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Efficient soluble expression of active recombinant human cyclin A2 mediated by *E. coli* molecular chaperones

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ABSTRACT

Bacterial expression of human proteins continues to present a critical challenge in protein crystallography and drug design. While human cyclin A constructs have been extensively characterized in complex with cyclin dependent kinase 2 (CDK2), efforts to express the monomeric human cyclin A2 in *Escherichia coli* in a stable form, without the kinase subunit, have been laden with technical difficulties, including solubility, yield and purity. Here, optimized conditions are described with the aim of generating for first time, sufficient quantities of human recombinant cyclin A2 in a soluble and active form for crystallization and ligand characterization purposes. The studies involve implementation of a His-tagged heterologous expression system under conditions of auto-induction and mediated by molecular chaperone-expressing plasmids. A high yield of human cyclin A2 was obtained in natively folded and soluble form, through co-expression with groups of molecular chaperones from *E. coli* in various combinations. A one-step affinity chromatography method was utilized to purify the fusion protein products to homogeneity, and the biological activity confirmed through ligand-binding affinity to inhibitory peptides, representing alternatives for the key determinants of the CDK2 substrate recruitment site on the cyclin regulatory subunit. As a whole, obtaining the active cyclin A without the CDK partner (referred as monomeric in this work) in a straightforward and facile manner will obviate protein – production issues with the CDK2/cyclin A complex and enable drug discovery efforts for non-ATP competitive CDK inhibition through the cyclin groove.

Introduction

Cyclin A is particularly interesting among the cyclin family due to its ability to activate different cyclin-dependent kinases (CDKs), in S phase (cyclin dependent kinase 2 – CDK2)[1] and mitosis (CDK1) [1]. Consistent with its role as a key cell cycle regulator, the expression of cyclin A has been found to be elevated in a variety of tumors, and inhibition of the CDK2/cyclin A complex activity, through blocking of the substrate recognition site ("the cyclin groove") in the cyclin A subunit, has been demonstrated to be an effective method for inducing apoptosis in tumor cells [2,3]. Non-ATP competitive inhibition through the cyclin groove is required for next generation inhibitors that specifically block the cell cycle CDKs and avoid activities on transcriptional regulating CDKs that contribute to toxicities of clinically evaluated compounds [4–6]. In our previous work, the REPLACE strategy has been validated and used for ligand optimization in designing fragment and non-peptidic alternatives, in the context of the binding peptide [7,8]. In application to CDK2/cyclin A, fragment alternatives for both the N-terminal tetrapeptide and the C-terminal dipeptide of an optimized p21WAF peptide (HAKKRLIF) have been identified [9]. More drug-like ligands obtained through REPLACE and their resulting affinity to the CDK2/cyclin A (174–432 fragment) complex have been previously characterized through fluorescence polarization binding and kinase assays, while further verified by co-crystallization of the protein–ligand complexes [9]. Further to this initial N-cap series [8], an additional class of ligand alternatives for the N-terminus was identified. These include 4-substituted benzoic acids and the optimized N-capped peptide: 4-((4-methylpiperazin-1-yl)methyl)benzoic acid ligated to the p21 C-terminus, RLIF (SCCP5921, this study). Our goal was to obtain sufficient quantities (>1 mg) of high purity (>95%) human cyclin A2, void of its CDK catalytic subunit, in order to facilitate the development of potential inhibitors of CDK activity through

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[1] *Abbreviations used:* CDK2, cyclin dependent kinase 2; REPLACE, REplacement with Partial Ligand Alternatives through Computational Enrichment.

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the cyclin groove [7]. Obtaining the monomeric construct in good yields would greatly simplify protein production required for biophysical and structural characterization of the binding of cyclin groove inhibitory ligands, instead of the currently employed expression of the CDK2/cyclin A complex [10,11]. Eukaryotic expression systems (*Baculovirus* transfected SF9 or SF21 cells) are preferable for production of kinases due to the requirement for activating post-translational modifications. Expression in bacterial systems is generally quicker and simpler than in eukaryotic systems and has been successfully applied to CDK/cyclin complexes [12–14]. A similar protein to the human 171–432 fragment, bovine cyclin A3, has been previously expressed in bacteria fused with a C-terminal tag and crystallized [15]. Since all the structures reported to-date of human cyclin A are in complex with the kinase subunit [10,11], the production of the monomeric cyclin counterpart represents a novel challenge.

A disadvantage of bacterial mass over-expression is the misfolding and aggregation of recombinant eukaryotic proteins within inclusion bodies, thus hindering their production in soluble, active form [16–19]. Protein recovery from such insoluble aggregates through refolding is also problematic, especially when the main goal is to crystallize protein complexes.

