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Profiling the Changes in Signaling Pathways in Ascorbic Acid/β-Glycerophosphate-Induced Osteoblastic Differentiation

Antonio Hernandes Chaves Neto,[1] Karla Cristiana Queiroz,[1,2] Renato Milani,[1] Edgar Julian Paredes-Gamero,[3] Giselle Zenker Justo,[1,3] Maikel P. Peppelenbosch,[2] and Carmen Veríssima Ferreira[1*]

[1] *Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Cidade Universitária Zeferino Vaz, Barão Geraldo, 13083-970 Campinas, SP, Brazil*

[2] *Department of Cell Biology, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, NL-9713 AV, Groningen, The Netherlands*

[3] *Departamento de Bioquímica, Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil*

ABSTRACT

Despite numerous reports on the ability of ascorbic acid and β-glycerophosphate (AA/β-GP) to induce osteoblast differentiation, little is known about the molecular mechanisms involved in this phenomenon. In this work, we used a peptide array containing specific consensus sequences (potential substrates) for protein kinases and traditional biochemical techniques to examine the signaling pathways modulated during AA/β-GP-induced osteoblast differentiation. The kinomic profile obtained after 7 days of treatment with AA/β-GP identified 18 kinase substrates with significantly enhanced or reduced phosphorylation. Peptide substrates for Akt, PI3K, PKC, BCR, ABL, PRKG1, PAK1, PAK2, ERK1, ERBB2, and SYK showed a considerable reduction in phosphorylation, whereas enhanced phosphorylation was observed in substrates for CHKB, CHKA, PKA, FAK, ATM, PKA, and VEGFR-1. These findings confirm the potential usefulness of peptide microarrays for identifying kinases known to be involved in bone development in vivo and in vitro and show that this technique can be used to investigate kinases whose function in osteoblastic differentiation is poorly understood. J. Cell. Biochem. 112: 71–77, 2011.

KEY WORDS: ASCORBIC ACID; β-GLYCEROPHOSPHATE; KINASES; OSTEOBLAST DIFFERENTIATION; SIGNALING

Mineralization is a fundamental step in osteoblast differentiation that involves the deposition of mineral particles in a collagen matrix. In MC3T3-E1 preosteoblasts (an osteoblastic cell line derived from normal mouse calvaria) differentiation is mediated by the expression of osteoblast-like differential markers and the formation of a mineralized extracellular matrix. Exposure to L-ascorbic acid (reduced vitamin C; AA) and β-glycerophosphate (β-GP) enhances this process by distinct mechanisms [Chung et al., 1992; Franceschi, 1992; Franceschi et al., 1994]. Chung et al. [1992] showed that β-GP favors mineralization by increasing the

Abbreviations used: ABL1, c-abl oncogene 1, receptor tyrosine kinase; Akt1, v-Akt murine thymoma viral oncogene homolog 1; ATF2, activating transcription factor 2; ATM, taxia-telangiectasia mutated; p38, p38 kinase; BCR, breakpoint cluster region; CHKA, choline kinase alpha; CHKB, choline kinase beta; ELK1, member of ETS oncogene family; ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian); ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; GSK3B, glycogen synthase kinase 3 beta; PAK1, p21-activated protein kinase 1; p90RSK, ribosomal protein S6 kinase, 90 kDa, polypeptide 2; p70 S6 Kinase, ribosomal protein S6 kinase; PIK3R1, phosphoinositide-3-kinase, regulatory subunit 1 (alpha); PKA, protein kinase, cAMP-dependent, catalytic, alpha; PAK2, p21-activated protein kinase 2; PKC, protein kinase C; PKD/PKCμ, protein kinase D1; PRKG1, protein kinase, cGMP-dependent, type I; SAPK/JNK, stress-activated protein kinase/Jun-amino-terminal kinase; Src, v-Src avian sarcoma (Schmidt-Ruppin a-2) viral oncogene; SYK, spleen tyrosine kinase; VEGFR-1, soluble vascular endothelial growth factor receptor-1.

