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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.

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Keywords

Recombinant human cyclin A2; Molecular chaperones; Soluble expression; Protein-ligand binding affinity; Tryptophan fluorescence titration

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) 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 (>1mg) 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 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 have 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 crystallised [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. *E.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 CyclinA2_{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 OD₆₀₀ ~0.5, 0.1 – 1 mM IPTG was added and incubation continued at temperatures that varied from 18–37°C, at times ranging from 3 hours to overnight incubation. Harvested cells were re-suspended in lysis buffer (50 mM NaH₂PO₄, 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 MgCl₂ (the concentration of MgCl₂ was gradually increased during exchange cycles to avoid precipitation). NaN₃ 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 overexpression. When cultures reached OD₆₀₀ of 0.5, 0.5 mM IPTG was added and the incubation proceeded for 6 hours to overnight at 18–25°C. Cells were collected by centrifugation, washed with solution buffer, weighted and stored at –20°C for subsequent lysis and protein purification. Approximately one gram of each of the six pellets (including the chaperone-less control) was treated with lysis buffer and an identical one-step purification of His-tagged cyclin A2 was subsequently performed for each transformant. Isolation of His-tagged recombinant human cyclin A2 was achieved by convenient one step affinity chromatography using pre-packed Ni-TED samples. The enriched elution fractions

were pooled together, gradually stripped from imidazole and salt and then dialyzed to fluorescence buffer. Concentrations were determined as previously described and samples were subjected to fluorescence assays.

Protein determination, gel electrophoresis and Western blot analysis

The concentration of protein in the samples was determined by the Bradford method [28] using bovine albumin as standard. Proteins were separated by electrophoresis in 12% (w/v) SDS-PAGE as previously described in [29]. For the quantitation of protein bands in gel images we used GelQuant.NET software provided by biochemlabsolutions.com. Western blot analysis immunoblots were performed as described previously [30]. Samples were run on a 12% (w/v) SDS-PAGE and electro-transferred in transfer buffer onto polyvinylidene fluoride (PVDF) membranes probed with anti-His antibody (GE Healthcare).

Determination of Dissociation Constant (K_d) from Fluorescence Measurements of Cyclin A2 Binding Activity

The dissociation constant is a measurement of binding between a protein and a ligand. For the reaction:

While K_d may be estimated by the equation:

$$K_d = \frac{[P][L]}{[PL]}$$

[P] is the concentration of unbound Protein, [L] is the concentration of the unbound ligand and [PL] is the Ligand-bound Protein.

Differences in fluorescence intensity at 345 nm between the complex (cyclin A2/5921) and free protein (excitation at 295 nm) were analysed as previously described in [31] (Eq. 2), in order to determine the dissociation constant (K_d) of recombinant cyclin A2 with 5921:

$$F_{obs} = F_{BG} + MF_{P_F} [P_F] + FR \cdot MF_{P_F} \cdot \frac{([L_T] + [P_T] + K_d) \pm \sqrt{([L_T] + [P_T] + K_d)^2 - 4[P_L][L_T]}}{2} \quad (2)$$

F_{obs} is the observed fluorescence intensity; F_{BG} is the fluorescence background signal; MF_{P_F} and P_F are the molar fluorescence and concentration of unbound protein, respectively; FR is the fluorescence ratio of bound protein; L_T and P_T are total concentrations of ligand and protein, respectively.

Fluorescence intensity was measured with a Hitachi F-2500 fluorescence spectrophotometer in 0.4×1 cm quartz cuvettes at 25°C. The excitation and emission wavelengths were 295nm and 345 nm respectively. The slits were set at 5 and 20 nm in the excitation and emission respectively. For the binding assay of 6xHis-CyclinA₂ with the 5921 ligand, measured intensities were corrected for blank signals, as previously explained [31]. Briefly, 1.5 ml of

protein solution in fluorescence buffer (0.1 to 0.65 μM) was placed in a cuvette. After equilibration at 25°C for 1 h, small increments (2 to 10 μl) of the ligand solution were injected. The fluorescence intensity was measured 2 min after each injection, time during which the shutter remained closed, in order to avoid protein deterioration. Fluorescence signals were corrected taking into account the dilution effect due to the added ligand volumes, as well as any possible fluorescence effect that might be caused by the unbound ligand. To this end, a blank sample containing Tryptophan, with a fluorescence signal of a similar level to our initial protein sample, was titrated with the addition of the same ligand injections. The sample absorbance was kept below 0.1 to minimize the inner filter effect [32]. The corresponding K_{d} s to the binding reactions were subsequently determined using Prism and the corrected fluorescence values (GraphPadSoftware, San Diego, CA).

