Acta Biochimica Polonica

Vol. 50 No. 1/2003

239–247

www.actabp.pl

Expression in *Escherichia coli* of human *ARHGAP6* gene and purification of His-tagged recombinant protein✪

Anna-Maria Ochocka, Marzena Czyżewska and Tadeusz Pawełczyk✉

Department of Molecular Medicine, Medical University of Gdańsk, Gdańsk, Poland

Received: 15 November, 2002; accepted: 12 February, 2003

Key words: ARHGAP6, His-tag, IbpA, IbpB

In this report we describe cloning and expression of human Rho GTPase activating protein (ARHGAP6) isoform 4 in *Escherichia coli* cells as a fusion protein with 6xHis. We cloned the ARHGAP6 cDNA into the bacterial expression vector pPROEX-1. Induction of the 6xHis-ARHGAP6 protein in BL21(DE3) and DH5α cells caused lysis of the cells irrespective of the kind of culture medium used. Successful expression of the fusion protein was obtained in the MC4100Δ*ibp* mutant strain lacking the small heat-shock proteins IbpA and IbpB. Reasonable yield was obtained when the cells were cultured in Terrific Broth + 1% glucose medium at 22°C for 16 h. The optimal cell density for expression of soluble 6xHis-ARHGAP6 protein was at A_{600} about 0.5. Under these conditions over 90% of the fusion protein was present in a soluble form. The 6xHis-ARHGAP6 protein was purified to near homogeneity by a two step procedure comprising chromatography on Ni-nitrilotriacetate and cation exchange columns. The expression system and purification procedure employed made it possible to obtain 1–2 mg of pure 6xHis-ARHGAP6 protein from 300 ml (1.5 g of cells) of *E. coli* culture.

Small monomeric G-proteins cycle between the GDP-bound inactive form and the GTP-bound active one. The activation signal causes GDP to be exchanged for GTP. Hydrolysis of GTP by an intrinsic GTPase activity returns the G-protein to the inactive state, but for typical small G-proteins the rate of GTP hydrolysis is extremely low (Sweet *et al.*, 1984). GTPase-activating proteins (GAPs) are accessory proteins catalyzing the formation of inactive G-protein by stimulating its GTPase activity (Trahey & McCormick, 1987).

Rho proteins are members of the superfamily of small G-proteins. At least three types

✪This work was supported by the State Committee for Scientific Research (KBN, Poland) grant No. 6P04B02620 to M.C.

✉To whom correspondence should be addressed: Department of Molecular Medicine, Medical University of Gdańsk, ul. Dębinki 7 paw. 29, 80-211 Gdańsk, Poland; tel.: (48 58) 349 2750, fax: (48 58) 349 2759, e-mail: tkpaw@amg.gda.pl

Abbreviations: GAPs, GTPase-activating proteins; PI, phosphatidylinositol.

of Rho protein are known, A, B, and C. Rho is involved in controlling of cytoskeletal organization. Microinjection of Rho into mammalian cells causes rapid and dramatic changes in cell shape, forming finger-like polarized cytoplasmic extensions (Paterson *et al.*, 1990; Miura *et al.*, 1993). Inactivation of endogenous Rho (by ADP-ribosylation *via* microinjection of botulin ADP-ribosyltransferase C3) causes dissolution of actin filaments, and cells to round up (Rubin *et al.*, 1988; Paterson *et al.*, 1990). In addition, Rho has been demonstrated to influence phosphatidylinositol (PI) metabolism by enhancing the activity of PI 4-phosphate 5-kinase (Chong *et al.*, 1994). On the other hand, the p122 GAP specific for Rho possesses the ability to interact with δ-1 isoform of phospholipase C (PLC δ1) and enhance its activity (Homma & Emori, 1995). Recently, a novel GTPase-activating protein gene named *ARHGAP6* specific for RhoA has been identified (Schaefer *et al.*, 1997; Prakash *et al.*, 2000).

In order to obtain reasonable quantities of pure ARHGAP6 protein needed to study its interaction with PLC δ1 we developed a bacterial expression system. Isoform 4 of human ARHGAP6 protein was expressed in *Escherichia coli* cells and purified to homogeneity.

