Research article

CHARACTERIZATION OF PROTEINS ASSOCIATING WITH 5' TERMINUS OF PGHS-1 mRNA

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Abstract: Induction of *Prostaglandin Endoperoxide H Synthase-1 (PGHS-1)* gene has been previously documented in a few studies during events such as development and cellular differentiation. However, molecular mechanisms governing the regulation of *PGHS-1* gene expression and contributing to changes in protein levels are poorly understood. Using the MEG-01 cell model of *PGHS-1* gene induction, our laboratory has previously demonstrated that the 5'UTR and the first two exons of PGHS-1 mRNA had a significant impact on decreasing the translational efficiency of a reporter gene and suggested that the presence of a secondary structure is required for conservation of this activity. This 5'end of PGHS-1 mRNA sequence has also been shown to associate with nucleolin protein. In the current study, we set to investigate the protein

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Abbreviations used: ARRE-2 – antigen receptor response element-2; ATCC - American Type Culture Collection; DRBP76 - double-stranded RNA-binding protein-76; dsRNA – double stranded RNA; FBS - fetal bovine serum; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; IL-2 – interleukin-2; IP – immunoprecipitation; IRES – internal ribosome entry site; LC-MS – liquid chromatography mass-spectrometry; Luc – luciferase; MKP-1 – mitogen-activated protein kinase phosphatase 1; MNEI - monocyte/neutrophil elastase inhibitor; mRNP - messenger ribonucleoprotein; NCL – nucleolin; NF45 - nuclear factor 45; NF90 - nuclear factor 90; NFAT - nuclear factor of activated T-cells; ORF - open reading frame; PG – prostaglandins; PGHS - prostaglandin endoperoxide H synthase; PMA - phorbol 12-myristate 13-acetate; SB1 or serpin B1 - serine protease inhibitor; TPA - 12-O-tetradecanoylphorbol -13- acetate; Tx - thromboxanes; UTR - untranslated region

composition of the mRNP (messenger ribonucleoprotein) associating with the 5'end of PGHS-1 mRNA and to identify its protein members. RNA/protein binding assays coupled with LC-MS analysis identified serpin B1 and NF45 (nuclear factor 45) proteins as potential members of PGHS-1 mRNP complex. Immunoprecipitation experiments using MEG-01 protein extracts validated mass spectrometry data and confirmed binding of nucleolin, serpin B1, NF45 and NF90. The RNA fraction was extracted from immunoprecipitated mRNP complexes and association of RNA binding proteins, serpin B1, NF45 and NF90, to PGHS-1 mRNA target sequence was confirmed by RT-PCR. Together these data suggest that serpin B1, NF45 and NF90 associate with PGHS-1 mRNA and can potentially participate in the formation a single or a number of PGHS-1 ribonucleoprotein complexes, through nucleolin that possibly serves as a docking base for other protein complex members.

INTRODUCTION

The enzyme prostaglandin endoperoxide H synthase (PGHS), also refered to as cyclooxygenase (COX), participates in a cascade of enzymatic reactions leading to the production of prostanoids from arachidonic acid [1]. Two distinct forms of PGHS have been identified and characterized; PGHS-1 and PGHS-2, both catalyzing the synthesis of prostaglandin H_2, the precursor of prostaglandins (PG) and thromboxanes (Tx). Historically, PGHS-1 is referred to as the constitutive isoform, whereas PGHS-2 is considered inducible with increased expression upon stimulation, particularly during inflammation [2, 3]. Contrary to initial observations, induction of *PGHS-1* gene has been documented in a number of studies such as during development and cellular differentiation [4-6] and examples continue to accumulate.

The molecular mechanisms governing the regulation of *PGHS-1* gene expression and contributing to an increase in protein levels are poorly understood. Our research group had previously documented regulation at the translational step of PGHS-1 enzyme synthesis within a context of MEG-01 cell differentiation following treatment with TPA [7, 8]. We noted a 2 to 3-day delay between an early rise in PGHS-1 mRNA and an increase in protein steady state levels [7, 8]. Our data are in agreement with other studies, which also demonstrate a delay between mRNA transcription and increase in the levels of PGHS-1 protein [9-11]. Induction of *PGHS-1* gene expression is not limited to hematopoietic cells but also has been documented in neuroblastoma cell lines and in human nasal mucosa cells, emphasizing the importance of regulation at the translational step for *PGHS-1* gene expression [12, 13]. Previous work from our laboratory has shown that the 5'UTR and the first two exons of PGHS-1 mRNA had a large

impact on decreasing the translational efficiency of a reporter gene [14]. The 5'UTR and first two exons sequence must be present in order to have a negative effect on translation and suggest the presence of a secondary structure required for this activity. This 5'end of PGHS-1 mRNA sequence has also been shown to directly interact with nucleolin protein [14]. Additional experiments have shown that mutation of the two nucleolin response elements (NRE) [15] found within PGHS-1 5'UTR and the first two exons, respectively, partially reduced the negative effects when reporter constructs were tested [14].

