A Combined Proteome and Microarray Investigation of Inorganic Phosphate-induced Pre-osteoblast Cells*‡§

Kelly A. Conrads‡§, Ming Yi§¶, Kerri A. Simpson‡§, David A. Lucas||, Corinne E. Camalier‡, Li-Rong Yu||, Timothy D. Veenstra||, Robert M. Stephens¶, Thomas P. Conrads||, and George R. Beck, Jr.‡**

Inorganic phosphate, which is generated during osteoblast differentiation and mineralization, has recently been identified as an important signaling molecule capable of altering signal transduction pathways and gene expression. A large-scale quantitative proteomic investigation of pre-osteoblasts stimulated with inorganic phosphate for 24 h resulted in the identification of 2501 proteins, of which 410 (16%) had an altered abundance ratio of greater than or equal to 1.75-fold, either up or down, revealing both novel and previously defined osteoblast-regulated proteins. A pathway/function analysis of these proteins revealed an increase in cell cycle and proliferation that was subsequently verified by conventional biochemical means. To further analyze the mechanisms by which inorganic phosphate regulates cellular protein levels, we undertook a mRNA microarray analysis of pre-osteoblast cells at 18, 21, and 24 h after inorganic phosphate exposure. Comparison of the mRNA microarray data with the 24-hour quantitative proteomic data resulted in a generally weak overall correlation; the 21-hour RNA sample showed the highest correlation to the proteomic data. However, an analysis of osteoblast relevant proteins revealed a much higher correlation at all time points. A comparison of the microarray and proteomic datasets allowed for the identification of a number of candidate proteins that are post-transcriptionally regulated by elevated inorganic phosphate, including Fra-1, a member of the activator protein-1 family of transcription factors. The analysis of the data presented here not only sheds new light on the important roles of inorganic phosphate in osteoblast function but also begins to address the contribution of post-transcriptional and post-translational regulation to a cell’s expressed proteome. The ability to accurately measure changes in both protein abundance and mRNA levels on a system-wide scale represents a novel means to extract data from previously one-dimensional datasets. Molecular & Cellular Proteomics 4:1284–1296, 2005.

Numerous factors determine both the initial cellular response and ultimate cellular adaptation to a given stimulus or insult. The terminal phenotype of a given cell response remains a poorly understood combination of changes in signal transduction pathways, promoter regulation, mRNA processing and subsequent protein abundance, and post-translational regulation of proteins. The challenge of large-scale gene expression profiling has been met, leaving the goal of fully understanding the cellular response to a given stimulus or changing environment limited by the inability to fully evaluate the proteome. The ability to conduct an analysis of changes in both protein abundance and RNA levels on a “systems level” scale sufficient to entirely enumerate the response of a cell will undoubtedly be a powerful tool in assessing not only complex cellular processes, such as differentiation, but should also provide clues as to how to manipulate the cell phenotype by intervention.

The process of osteoblast differentiation leading to bone mineralization is a poorly understood series of temporally and spatially coordinated events occurring on the order of weeks and months. A significant end point in this process is the creation of a mineralized matrix that consists mainly of collagen, non-collagenous proteins and hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂). During this process, osteoblasts are exposed to high levels of inorganic phosphate. Using the MC3T3-E1 osteoblast differentiation model (1–3), we have recently described the significance of inorganic phosphate, which is generated during differentiation, as a signaling molecule capable of altering specific signal transduction pathways, gene expression, and ultimately mineralization (4, 5). Furthermore, a number of studies have demonstrated the ability of inorganic phosphate to alter gene expression and or cell function in other cell types, including parathyroid (6), neurons (7), kidney (8), vascular smooth muscle (9), chondrocytes (10), and osteoclasts (11). Hence, a more complete understanding of the consequences of elevated inorganic phosphate on cell function may be relevant not only to the...
process of osteoblast differentiation but also to various cell types with a wide range of functions. Toward this end, we were interested in determining how inorganic phosphate affects protein abundance levels and how these effects correlate with changes in gene expression at transcriptional levels in the MC3T3-E1 osteoblast differentiation model.

The aims of this study were to determine the affect of elevated phosphate on the osteoblast proteome and to evaluate this effect at a systems level by comparing microarray and proteomic datasets. Herein, we report the identity and comparison of changes of relative abundance of more than 2,500 proteins measured in control and inorganic phosphate-treated MC3T3-E1 pre-osteoblast cells using cleavable isotope-coded affinity tag reagents and mass spectrometry. The methods used to derive this dataset have been described previously (12). Four hundred and ten proteins (corresponding to ~16% of all proteins identified) showed a change in relative abundance of ≥1.75-fold, either up or down, when the cells were treated with inorganic phosphate. This large number of differentially abundant proteins underscores the significance of inorganic phosphate as a signaling molecule. A comparison of the proteomic dataset with multiple mRNA microarray analysis at time points preceding the cICAT data revealed an overall low correlation, although specific subgroups of proteins/genes based on functional categorization or pathway analysis demonstrated much higher correlations. The comparison of genomic and proteomic datasets led to the identification of Fra-1, an important bone regulatory transcription factor, as an inorganic phosphate regulated protein. The datasets reported here not only shed light on the effects of inorganic phosphate on osteoblast function but also reveal novel systems level relationships as to the nature of transcription and translation.

EXPERIMENTAL PROCEDURES

Materials—Dibasic sodium phosphate (Na2HPO4), monobasic sodium phosphate (NaH2PO4), cycloheximide, and actinomycin D were purchased from Sigma. Sodium sulfate (Na2SO4) was purchased from Valeant.

