A fully automated procedure for the parallel, multidimensional purification and nucleotide loading of the human GTPases KRas, Rac1 and RalB

Christopher H. Gray a,*, Jennifer Konczal a, Mokdad Mezna a, Shehab Ismail b, Justin Bower a, Martin Drysdale a

a Drug Discovery Program, CRUK Beatson Institute, Garscube Estate, Switchback Road, Glasgow, G61 1BD, UK
b CRUK Beatson Institute, Garscube Estate, Switchback Road, Glasgow, G61 1BD, UK

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Abstract
Small GTPases regulate many key cellular processes and their role in human disease validates many proteins in this class as desirable targets for therapeutic intervention. Reliable recombinant production of GTPases, often in the active GTP loaded state, is a prerequisite for the prosecution of drug discovery efforts. The preparation of these active forms can be complex and often constrains the supply to the reagent intensive techniques used in structure base drug discovery. We have established a fully automated, multidimensional protein purification strategy for the parallel production of the catalytic G-domains of KRas, Rac1 and RalB GTPases in the active form. This method incorporates a four step chromatography purification with TEV protease-mediated affinity tag cleavage and a conditioning step that achieves the activation of the GTPase by exchanging GDP for the non-hydrolyzable GTP analogue GMPPnP. We also demonstrate that an automated method is efficient at loading of KRas with mantGDP for application in a SOS1 catalysed fluorescent nucleotide exchange assay. In comparison to more conventional manual workflows the automated method offers marked advantages in method run time and operator workload. This reduces the bottleneck in protein production while generating products that are highly purified and effectively loaded with nucleotide analogues.

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1. Introduction

Cellular processes rely on small GTPases for the regulation of diverse, key signalling cascades and networks. Signal transduction relies on the activation of these GTPases when the bound nucleotide is swapped from GDP to the more abundant GTP through interaction with Guanine Nucleotide Exchange Factors (GEFs) [1,2]. The small GTPase KRas is the most frequently mutated gene in cancer and is a critical driver and negative prognostic factor for major presentations including colorectal, pancreatic and non small cell lung cancer [3]. Rac1 is often implicated in cancer progression, promoting angiogenesis and metastasis [4]. Similarly, the Ral GTPases promote tumorigenesis with RalB emerging as a particular driver of invasion and metastasis [5]. As a consequence, the small GTPases are established as strongly validated targets for therapeutic intervention.

Historically, efforts to target GTPases with small molecules have proved extremely difficult with several declaring these proteins to be undruggable. However, persistence on these high value targets has led to recent encouraging progress, including the direct targeting of mutant KRas [6]. A series of small molecules have been characterized that directly bind KRas to inhibit SOS1 catalysed nucleotide exchange in vitro [7,8]. Other small molecules covalently interacts with the KRas G12C mutant and show modulation of KRas G12C in a cellular setting by blocking nucleotide exchange and trapping the KRas in the inactive GDP form [9]. Additionally, some cyclic peptides have been reported to affect KRas, blocking protein-protein interactions with downstream effectors [10].

As a consequence of this high level of target validation in disease, and encouraging signs of tractability, the small GTPases are once again the subject of intensifying drug discovery efforts. These programs often require significant amounts of purified recombinant proteins to prosecute their programs, and often this includes

* Corresponding author.
E-mail address: c.gray@beatson.gla.ac.uk (C.H. Gray).
SPR3 were con...
uncleaved GTPase and the H8-TEV S219V. GTPases were then conditioned by a nucleotide exchange reaction, and the protein was separated from excess unbound nucleotide by a 26/10 desalting column in Buffer E (10 mM Tris HCl pH7.5, 50 mM NaCl, 2 mM MgCl2).

2.6. Nucleotide exchange

The exchange of GDP (guanosine diphosphate) for the nucleotide analogues was achieved by methods adapted from Scherer et al. [19]. For the exchange of GDP to GMPPnP (guanosine 5'-[β,γ-imido]triphosphate), an appropriate volume (typically 3 ml) of 10× exchange buffer (containing at minimum five-fold molar excess of GMPPnP; 2M (NH₄)₃PO₄, 1 mM ZnCl₂, 5 mM GMPPnP) was delivered to the GTPase. This was achieved by either an automated step, supplying reagent from a reservoir attached to a loop valve, or by manual addition. Also, 10U per milligram of alkaline phosphatase agarose (Roche) was included by either pre-positioning in the collection tube for the SEC output, or by manual addition. GDP to GMPPnP nucleotide exchange was performed to completion on incubation for a minimum of 4 h at 4°C.

