

Biophysical interaction between phospholamban and protein phosphatase 1 regulatory subunit GM

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Abstract Regulation of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA 2a) depends on the phosphorylation state of phospholamban (PLB). When PLB is phosphorylated, its inhibitory effect towards SERCA 2a is relieved, leading to an enhanced myocardial performance. This process is reversed by a sarcoplasmic reticulum (SR)-associated type 1 protein phosphatase (PP1) composed of a catalytic subunit PP1C and a regulatory subunit GM. Human GM and PLB have been produced in an *in vitro* transcription/translation system and used for co-immunoprecipitation and biosensor experiments. The detected interaction between the two partners suggests that cardiac PP1 is targeted to PLB via GM and we believe that this process occurs with the identified transmembrane domains of the two proteins. Thus, the interaction between PLB and GM may represent a specific way to modulate the SR function in human cardiac muscle.

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Key words: G subunit; Protein phosphatase 1 regulatory subunit; Phospholamban; Interaction; Co-immunoprecipitation; Surface plasmon resonance

1. Introduction

Phospholamban (PLB) is a 52-amino acid protein that regulates the activity of the cardiac sarco(endo)plasmic reticulum calcium ATPase (SERCA 2a). The physiological role of PLB on the activity of the calcium ATPase has been demonstrated using transgenic models in which the intracellular calcium transient and the myocardial relaxation are directly related to the level of expressed PLB [1]. Similarly, agents that inhibit the interaction between PLB and SERCA 2a, such as ellagic

acid, can increase the activity of the calcium pump [2]. In a dephosphorylated form, PLB decreases the affinity of SERCA 2a for calcium and phosphorylation of PLB removes its inhibitory effect towards SERCA 2a [3] which is able to pump more rapidly the Ca^{2+} from the cytosol into the SR [4]; this latter effect is correlated with an enhanced relaxation of the heart [5]. Phosphorylation of PLB at distinct sites by cAMP-dependent (at serine 16) and calmodulin-dependent (at threonine 17) protein kinases is reversed by a sarcoplasmic reticulum (SR)-associated protein phosphatase. MacDougall et al. [6] have shown that the major protein phosphatase associated with the cardiac SR is a type 1 protein phosphatase, one of the most important serine/threonine protein phosphatases in eukaryotic cells. Inhibitors of such phosphatases, like calyculin A, okadaic acid or tautomycin, have been shown to improve cardiac relaxation and calcium uptake by the SR [7,8]. Furthermore, we have recently reported that this effect is directly associated with an increased amount of phosphorylated PLB [9].

Although type 1 protein phosphatases are ubiquitously distributed, several lines of evidence suggest that like other enzymes they do not reach their physiological substrates by simple diffusion within cells. In contrast, it appears that phosphatases are frequently directed to particular loci in the vicinity of their substrates by interaction with targeting subunits [10]. Type 1 protein phosphatases are typical examples of heterodimer enzymes involved in controlling diverse cellular functions including glycogen metabolism, exit from mitosis, splicing of RNA and muscle contraction [11] where the same catalytic subunit (PP1C) is complexed to different targeting or regulatory subunits conferring substrate specificity.

In the heart, this heterodimer called PP1 (also known as PP1G) is composed of a 37-kDa catalytic subunit PP1C complexed to a larger subunit called GM (also known as G, RG1, PPP1R3) which is responsible for the association of PP1 with SR membranes [6]. In human, GM is one of the several regulatory subunits which have been isolated and characterised. Although GM, a 1122-residue protein, binds directly to glycogen, the mechanism by which it interacts with SR membranes is still unknown. It has been suggested that it could be due to a membrane-associated domain or a domain for interaction with a protein that binds to SR membranes [10,12,13].