Immunoblotting—MC3T3-E1 osteoblast cells, cultured as described previously (12), were treated with 10 mM inorganic phosphate or 10 mM sodium dihydrogen phosphate, 250 mM sodium chloride, 30 mM sodium pyrophosphate, 0.1% Nonidet P-40, 5 mM EDTA, and 10 mM sodium fluoride, adjusted to pH 7.0) supplemented with the following protease inhibitors at the indicated final concentrations: 2.0 μg/ml aprotinin, 2.0 μg/ml leupeptin, 1.0 μg/ml pepstatin, 100 μM sodium orthovanadate, and 1 mM dithiothreitol. For nuclear and cytoplasmic fractionation, MC3T3-E1 cells were treated as above and harvested in PBS. To obtain the cytoplasmic fraction, cells were lysed in 10 mM Tris HCl, 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40 supplemented with protease inhibitors as described above. After centrifugation (2000 rpm for 10 min) and removal of the cytoplasmic fraction, the nuclear pellet was lysed in p300 lysis buffer as described above and centrifuged at 13,200 rpm for 10 min. The protein concentration of the cell lysates was determined with a BCA protein assay reagent kit (BioRad Laboratories). 50 μg of lysate was resolved on a NuPAGE Bis-Tris gel (Invitrogen). After electrophoresis, proteins were transferred to Hybond-P membranes (Amer sham Biosciences) according to the manufacturer’s protocol. The membrane was blocked with 3% milk in Tris-buffered saline/Tween 20 (20 mM Tris, pH 7.5, and 150 mM NaCl) before addition of antibody, except osteopontin, which was blocked in 5% milk. The blots were visualized by chemiluminescence development using a Western blotting Detection System (Amer sham Biosciences). Antibodies, Fra-1, histone deacetylase 1, proliferating cell nuclear antigen, protein kinase Cα, and actin were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA), p21 and PKC alpha from Pharmingen (BD Biosciences, Franklin Lakes, NJ), ERK1/2 from Promega, Madison, WI) and cyclin D1 antibody (Cell Signaling Technologies Inc., Beverly, MA).

Quantitation of Western Blot—Densitometric analysis of the Western blot was performed using the Kodak Digital Science 1D program. The region of interest was defined using a rectangular box with identical interior pixel area for both sulfate and phosphate-treated samples, although the pixel area varied for different proteins. The dimension of the box was fitted to the larger of either the sulfate or phosphate bands by hand. The net intensity as determined by the program was used to calculate fold induction or repression = [phosphate (net)/sulfate (net)].

Function Analysis—Data were analyzed using Ingenuity pathway analysis application (www.ingenenuity.com). Only the cICAT datasets representing proteins with changes in abundance of 1.75 or more were used (Supplemental Tables III and IV) for this analysis.

Proliferation Assay—MC3T3-E1 cells were plated in 96-well plates at a density of 2,500 cells per well. Seventy-two hours after plating, cells were treated with 10 mM inorganic phosphate or sulfate (control). After treatment for the indicated length of time, cell titer reagent (Promega’s Celltiter 96 aqueous one solution cell proliferation assay) was added to each well following the manufacturer’s protocol. After addition of reagent, plate was incubated at 37 °C and absorbance readings at 490 nm were taken at 2 h (BioRad Luminark microplate reader).

Flow Cytometry—Seventy-two hours after plating, MC3T3-E1 cells were treated with 10 mM inorganic phosphate or sulfate for the indicated times. Cells were then rinsed with ice-cold PBS, scraped off the culture dishes, and pelleted by centrifugation at 2,000 rpm for 2 min. Cell pellets were resuspended in 0.3 ml of ice-cold PBS followed by addition of 0.7 ml of ice-cold 100% ethanol. Samples were washed twice with 1 ml of ice-cold of PBS and resuspended in 0.9 ml of PBS. RNase A (Invitrogen) was added to each sample and incubated for 15 min. Propidium iodide (20 μl of 0.5 mg/ml) was added to each sample 1 hour before analysis by flow cytometry (Laboratory of Experimental Immunology, NCI-Frederick).

Cleavable ICAT Labeling and Affinity Purification—The methods used in the derivation of this particular dataset are described in more detail in Ref. 12. In brief, MC3T3-E1 osteoblast cell proteins (~750 μg each) were labeled either with the light (control, cICAT-12C5) or the heavy (phosphate-treated, cICAT-13C5) isotopic versions of the cICAT reagent using a method modified from that recommended by the manufacturer. An UltraLink immobilized monomeric avidin column was slurry-packed in a glass Pasteur pipette and equilibrated. The
column was blocked, the biotin was stripped from the reversible binding sites of the column per the manufacturer’s instructions, and the column was re-equilibrated. The cICAT-labeled peptides were boiled, cooled to room temperature, and loaded onto the avidin column and allowed to incubate for 15 min at ambient temperature. After the column was washed, the cICAT-labeled peptides were eluted and lyophilized to dryness. The biotin moiety was cleaved from the cICAT-labeled peptides by treatment with the cleaving reagents provided by the manufacturer and lyophilized to dryness.

Strong Cation Exchange Fractionation and Microcapillary Reversed-phase LC-MS/MS of cICAT Labeled Peptides—Again, these methods are described in more detail in Ref. 12. The lyophilized MC3T3-E1 osteoblast cICAT-labeled peptides were dissolved and injected onto a strong cation-exchange LC column (PolyLC Inc., Columbia, MD). A multistep gradient was used to elute the cICAT-labeled peptides from the column. Each strong cation-exchange LC fraction was lyophilized and reconstituted before microcapillary reversed-phase LC-MS/MS. 10-cm microcapillary reversed-phase LC ESI columns were coupled online with an ion-trap MS (LCQ Deca XP, ThermoElectron, San Jose, CA) to analyze the cICAT-labeled peptides from the MC3T3-E1 osteoblast cells. After loading the sample, the cICAT-labeled peptides were eluted using a linear step gradient. The ion-trap MS was operated in a data-dependent tandem MS (MS/MS) mode in which each full MS scan was followed by three MS/MS scans. The MS spectrum for the fragment ions was acquired using three microscans. Dynamic exclusion was used to minimize redundant acquisition of peptides previously selected for MS/MS. The heated capillary temperature and electrospray voltage were set at 160 °C and 1.7 kV, respectively.