Synthesis of 4-((4-methylpiperazin-1-yl)methyl)benzoic acid

To a solution of 4-Formyl benzoic (0.15 g, 1 mmol) and 1-methyl Piperazine (0.1 g, 1 mmol) in 25 ml of dichloromethane, sodium triacetoxy borohydrate (0.21 g, 1.4 mmol) was added. The reaction mixture was stirred overnight at RT and the reaction was monitored by TLC (ethylacetate:hexanes-35:65). After the reaction was completed the reaction mixture was quenched with water and the reaction mixture was stirred for 10 min. The layers were separated; the aqueous layer was evaporated to get the crude product which was purified by flash chromatography (Biotage SP4) using a SNAP 10 g column with a gradient run starting from 6 % ethylacetate: 94 % hexanes to 25 % ethyl acetate and 75 % hexanes over 10 column volumes to yield a white solid (0.19 g, 80.2 %), Fig. 1.

Peptide Synthesis

Peptides and the FLIP molecule, SCCP5921, were assembled by using standard solid-phase synthesis methods [7]. A sample procedure is given as follows: A Rink resin (0.15 mmol, 750 mg) was swollen in DMF for about 20 min. 5 equivalents of the C-terminal amino acid (Fmoc-Phe, 2027 mg) were coupled to the resin using DIEA (0.082 mL) and HBTU (189.6 mg) in 5 mL of DMF for 1 h. The N-terminus was deprotected using 20% piperidine in 5 mL of DMF for 10 min prior to addition of the next amino acid (Fmoc-Ile). Wash cycles (5 $\times$ 10 mL of DMF + 5 $\times$ 10 mL of DCM) were applied to each step in between coupling and de-protection of Fmoc. The subsequent amino acids (Fmoc-Leu and Fmoc-Arg(pmc) and N-terminal capping group (4-((4-methylpiperazin-1-yl)methyl)benzoic acid) were coupled and de-protected in a similar fashion. Upon completion of the assembled peptide or FLIP, side chain protecting groups were removed, and the molecule liberated from the resin using TFA/H₂O/TIPS (95:2.5:2.5). Crude peptides were purified using reverse-phase flash chromatography or semi-preparative reverse-phase HPLC methods. Pure peptides were lyophilized and characterized using mass spectrometry and analytical HPLC.

Results

Purification of the Poorly Soluble Fraction of Recombinant Monomeric Human Cyclin A2

Factors considered important during bacterial expression and protein synthesis, include choice of expression vector and induction parameters, selection of an appropriate solubilization tag, and furthermore, which cultivation conditions are used [19]. In the first

instance, tags known for their contribution to soluble expression including GST (glutathione S-transferase) [33] may be implemented. In our initial studies, a pET49(+) construct expressing the GST tagged truncated cyclin A2 was used, however resulted in expressed protein which rapidly precipitated after removal of the tag via TEV proteolysis (6× His-tag), even though CDK2 was added to stabilize the cyclin subunit.

In order to monitor the over-expression and solubility of His-tagged recombinant cyclin A2, we employed the established protocol of IPTG induction. Whereas expression was considerably enhanced after scrutinizing several induction conditions, a significant amount of cyclin A2 production remained aggregated in inclusion bodies (Fig. 2A). The soluble fraction of the expressed cyclin A2 was estimated to represent only the 5% of the amount of the induced protein, even after lowering the concentration of the inducer and further tuning the induction temperature (the presence of His-tagged recombinant product was determined using western blot, as demonstrated in Fig. 2B). The expression profile revealed a level of production sufficient for ligand-binding assays, following the single-step affinity purification. However, the yield would prove insufficient were we to perform further purification steps required to achieve the quantity and purity required for crystallization purposes [15]. Recombinant cyclin A2 was produced and purified via affinity chromatography. Elution fractions (Fig. 2C) were pooled, dialyzed in fluorescence buffer and subjected to fluorescence titration with cyclin groove inhibitory ligands.

