added (Pierce Biotechnology, Rockford, IL, USA), and plates were read in a multiwell spectrophotometer at 595 nm after 10–30 minutes. Endostatin-Fc was used as a negative control for the recombinant BMP receptor-Fc fusion proteins. Data are presented as the average background subtracted absorbance ± SD (*n* = 4). Furthermore, to compete with bound complexes of BMPR1B-Fc and B2A2 peptide, free BMP-2 was added to wells in the concentrations shown in the figures, before addition of the receptor-detecting anti-Fc antibody.

Immunoblot analysis

Cells were washed with ice-cold PBS and lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C. Cellular proteins were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h. The blots were incubated with monoclonal anti-phosphorylated MAP kinase antibody or polyclonal anti-phosphorylated Smad1(Ser463/465)/Smad5(Ser463/465)/Smad8(426/428) antibody overnight at 4°C. After washing in TBST, blots were incubated with anti-mouse Ig or anti-rabbit Ig HRP-conjugated antibodies for 1 h at room temperature and visualized with chemiluminescence reagents. Films were scanned and bands were quantitatively assessed by Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA).

RESULTS

B2A2 enhancement of BMP-2–induced ALP

By itself, B2A2 had little if any effect on ALP activity in C3H10T1/2 cells or C2C12 cells, in the dose range shown in Fig. 1 (0.075–10 µg/ml). However, when these cells were treated with 100 ng/ml of BMP-2 together with B2A2, the induction of ALP activity was found to be greatly enhanced—co-treatment was not additive, but was synergistic. In both cell types, the synergistic effect of B2A2 begins to be observable at doses around 300 ng/ml. In C3H10T1/2 cells, a maximal augmentation of 5- to 6-fold is seen at a B2A2 dose of 5000 ng/ml (Fig. 1A). In C2C12 cells, an augmentation of 27-fold is seen at the same B2A2 dose of 5000 ng/ml (Fig. 1B), and an even higher augmentation of 40-fold at the highest B2A2 dose tested (10 µg/ml). It is notable that, whereas the absolute ALP levels were roughly equivalent in this series of experiments, the baseline ALP levels were different in the two cell lines, accounting for the different fold-augmentation observed.

B2A2 consists of three domains, and to confirm that the synergistic effect of B2A2 is related to the specific BMP-2 sequences contained in the receptor-targeting domain, we synthesized several peptides that contained sequences derived from other nonrelevant growth factors as well as sequences from other portions of human BMP-2. One notable control was the peptide designated "B2A2-Rev." This peptide contains the same hydrophobic domain and heparin-binding domain as B2A2 but whose receptor targeting domain consists of reversed amino acid sequence. B2A2-Rev failed to show any enhancement of BMP-2 at any dose (Fig. 1B). In addition, to confirm that the entire multidomain structure of B2A2 was required for activity, we tested a linear peptide consisting of the receptor-targeting domain sequence only (AISMLYLDENEKVVL), lacking the spacer and the heparin-binding domains. This and all other control peptides failed to enhance BMP-2 activity, and they did not have direct activity on their own (data not shown). This data suggests a requirement for a specific sequence (or closely related sequences) in the B2A2 receptor targeting domain.

In the experiments above, the concentration of BMP-2 was held constant while varying the B2A2 concentration.
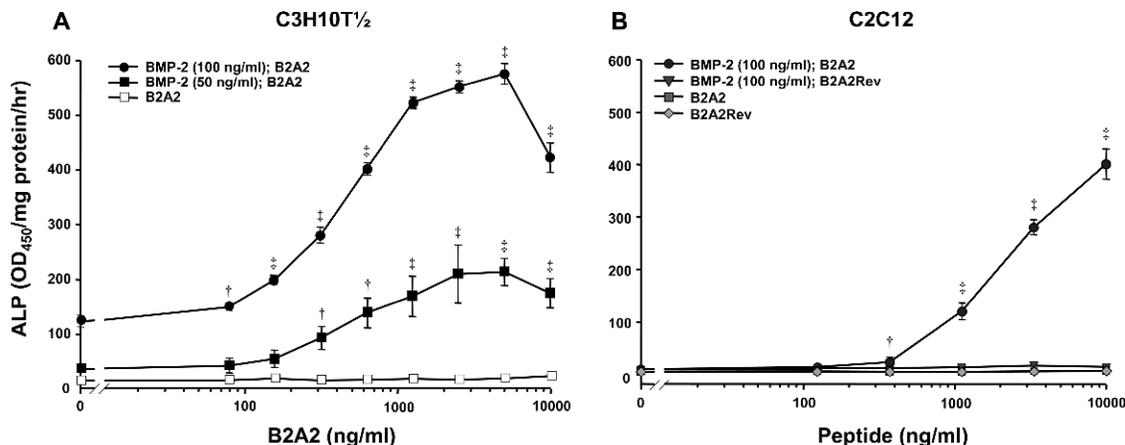
FIG. 1. B2A2 enhances BMP-2 induction of ALP activity. (A) C3H10T1/2 cells and (B) C2C12 cells were seeded in 96-well plates at 104/well in medium with 10% FBS overnight, changed to a medium with 2% FBS containing BMP-2 at the concentrations indicated in the figures, with or without B2A2, and cultured for 5 days, and ALP activity was determined. In B, B2A2Rev, a peptide contains the same amino acid composition but in a reversed sequence, was included as negative control. The results are the means of three independent determinations ± SD ($\dagger p < 0.05$ and $\ddagger p < 0.01$ compared with B2A2 alone control).