KRas was loaded with mantGDP by incubating the protein with a molar excess of nucleotide (800 μM) and 10 mM EDTA for 2 h at 4°C. Reactions were then supplemented with 10 mM MgCl₂ and incubated for a further 1 h at 4°C prior to desalting into Buffer E. Automation of mantGDP loading was achieved by positioning of a 10× stock solution, of 8 mM mantGDP plus 50 mM EDTA, in a reservoir attached to position 1 of the loop valve. Similarly, MgCl₂ was delivered from a 200 mM stock attached to loop valve position 2.

2.7. Workflow configurations

GTPase purifications were performed either as single runs or as parallel batches where all three targets were processed in each step using the Unicorn 7 software “scouting” functionality.

In addition, workflows were designated as either “manual” or “automated”. Manual workflow involved conventional practices where proteins were applied to a single chromatography technique and purified material was subsequently collected from the AKTA fraction collector by the operator. The protein was then pooled and prepared for application to the next step in the production procedure, including manual addition of protease or nucleotide loading reagents.

Automated workflow made use of the Unicorn software’s “method queueing” functionality where all sequential chromatography steps can be programmed to run in sequence with no operator intervention. The desired peaks from automated purification steps were sent to 50 ml tubes via the AKTA outlet valve. Sample inlet lines were pre-positioned in these tubes to allow onward delivery to downstream steps. H8-TEV S219V and alkaline phosphatase agarose were pre-positioned in the appropriate intermediate tubes during the run setup. Other reagents for nucleotide exchange buffer were delivered to the pool of protein from a 10 ml sample loop attached to an AKTA loop valve.

2.8. HPLC assessment of GMPPnP loading

A 500 μl sample of protein at approximately 50 μM was heated to 95°C for 2 min to denature the protein and release bound nucleotide. The precipitated protein was removed by centrifugation...
at 13,000 rpm for 2 min and the supernatant was loaded into a HPLC auto-sampler. Analysis was carried out on a Shimadzu HPLC using a Xselect® C18 CS 2.5 μm column (4.6 mm × 7.5 cm) (Waters) running at 1.5 ml per minute in a buffer of 100 mM K3PO4 pH 6.5, 10 mM tetra-butyl-ammonium-bromide and 7.5% acetonitrile. Detection of nucleotide was achieved by monitoring absorbance at 254 nm and the nucleotide was identified with reference to 100 μM GDP or GMPnP standards.

2.9. SOS1 catalysed nucleotide exchange of KRas

A fluorescent based nucleotide exchange assay was performed using mmtGDP loaded KRas 1–169. This assay monitors the quenching of mmtGDP fluorescence as it is displaced from KRas by GTPγS on interaction with the GEF SOS1. KRas 1–169 (mmtGDP loaded) and SOS1 (564–1049) were mixed in a black, 96 well plate to concentrations of 500 nM and 100 nM respectively and a volume of 23 μl. Samples were mixed on a shaking platform for 2 min and baseline fluorescence was established by an Infinite M1000 Pro fluorimeter (Tecan) (excitation wavelength, 360 nm; emission wavelength 430 nm). The reaction was initiated by the addition and mixing of 2 μl of GTPγS to a final concentration of 200 μM. Nucleotide exchange was measured by the decrease in emission at 430 nm over a period of 90 min measuring every 60 s.

3. Results

3.1. Validation of expression of soluble human GTPases from pBDPP-SPR3

As part of a workflow optimisation program we decided to introduce routine small scale analysis of expression cultures prior to protein purification of the batch. In this way we would validate batches for onward purification or discard batches that were unsatisfactory and inefficient for further work. We determined the level of recombinant protein by noting the appearance of the target in expression cultures following IPTG induction, and confirming the presence of soluble GTPase that could be purified by IMAC resins.

Cultures were incubated and induced as per our most common laboratory protocols, and these proved satisfactory for recombinant expression. A small sample of cells were harvested at the time of induction (T = 0) and at harvesting (endpoint) and resuspended in TBS buffer. A simple formula related culture OD600 to the volume of resuspension buffer to generated a sample that gave an appropriate concentration of total cell protein on an SDS-PAGE gel. In this way we could typically ensure a gel lane of well separated bands with a clear indication of the overexpressed target GTPase. In the vast majority of expression cultures the G-domains of KRas, Rac1 and RalB expressed well in pBDPP-SPR3. In addition an acceptably clean small scale Co-NTA purification confirmed the presence of all three GTPases as soluble proteins at appropriate molecular weights and with intact His-tags, thus confirming suitability of the batches for onward FPLC purification (Fig. 2).

