Using Cloning and Transfection to Develop Traceable Pit1 and Pit2 Proteins

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Abstract: Vascular calcification is a prevalent and deleterious disease occurring as a natural side effect of aging, diabetes, menopause, and osteoporosis and contributing to cardiovascular disease as well as the failure of native and bioprosthetic cardiac valves. One important regulator of calcification is phosphorous. It has been found that elevated extracellular phosphorus levels cause vascular smooth muscle cells (VSMCs) to calcify and that phosphorous is transported into human VSMCs by the type III sodium-phosphate cotransporters Pit1 and Pit2. While antibodies to these cotransporters have been developed, attempts to characterize them using Western blots and cell lysates expressing endogenous levels of the cotransporters have yielded inconclusive results. This study created traceable Pit1 and Pit2 proteins by cloning their full-length cDNA into pIREShrGFP2a, a vector with a hemagglutinin (HA) fusion protein and green florescence protein (GFP) coexpression. By transfecting these traceable plasmids into human HELA cells, cells expressing higher levels of these cotransporters were produced. Since the HA tag and cotransporter proteins are fused together, the reliable and well-characterized anti-HA antibody should recognize the same protein as the cotransporter antibodies, thus providing a way to verify the specificity of the cotransporter antibodies. Western blots were run on these transfected cells comparing the HA antibody with Pit1 and Pit2 antibodies. 293 cell lysate transfected with a 55 kB HA gene (obtained from Abcam) revealed a distinct band at 55 kD corresponding with the reported protein size, only nonspecific bands were detected in HeLa cell lysates. The anti-Pit1 antibody recognized a specific band at 88 kD and the anti-Pit2 antibody recognized a multimeric band between 90 kD and 121 kD. Definitively characterizing these antibodies enables future lines of study which pursue the prevention of Pit1 and Pit2 mediated vascular calcification.

1. INTRODUCTION

Vascular calcification, or the deposition of calcium phosphate in the blood vessels, myocardium, and cardiac valves, is an extremely common and deleterious condition [6]. It occurs as a natural side-effect of aging, diabetes, menopause, and osteoporosis and contributes to the initiation and progression of cardiovascular disease as it increases atherosclerotic plaque burden and blood pressure [1,6,7,12]. Furthermore, it is the major cause of failure for native and bioprosthetic cardiac valves [7].

Phosphorous has been pin-pointed as an important regulator of vascular calcification [9]. Under normal conditions, vascular smooth muscle cells (VSMCs) do not spontaneously mineralize, but when grown with elevated extracellular phosphate levels, smooth muscle cells (SMCs) undergo the precise phenotypic transition observed in calcified cells [8,14]. The SMC specific gene expression of these cells is replaced with expression of osteochondrogenic genes including osteopontin, Runx2, alkaline phosphatase, and osteocalcin [7]. Phosphorous is transported into cells by sodium-phosphate (NaPi) cotransporters [9]. The quantity of phosphorous transported is directly linked with the abundance of extracellular phosphorous [6]. There are three types of NaPi cotransporters: Type I, Type II, and Type III. Type III NaPi transporters are universally expressed in many tissues including the kidney, heart, lung, brain, liver, and bone and function to supply phosphorus for oxidative phosphorylation [7]. They do not share homology with Types I and II, which are predominately expressed in intestine and kidney and control phosphate reabsorption into these tissues [11]. Only Type III cotransporters, consisting of Pit1 and Pit2, are expressed in human VSMCs [7]. Pit1 and Pit2 are multipass transmembrane glycoproteins with multipass N and C termini [5,11].

Many studies have convincingly shown that Pit1 activity is responsible for VSMC calcification. Pit1 has been identified as the predominant NaPi cotransporter in VSMCs, with eight times higher expression than Pit2 [9]. The inhibition of Pit1 mRNA, protein, and activity by small interfering double-stranded RNA
significantly inhibits phosphate induced SMC calcification whereas the overexpression of Pit1 restores phosphate intake and phosphate induced calcification [9]. The under-expression and over-expression of Pit1 in osteoblast cultures causes the respective down-regulation and up-regulation of mineralization [15].