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*Correspondence to: Prof. Carmen Veríssima Ferreira, PhD, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), C.P. 6109, CEP 13083-970, Campinas, SP, Brazil.
E-mail: carmenv@unicamp.br

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availability of phosphate ions, whereas AA has a role in reducing the iron prostethic group of hydroxylase enzymes responsible for collagen biosynthesis. Exposure to AA/β-GP stimulates procollagen hydroxylation, processing and fibril assembly followed by the dramatic induction of specific genes associated with the osteoblast phenotype, including alkaline phosphatase, osteopontin, osteocalcin, and the PTH/PTH-related protein receptor [Franceschi and Iyer, 1992; Quarles et al., 1992]. Although several reports have addressed various aspects of differentiation in MC3T3-E1 preosteoblasts, there is little information about the molecular mediators involved in the signal transduction that culminates in the osteoblast phenotype. Although multiple biological functions have been identified for AA/β-GP, the precise molecular mechanisms underlying these actions have not yet been fully elucidated. In this context, the involvement of activated protein kinases in distinct stages of cell differentiation is critical in determining the quality of newly formed bone.

Although the ability of AA/β-GP to stimulate the differentiation of preosteoblasts is very well-known, the molecular mechanisms involved still need to be elucidated. The activities of differentially expressed proteins have often remained undefined because of incomplete characterization of the relevant post-translational modifications that can modulate protein activity. Of these modifications, reversible phosphorylation catalyzed by kinases is probably the most important regulatory mechanism in eukaryotes. Protein kinases play a crucial role in a variety of cellular processes, including cytoskeletal reorganization, cell cycle progression, cell survival, and differentiation.

In view of this important role, in the present study we used peptide arrays (PepChip) containing 1,024 kinase-specific substrate motifs for a variety of kinases to obtain a comprehensive overview of cellular kinase activity during differentiation in osteoblasts cultured for 7 days in the presence of AA/β-GP. This approach, first described by Diks et al. [2004], allows the detection of signaling pathways in cell lysates. Our findings confirmed the usefulness of peptide microarrays for identifying kinases already known to play roles in bone development in vivo and in vitro after exposure to osteogenic inducers and revealed a complex biological network during differentiation in AA/β-GP-stimulated preosteoblast cells.

MATERIALS AND METHODS

MATERIALS

Ascorbic acid and β-GP were purchased from Sigma. MC3T3-E1 preosteoblast cells were from the American Type Culture Collection (ATCC, Rockville, MD). Antibodies against phospho-ATF-2 Thr^{71}, phospho-Akt Thr^{308}, phospho-Elk-1 Ser^{383}, phospho-GSK3β Ser^{9}, phospho-p38 Thr^{180}/Tyr^{182}, phospho-p42/44 (ERK1/2), phospho-p70 S6 kinase Thr^{389}, phospho-p90RSK Ser^{380}, phospho-Src Tyr^{416}, phospho-PAK2 Ser^{20}, phospho-(Tyr)-PI3K p85, phospho-PKC (pan) Ser^{660}, phospho-PKD/PKCμ $Ser^{744/748}$, phospho-SAPK/JNK Thr^{183}/Tyr^{185}, and phospho-Syk $(Tyr^{525/526})$, anti-rabbit, anti-goat, and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against PAKα and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-FAK Tyr^{397} and β-catenin were from Upstate Biotechnology, Inc. (Lake Placid, NY). Kinase array slides (PepChip™) were purchased from Pepscan Systems (The Netherlands). γ-^{33}P-ATP (adenosine triphosphate) was purchased from Amersham Biosciences.