Once exported to the cytoplasm, not all mRNAs immediately enter the translationally active pool, some are held in a translationally silent state awaiting either proper subcellular localization or a signal that the appropriate protein synthesis is now required [16]. Specific regulation of mRNA translation is often mediated by, but not limited to, regulatory proteins that bind to the 5' and/or 3'UTR of the target mRNA [16, 17]. These mRNA binding proteins, in their majority, target structures or sequences specific to some mRNAs [16]. We designed an experiment to identify additional RNA binding proteins that might have an effect on the efficiency of PGHS-1 mRNA translation.

Generally, traditional affinity purification methods are targeted towards identification of complex partners associated with a specific RNA-binding protein such as co-purifying RNA or proteins, and not partners associated with a specific RNA transcript. A disadvantage of such techniques include the fact that they can demonstrate interactions that are initiated prior to lysis of the cells but can also include *in vitro* artificially generated complexes due to high binding ability of its partners [18]. In addition, contamination of the immunoprecipitate with nonspecific RNA transcripts is a problem. Increasing the salt concentration of the washes might enhance the stringency of association but may also disrupt weak and low affinity RNA-protein interactions. Use of formaldehyde cross linking can strengthen associations and aid in identification of specific RNA-protein interactions [19]. A downside of formaldehyde use is that it leads to the formation of multi-molecular bridges, creating artifacts of macromolecules that are pulled down as a single complex and limit the identification of direct and indirect protein-RNA interaction [20].

To overcome problems associated with traditional affinity purification methods, we employed a newly reported protocol of RNA affinity tag purification [21-23] facilitating the detection of ribonucleoprotein complexes interacting with the 5' end of PGHS-1 mRNA sequence, which was previously shown to have an impact on the translational efficiency. The advantage of the S1 affinity tag protocol is the use of small RNA which serves as an affinity tag that binds with high affinity and specificity to streptavidin affinity resins even in high salt concentrations. Ribonucleoprotein complexes can be eluted under mild, native conditions allowing the retention of an intact complex structure [21, 22]. Therefore, in the current study, we set to investigate the composition of protein complexes associating with the 5'end of PGHS-1 mRNA using S1 tag protocol.

MATERIALS AND METHODS

Constructs and *in vitro* transcription of S1-tagged transcripts

Constructs (pLuc135 and pLuc135GAPDH) used in the experiments were prepared as described previously [14]. Briefly, to produce pLuc135 reporter constructs, the PGHS-1 sequences obtained as PCR products corresponding to the 5′UTR and the first two exons were cloned into pLuc (pcDNA3.1(-)) (Invitrogen) vector containing Luciferase (Luc) ORF inserted between the NheI and XhoI restriction sites located upstream from the *Luciferase* gene. A construct containing a random *GAPDH* gene sequence (lacking AUG or STOP codons) was prepared by introducing a SacII restriction site into the pLuc135 construct using the Quick Change® Site Directed Mutagenesis kit (Stratagene) to allow for the insertion of a random 100 nucleotide long GAPDH sequence, two nucleotides downstream from the AUG start codon of PGHS-1. DNA template for *in vitro* transcription were generated by linearizing pLuc135 and pLuc135GAPDH constructs with NheI and XhoI restriction enzymes and adding an S1-tag [21, 22] template up-stream of the PGHS-1 5'UTR sequence by using forward (GGAATTCTAATACGACTCACTATAGGGACCGACCAGAATCATG CAAGTGCGTAAGATAGTCGCGGGCCGGGCTGCACTCTGCGTCCCGCAC) and reverse (TTCACTGGCGTGGGCGCCCCTGG) primers and Platinum DNA Taq polymerase (Invitrogen) in a PCR reaction. PCR conditions were the following: 1 cycle of 94°C for 2 min; 40 cycles of 94°C for 30 s, 50°C 30 s and 72°C 1 min; using an automated thermal cycler (Mastercycle® personal; Eppendorf). PCR products were gel purified with QIAquick gel extraction kit (QIAGEN) and used as DNA templates for *in vitro* transcription of S1-tagged cRNAs using MEGAshortscript kit (Ambion). Template DNA was eliminated with 10 U of DNase I (Amersham Biosciences). RNA transcripts were purified using RNeasy mini kit (QIAGEN) according to manufacturer's protocol. Purified transcripts were then quantified by spectrophotometry and used for S1-tagged mRNP purification assays.

Cell culture and protein extract preparation

MEG-01 cells were purchased from the American Type Culture Collection (ATCC). Cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) in the presence of 100 units/ml Penicillin and 100 μg/ml Streptomycin in 75 cm^2 tissue culture flasks. The cultures were maintained at 37°C in an incubator with a 5% CO_2 atmosphere. The media was partially replaced with fresh RPMI 1640 supplemented with 10% FBS and Penicillin/Streptomycin every 4 days. For preparation of total MEG-01 protein extracts, the cells were harvested by mechanical detachment and collected by centrifugation at 4°C, 855×g for 5 min. Cell extract preparation was carried out using PARIS Protein and RNA isolation system (Ambion) according to manufacturer's instructions.