The converse was done in the following experiment (Fig. 2), in which B2A2 was held constant while varying the BMP-2 concentration. The data are displayed in a double log plot to highlight that the synergistic effect is significant over a wide dose range. In this culture system, the threshold for BMP-2 induction of ALP starts at ~30 ng/ml (Fig. 2, ○). However, when B2A2 is added, the threshold of ALP induction is significantly lowered, starting at ~3 ng/ml BMP-2 (Fig. 2, ●). The synergistic effect of B2A2 was observed throughout the BMP-2 concentration range tested, up to 1000 ng/ml. Similar synergistic effects were also observed in C2C12 (data not shown).

Currently, human BMP-2 is commercially produced by bacterial (*E. coli*) or eukaryotic (CHO cell) recombinant protein expression systems. To date, only CHO-produced rhBMP-2 is approved by the FDA for human use. Reports suggest that CHO-produced BMP-2 is more potent than *E. coli*–produced BMP-2, probably because of differences in post-translational modifications of the recombinant protein arising from eukaryotic versus prokaryotic expression.[34] We tested whether B2A2 enhances the biological effects of both types of commercial rhBMP-2. Figure 3 shows that CHO rhBMP-2 was more effective than *E. coli* rhBMP-2 in inducing ALP activity in C2C12 cells, consistent with others' results. The activity of *E. coli* BMP-2 was increased to a similar level as CHO BMP-2 when combined with 1000 ng/ml of B2A2. Similar to the results of *E. coli* BMP-2, the effects of CHO BMP-2 were further increased by 10-fold when combined with B2A2. Because the B2A2 synergistic effects could be observed in rhBMP-2 from both sources, *E. coli* rhBMP-2 was used for subsequent experiments.

FGF-2 and TGF-β have been shown to enhance ALP induced by BMP-2 or vitamin D_3, respectively.[35,36] We tested B2A2 in combination with other growth factors including FGF-2, TGF-β, and VEGF for induction of ALP in C2C12 cells. Not associated with osteoinduction, VEGF is a heparin-binding cytokine of similar size to FGF-2 and is thus a useful comparison. Treatments of FGF-2, TGF-β,
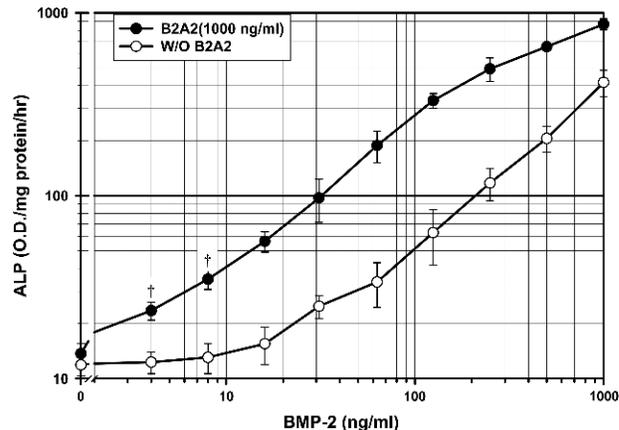

FIG. 2. B2A2 enhances BMP-2 induction of ALP activity in C3H10T1/2 cells over a wide range. Multipotent mouse mesenchymal C3H10T1/2 cells were seeded at 104 cells/well in 96-well plates in medium with 10% FBS overnight, changed to a medium with 2% FBS containing BMP-2 or/and B2A2 for 5 days, and assayed for ALP activity. Statistically significant differences between groups treated with or without B2A2 were detected throughout the entire dose range (all points, $p < 0.01$; except where indicated $\dagger p < 0.05$).

and VEGF alone failed to induce ALP in C2C12 cells. Combination of B2A2 with these growth factors also failed to activate ALP (Fig. 4), suggesting that BMP-2-is the effector in the combination of B2A2 and BMP-2.

