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Peptide-Based Activation of Alpha5 Integrin for Promoting Osteogenesis

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ABSTRACT

Promoting osteoblastogenesis remains a major challenge in disorders characterized by defective bone formation. We recently showed that the alpha 5 integrin subunit (ITGA5) is critically involved in human mesenchymal cell osteoblast differentiation. In this study, we determined the potential of pharmacological ITGA5 activation by a synthetic cyclic peptide (GA-CRRETAWAC-GA) on murine osteoblast differentiation and function in vitro and bone formation in vivo. Peptide-mediated activation of ITGA5 in murine C3H10T1/2 mesenchymal cells resulted in the generation of the integrin-mediated cell signals FAK and ERK1/2-MAPKs. In vitro, peptide-based activation of ITGA5 protected from cell apoptosis but did not affect cell adhesion or replication, while it enhanced the expression of the osteoblast marker genes Runx2 and type I collagen and increased extracellular matrix (ECM) mineralization as also found with bone morphogenetic protein-2 (BMP2), a standard bone anabolic factor. When injected on adult mouse cranial bone for 3 weeks, the peptide-mediated activation of ITGA5 increased bone thickness by twofold, an effect also induced by BMP2. Histomorphometric analysis showed that this anabolic effect resulted from decreased cell apoptosis and increased bone forming surfaces and bone formation rate (BFR). We conclude that pharmacological activation of ITGA5 in mesenchymal cells is effective in promoting de novo bone formation as a result of increased osteoprogenitor cell differentiation into osteoblasts and increased cell protection from apoptosis. This peptide-based approach could be used therapeutically to promote the osteogenic capacity of osteoblast progenitor cells and to induce de novo bone formation in conditions where osteoblastogenesis is compromised. J. Cell. Biochem. 113: 3029–3038, 2012.

KEY WORDS: INTEGRIN ALPHA5; SIGNALING; OSTEOBLAST; BONE FORMATION

Defective bone formation relative to bone resorption is a hallmark of bone loss associated with aging [Khosla and Riggs, 2005]. The decreased bone formation relative to resorption results from reduced number and activity of osteoblasts [Manolagas, 2000; Marie and Kassem, 2011a]. While anti-resorptive agents are being used as therapeutic agents for age-related bone loss [Riggs and Parfitt, 2005], improving osteoblastogenesis, and promoting bone formation remain major therapeutic challenges in conditions where the number of osteo-competent cells is compromised [Marie and Kassem, 2011b]. The osteogenic process is characterized by the progressive differentiation of mesenchymal stem cells (MSC) into pre-osteoblasts and osteoblasts. Osteoblast differentiation begins with the expression of the osteoblast transcription factor Runx2, followed by the expression of osteoblast markers such as alkaline phosphatase (ALP) and type I collagen (Col1A1), and is typified by extracellular matrix (ECM) mineralization [Aubin, 1998; Karsenty and Wagner, 2002; Lian et al., 2004]. Up to now, a limited number of factors have been shown to promote osteogenic differentiation in MSCs [Marie and Fromigué, 2006]. In most species, osteogenic differentiation of MSCs can be promoted in vitro by dexamethasone, bone morphogenetic protein-2 (BMP2), or activation of the canonical Wnt pathway which acts by increasing the expression

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or activity of the transcription factor Runx2 and downstream genes. However, the number of available factors promoting osteogenic differentiation of MSCs in vivo remains limited. The identification of novel factors that can optimally induce osteogenic differentiation in osteoblast progenitor cells is therefore of major importance to promote osteoblastogenesis and bone formation.

During osteoblastogenesis, osteoblast commitment, differentiation, and lifespan are tightly controlled by ECM interactions [Franceschi, 1999; Marie, 2009]. Osteoblasts interact with ECM components such as type I collagen and fibronectin through integrins, a family of transmembrane αβ heterodimer proteins that generate intracellular signals [Hynes, 2002]. The α5β1 integrin is a cell surface receptor for fibronectin. In non-skeletal cell types, the integrin has been implicated in cell spreading, proliferation, differentiation, migration, and survival [Meredith et al., 1993; Ruoslahti and Reed, 1994; Zhang et al., 1995; Giancotti, 1997; Cao et al., 1998; Matter and Ruoslahti, 2001; Grossmann, 2002; Howe et al., 2002; Pulai et al., 2002; Reginato et al., 2003]. In bone, osteoblasts highly express α5β1 integrin which is involved in osteoblast adhesion [Moursi et al., 1996; Moursi et al., 1997] and survival [Kaabeche et al., 2005; Dufour et al., 2008a]. Using genome wide and functional analyses, we previously found that overexpression of the α5 integrin subunit (ITGA5) triggers osteoblast differentiation in human MSC [Hamidouche et al., 2009; Hamidouche et al., 2010]. We also recently showed that overexpression of ITGA5 in human MSCs promotes bone repair in vivo [Srouji et al., 2012]. These studies identified a critical role for ITGA5 in osteogenic differentiation of human MSC. This prompted us to determine whether targeting ITGA5 by pharmacological means may lead to promote osteoblast function and bone formation in vivo. To this goal, we used a cyclic peptide (GA-CRRETAWAC-GA) that was shown to prime ITGA5 [Mould et al., 2000]. The active peptide CRRETAWAC acts as a direct competitive inhibitor of the binding of Arg-Gly-Asp (RGD)-containing fibronectin fragments to the α5β1integrin. It was proposed that the Ser156-Trp157 in ITGA5 is responsible for the specific recognition of RRETAWA by α5β1 [Mould et al., 1998]. We previously showed that the peptide CRRETAWAC acts as an agonist of ITGA5 in human MSC [Hamidouche et al., 2009]. However, this peptide may also act as antagonist of ITGA5 since it can block cell attachment to fibronectin in other cell types [Koivunen et al., 1994; Mould et al., 2009]. In the present study, we tested whether pharmacological activation of ITGA5 using this peptide in murine mesenchymal cells may lead to increase integrin-mediated cell signaling, osteoblast differentiation, and function in vitro. We also analyzed whether the peptide-mediated activation of ITGA5 may trigger new bone formation in vivo in a mouse model. The data indicate that pharmacological activation of ITGA5-mediated signals using this agonist peptide is effective in promoting osteoblast differentiation and function in vitro and bone formation in vivo.