Purification of S1-tagged mRNPs

Protocol used for isolation of S1-tagged mRNP complexes was modified from the one previously described [21, 22]. All the steps of affinity binding were performed at 4°C. Streptavidin agarose beads (Invitrogen) were pre-washed several times with binding buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl) and pre-incubated with MEG-01 protein extract partially equilibrated in binding buffer for 1 hour at 4°C. Following three washes in binding buffer of 15 minutes each, the beads were allowed to bind to the added *in vitro* transcribed cRNA for 1 hour. MEG-01 protein extract was added to RNA coated streptavidin beads and incubated for an additional 1 hour, followed by two 1.5 hour long washes with binding buffer. Samples were centrifuged to remove unbound proteins. S1-tagged mRNP complexes were eluted under native conditions by incubating with binding buffer containing 5 mM δ-biotin (USB) at 4°C for 45 minutes. The bound proteins were precipitated from eluted complexes by adding 2× Laemmli buffer and incubating at 90°C for 5 min. Proteins bound to RNA were visualized after resolution on 10% SDS-PAGE stained with Silver Stain. Protein bands were excised and sent to the Dalhousie University Dr. Doucette's group and subjected to LC-MS analysis.

Immunoprecipitation

MEG-01 cell lysates were used for immunoprecipation (IP) assay to detect protein-associated PGHS-1 mRNA as described previously [24] and with slight modifications. Growing MEG-01 cells were stimulated with 10 ng/ml TPA overnight, harvested by mechanical detachment and collected by centrifugation at 4°C, 855 x g for 10 min. Cells were washed with ice cold 1x PBS, snap frozen in liquid nitrogen and resuspended in 1 mL hypotonic buffer (10 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.3 μl/ml Protease Inhibitors and 1 U/ml RNase Inhibitors), followed by 5 min incubation on ice and final lysis with Nonidet P-40 to a 1% final concentration, while incubating on ice for additional 10 minutes. Cell lysates were vortexed, centrifuged at 4000 x g at 4°C for 15 minutes and supernatant was removed and used for immunoprecipitation. Protein G-Agarose beads were washed in 0.1% BSA in PBS, pH 7.4, three times to remove storage buffer prior to addition to cell lysate supernatant. Each 250 μl of supernatant prepared from cell lysate was pre-cleared with 25 μl of Protein G-agarose beads (Thermo Scientific) at 4°C for 30 min. Antibodies were covalently coupled to protein G-agarose beads (Thermo Scientific) according to protocol described by Trinkle-Mulcahy *et al.* [25]. Precleared lysates were added and incubated for 1 hour at 4°C with rotation. The immune complexes were washed five times with wash buffer (10 mM Tris, pH 7.6, 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 1 U/ml RNase inhibitors) and collected by centrifugation. The formed complexes were eluted with elution buffer (50 mM Tris, pH 8.0, 100 mM KCl, 1% SDS, 10 mM EDTA, 1 U/ml RNase inhibitors) at 65°C for 10 min. Samples were split into two. One half was used for western blot analysis. Another half was first treated with 1 μl DNase I (Amersham

Biosciences) and then with 100 μg of Proteinase K (New England Biolabs) at 37°C, RNA extracted using TRIZOL (Invitrogen) according to manufacturer's protocol and used for RT-PCR.

Antibodies
The anti-nucleolin rAb was purchased from Sigma, the anti-serpinB1 rAb was purchased from Santa Cruz Biotechnology, the anti-NF45 mAb was purchased from Abnova, and the anti-NF90 (anti-DRBP76) mAb was purchased from BD Biosciences. Secondary anti-mouse and anti-rabbit antibodies were purchased from Promega.

Western blot
Protein complexes obtained following immunoprecipitation procedure were denatured by boiling in Laemmli buffer and resolved using 10% SDS-PAGE as described previously [6]. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane and incubated with a corresponding primary antibody. Immunoreactive bands were detected by incubation of the membrane with a secondary antibody followed by incubation with the chemiluminescence reagents (Boehringer Ingelheim).

RT-PCR
RT-PCR was performed on extracted RNA obtained from immunoprecipitation samples using the SuperScript One-Step RT-PCR kit (Invitrogen) according to manufacturer's protocol. Specific PGHS-1 primers (sense 5' CACCCCAGCAG CCGCGCCATGAGCC 3' and anti-sense 5' GGTGTGGGGCAATCTTTAGG CACAG 3') were used to amplify a 500 bp fragment in the entire 5'UTR and the ORF. RT-PCR conditions were: 1 cycle of 50°C for 30 min and 94°C for 2 min; 30 cycles of 94°C for 15 s, 55°C 30 s and 72°C 1 min; and final extension cycle of 72°C for 10 min using an automated thermal cycler (Mastercycle® personal; Eppendorf).

