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Phosphoproteome Reveals an Atlas of Protein Signaling Networks During Osteoblast Adhesion

Renato Milani,[1] Carmen V. Ferreira,[1] José M. Granjeiro,[2] Edgar J. Paredes-Gamero,[3] Rodrigo A. Silva,[1] Giselle Z. Justo,[3] Helena B. Nader,[3] Eduardo Galembeck,[1] Maikel P. Peppelenbosch,[4] Hiroshi Aoyama,[1] and Willian F. Zambuzzi[1*]

[1] *Department of Biochemistry, Institute of Biology, University of Campinas (UNICAMP), 13083-970 Campinas, São Paulo, Brazil*

[2] *Department of Cellular and Molecular Biology, Institute of Biology, Federal Fluminense University (UFF), 24050-120 Niterói, Rio de Janeiro, Brazil*

[3] *Department of Biophysics, Federal University of Sao Paulo, 04044-020 São Paulo, Brazil*

[4] *Kinome Profiling Unit, Department of Cell Biology, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, the Netherlands*

ABSTRACT

Cell adhesion on surfaces is a fundamental process in the emerging biomaterials field and developmental events as well. However, the mechanisms regulating this biological process in osteoblasts are not fully understood. Reversible phosphorylation catalyzed by kinases is probably the most important regulatory mechanism in eukaryotes. Therefore, the goal of this study is to assess osteoblast adhesion through a molecular prism under a peptide array technology, revealing essential signaling proteins governing adhesion-related events. First, we showed that there are main morphological changes on osteoblast shape during adhesion up to 3 h. Second, besides classical proteins activated upon integrin activation, our results showed a novel network involving signaling proteins such as Rap1A, PKA, PKC, and GSK3β during osteoblast adhesion on polystyrene. Third, these proteins were grouped in different signaling cascades including focal adhesion establishment, cytoskeleton rearrangement, and cell-cycle arrest. We have thus provided evidence that a global phosphorylation screening is able to yield a systems-oriented look at osteoblast adhesion, providing new insights for understanding of bone formation and improvement of cell–substratum interactions. Altogether, these statements are necessary means for further intervention and development of new approaches for the progress of tissue engineering. J. Cell. Biochem. 109: 957–966, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: OSTEOBLAST; CELL ADHESION; SIGNAL TRANSDUCTION; SURFACES; KINOME; PKC; RAP1; BIOENGINEERING

Improved hard-tissue repair, augmentation, or replacement has become a very significant challenge for orthopedic biomaterials and surgery. Adhesion and proliferation are fundamental cellular processes involved in broad biological events, such as tissue maintenance and wound healing [Altankov et al., 1996]. In this sense, cell adhesion of anchorage-dependent cells are among the first phases of cell–material interactions [Grinnell, 1978] and profoundly influence integration with host tissue and eventual success or failure of implanted biomaterials [Altankov et al., 1996]. For these reasons, as well as a compelling need to understand the molecular mechanisms under osteoblast adhesion, determining the mechanisms behind cell adhesion has been a focus of research for nearly 50 years.

We have focused on understanding osteoblast adhesion at the molecular level in order to better comprehend this biological event [Zambuzzi et al., 2009a]. Although many specific points in this field have been reached, global molecular events guiding osteoblast adhesion remain poorly understood.

Although methods have been developed to measure cell responses during deformation, cell adhesion, locomotion, and mitosis [Capulli et al., 2009], reliable experimental tools are currently unavailable for quantifying distribution and modulation of signaling protein

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*Correspondence to: Dr. Willian F. Zambuzzi, PhD, Division of Cell Signaling and Bioassays, University of Campinas, Cidade Universitária "Zeferino Vaz," Campinas, SP 13083-970, Brazil. E-mail: wzamba@unicamp.br

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networks amongst various subcellular structures. Additionally, proteins are complex entities, performing a diverse array of tasks in living systems, including signal transduction, metabolic and catalytic functions, and also mechanical support.

More recently, proteomic and genomic fields have been offering new insights into changes in the regulation of the cell machinery. Altogether, these tools offer a range of methodologies able to guide the study of events occurring from gene activation to protein synthesis, but currently not of protein activity. Thus, a more comprehensive approach is needed in order to reveal signaling pathways active in nucleate cells. Towards this end, kinome analysis techniques using peptide arrays have begun to be applied with substantial success in a variety of organisms from all branches of eukaryotic life [Diks et al., 2007], generating descriptions of cellular signaling without a priori assumptions as to possible pathways related to a particular process. From our point of view, this information will revolutionize our understanding of cell adhesion at the intra-cellular level.

In this context, a peptide microarray able to identify multiple kinase activity was developed, allowing the emergence of functional kinome studies [Parikh et al., 2009]. In turn, kinases are pivotal regulators of cellular physiology [Manning et al., 2002], whose protein products exert their action via phosphorylation of specific substrates by recognition of specific sites.

Among post-translational modifications, reversible protein phosphorylation is capable of mediating most of signal transduction processes in living cells. Since these processes are of the utmost importance to adequate cell development and differentiation, intercellular communication, homeostasis and apoptosis, they are usually under tight and transient regulation, whereas abnormal phosphorylation events lead to unwanted substrate activity and have a causal role in many diseases, such as cancer and neurological disorders.

In this work we investigated the kinome profiling (also called phosphoproteome) of osteoblast adhesion by using a peptide array (PepChip®). This approach brings out a wide range of information not only about signaling proteins but about their interacting partners as well, providing means to improve our understanding about osteoblast adhesion under signaling outcomes and allowing a knowledge leap in the biomaterials field.