MATERIALS AND METHODS

CELLS

Established murine multipotent mesenchymal cells (C3H10T1/2) were obtained from ATCC (LGC Standards, Molsheim, France). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corporation, Paisley, Scotland) supplemented with 10% heat inactivated Fetal Calf Serum (FCS; PAA Laboratories, Les Mureaux, France), L-Glutamine (292 mg/L) and antibiotics (10,000 U/ml Penicillin and 10,000 μg/ml Streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2 in air. Culture media were changed three times a week.

REAGENTS AND PEPTIDES

Trizol reagent, was from Life Technologies SAS (Saint Aubin, France). Ascorbic acid, fibronectin, phalloidine, calcein and tetracycline were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Recombinant human BMP2 was from R&D Systems (Lille, France). The cyclic peptide GA-CRRETAWAC-GA was synthesized on an Applied Biosystems Model 433A peptide synthesizer using standard automated continuous-flow SPPS methods. After peptide cleavage, cyclisation of the agonist peptide was performed under conditions of high dilution (5.10^{-4} M) in water to avoid formation of the corresponding dimeric cyclopeptide. The cyclic peptide was purified by reverse-phase HPLC using a Shimadzu semi-preparative HPLC system on an RP-HPLC C18 column (Prosphere® C18, 100 Å 15 μm, 25 × 100 mm) with a mixture of aqueous 0.1% (v/v) trifluoroacetic acid (A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile/water mixture (80/20, v/v) (B) as the mobile phase (flow rate of 3 mL/min) and employing UV detection at 220 nm. Characterization of the peptide was performed by mass spectrometric on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight instrument. The predicted and observed high resolution masses (HRMS) for GA-CRRETAWAC-GA ($C_{54}H_{84}N_{20}O_{17}S_2$) were 1349.5844 and 1349.5778, respectively.

CELL PROLIFERATION ASSAYS

Cells were plated at 3,000 cells/cm^2, treated with or without the synthetic cyclic peptide GA-CRRETAWAC-GA (100 μg/ml in PBS) and cell replication was determined using the BrdU ELISA assay (Amersham, Cell Proliferation Biotrak ELISA System) according to the manufacturer's recommendations. In parallel, total cell number was evaluated by crystal violet staining at various time points on fixed cells. Optical density measure at 595 nm was performed after dye solubilization in 1% SDS buffer.

ADHESION ASSAY

Plates were coated with PBS, the synthetic cyclic peptide GA-CRRETAWAC-GA (670 μg/ml in PBS) or fibronectin (100 μg/ml in PBS). Cells were seeded at 10,000 cells/cm^2 and incubated for 45 min. After two washes with PBS, adherent cells were fixed in 75% ethanol. Adherent cells were detected by crystal violet staining.

ACTIN FIBERS STAINING

Plates were coated with PBS, the synthetic cyclic peptide GA-CRRETAWAC-GA (670 μg/ml in PBS) or fibronectin (100 μg/ml in PBS). Cells were seeded at 10,000 cells/cm^2 and incubated for 24 h. After two washes with PBS, adherent cells were fixed in PFA 3.7%. F-actin fibers were detected by incubation with fluorescein-labeled phalloidin (0.5 U/ml) for 20 min. Nucleus staining was performed

using DAPI (300 mM). All images were microphotographed using an Apotome microscope (Zeiss, Germany).

CASPASES ACTIVITY

Cells were plated at 26,000 cells/cm^2. The next day, the cells were incubated in the presence or absence of serum for 24 h, then treated with or without the synthetic cyclic peptide GA-CRRETAWAC-GA (100 μg/ml in PBS) for further 48 h. Effector caspases activity was determined using Ac-DEVD-pNA as substrate and Ac-DEVD-CHO as inhibitor (Alexis Biochemicals, CA) as previously described [Marie and Fromigué, 2006].

WESTERN BLOT

Cell lysates were prepared as described [Marie and Fromigué, 2006]. Briefly, proteins (40 μg) were resolved on 10% SDS–PAGE and electro-transferred onto PVDF nitrocellulose membranes (Millipore Corporation, Bedford). Filters were incubated for 1 h in blocking buffer (Sigma Aldrich), then overnight at +4°C on a shaker with specific primary antibodies (1/1,000). Membranes were washed twice with [50 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20] (TBST) and incubated for 2 h with appropriate HRP-conjugated secondary antibody (1/20,000). After final washes in TBST, the signals were visualized with enhanced chemiluminescence western blotting detection reagent (ECL, Amersham Biosciences, Piscataway, NJ) and autoradiographic film (X-OMAT-AR, Eastman Kodak Company, Rochester, NY). Densitometric analysis using QuantityOne software (BioRad Laboratories) was performed following digital scanning (Agfa, Japan). Representative images of immunoblots are shown. Rabbit monoclonal anti-phospho-ERK1/2-MAPKs and anti-phospho-FAK were from Cell Signalling (Danvers, MA), mouse monoclonal anti-ERK1/2-MAPKs and anti-FAK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