3.2. Design of an automated, multidimensional, parallel production strategy

In order to increase throughput and productivity we devised a strategy for the parallel, automated production of several activated GTPases by multi-dimensional chromatography. Purification was achieved using standard enrichment and purification approaches, principally IMAC and SEC. Desalting columns were incorporated to facilitate buffer exchange. In our hands, these GTPases aggregate after sufficient exposure to imidazole, resulting in a loss of yield and quality. Buffer was exchanged immediately after IMAC to ensure no imidazole persisted during the 6 h incubation with TEV protease. A second buffer exchange was required following nucleotide loading, as the GMPnPP is applied at significant molar excess so the unbound nucleotide must be removed from the final product and exchange buffer conditions are not ideal for downstream applications. Overall, the sequence of the workflow was IMAC->Desalting->TEV cleavage (6 h pause)->S75 SEC->Nucleotide exchange (4 h pause)->Desalting (panel, Fig. 3). (*SEC had a 5 ml nickel column in line to trap uncleaned product and TEV protease.)

For tag cleavage, TEV protease was pre-placed in the tube allocated to receive the appropriate desalting column elution peak. To produce active GTPases the nucleotide was exchanged from GDP to a GTP analogue as the protein emerges from E. coli in the inactive GDP bound form. In our workflow we incorporated the nucleotide exchange of GDP with the non-hydrolyzable GMPnP (or other required analogue) following elution from the SEC column. Alkaline phosphatase agarose reagent was pre-placed in the tubes receiving the material for nucleotide exchange. A 10× exchange buffer, containing the concentrated ammonium sulfate and GMPnP, was held apart from the agarose until required. (The high salt concentration of the 10× buffer eliminated the phosphatase activity and it was preferable to keep the GMPnP and phosphatase separate in case some low level hydrolysis did occur.) A loop valve was added to the AKTA configuration to hold a reservoir of 10× exchange buffer stock. Following the completion of the SEC column, a short method was included in the method queue to deliver 3 ml of 10× exchange buffer to the protein in the waiting tubes. This material was applied at a flow rate of 10 ml per minute and this successfully agitated the phosphatase agarose in the tube to disperse it throughout the

![Fig. 2. Small scale expression analysis from pBDPP-SPR3 constructs](image-url)
mixture. (Similarly, reagents for the loading of KRas with mantGDP were held in two 10 ml sample loops on the loop valve and appropriate volumes delivered to initiate and stabilise exchange.)

TEV protease, exchange buffer, and nucleotide exchange reagents were applied at concentrations sufficient to achieve the desired modification of the maximum anticipated protein yield. As there was some variation in yields between replicate batches, it was not possible to be precise with ratios but we observed no negative effects on the product on occasions where the modifying reagents were in unnecessary excess.

The multi-dimensional flowpath and run scheduling were established using the method queuing functionality in GE Healthcare’s Unicorn software incorporating pauses to allow for tag cleavage or nucleotide exchange. The parallel purification of multiple GTPases used the Unicorn 7 scouting option, where the processing of several samples can be included in the same chromatography method. To prosecute the three production runs, an integrated multidimensional flow path was established utilising 12 ports of the extended sample inlet valve, 5 ports of the column selection valve, 9 ports of the outlet valve and the fraction collector (Fig. 3). All intermediate products were successfully collected in 50 ml tubes via the outlet valve ensuring the appropriate peak was selected and positioned for onward processing.

In order to restrain the early sample volume, the elution gradient from the IMAC was steep to compress the volume of the first elution peak and restrain requirements to one SEC column run per GTPase. However, sample dilution was noted through the run and it was common to run two final desalting columns after nucleotide exchange to process the entire final sample volume.

Fig. 3. Multidimensional schematic for parallel GTPase production. An example flow diagram of the multidimensional purification configuration on the ÄKTA AVANT 25 system for the parallel purification of three active, GMPPnP loaded GTPases. Flow of samples to and from the columns was achieved using tubing connected to sample inlet or outlet valves respectively. Each purification included four column steps and integrated both TEV protease tag cleavage and a nucleotide loading reaction. GTPases were purified on a dedicated 5 ml HiTrap FF IMAC column followed by a 26/10 desalting column to remove imidazole. H8-TEV S219V protease then cleaved the tag from the GTPases during a 6 h incubation. Cleaved proteins were purified further on a 26/10 575 Superdex Prep Grade Column with a 5 ml HiTrap FF column attached downstream (to capture H8-TEV S219V and any uncleaved material). Finally the protein was delivered to an aliquot of alkaline phosphatase agaroze. The 10x nucleotide exchange buffer, containing an excess of GMPPnP, was delivered to from a reagent reservoir connected to a loop valve. Nucleotide loading was allowed to proceed for 4 h and a final 26/10 desalting run removed excess nucleotide and collected the material in the integrated fraction collector prior to delivery. All intermediate product collection, protease reactions and nucleotide loading was performed in prepositioned 50 ml tubes. GE Healthcare Unicorn 7.0 software was programmed with a series of “scouting” runs and “method queues” to automatically schedule all steps in the production workflow running in sequence and without any user intervention.
3.3. Parallel purification and activation using the multidimensional system