The objective of the present study was to investigate whether PLB, one of the substrates of PP1, can also represent a molecular target for GM. To this end we have produced the human GM and PLB recombinant proteins and used co-immunoprecipitation and biosensor methodologies to evidence the physical interaction between GM and PLB. A method for predicting transmembrane domains was performed on

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Abbreviations: GM, G subunit = RG1 = PPP1R3 = protein phosphatase 1 (PP1) that targets the catalytic subunit (PP1C) to glycogen particles and SR; SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum calcium ATPase; ATPase, adenosine triphosphatase; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propane sulfonic acid; EGTA, ethylene glycol bis-(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS-T, phosphate buffer saline-Tween 20; Ig, immunoglobulin; Ig-HRP, Ig complexed to horseradish peroxidase; ECL, enhanced chemiluminescence; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HBS, HEPES buffer saline; RU, resonance unit; GL, targeting subunit for liver glycogen; PTG, for protein targeting to glycogen; M110, myofibrils; PCR, polymerase chain reaction

the 1122-residue sequence of GM and confirmed that the protein is able to anchor to the SR membrane via its C-terminal stretch. Together these data have led to a new way of understanding the molecular events involved in SERCA 2a activity.

2. Materials and methods

2.1. Antibodies and reagents

Anti-canine PLB monoclonal antibodies were obtained from Euro-medex, France and Dr Philip Cohen (University of Dundee, UK) provided sheep antibodies raised against GM subunit. Anti-mouse Ig-HRP-linked whole antibody (from sheep) and anti-rabbit Ig-HRP-linked whole antibody (from donkey) were purchased from Amersham Life Science, France. Anti-sheep IgG-HRP-linked whole molecule (from donkey) was purchased from Sigma. Protein A and G Sepharose were purchased from Pharmacia Biotech, France.

2.2. Human PLB cDNA cloning

PLB encoding cDNA was generated according to the sequence published by Fujii et al. [14], GenBank accession number m63603. The sequence was modified by the addition of a *HindIII* site at the 5' end and an *EcoRI* site at the 3' end. Eight oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser using standard cyanoethyl phosphoramidite chemistry. The oligonucleotides were gel-purified and assembled by pairs (OL1+OL2, OL3+OL4, OL5+OL6 and OL7+OL8) into the full-length species (159 bp):

OL1: 5'-AGCTTCCCGGATGGAGAAAGTCCAATACCTCACT-
CGCTCAGC-3'
OL2: 5'-CTTCTTATAGCTGAGCGAGTGAGGTATTGGACTTT-
CTCCATCCCGGGA-3'
OL3: 5'-TATAAGAAGGCCTCAACCATTGAAATGCCTCAAC-
AAGCAGTCA-3'
OL4: 5'-TGTAAGCTTTTGACGTGCTTGTGAGGCATTTCAT-
GGTTGAGGCT-3'
OL5: 5'-AAAGCTACAGAATCTATTTATCAATTTCTGTCTCAT-
CTTAATAT-3'
OL6: 5'-GCAAGAGACATATTAAGATGAGACAGAAATTGAT-
AAATAGATTC-3'
OL7: 5'-GTCTCTTGCTGATCTGTATCATCGTGATGCTTCTCT-
GAG-3'
OL8: 5'-AATTCTCAGAGAAGCATCACGATGATACAGATCA-3'

The sequence of PLB was then cloned into *HindIII/EcoRI*-digested pCDNA3 vector (Invitrogen, Carlsbad, CA) to give pCDNA3-PLB plasmid. PLB cDNA (173-bp fragment) was amplified by PCR from pCDNA3-PLB plasmid using a specific set of primers and subcloned into pCITE 2b vector (Novagen, Madison, WI) digested by *NcoI-EcoRI* in order to gain some efficiency in producing PLB protein in the rabbit reticulocyte translation/traduction system. A *NcoI* restriction site was added to the sense primer at the 5' end overlapping with the ATG initiation codon and allowing the PLB cloning in frame with IRES (internal ribosomal entry site) in pCITE plasmid. The *EcoRI* restriction site at the 5' end of the antisense primer was conserved. The primers used were as follows: PLB-*NcoI*, 27 mer: 5'-TAGGATC-CATGGAGAAAGTCCAATACC-3'; PL-*EcoRI*, 20 mer: 5'-CGAA-TTCTCAGAGAAGCATC-3'.

2.3. GM plasmid construct

cDNA encoding GM [15], GenBank accession number X78578, was provided by Dr Philip Cohen. The corresponding sequence (3366 bp) was inserted into pBluescript KS⁺ using the *Clal* site at the 5' end and the *BamHI* site at the 3' end.