REAL TIME QUANTITATIVE PCR

Total RNA was isolated using Trizol reagent according to the manufacturer's recommendations. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Briefly, each reaction containing 3 μg of total RNA, 1X RT buffer, 1 mM dNTP mix, 1X random primers and 50 U multiscribe reverse transcriptase in a total volume of 20 μl. The reverse transcription reactions were run under the following conditions: 25°C for 5 min, 37°C for 120 min, and 95°C for 2 min. Products of reverse transcription were analyzed by quantitative PCR using LightCycler (Roche Applied Science, Indianapolis Ind.) and SYBR Green PCR kit (ABGen, Courtabœuf, France) supplemented with 0.5 μM of specific primers. Primers were as follows: COL1A1 sense 5′-CTTGGTGGTTTTGTATTCGATGAC-3′; COL1A1 antisense 5′-GCGAAGGCAACAGTCGCT-3′; RUNX2 sense 5′-AGGTTGGAGGCACACATAGG-3′; RUNX2 antisense 5′-TTGACCTTT GTCCCAATGC-3′; GAPDH sense 5′-ACACATTGGGGGTAGGAACA-3′; GAPDH antisense 5′-AACTTTGGCATTGTGGAAGG-3′. Reactions were performed on LightCycler 480 Instrument (Roche Applied Science, Indianapolis, OH) using the following thermal conditions: Activation at 95°C for 15 min then 40 cycles of denaturation at 95°C for 20 s, 58°C annealing for 15 s, and 72°C extension for 15 s. Melting curve analysis was included to assure that only one PCR product was formed. The relative amount of RNA was calculated by the $2^{-\Delta\Delta Ct}$ method. Signal was normalized to GAPDH as internal control.

IN VITRO OSTEOGENIC ASSAY

For in vitro matrix mineralization, the medium was supplemented with ascorbic acid (25 μg/ml) and inorganic phosphate (NaH_2PO_4, 3 mM) to induce collagenous matrix synthesis and mineralization [Hamidouche et al., 2009]. After 10 days of culture, cell layers were fixed in 70% ethanol. Matrix mineralization was detected by alizarin red staining (40 mM, pH 4.2) and microphotographed using an Olympus microscope (Japan). Alizarin red content was quantified spectrophotometrically after dissolution in cetylpyridinium chloride (10% in PBS).

IN VIVO BONE FORMATION ASSAY

Experiments were approved by the local institutional Lariboisière-Villemin Ethical Board (approval No: CEEALV/2012-04-03). ICR/SWISS mice (Charles River) aged 4 weeks were injected subcutaneously on the calvaria daily for 5 days with PBS (20 μl) or GA-CRRETAWAC-GA (25 mg/kg/20 μl PBS/day, a dose based on our in vitro data and blood volume), recombinant human BMP2 (10 μg/kg/20 μl PBS/day, a dose known to be effective in vivo [Mundy et al., 1999], used as a positive control. In a first set of experiments, mice were injected with calcein (10 mg/kg) or tetracycline (20 mg/kg) at days 4 and 1, respectively, before sacrifice to ensure double labeling of the new bone matrix formed [Parfitt et al., 1987]. In vivo new bone formation was determined by histomorphometric analysis on 5 μm thick sections of calvaria embedded in methyl methacrylate. Sections were stained with toluidine blue or left unstained for fluorochrome evaluation. The mineral apposition rate (MAR) was measured using image analyzer (Biocom) on double labeled surfaces. The single (MS) and double-labeled mineralizing surfaces (DLS) were measured in the same area using the objective eyepiece Leitz integrate plate II, and the bone formation rate (BFR) was derived from the product of MAR by MS. All readings were performed without knowledge of the treatment.

In a second set of experiments, mice were sacrificed at day 21 after the first injection in order to perform immunohistochemical analyses. Calvaria were fixed overnight with neutral buffered 3.7% PFA at +4°C then paraffin embedded. Five μm sections were deparaffinised in xylene, rehydrated through graded washes of ethanol in water then incubated overnight at 70°C in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Immunohistochemistry was performed using the peroxidase method (Vectastain Elite ABC kit, Vector Lab., Burlingame, CA) following the manufacturer's instructions. Tissue sections were incubated for 15 min at 37°C in type IV Hyaluronidase (1 mg/ml in PBS). Endogenous peroxidases were blocked with 3% H_2O_2 for 15 min at RT and non-specific binding inhibited with 5% normal goat serum. Slides were incubated with rat monoclonal anti-mouse anti-Ki67 antibody (Abcam, UK) (1/100 in blocking buffer) overnight at +4°C, washed twice in PBS then incubated with the goat anti-rabbit HRP-conjugated secondary antibody for 1 h at RT before visualization with DAB. Slides were counterstained with toluidine blue. Negative controls were prepared by omitting the primary antibody. Cell proliferation quantification was obtained by scoring the frequency of Ki67 positively stained

cells. A TUNEL assay was performed using an in situ cell apoptosis detection kit (Chemicon) and revealed according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All in vitro experiments were repeated at least three times. The results are expressed as mean ± SD of at least five samples. Comparisons between data were performed using the Student's *t*-test with $P < 0.05$ considered as significant.

RESULTS

PEPTIDE-BASED ACTIVATION OF ITGA5 GENERATES SIGNALING IN MOUSE CELLS

The cyclic peptide (GA- CRRETAWAC-GA) was previously shown to prime ITGA5 [Mould et al., 2000]. Because integrins generate cell signaling after activation [Hynes, 2002], we determined whether treatment of murine mesenchymal cells with this cyclic peptide can efficiently generate signaling mechanisms linked to activation of ITGA5. We found that treatment of osteoblast progenitor cells with the cyclic peptide GA-CRRETAWAC-GA (100 μg/ml) effectively increased FAK phosphorylation by twofold (Fig. 1A,B). This effect was associated with increased ERK1/2-MAPKs phosphorylation (Fig. 1C,D). These results demonstrate that the cyclic peptide efficiently activates ITGA5 which translates into generation of integrin-mediated signaling in murine mesenchymal cells. Since integrins are known to control cell adhesion [Hynes, 2002], we next determined whether the peptide may modulate cell adhesion. Cells cultured on the peptide showed actin-positive focal contact points similar to cells cultured on fibronectin (Fig. 1E). Furthermore, there was no detectable difference in cell adhesion between cells cultured on surface coated with the peptide or coated with fibronectin (Fig. 1F). These results indicate that cell adhesion is not significantly affected by the peptide in the culture conditions that we used to test the effectiveness of the peptide on osteoblastogenesis.