CELL CULTURE

MC3T3-E1 preosteoblast cells were routinely grown in modified alpha minimum essential medium (α-MEM) without AA, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, in a humidified atmosphere at 37°C and at 5% CO_2. For all experiments, the cells were plated at an initial density of 50,000 cells/cm^2. All treatments were done after allowing 24 h for cell adherence (day 0). Differentiation was induced by adding osteogenic medium containing 50 μg of AA/ml and 10 mM β-GP and the medium was changed every 3 or 4 days. The profile of signaling proteins was analyzed by kinome microarrays and Western blotting on the 7th day of treatment. Cells incubated with medium alone were used as a control (Ctrl) for all experiments.

KINOME ARRAY ANALYSIS

A kinase peptide substrate array consisting of 1,024 peptides with specific phosphorylation sites was used to comprehensively evaluate the kinome during osteoblastic differentiation, as previously described [Diks et al., 2004; Löwenberg et al., 2005; Tuynman et al., 2008; Parikh et al., 2009]. After treatment with AA/β-GP for 7 days, the cells were washed in ice-cold phosphate-buffered saline (PBS) and harvested in lysis buffer (Pierce Biotechnology, Inc.) supplemented with protease and phosphatase inhibitors (Roche). Protein concentrations were determined by the Bradford dye-binding assay (Bio-Rad). The protocol of the kinome array is described in detail on the website (http://www.pepscanpresto.com/files/PepChip%20Kinase%20Lysate%20Protocol_v5.pdf). Stimulations were terminated by a wash with ice-cold PBS and the cells were lysed as described by Diks et al. [2004]. The kinomic analyses were done in triplicate using at least three independent cell cultures.

DATA ACQUISITION AND STATISTICAL ANALYSIS OF THE PEPCHIP™ ARRAY

After drying, the glass slides were exposed to a phospho-imager plate for 72 h. Acquisition of the peptide array was done using a phospho-imager scanner (Storm™, GE Life Sciences, Piscataway, NY). The level of incorporated radioactivity, which reflects the extent of phosphorylation, was quantified with specific array software (EisenLab ScanAlyze, version 2.50) as described elsewhere [Diks et al., 2004; Löwenberg et al., 2005; Tuynman et al., 2008]. Datasets from chips were then analyzed statistically using PepMatrix, as described by Milani et al. [2010]. Basically, spot replications were scrutinized for consistency using two indexes: one being the standard deviation:average (SD/A) ratio and the other being the ratio between the average and the median (A/M) of all three replications for each chip. Parameters applied to the indexes were an $SD/A < 20\%$ and $80\% < A/M < 120\%$. The fold change in phosphorylation between control and treated cells was assessed using Student's t-test, with $P < 0.05$ indicating significance.

WESTERN BLOT ANALYSIS

Following treatment of cells for 7 days, the medium was aspirated and the cells were washed with cold physiological solution. The cells were then incubated in 200 μl of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 20 mmol/L NaF, 1 mM Na_3VO_4, 0.25% sodium deoxycholate, and protease inhibitors – 1 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride) on ice for 30 min. Protein extracts were centrifuged and the protein concentrations of the supernatants were determined as described by Lowry et al. [1951], with bovine serum albumin as the standard. An equal volume of 2× sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to the samples that were subsequently boiled for 10 min. Aliquots of cell extracts (75 μg of protein) were separated by SDS–PAGE in 12% gels and transferred to PVDF membranes. The membranes were subsequently blocked in 1% fat-free dried milk or 1% bovine serum albumin in Tris-buffered saline–0.05% Tween 20 (TBS-T) and incubated overnight at 4°C with appropriate primary antibody diluted 1:1,000. After washing in TBS-T, the membranes were incubated for 1 h with anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase-conjugated secondary antibodies diluted 1:2,000 in blocking buffer (the same dilution for all Western blots). Immunoreactive bands were detected using enhanced chemiluminescence (ECL). Western blots were obtained for three experiments that were independent of those used in the kinome microarrays.

