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The RNA binding protein hnRNP-K mediates post-transcriptional regulation of uncoupling protein-2 by angiopoietin-1

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ABSTRACT

Angiopoietin-1 (Ang1) is a ligand for the receptor tyrosine kinase Tie2 and has key roles in the development of the vascular system and vascular protection. In a screen to define signalling pathways regulated by Ang1 in endothelial cells we found the RNA-binding protein hnRNP-K to be phosphorylated in response to Ang1. The ligand stimulated both tyrosine phosphorylation of hnRNP-K and recruitment of the tyrosine kinase Src to the RNA-binding protein. In endothelial cells hnRNP-K was found bound to mRNA encoding the mitochondrial protein uncoupling protein-2 (UCP2). Ang1 stimulation of cells resulted in the release of UCP2 mRNA from hnRNP-K. Using *in vitro* assays we confirmed direct binding between hnRNP-K and UCP2 mRNA. Furthermore Src induced phosphorylation of purified hnRNP-K and prevented UCP2 mRNA binding. Tyrosine 458 in the RNA-binding protein was found to be required for suppression of UCP2 mRNA binding by Src phosphorylation. In addition to releasing UCP2 mRNA from hnRNP-K, Ang1 induced an increase in UCP2 protein expression in endothelial cells without affecting total UCP2 mRNA levels. Consistent with the known effects of UCP2 to suppress generation of reactive oxygen species, Ang1 limited ROS production in endothelium stimulated with tumour necrosis factor-α. Taken together these data suggest that UCP2 mRNA is present in endothelial cells bound to hnRNP-K, which holds it in a translationally inactive state, and that Ang1 stimulates Src interaction with hnRNP-K, phosphorylation of the RNA-binding protein, release of these transcripts and upregulation of UCP2 protein expression. This study demonstrates a new mechanism for post-transcriptional regulation of UCP2 by the vascular protective ligand Ang1. The ability to rapidly upregulate UCP2 protein expression may be important in protecting endothelial cells from excessive generation of potentially damaging reactive oxygen species.

1. Introduction

Angiopoietin-1 (Ang1) is a secreted ligand essential for development of the vascular system as well as maintenance and protection of the mature vasculature [1,2]. This ligand is a glycoprotein of approximately 70 kDa [3] and, together with angiopoietins 2, -3 and -4, is a member of the angiopoietin family of proteins [1,2]. In development Ang1 is required for the latter stages of blood vessel formation, where it acts to regulate branching and interaction of endothelial cells in nascent vessels with extracellular matrix and perivascular support cells [4,5]. Post-development effects of Ang1 are broadly protective and the ligand has been shown to inhibit microvessel regression, suppress vascular inflammation and inhibit vessel leakage [1,2,6]. The protective effects of Ang1 are important in maintaining a quiescent and stable vasculature [1]. At the cellular level Ang1 acts to suppress apoptosis, enhance migration, promote monolayer integrity and inhibit inflammatory gene expression in endothelial cells [1,2,6].

The primary receptor for Ang1, and the other angiopoietins, is the receptor tyrosine kinase Tie2 [3]. Binding of Ang1 to Tie2 results in phosphorylation and activation of the receptor and stimulation of downstream signalling cascades including the phosphatidylinositol-3-kinase pathway [7]. This pathway has key roles in the anti-apoptotic actions of Ang1 [8,9]. Phosphatidylinositol-3-kinase also contributes to Ang1 suppression of endothelial inflammation [10], as does ABIN2, an intermediate that is recruited to activated Tie2 and acts as an inhibitor of the inflammatory transcription factor NFkB [11,12]. Another signalling intermediate involved in Ang1 action is the protein Dok-2 [13]. This adaptor protein is recruited to the activated receptor where it gets phosphorylated leading to binding of Nck and p21-activating kinases that are involved in Ang1 activated endothelial migration [14].

Although a number of signalling pathways have been implicated in mediating Ang1 actions, our understanding of how this ligand regulates cellular function is still comparatively poor. In particular we know relatively little about the pathways involved in the cellular protective effects of Ang1. In order to uncover signalling components mediating

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Ang1 effects, therefore, we performed a proteomic screen to identify proteins that become tyrosine phosphorylated in response to Ang1 in endothelial cells. Here we report the RNA-binding protein heterogeneous nuclear ribonucleoprotein-K (hnRNP-K) as a new intermediate in Ang1 signalling involved in post-transcriptional control of the antioxidant mitochondrial uncoupling protein-2.

2. Materials and methods

2.1. Materials

Recombinant human Angiopoietin1 was purchased from R&D Systems and used at a concentration of 2.8 nM (monomer). UCP2 antibodies were from Biolegend, and hnRNP-K antibodies were from Abcam. All other reagents were as previously detailed [11]. hnRNP-K cDNA was a kind gift from Professor Sui Huang (Northwestern University Medical School, Chicago, IL, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Promocell and maintained in medium 199 supplemented with 20% fetal calf serum (FCS), heparin 5 units/ml and endothelial cell growth supplement (50 μg/ml). Only passages 3–5 were used in experiments. The EA.hy.926 endothelial cell line [15] was a generous gift from C. J. Edgell (University of North Carolina) and were maintained Dulbecco's modified Eagle's medium supplemented with 10% FCS. Chinese Hamster Ovary (CHO) cells were maintained in minimum essential media supplemented with 10% FCS. All cells were cultured at 37 °C in a humid atmosphere containing 5% CO_2 in air. For transfection, 80% confluent cells were transfected with plasmid DNA using lipofectamine 2000 according to manufacturer's protocol. Cells were cultured for 18 h post-transfection before lysis.