RESULTS

Previous studies from our laboratory demonstrate that translation of PGHS-1 mRNA is delayed after induction of gene expression [8]. Furthermore, this translational regulation is influenced by the presence of both the 5'UTR and the first two exons of PGHS-1 and nucleolin protein that binds to the 5'end of PGHS-1 mRNA [14]. It is also important that both the 5'UTR and the first two exon sequences are in continuity to each other since insertion of a random sequence separating these two regions resulted in elimination of translational down-regulation, possibly due to disruption of original RNA secondary structure [14]. We examined the possibility that protein complexes, consisting of several proteins in addition to already identified nucleolin protein, may bind to the 5'terminus of PGHS-1 transcript with potential influence on translational efficiency. To characterize the protein complexes binding to the PGHS-1 5'UTR

and the first two exons sequence and identify the mRNA binding proteins involved, we performed RNA-protein interaction studies.

Partial PGHS-1 RNA corresponding to the 5'UTR and first two exons and preceded by an S1-tag RNA sequence, which has a high affinity for streptavidin, was generated by *in vitro* transcription and linked to streptavidin coated beads (Fig. 1A). Total MEG-01 protein extracts were used as a source of proteins. Following incubation of RNA coupled beads with MEG-01 protein extracts, protein complexes which remained bound to the PGHS-1 sequence were eluted, resolved using SDS-PAGE and visualized using silver stain. Controls for the RNA-protein interaction studies included a sample without RNA linked to streptavidin beads, as well as one with the PGHS-1 RNA sequence interrupted with GAPDH sequence separating the 5'UTR from the first two exons (Fig. 1A)

Fig. 1. Protein binding to the 5′end of PGHS-1 mRNA. A − The 135-nucleotide sequence of the 5′ end of PGHS-1 mRNA corresponding to the entire 5′UTR and first 2 exons and coupled to S1 tag was generated by *in vitro* transcription. Analogous construct, also containing GAPDH sequence lacking AUG or STOP codons, inserted immediately downstream from the PGHS-1 AUG initiation codon and before the first two exons, was *in vitro* transcribed and used as control. B − Total MEG-01 protein extracts were used as a protein source and incubated with RNA coupled streptavidin beads. Proteins bound to the PGHS-1 mRNA sequence were eluted with biotin containing buffer, resolved on SDS-PAGE and revealed by silver staining. Note the band detected with the PGHS-1 135 nts sequence (third lane from the right, indicated by arrow) and absent when the PGHS-1 sequence interrupted with a GAPDH sequence (135GAPDH, second lane from the right) is used or when no RNA probe is included in the experimental protocol (beads only elution, first lane on the right). The band was sent for LC-MS analysis. SDS gel is a representative of 3 independent experiments.

which lacks the ability to negatively regulate translation when tested with a reporter construct [14]. Experimental samples along with controls were resolved on a gel and examined for differences in band pattern. A band migrating at approximately 45 kDa was specific for the PGHS-1 RNA sample (Fig. 1B, third lane from the right, indicated by arrow) and was absent from the control lanes: sample containing elution from streptavidin beads only (Fig. 1B, first lane from the right) and sample containing PGHS-1 RNA sequence interrupted by GAPDH random sequence which disrupts original secondary structure of PGHS-1 RNA (Fig. 1B, second lane from the right). Hence, this protein band is specific to the predicted secondary structure formed by the PGHS-1 5'UTR and partial ORF. This band was excised and sent for sequencing by LC-MS technique and determination of protein identity. An identically sized piece of gel excised at the same molecular weight range (~45 kDa) in the lane containing no RNA served as a negative control for LC-MS analysis and protein sample containing 100 ng BSA run on the same SDS-PAGE served as a positive control (not shown). MS analysis yielded a list of peptides and proteins. This list has been shortened to include only those proteins for which two or more unique peptides were identified by LC-MS to eliminate possible false positive hits and excluded keratin group of proteins. Top two proteins from the list, serpin B1 and NF45, for which the maximal number of peptides were identified, were chosen for further analysis and binding activity to PGHS-1 mRNA. Condensed results of protein identification are summarized in Tab. 1. Additionally, a common complex partner of NF45 and a known dsRNA binding protein [26-28], NF90, was predicted to be part of the mRNP complexes and was tested for its ability to bind PGHS-1 mRNA sequence.

Tab. 1. Proteins identified by MS following analysis of ribonucleoprotein complex obtained from RNA/protein interaction study using S1 tagged 5'end of PGHS-1 RNA and MEG-01 protein extracts. Complete list of identified proteins available in supplemental materials, http://dx.doi.org/10.2478/s11658-010-0005-5.