Despite their 60% amino acid sequence homology, Pit1 and Pit2 have a number of differences [4]. The highest expression of Pit1 is found in the bone marrow while the highest expression of Pit2 is found in the heart [4]. Pit1 and Pit2 also use different mechanisms of Pi transport [2]. In *Xenopus laevis* oocytes, Pit1 can use Mg$^{2+}$ in place of Ca$^{2+}$ for NaPi transport but Pit2 cannot and Pit2 can uncouple NaPi symport functions while Pit1 cannot [2]. It is thought that Pit1 has ten transmembrane regions while Pit2 has twelve [3,11]. The fact that Pit1 mRNA is up-regulated in osteogenic cells by BMP-2, a bone morphogenetic protein, while Pit2 mRNA is unaffected, suggests that in addition to having different mechanisms, the cotransporters are regulated differently [13]. The evidence implicating Pit1 in vascular calcification, the lack of studies done on Pit2, and the fact that these cotransporters perform the same function with slight but potentially significant differences necessitate the study of both of them.

As it is thought that increased intracellular phosphorous levels induce calcification and that intracellular phosphorous levels are regulated by Pit1 and Pit2, manipulation of these cotransporters could be a means to prevent calcification [2,8]. Thus, it is important to investigate the effects of these cotransporters under different experimental conditions, a process conveniently done with antibody visualization. Although antibodies to Pit1 and Pit2 had been developed, they were not functional due to non-specificity. When used in Western blots, these antibodies identified multiple protein bands rather than bands specific to the cotransporter.

This study created traceable Pit1 and Pit2 proteins by cloning their full-length cDNA into pIREShrGFP2a, a vector with a hemagglutinin (HA) fusion protein and green fluorescence protein (GFP) coexpression. Transfecting these cotransporter plasmids into human cells created a population of cells known to have enhanced cotransporter abundance. As the anti-cotransporter antibodies detected multiple bands on Western blots run with endogenous protein cotransporter levels, this overexpression should enhance cotransporter protein levels enough that there is a difference in the intensity of the specific cotransporter band between the cells transfected with the vector backbone and cells transfected with the cotransporter plasmids. Since the HA tag and cotransporter proteins are fused together, the reliable and well-characterized anti-HA antibody should recognize the same protein as the cotransporter antibodies, thus providing a way to verify anti-cotransporter antibody specificity.

### 2. MATERIALS AND METHODS

#### 2.1 Insertion of cotransporter DNA into pIREShrGFP2a

Pit1 cDNA (3.3 kB) was obtained from ATCC as an insert in a PCMVSport6 vector (4.4 kB) and Pit2 cDNA (3.7 kB) was obtained from Invitrogen as an insert in an unknown MGC clone (2.8 kB by experimentation). The pIREShrGFP2a Vitality vector (5.0 kB) was produced by Stratagene.

All restriction enzymes were acquired from New England Biolabs. Restriction digest reactions were assembled, run, and heat inactivated according to standard protocols unless otherwise noted. Temperature was controlled using an Authorized Thermal Cycler or a PTC-100 Programmable Thermal Controller from MJ Research, Inc. DNA fragment ends were blunted with 5U of DNA Polymerase I Large (Klenow) at 30°C for 15 min and heat inactivated 75°C for 10 min and dephosphorylated by 1U of Calf Intestinal Alkaline Phosphatase (CIAP) at 37°C for 60 min.

The 5’ and 3’ ends of the Pit1 cDNA insert were simultaneously excised from PCMVSport6 by EcoRV (10U) and Xho1 respectively (Figure 1). pIREShrGFP2a was linearized for Pit1 with Sma1 (5’ end) and Xho1 (3’ end) at 37°C for 16 hr and heat inactivated at 80°C for 20 min. Another 20U of Sma1 were added and the reaction was incubated at 25°C for 4 hr and heat inactivated at 65°C for 20 min. The linearized
pIREShrGFP2a vector was dephosphorylated with CIAP. To verify enzyme efficacy, PCMVSport6 was singly cut with Xho1 and pIREShrGFP2a was singly cut with Sma1.

The MGC clone was linearized with a cut at the Pit2 cDNA insert’s 3’ end by BglI (Figure 2). The resulting ends were blunted with Klenow before a cut was made at Pit2’s 5’ end with EcoRI, releasing the Pit2 insert. Similarly, to prepare pIREShrGFP2a for Pit2, the vector was linearized with Sal1. These ends were blunted with Klenow before the 5’ end was trimmed

Figure 1. Schematic of Pit1 cDNA insert excision from its vector (PCMVSport6), linearization of pIREShrGFP2a for Pit1, and ligation of the Pit1 cDNA insert into the linearized pIREShrGFP2a.

Figure 2. Schematic of Pit2 cDNA insert excision from its vector (unknown MGC clone), linearization of pIREShrGFP2a for Pit2, and ligation of the Pit2 cDNA insert into the linearized pIREShrGFP2a.
with EcoRI. The linearized vector was dephosphorylated with CIAP.