RESULTS

KINOME PROFILING OF AA/β-GP-INDUCED OSTEOBLASTIC DIFFERENTIATION

Peptide arrays with specific consensus sequences (potential substrates) for protein kinases were used to obtain a global analysis of cellular kinase activity in osteoblasts. The phosphorylation of peptide arrays by cell lysates showed that osteoblasts contain substantial kinase activity since almost all of the peptide substrates incorporated [γ-^{33}P]ATP. To our knowledge, the results presented below represent the first analysis of the kinomic profile of cultured preosteoblastic cells incubated with osteogenic inducers.

Since almost all cellular biochemical pathways are strictly controlled by the reversible phosphorylation of rate-limiting enzymes, we assumed that alterations in the level of phosphorylation following exposure to AA/β-GP would lead to changes in the activities of kinases necessary for development of the mature osteoblast phenotype. Analysis of the pattern of peptide phosphorylation in osteoblasts exposed to AA/β-GP for 7 days revealed substantial changes in kinase activity, with 18 kinase substrates showing a significant increase or decrease in phosphorylation (Table I). There was a significant decrease in PI3K-associated signal transduction, including reduced phosphorylation of the substrates for Akt and PKC. In agreement with this, there was a concomitant increase in the phosphorylation of a substrate for GSK3β. There was a significant reduction in the phosphorylation of substrates for BCR, ABL, PRKG1, PAK1, PAK2, ERK1, ERBB2, and SYK, but an increase in the phosphorylation of substrates for CHKB, CHKA, PKA, FAK, ATM, PKA, and VEGFR-1.

TABLE I. Peptide Substrates With Significantly Altered Phosphorylation in Lysates From Preosteoblastic Cells Treated With AA/β-GP for 7 Days

PEP_NR	Fold change	*P*-value	Sequence	PH site	Kinase
779	2.71	0.015	DSTNEYMDMKP	Y721	CHKB, CHKA
920	2.32	0.007	GKRHRYRVLSS	Y80	PKA
733	2.12	0.004	RKMKDTDSEEE	T79	FAK
394	2.00	0.008	GKKATQASQEY	S140	ATM
344	1.76	0.042	RRGDSYDLKDF	S440	PKA
891	1.71	0.045	SPPRSSLRRSS	S36	GSK3B
523	1.60	0.036	SDDVRYVNAFK	Y1213	VEGFR-1
676	0.82	0.030	LTRIPSAKKYK	S104	PKC
910	0.76	0.048	SSDDDYDDVDI	Y381	SYK
68	0.76	0.045	AEKPFYVNVEF	Y177	BCR
416	0.74	0.043	KVVALYDYMPM	Y222	ABL
805	0.67	0.023	YVEKFSYKSIT	S415	PKC
356	0.64	0.042	KKRKRSRWNQD	S20	PRKG1
304	0.62	0.007	RGQRDSSYYWE	S338	PAK1, PAK2
183	0.61	0.019	YGGLTSPGLSY	T431	ERK1
885	0.55	0.024	RDRSSSAPNVH	S363	AKT
185	0.53	0.003	GTRRGSPLLIG	S205	PKC
961	0.51	0.002	AENPEYLGLDV	Y1248	ERBB2
364	0.49	0.029	TEDQYSLVEDD	Y607	PIK3R1

The fold induction of each substrate, their corresponding protein kinases and the chip peptide number are provided.

QUALITATIVE VALIDATION OF THE KINOME PROFILE RESULTS

The peptide array results indicated that AA/β-GP decreased PI3K and Akt activity. To confirm this finding, the level of phosphorylation of downstream kinases in the PI3K signaling pathway was evaluated by Western blotting (Fig. 1A). In agreement with the peptide array analysis, there was significant reduction in the phosphorylation of PI3K, Akt, GSK3β, p70 S6 kinase, p90RSK, PKC Pan, and PKD/PKCμ. The increase in GSK3β activity, an event associated with decreased phosphorylation of Ser^9 by Akt, was confirmed by the reduction in the level of active (unphosphorylated) β-catenin, a GSK3β substrate involved in Wnt signaling.