Titration of Recombinant Cyclin A2 with Cyclin Groove Inhibitors

The method of choice for direct K_d measurement was tryptophan fluorescence titration [5] rather than the previously implemented competitive binding with a fluorescent peptide [9]. Direct binding was preferred in this context due the more accurate reflection of this assay for protein folding and activity since competitive binding would involve a more subjective result. Measurement of ligand binding to the monomeric constructs expressed under various conditions was performed by observing variation in intrinsic fluorescence mostly contributed from Trp217 in the cyclin groove of cyclin A. The quantification cannot be performed with the intact CDK2/cyclin A complex, due to the presence of multiple tryptophan residues in CDK2. These would result in a high background, which would dilute the fluorescence signal, thus masking the effect of ligand binding to cyclin A. The octapeptide HAKRRLIF [9], the pentapeptide RRLIF[34] and the N-capped peptide SCCP5921 (this study), were the three ligands sampled for titration, representing high, low and putatively intermediate affinity compounds. HAKRRLIF represents an optimized version of the cyclin binding motif found in the endogenous CDK2 inhibitor, p21Waf, whereas RRLIF represents a truncated version of this sequence retaining respectable activity at a lower molecular weight and is therefore considered as a good compromise between potency and size [15, 35]. Due to these parameters, the pentapeptide represents a useful template for application of the REPLACE strategy, in order to convert it into a more drug-like compound. To this end, the strategy was applied in order to discover 4-substituted benzoic acid capping groups that mimic the interactions of the basic residues in the N-terminal tetrapeptide part of HAKRRLIF. The 4-((4-methylpiperazin-1-yl)methyl)benzoic acid capping group was appended onto the C-terminal tetrapeptide, RLIF and was found to be an effective fragment alternative primarily for the first arginine. This capping group

interacts with three acidic residues in the cyclin groove (Fig. 3) namely Glu220, Glu224 and Asp283 through ion pairing interactions and mimics the contacts of Lys3 and Arg4 of the octapeptide. The respective K_{ds} for HAKRRLIF (0.019 μ M), RRLIF (23.98 μ M) and SCCP5921 (3.212 μ M) were determined (Figure 2 D–G), with the latter being chosen for K_d estimation in this study, due to its potency and reproducible binding data.

Molecular Chaperones Co-expression Enhances the Solubility of Recombinant His-tagged cyclin A2

To increase the fraction of soluble recombinant His-tagged cyclin A2 protein, co-expression with molecular chaperones derived from *E. coli* was implemented in various combinations (Table 1). The BL21 (DE3) strain of *E. coli*, initially transformed with Cyclin A2_{174–432}-pET-16b(+), was re-transformed with plasmids expressing molecular chaperones, including: pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16. Following induction, cell pellets were re-suspended in lysis buffer and partial purification was carried out to determine the amount of protein in soluble and aggregated form. Qualitatively, comparing the samples of soluble fractions of all five preparations in Fig. 4 to that of unassisted expression in Fig. 2, it can be clearly visualized that the presence of chaperones significantly enriches the soluble cyclin A2 fraction.

Recovery estimation verified the apparent rise, both in the total amount of purified protein and in the enrichment of the soluble fraction (Table 2). The protein amount in the soluble fraction was estimated to increase significantly, when co-expressed with pG-KJE8 (to approximately 15 % of the induced protein) and even more so in the case of pGro7 (to approximately 23 %) plasmid (Fig. 4; Table 2), as observed from previous co-expression profiles. Solubility levels maintained a similar proportion (22.7 %) in the presence of the pTf16 plasmid, albeit at lower levels of total protein production (Fig. 4; Table 2). Other plasmids that resulted in a visible enhancement in soluble production of the His-tagged cyclin A2 included pG-Tf2 and pKJE7 (to approximately 10 % in both cases) (Fig. 4; Table 2). Overall, these results demonstrate that the presence of certain chaperone groups, especially those containing GroES and GroEL and/or dnaK/DnaJ/GrpE (e.g. plasmids pG-KJE8, pGro7) combinations, exhibited a higher impact in increasing the yield of soluble recombinant cyclin A2. The pTf16 plasmid, expressing the Tig chaperone also resulted in a significant enhancement in the solubility of the His-tagged protein. The other plasmids used (pKJE7 and pG-Tf2) improved levels of soluble protein as well, exhibiting a two-fold increase, when compared to the auto-induced expression.