MATERIALS AND METHODS

Antibodies and chips: phospho-PKA C (Thr197) antibody #4781, GSK-3β (27C10) rabbit mAb #9315, phospho-GSK-3α/β (Ser21/9) antibody #9331, FAK antibody #3285, phospho-FAK (Tyr576/577) antibody #3281, phospho-FAK (Tyr397) antibody #3283, Src (36D10) rabbit mAb #2109, phospho-Src family (Tyr416) antibody #2101, pan-actin antibody #4968, PKA C-α antibody #4782, Cofilin antibody #3312, phospho-Cofilin (Ser3) antibody #3311, anti-mouse, anti-rabbit and anti-goat IgGs antibodies were purchased from Cell Signaling Technology (Boston, MA). PepChip® Kinomics™ slides were obtained from Pepscan Presto BV (Lelystad, the Netherlands).

CELL LINE AND CULTURE CONDITIONS

MC3T3-E1 cells (sub-clone 4), a mouse pre-osteoblast cell line, were obtained from American Type Culture Collection (ATCC, Rockville, MD). These cells were grown at 37°C in α-MEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin under a humidified 5% CO_2 atmosphere. In order to check the capacity of mice primary osteogenic cells (mpOC) in acquiring mature osteoblast phenotype we confirmed alkaline phosphatase activity (data not shown) under osteogenic condition as previously described by Zambuzzi et al. [2009b].

MICE AND PERIOSTEAL-DERIVED CELL CULTURE

Mice used in this work were kindly donated by Dra. Camila P. Buzalaf, Bauru Dental School, University of Sao Paulo and all procedures were under the principles of the Brazilian College of Animal Research (COBEA). Briefly, periosteal explants were harvested from skull of *Swiss* female mice (*mus musculus*). Periosteum tissue was stripped off, intensively washed (milli-Q plus antibiotics) and cut into smaller pieces. Several small pieces were placed in a 100-mm culture dish and cultured in alpha-MEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 95% humidified air and 5% CO_2. Reaching semiconfluence after 2 weeks, mpOC were trypsinized for 5 min, centrifuged for 5 min at low rotation, and expanded. After passage 2, periosteal cells were seeded in 100-mm culture dishes and the sample collected for providing Western blotting experimentation.

EXPERIMENTAL DESIGN

In order to assess the kinome profiling of pre-osteoblast adhesion on polystyrene surfaces, MC3T3-E1 cells were seeded on polystyrene surfaces and the samples collected after 2 h and 10 days later. To verify initial cell morphology, osteoblasts were seeded and the confocal images were captured at 30, 60, 120, and 180 min.

IMMUNOBLOTTING ASSAY

MC3T3-E1 pre-osteoblast cells were cultured and protein extracts were obtained using lysis cocktail (50 mM Tris [tris(hydroxymethyl)aminomethane]–HCl [pH 7.4], 1% Tween-20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM *O*-Vanadate, 1 mM NaF, and protease inhibitors [1 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl)-benzolsulfonyl-fluorid-hydrochloride]) for 2 h on ice, as used previously [de Souza Queiroz et al., 2007; de Fátima et al., 2008]. After clearing by centrifugation, protein concentration was determined using the Lowry method [Hartree, 1972]. An equal volume of 2× sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris–HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to samples and boiled for 5 min. Protein extracts were resolved by SDS–PAGE (10% or 12%) and transferred to PVDF membranes (Millipore). Membranes were blocked with either 1% fat-free dried milk or bovine serum albumin (2.5%) in Tris-buffered saline (TBS)–Tween-20 (0.05%) and incubated overnight at 4°C with appropriate primary antibody at 1:1,000 dilutions. After washing in TBS–Tween-20

(0.05%), membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse IgGs antibodies, at 1:2,000 dilutions (in all immunoblotting assays), in blocking buffer for 1 h. Detection was performed by using enhanced chemiluminescence.

CONFOCAL MICROSCOPY

3×10^4 cells were seeded at different times on coverslips under conditions previously described [Zambuzzi et al., 2009a]. Subsequently, the cells were fixed with 2% formaldehyde for 30 min, washed with 0.1 M glycine, permeabilized with 0.01% saponin for 15 min, and washed with PBS, three times. The cells were incubated for 40 min with 4 mg/ml, alexa fluor 488-labeled phalloidin (Invitrogen/Molecular Probes, USA). Nuclei were stained with DAPI (20 mg/ml, Sigma, USA) for 20 min. Coverslips were mounted on glass microscope slides using an mounting medium (fluoromount-G). Cells were viewed on a confocal laser scanning microscope (Zeiss, LSM 510 META) using a 40× oil-immersion lens.

PHOSPHOPROTEOME (PEPTIDE ARRAY)

Kinome array analysis was done as described by Diks et al. [2004] and Lowenberg et al. [2005]. Furthermore, the protocol of the kinome array is described in detail on the Web site (http://www.pepscan.nl/pdf/Manual%20PepChip%20Kinase%200203.pdf). In short, cells were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan Presto BV), containing up to 1,024 different kinase substrates (in triplicate), were incubated with cell lysates for 2 h in a humidified stove at 37°C plus ^{33}P-γ-ATP. Subsequently, the arrays were washed in 2 M NaCl, 1% Triton-X-100, PBS, 0.1% Tween, and H_2O, where after arrays were exposed to a phospho-imaging screen for 72 h and scanned on a phospho-imager (Fuji Storm 860, Stanford, GE). The density of the spots was measured and analyzed with array software.