To overcome these limitations different strategies have been employed in the production of natively folded protein including appropriate selection of the fusion tag, refinements in the purification method and optimization of the induction temperature [17]. Auto-induction methods can be therefore optimized for easier handling of cultures to generate higher protein yield [20], while co-expression with specific bacterial molecular chaperones can assist the proper folding process. *Escherichia coli* bacteria embody a variety of proteins characterized as chaperones, including the GroEL/GroES and DnaK/DnaJ/GrpE classes. Normally expressed at low levels in prokaryotic cells, such chaperones have been shown to improve heterologous, soluble over-expression of eukaryotic proteins [21]. Of the various chaperones found in *E. coli*, some drive protein folding directly, while others are known to prevent protein aggregation [22–24]. Co-expression of molecular chaperones with the client protein is therefore a possible strategy for the prevention of inclusion body formation [22–24]. A chaperone expression strategy previously described [25], was exploited to achieve elevated amounts of heterologous soluble over-expression of a 6-His tagged-human protein with the two groups of molecular chaperones from *E. coli* (GroES/GroEL and DnaK/DnaJ/GrpE). This strategy resulted in higher yield of the soluble, active and natively folded form of ALDH3A1 [26]. In addition, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS, from *Actinosynnema pretiosum* ssp. *Auranticum*) represents another example for successfully expressed, soluble production with GroEL/GroES (*E. coli*) [21]. High level production of an active ribonuclease inhibitory protein (RI) in E. coli was also obtained by its co-overexpression with GroELS [27].

As outlined above and in order to overcome the issues inherent with insoluble expression of monomeric human cyclin A2 in bacteria, we investigate optimized protocols, taking into account various determinant factors. In this study, a chaperone-facilitated methodology has been applied, in order to over-express and purify a soluble and active His-tagged form of monomeric human cyclin A2. The relative activity of the protein products was assayed by direct determination of dissociation constant (K_d) with established and potential inhibitors. The results of this study showed that, while expression products of variable purity were obtained, recombinant chaperone mediated expression of cyclin A2 resulted in a similarly folded protein to that observed in the heterodimeric complex with CDK2. Peptide ligands and a novel optimized N-capped cyclin groove inhibitor, identified using REPLACE, were used as positive controls, in order to verify the correct folding of the monomeric cyclin A protein.

Materials and methods

Materials

The chaperone plasmid set was purchased from Takara (Shiga, Japan). Protino™ Ni–NTA agarose beads and Ni–TED pre-packed columns were purchased by Macherey–Nagel (Germany). Materials for the bacterial cultures medium were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany), lach:ner (Czech Republic) and Lab M Limited (United Kingdom), while antibiotics, imidazole, agarose and inducers were purchased from Sigma–Aldrich Co. (Taufkirchen, Germany). L-arabinose was purchased from Alfa Aesar & Co. KG (Karlsruhe, Germany). For western blotting, PVDF membranes were purchased from Millipore (Bedford, MA, USA), whereas the monoclonal anti-His antibody was obtained from Abgent (San Diego, CA, USA) and the goat anti-rabbit IgG horseradish peroxidase conjugated antibody was purchased by Millipore (Bedford, MA, USA).

Cyclin A2 His-tagged over-expression via auto-induction screening and purification

The human cyclin A2 (sequence 174–432) was sub-cloned in the Cyclin $A2_{174-432}$-pET16b resulting plasmid, also implementing a TEV protease recognition site for subsequent removal of the His-tag. The construct was then transformed into competent *E. coli* BL21 (DE3) strain, and selected on LB agar plates containing 100 μg/mL ampicillin. Positive transformants with the Cyclin $A2_{174-432}$-pET16b expression plasmid were selected and the sequence of the isolated plasmid was appropriately verified and furthermore exploited. An overnight culture of BL21 (DE3) *E. coli* transformed with Cyclin $A2_{174-432}$-pET16b, in standard LB Medium (1% tryptone, 0.5% yeast extract, 1% NaCl, w/v, pH 7.0) was used for 1:500 inoculation at 37 °C, in the presence of 100 mg/ml ampicillin. At OD_{600} ~0.5, 0.1–1 mM IPTG was added and incubation continued at temperatures that varied from 18 to 37 °C, at times ranging from 3 h to overnight incubation. Harvested cells were re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) in the presence of 100 mM PMSF and 1 mg/ml lysozyme. Purification was conducted after sonication and separation from the insoluble material via affinity chromatography through Ni–NTA column with gradient elution. The cyclin-enriched elution fractions were pooled and subjected to gradient buffer exchange through Millipore Centrifugal Filter Units to crystallization/fluorescence buffer containing 50 mM Tris pH 8.0, 100 mM $MgCl_2$ (the concentration of $MgCl_2$ was gradually increased during exchange cycles to avoid precipitation). NaN_3 and Monothioglycerol were added to final concentrations of 0.01% each [15].

Molecular chaperone cyclin A2 co-expression and purification

The Cyclin $A2_{174-432}$-pET-16b(+) transformed BL21 (DE3) *E. coli*, were re-transformed with the chaperone-expressing plasmids: pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16 (Table 1) and cultured in LB broth with 20 μg/mL chloramphenicol, along with 100 μg/mL ampicillin for the selection of the transformed clones. For chaperones-facilitated expression, cells were inoculated in shaking cultures at 37 °C, in the presence of the appropriate chaperone inducers (0.5 mg/ml L-arabinose and/or 5 ng/ml tetracycline), in order to ensure that requisite amounts of chaperones would be present during IPTG induction and cyclin A2

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