Determination of dissociation constant through Fluorescence Measurements of cyclin A2 Binding Activity to a Ligand Partner

The binding of the 5921 ligand to each purification product was estimated by Trp fluorescence titration. As visualised in Fig. 4 and shown in table 2, each of the sampled co-expression products of cyclin A2 exhibited ligand binding activity, determined by the calculation of the respective K_{ds} , albeit at a lower level of affinity than the auto-induced protein control. The values obtained reflect the specific activity of the product, which is related to the purity of the product, as well as the effectiveness of the chaperones in refolding. The direct correlation between the ligand affinity -quantified by the K_d values and

the amount of protein in the soluble fraction can be observed through the values in table 2 and the plots in Figure 5. The products with higher amount of soluble protein (e.g. co-expressed with plasmids pG-KJE8, pGro7 and pTf16) typically exhibited binding affinity closer to that of the auto-induced protein (e.g. K_d lower than 5.5 μ M). The same cannot be said regarding expression in the presence of the pKJE7, pG-Tf2 plasmids, where the dissociation constants for ligand binding appear to be significantly higher (K_d s above 6.5 μ M). As a necessary control, auto-induced recombinant cyclin A2 was produced under the same bulk conditions and the inhibitor SCCP5921 exhibited a K_d of 3.546 ± 0.361 μ M, a value that is well inside the error margins. These results demonstrate that production mediated by certain groups of molecular chaperones not only enhances the protein's solubility, but also result in properly folded products that retain their biological activity (in this case the ability to bind tightly to cyclin groove inhibitory compounds).

Discussion

Previous attempts to express soluble, active recombinant human cyclin A2 in *E. coli*, avoiding refolding from inclusion bodies and without the structural stabilization of the cognate CDK partner, have failed primarily due to issues of low soluble expression, yield and insufficient purity. To the best of available knowledge, there is no literature precedent for the production of the truncated human cyclin A2 in soluble form in *E. coli*, in order to target its crystallization as a monomer. Even when a bovine construct similar to human cyclin A, was successfully engineered into the appropriate vectors and the recombinant protein abundantly expressed in *E. coli* [15], insoluble production remained a critical issue. This is due to their heterogeneity and aggregation-orientated nature and a partially folded state during expression, therefore resulting in inactive, inclusion body conformations. In the present work, different *E. coli* fusion expression strategies have been implemented under various conditions, with the intent of increasing efficiency soluble expression of human cyclin A2 [19]. In the first instance, the culture temperature subsequent to IPTG-induction, was lowered in increments below 20°C. Temperature variation is known to facilitate the production of active protein through a variety of mechanisms. Hydrophobic interactions, the basic driving force of inclusion body formation can be decreased through lowering of the temperature. Furthermore, the temperature-dependent expression of molecular chaperones, reduction of protein synthesis rate, different folding kinetics and lower activity of specific proteases [36–39] can also contribute to the enhanced yield of active recombinant proteins. Consistent with these prior observations, a temperature decrease, in combination with prolonged incubation time, resulted in an enhancement of the limited soluble expression of His-tagged cyclin A2 observed under standard conditions.