2.4. Production of human PLB and GM in a rabbit reticulocyte lysate system

Protein synthesis was achieved using an in vitro transcription/translation system according to the manufacturer's recommended conditions (TNT T7/T3-coupled reticulocyte lysate system, from Promega, Madison, WI). Reactions were done including 60 μ Ci [³⁵S]methionine. Sense mRNA translation was performed using T7 RNA polymerase coupled with T7 promoter (PLB protein synthesis) and T3 RNA

polymerase coupled with T3 promoter (GM protein synthesis). Negative control experiments were run by doing antisense translation using alternative T3/T7 promoters with corresponding RNA polymerase enzymes and a positive control experiment was done using luciferase T7 control DNA. In vitro transcription-translation was performed in a reaction volume of 25 μ l with 1 μ g of each construct according to conditions recommended by manufacturer. The reaction was incubated at 30°C for 120 min and a 5- μ l aliquot of the synthesised proteins was analysed by SDS-PAGE.

2.5. Immunoprecipitation of PLB and GM

6 μ l of [³⁵S]PLB lysate (10⁷ cpm) was immunoprecipitated with 5 μ l of anti-canine PLB antibodies in a total volume of 1 ml PBS buffer at 4°C for 2 h under stirring conditions. Then 50 μ l of protein A Sepharose was added to the medium and incubated at 4°C for 45 min under stirring conditions. Five successive washing steps were done, Laemmli buffer (v/v) was added to the pellet, the solution was then boiled 5 min and finally centrifuged. The supernatant was loaded on the gel and run on a 15% polyacrylamide gel. The same protocol was applied for [³⁵S]GM lysate (10⁷ cpm), with sheep antibodies raised against GM subunit (anti-GM) and with protein G Sepharose instead of protein A Sepharose.

2.6. Co-immunoprecipitation of PLB and GM

The putative interaction between PLB and GM was investigated in the following conditions: 2 μ l of PLB T7 lysate and 50 μ l GM T3 lysate were incubated with 943 μ l PBS at 4°C for 1 h, 5 μ l of PLB monoclonal antibody was added to the medium and incubation was done at 4°C for 2 h. Then, 50 μ l of protein A Sepharose was added and incubated at 4°C for 45 min under gentle stirring. After three washes with PBS, the Laemmli buffer (v/v) was added to the pellet, boiled for 5 min, centrifuged and the supernatant was loaded on the gel and electrophoresed.

2.7. Revelation method for fluorographies

Proteins were fixed on the gel after migration. To increase detection sensitivity of fluorography, the gel was then incubated with Amplify reagent from Amersham. After drying, the gel was kept at -70°C overnight in contact with a hyperfilm before revelation.

2.8. Biomolecular interaction using surface plasmon resonance

2.8.1. Covalent immobilisation of anti-canine PLB monoclonal antibodies on biosensor Cm-5 chip. The aminosilane surface of the sensor chip was activated by injection of a 1/1 mixture of *N*-hydroxysuccinimide (NHS) and ethylenecarbodiimide (EDC) in order to form reactive esters. After activation, monoclonal anti-PLB antibodies (1–5 μ g (50 μ g/ml) in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% v/v surfactant P20 (HBS buffer)) were injected on the Cm-5 sensor chip and immobilised on the surface for 10 min, then washed with HBS buffer. Injection of a solution of ethanolamine in HBS buffer deactivates the excess of reactive esters. Anti-myoglobin was immobilised as reference flow cell in order to control non-specific binding.

2.8.2. Capture of PLB to immobilised anti-PLB. Once covalently coupled to the Cm-5 sensor chip, anti-PLB antibodies were used to capture PLB; 30 μ l of 1/5 diluted PLB T7 lysate was injected at a flow rate of 10 μ l/min. When PLB binds the refractive index of the medium at the surface of the sensor is changed in direct proportion to the bound mass. This change was detected according to the principle of surface plasmon resonance and was expressed in resonance units (RU). Regeneration was done using 2.5% zwittergent in H₂O at a flow rate of 10 μ l/min. Both anti-myoglobin flow cell and antisense PLB lysate were used as negative controls.