PEPCHIP DATA ANALYSIS

Datasets from chips were generated by EisenLab ScanAlyze, version 2.50, array software, and then submitted to a statistical spot reliability approach called PepMatrix (article does not published). Basically, spot replications were scrutinized for consistency using two indexes: one of them is the ratio standard deviation/average (SD/A) and the other is the ratio between the average and the median (A/M) of all three replications for each chip. Parameters applied to indexes are $SD/A < 20\%$ and $80\% < A/M < 120\%$. Standard statistical analysis follows with calculated fold changes and *t*-tests for assessing significantly different averages between chips.

RESULTS

MORPHOLOGICAL CHANGES UP TO 3 H OF ATTACHMENT

We observed that osteoblast morphology is mainly changed up to 3 h of adhesion (Fig. 1), recruiting mechanics of subcellular structures ranging from stress fibers and the filopodium (specially at 2 h after seeding) to activation of focal adhesion complexes (FACs), formed mainly by integrin, FAK, talin, vinculin, α-actinin, paxillin, and tensin.

Many kinases are known to govern the cell adhesion process by integrating molecular complexes close to the plasma membrane, such as integrins, FAK, and Src. Therefore, their activity status is a good indicator of whether and how this pathway is being regulated. Within the first 2 h of interaction with a polystyrene surface, there were already significant morphological modifications in the cell structure as seen in Figure 1. Using a peptide array, we looked at several kinase activities at 2 h of cell adhesion as well, and it was compared with those ones at 10 days from seeding.

After checking cellular changes at morphological aspect, we decided to verify the global kinome involved in this condition. For further analysis, our results were grouped and disposed as follows.

GLOBAL KINASE ACTIVITY DURING OSTEOBLAST ADHESION

To investigate thoroughly the signaling events associated with the pre-osteoblast adhesion process, we regarded the chip as a whole. Subsequent analysis of the kinome profiles revealed significant differential activity during osteoblast adhesion ($P < 0.05$; Supplementary Data, Table S1).

The dot plot depicted in Figure 2A shows the median intensity of all 1,024 spots for 2 h-adhering cells versus control conditions (MC3T3-E 1 cells cultured for 10 days, for details see, Experimental Design Section), normalized from 0 to 100 tailed by 5%. Kinases shown have survived the replication consistency approach. Overall, we found more intensely active kinases during the adhesion phase. We disregard the possibility of specific phosphorylations in spots above the normalization tail and under the 100-horizontal line because in control conditions the 100 cutoffs were not crossed.

Figure 3A brings an individual activity for each kinase based on their respective spot intensity. Thus, Src, PKA, FAK, and GSK3β present strong evidence of specific activation during adhesion.

In order to validate those results from PepChip®, we investigated some proteins by performing Western blotting as previously proposed by Diks et al. [2007]. In fact, our results from PepChip® were consistently confirmed, as seen in Figure 3B.

SRC, PKC, AND FAK ARE HOTSPOTS OF FOCAL ADHESION FORMATION AND ACTIN CYTOSKELETON REARRANGEMENT

It is well accepted that FACs relate to the cytoskeleton coupling actin remodeling to cell surface contact signaling mediated by integrins. Our results from kinomic analysis obtained from osteoblast adhesion showed differential activity of Src, PKC, PAK1, FAK, and GSK3β, all up-regulated by twofold or more (Fig. 2B) when compared to a control group (those cells at 10 days). As it has been reported previously, FAK and Src are pivotal regulators of cell adhesion upon integrin activation. As a novelty for osteobalsts, PKC can interact with Src and FAK, promoting a downstream cascade affecting activation of Rho, ROCK, paxillin, and talin (schematized in Fig. 6). These signaling proteins are able to guide actin rearrangement by lastly modulating cofilin activity. In turn, cofilin is responsible for mediating actin severing and thereby defining the average length of actin filaments, subsequently rearranging the cytoskeleton [Yang et al., 1998]. These speculations were assayed by determining cofilin activation status (phosphorylation at Serine 03). In fact, our results showed that cofilin was active (Fig. 4) at 2 h as proposed. Of note, the specific antibody used for verifying cofilin phosphorylation