In further efforts to improve the amount of protein expressed in the soluble fraction, co-expression of chaperones with human cyclin A was undertaken. This methodology previously has been demonstrated to be a highly effective way to increase the soluble expression of various recombinant proteins in *E. coli* [25, 26], and furthermore using a chaperone transformation strategy [25, 40]. The resulting expression studies showed that two groups of molecular chaperones from *E. coli* (GroES/GroEL and DnaK/DnaJ/GrpE) were the most effective with greater than 20% of the monomeric cyclin A being expressed in the soluble fraction. These two groups are the most commonly used systems for the

expression of soluble proteins [19] and are known to be ATP dependent chaperones which function by inducing the partial unfolding and subsequent re-folding of non-native protein conformations. The native correctly folded states of human monomeric cyclin A induced using these expression conditions were validated by testing with known cyclin groove ligands and the resulting K_d values confirmed that the protein is functionally relevant to a similar level observed in the fully active heterodimeric CDK2/cyclin A complex [9]. The combined result of co-expression of a soluble and active product that stands out among the chaperones plasmids used in this study can be attributed to the pGro7 plasmid, expressing the GroES/GroEL chaperones. Overall, these results demonstrated that the optimized conditions identified in this study would increase the quantities of soluble protein expression, paired by the retained binding activity. This in turn will enable monomeric cyclin A2 to be purified through convenient methods to sufficient levels of homogeneity and in the amounts required for intensive crystallization experiments and for high-throughput functional.

Conclusions

In summary, His-tagged recombinant monomeric human cyclin A2 protein was successfully expressed in *E. coli* and purified in a stable, soluble form. The increased yield of pure and natively folded protein, that retained binding to cyclin groove ligands with similar affinity to that of the CDK2/cyclin A2 complex, was facilitated through the co-expression with certain groups of molecular chaperones. The fusion protein product of each of the double transformants was efficiently purified and found to retain native biological activity, especially when co-expressed with the GroEL/GroES chaperones (plasmid pGro7). This confirmed the primary goal, that the monomeric cyclin A protein with a natively folded conformation can be obtained in a soluble, active form, leading to a next step of high yields and degree of purity required for crystallization attempts (ongoing experiments) with the appropriate ligands. The methods described in this study, therefore permit the production of required amounts of the recombinant human cyclin A2 for conducting high-throughput binding assays with putative cyclin groove inhibitors and also render possible the thorough investigation of its potential for structure determination, through crystallography. As a result, this work helps to address a major challenge in drug discovery of non-ATP competitive CDK inhibitors as anti-tumor therapeutics to convert peptidic compounds to more pharmaceutically relevant compounds. It also contributes to further validation of the REPLACE methodology as an improved strategy for targeting protein-protein interactions.

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Abbreviations

CDK2	Cyclin dependent kinase 2
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REPLACE Replacement with Partial Ligand Alternatives through Computational Enrichment

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Highlights

- Truncated human recombinant cyclin A2 was over-expressed in *E. coli*
- Molecular chaperone plasmids enhanced the cyclin A2 soluble expression
- Cyclin groove-ligand alternatives generated, including optimized N-capped peptides
- 4-((4-methylpiperazin-1-yl)methyl)benzoic acid ligated to p21 C-terminus was tested
- Chaperone-induced products retain their cyclin groove-inhibitors binding activity

ES +235

Figure 1. Chemical Synthesis of the SCCP5921 Ligand

Figure 2. Expression and purification of recombinant human cyclin A2 and determination of its dissociation constant with HAKRRLIF and potential inhibitor SCCP 5921

(A) Samples of protein purification stages were subjected to SDS-PAGE and stained with Coomassie blue. -IPTG: Total cell extract prior to induction, IB: Inclusion Bodies re-suspended precipitate, SF: Soluble Fraction of the lysed cells after lysozyme treatment, sonication and centrifugation to separate the insoluble materials. (B) Anti-His western blot of the two bands corresponding to the IB: Inclusion Bodies and SF: Soluble Fraction. (C) Purification of recombinant His-tagged cyclin A2 using a gradient eluted Ni-NTA column (Coomassie blue staining), various elution fractions of the eluted cyclin A2/6xHis. (D) The ligand binding experiment was performed in fluorescence buffer containing 50 mM Tris pH 8.0, 100 mM MgCl_2 , NaN_3 and Monothioglycerol 0.01 %. (D) SCCP 5921: Direct plot of fluorescence intensity against ligand total concentration. Sequential additions of ligand were made into a cuvette containing cyclin A2 and the dissociation constant was calculated by fitting the fluorescence intensity corrected values to a quadratic equation. (E) SCCP 5921:

Saturation plots after calculation of free (L) and bound (PL) ligand concentrations. Inset: Scatchard plots. The mean values of three independent measurements are presented.