ACTIVATION OF ITGA5 PROMOTES OSTEOBLAST COMMITMENT AND DIFFERENTIATION

Having shown that the cyclic peptide activates FAK and ERK1/2 signaling in murine mesenchymal cells, we analyzed whether this effect translated into functional expression of osteoblast genes. As expected, we found that BMP2 (100 ng/ml) which is a standard agent known to promote osteoblast differentiation [Chen et al., 2004; Phimphilai et al., 2006], increased Runx2 and type I collagen expression (Fig. 2A,B). We found that the cyclic peptide GA-CRRETAWAC-GA (100 μg/ml) significantly increased the expression of Runx2 (Fig. 2A). This effect was associated with increased type 1 collagen expression (Fig. 2B), which typifies osteoblast function.

Since matrix mineralization is the hallmark of terminal osteoblast differentiation [Aubin, 2001], we determined the effect of the peptide on matrix mineralization. We found that treatment of murine osteoblast progenitor cells with the cyclic peptide GA-CRRETAWAC-GA (100 μg/ml) increased matrix mineralization (Fig. 2C,D). These results indicate that the cyclic peptide has a high potential to promote osteoblast differentiation and function in murine mesenchymal cells in vitro.

Activation of FAK was shown to activate osteoblast differentiation [Salasznyk et al., 2007] and ERK1/2-MAPKS signaling is known to activate cell proliferation, differentiation, or survival in osteoblastic cells [Jaiswal et al., 2000; Xiao et al., 2000; Almeida et al., 2005; Ge et al., 2011]. Since the cyclic peptide modulates FAK and ERK1/2-MAPKs activity (Fig. 1), we determined whether the cyclic peptide altered cell replication and number. The addition of 100 μg/ml cyclic peptide, a dose that promoted osteoblast gene expression and matrix mineralization (Fig. 2), had no effect on cell replication, as shown by BrdU incorporation assay (Fig. 3A) and cell number evaluation (Fig. 3B).

We previously showed that disruption of ITGA5 in osteoblasts leads to cell apoptosis in vitro [Kaabeche et al., 2005] and in vivo [Dufour et al., 2008b]. We therefore determined whether, in contrast, an activation of ITGA5 by the cyclic peptide may translate into changes in cell death. We found that the peptide (100 μg/ml) had no effect on cell apoptosis in basal culture conditions, as evaluated by caspases activity (Fig. 3C). In contrast, cell apoptosis induction by serum deprivation was reduced by the addition of cyclic peptide (Fig. 3C). Overall, these results indicate that activation of ITGA5 by the cyclic peptide GA-CRRETAWAC-GA in murine mesenchymal cells generates intracellular signals that lead to protect against cell apoptosis induction and to promote osteoblast progenitor cell differentiation and osteoblast function in vitro.

PEPTIDE-BASED ACTIVATION OF ITGA5 PROMOTES BONE FORMATION IN VIVO

To determine whether activation of ITGA5 by the cyclic peptide may translate into bone tissue formation in vivo, we used an established assay allowing evaluation of anabolic agent effectiveness in rodents [Mundy et al., 1999]. Strikingly, we found that subcutaneous injection of the peptide (25 mg/kg/day for 5 days) increased bone matrix apposition compared to the control bone, as shown by the mean distance between double labeled surfaces documenting active bone forming surfaces (Fig. 4A). Quantification of de novo bone formation revealed that the cyclic peptide tended to increase the matrix appositional rate (MAR) (Fig. 4B), and significantly increased the single and double labeled surfaces (Fig. 4C,D) which are hallmarks of osteoblast activity, compared to the control bone. This indicates that the peptide increased the extent of functional osteoblasts which resulted in increased BFR (Fig. 4E). Interestingly, the positive effect of the peptide on bone formation was close to the effect of BMP2 at the dose tested (Fig. 4B–E).

We next determined whether the positive effects of the cyclic peptide on bone formation in vivo resulted from changes in cellular replication or survival. We found that the cyclic peptide had no significant effect on osteoblast replication as indicated by Ki67 positive cells quantification (Fig. 4F,G), which supports the lack of effect of the peptide on cell proliferation in vitro (Fig. 3A,B). In contrast, the cyclic peptide reduced cell death as indicated by TUNEL assay compared to the control bone (Fig. 4F,H), indicating that the peptide reduced osteoblast apoptosis. These in vivo results are consistent with our in vitro data and indicate that activation of ITGA5 by the cyclic peptide GA-CRRETAWAC-GA promotes bone