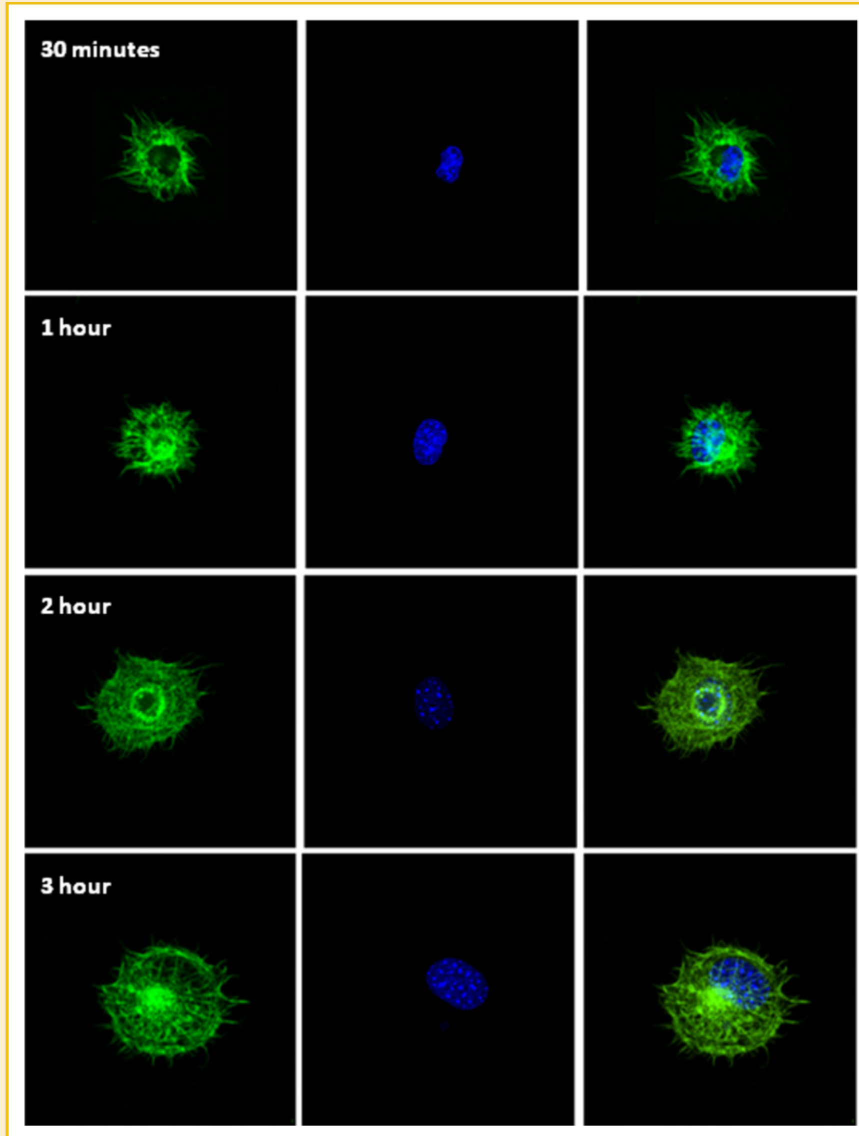

Fig. 1. Cytoskeleton dynamics in osteoblasts, as illustrated by changes in the actin filament network during cell spreading and the rearrangement of fibers. When a rounded osteoblast cell was placed on a polystyrene surface, its spreading was accompanied by marked changes in actin filaments (phalloidin, green) as recorded after 30 min, 1, 2, 3 h. The nucleus was stained with DAPI (blue). Images were obtained by confocal microscopy.

recognizes its phosphorylation at serine-03, which promotes cofilin inhibition [Arber et al., 1998].

In order to extend these results for others kind of cells we decided to explore the presence of Src (Y416) and PKA (Thr197) during mpOC adhesion by performing Western blotting as tool (Supplementary Data, Fig. S2).

OSTEOBLAST ADHESION REQUIRES SURVIVAL SIGNALING AND INHIBITION OF CELL PROLIFERATION AS WELL

Using peptide arrays, we found that GSK3β had increased activity phosphorylating c-myc in a motif containing Thr-58 (Fig. 3A, Supplementary Data, Table S1), leading to its down-regulation and subsequent inhibition of cell proliferation.

Interestingly, a Ras-related protein 1A (Rap1A) motif pseudo-substrate was found to be phosphorylated more than eightfold during this early phase of pre-osteoblast adhesion by PKA (Supplementary Data, Table S1). Lastly, we verified that PAK1 showed an almost twofold increase in phosphorylation (Fig. 2B) during osteoblast adhesion. In turn, PAK plays a dual role: while important to cell adhesion through promotion of focal adhesion turnover, it can also stimulate cell survival by the same pathway described for Rap1A.

SERINE-BASED MOTIFS ARE MARKEDLY INCREASED IN NUMBER IN RELIABLE SIGNIFICANTLY DIFFERENT SPOTS VERSUS THE WHOLE CHIP

Figure 5 shows that for reliable replicate spots showing differential phosphorylation the profile of serine-based motifs is notably higher than this same measure regarding the whole chip. Apparently, this is a reflex of several AGC group kinases (i.e., PKC and PKA) appearing