B2A2 promotes Smad activation through BMP receptors

BMPs are members of the TGF-β superfamily of growth regulators that bind to members of the TGF-β receptor superfamily, specifically type I and type II BMP receptors (BMPR-I and BMPR-II)[7,8,12,13,37–40] as well as Activin receptors (ActR).[9,10] Whereas B2A2 was designed to bind to BMP receptors, the observation that co-administration with BMP-2 was required suggested the possibility that
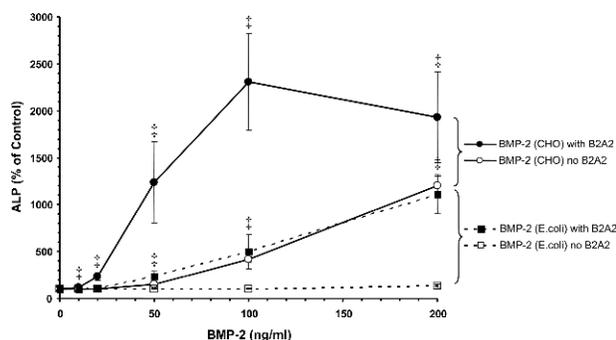
FIG. 3. B2A2 enhances the activity of recombinant human BMP-2 obtained from CHO cell and *E. coli* commercial production methods. Mouse C2C12 cells were seeded onto 96-well plates, treated with B2A2 in combination with BMP-2 (CHO [circles] vs. *E. coli* [squares]), incubated for 4 days, and assayed for ALP activity. B2A2 was applied at 1000 ng/ml and BMP-2 at the concentrations indicated in the graph. B2A2 increases the efficacy of *E. coli*–derived BMP-2 to level similar to that of CHO cell–derived BMP-2. CHO-derived BMP-2 is further increased by B2A2. Points represent means of quintuplet determinations ± SD ($†p < 0.05$; $‡p < 0.01$).
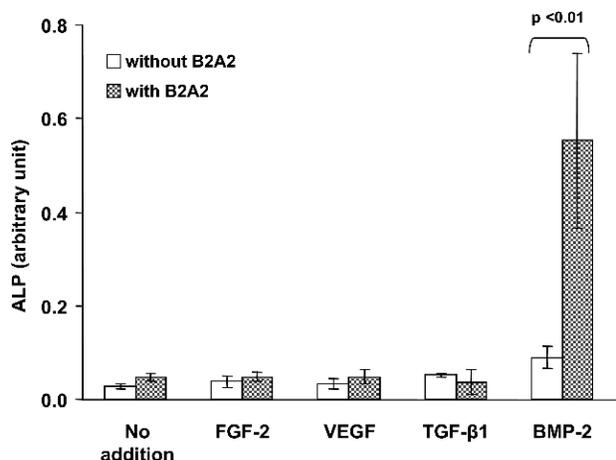

FIG. 4. The synergistic effect of B2A2 is specific to BMP-2. Mouse C2C12 cells were cultured as above, treated with a combination of various growth factors plus or minus B2A2, and assayed for ALP. FGF-2 was used at 50 ng/ml, VEGF at 25 ng/ml, TGF-β1 at 50 pg/ml, BMP-2 at 50 ng/ml, and B2A2 at 1000 ng/ml. Bars represent means of quintuplet determinations ± SD.

B2A2 may interact with the BMP-2 ligand instead. A simple way to address this issue was to temporally dissociate the administration of B2A2 and BMP-2. In pulse-chase experiments, cells were first treated with B2A2, incubated for 45 minutes, and washed out (Fig. 5). After varying amounts of additional time elapsed, BMP-2 was added. In this serial treatment scheme, the synergistic effect was still observed, although the magnitude was less than when both were added simultaneously as in Fig. 1. Conversely, no synergistic effect was observed when cells were treated with BMP-2 first and then exposed to B2A2 later (data not shown). This suggests that, whereas B2A2 acts on constituents present on the cells themselves (e.g., BMP receptors), again, BMP-2 is the effector of the co-treatment.
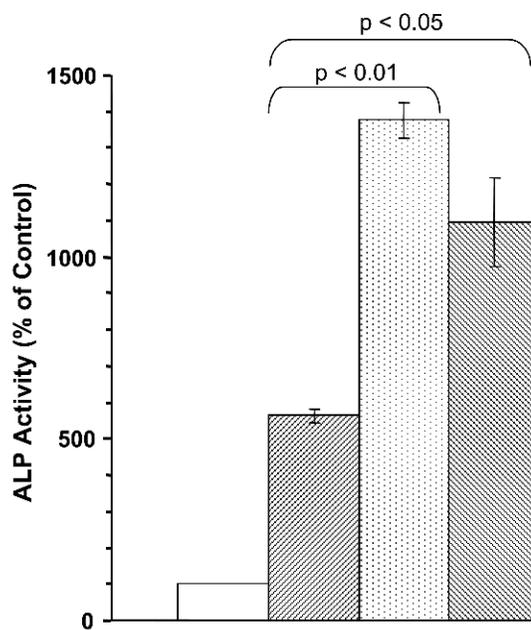
B2A2 (1000 ng/ml)	−	−	+	+
BMP-2 (200 ng/ml)	−	+	+	+
Time between two treatments (min)			30	60

FIG. 5. Direct interaction between BMP-2 and B2A2 is not required for enhancement. C2C12 cells were seeded on 96-well plates. Twenty-four hours later, cells were treated with B2A2 for 45 minutes. At the end of B2A2 treatment, cells were incubated with fresh medium for the period indicated in the figure, and BMP-2 was added to the culture medium. ALP activity was measured after 5 days. Data are the means of three independent determinations ± SD.