MATERIALS AND METHODS

Leupeptin, isopropyl β-D-thiogalactopyranoside, ampicillin, alkaline phosphatase-conjugated goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate, and Nitro Blue Tetrazolium were from Sigma-Aldrich Sp. z o.o. (Poznań, Poland). Pefabloc SC was from Roche Applied Science (Mannhein, Germany). *E. coli* QC771Δ*ibpA/B* and MC4-100Δ*ibpA/B* were kindly provided by Dr. Ewa Laskowska (Biochemistry Department, University of Gdansk). *E. coli* strains BL21(DE3), DH5α, pPROEX-1 vector, Ni^{2+}-NTA resin, rTEV protease, oligo(dT), and dNTPs were from Invitrogen (Carlsbad, CA, U.S.A.). Immobilon-P Transfer Membrane was from Millipore Corp. (Bedford, MA, U.S.A.). *E. coli* Qiagen EZ cells and PCR Cloning Kit were from Qiagen GmbH (Hilden, Germany). All primers were from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.). Total RNA Prep Plus Kit, Plasmid Miniprep Plus and DNA Clean-Up Kit were from A&A Biotechnology (Gdansk, Poland). Moloney murine leukemia virus reverse transcriptase (MMLV-RT), and Tth DNA polymerase were from Epicentre Technologies (Madison, WI, U.S.A.). RNasin was from Promega (Madison, WI, U.S.A.). *Nde*I and *Xho*I were from Fermentas AB (Vilnius, Lithuania). All other reagents were of analytical grade.

RNA extraction and reverse transcription. Total RNA was extracted from human blood cells with the use of Total RNA Prep Plus Kit and stored at –40°C. Reverse transcription was performed in 20 μl final volume of 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM dNTPs, 250 ng oligo(dT), 14 units of reverse transcriptase (MMLV-RT), 10 units of RNasin, and 1–5 μg of RNA. Reactions were incubated for 45 min at 42°C and 5 min at 95°C.

Cloning of the* ARHGAP6 *gene. Unless otherwise indicated, the recombinant DNA methods used were those of Sambrook *et al.* (1989). In order to obtain cDNA for ARHGAP6 we ran PCR reaction with the primers GAPB3 (GGTGATTTCACCGGATCCAGCATGT, forward) and GAPE2 (GTGCCAGTGGAATTCACCCACGG, reverse). The primers were based on the human cDNA sequence (GeneBank, accession No. AF177663). The PCR reaction was performed in 20 μl final volume in 50 mM Tris/HCl, pH 9.0, 20 mM ammonium sulfate, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 1.5 U of Tth DNA polymerase, 0.5 μM primers and 1 μg of human blood cells cDNA. The PCR consisted of an initial denaturation at 95°C for 3 min and 35 cycles of 45 s at 95°C, 60 s at 63°C, 150 s at 72°C, and a final extension of 10 min at 72°C. The PCR product

(2418 bp) was directly cloned into pDrive Cloning Vector using Qiagen PCR Cloning Kit according to the manufacturer's protocol. The pDrive-*ARHGAP6* plasmid was produced in Qiagen EZ cells. The cloned cDNA was sequenced and was confirmed to be complete ARHGAP6-encoding cDNA.

In order to clone the ARHGAP6 cDNA into the bacterial expression vector pPROEX-1 (Fig. 1) we generated a restriction enzyme

Cell growth and expression of ARHGAP6. pPROEX-*ARHGAP6* was used for the expression of ARHGAP6 as a fusion protein with the 6xHis peptide at the amino-terminus. Appropriate *E. coli* strain was transformed with the above-mentioned plasmid and colonies were grown on agar plates. For BL21(DE3) and DH5α the agar contained 100 μg/ml ampicillin. For QC771Δ*ibpA/B* and MC4100Δ*ibpA/B* cells