Protein identified	Accession number	Known function	Identified peptides
Leukocyte elastase inhibitor (serpin B1)	P30740	Protease inhibitor	8
Interleukin enhancer-binding factor 2 (NF45)	Q12905	Transcription factor RNA binding protein	5

Direct binding of the individual proteins serpin B1, NF45 and NF90, to the PGHS-1 mRNA sequence was investigated by the means of immuno-precipitation assays. In this experiment, anti-nucleolin, anti-serpin B1, anti-NF45 and anti-NF90 antibodies were used to immunoprecipitate ribonucleoprotein complexes from MEG-01 cell lysates. Immunoblotting with the anti-nucleolin antibody revealed that both serpin B1 and NF90 associate with 90 kDa form of nucleolin when this complex is precipitated from cell lysates with corresponding

antibodies (Fig. 2 top panel). The 90 kDa isoform of nucleolin has been previously reported to be bound to PGHS-1 mRNA 5'terminus [14]. Similarly, nucleolin was found to reciprocally co-immunoprecipitate with serpin B1 and NF90 when anti-nucleolin antibody was used for immunoprecipitation (Fig. 2, second and forth panels from the top). Interestingly, these two proteins, serpin B1
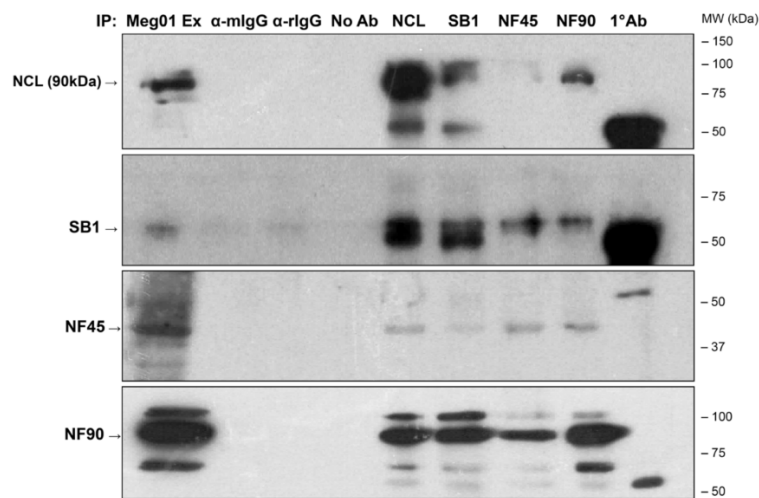

Fig. 2. Binding of serpin B1, NF45 and NF90 proteins to nucleolin. Immunoprecipitations (IP) from MEG-01 cell lysates with nucleolin, serpin B1, NF45 and NF90 antibodies (lanes 5, 6, 7 and 8 from the left, respectively) and subsequent immunoblotting with all four antibodies as indicated on the left. Positions of the molecular weight markers are indicated on the right. Proteins of interest are indicated by arrows. An aliquot of primary antibody used for immunoblotting (lane 9 from the left) was used as a control to distinguish precipitated protein from the heavy and the light chains of the antibody used in the immunoprecipitation. Anti-mouse IgG, anti-rabbit IgG and "no antibody" (lanes 2, 3 and 4 from the left, respectively) were used as a control for nonspecific binding in the immunoprecipitation reactions. No protein immunoprecipitation was observed with negative controls. All gels are representative of 3 independent experiments.

and NF90, were also found to co-immunoprecipitate with each other as well as NF45 (Fig. 2 three bottom panels). On the other hand, direct interaction between NF45 and nucleolin is uncertain. When the protein complexes are precipitated from cell lysates with anti-nucleolin antibody and then detected with anti-NF45 antibody, a band is observed implying association between nucleolin and NF45 (Fig. 2 third panel from the top). Alternatively, when anti-NF45 antibody is used for immunoprecipitation and anti-nucleolin antibody used for immunoblotting no band is observed, which is indicative of either a weak or an indirect association between NF45 and nucleolin and/or a weakened ability of the antibody to affinity purify protein complexes in comparison to other antibodies used. An additional control consisting of loading a primary antibody (Fig. 2 first lane from the right on all panels), consecutively used for immunoblotting,

ensured the bands observed were not due to nonspecific interaction of a secondary antibody with the primary antibody used in immunoprecipitation and eluted off the protein G coated beads during SDS-PAGE but rather represented specific proteins precipitated from cell lysates. The presence of protein specific bands and the absence of these bands in the control lanes indicate that co-immunoprecipitation was not an artifact of protein affinity for the materials used in immunoprecipitation and suggest formation of a single or several protein complexes comprising of two or more proteins. These proteins, as demonstrated here, include any of the following: nucleolin, serpin B1, NF45 and NF90.