To confirm that B2A2 directly interacts with BMP receptors, solid phase receptor binding assays were performed using purified receptor/Fc chimeric molecules. These chimeras are recombinant constructs of the soluble ectodomain of various receptor molecules (BMPR and ActivinR isoforms) fused to the carboxyl-terminal of the human IgG$_1$ Fc region through a polypeptide linker. ELISA plates were coated with B2A2 or control compounds, soluble chimeric receptor/Fc molecules were added as indicated in Fig. 6A, and the captured receptor was detected by anti-Fc antibody and quantified with a colorimetric ELISA. B2A2 was shown to bind preferentially to BMPR-Ib and ActivinR-II, as well as other isoforms in the following order: BMPR-Ib = ActR-II >> BMPR-Ia = ActR-IIb > BMPR-II. Insulin, used as a control growth factor, bound to the solid phase did not bind any receptor tested, and endostatin-Fc, used as control for the receptor chimeras did not bind either B2A2 or BMP-2 (data not shown). In addition, BMP-2 added in large molar excess with the receptors blocked binding to B2A2. When BMP-2 was added in varying concentrations, the resulting displacement curve suggests two-stage binding kinetics of B2A2 to BMPR-Ib (Fig. 6B).

When BMPR-I receptors are triggered in cells competent for osteogenic differentiation, the transcription factor
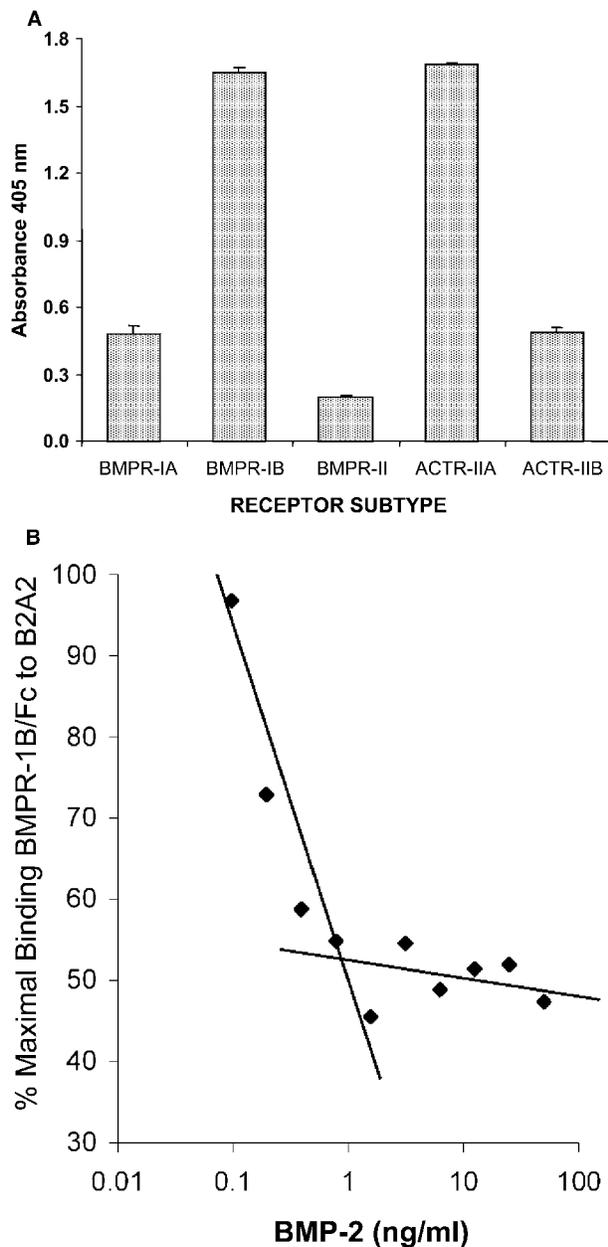
FIG. 6. Binding of B2A2 to BMP receptors in a solid phase binding assay. B2A2 was absorbed onto ELISA plates to saturation, soluble BMP receptor-immunoglobin Fc fusion proteins were added, and bound receptor was detected by HRP-conjugated anti-Fc antibody and colorimetric assay; values shown are background subtracted. (A) Specific binding of B2A2 to different receptor isoforms of the BMPR and Activin receptor family were tested, employing the receptor-Fc chimeras shown. Negative controls establishing specificity including unrelated polypeptide (e.g., insulin) was adsorbed to the plates and incubation of unrelated chimeric protein (Endostatin-Fc); neither resulted in binding of B2A2 (data not shown). (B) Apparent two-stage binding to BMPR-Ib was revealed by receptor displacement experiments. Bound receptor was displaced by the addition of rhBMP-2 at the levels indicated in the graph.