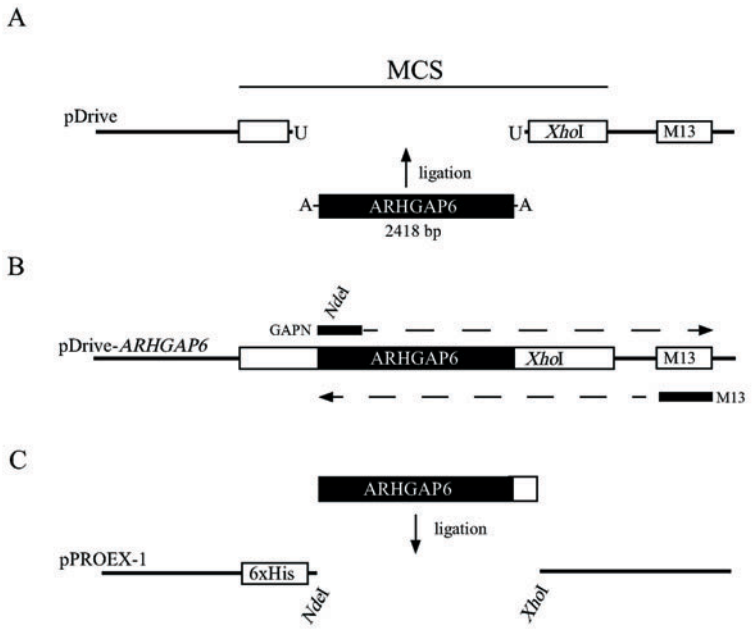

Figure 1. Cloning of ARHGAP6 cDNA into pPROEX-1 vector.

A. Product of PCR (2418 bp) performed on human blood cells cDNA was directly ligated into pDrive cloning vector. MCS, multiple cloning site; M13, M13 primer binding site. The *Xho*I restriction site in MCS of pDrive is indicated. **B.** Generation of *Nde*I restriction site by PCR with GAPN and M13 primers. **C.** Ligation of ARHGAP6 cDNA into pPROEX-1 vector.

cleavage site for *Nde*I by running PCR reaction with the primers GAPN (ACCGGATCCCATATGTCAG, forward) and M13F (GTAAAACGACGGCCAGT, reverse). The PCR reaction was performed as above with 10 ng of pDrive-*ARHGAP6*. The PCR consisted of an initial denaturation at 95°C for 3 min and 35 cycles of 45 s at 95°C, 60 s at 53°C, 180 s at 72°C, and a final extension of 10 min at 72°C. The ARHGAP6 cDNA was then cut with appropriate enzymes and ligated into the unique *Nde*I and *Xho*I sites in pPROEX-1 (Fig. 1).

the agar contained 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 30 μg/ml streptomycin. Picked colonies were grown overnight at 37°C in ampicillin-containing Luria-Bertani (LB) or Terrific Broth supplemented with 1% glucose (TBG) medium, and 1 ml of this culture was inoculated into 1 l of fresh medium, and incubated at the desired temperature to an absorbance of 0.5–0.8 (measured at 600 nm). Expression of the 6xHis-ARHGAP6 protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following induction, the

cells were grown for an appropriate time and harvested by centrifugation.

Purification of ARHGAP6. All steps were done at 4°C. Cell pellet from 1 l culture was suspended in 40 ml of buffer A (50 mM Tris/HCl buffer, pH 8.5, 0.5 μg/ml leupeptin, 0.2 mM Pefablock SC), placed on powdered dry ice and sonicated. The crude cell extract was clarified by centrifugation at 20 000 × ***g*** for 30 min. Clear supernatant was passed through a 5 ml Ni-nitrilotriacetate (Ni-NTA) column preequilibrated with buffer A containing 300 mM KCl (buffer A1). The column was washed with buffer A1 containing 20 mM imidazole until no more protein was eluted. The 6xHis-ARHGAP6 protein was then eluted with 7 ml of buffer A1 containing 100 mM imidazole. Fractions containing high protein concentration were combined, desalted on Sephadex G-25 M (Pharmacia columns PD-10) previously equilibrated with buffer B (50 mM phosphate buffer, pH 7.0, 1 mM dithiothreitol, 0.5 μg/ml leupeptin, 0.2 mM Pefablock SC, 20% glycerol), and applied to a UnoS cation exchange FPLC column equilibrated with buffer B. The column was washed with 10 ml of buffer B and eluted with a linear gradient from 0 to 2 M KCl over 20 ml. The active fractions were pooled and stored at –20°C.