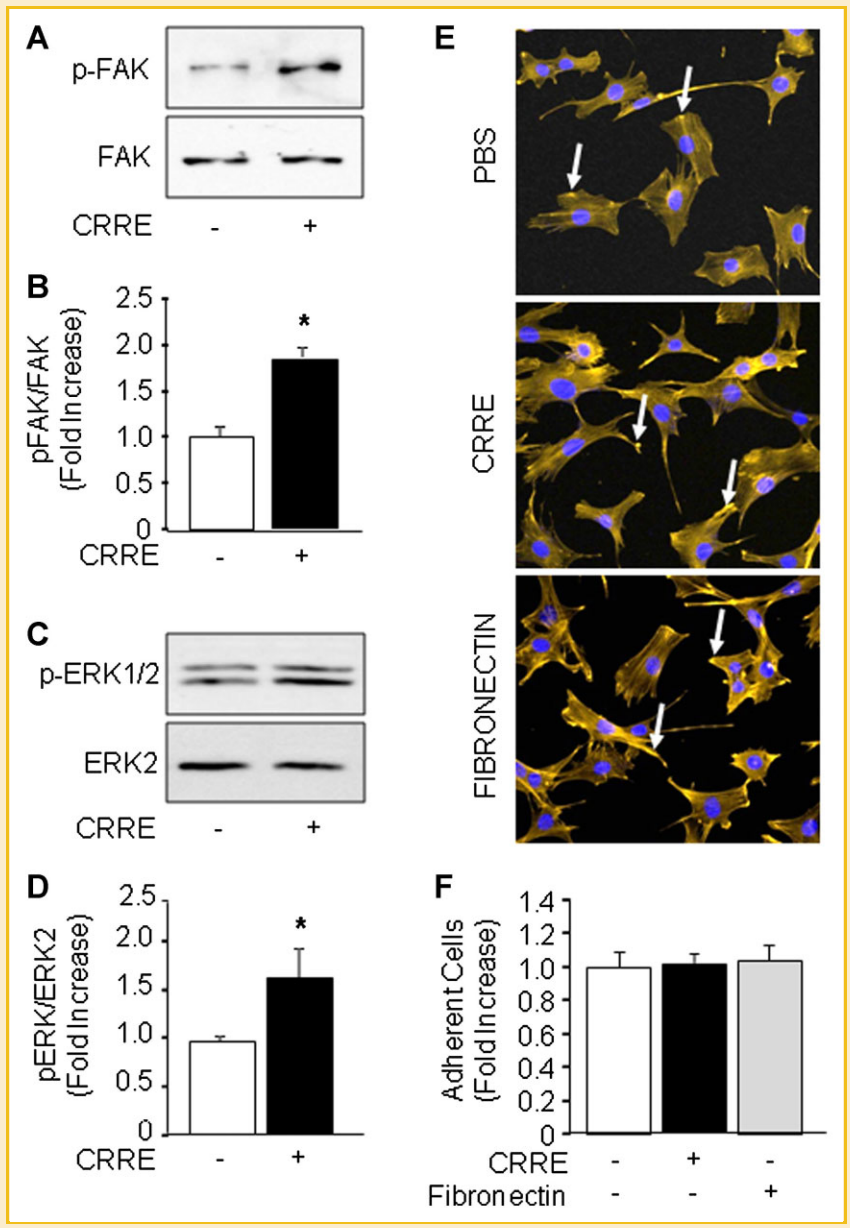

Fig. 1. ITGA5 activation generates cell signaling in murine mesenchymal cells. A–D: C3H10T1/2 cells were treated with the cyclic peptide GA-CRRETAWAC-GA (CRRE; 100 μg/ml) for 30 min and protein lysates were processed for western blot analysis of FAK and ERK1/2 signaling and quantification was performed. E: C3H10T1/2 cells were cultured for 24 h on surfaces coated with the cyclic peptide GA-CRRETAWAC-GA (670 μg/ml) or fibronectin (100 μg/ml). F-actin fibers were revealed with fluorescein-labeled phalloidin staining (yellow) whereas nuclei were stained with DAPI (blue). White arrows indicate actin-positive focal contact points. F: C3H10T1/2 cells were cultured for 45 min on surfaces coated with the cyclic peptide GA-CRRETAWAC-GA (670 μg/ml) or fibronectin (100 μg/ml) and the number of adherent cells was determined. Data represent mean values of triplicate experiments ± SD ($^{*}P < 0.05$ vs. Control). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

formation mainly by increasing the extent of bone forming surfaces and reducing osteoblast apoptosis.

PEPTIDE-BASED ACTIVATION OF ITGA5 INCREASES BONE MASS IN VIVO

Finally, we examined whether the increased extent of bone forming cells induced by the cyclic peptide GA-CRRETAWAC-GA translated into bone gain in vivo. We found that the local injection of the cyclic peptide increased de novo bone tissue formation compared to the control bone (Fig. 5A). Quantitative analysis of the average distance between the new bone formed and the bone border showed that the anabolic effect of the cyclic peptide (25 mg/kg/day for 5 days) on bone formation was close to that induced by the local injection of BMP2 at the dose tested (10 μg/kg/day for 5 days), a standard anabolic agent that promotes bone formation locally [Rosen, 2009] (Fig. 5B). Overall, the results indicate that peptide-mediated activation of ITGA5 by a cyclic peptide in murine osteoblast progenitor cells promotes FAK-ERK1/2 signaling,