Cotransporter DNA and linearized pIREShrGFP2a vectors were purified using 0.8% agarose gel electrophoresis and Qiagen’s QIAquick gel extraction kit.

A 3:1 molar ratio of the cotransporter insert to linearized pIREShrGFP2a vector was combined in a ligation reaction catalyzed by T4 DNA Ligase. As controls, reactions were prepared containing the linearized vectors but lacking the cotransporter DNA. The ligation reactions progressed for 16 hr at 16ºC. Ligase was heat inactivated for 10 min at 65ºC.

2.2 Amplification of cotransporter pIREShrGFP2a plasmids using bacteria

The cotransporter pIREShrGFP2a plasmids created by the ligation reactions were transformed into Stratagene XL-1 Blue Supercompetent cells. For each reaction, 100 µl of the XL-1 Blue Supercompetent cells and 1.7 µl of β-mercaptoethanol were incubated on ice for 10 min and gently mixed every 2 min. Two µl and 5 µl of each ligation reaction were each added to these cells. PUC18 plasmids were used as a control. The tubes were iced for 30 min and heat pulsed in a 42ºC water bath for 45 s. After 2 min on ice, 0.9 ml of room temperature SOC medium was added. The tubes were shaken between 200 and 220 rpm at 37ºC for 1 hr. Fifty µl and 200 µl of each reaction were plated onto LB with 100 µg/ml ampicillin agar and the plates were incubated overnight at 37ºC.

PCR screening verified the presence of cotransporter DNA in the bacteria colonies. Pit1 reactions were primed in the forward direction at 5'-AAGCGAATTCGAATGGACAG-3' and in the reverse direction at 5'-TGTGACAAACCAGGCCATAA-3'. Pit2 reactions were primed in the forward direction at 5'-GGCTATGATGACAGCACA-3' and in the reverse direction at 5'-ACCTGCAGGAAATGGAACAG-3'.

Elongation was achieved by Promega’s GoTaq Flexi DNA polymerase. Reactions were subjected to a 4 min 94ºC initial denaturing, followed by 30 cycles of 30 s of 94ºC denaturing, 53ºC annealing, and 72ºC extension.

The DNA concentration of each sample was determined by measuring the optical density of the 1 to 70 diluted samples at 260 nm and 280 nm using a Cecil CE 2041 2000 Series spectrophotometer.

Plasmid isolation was verified by sequencing and restriction digests. To verify by restriction digest, the isolated Pit1 pIREShrGFP2a and Pit2 pIREShrGFP2a plasmids were incubated with Not1 and Xho1. For controls, the Pit1 pIREShrGFP2a plasmids were incubated with Sma1 and the Pit2 pIREShrGFP2a plasmids with Sal1. The resulting DNA fragments were separated with 0.8% agarose gel electrophoresis.
2.4 Transfection of cotransporter pIREShrGFP2a plasmids into HeLa cells

HeLa cells were transfected with plasmid DNA using Invitrogen’s Lipofectamine 2000 Plasmid DNA Transfection Protocol. Lipofectamine 2000 was applied in a 3 to 1 ratio to micrograms of DNA and 5x10^4 Hela cells per ml were plated in 12-well plates in 1 mL of growth medium without antibiotics. DNA and lipofectamine were each diluted in 100 µl of Opti-MEM and after 5 min, the two solutions were combined and incubated at room temperature for 20 min. These solutions were then added to cells that had been plated the previous day. Media was changed 4 to 6 hr later. Cells were incubated at 37 ºC and harvested 24 or 48 hr later in lysis buffer containing pepstatin (2 µg/ml), PMSF (1 mM), and leupeptin (2 µg/ml), and aprotinin (1 µg/ml). The cell lysate was homogenized before use in Western blots.

2.5 Characterization of anti-cotransporter antibodies using Western blotting

BCA assays analyzed by a Packard Spectracount were used to assess protein quantity in the harvested cell lysates. Western blots were run with four types of cell lysates to characterize the anti-cotransporter antibodies: HeLa cells transfected with Pit1 pIREShrGFP2a, with Pit2 pIREShrGFP2a, and with pIREShrGFP2a as well as 293 cells transfected with a 55 kB HA gene (obtained from Abcam). Ten µg of protein was loaded for samples probed with the anti-HA antibody and 30 µg of protein was loaded for samples probed with the anti-cotransporter antibodies. One percent β-mercaptoethanol was added to the samples before they were loaded into 5% stacking and 10% resolving polyacrylamide gels.