2.8.3. Binding of GM protein to captured PLB. 25 μ l of pure GM lysate was injected over the Cm-5/anti-PLB/PLB surface. Regeneration was done as mentioned above. Both anti-myoglobin flow cell and antisense GM lysate were used as negative controls.

2.9. Sequence analysis of GM

GM was analysed using the neural network-based system PHD (Profile fed neural network systems from HeiDelberg) in order to identify a putative transmembrane domain. The method generated several parameters like prH which represents the probability for assigning helical transmembrane regions as well as an index of reliability, Rel. Details of the methodology have been reported by Rost et al. [16].

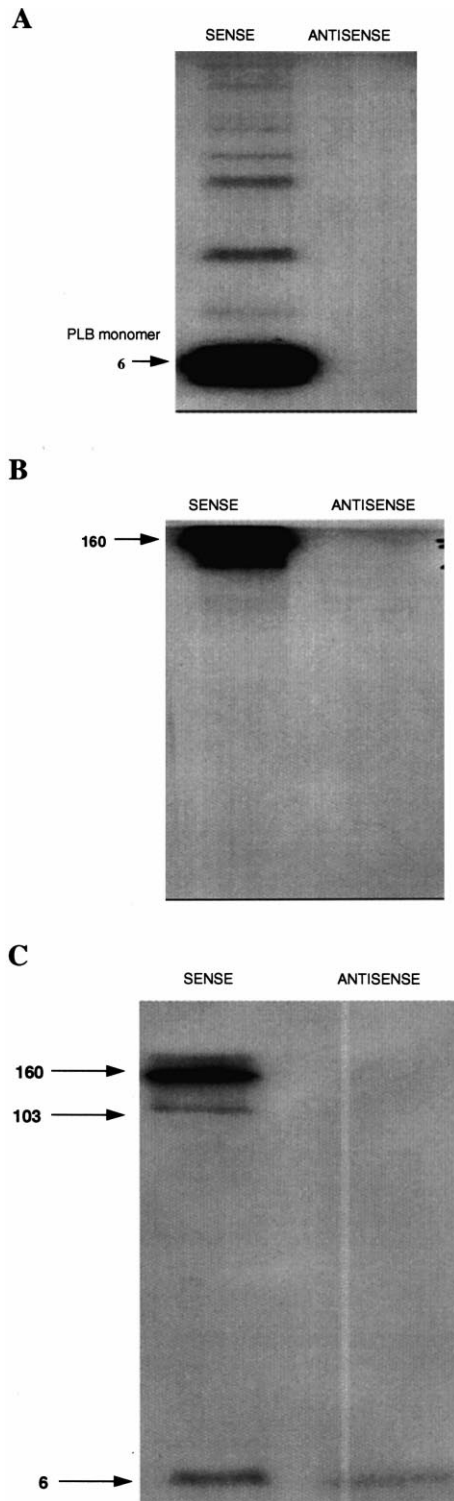


Fig. 1. Co-immunoprecipitation experiments. A: Immunoprecipitation of PLB. PLB synthesis was performed using an in vitro transcription/translation system (rabbit reticulocyte lysate). 6 μ l of [35 S]PLB lysate was subjected to immunoprecipitation using 5 μ l of anti-PLB antibodies at 4°C for 2 h. Then 50 μ l of protein A Sepharose was added to the medium and incubation was done at 4°C for 45 min. After washing steps, Laemmli buffer (v/v) was added to the pellet, the solution was boiled 5 min and finally centrifuged. The supernatant was loaded on the gel, run on polyacrylamide gel electrophoresis and autoradiographed. Sense mRNA translation was obtained using T7 RNA polymerase coupled with T7 promoter. Negative controls (antisense) were run with T3 RNA polymerase. PLB is mainly detected as a monomer of 6 kDa as indicated with an arrow. Additional spots represent traces of oligomeric forms (from dimer to pentamer). B: Immunoprecipitation of GM. GM synthesis was performed in rabbit reticulocytes lysate as described for PLB. 6 μ l of [35 S]GM lysate was subjected to immunoprecipitation using 5 μ l of anti-GM antibodies at 4°C for 2 h as described for PLB immunoprecipitation (A). Sense mRNA translation was obtained using T3 RNA polymerase coupled with T3 promoter. Negative controls (antisense) were performed using T7 RNA polymerase. The 1122-residue protein is detected at a molecular weight of 160 kDa although a truncated form, already reported in the literature, can also be observed at 103 kDa. C: Co-immunoprecipitation of PLB (6 kDa) and GM (160 kDa) from lysates. PLB T7 lysate was incubated with GM T3 lysate with relative concentrations of 2 μ l PLB and 50 μ l GM at 4°C for 1 h. The resulting complex was incubated with 5 μ l of anti-PLB antibodies and then precipitated by 50 μ l of protein A Sepharose at 4°C for 45 min, washed, boiled and subjected to SDS-PAGE (15% gel polyacrylamide). Autoradiography analysis was performed by molecular analyst software from Bio-Rad. All antibodies failed to reveal immunoreactive bands in lysates prepared with antisense mRNA translation products for GM and PLB.