Important regulatory kinases involved in other pathways, such as Syk, Src, and ERK 1/2, were down-regulated, as shown by their degree of phosphorylation (Fig. 1B). The reduction in PAK2 activity suggested by the peptide array was confirmed by the reduced autophosphorylation at Ser^{20}. The activities of PAK2 and Syk positively regulate the cellular response to stress by activating SAPK/JNK. The decrease in the phosphoryation of SAPK/JNK was reflected in the suppression of PAK2 and Syk (Fig. 1B,C). FAK and PKA were consistently up-regulated in the peptide array (Fig. 1D).

DISCUSSION

Gene array technologies have allowed researchers to define the transcriptome of MC3T3-E1 preosteoblastic cells treated with AA [Carinci et al., 2005] and AA plus β-GP [Beck et al., 2001]. However, many aspects of differentiation in this osteogenic lineage involve complex genetic and biochemical regulatory mechanisms [Harada and Rodan, 2003; Lian and Stein, 2003]. Consequently, experimental procedures that can provide information on the functional/activity status of a set of proteins involved in specific physiological processes are particularly valuable. As shown here, kinomic profiling of preosteoblastic cells cultured with AA/β-GP revealed

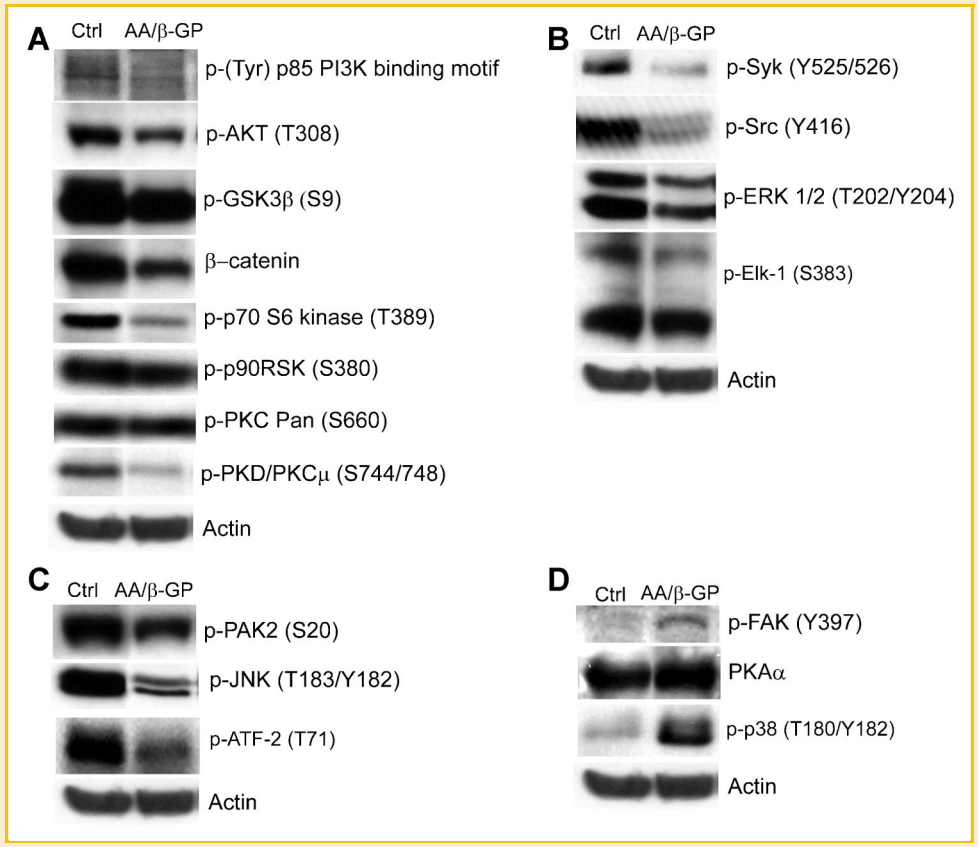

Fig. 1. Qualitative validation of the kinomic profile for kinases involved in AA/β-GP-induced osteoblastic differentiation. Western blot analysis confirmed the kinomic results and identified PI3K, AKT, GSK3β, PKC, Syk, PKA, FAK, PAK2 as targets for modulation by AA/β-GP. A: Down-regulation of the PI3K/AKT pathway with markedly reduced phosphorylation of the p70 S6 kinase, p90RSK, PKC Pan, and PKD/PKC2; enhanced GSK3β activity was seen as a decrease in active β-catenin. B: Down-regulation of the Syk and Src/ERK pathways. C: Reduced phosphorylation and activity of PAK2 and SAPK/JNK. D: Enhanced FAK and P38 activity and PKA expression. Protein phosphorylation and expression in cell lysates were assessed by Western blotting using phospho-specific monoclonal Abs. Actin expression was used as an internal (housekeeping) control.

the modulation of a broad range of kinases involved in a variety of signaling pathways. Kinases already known to play roles in osteogenesis in vitro and/or in vivo, such as PI3K, AKT, GSK3β, and ERK, were identified in this analysis, as were kinases such as CHK, SYK, PRKG1, ABL, and BCR that have well-known roles in the metabolic responses to many stimuli but whose function in osteoblastic differentiation is unclear.