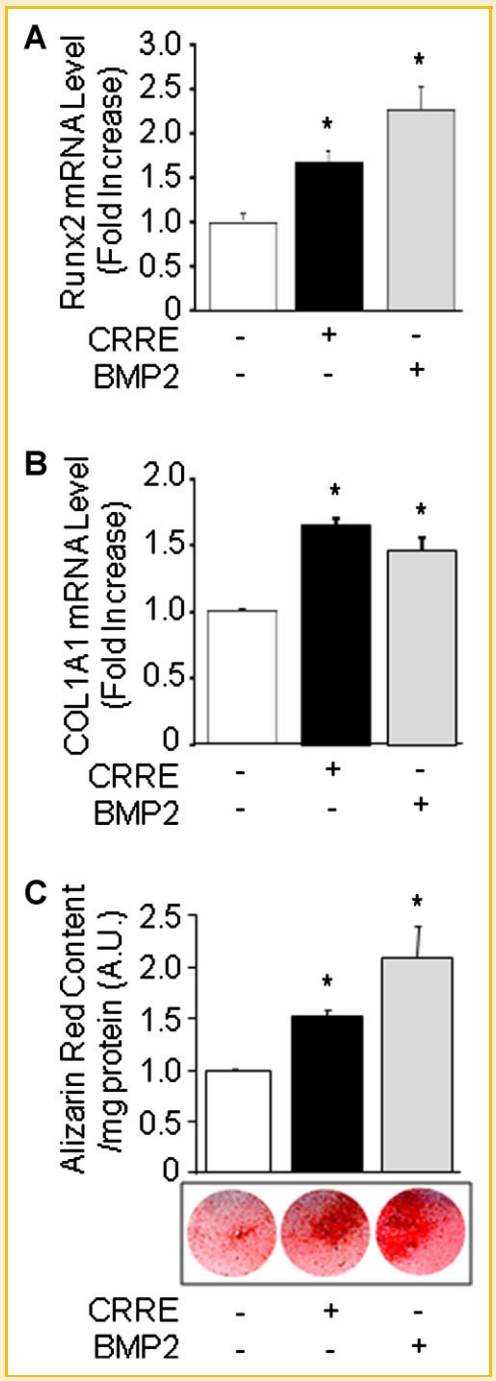

Fig. 2. Peptide-mediated activation of ITGA5 signaling translates into functional osteoblast differentiation activity. A,B: C3H10T1/2 cells were treated for 24 h with the cyclic peptide GA-CRRETAWAC-GA (CRRE; 100 μg/ml), recombinant BMP2 (100 ng/ml) or the solvent (PBS) and the expression of phenotypic osteoblast marker genes Runx2 and type I collagen (COL1A1) was determined by RT-qPCR analysis. C,D: The cyclic peptide GA-CRRETAWAC-GA (CRRE; 100 μg/ml) increased extracellular calcified matrix formation in vitro, as revealed by alizarin red staining and quantification. Data represent mean values of triplicate experiments ± SD (*$P < 0.05$ vs. Control). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

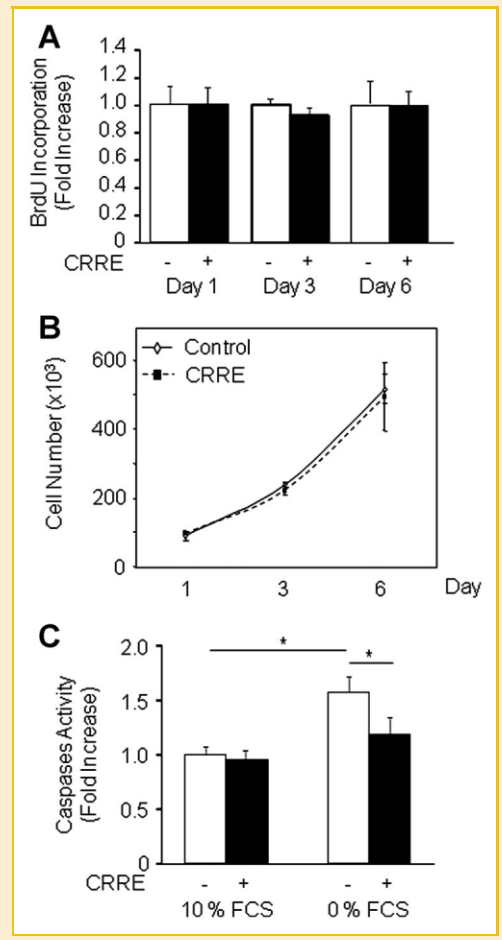

Fig. 3. Peptide-induced activation of ITGA5 in murine mesenchymal cells protects from apoptosis in vitro. The addition of the cyclic peptide GA-CRRETAWAC-GA (CREE; 100 μg/ml) had no effect on cell replication in C3H10T1/2 cells, as shown by BrdU incorporation (A) and cell number (B). The cyclic peptide protected from cell apoptosis induced by serum deprivation in vitro, as evaluated by effector caspases activity assay at 48 h in C3H10T1/2 cells (C). Data represent mean values of triplicate experiments ± SD (*$P < 0.05$ vs. untreated cells).

osteoblastogenesis and bone tissue formation in vitro and in vivo (Fig. 6).

DISCUSSION

Preclinical and clinical studies indicate that the use of mesenchymal cells with osteogenic potential may prove to be useful for bone regeneration [Bianco et al., 2001; Kassem, 2004; Prockop, 2009]. A remaining challenge for efficient bone formation is to identify the factors that can optimally promote the osteogenic differentiation program. Our recent studies indicate that forced ITGA5 expression results in increased osteoblast differentiation in human MSC [Hamidouche et al., 2009; Hamidouche et al., 2010]. In this study, we investigated the potential of pharmacological activation of ITGA5 in murine mesenchymal cells to trigger osteoblastogenesis