Peptide Identification and Quantitation—The raw MS/MS data acquired on the ion-trap MS were searched using SEQUEST against the Mus musculus proteome database (27,612 entries) downloaded from the European Bioinformatics Institute (EBI; www.ebi.ac.uk/proteome/index.html). The Archaea-derived database (12,038 entries) used in the false-positive bioinformatic analysis was constructed using genomic sequence information from the following organisms: Aeropyrum pernix, Archaeoglobus fulgidus, Pyrococcus aerophilum, Sulfolobus tokodaii, and Thermoplasma volcanium. Dynamic modifications for cysteiny1 (Cy5) residues were set by mass addition of the cleaved cICAT labels (227.13 Da for the light label, 236.16 Da for the heavy label) in a single search. SEQUEST criteria were set as Xcorr ≥ 1.9 for [M + H] + ions, Xcorr ≥ 2.2 for [M + H] 2+ ions, and Xcorr ≥ 2.9 for [M + H] 3+ ions, and DeltaCn ≥ 0.08 for the identification of fully tryptic peptides within the cICAT-labeled samples. The identified peptides were quantified using XPRESS (Thermo Electron, San Jose, CA), which calculates the relative abundances of (13C9/13C0, in this dataset) of peptides based on the area of their extracted ion chromatograms.

RNA Isolation and Northern Blotting—Total cell RNA was prepared using TRIzol reagent (Invitrogen) according to manufacturer’s recommendations. 10 μg of RNA was loaded per lane and separated by electrophoresis through a 1% formaldehyde-agarose gel. The RNA was transferred to a Hybond-N nylon membrane (Amersham Biosciences) and cross-linked by UV irradiation and baking at 80 °C. 32P-labeled probes were prepared using a random prime labeling kit (Roche Applied Science). Between successive probes, blots were stripped by treatment with boiling 0.1% SDS.

Plasmids Used for Northern Probes—The Fra-1 plasmid was provided by Nancy Colburn (NCI-Frederick) and described in Ref. 13. The osteopontin plasmid, mop3, was provided by Marian Young and described in Ref. 14. The actin probe has been described previously (15).
In this study, we report a functional characterization and correlation analysis of more than 2501 proteins in MC3T3-E1 pre-osteoblasts and the relative change in abundance in response to elevated inorganic phosphate identified by cICAT. The technique used to derive this dataset is described in more detail in Ref. 12. A complete summary of the proteins identified in this study with the average change in abundance is presented in Supplemental Table I, and a complete summary of all peptides identified is presented in Supplemental Table II.

The generation of such a large dataset, while revealing the complexity of protein expression and function, will differ for most proteins. We have chosen to highlight proteins that have an altered abundance ratio of greater than 1.75-fold in response to inorganic phosphate treatment for 24 h relative to control (sulfate). The number of times a peptide was identified was counted for each protein and the average change in abundance are listed. These peptides may represent the same or unique proteins, and in turn regulate cell function is through a correlation analysis of more than 2501 proteins in MC3T3-E1 pre-osteoblasts and the relative change in abundance in response to elevated inorganic phosphate identified by cICAT.

| Protein description | n | Avg. | S.D. | Accession no. |
|---------------------|---|------|------|---------------|
| Extracellular matrix | | | | |
| α-2-macroglobulin-P | 4 | 51.25 | 38.81 | Q81150 |
| AMBP protein (Bikunin) | 1 | 20.00 | | Q07456 |
| ADAMTS-2 | 1 | 10.00 | | Q8C9W3 |
| Extracellular matrix protein 1 | 10 | 5.01 | 9.96 | Q61508 |
| Polycystin 2 | 1 | 5.00 | | Q35245 |
| Metallopr. Inhib.1 (TIMP-1) | 5 | 3.15 | 0.81 | P12032 |
| α-2-HS-glycoprotein | 7 | 1.75 | 1.10 | P29699 |
| Transcriptional regulators | | | | |
| Zinc finger protein 30 | 1 | 10.00 | | Q60585 |
| Steroid receptor coactivator-1 | 1 | 9.09 | | P70366 |
| Fos-related antigen-1 (FRA-1) | 2 | 3.15 | | Q8C9W3 |
| Signaling | | | | |
| Plecine | 17 | 5.89 | 9.21 | Q9QXS1 |
| GXII sPLA2 | 2 | 4.66 | 3.51 | Q8EP2 |
| MAPKK | 4 | 3.35 | 2.54 | Q99110 |
| Citron protein | 1 | 3.13 | | P49025 |
| Receptors/transporters | | | | |
| Na<sup>+</sup>-dependent dopamine transport | 1 | 11.11 | | Q61327 |
| Thrombin receptor (PAR-1) | 1 | 4.55 | | P30558 |
| Mitochondrial (OXPHOS) | | | | |
| Cytochrome b<sub>6</sub> reductase | 4 | 8.54 | 11.15 | Q8DCN2 |
| NADH-ubiquinone oxi. B17.2 | 1 | 8.33 | | Q7TMF3 |
| Ubiquinol-cytochrome c reductase | 2 | 4.98 | 5.81 | Q9DB77 |
| NADH-ubiquinone oxi PDSW | 4 | 4.36 | 6.62 | Q9DCS9 |
| Adenosine kinase | 1 | 4.35 | | Q91V3 |
| Cytochrome c<sub>1</sub> | 3 | 3.15 | 3.46 | Q9D0M3 |
| ADP,ATP carrier protein | 8 | 2.80 | 2.70 | P51881 |
| Hexokinase type II | 3 | 2.58 | 2.10 | O08528 |
| NAD(P) transhydrogenase | 9 | 2.28 | 3.89 | Q61941 |

### RESULTS

The identification and localization of protein expression and function provide insight into the molecular mechanisms that regulate cell function. The translation of these novel phosphate responsive proteins in the process of osteoblast differentiation and mineralization but also the relative roles of transcription and post-translational modifications in protein abundance.