In the second series of experiments, RNA was extracted from immuno-precipitated ribonucleoprotein complexes and the presence of PGHS-1 transcript in immunoprecipitates was detected by RT-PCR. Using PGHS-1 specific primers, a 500 bp product was amplified from MEG-01 extracted total RNA and the sample immunoprecipitated with the anti-nucleolin antibody that served as a positive control and previously shown to be bound to PGHS-1 mRNA (Fig. 3, lanes 2 and 5 from the left respectively). No PCR products were detected, on the gel depicted in Fig. 3, in the absence of protein specific antibodies (No Ab, lane 11), in the presence of unspecific anti-mouse IgG (α-mIgG, lane 9) or anti-rabbit IgG (α-mIgG, lane 10), by skipping the RT step (No RT, lane 3) or by omitting
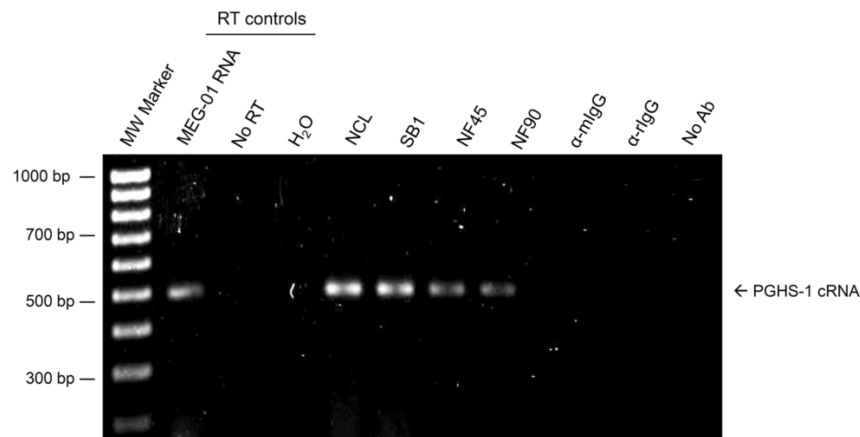

Fig. 3. Binding of serpin B1, NF45 and NF90 proteins to PGHS-1 mRNA. Protein-antibody complexes formed using anti-nucleolin, serpin B1, NF45 and NF90 antibodies were immunoprecipitated from MEG-01 cell lysates and used for RT-PCR reactions with primers designed to amplify a segment of the PGHS-1 ORF. Amplified PCR fragments corresponding to PGHS-1 mRNA were detected in MEG-01 total RNA sample and in the Anti-NCL, anti-serpin B1, anti-NF45 and anti-NF90 Ab samples (lanes 2, 5, 6, 7 and 8 from the left) but not in the control samples: No RT, H_2O, anti-mouse IgG, anti-rabbit IgG, No Ab (lanes 3, 4, 9, 10 and 11 from the left). Gel is a representative of 3 independent experiments.

template (H_2O, lane 4). PCR products were presents in samples immunoprecipitated using anti-serpin B1, anti-NF45 and anti-NF90 antibodies (Fig. 3, lanes 6, 7 and 8, respectively). These experiments suggest that nucleolin, serpin B1, NF45 and NF90 proteins are associating with PGHS-1 mRNA either separately or by participating in the formation of ribonucleoprotein complexes that associate with the 5' end of PGHS-1 mRNA, possibly through nucleolin that serves as a docking base for other complex members (Fig. 4).
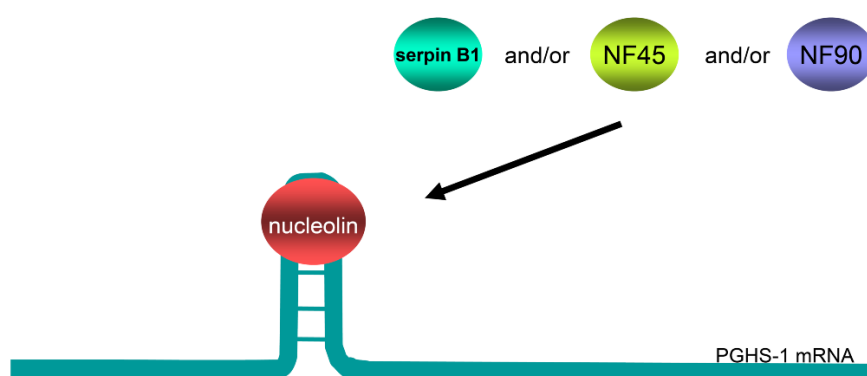

Fig. 4. Proposed model of PGHS-1 mRNA-protein interactions. The 5'end of PGHS-1 mRNA forms a stable stem loop structure providing the basis for the docking of mRNA binding proteins including nucleolin, serpin B1, NF45 and NF90, which bind individually or form complexes and are potentially involved in translational regulation of PGHS-1 transcript.