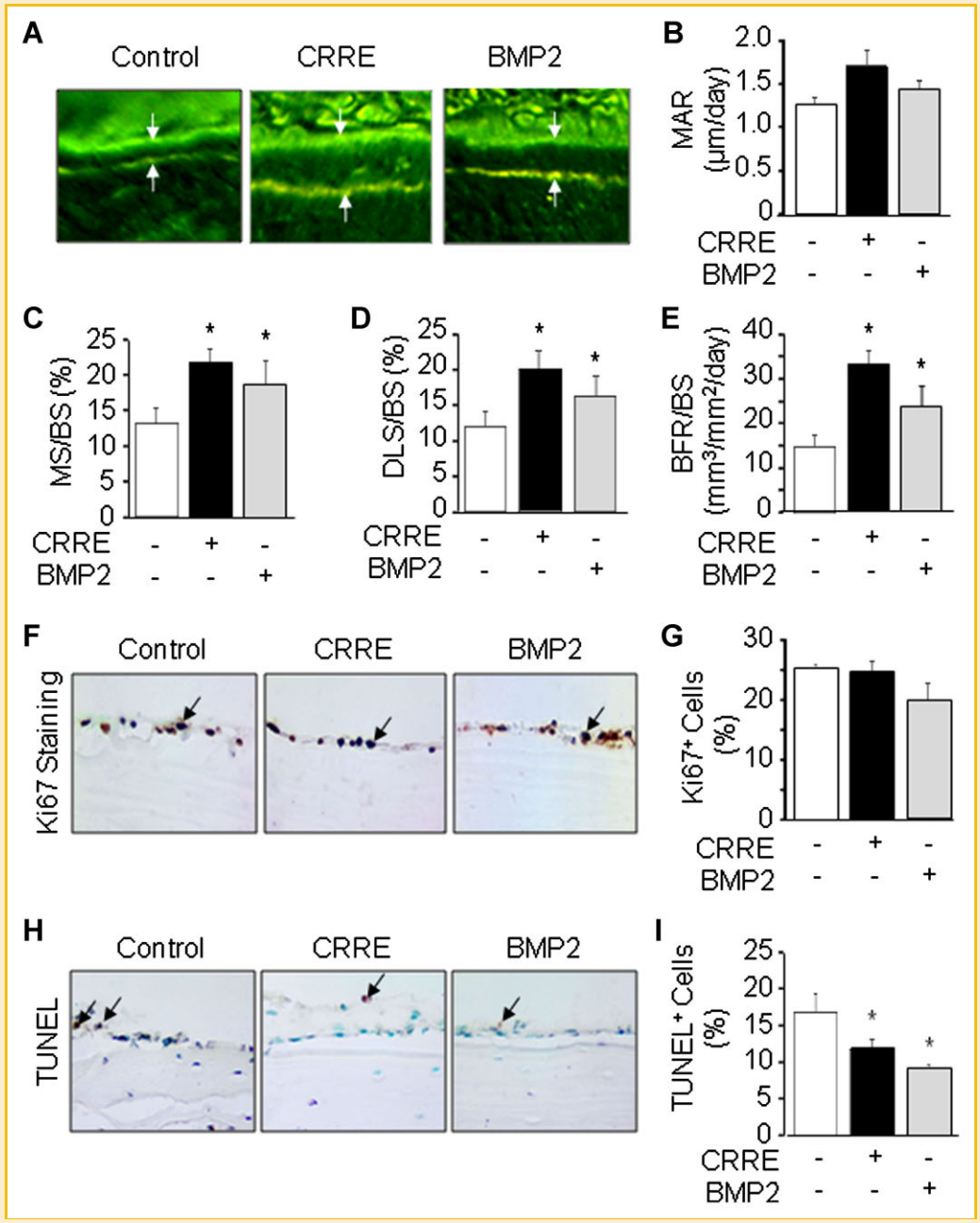

Fig. 4. Peptide-based activation of ITGA5 promotes bone formation in vivo. Subcutaneous injection of the cyclic peptide GA-CRRETAWAC-GA (CRRE; 25 mg/kg/day) near the mouse cranial bone increased bone formation compared to the control bone (PBS), as shown by the mean distance between calcein and tetracycline labeled surfaces (arrows) (A). Quantification of the mean appositional rate (MAR) (B), simple labeled surface (MS/BS) (C), double labeled surface (DLS/BS) (D), and bone formation rate (BFR) (E) induced by the cyclic peptide or BMP2 (10 μg/kg/day) compared to the control bone. In vivo, cell replication and cell death were evaluated by immunohistochemistry using Ki67 antibody and TUNEL assay, respectively (arrows: Ki67 or TUNEL positive cells) (F). Quantification of positive cells (G,H). Data represent mean values of 5–6 animals ± SD (*$P < 0.05$ vs. Control). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and new bone formation in vivo. We used a cyclic peptide that was shown to prime ITGA5 [Mould et al., 2000]. We found that this peptide activates ITGA5 in murine cells as it triggers FAK and ERK1/2-MAPKS pathways that are hallmarks of integrin-mediated signaling [Hynes, 2002]. We previously demonstrated that silencing FAK and ITGA5 using specific siRNA, or reducing ERK signaling actually abolishes the in vitro osteogenic activity of the cyclic peptide in human mesenchymal cells [Hamidouche et al., 2009], indicating that the observed effect is mediated by ITGA5 and its downstream FAK and ERK signaling. The present data thus indicate that the peptide acts as agonist of ITGA5 in the present context. In contrast, others have reported that this peptide may act as a potential antagonist of ITGA5 since it could block cell attachment to fibronectin in other cell types [Koivunen et al., 1994; Mould et al.,