Smad1 is phosphorylated on a SSVS motif in its C terminal region, causing Smad1 translocation to the nucleus and activation of its transcriptional activity.[41] In contrast, ERK-

mediated phosphorylation of Smad1 at a PXSP motif in its linker region prevents Smad1 nuclear accumulation and inhibits its transcriptional activity. Thus, for osteogenic differentiation, not only must Smad be activated, but the ERK pathway needs also to be shut down.[27] We analyzed this duality in C2C12 cells after the treatment with BMP-2/B2A2. A near subthreshold dose of BMP-2 alone (200 ng/ml) did not change the basal level of EKR phosphorylation, and hence, ERK activation (Fig. 7A, compare lanes 1 and 2). However, with the combination of BMP-2 and B2A2, ERK phosphorylation was repressed below basal levels (compare lanes 1 and 4). Unexpectedly, B2A2 alone seemed to cause ERK phosphorylation (lane 3). However, the combination of BMP-2 and B2A2 shut down ERK phosphorylation far below this elevated level (compare lanes 3 and 4) and below the baseline of untreated cells. Overall, this experiment supports the notion that BMP-2 signal transduction is intact and is potentiated by B2A2. However, it also shows that B2A2 has some effects on its own, not related to BMP-2.

Next we analyzed Smad activation using an antibody recognizing Smad that is phosphorylated at its C terminus. A slight activation of Smad was observed after a low dose of BMP-2; however, the combination of BMP-2 with B2A2 caused substantial phosphorylation of Smad (Fig. 7A), consistent with findings from other groups.[8] Treatment of B2A2 alone failed to induce Smad phosphorylation in the C terminus, consistent with the observation that only the combination of B2A2 with BMP-2 is effective. However, in the previous experiment, B2A2 alone was capable of activating ERK, thus one might predict that Smad would be phosphorylated in its linker regions. Because the antibody used in this study does not recognize phosphorylation in the linker region of Smad, we are not able to determine whether these phosphorylation states were changed because of the B2A2 treatment. Figure 7B shows in a parallel experiment that the early signaling events of suppressed ERK phosphorylation and induced Smad C-terminal phosphorylation is correlated with the later-term differentiation associated with ALP activity.

B2A2/BMP-2 treatment by surface coatings

It was found that not only could the administration of B2A2 and BMP-2 be temporally separated, they could be spatially separated as well. The heparin-binding domain engineered into the B2A2 allows the molecule to be noncovalently adsorbed onto heparin-coated surfaces or matrices. One such heparin coating is silyl-heparin, which can be used as a loco-regional delivery vehicle for bioactive molecules.[32] For example, we have previously achieved delivery of bFGF with silyl-heparin adsorbed onto hydrophobic surfaces, including biodegradable polymers in vivo.[32] In these experiments, we sought to study whether B2A2 attached to a silyl-heparin–coated surface remained competent for enhancing BMP-2. The polystyrene surface of 96-well plates were first coated by silyl-heparin, followed by B2A2, C2C12 cells were seeded and allowed to attach, and 50 ng/ml BMP-2 was added to the culture medium. B2A2, delivered as the sole surface coating, did not induce ALP in

A

| Induction | 1 | 1.2 | 11.7 | 0.6 |

pSmad1/5/8 →

| Induction | 1 | 2.3 | 0.1 | 6.2 |

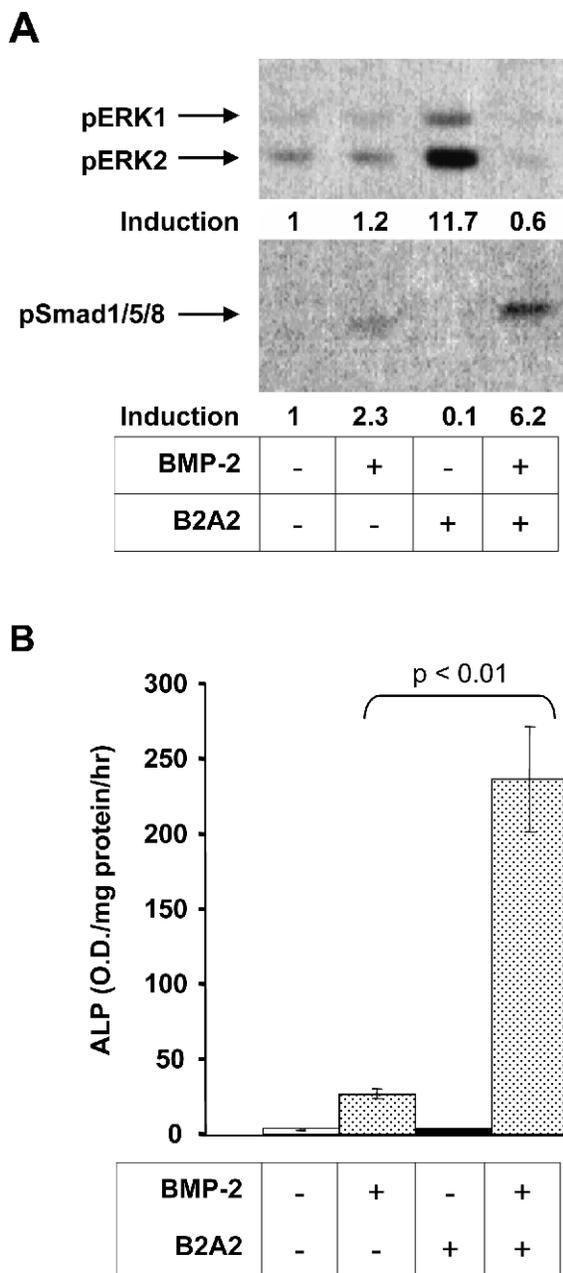| BMP-2 | - | + | - | + |
| B2A2 | - | - | + | + |