cDNAs. Immunoprecipitation of human PLB protein in the presence of specific monoclonal antibody shows that PLB was produced in its monomeric form and to a lesser extent in its pentameric form (Fig. 1A) whose full dissociation occurs in SDS by application of heat [17]. In the presence of the polyclonal antibody raised against GM, human GM was detected mainly as the expected 160-kDa protein (Fig. 1B), but to a lower extent as a truncated 103-kDa protein. Antisense mRNA translation products for PLB and GM used as control experiments did not yield any immunoprecipitate suggesting that native reticulocyte lysates are free of endogenous PLB and GM proteins. Finally, the experiment illustrated in Fig. 1C and performed with monoclonal antibodies raised against

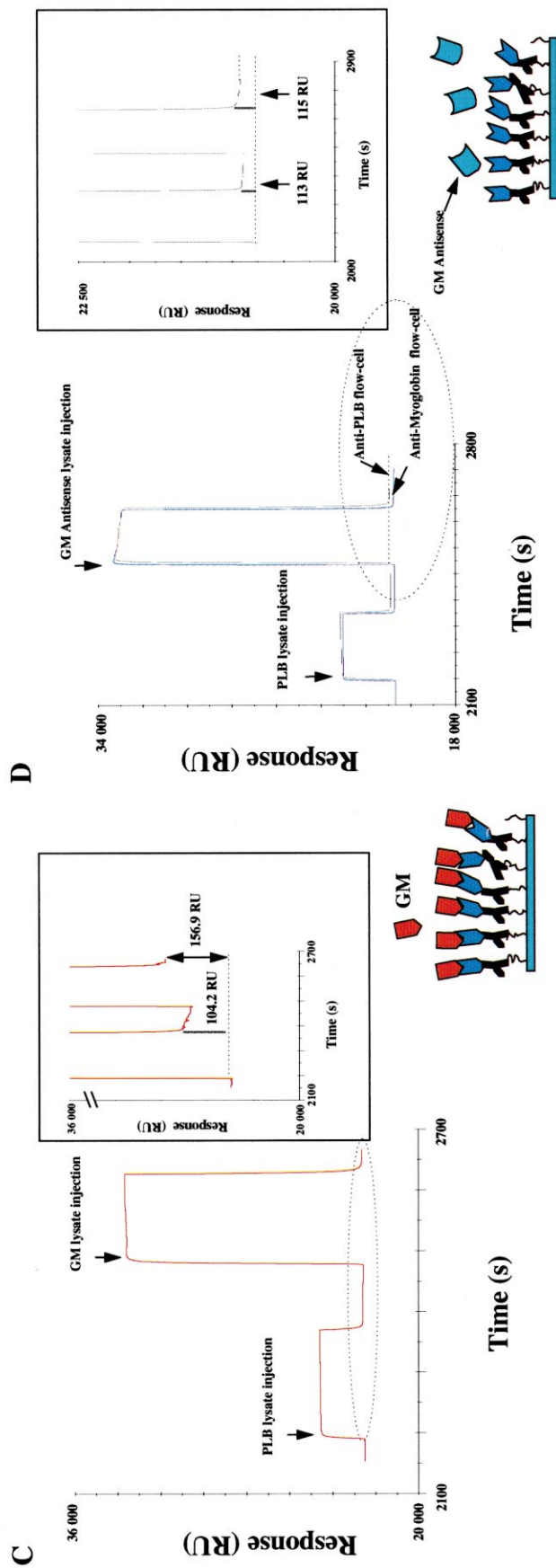
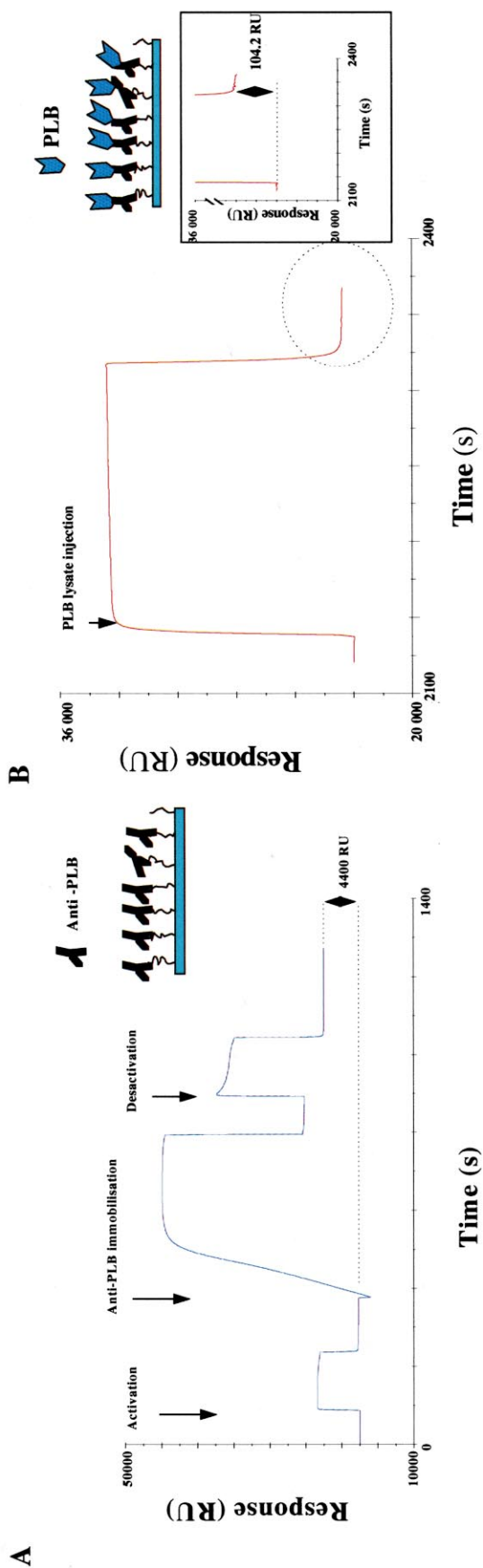
3. Results

3.1. GM interacts specifically with PLB

We have performed experiments to investigate if a biophysical interaction occurs between the human proteins PLB and GM using two different approaches.

First, immunoprecipitation followed by co-immunoprecipitation experiments were carried out with the human proteins [35 S]GM and [35 S]PLB produced in vitro from their respective

Fig. 2. Biomolecular interaction of PLB and GM detected by surface plasmon resonance. A: Immobilisation of anti-PLB antibodies. Anti-PLB was immobilised on a dextran surface using EDC/NHS chemistry as described in Section 2. Anti-PLB immobilisation induces a marked increase in response which is reduced during washout. The observed relevant signal is measured after deactivation by ethanolamine and compared to the initial baseline. B: Interaction of PLB to captured specific antibodies. Once covalently coupled to the Cm-5 sensor chip, anti-PLB antibodies were used to capture PLB; 30 μ l of 1/5 diluted PLB T7 lysate were injected at a flow rate of 10 μ l/min. Regeneration was performed using 2.5% zwittergent in H₂O at a flow rate of 10 μ l/min. C: Binding of GM to PLB. 25 μ l of pure GM lysate was injected over the Cm-5/anti-PLB/PLB surface. Regeneration was done using 2.5% zwittergent in H₂O at a flow rate of 10 μ l/min. D: Binding on non-specific flow cell. Anti-myoglobin has been immobilised as a control of non-specific binding or with GM antisense lysate injected as negative control of the interaction with PLB.