Gels were transferred to PVDF membranes at 100 volts for 1.5 hr. The membranes were blocked in TBST containing 10% w/v nonfat dry milk powder and 1% w/v BSA. All antibodies were diluted in this blocking buffer. The membranes were incubated in primary antibodies for one hr at room temperature or overnight at 4°C. A rabbit polyclonal antibody to HA tag (1 mg/ml; obtained from Abcam: ab9110-100) was used at a 1:4000 dilution and anti-Pit1 and anti-Pit2 cotransporter antibodies developed by Li et al. [9], were used at 1:500 dilutions. After thorough washes (once for 15 min and four times for 7 min in TBST), the membranes were then incubated in Pierce’s stabilized goat anti-rabbit HRP-conjugated (10 μg/ml) at a 1:500 dilution for 1 hr at room temperature. After the primary and secondary antibody incubations, the membranes were washed as mentioned above. Membranes were then incubated with Pierce’s Supersignal West Dura Stable Peroxide Buffer and Pierce’s Supersignal West Dura Luminal/Enhancer solution (1:1 ratio) for 5 min. Film was exposed for time intervals ranging from 30 s to overnight.

3. RESULTS AND DISCUSSION

2.1 Insertion of cotransporter DNA into pIREShrGFP2a

The sizes of the DNA fragments generated from the restriction digests used to excise cotransporter DNA from their original vectors and to linearize pIREShrGFP2a for each cotransporter and revealed by gel electrophoresis confirmed the success of these goals (Figure 3).

The PCMVSport6 restriction digest to remove the Pit1 cDNA insert resulted in fragments of 3.3 kB and 4.4 kB. Since the Pit1 insert is 3.3 kB and the combined backbone and Pit1 insert is 7.7 kB, these band sizes indicate the successful removal of Pit1.

The Pit2 MGC clone restriction digest to remove Pit2 yielded bands at 1.3 kB, 1.5 kB, and 3.7 kB. The 3.7 kB band corresponds to the Pit2 insert, thus indicating the successful excision of Pit2, and implying that the Pit2 cDNA’s original vector’s backbone had an internal Xho1 site. This finding also reveals that the Pit2 cDNA insert’s original vector was not the one reported.

The restriction digests linearizing pIREShrGFP2a for Pit1, for Pit2, and as a control (Sma1) all yielded one band at 5 kB, matching its reported value. This was expected as the pIREShrGFP2a vector is 4936 bps and only approximately 25 base pairs were removed by its linearization for each insert.
2.2 Amplification of cotransporter pIREShrGFP2a plasmids using bacteria

Plating of the E. coli transformed with the cotransporter plasmids onto agar plates with ampicillin yielded 93 bacteria colonies potentially containing Pit1 pIREShrGFP2a and 33 potentially containing Pit2 pIREShrGFP2a. Plating of the E. coli transformed with the linearized pIREShrGFP2a for each cotransporter lacking the cotransporter insert yielded only 11 colonies for Pit1 and 18 colonies for Pit2, indicating an adequately low rate of recircularization. DNA from 17 potential Pit1 pIREShrGFP2a colonies and 13 potential Pit2 pIREShrGFP2a colonies were amplified by PCR and tested for cotransporter presence by gel electrophoresis. DNA from nine Pit1 and five Pit2 colonies yielded bands around 650 bps, while the other reactions did not yield bands (Figure 4), demonstrating that these colonies were successfully transformed with Pit1 pIREShrGFP2a or Pit2 pIREShrGFP2a.

2.3 Isolation of cotransporter pIREShrGFP2a plasmids

The ratio of sample absorbance at 260 nm versus the absorbance at 280 nm of the purified plasmids ranged from 1.74 to 2.45, an acceptable range. The DNA concentration of the samples ranged from 0.6 µg/µl to 3.21 µg/µl.

The identity of all plasmids was confirmed by sequencing and restriction digests (Figure 5). When Pit1 pIREShrGFP2a was linearized with Smal1, it yielded bands at 2.6 kB and 5.7 kB. As Smal1 cuts Pit1 106 and 2686 bps into the sequence, the 2.6 kB band is the middle of the Pit1 insert and the 5.7 kB band includes the 5.0 kB pIREShrGFP2a vector, the first 0.1 kB of Pit1, and the last 0.6 kB of Pit1. On the other hand, when Pit2 pIREShrGFP2a was digested with Sal1, it resulted in the same size DNA fragments as the uncut Pit2 pIREShrGFP2a plasmid, thus indicating that it was not cut. This
makes sense as Pit2 does not have a Sal1 site and as the Sal1 restriction enzyme site on the pIREShrGFP2a vector was destroyed by Klenow.