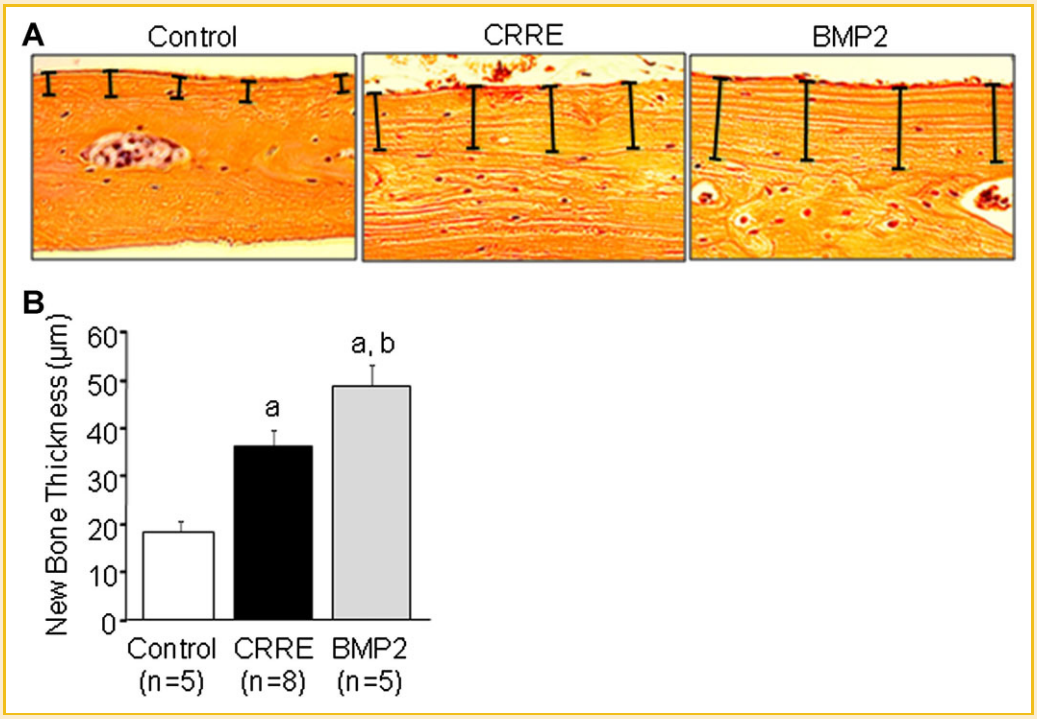

Fig. 5. Peptide-based activation of ITGA5 increases bone mass in vivo. The injection of the cyclic peptide GA-CRRETAWAC-GA (CRRE; 25 mg/kg/day) near mouse cranial bone increased de novo bone formation (A), as demonstrated by bone thickness (bars) and quantitative determination (B) compared to BMP2 (10 μg/kg/day) and control bone. Data represent mean values of 5–8 animals ± SD (a: $P < 0.05$ vs. PBS; b: $P < 0.05$ vs. CREE). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

2009]. Here we found that the signals triggered by the peptide led to promote Runx2 and COL1A1 gene expression without affecting cell adhesion, indicating that the peptide can activate osteoprogenitor cell commitment and osteoblast differentiation without significantly affecting cell attachment in our culture conditions. Furthermore, the positive effect of the peptide on gene expression was functional since it resulted in increased matrix mineralization in vitro, a hallmark of osteoblast function. This suggests that activating ITGA5 using this peptide may be a potent tool for promoting osteoblast function.

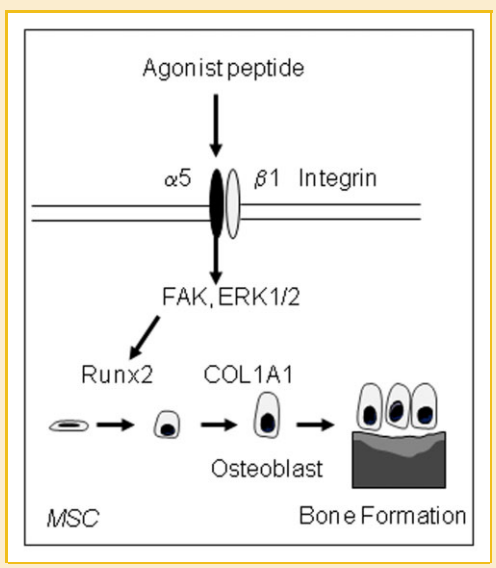

Fig. 6. Schematic representation showing that activation of ITGA5 by the cyclic peptide acting as agonist promoted FAK-ERK1/2 signaling, osteoblast commitment, function and survival, resulting in increased bone tissue formation in vivo.

Several cellular mechanisms may be involved in the positive effect of the peptide on osteoblastogenesis. The rise in Runx2, type I collagen expression, and matrix mineralization induced by the peptide indicates that it promoted osteoblast commitment and function in murine mesenchymal cells. Additionally, we found that the peptide at the dose that is efficient to trigger osteoblast gene expression and matrix mineralization also protected cells against apoptosis induced by serum deprivation, indicating that the peptide may promote osteoblastogenesis in part via increased cell survival. This is consistent with our previous finding that the expression of ITGA5 correlates with osteoblast survival in vitro [Kaabeche et al., 2005] and in a rat model of osteopenia [Dufour et al., 2008b]. Thus, our in vitro data indicate that the pharmacological activation of ITGA5 by the peptide increased matrix formation in vitro by acting on osteoblast commitment, function and survival.

One important issue was to determine whether the increased osteoblast differentiation and survival induced by the peptide-mediated ITGA5 activation in vitro may translate into efficient bone formation in vivo. To this goal, we used an established murine model of bone formation that allows measuring the activity of bone forming agents [Mundy et al., 1999]. Our histomorphometric study showed that activation of ITGA5 using our peptide-based strategy

increased bone formation, as revealed by the increased extent of simple and double-labeled surfaces and the increased BFR. In this model, the peptide-based activation of ITGA5 promoted bone formation, indicating the potential of this strategy to enhance de novo bone formation. Consistent with our in vitro findings, we found that activation of ITGA5 by the cyclic peptide had no effect on cell replication whereas it decreased cell apoptosis in vivo, indicating that the de novo bone formation resulted in part from increased osteoblast survival. Overall, our in vitro and in vivo data are consistent with a model in which activation of ITGA5 by the peptide promotes bone formation as a result of increased osteoprogenitor cell differentiation into osteoblasts and increased cell protection from apoptosis.

CONCLUSION

This study shows that pharmacological activation of ITGA5 in murine mesenchymal cells promotes osteoblastogenesis and de novo bone formation, which supports the concept of targeting ITGA5 for increasing bone matrix formation. We propose that strategies targeting ITGA5 activation could be therapeutically exploited to increase the osteogenic capacity of autologous osteoblast progenitor cells and to promote bone formation in conditions where intrinsic osteoblastogenesis is compromised.

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