Change in Relative Abundance after Exposure to Elevated Inorganic Phosphate—One mechanism by which proteins are regulated, and in turn regulate cell function is through changes in relative abundance. At present, it is unclear to what extent the relative abundance of a protein must be altered to result in a physiologically relevant response. It is likely that the physiologically relevant change in abundance will differ for most proteins. We have chosen to highlight proteins that have an altered abundance ratio of greater than 1.75-fold because we have previously determined that this -fold change is significantly detectable by both Northern (5) and Western (12) blotting. Table I represents a partial list of identified proteins representing multiple functional classes that are either increased or decreased by ≥1.75-fold in response to inorganic phosphate treatment for 24 h relative to control (sulfate). The number of times a peptide was identified for each protein and the average change in abundance are listed. These peptides may represent the same or unique peptides within a given protein. All proteins with increased or decreased relative abundances ≥1.75-fold are accessible in

### Table I—continued

| Protein description | n | Avg. | S.D. | Accession no. |
|---------------------|---|------|------|---------------|
| Intracellular Regulators | | | | |
| JAZ (Zfp346) | 1 | 0.16 | | Q9R0B7 |
| ECSI | 1 | 0.18 | | Q9QZ6H |
| SDF2L1 | 2 | 0.20 | 0.00 | Q9ESP1 |
| Receptors/transporters | | | | |
| Fgr2 | 1 | 0.15 | | P21803 |
| DAAM 2 | 1 | 0.33 | | Q8OUI9 |
| Mitochondrial (β-oxidation) | | | | |
| Carnitine O-palmitoyltransferase | 1 | 0.31 | | P52825 |
| Putative D2,D4-diolenyl-CoA | 1 | 0.40 | | Q99M71 |
| Propionyl CoA carboxylase α | 1 | 0.46 | | Q91Z3 |
| Acyl-CoA acyltransferase | 4 | 0.48 | 0.30 | Q8QZ71 |
| D2,D4-diolenyl-CoA reductase | 2 | 0.56 | 0.03 | Q8WV68 |
| Acyl-CoA dehydrogenase | 2 | 0.59 | 0.02 | Q9DBL1 |
Supplemental Tables III and IV, respectively. It is clear from the results that the cICAT technique is capable of identifying classes of proteins ranging in function from extracellular matrix proteins to transcription factors, making this a powerful technique to survey protein changes on a proteome wide scale.

Validation of cICAT Quantitation by Western Blotting — Few large datasets have been generated using protein quantification techniques that will identify changes in relative protein abundance of treated samples. To determine the validity of the cICAT-quantified protein abundance described here, traditional PAGE followed by immunoblotting was performed on a series of selected proteins. Samples harvested at 24 h in parallel with the sample used for cICAT labeling were either separated into nuclear and cytoplasmic fractions or lysed as a total cell fraction and separated by polyacrylamide gel electrophoresis and electroblotted onto a membrane, which was then probed with a series of antibodies (Fig. 1). We chose to analyze proteins predicted to be either increased, decreased, or unchanged by the cICAT study, as well as proteins with different cellular locations and varying functions. In addition, we chose proteins that were identified through only a single or few peptides by cICAT (i.e. p21: n = 1) as well as proteins that were identified by a large number of peptides (i.e. actin: n = 306). Quantitation of the protein abundances by densitometric analysis of the Western blot revealed a good agreement with the cICAT measured ratio in response to elevated inorganic phosphate for all conditions examined (Fig. 1) and suggests that, in fact, such global quantitative proteomic measurements can be used to accurately predict changes in relative protein abundance from complex samples in an equivalent manner to more traditional biochemical methods.

Pathway/Function Analysis of Inorganic Phosphate Regulated Proteins: Cell Cycle and Proliferation — The global scale of the cICAT labeling technique has the potential to reveal insights into entire pathways or functional relationships in response to a given stimuli. An analysis of the dataset representing all proteins with altered changes in abundance greater than 1.75, either up or down, using Ingenuity’s pathway analysis application revealed a number of significant changes in functionally related processes (Table II). This analysis revealed that the expression of proteins related to cell cycle change significantly in cells exposed to inorganic phosphate. In essence, all of the highly significant functional relationships determined by the analysis represent an increase in cellular metabolism and proliferation. An interactive network of some of these proteins, generated by the pathway analysis software, is represented in Fig. 2A. All proteins identified in this network were up-regulated by exposure to inorganic phosphate, and the -fold increase is listed below the respective protein.

To determine whether this analysis represented a biologically relevant output, we examined the effect of inorganic phosphate on cell cycle and proliferation. MC3T3-E1 cells were treated with inorganic phosphate and harvested for analysis of cell cycle status by flow cytometry. Inorganic phosphate caused a >50% increase in the number of cells in S phase by 24 h (Fig. 2B). The increase in cell cycle should be accompanied by an increase in proliferation. To determine whether inorganic phosphate caused an increase in proliferation, the treated cells were analyzed by a celltiter assay as
described under “Experimental Procedures.” The celltiter results from multiple determinations (Fig. 2C) reveal an increase in proliferation starting at 24 h and extending through 48 h. The data are in good agreement with the approximate 24-h doubling time of these cells. This analysis reveals that exposure to elevated inorganic phosphate results in an increased number of cycling cells and an overall increase in proliferation. In addition, these results suggest that a global analysis of changes in relative protein abundance can in fact reveal significant and biologically relevant changes in cell function.