B

FIG. 7. The synergistic effects of B2A2 correlated with repression of MAP kinase ERK1/2 and increase of Smad activation. (A) C2C12 cells were treated with BMP-2 and/or B2A2 for 30 minutes. Cells were collected and lysed, and equal amounts of cellular proteins were analyzed by Western blot using antibodies recognizing phosphorylated ERK1/2 and Smad. The films was scanned, and the intensity of bands was quantitatively assessed by Image-Pro Plus Software. Level of induction was expressed as the ratio of treated vs. nontreated samples. (B) C2C12 cells were seeded on 96-well plates. Twenty-four hours after seeding, cells were treated with BMP-2 or/and B2A2, and ALP was measured 5 days later. Results are the means of three independent determinations.

the absence of BMP-2 (Fig. 8A, second data series bar), consistent with all previous data in this report where soluble B2A2 was delivered in solution. Surfaces coated with silyl-heparin alone promoted a modest enhancement of BMP-2

activity (Fig. 8A, fourth bar), consistent with previous reports from other groups that soluble heparin potentiates BMP-2.[42] In the wells coated with B2A2 adsorbed onto silyl-heparin (Fig. 8A, fifth bar), BMP-2 produced higher ALP activity (165% over control levels) than in the wells coated with silyl-heparin alone (85% over control), suggesting that surface coating by silyl-heparin could be a viable alternative for B2A2 locoregional delivery. As part of a noncovalent association, B2A2 is slowly desorbed (released) from silyl-heparin. However, under the conditions and time frame of these experiments, the vast majority of B2A2 presented to the cells remained on the coated surfaces, and the contribution of desorbed B2A2 was judged to be minimal, based on data obtained with other polypeptides bound to silyl heparin (X Lin, PO Zamora, LA Peña, unpublished data, 2004).[32] Furthermore, we studied other substrates such as stainless steel (Fig. 8B), a common material used in bone implant devices. Again, silyl-heparin + B2A2–coated wafers did not induce ALP by themselves, but adding BMP-2 to the culture medium caused dose-dependent induction of ALP activity in the coated wafer cultures (Fig. 8B, shaded bars). It should be noted that, in the uncoated wafer cultures (Fig. 8B, open bars), the addition of BMP-2 did not statistically increase ALP induction except at the highest concentration (200 ng/ml)—an effect not obvious with the scale of graph. In any case, the enhancement of BMP-2 by B2A2 on stainless steel was profound, and the same silyl-heparin + B2A2 coating on titanium wafers showed similar results (data not shown). These results suggest that surface-coated B2A2 maintains its properties of enhancing BMP-2 activity and has the potential to do so in in vivo implants of various configurations.

DISCUSSION

We have developed a synthetic, peptidergic BMP-2 enhancer designated B2A2. By biochemical and physiological criteria, B2A2 interacts directly with BMP receptors to positively modulate BMP-2 induced osteogenic differentiation. Synergistic effects between B2A2 and BMP-2 were observed in two cell lines, a multipotential mesenchymal stem cell C3H10T1/2 and a myogenic cell C2C12, as determined by at least two biological endpoints: ALP activity and phosphorylation of Smad. The augmentation of ALP activity at 100 ng/ml of BMP-2 was a 4- to 40-fold increase (Figs. 1A and 1B). Synergism is not unprecedented, having been observed between different members of the BMP family, namely BMP-4 and BMP-6.[11] Whereas researchers have identified other BMP-2 modulators that have either been negative regulators or agents that fail to work under relatively normal physiological conditions, B2A2 seems to be the first specific regulator that positively modulates BMP-2.