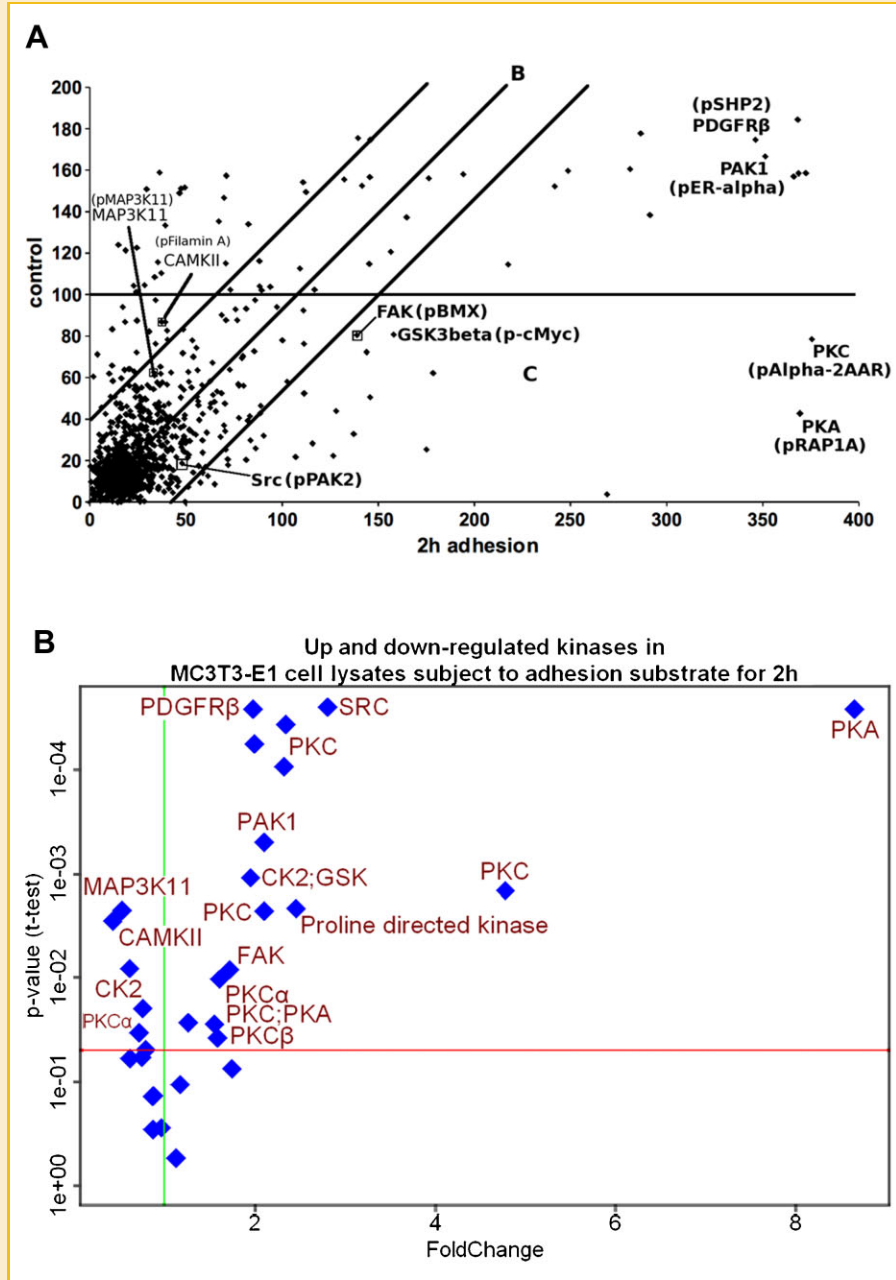

Fig. 2. A: Global kinome analysis of pre-osteoblast adhesion for 2 h in MC3T3-E1 cells. This dot plot ranks each spot representing the phosphorylation status of a 11-residue motif specific for a kinase. Spots can present highly decreased (A) or highly increased (C) phosphorylation statuses during the adhesion process versus a control condition. B-ranked spots show a less pronounced variation. C-ranked spots below the 100-horizontal line are the most phosphorylated spots during the adhesion phase while probably ruling out specific phosphorylation, since control intensity values kept below the normalization threshold. B: Up- and down-regulated kinases in MC3T3-E1 cell lysates subject to adhesion substrate for 2 h. Fold changes for PAK1, Src, PKC, PKA, and FAK stand above twofold.

with multiple pseudo-substrates exhibiting differential activities during adhesion, indicating a fundamental role of this kinase family in the aforementioned biological process. Symbols Y, S, and T (showed in Fig. 5) mean residues of tyrosine, serine, and threonine, respectively.

In order to summarize our results, Figure 6 brings a schematization of signaling network found under osteoblast adhesion by the peptide array.

DISCUSSION

The understanding of osteoblast adhesion represents a valuable stride for comprehension of bone development and improvement of biomedical materials performance. Understanding and improving cell adhesion on implanting materials to extend lifespan and self-regeneration properties are therefore substantially important for their osteointegration into host tissues. In this way, to fathom