Comparison with Microarray Analysis—To date, because of the technical limitations of generating large proteome data-
bases, there are limited data regarding the correlation of protein and mRNA levels on a global scale. The ability to conduct such correlation analyses may represent an additional and novel means to generate discrete and testable biological hypotheses from these global and discovery driven measurements. To investigate the relationship of transcription with our cICAT database, MC3T3-E1 cells were treated with 10 mM inorganic phosphate for 18, 21, and 24 h, in addition to a separate 24-hour sample harvested in parallel with the cICAT sample. The extracted mRNA samples were used to perform a NCI 22,000 oligonucleotide mRNA microarray analysis. Of the 2501 proteins identified by cICAT, 1900 could be aligned with the corresponding genes identified in the mRNA microarray analysis, and the entire database is presented in detail in Supplemental Table V, with the 24-hour sample in parallel with cICAT listed as “array.” The correlations between global mRNA levels at different time points and corresponding protein levels at 24 h after inorganic phosphate exposure were measured by Pearson’s method and are represented graphically in Fig. 3A. As determined by this statistical analysis, there is little correlation between RNA and protein abundance levels identified and predicted by cICAT.

To determine whether the general lack of correlation of the entire dataset also applied to tissue specific gene expression, 40 osteoblast relevant genes/proteins were chosen for correlation analysis. The genes/proteins were chosen based on previous descriptions of relevance to the osteoblast differentiation process and are listed in Supplemental Table VI. This analysis (Fig. 3B) revealed a much higher degree of correlation between the osteoblast specific genes/proteins than the global proteome/transcriptome correlation. The potential reasons for the general lack of correlation over the entire dataset but high correlation within the osteoblast relevant dataset are more fully explored under “Discussion.”

**Fig. 3.** Pearson correlation of relative RNA and protein abundance at various time points after inorganic phosphate treatment. A, RNA samples generated at 18, 21, and 24 h and parallel samples with cICAT (array) at 24 h after exposure to 10 mM inorganic phosphate were correlated with the relative protein abundance. B, osteoblast-specific genes were compared by Pearson correlation as described in A.
Functional groupings regulated through transcription/translational type of analysis is a valid method for identifying pathways or functional groupings, such as the "ucalpain and friends" subset at multiple time points also suggests that this pathway/function groupings, such as the "ucalpain and friends in Cell spread (n = 10)" reveals a previously defined method of regulation, which was reflected in the correlation of certain genes/proteins that when correlated have a p value of less than 0.05 and positive correlation from 1000 permutation; Smallest ≤ 0.05, smallest positive correlation with p value =< 0.05.

| BioCarta Pathway                              | Original Cor/p value (vsiCAT) | Time point of correlation | No. of cases | Smallest ≤ 0.05 | p Value |
|-----------------------------------------------|-------------------------------|---------------------------|--------------|-----------------|---------|
| Cell Cycle, G1-S Check Point (n = 6)          | 0.926642/0.007875             | 24 h                      | 19           | 0.816           | 0.048   |
| Cyclins and Cell Cycle Regulation (n = 4)     | 0.967958/0.03205              | 24 h                      | 32           | 0.951           | 0.049   |
| Influence of Ras/Rho on G1 to S (n = 10)     | 0.6871/0.02816                | 24 h                      | 27           | 0.639           | 0.047   |
| Influence of Ras/Rho on G1 to S (n = 7)      | 0.7885/0.03511                | Array                     | 26           | 0.757           | 0.049   |
| uCalpain and friends in Cell spread (n = 10)  | 0.7009515/0.02393             | 18 h                      | 28           | 0.636           | 0.048   |
| uCalpain and friends in Cell spread (n = 10)  | 0.710019/0.02142              | 21 h                      | 25           | 0.635           | 0.048   |
| uCalpain and friends in Cell spread (n = 8)   | 0.8931876/0.02808             | 24 h                      | 23           | 0.715           | 0.046   |

**GO-Term**

| GO-Term                                      | Original Cor/p value (vsiCAT) | Time point of correlation | No. of cases | Smallest ≤ 0.05 | p Value |
|----------------------------------------------|-------------------------------|---------------------------|--------------|-----------------|---------|
| Cell Cycle (n = 22)                          | 0.5011/0.01751                | 21 h                      | 36           | 0.429           | 0.047   |
| Integrin-mediated signaling (n = 15)         | 0.8295/0.04115                | Array                     | 20           | 0.816           | 0.048   |
| G-protein coupled receptor signaling (n = 15)| 0.7639/0.009158              | Array                     | 25           | 0.950           | 0.050   |
| Induction of Apoptosis (n = 4)               | 0.9636/0.03637                | Array                     | 25           | 0.950           | 0.050   |
| Mitosis (n = 7)                              | 0.8256/0.02214                | 21 h                      | 26           | 0.755           | 0.050   |
| Rho protein signal transduction (n = 7)      | 0.8316/0.020356               | 21 h                      | 26           | 0.755           | 0.050   |
| Rho protein signal transduction (n = 7)      | 0.82235/0.02316               | 21 h                      | 26           | 0.755           | 0.050   |