2.3. Immunoprecipitation and Immunoblotting

Before lysis cells were washed with PBS then lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.0, and 0.5% Nonidet P-40 detergent, 1 mM DTT, protease inhibitor cocktail) added. The cells were scraped and the lysate centrifuged at 10,000 *g* for 10 min at 4 °C. For immunoprecipitation, relevant antibodies were added to the supernatants and incubated at 4 °C on a roller for 3 h after which protein-A sepharose was added and left for a further 3 h on the roller at 4 °C. The immunoprecipitate was centrifuged at 3000 *g* for 1 min and washed in lysis buffer 3 times. Proteins were eluted by the addition of Laemmli sample buffer containing 100 mM dithiothreitol and heated to 95 °C for 5 min before being resolved by SDS–PAGE. For the analysis of whole cell protein extracts, lysates were mixed with Laemmli buffer as above. Proteins were transferred to nitrocellulose membranes, probed with the relevant antibodies and visualized with peroxidase-conjugated secondary antibodies and chemiluminescent detection. Optical densities of bands on immunoblots were quantified using ImageJ software.

2.4. RNA co-immunoprecipitation

RNA was isolated from immunoprecipitates using the protocol of Tenenbaum [16] with a few modifications. Cells were lysed directly in lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.6, and 0.5% Nonidet P-40 detergent, 1 mM DTT, 100 U/ml SuperaseIn RNase inhibitor and protease inhibitor cocktail). Cells were scraped and centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant was removed and re-centrifuged at 14,000 *g* for 10 min at 4 °C. Antibodies were allowed to bind to protein-A beads for 2 h at 4 °C in NT2 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.05% Nonidet P-40 before addition to the supernatant. The supernatant was added to antibody coated protein-A beads with the addition of 0.75 ml NT2 buffer, 1 mM DTT, 15 mM EDTA and an extra 100 U/ml of RNase inhibitor. The immunoprecipitation was allowed to proceed for 3 h at room temperature with agitation after which immunoprecipitates were centrifuged at 3000 *g* for 20 s. The beads were washed in NT2 buffer 3 times. The beads were then resuspended in NT2 buffer with the addition of 0.1% SDS and 30 μg of proteinase K and incubated at 55 °C for 30 min. Immunoprecipitated RNA was isolated using phenol:chloroform: isoamyl alcohol extraction and ethanol precipitation.

2.5. PCR

Isolated RNA was reverse transcribed using RETROscript (Ambion) and random decamer primers. The cDNA was used as template for subsequent PCR to screen for the presence of UCP2 using the primers UCP2 forward (5′ATGGTTGGGTTCAAGGCCACAGAT3′) and UCP2 reverse (5′CATGCTCAGAGCCCTTGGTGTAGA3′).

2.6. Subcloning, expression and purification of hnRNP-K

Full-length human hnRNP-K was cloned into the *Escherichia coli* expression vector pLEICS-01 (University of Leicester) encoding an N-terminus His6-tag using the primers; forward 5′TACTTCCAATCCATGG AAACTGAACAGCCAGAAGAAACCT3′ and reverse 5′TATCCACCTTTACT GTCATTAGAATCCTTCAACATCTGCA3′.

BL21 (Novagen) were transformed with hnRNP-K expression vector using the manufacturer's instructions. Transformed *E. coli* was grown in Luria-Bertani broth at 37 °C, 250 rpm to an optical density of 0.6 (600 nm) after which cells were induced for expression of hnRNP-K using Isopropyl β-D-1-thiogalactopyranoside at 1 mM at 25 °C, 250 rpm for 4 h. Cells were harvested and lysed using 10 ml of lysis buffer (50 mM Tris pH8.0, 150 mM NaCl, 5% glycerol, 10 mM imidazole pH 8.0, protease inhibitor cocktail from Roche). The cells were sonicated in the lysis buffer. The debris was pelleted at 15,000 g at 4 °C for 30 min. The supernatant was incubated with 1 ml of nickel beads (Ni-NTA superflow from Qiagen) for 16 at 4 °C under gentle rocking. The beads were then gently transferred to a 10 ml polypropylene column. The beads were washed with 10 column volumes of wash buffer (2 M NaCl, 20 mM Tris pH 6.8, 60 mM imidazole and 1% triton). hnRNP-K protein was eluted using 5 ml elution buffer (1 M imidazole 5% glycerol, pH 8.0). The hnRNP-K protein was dialysed against phosphate buffered saline. The purity and concentration were analysed using coomassie stained gels and absorbance at 280 nm respectively.

2.7. Site-directed mutagenesis

Tyrosine 458 of hnRNP-K was mutated to phenylalanine using the primers; forward 5′CAGTGTGAAGCAGTTTGCAGATGTTGAAG3′ and reverse 5′CTTCAACATCTGCAAACTGCTTCACACTG3′. Mutations were introduced using Quick-Change mutagenesis (Agilent Technologies).

2.8. In vitro transcription and biotinylation of UCP2 RNA

1 μg of plasmid containing full length UCP2 with 5′ and 3′ UTR's (1–1647 nt) was linearized with Eco RV. The linearized plasmid was purified using acetate/ethanol precipitation. UCP2 was *in vitro* transcribed using MAXIscript *in vitro* transcription kit (Ambion). Transcription was confirmed by gel electrophoresis and concentration was determined using an absorbance at 260 nm. The *in vitro* transcribed RNA was biotinylated using the Pierce RNA 3′-biotinylation kit from Thermo Scientific according to the manufacturer's instructions.

2.9. Src-mediated phosphorylation of hnRNP-K

Purified hnRNP-K was phosphorylated by activated Src (Sigma). Briefly, 500 ng of purified hnRNP-K was incubated with 1 ng of active src in kinase assay buffer (5 mM MOPS, pH 7.2, 2.5 mM glycerol 2-