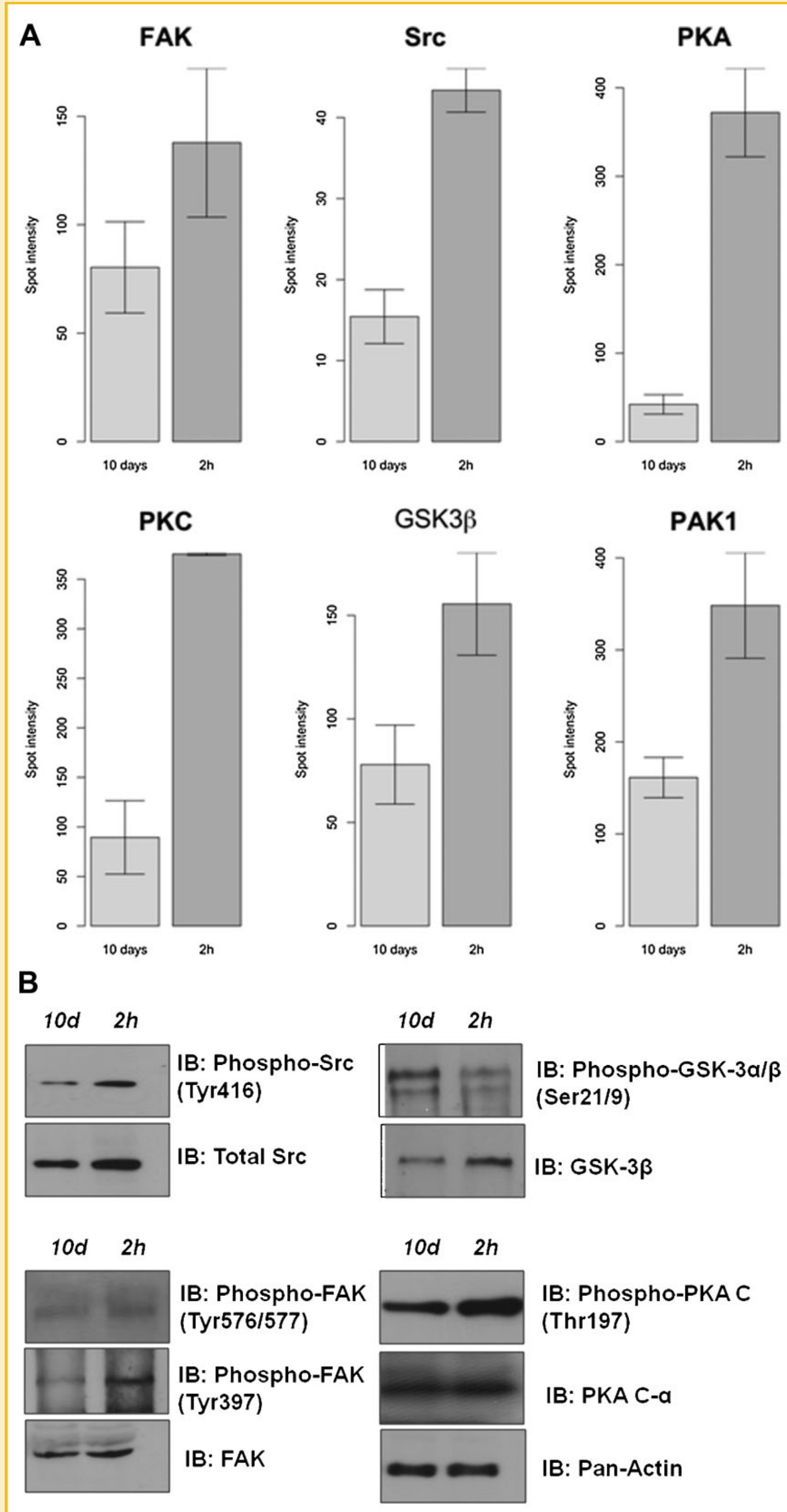

Fig. 3. A: Profile of kinase activities from peptide array spots. These graphs bring out activity intensities of Src, FAK, PKA, PKC, GSK3, and PAK. B: Kinase activity was valided by specific antibodies. In order to validate PepChip® data we used specific antibodies which are able to recognize specific sites of phosphorylation. All immunoblotting assays confirmed phosphorylation events observed on the chip.

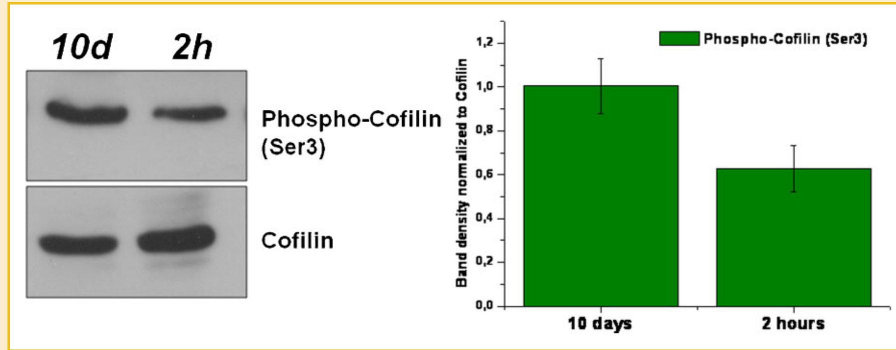

Fig. 4. Cofilin is activated at 2 h of osteoblast adhesion. In order to check cofilin involvement during osteoblast adhesion, we tested Serine 03 phosphorylation profile by using specific antibody. The graph brings a band densitometries normalized to total cofilin. This figure supports that there is a significant activation of cofilin ($^{*}P < 0.01$) at 2 h in osteoblasts.

osteoblast adhesion is a pivotal condition to look for ideal material surfaces [Bertazzo et al., 2009]. Thus, the proposed mechanism in this work could be exploited, under molecular aspects, to engineer materials able to control "smart molecules" binding specifically to elicit desired cellular activities. In this report, we proposed to investigate osteoblast adhesion on polystyrene because it is considered as gold standard to favor eukaryotic cell adherence and maintenance of their native characteristics in vitro.

Our results suggested that GSK3β is a central modulator during osteoblast adhesion. GSK-3 is a ubiquitously expressed serine/threonine protein kinase that phosphorylates and inactivates glycogen synthase. GSK-3 is a critical downstream element of the PI3 kinase/Akt cell survival pathway whose activity can be inhibited by Akt-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β [Welsh et al. 1996; Srivastava and Pandey, 1998]. Among their roles on the cell metabolism, we highlight that GSK3β is able to control cell proliferation by modulating both β-catenin and c-myc activations. Through peptide arrays, we showed that GSK3β phosphorylates c-myc. Also based on high level of GSK3β activity, we suggest its involvement in other signaling pathways, such as in