An example of the set of chromatograms is presented in Fig. 4, illustrating the purification of tag cleaved, activated KRas 1–169. A robust workflow was achieved following trial and optimisation that allowed the successful isolation of all the GTPase targets. Proteins isolated by both automated and manual methods exhibited mobility on SDS-PAGE consistent with a species cleaved by TEV protease. Comparison of products from the automated and manual workflows showed comparable levels of purity on coomassie stained gels. However, using silver stain revealed that the material from the multidimensional workflow appeared to be marginally more purified (Fig. 5).

An initial concern was that stringent peak recognition parameters and rigid adherence to injection volumes within runs would result in a reduced yield from the automated method versus the manual approach. Surprisingly we routinely encountered the opposite. As expected the comparative yields from the first IMAC step showed the lowest discrepancy between approaches with average outputs from both methods approximately equal (n = 3) (Fig. 6a). However, greater losses are consistently observed with the manual method. It would appear that a combination of extended processing time and the manipulation and transfer of intermediate products between plasticware results in more attrition than the automated approach. Marginally, the greatest point of loss is seen in the yields from the SEC column, possibly as result of increased protein aggregation following prolonged storage in the fraction collector or from manual handling. Ultimately, substantial multimilligram quantities were obtained by all methods but the automated approach invariably returned the higher yields with the enhancement ranging between 8.5% and 16.9% when compared to the manual workflow (Table 1).

3.4. Nucleotide loading and SOS1 catalysed nucleotide exchange

GMPPnP status was ascertained by HPLC. Both manual and automated loading showed complete incorporation of GMPPnP in all GTPases with a positive GMPPnP peak, at amplitudes consistent with the analyzed protein concentration, and a complete absence of any signal for the GDP species (Fig. 7). The utility of KRas 1–169 loaded with mantGDP was assessed by application in a SOS1 dependant fluorescent nucleotide exchange assay (Fig. 8a). Both methods produced loaded material that
showed very similar exchange kinetics of SOS1 dependant exchange (automated, $1.15 \pm 0.032 \times 10^3$ sec$^{-1}$; manual $1.20 \pm 0.010 \times 10^3$ sec$^{-1}$) demonstrating that automated preparation of mantGDP loaded material was suitable for downstream applications (Fig. 8b).

Table 1
A comparison of final yields (mg) from each approach revealed an enhancement of between 8.5% and 16.9% through the use of the automated workflow.

|                     | Automatic | Manual | Manual vs Auto |
|---------------------|-----------|--------|---------------|
| KRas 1–169          | 48.0      | 39.9   | 83.1%         |
| Rac1 2–177          | 61.4      | 55.5   | 90.2%         |
| RalB 12–186         | 46.9      | 42.9   | 91.5%         |

3.5. Evaluation of workflow enhancements from the automated system

Often individual process steps in a manual method will complete outside routine working hours leaving the equipment idle or extending the incubation times with TEV protease or nucleotide exchange reagents. This is not the case in the automated method which has substantial time benefits compared to the manual approach. We established typical processing times for each workflow and demonstrated that the parallel purification of the three GTPases was over 29 h faster using the automated multidimensional method. When comparing the single GTPase run data, the automated preparation of a one GTPase domain almost 24 h quicker (Fig. 9). The net result is quicker delivery, increased FPLC throughput and a fresher protein product delivered to the downstream application.

4. Discussion

Many research programs, including structure based drug discovery, utilize a lot of protein intensive techniques, such as x-ray crystallography, nuclear magnetic resonance or isothermal titration calorimetry. As a consequence it is very easy for protein production to become a rate limiting factor in the discovery process. Whilst it is
not always possible to completely eliminate this, it is always advisable to monitor bottlenecks and be mindful of opportunities to reduce or eliminate them. To achieve efficiencies in this area we decided to design a flexible, fully automated system for the parallel production of multiple activated GTPases.

We developed pBDDP-SPR3 expression vector by replacing the tag and multiple cloning site of pET28a with our own bespoke configuration. This encodes for two His8 motifs, a TEV protease cleavage sequence and a simplified multiple cloning site, and offers an easy to use flexible expression vector tailored to our laboratory’s activities. This vector has now been used in the successful overexpression of scores of proteins in addition to those described in this paper.