As shown here, AA/β-GP modulated a variety of kinases that have already been shown to be involved in osteoblast differentiation in vitro and/or in vivo. We previously reported a decrease in Src activity in AA/β-GP-induced preosteoblast differentiation [Zambuzzi et al., 2008a], and this was confirmed here. Likewise, in a temporal analysis of the alterations caused by AA/β-GP during MC3T3-E1 differentiation [Zambuzzi et al., 2008b], we observed down-regulation of the ERK 1/2 and PI3K/Akt pathways followed by the activation of GSK3β. Previous reports have associated inhibition of the PI3K pathway with differentiation in B16 melanoma cells [Buscà et al., 1996], human promyelocytic HL60 leukemia cells [Peiretti et al., 2001], and the myogenic cell line C2C12 [Viñals et al., 2002], as well as in chondrocyte terminal differentiation [Kita et al., 2008]. On the other hand, PI3K/Akt/GSK3β signaling pathway is one of the key players in the signaling of potent anabolic factors in bone. For instance, growth factors such as PTH and IGF-1 can activate the PI3K pathway leading to the activation of Akt [Yamamoto et al., 2007; Nakasaki et al., 2008], whereas Wnt proteins prolong the survival of osteoblasts and uncommitted osteoblast progenitors via activation of the Src/ERK and PI3K/Akt signaling cascades [Almeida et al., 2005].

The Wnt/β-catenin signaling pathway is involved in osteogenesis [Krishnan et al., 2006]. This pathway is antagonized by GSK3β through the phosphorylation of β-catenin. GSK3β activation and inactivation are regulated by phosphorylation at Ser^9, a critical event in many receptor-coupled signaling processes, and also by selective intracellular compartmentalization. Although work in rats [Kulkarni et al., 2006] and MC3T3-E1 cells treated with glucocorticoids [Wang et al., 2009] has shown that the inhibition of GSK3β enhances osteoblast differentiation, our results shown that GSK3β was active in AA/β-GP-induced osteoblast differentiation. This was confirmed by immunoblots which showed a reduction in active β-catenin. The enhanced GSK3β activity may be related to the inactivation of PRKG1 which negatively regulates GSK3β activity by phosphorylating the latter protein at Ser^9 in vitro and in vivo [Zhao et al., 2005]. The discrepancies between our findings for GSK3β and those of other investigations may reflect the different

osteogenic inducers and/or cell cultures used to study osteoblast differentiation.