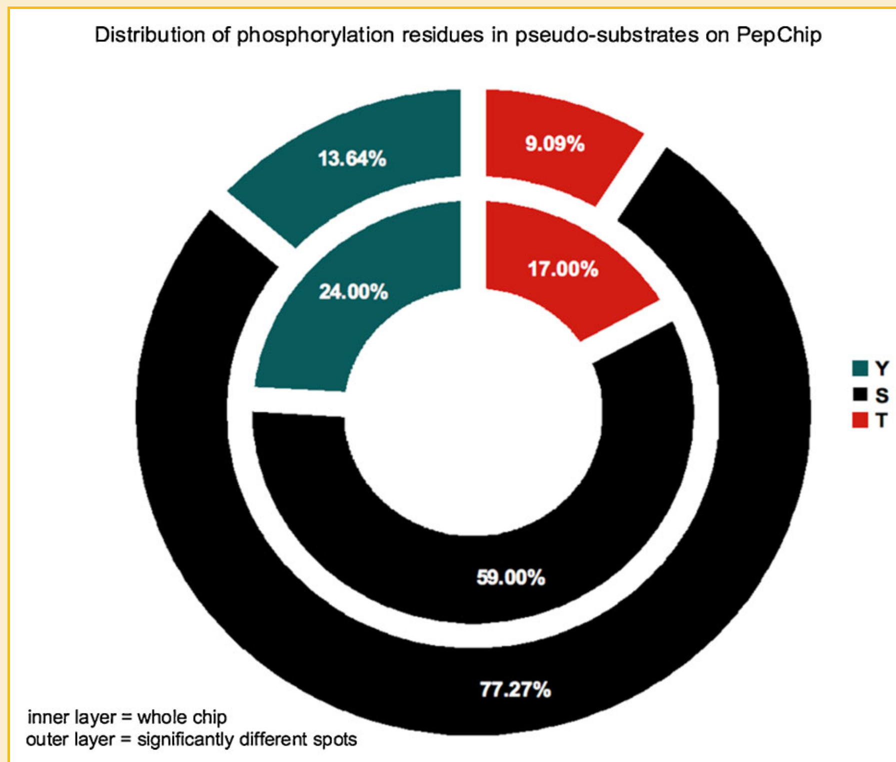

Fig. 5. Phosphorylated residues are unevenly distributed in reliable replication spots. A significant increase in number can be seen in serine-phosphorylated motifs when considering only substrates with significantly different phosphorylation statuses against the motif distribution seen in the whole chip. Note: Y, S, and T mean residues of tyrosine, serine, and threonine, respectively.

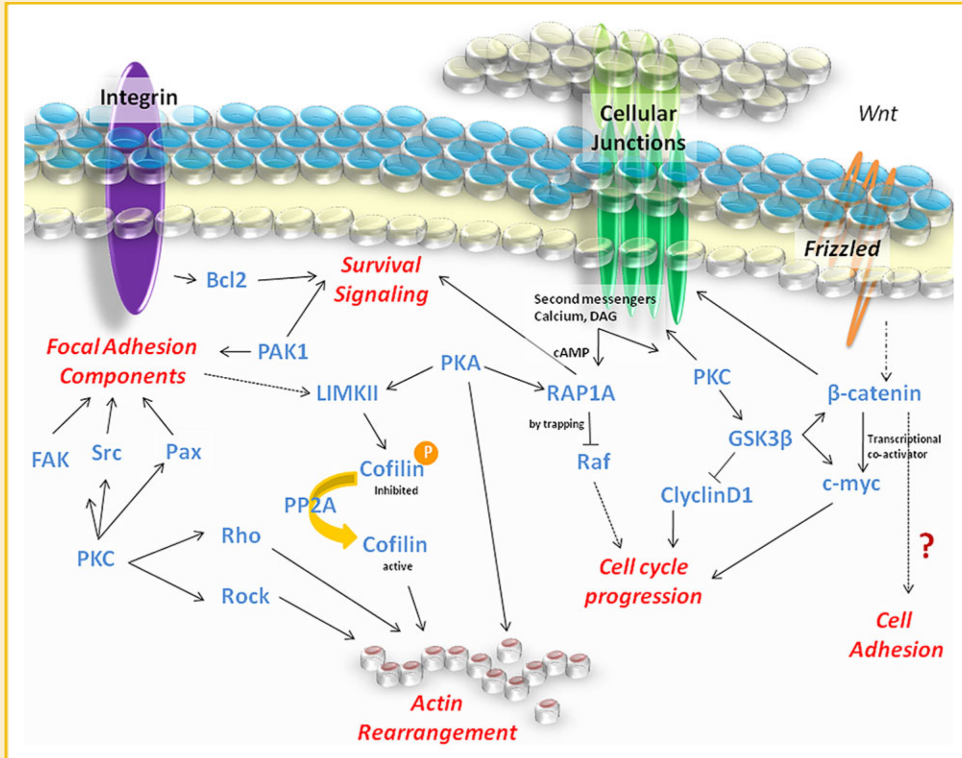

Fig. 6. Schematic representation. This schematization summarizes all events obtained from PepChip during osteoblast adhesion. Specifically, this figure brings all destinations of kinases cascades involved, such as: survival signaling, focal adhesion, cell cycle progression, actin rearrangement.

regulating cyclin D1 proteolysis and subcellular localization [Diehl et al., 1998]. Cook et al. [1996] described that the Wingless protein from *Drosophila* could inactivate the GSK3β kinase in murine fibroblasts. In addition, these authors suggested the involvement of an upstream TPA (12-*O*-tetradecanoylphorbol-13-acetate)-sensitive PKC isoform in this process. Inactivation of GSK3β should lead to stabilization of β-catenin and maybe increased cell adhesion besides signaling to the nucleus [Miller and Moon, 1996].