**Analysis of BioCarta Pathways and GO-Term Associated Groups Reveals a Higher Correlation between Protein and mRNA Levels within Functional Subsets**—The general lack of overall correlation prompted us to examine whether subsets of genes/proteins based on function/pathway (Gene Ontology “GO” or BioCarta databases) would show higher correlations. As described under “Experimental Procedures,” this analysis uses an unbiased means to determine correlation based on previously defined gene groupings. The output, a partial list of which is detailed in Table III, represents a strong correlation between protein and mRNA levels within certain pathways or functional groupings as evidenced by detection of least four genes/proteins that when correlated have a p value of less than 0.05 at any time point analyzed. The full analysis is found in Supplemental Table VII. In addition, we evaluated the likelihood that these correlations were statistically significant by permutation analysis. The analysis (Table III) revealed that for all pathwayfunctional groupings that demonstrated a significant correlation in the change in mRNA and protein abundance in osteoblasts exposed to inorganic phosphate, the permutation analysis showed less than 5% of permutations with significant correlations compatible with the original correlations and therefore strengthens and validates the conclusion that these groupings are significantly correlated. The identification of high correlation between protein and mRNA in the cell cycle category of both GO and Biocarta databases reflects a previously defined method of regulation, which was revealed as a regulation mechanism in many basic biological processes in many lower organisms. The correlation of certain pathwayfunction groupings, such as the “ucalpain and friends” subset at multiple time points also suggests that this type of analysis is a valid method for identifying pathways or functional groupings regulated through transcription/translation. These data suggest that although there is within the total proteome and transcriptome at any given time point a general lack of correlation, functional subsets of genes/proteins may still be tightly coordinately regulated. Likewise, other subsets are not coordinately regulated and may represent pathways regulated by post-translational modification.

**Subtraction of Proteome and Transcriptome Databases Reveals Post-translationally Regulated Proteins: Fra-1**—The ability to compare large datasets of protein and mRNA levels represents an opportunity to extract biologically meaningful data not previously possible. A comparison of the protein and mRNA data generated in this analysis revealed a number of proteins whose relative abundance was substantially increased in osteoblasts after exposure to elevated inorganic phosphate, whereas the corresponding mRNA levels show little change. Table IV reveals proteins detected with changes in abundance greater than 3.0-fold, whereas the corresponding RNA, detected by microarray, was not altered by more than 1.5-fold at any time point investigated. These proteins represent potential targets of post-translational regulation by elevated inorganic phosphate.

To experimentally validate this notion, we chose to analyze in more depth Fra-1 (fosl1). Fra-1 is of particular interest in that it is a member of the activator protein (AP)-1 family of transcription factors, which is important in both bone development and the regulation of osteoblast function (reviewed in Ref. 16). A Northern blot analysis of samples used for the cICAT microarray revealed no detectable changes in Fra-1 RNA (data not shown) in agreement with microarray results (Supplemental Table V). To test the hypothesis that the increase in Fra-1 protein is the result of post-translational regulation in response to elevated inorganic phosphate, we treated the pre-osteoblasts with either the translation inhibitor cycloheximide or the transcription inhibitor actinomycin D.
followed by treatment with 10 mM inorganic phosphate for 24 h. The results (Fig. 4A) revealed an increase in Fra-1 protein in the presence of either the translation or transcription inhibitor and suggest that in fact Fra-1 is post-translationally regulated in response to inorganic phosphate. Analysis of samples harvested in parallel for mRNA levels revealed that Fra-1 is not highly regulated by transcription at this time point (Fig. 4C). Quantification of the Northern blot revealed an approximate 1.2-fold increase in response to phosphate (data not shown) in good agreement with the 1.23-fold increase determined in the mRNA microarray analysis (Table III). Immunoblotting for osteopontin, a positive control, revealed a strong up-regulation of both protein and RNA that is completely blocked by treatment with either inhibitor. This result also demonstrated that both the cycloheximide and actinomycin D were given at a functionally relevant concentration (Fig. 4, B and C).

In addition, we sought to determine whether another known stimulator of Fra-1 protein functioned in this manner. MC3T3-E1 cells were treated as above, but the phorbol-ester TPA was used as an inducer. The results revealed a somewhat different mode of regulation relative to inorganic phosphate (Fig. 4, A–C). The translation and transcription inhibitors both caused a more pronounced inhibition of TPA-induced Fra-1 levels and suggested that transcription plays a more significant role in the regulation of Fra-1 in response to TPA than inorganic phosphate at the time points analyzed. This is also evident in the larger increase in Fra-1 RNA in response to TPA than inorganic phosphate (Fig. 4C). Taken together, these results not only provide evidence that Fra-1 is post-translationally regulated by inorganic phosphate but also serve as a proof of principle that post-translationally regulated proteins can successfully be identified by a systems level analysis such as presented herein. Furthermore, these experiments identify inorganic phosphate as a potential key regulator of an important transcription factor, Fra-1, during osteoblast development.