When Pit1 pIREShrGFP2a was cut with Xho1 and Not1, it yielded bands at 3.3 kb and 5.0 kb, indicating the direct excision of the Pit1 insert, and thus verifying its presence in the plasmid. Pit2 pIREShrGFP2a was also cut with Xho1 and Not1, yielding bands at 1.6 kb, 2.1 kb, and 5.0 kb. These DNA fragments indicate that Pit2 was successfully removed from the pIREShrGFP2a vector and internally cut by Xho1, a conclusion supported by the fact that Xho1 cuts Pit2 1668 bps into its sequence. These results show that both cotransporters’ cDNA was successfully inserted into pIREShrGFP2a.

2.4 Transfection of cotransporter pIREShrGFP2a plasmids into HeLa cells

As the pIREShrGFP2a backbone coexpresses GFP with the cotransporter protein, successful transfection could be assessed by fluorescence microscopy (Figure 6). pIREShrGFP2a (5.0 kb) had a higher transfection efficiency than Pit1 pIREShrGFP2a (8.3 kb), which, in turn, had a higher transfection efficiency than Pit2 pIREShrGFP2a (8.7 kb). This pattern can be explained by plasmid size, as smaller plasmids are easier to uptake.

2.5 Characterization of anti-cotransporter antibodies using Western blotting

All HeLa cells, including the nontransfected and lipofectamine controls, picked up a nonspecific HA signal whereas mouse cells (NIH3T3) did not (Figure 7). The 293 cells transfected with HA yielded a band at 55 kD, matching the purported protein size. As this evidence indicates that the anti-HA antibody was working correctly, these results could be due to a previous exposure of these HeLa cells to HA. As a result, HA could not be used to characterize the cotransporter antibodies.
On the other hand, specific bands were distinguished when Western blots of cell lysates over-expressing their respective cotransporter and cell lysates endogenously expressing the cotransporters were treated with the anti-cotransporter antibody and compared. When the anti-Pit1 antibody was used on cell lysates over-expressing Pit1, one band at 88 kD was significantly darker than the rest (Figure 8). Similarly, when the anti-Pit2 antibody was used on cell lysates over-expressing Pit2 a multimeric band between 90 and 121 kD was specifically darkened (Figure 9).

4. CONCLUSIONS

Now that anti-Pit1 and anti-Pit2 antibodies have been characterized, they can be used in future experiments to determine the distribution of cotransporters in different populations of cells and in the same population of cells exposed to different experimental conditions, as well as cotransporter movement in matrix vesicles.

The success of this characterization demonstrates the applicability of cloning procedures in antibody verification as it has now been shown that creating populations of cells expressing higher than endogenous levels of the protein of interest can produce differences in protein quantity such that antibodies can detect these levels in Western blots, and thus be characterized. This same procedure can also be used with different fusion proteins for different purposes. One future application is to repeat this procedure with a GFP fusion protein vector backbone. This would cause the individual cotransporters to fluoresce, thus providing a more sure way to assess cotransporter distribution in the previously mentioned conditions as well as a way to track cotransporter movement in matrix vesicles.
Vesicles released by VSMCs after exposure to elevated phosphorous conditions calcify while vesicles released under normal conditions do not, indicating that hyperphosphatemia promotes VSMC matrix vesicle calcification in addition to cellular calcification [10]. Being able to track the movement of these cotransporters in matrix vesicles will help determine how the cotransporters are involved in matrix vesicle mediated calcification and the pathway taken by the matrix vesicles once they leave VSMCs.

Although the anti-HA antibody could not verify our Western blotting results, these plasmids could be transfected into other cell lines to confirm these results. The HA tag would also be useful for immunocytochemistry. The anti-HA antibody could be used in conjunction with a fluorescent secondary antibody so that the actual cotransporters fluoresce, performing the same function as a GFP fusion protein backbone.

In conclusion, this study demonstrated that the methods of subcloning cDNA into a specialized vector backbone is a useful tool that can confer additional properties on the subsequent protein and that the method of overexpressing proteins in cell populations is an effective method to verify the efficacy of their antibodies. This successful characterization of anti-Pit1 and anti-Pit2 antibodies allows for their use in many future investigations that should expand our knowledge of type III cotransporters and their role in vascular calcification.

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