In addition, Nemeth et al. [2009] attempted to determine the effects of deletion of β-catenin specifically within osteoblasts. They observed that a β-catenin-deficient bone marrow microenvironment maintained hematopoietic stem cells but exhibited a decreased capacity to support primitive hematopoietic cells. These results were correlated with decreased numbers of osteoblasts. Based on these statements, we suggest an essential role of β-catenin during osteoblast adhesion and maintenance. A detailed analysis of our data is complicated by the transient nature of PKC activation.

In addition to cell adhesion, PKC activity seems to be involved on activation of junctional complexes. Down-regulation of PKC by sustained TPA treatment is well established and should be considered when comparing our results with the often contradictory literature on PKC activation in epithelial cells: Disruptions [Ben-Ze'ev, 1986; Dong et al., 1993; Fabre and de Herreros, 1993; Lampe, 1994; Nilsson and Ericson, 1995] as well as assembly of various junctional complexes in epithelial cells [Sheu et al., 1989; Winkel et al., 1990; Balda et al., 1993; Williams et al., 1993; Denisenko et al., 1994; Lewis et al., 1994, 1995; Stuart and Nigam, 1995] have been described to result from PKC activation. In addition, down-regulation of PKC has been forwarded as a fair explanation for transient effects on cell junction functionality [Sheu et al., 1989; Winkel et al., 1990; Lewis et al., 1995]. van Hengel et al. [1997] showed that the underlying mechanism comprises up-regulation of desmosomes and tight junctions by activation of the PKC signaling pathway, whereas E-cadherin remains essential for basic cell–cell adhesion, even in the absence of α-catenin.

For osteoblasts, despite this complexity, it might be clear from our findings that remarkably genuine PKC activation is able to control both cell adhesion and the beginning of proliferative events.

In this way, PKA activity also was mainly increased at the beginning of osteoblast adhesion. Mechanistically, PKA is composed of two catalytic subunits and a homodimer of two regulatory subunits that can dissociate upon activation by cAMP. It has been shown that PKA is involved with modulation of cytoskeleton remodeling under several external responses [Howe, 2004]. We have reported previously that cytoskeleton rearrangement is needed for osteoblast adhesion up to 6 h from seeding [Zambuzzi et al., 2009a].

Other molecule that deserves attention during osteoblast adhesion is ras-related protein 1A (Rap1A). Rap1A was found to be phosphorylated more than eightfold during this early phase of pre-osteoblast adhesion, pointing out its importance in this process. In agreement to Rap1A phosphorylation increase we reported an increase of PKA activity. From these data we can strongly suggest

that PKA, besides controlling cytoskeleton rearrangement, is also able to phosphorylate Rap1A, establishing a possible route for maintaining cell viability through MAPK [Pizon and Baldacci, 2000] and BCL-2 signaling upon integrin activation. Also in this sense, Nadella et al. [2009] reported that PKA is crucial in maintaining cell morphology and controlling cell migration through its ability to modulate directly the activity of LIM kinase. In fact, LIMK is able to phosphorylate cofilin at serine 03, modulating cofilin activity. In relation to cytoskeleton maintenance these statements are interesting because cofilin is responsible for controlling actin assembly and it seems to be affected by PP2A activity in osteoblasts [Zambuzzi et al., 2009a].

Although Rap1 protein function remains unclear, several observations indicate that Rap1 is involved in signal transduction [Bos, 1998]. Activated (GTP-bound) Rap1 inhibits Ras-dependent LPA (1-oleoyl-lyso-phosphatidic acid) activation of ERK [Cook et al., 1993] and at least three second messengers [namely calcium, diacylglycerol (DAG), and cAMP] can activate Rap1 in different cell types [Altschuler et al., 1995; Franke et al., 1997; Dremier et al., 2000]. In addition, a growing body of evidence supports the idea that Rap1 could interfere with MAP kinase pathways by trapping Raf protein [Boussiotis et al., 1997; Vossler et al., 1997; Okada et al., 1998].

As mentioned above, both calcium and DAG are second messengers responsible for activating Rap1. Importantly, calcium and DAG are required also for activation of PKC activity. Based on second messenger-dependent activation and activities of PKC and Rap1A (schematized in Fig. 6), we can predict that both second messengers play a critical role during osteoblast adhesion.

Besides new signaling pathways, data from peptide array pointed out classical signaling proteins involved with canonical pathways, as those ones active upon integrin activation, such as Src, FAK, and paxillin. FAK is a widely expressed cytoplasmic protein tyrosine kinase involved in integrin-mediated signal transduction. It plays an important role in the control of several biological processes, including cell spreading, migration, and survival [Parsons et al., 2000]. Sequentially, activation of FAK by integrin clustering leads to autophosphorylation at Tyr397, which is a binding site for the Src family kinases [Schaller et al., 1994]. In addition, we have proposed a low molecular weight protein tyrosine phosphate as a modulator of Src activity in osteoblast metabolism [Zambuzzi et al., 2008; de Souza Malaspina et al., 2009].

To our understanding, the elucidation of molecular events triggered during adhesion and spreading of pre-osteoblasts are important to provide new insights to understand both bone formation and improve cell–substratum interactions. This is one of the major goals in the fields of dental and orthopedic implants and bone repair research. Thus, control of the strength, dynamics, and mechanics of osteoblast adhesion offers an opportunity for designing novel biomaterials for tissue engineering and biotechnology advances. Finally, although other investigators have assessed the ability of different surfaces to support primary osteoblast adhesion, growth, and differentiation, this is the first article to our knowledge that screens the global network of signaling proteins responsible for osteoblast adhesion.

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