### Table IV

| Protein description                | ICAT 18 | 21  | 24  | Unigene   |
|-----------------------------------|---------|-----|-----|-----------|
| II iodothy. deiodin.              | 25.0    | 0.63|     | Mm.21389  |
| AMBP protein (bikinun)            | 20.0    | 1.22|     | Mm.2197   |
| 7-dehydro. red.                   | 12.9    | 0.76| 0.55| Mm.249342 |
| 150004014Rik protein              | 11.1    | 1.06| 1.16| Mm.306482 |
| 493044312Rik protein              | 11.1    | 1.01|     | Mm.307948 |
| Na-dep. dopamine trans.           | 11.1    | 0.61| 1.04| Mm.41993  |
| Nucleophosmin (NPM)               | 10.4    | 1.04| 1.33| Mm.6343   |
| Steroid coactivator-1             | 9.09    | 0.84| 0.68| Mm.329196 |
| Collagen a1 XIX chain             | 9.09    | 0.74| 1.34| Mm.329196 |
| Cyto. b-5 red. (NADH)             | 8.54    | 0.69| 0.81| Mm.22560  |
| 5’-3’ exoribo. 2 (Dhm1)           | 8.27    | 0.79| 1.01| Mm.3065   |
| P52rIK                             | 7.69    | 0.78| 1.30| Mm.4428   |
| 0610006A11Rik protein             | 6.67    | 1.05|     | Mm.2202   |
| 1500016H10Rik protein             | 5.76    | 1.05| 1.26| Mm.218533 |
| PRP31                             | 5.56    | 1.13| 1.41| Mm.246863 |
| Polycystin 2                      | 5.00    | 1.07| 0.56| Mm.6442   |
| Ubic.-cyto. C reduct.             | 4.98    | 0.74| 1.03| Mm.334206 |
| Thymidine kinase                   | 4.86    | 1.06| 1.13| Mm.2661   |
| 3110031G15Rik protein             | 4.76    | 1.18| 0.59| Mm.208618 |
| Sim. to cylindromatosis           | 4.76    | 0.93| 0.62| Mm.24282  |
| XII secr. Phopholip. A2           | 4.66    | 1.29| 1.22| Mm.151951 |
| Myotrophin (V-1)                  | 4.60    | 0.75| 0.61| Mm.182746 |
| Chromobox pro. hom. 5             | 4.55    | 1.01| 1.06| Mm.262059 |
| Sim. to SUMO-1 prot. 2            | 4.55    | 0.93| 0.83| Mm.255784 |
| NADH-ubi. oxi. PDSW               | 4.36    | 0.93| 1.23| Mm.1129   |
| 5730455C01Rik protein             | 4.35    | 0.80| 0.66| Mm.22379  |
| 4921529018Rik protein             | 4.17    | 0.55|     | Mm.266280 |
| Pentaxin-related protein           | 4.17    | 0.30| 0.38| Mm.276776 |
| Histone H3 (H3.2)                 | 3.52    | 0.75| 0.49| Mm.261670 |
| Nucleoprin-ip-1 iso...T           | 3.45    | 0.71| 0.95| Mm.45513  |
| CUG repeat RNA-bp 1               | 3.40    | 0.95| 0.99| Mm.29495  |
| Serum albumin                     | 3.24    | 0.94|     | Mm.16773  |
| l-lactate dehydrog. A             | 3.20    | 0.95| 1.18| Mm.29324  |
| FRA-1                             | 3.15    | 0.71| 1.22| Mm.6215   |
| 70 kDa WD-repeat                  | 3.03    | 1.18| 0.83| Mm.325512 |
Proteomic/RNA Analysis of Osteoblasts

**DISCUSSION**

The spatial and temporal coordination of osteoblast differentiation and mineralization is a complex process requiring exquisite communication between the cell and the extracellular environment. We have described previously the significance of inorganic phosphate that is generated during osteoblast differentiation as a key signaling molecule in the process of regulated gene expression leading to mineralization (reviewed in Ref. 17). In an attempt to understand the effect of this signaling molecule on cell function at a systems level, we conducted both quantitative proteomic and gene expression analyses of inorganic phosphate-treated osteoblasts with the goal of conducting a global analysis of the relationship between regulation of the osteoblast transcriptome and the proteome in response to inorganic phosphate exposure.

Analysis of the cICAT data alone has revealed insights into the mechanism by which inorganic phosphate influences osteoblast function. A number of proteins involved in the oxidative phosphorylation pathway were strongly increased, suggesting a possible important role of mitochondria in the phosphate response. In fact, a recent study has defined inorganic phosphate as a regulator of oxidative phosphorylation (18). Furthermore, a number of studies have identified the requirement of mitochondrial function and increased ATP in osteoblast mineralization and differentiation (19–21). These results in combination with the data presented here suggest that inorganic phosphate may be an important regulator of metabolic function during osteoblast differentiation. It is interesting that the results identify a general decrease in many proteins involved in β-oxidation, suggesting the ability of elevated inorganic phosphate to preferentially regulate particular metabolic pathways. The increase in metabolism agrees with our data revealing for the first time that inorganic phosphate causes an increase in the number of cells actively cycling and overall proliferation. Although more work is required to determine the significance of phosphate induced proliferation in osteoblast differentiation, this line of inquiry may also prove relevant to other cell types as suggested by an increase in thymidine incorporation in parathyroid cells exposed to elevated inorganic phosphate (6).

A complete understanding of how osteoblasts generate a mineralized matrix will undoubtedly prove useful in developing interventions to disorders of bone metabolism. This study has identified a number of proteins responsive to inorganic phosphate and previously demonstrated relevant to osteoblast function including periostin (osf-2), α2-HS glycoprotein, bFGFR2, decorin, osteoglycin (mimecan precursor), TIMP-1, GFR2, decorin, osteoglycin (mimecan precursor), TIMP-1, AMTS-2, polycystin 2, Chondroitin 4 sulfotransferase, calgizzarin, copine I, and DAAM2, among others. The response of these proteins to inorganic phosphate suggests that inorganic phosphate may be an important signal for the transition of the matrix to a mineralization competent state. The quantitative proteomic analysis has also identified a number of proteins regulated by inorganic phosphate that were not previously known to be associated with osteoblast function including α1- and α2- microglobulin, ADAMTS-2, polycystin 2, Chondroitin 4 sulfotransferase, calgizzarin, copine I, and DAAM2, among others. The response of these proteins to inorganic phosphate suggests that they may play important roles in the mid to late phases of osteoblast differentiation when inorganic phosphate levels are higher. The increased abundance of a number of proteins involved in calcium regulation agrees with previous results suggesting that as inorganic phosphate levels rise, the cell must balance the calcium-to-phosphate ratio or risk apoptosis (22).

Although much useful information can be gleaned from proteomic data alone, the ability to combine the gene expression data with corresponding protein levels on a large scale represents a novel mechanism for extracting biologically relevant results. Because the temporal relationship between transcription and translation on a large scale is not well defined, we chose to not only analyze RNA samples corresponding to the 24-h time point used for the quantitative proteomic analysis but also two time points preceding this, 18 and 21 h.
This analysis resulted in a generally low level of correlation. The lack of correlation between quantitated protein and RNA levels has been reported previously in a number of models although with smaller datasets (23–30). There may be multiple reasons for the lack of overall correlation. First, there is an inherent variability within all scientific assays and biological systems, especially in high throughput technologies such as those used in the present studies; therefore, a pure statistical comparison may not be most representative. In addition, the higher correlation between the 21-hour-treated RNA sample with the 24-h protein sample suggests that analyzing a single static time point in a dynamic system such as a cellular response to a stimulus may not be optimal. Because the temporal relationship between transcription and translation is likely to vary based on the individual gene/protein, it may not be realistic to expect a high degree of correlation between RNA and protein levels when attempting to correlate dynamic changes in RNA with a static picture of the proteome. Finally, the results described here represent a time point 24 h after stimulation, and it is possible that the longer the time from stimulation, the less likely it is that RNA and protein levels will correlate. Experiments are currently underway to address this possibility.