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Figure 3. Modeled structure of SCCP5921 in complex with cyclin A2

A Connolly surface representation of cyclin A2 groove binding surface visualized with the bound SCCP 5921 ligand. This model was generated through modification of the crystal structure of RRLIF (1OKV) in Accelrys DiscoveryStudio 3.0. Since only the capping group changes in the SCCP5921 structure, and the ion pairing interactions are important for binding, it is relatively straightforward to model its conformation.

Figure 4. Co-expression profile of cyclin A2 with molecular chaperones and subsequent purification

(A) Co-expression of pG-KJE8, pGro7, pKJE7/, pG-Tf2 and pTf16 plasmids' chaperones. Samples were subjected to SDS-PAGE and stained with Coomassie blue. -IPTG: Total cell extract prior to induction, +IPTG: Total cell extract 6 hours after induction, SF: Soluble Fraction of the lysed cells after lysozyme treatment, sonication and centrifugation to separate the insoluble materials, IB: Inclusion Bodies re-suspended precipitate. The protein bands of interest expression are highlighted with the rectangular.

Figure 5. Determination of its dissociation constant with the potential inhibitor SCCP 5921 of all chaperone co-expressed cyclin A2 products

The ligand binding experiment for each of the five partially purified samples was performed in fluorescence buffer containing 50 mM Tris pH 8.0, 100 mM MgCl_2 , NaN_3 and Monothioglycerol 0.01 %. For each purified protein sample from co-transformed cells with (A) pG-KJE8, (B) pGro7, (C) pKJE7, (D) pG-Tf2 and (E) pTf16 plasmids, a direct plot of fluorescence intensity against ligand total concentration -where sequential additions of ligand were made into a cuvette containing cyclin A2- was designed and the dissociation constant was calculated by fitting the fluorescence intensity corrected values to a quadratic

equation. Saturation plot after calculation of free (L) and bound (PL) ligand concentrations. Inset: Scatchard plots. The mean values of three independent measurements are presented.

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Table 1

Citation of the chaperone-expressing plasmids detailed description.

Plasmid	Antibiotic	Inducers	Expressed Chaperones	M. W.s
pG-KJE8	Chloramphenicol (20 µg/mL)	L-arabinose (0.5 mg/ml) tetracycline (5 ng/ml)	DnaK-DnaJ-GrpE/GroES-GroEL	DnaK-70 kDa DnaJ-40 kDa GrpE-22 kDa GroES-10 kDa GroEL-60 kDa
pGro7	Chloramphenicol (20 µg/mL)	L-arabinose (0.5 mg/ml)	GroES-GroEL	GroES-10 kDa GroEL-60 kDa
pKJE7	Chloramphenicol (20 µg/mL)	L-arabinose (0.5 mg/ml)	DnaK-DnaJ-GrpE	DnaK-70 kDa DnaJ-40 kDa GrpE-22 kDa
pG-Tf2	Chloramphenicol (20 µg/mL)	tetracycline (5 ng/ml)	GroES-GroEL/Tig	GroES-10kDa GroEL-60 kDa Tig-56 kDa
pTf16	Chloramphenicol (20 µg/mL)	L-arabinose (0.5 mg/ml)	Tig	Tig-56 kDa

Table 2

Soluble production and ligand-binding activity of recombinant cyclin A2 mediated by co-expression with chaperones.

Expression Profile	Production levels (mg/gr of cells)	Solubility (aprox. % of the induced product)	Dissociations Constants K_d (μ M)
Cyclin A2/6×His (IPTG)	0.05	5%	3.546 ± 0.361
Cyclin A2/6×His (IPTG) / pG-KJE8	0.45	15 %	5.282 ± 0.395
Cyclin A2/6×His (IPTG) / pGro7	0.3	23 %	5.440 ± 0.257
Cyclin A2/6×His (IPTG) / pKJE7	0.132	9.6 %	6.589 ± 0.640
Cyclin A2/6×His (IPTG) / pG-Tf2	0.12	10%	6.772 ± 0.424
Cyclin A2/6×His (IPTG) / pTf16	0.08	22.7 %	4.771 ± 0.417