The scheduling of the purifications on FPLC systems also represented an opportunity for process optimisation. The FPLCs were inactive for significant amounts of time during the purification if the operator was committed to another task or a process step completed outside of core working hours. It was clear that a reduced requirement for frequent manual intervention could enhance throughput by potentially reducing run times, uplifting capacity and may also provide the added reproducibility and reliability that can accompany automation. By enhancing the capability of our AKTA AVANT 25 systems with extra sample inlets and a loop valve we were able to develop a parallel multidimensional process for the fully automated production of nucleotide loaded GTPases.

As the overall purification sequence between automated and manual methods was the same, there was no expectation that moving to a multidimensional system would result in an improvement in protein purity. Comparison of products on coomassie stained gels suggested that purity levels between approaches was equivalent. However silver stained gels did suggest that proteins isolated by the automated workflow may be marginally purer (Fig. 5). Any degree of improved purification may seem slight but it can have significant effects on the success and performance level of techniques like protein crystallography and NMR.

The principal motivation for this method development was to reduce run times and there were some initial concerns that yields would be reduced as a result of incomplete sample application or peak collection. Indeed, in order to minimize sample volumes for subsequent steps, and to avoid the collection of small unwanted peaks, the criteria for peak recognition was tightly specified in the software with relatively strict requirements in terms of both peak height and slope. However, our early concerns about reduced yields did not seem to materialize, with automated processing invariably producing higher yields than the manual equivalent. It would appear that any losses incurred as a result of strict peak recognition in automation were less than losses observed by manual collection and reformatting of intermediate products.

While others have described the use of multidimensional chromatography for protein purification there is less precedent for the incorporation of protein conditioning and modification steps. By attaching a reservoir to a loop valve the method was able to deliver appropriate reagents to purified proteins and achieve a complete exchange of GDP to selected nucleotide analogues. Buffer chase steps were included to push the complete volume of exchange reagents beyond the outlet valve and into the waiting protein sample. A comprehensive recording of tubing and component dead volumes and precise chaging of the reaction reagents with buffer allowed us to confidently deliver accurate triple digit microliter volumes to intermediate proteins. This component has the potential to automate a broad range of protein conditioning events including co-factor delivery, chemical modification, and the introduction of other proteins for complex formation or the delivery of enzymes to manipulate post-translational modifications.

By far the main advantage offered by automated purification is reduced run time, with a three protein parallel GTPase purifications taking approximately 44 h in contrast to the 96 h run time of the manual workflow (Fig. 9). Indeed the run time can be compressed further when producing proteins with compatible buffer...
Fig. 8. KRas/SOS1 nucleotide exchange assay. (A) Equimolar concentrations of KRas 1–169 loaded with mantGDP by both production methods and applied to SOS1 catalysed nucleotide exchange which measures a quenching of mant fluorescence when it is displaced from KRas by GTPyS. (B) The mean rates of exchange kinetics of both preparations were almost identical (automated, 1.15 ± 0.032 × 10^3 sec^{-1}; manual 1.20 ± 0.010 × 10^3 sec^{-1}) demonstrating that the multidimensionally prepared mantGDP loaded KRas was suitable for downstream applications. Data was analyzed and the rates derived by fitting to a single exponential decay equation. The red line joining the data illustrates the fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 9. Run times for purifications by manual and automated workflows. Recording of purification periods during both automated and manual workflows reveal substantial compression of run times using the automated method with 50% reductions in duration in both single protein and three protein parallel productions.
requirements. This allows a single equilibration of desalting and SEC columns, and then all parallel proteins can be repeatedly applied without re-equilibration between sample injections. This significantly reduces the run time - in these circumstances parallel purification of GTPases by the method described would be further reduced to only 31 h and 20 min. If it were practical to keep this system supplied with lysates when required, each FPLC could theoretically establish a throughput of 5 parallel multidimensional runs per week and an output of 15 purified, activated GTPases. Although this ideal is difficult to achieve, the methodology has greatly increased our ability to respond quickly to increases in demand while maintaining or even enhancing the yield and quality of the product. It is especially satisfying that it is now possible to incorporate a sensitive reaction, like GTPase nucleotide exchange classification, and quality assessment for immobilization of protein onto surface plasmon resonance sensorchips, Anal. Chem. 83 (5) (2011) 1800–1807.

In summary, the implementation of parallel multidimensional workflows, even for relatively complex production protocols, offers busy protein production teams the opportunity to increase routine output, improve product quality and free up human resource for other tasks thus loosening the bottleneck and accelerating the discovery process.

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