Recently, several BMP-specific regulators have been identified. Antagonists like noggin, chordin, and gremlin have been shown to bind to BMPs with the same affinity as BMP receptors, and thus competitively inhibit BMPs.[43,44] Enhancers like the cytokine bFGF have been shown in a rat marrow cell culture to act synergistically with BMP[24,45]; however, higher doses of bFGF caused profound inhibitory effects in vivo. Spinella-Jaegle et al.[20] reported that Sonic

A

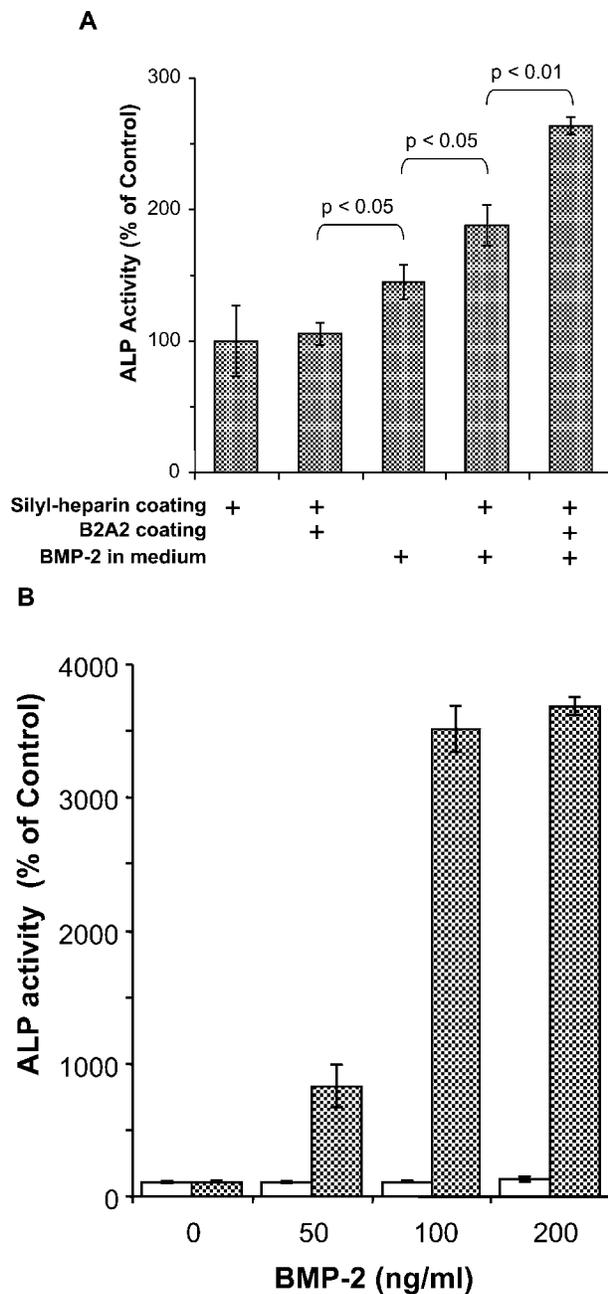| Silyl-heparin coating | + | + | | + | + |
| B2A2 coating | | + | | | + |
| BMP-2 in medium | | | + | + | + |

B

FIG. 8. BMP-2 enhancement with B2A2-coated surfaces. (A) Tissue culture wells (0.32 cm^2) were coated with silyl-heparin and/or a coating of B2A2 (10 μg/ml, 100 μl/well) as indicated. C2C12 cells were seeded in medium containing 2% FBS, and 2 h later BMP-2 (50 ng/ml) was added to some wells as indicated. ALP activity was measured 5 days later. Data are presented as quadruplicate means ± SD. (B) Stainless steel wafers (1 cm^2) were coated with silyl-heparin, followed by a B2A2 solution of 100 μg/ml, in wells of a 24-well plate and transferred to a fresh untreated plate before cell seeding. C2C12 cells were treated with BMP-2 as indicated, and ALP activity was measured after 5 days (uncoated, open bars; coated, shaded bars). A similar response was observed on titanium wafers (data not shown).

hedgehog (Shh) enhanced BMP-2 effects in C3H10T1/2 and ST2 cells, but it failed to enhance BMP-2 activity in C2C12 and MC3T3-E1 cells. They further showed that the

enhancing effect seemed to be a priming effect in which Shh increased the percentage of cells responding to BMP-2, whereas Shh itself is able to induce ALP activity in C3H10T1/2.[46–49] The physiological regulators in these examples do not act directly on BMP receptors.

In another line of study, attempts to generate peptides that possess BMP activity have been less than satisfactory. Osteoinductive effects were reported by White et al.[50] for a stretch of BMP-7 sequence and by Saito et al.[51] and Suzuki et al.[52] for two overlapping stretches of BMP-2 sequence. These results, however, were obtained in supra-normal experimental systems with peptides at extremely high concentrations and/or covalently attached to a substrate that kept them in contact with cells for a period of weeks. For example, the peptide reported to have the highest BMP-2-like activity[51] works only at concentrations >1–2 mg/ml (100–200 μg/well)—this is >2000 times higher than BMP-2's lower threshold (at 30–50 ng/ml, depending on the culture system) and 200 ~ 400 times higher than B2A2 at a dose that produces the highest synergistic effect (5 μg/ml). Moreover, at concentrations that high, peptides can completely displace BMP-2 from cell surface receptors and can be competitors of BMP-2. Furthermore, those were linear synthetic peptides (unlike the soluble multidomain synthetic constructs described in this report) that were precipitated onto surfaces, thus raising the effective concentration locally at the site of the cell membrane. In our studies, linear peptides comprising only the receptor-targeting domain of B2A2 that contained sequences derived from BMP-2 had no direct or synergistic effect at any concentration tested. In any case, the synergistic effects of B2A2 were observed when BMP-2 was in a presumptive physiological concentration range (<10 ng/ml), consistent with the range seen with other with native cytokines in vitro and in tissues.