DISCUSSION

The expression of *PGHS-1* gene and mechanisms leading to the regulation of PGHS-1 enzyme levels are poorly understood. Our previous work has identified the step of PGHS-1 mRNA translation as regulated during the differentiation of MEG-01 cells. The present study stemmed from our previous findings which demonstrated a decrease in translational efficiency exerted by the 5' end of PGHS-1 mRNA sequence also shown to directly associate with nucleolin protein [14]. In the current study we used an S1 RNA tag affinity purification protocol to identify additional proteins which may be members of a putative mRNP complexes forming at the 5'end of PGHS-1 mRNA. Two proteins, serpin B1 and NF45, with maximum number of unique peptide hits identified by LC-MS (Tab. 1) after affinity purification, were used for validation experiments. An additional protein, NF90, was included in the investigation since it is a common binding partner of NF45 and a known dsRNA binding protein [26-28]. The affinity purification results were further validated by immunoprecipitation assays using antibodies specific to nucleolin, NF45, NF90 and serpin B1 followed by RT-PCR experiments to detect PGHS-1 mRNA. Our data demonstrate that

NF45, NF90 and serpin B1 are indeed associating with the 5' end of PGHS-1 mRNA either as a part of bigger protein complexes or individually.

Following the S1 RNA tag affinity purification experiment, we selected a single and intense band of ~45 kDa for LC-MS analysis as the 45 kDa band was absent from the control sample lacking translational activity (135GAPDH) as well as from the sample lacking RNA. A piece of gel of identical size was excised at the same molecular weight range (~45 kDa) from the lane containing no RNA and was used as a control sample to eliminate identification of proteins with affinity for streptavidin beads and not specifically binding to PGHS-1 mRNA. Additional controls for mass spectrometry analysis could be used to explore the nature of protein and PGHS-1 mRNA interactions and to determine if more than one type of protein complex form with the PGHS-1 mRNA sequence. Such controls can include but not limited to the following: 1. Mutants of 135 PGHS-1 mRNA sequence in which the primary sequence is mutated to disrupt the previously predicted secondary structure [14] and could demonstrate the importance of the stem loop for mRNP binding; 2. A non PGHS-1 nucleic acid sequence known to bind identified proteins can be a useful positive control and 3. RNA sequence corresponding to PGHS-1 5'UTR and first two exons and treated with RNase prior to addition of streptavidin beads could serve as a good control in removing proteins with a high affinity for streptavidin and not specific to the RNA. The protocol we used allowed us to demonstrate specific association between the 5' end of PGHS-1 mRNA, and a group of proteins including nucleolin, NF90, NF45 and serpin B1 proteins identified by mass spectrometry analysis. To confirm the association of the identified proteins to the PGHS-1 mRNA sequence, we conducted immunoprecipitation and RT-PCR experiments.

Serpin B1 is a 42 kDa protein also referred to as monocyte/neutrophil elastase inhibitor (MNEI). Initially identified as a fast-acting elastase inhibitor, serpin B1, found in high levels in neutrophils and monocytes [29]. Studies show that serpin B1 efficiently inhibits proteases with elastase- and chymotrypsin-like specificities through efficient reactions at two active sites [30]. Serpin B1 belongs to a large family of serpin proteins that regulate a wide range of physiological processes including blood coagulation, complement activation, inflammation, extracellular matrix remodeling, and tumor suppression [31, 32]. More specifically, serpin B1 belongs to the clade B of serpin protein family, with nucleocytoplasmic localization. While lacking an obvious classical nuclear import signal, serpin B1 is imported into the nucleus through a facilitated (active) pathway [33]. Accumulation of serpin B1 within the cell nuclei has been linked to terminal differentiation of U937 cell line induced by retinoic acid [34]. By contrast, stimulation of U937 cells with a phorbol ester, PMA, which also caused cell differentiation, had very little effect on changes in serpin B1 expression levels [35]. This study also demonstrates that serpin B1 has a temporal pattern of expression with predominantly higher levels of transcript in early haemopoietic progenitor cells and decreased levels in more mature populations

of cells. One of the limitations of this study is that evidence for gene expression is based on a semi-quantitive RT-PCR without any reference to actual protein production levels. Studies using a MEG-01 model of hematopoietic cell differentiation in our own laboratory have shown a similar pattern for *PGHS-1* gene expression, with the mRNA levels starting high within the adherent megakaryocyte cell population and decreasing in the detaching platelet-like population of cells [6].

NF45 (nuclear factor of activated T cells 45 kDa, also referred to as ILF2) and NF90 (nuclear factor of activated T cells 90 kDa, also referred to as ILF3) are transcription factors originally discovered in Jurkat T-cells [36]. NF45 and NF90 were initially purified as components of an NFAT complex that interacts with the antigen receptor response element-2 (ARRE-2) sequence within the promoter region of the *interleukin-2 (IL-2)* gene and act as transcriptional enhancer [37, 38]. It is suggested that NF45 acts as a regulator of NF90 activity [28]. Originally identified as DNA-binding proteins [26, 36, 37], NF90 and NF45 do not contain a recognized sequence-specific DNA-binding domain and the complex does not appear to interact with DNA directly [26, 39]. Nevertheless, NF45 and NF90 were shown to bind to dsRNA as well as to proteins [26, 39]. In neuronal cells, the NF45/DRBP76 heterodimer complex can inhibit translational initiation by binding to the 5'UTR of human rhinovirus type 2 that encoding the internal ribosome entry site (IRES) [40]. NF90 has been shown to increase the half-life of IL-2 mRNA following T-cell activation [41]. The increase of mRNA half-life mediated by NF90 has also been documented for MKP-1 mRNA in HeLa cells upon H_2O_2 treatment [42]. This group also reported that the binding of NF90 also appeared to suppress MKP-1 translation [42]. These effects of NF90 are consistent with an earlier report showing that the alternatively spliced form of NF90, TCP80, suppresses the translation of beta-glucosidase mRNA [43] suggesting a broad role of NF90 in translational repression. More recently, experiments aiming at depleting NF45 or NF90 in HeLa cells resulted in a mitotic defect resulting in an accumulation of multinucleated cells [39]. Interestingly, megakaryocytes, used in our study, are notorious for their polyploidy state during their differentiation process, with ploidity sometimes reaching 64 [44]. Moreover, a study by Raslova *et al.* [47] examining gene expression during polyploidization of megakaryocytes using microarray analysis approach had reported the down-regulation of *NF90* gene expression during polyploidization of human megakaryocytes.