There was marked down-regulation of PAK2 and Syk kinases during AA/β-GP-induced osteoblast differentiation, although little is known about the biological functions of these kinases during osteogenesis. p21-activated protein kinase (PAK) was first identified as Rac/Cdc42 GTPase activated kinases [Manser et al., 1994] and can be activated in response to hyperosmolarity, irradiation, UV light, and DNA-damaging chemotherapeutic drugs such as cytosine β-D-arabinofuranoside and cisplatin. Syk is a non-receptor protein tyrosine kinase ubiquitously expressed by hematopoietic cells and also found in epithelial, endothelial [Kurosaki, 2000; Inatome et al., 2001] and osteoblastic [Rezzonico et al., 2002] cells. Syk has been suggested to play a critical role in cell morphogenesis, growth, migration, and survival [Inatome et al., 2001]. This kinase is a major upstream effector of the PI3K/Akt pathway and participates in PI3K activation, which in turn has a significant role in regulating cancer cell motility [Jiang et al., 2002; Mahabeleshwar and Kundu, 2003]. Recent studies suggest that PAK2 and Syk kinases act as upstream regulators of the JNK and p38 MAPK pathways [Zhang et al., 1995; Frost et al., 1996]. Miah et al. [2004] demonstrated that PAK2 and Syk cooperate positively to regulate cellular responses to stress by activating JNK under hyperosmotic conditions. Rezzonico et al. [2002] showed that Src could mediate the interaction of Syk with large conductance Ca^{2+}-dependent K^+ channels to promote alterations in bone remodeling induced by PGE_2 in osteosarcoma cells. Our findings suggest that the effects of AA/β-GP in down-regulating PI3K and JNK may be related to the decreased activity of Syk and PAK2. However, since kinase cascades are complex networks rather than linear pathways, it is possible that there are other interactions among the kinases mentioned above.

Exposure to AA/β-GP resulted in the activation of choline kinases (CHK) A and B that catalyze the phosphorylation of choline to phosphocholine during phosphatidylcholine biosynthesis. The accumulation of phosphocholine in cancer cells is required to provide a supply of phosphatidylcholine necessary for the feed-forward amplification of signaling pathways related to cancer survival [Iorio et al., 2010; Yalcin et al., 2010]. Likewise, the enhanced 1,2-diacylglycerol production from phosphatidylcholine hydrolysis in preosteoblast MC3T3-E1 cells is important for maintaining cellular proliferation [Sakai et al., 2004].

An elevation in intracellular cyclic adenosine monophosphate (cAMP) and the activation of PKA regulate osteogenesis in response to a variety of osteogenic inducers. We observed an increase in PKA expression and activity. This finding agreed with Wu et al. [2007] who reported that an increase in AA uptake and stimulation of preosteoblast MC3T3-E1 differentiation was associated with PKA-mediated phosphorylation of the sodium-dependent vitamin C transporter 2 (SVCT2). As with BMP2-induced osteoblast differentiation in pluripotent C2C12 cells, elevated cAMP levels and PKA activation may be associated with AA/β-GP-induced osteoblast differentiation and the suppression of ERK (p44/p42) phosphorylation in MC3T3-E1 cells [Ghayor et al., 2009].

Preosteoblast MC3T3-E1 cells secrete extracellular matrix, particularly type I collagen (Col-I), during AA/β-GP-induced osteoblast differentiaton [Franceschi and Iyer, 1992; Quarles et al., 1992], which facilitates differentiation via the activation of FAK by Col-I-α2β1 integrin interaction [Takeuchi et al., 1997]. This activity agrees with the up-regulation of FAK seen here and suggests a possible interaction between cell surface integrins and components of the extracellular matrix.

ATM is a serine/threonine kinase involved in the response to DNA damage and is therefore required for genome integrity and stem cell renewal. The up-regulation of ATM during osteoblast differentiation in response to AA/β-GP suggests a role for this kinase in cellular development and supports the findings of Rasheed et al. [2006], who showed that the reduced bone mass in $ATM^{-/-}$ mice probably reflected compromised regulation of the expression of osteoblast-specific transcription factor (osterix) and subsequent alterations in osteoblast differentiation.