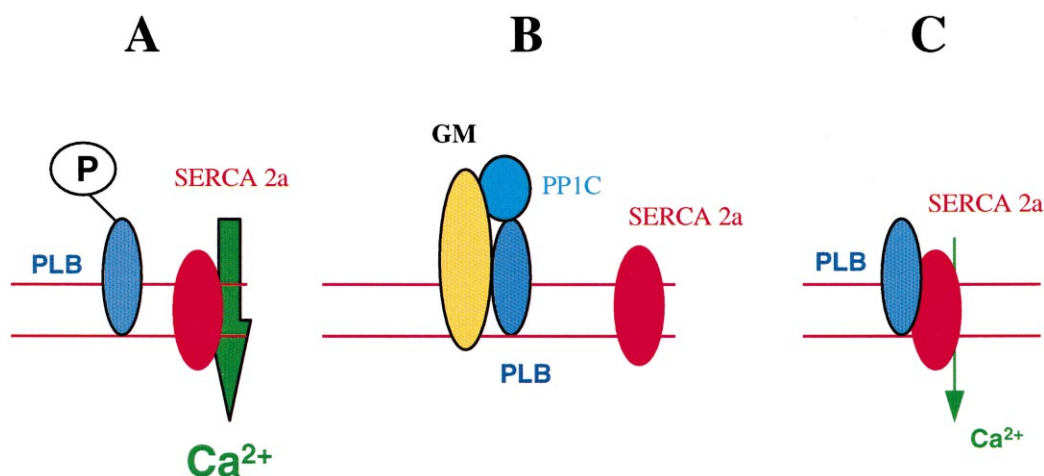


Fig. 4. Proposed three-step pathway for regulation of SERCA 2a via PP1/PLB interaction. A: SERCA 2a is displayed in an active form as PLB is in its phosphorylated state. B: Heterotrimeric complex PP1C/GM/PLB involved in the dephosphorylation process of PLB: GM directs PP1C towards the membrane protein PLB via its transmembrane domain. C: Dephosphorylated PLB interacts with SERCA 2a making it less active and thus reducing the calcium uptake.

products used as negative controls for GM and PLB are devoid of binding capacity (115–113 = 2 RU; Fig. 2D).

3.2. GM as an integral membrane protein

The neural network-based analysis performed on the 1122 residues of GM gave rise to the identification of a putative unique transmembrane domain located at the very end of the C-terminal stretch of the protein as indicated by prH values ranging from 5 to 9. This domain is comprised of about 25 hydrophobic residues. Reliability of the prediction method is monitored by the Rel index showing that the identified segment PYFLLFLIFLITVYHYDLMIGLTFYVL is likely to span the lipid bilayer (Fig. 3).

4. Discussion

Several mammalian regulatory proteins have been isolated and characterised. Amongst them, GL targets PP1C to liver glycogen [18,19], M110 is responsible for the association of PP1 with the myofibrils of skeletal muscle [20,21] and spinophilin is a novel PP1 binding protein identified recently in dendritic spines [22]. In fact, at least 15 regulatory subunits able to interact with the same catalytic subunit PP1C have been identified to date in mammals [11], allowing PP1 to regulate diverse functions. It is conceivable that the interaction of regulatory subunits with PP1C may be transitory and mutually exclusive, leading to the formation of different complexes in a dynamic way. Recently, the novel PP1 glycogen-associated regulatory subunit identified in 3T3-L1 adipocytes and termed PTG (for protein targeting to glycogen) has been shown to exhibit a dual activity similarly to GM [23]. In addition to targeting PP1 to the glycogen particle, PTG can also form complexes with PP1 substrate enzymes that regulate glycogen metabolism, namely glycogen synthase, glycogen phosphorylase, and phosphorylase kinase. In this case, PTG not only binds PP1C and glycogen but also co-localises PP1 with its substrate at the glycogen particles suggesting that PTG is responsible for assembling metabolic enzymes for the localised reception of intracellular signals [23,24]. In a similar way, the present experiments suggest that GM, which targets PP1C to both glycogen particles and the SR of striated