According to a recent report, NF45 is amongst the list of proteins that could potentially be a contaminant which binds non-specifically to the sepharose affinity matrix [25] which was used in our experiments to identify protein partners associated with the 5' terminus sequence of PGHS-1 mRNA. However, validation of LC-MS results by immunoprecipitation experiment demonstrates the association between nucleolin, serpin B1, NF45 and NF90 with PGHS-1 mRNA. Previous studies have listed nucleolin, NF90 and NF45 as members of the same protein complex in HeLa S3 and HEK 293 cells [39]. Our data do not

allow for concluding if one or more complexes are formed between the PGHS-1 mRNA sequence and the identified proteins or whether these proteins bind individually to PGHS-1 mNRA. Nucleolin-binding ribonucleoprotein complexes have been characterized in human 293EBNA cells and similarly to our findings, NF90 was found to be present in the immunopurified complexes [45]. In addition, the same authors provided evidence that isolated RNP complexes represent endogenous nucleolin-binding RNP complexes [45]. Therefore, it is reasonable to assume that NF45, NF90 and serpin B1 co-precipitate with nucleolin due to specificity of association and function, and suggesting that the association in not only occurring *in vitro* but can potentially occur in cells. Our data, however, are limited to protein binding observed in immunoprecipitates generated from MEG-01 cell lysates. We also acknowledge that our data so far do not indicate whether the identified PGHS-1 mRNA binding proteins are part of a large multi-component PGHS-1 mRNP complex or whether the identified proteins bind to PGHS-1 mRNA as a series of smaller complexes including some but not all of the identified proteins. The absence of nucleolin protein in the immunoprecipitate obtained with anti-NF45 antibody could indicate an indirect or a weak interaction between those two proteins. Data from other laboratories indicate that NF45 associates with nucleolin through NF90, which is a common complex partner of NF45 and also binds to nucleolin [39]. Dissecting the nature of complex composition and association between RNA binding proteins requires extensive experimentations.

Reverse transcription of mRNA species associated with the eluted protein complexes confirm that PGHS-1 mRNA interacts with serpin B1, NF45 and NF90 proteins. However, there is insufficient evidence as of yet to presume a direct interaction between PGHS-1 mRNA and serpin B1, NF45 or NF90 proteins. Individual mRNP components can serve as adaptors that allow different mRNA transcripts to interact with numerous intracellular machineries mediating their subcellular localization, translation, and decay [16]. Some protein adaptors are involved in positive interactions and serve as activators of a particular process, whereas others disrupt the positive interactions and act as repressors [16]. By containing a variety of different adaptors bound to the transcript, individual mRNAs can respond to numerous inputs, allowing their expression to be fine-tuned to changing conditions and cellular events. Indeed, nucleolin, can serve as such an adaptor, or rather a docking protein [46], that is able to recruit specific cofactors that serve as activators or repressors. We previously reported on the presence of highly stable secondary structures formed by the 5'UTR and the first two exons of PGHS-1 mRNA [14] which can potentially serve as the docking site for the attachment of nucleolin or a bigger protein complex. We therefore hypothesize that while Serpin B1, NF45 and NF90 might not bind directly to PGHS-1 mRNA, they can potentially participate in the formation of a single or several ribonucleoprotein complexes through nucleolin that possibly serves as a docking base for other protein complex members (Fig. 4).

These data provide evidence for the association of serpin B1, NF45 and NF90 proteins with PGHS-1 mRNA and nucleolin protein. However, further studies are necessary to explore the nature of this association and whether this is a direct or an indirect RNA-protein interaction. It is also essential to explore the effects of these proteins on the efficiency of PGHS-1 mRNA translation and elucidate what roles these proteins play during megakaryocyte maturation and platelet formation. The original discovery of NF45, NF90 and serpin B1 in hematopoietic cells taken together with the temporal pattern of serpin B1 expression suggest a possible role in megakaryocyte differentiation which consequently affects *PGHS-1* gene expression.

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