We have directly shown (Fig. 6) that B2A2 binds to BMP receptors and that receptor activation is associated with the expression of the transcription factor Smad and repression of MAPK (Fig. 7), followed by a phenotypic transformation in which ALP is induced. One possible mechanism of the synergism could be that B2A2 binds directly to BMP-2 and the resulting complexes are more active than BMP-2 alone. For instance, heterodimers of BMP-2/7 and BMP-4/7 were found to be more active than BMP-2, -4, and -7 homodimers.[53,54] However, the evidence clearly rules out direct interaction between B2A2 and BMP-2 (Fig. 5). Rather, it points to B2A2 acting on BMP receptors at a lower affinity than BMP-2 because synergistic effects seems to plateau past doses of 5 μg/ml and decline at 10 μg/ml (Fig. 1B), presumably because of the high concentration of B2A2 displacing BMP-2 from cell surface receptors by mass action. In one isolated instance, B2A2 by itself seems to engage an undefined receptor kinase (Fig. 7A), but every experiment involving the biology of osteodifferentiation suggests that B2A2 acts on BMP receptors in concert with BMP-2.

Initial characterizations of the direct binding of B2A2 to BMP receptors in a cell-free system (Fig. 6) show that B2A2 has a strong association with receptor isoform

BMPR-Ib. For the receptor targeting domain, we chose a sequence that had the potential to bind to BMPR-I or BMPR-II. Residues at the N- and C-terminal ends of the AISMLYLDENEKVVL sequence have been shown to appear on a surface of BMP-2 that contacts BMPR-II, and site-directed mutagenesis studies by Sebald and colleagues[39,55] reveal these to be essential for BMPR-II binding. The middle portion of this sequence loops back to a different surface of BMP-2 that contacts BMPR-I, and residues adjacent to this loop were shown to be essential for BMPR-I binding. Thus, it was entirely predicted that B2A2 would bind strongly to one of the BMP receptor isoforms, but it was not clear to which one until the actual experiments were done. Because less information is known about BMP-2 binding to Activin receptors, further study will be required to understand the context of B2A2 binding to this class of TGF-β receptor family members. In any case, this information leads us to suspect that B2A2 promotes the formation of BMP receptor complexes (and perhaps BMP receptor/Activin receptor complexes), but by itself is insufficient to trigger signal transduction—again, phosphorylation of BMPR-I by activated BMPR-II is required for signaling. In this model, BMP-2 is the effector molecule when added in combination that initiates the phosphorylation events on BMPR-II, but much less BMP-2 is required to cause the appropriate trigger in these preformed receptor complexes. Further confirmation of this hypothesis is beyond the scope of these studies, but is an area for future study.

Different sources of BMPs present different attributes to consider for human applications. BMPs have been purified from bone, but with very low yields, and potential health risks associated with isolation from allogenic donor bone also limit clinical application of BMP from this source. Most of the BMP in clinical use is recombinant protein obtained from eukaryotic cell culture expression systems. Complications of post-translational modification and low yield result in a very high cost of these recombinant proteins. Moreover, the amounts required for efficacy in human applications turned out to be unexpectedly high.[5,6] Thus, the development of BMP receptor agonists remains a very attractive idea because a true agonist could replace BMPs in clinical applications.

However, a BMP-specific enhancer could also have unique clinical significance. Practically, a BMP2 enhancer may be useful in reducing the amounts of BMP-2 required because, as a synthetic peptide, B2A2 is (1) less expensive to produce, (2) vastly more chemically stable, and (3) easy to chemically modify for enhanced drug delivery. Biologically, there may be other advantages. For example, the process of spinal fusion involves a sequence of events associated with a temporal and spatial pattern of osteogenic-related gene expression. Morone et al.[56] studied the expression of the mRNA of several BMPs in spinal fusion and found that BMP-2 and others were increased at different levels at different times. It is daunting to match exogenous application of recombinant BMP-2 to the biologically optimal schedule. Similarly, BMPs can occur as homo- and heterodimers. A BMP-2 enhancer might be effective by augmenting the natural endogenous expression of BMPs as they occur in situ.

In these studies, B2A2 was added in both soluble and immobilized states (Fig. 8), indicating a broad potential for bioengineering applications. B2A2 could be used to reduce the effective dose of recombinant BMP-2 on or in a medical device. Silyl-heparin, in particular, could be of particular use for coating B2A2 onto device surfaces. B2A2 could also maximize the biological activity of biological preparations like demineralized bone matrix (DMB). Furthermore, B2A2 could augment the endogenous levels of BMP-2 generated by host tissue during bone healing. The discovery of BMP-2 enhancers therefore opens up a wide variety of practical applications to further study.

ACKNOWLEDGMENTS

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