muscle [13], may localise PP1C close to its PLB substrate in order to control specifically the activation of the calcium ATPase. Indeed, PLB is commonly viewed as a regulatory protein which, when dephosphorylated, reduces or even suppresses the activity of SERCA 2a by decreasing its affinity for calcium. Phosphorylation either on serine 16 or threonine 17 or on both of them relieves the inhibitory effect of PLB towards SERCA 2a allowing the ATPase to pump calcium more rapidly from the cytosol to the SR as illustrated in Fig. 4A [4].

By analogy with skeletal muscle, it has been suggested that the complex of PP1C and GM associated with SR membranes [25,26] likely represents the major PLB phosphatase in the heart [6]. MacDougall et al. [6] showed that in a rabbit cardiac muscle fraction enriched in SR membranes, PP1 accounted for 90% of the associated PLB (serine 16 or threonine 17) phosphatase activity. In heart tissue, the protein previously identified in skeletal muscle has been reported to be a heterodimer composed of a 37-kDa catalytic PP1C complexed to a much larger subunit (GM) of about 160 kDa [6]. Another study has shown that the glycogen-bound form of GM has been previously purified as a heterodimer composed of the 37-kDa catalytic subunit described above and of a proteolytically sensitive 103-kDa glycogen binding subunit [27]. Moreover, Hubbard et al. [28] demonstrated that the intact GM subunit is a 161-kDa protein and that the 103-kDa species they called G' was a product of proteolysis. Interestingly, we observed the presence of a 103-kDa peptide in our experimental conditions, which is also able to interact with PLB as shown in the co-immunoprecipitation experiments. Although we cannot exclude that it is a proteolytic product of the reaction, the 103-kDa peptide is more likely to result from an internal translation initiation process commonly observed *in vitro*.

Furthermore, the present experiments indicate that PP1 regulatory subunit GM, which is responsible for targeting the catalytic subunit to its substrate ensuring subsequent dephosphorylation, is tightly and specifically associated with PLB. Human PLB is comprised of two main domains: an N-terminal segment of about 30 residues extending in the cytosol and a C-terminal domain of 22 hydrophobic residues which spans membrane bilayers [29,30]. These two domains are involved in

cytosolic and intramembrane interactions with the calcium pump and it has been reported that the inhibitory interaction site lies entirely in the transmembrane sequences of PLB and SERCA 2a [31]. When compared to PLB, GM is a large protein of 1122 amino acids and it is established that its N-terminal stretch is responsible for the binding of the catalytic subunit PP1C [32,33]. A previous hydropathy analysis of the amino acid sequence of GM had revealed a hydrophobic region likely to anchor the protein to the membrane of the SR [12]. Therefore to further understand what structural features could be involved in the interaction between GM and PLB, an additional sequence analysis was performed on GM. It showed that the hydrophobic residue-rich domain located between amino acids 1077 and 1103 possesses criteria of a putative transmembrane region able to anchor PP1 to the SR lipid bilayer (Fig. 3), establishing a connection between PP1C and PLB (Fig. 4B). Thus, a biophysical interaction should exist in cardiac human cells between the regulatory subunit GM and PLB as evidenced by this study with human recombinant proteins both by co-immunoprecipitation and by the biosensor technology and we believe that such an interaction may occur at least through the transmembrane domain of the two proteins as reported for the binding of PLB and SERCA 2a [31]. All together, these results clearly demonstrate that PLB is a molecular target for GM and this is consistent with the role of the regulatory protein providing specificity to a ubiquitously distributed catalytic PP1C.

Finally, the present work suggests that the disruption of the interaction between GM and PLB would be highly specific; in this way PLB will remain in a phosphorylated state avoiding its inhibitory effect towards SERCA 2a. Therefore, the study of GM/PLB interaction could represent a completely novel pharmacological approach to increase calcium uptake by the SR and thus to improve cardiac relaxation.

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