Although overall there was a generally low correlation, a stronger correlation was observed between several of the known osteoblast associated proteins and their corresponding RNA levels, such as periostin, osteoglycin, and TIMP-1, among others. Other studies have found some degree of correlation when looking at particular subsets of proteins (31–33). We also investigated the possibility that selected pathways and/or Gene Ontology groups may have higher or lower relative correlations. Indeed, the analysis revealed a number of subsets of proteins/RNAs that demonstrated a high degree of correlation that were subsequently validated by permutation analysis. Furthermore, the observation that the correlation of certain pathway/function groupings, such as the “ucalpain and friends” biocarta pathway exists at multiple time points may imply that there is a time-persistent type of coordination between transcription and translation that is maintained during a biological process, which might be critical for osteoblast differentiation. The identification of a high correlation between protein and RNA in certain “critical or specific” pathways or GO-groups such as cell cycle, may reflect a previously defined method of regulation or existence of global attractors of the dynamics, which has been revealed by recent biological network study in yeast as attractors or attracting trajectory (34). This may indicate that genes for certain specific biological processes may need to be better synchronized both transcriptionally and translationally for synergistic purposes. It may also suggest that “specific” effects that are a direct response of a given stimuli to the particular cell system will correlate in a more temporal manner than “bystander” or “housekeeping” effects that are activated to maintain cell homeostasis. Information surrounding the degree of correlation between transcription and translation within certain functional groupings or pathways may prove useful in designing strategies for therapeutic intervention.

Although many of the osteoblast specific genes/proteins correlated well, a few did not. Subtraction of the RNA and protein databases has revealed a number of proteins regulated without concurrent increases in RNA at any time point analyzed. One such protein, Fra-1, is a member of the AP-1 family of bzip transcription factors. Genetic manipulation of AP-1 genes through either transgenic or knockout mice, have revealed that most AP-1 family members play important roles in osteoblast development and bone metabolism (reviewed in Ref. 35). In particular, Fra-1 has been demonstrated to be a key transcription factor in bone metabolism either by transgenic expression (36) or conditional loss of function (37). The recently reported conditional loss-of-function mice developed osteopenia characterized by progressive loss of bone mass, suggesting an important role for Fra-1 in osteoblast function. Furthermore, these authors suggest that Fra-1 is likely to function at the mid to late stages of osteoblast maturation, a time that corresponds to elevated inorganic phosphate. Transgenic expression of Fra-1 revealed a dramatic increase in postnatal bone mass of the entire skeleton, in part because of a increased number of osteoblasts (36). These studies clearly demonstrate the significance of Fra-1 in osteoblast function as it relates to bone metabolism.

Although many of the AP-1 genes/proteins have been linked to bone metabolism, little is known about the forces that regulate this family of transcription factors during bone metabolism. Data presented here identify post-translational modification as a regulator of Fra-1 in osteoblasts. Previous studies suggest a mechanism by which Fra-1 is post-translationally regulated. The phosphorylation of Fra-1 by the mitogen-activated protein kinases ERK1/2 has been demonstrated to protect Fra-1 from proteosomal degradation (38) and to result in post-translational stabilization (39). We have previously demonstrated that ERK1/2 is activated by elevated inorganic phosphate at 24 h (4), which suggests a possible mechanism by which Fra-1 is post-translationally regulated in this model. The identification of inorganic phosphate as a regulator of Fra-1 during osteoblast differentiation represents a novel mechanism by which the activity of the translational but not transcriptional machinery is coordinated with the extracellular environment, and represents one of the first identifications of a regulator of the AP-1 family of transcription factors in bone metabolism.

In conclusion, the studies presented here describe the successful combination of large scale proteomic and microarray datasets to determine at least three novel mechanisms by which inorganic phosphate regulates cell function (Fig. 5). 1) Analysis of the cICAT data defined novel phosphate responsive proteins and revealed the regulation of pathways or functional networks involved in cell cycle, proliferation and energy metabolism. 2) A comparison of the cICAT-derived protein...
Mechanisms of inorganic phosphate affect on cell function. The combined proteomic and microarray studies presented here suggest at least three novel mechanisms by which inorganic phosphate affects cell function: an increase in cellular energy metabolism, increased transcription/translation, and targeted post-translational regulation. The significance is 2-fold: 1) the ability to identify these regulatory mechanisms was made possible only by combining these global analyses and emphasizes the power of such an approach, and 2) data generated from this approach have identified biologically relevant consequences of elevated inorganic phosphate in the process of differentiation and mineralization.

data with microarray samples has led to the determination that osteoblast-specific genes are more likely to be tightly regulated by the traditional transcription/translation pathways. 3) Furthermore, this type of comparison can lead to novel methods of data extraction, including the identification of post-translationally regulated proteins as evidenced by Fra-1. A future challenge will be to determine how these signals are integrated in a complex process that spans weeks and months. The data presented here not only reveal novel insights into the role of inorganic phosphate in altering cell function but also provide an initial glimpse at the power of combining large scale protein and mRNA analysis for a systems level understanding of the cell.

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** To whom correspondence should be addressed. Tel.: 301-846-1651; Fax: 301-846-8807; E-mail: gbeck@ncifcrf.gov.

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