The PKC family consists of serine–threonine kinases that play pivotal roles in the regulation of cell proliferation, differentiation, and survival [Newton, 1995]. The different patterns of PKC isoform expression suggest that these proteins play an important role in regulating osteoblastic differentiation [Lampasso et al., 2006]. Analysis of the peptide microarray indicated down-regulation of the PKC pathway during AA/β-GP-induced osteoblastic differentiation, a finding confirmed by Western blotting using phospho-PKC (pan) (βII Ser^{660}) and PKD/PKCμ ($Ser^{744/748}$) antibodies; the latter protein is a serine/threonine kinase that is activated via phosphorylation by different PKC isoforms [Johannes et al., 1994]. In contrast, PKC activation is important for cell replication [Caverzasio, 2008], apoptosis [Haÿ et al., 2001], actin cytoskeletal reorganization [Higuchi et al., 2009], and osteoblast differentiation [Cheung et al., 2006], although these effects were observed in distinctly different treatment regimes and cell cultures. Overall, our findings suggest that the exposure of preosteoblasts to AA/β-GP results in the suppression of proliferation and survival signaling pathways.

One interesting aspect of this study was the finding that ABL (a non-receptor protein tyrosine kinase) and BCR (a serine/threonine kinase) were down-regulated during AA/β-GP-induced osteoblast differentiation. Although BCR/ABL fusion proteins have often been found in patients with chronic myelogenous leukemia (CML), the function of normal BCR and ABL is unclear. Long-term therapy with imatinib mesylate, a rationally designed tyrosine kinase inhibitor (PDGFR, c-ABL) has been associated with enhanced bone formation in CML patients [Fitter et al., 2008; Vandyke et al., 2010], whereas dasatinib, a dual Src family kinase and Abl inhibitor, enhanced the differentiation of preosteoblast MC3T3-E1 cells [Lee et al., 2010]. Our data therefore reinforce the evidence that under normal conditions ABL and BCR may have distinct roles in cell signaling during osteoblast differentiation and bone development.

VEGFR-1 (also known as Flt-1) is expressed in osteoblasts during osteogenesis in vitro [Deckers et al., 2000; Street and Lenehan, 2009] and in vivo [Byun et al., 2007; Otomo et al., 2007]. The expression of this receptor is dependent on differentiation and coincides with the expression of its ligand [Deckers et al., 2000]. Our kinomic results support the notion that enhanced VEGFR-1 signaling plays a role in AA/β-GP-induced osteoblast differentiation and also reinforces the work of Street and Lenehan [2009], who described an important autocrine feedback mechanism central to osteoblast survival.

Epidermal growth factor (EGF) receptor tyrosine kinases (EGFR/ErbB1, ErbB2, ErB3, and ErB4) are cell surface receptors that regulate many important intracellular responses. Salvatori et al. [2009] showed that down-regulation of the expression of EGFR induced by estrogens and phytoestrogens led to the differentiation of U2OS human osteosarcoma cells, whereas ErbB2/ErbB signaling was required for cartilage and bone development by maintaining chondrocyte and osteoblast proliferation in vivo [Fisher et al., 2007]. As shown here, the incubation of osteoblasts with AA/β-GP led to a reduction in ErbB2 activity, in addition to suppressing EGFR-activated phospholipase Cγ-protein kinase C (PLC-PKC), PI3K/Akt, and ERK1/2 signaling pathways. However, additional studies of ErbB2/ErbB signaling in other stages of osteoblast development are required since the expression and activation of EGFR vary with the degree of cell proliferation, differentiation, and survival, and this may in turn influence the signaling responses to different stimuli.

CONCLUSION

To our knowledge, this is the first study to report a kinomic analysis of the intracellular signaling responses to AA/β-GP in osteoblasts. The results described here clearly demonstrate the potential usefulness of peptide microarrays for analyzing kinases known to play roles in bone development in vivo and in vitro and open the field for further investigations into the role of poorly understood kinases in osteoblastic differentiation.

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