Antibodies. Polyclonal antibodies to the 6xHis-ARHGAP6 protein were generated in rabbits. Rabbits were subcutaneously injected in the back of the neck with 400 μg of purified recombinant 6xHis-ARHGAP6 protein in Freund's adjuvant followed by three boosts with 200 μg of the antigen each every 3 weeks. The antibodies were purified by chromatography on a protein A-agarose column.

SDS/PAGE and immunoblotting. Samples (100 μg of protein) were separated by polyacrylamide gel electrophoresis (8% acrylamide) in the presence of sodium dodecyl sulfate (SDS/PAGE) (Laemmli, 1970) and then electrophoretically transferred to Immobilon poly(vinylidenedifluoride) (Millipore) transfer membrane. The membrane was blocked with 3% bovine albumin (fraction V) in phosphate buffered saline (PBS) with 0.02% NaN_3 and then washed with PBS. The blocked membrane strips were incubated with rabbit anti-ARHGAP6 polyclonal antibodies (dilution 1:10000). Immunostaining was done using alkaline phosphatase-conjugated goat anti-rabbit IgG (dilution 1:20000), the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium.

E. coli *strains*. BL21(DE3): F^- *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3); DH5α: F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyrA96 rel*A1; Qiagen EZ: [F'::Tn*10*(Tc^r) *proA*$^+$*B*$^+$ *lacl*^{q}ZΔ*M15*] *recA1 endA1 hsdR17* (r_{K12}^- m_{K12}^+) lac *glnV44 thi-1 gyrA96 relA1*; QC771: F^- Δ(*lac-arg*F) U169 *rps*L179 inversion (*rrnD-rrnE*)1 sup *amb* P1 (Δ*ibpA/B::cm*); MC4100*A/B*: [*araD139* Δ(*lac POZYA argF*) *U169 fla relA rpsL*] Δ*ibp::cmA/B*.

Analytical. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. DNA and RNA concentrations were determined by measuring the absorbance at 260 nm.

RESULTS

Expression of 6xHis-ARHGAP6 protein in *E. coli* cells

The cDNA of human ARHGAP6 isoform 4 (GeneBank accession No. AF177663) was cloned into pPROEX-1 vector (Fig. 1), which in our laboratory proved to be useful in obtaining several mammalian proteins in *E. coli* cells (Pawelczyk & Matecki, 1997; Pawelczyk *et al.*, 2000; Sakowicz *et al.*, 2001). Initially, in order to obtain human recombinant ARHGAP6 protein we used the BL21(DE3) cells. However, after transformation with the pPROEX-*ARHGAP6* plasmid the cells lysed on LB agar and occasionally single colonies were observed on TB +1% glucose (TBG) agar

plates. Our attempts to culture cells from these colonies in TBG medium at 37°C were unsuccessful, because the cells lysed. Similar results were obtained for DH5α cells. These observations indicated that human ARHGAP6 protein is highly toxic to *E. coli* cells. Such a toxicity could be the result of accumulation of native and/or denatured and aggregated ARHGAP6 protein. Recently, it has been demonstrated that small heat-shock proteins (IbpA, IbpB) stabilize denatured and aggregated proteins in *E. coli* cells (Kuczynska-Wisnik *et al.*, 2002). Therefore, for the expression of human ARHGAP6 protein we tested the QC771Δ*ibpA/B* and MC4100-Δ*ibpA/B* mutant strains, which do not express both the IbpA and IbpB proteins. After transformation with pPROEX-*ARHGAP6* both mutant strains grew as colonies on LB and TBG agar plates (not shown). Induction of the 6xHis-ARHGAP6 protein in MC4100-Δ*ibpA/B* cells cultured in LB or TBG medium at 37°C resulted in obtaining proteins with lower molecular masses than the predicted mass of 6xHis-ARHGAP6 (Fig. 2). Under such conditions no induced proteins were visible in extracts of QC771Δ*ibpA/B* cells. A reasonable yield of the 6xHis-ARHGAP6 protein was obtained when MC4100Δ*ibpA/B* cells were cultured in TBG medium at 22°C for 16 h (Fig. 2). The optimal cell density for expression of soluble protein was at a A_{600} about 0.5; at greater cell densities the amount of the expressed protein decreased sharply. Under these conditions over 90% of 6xHis-ARHGAP6 was present as a soluble protein (Fig. 3). There were no

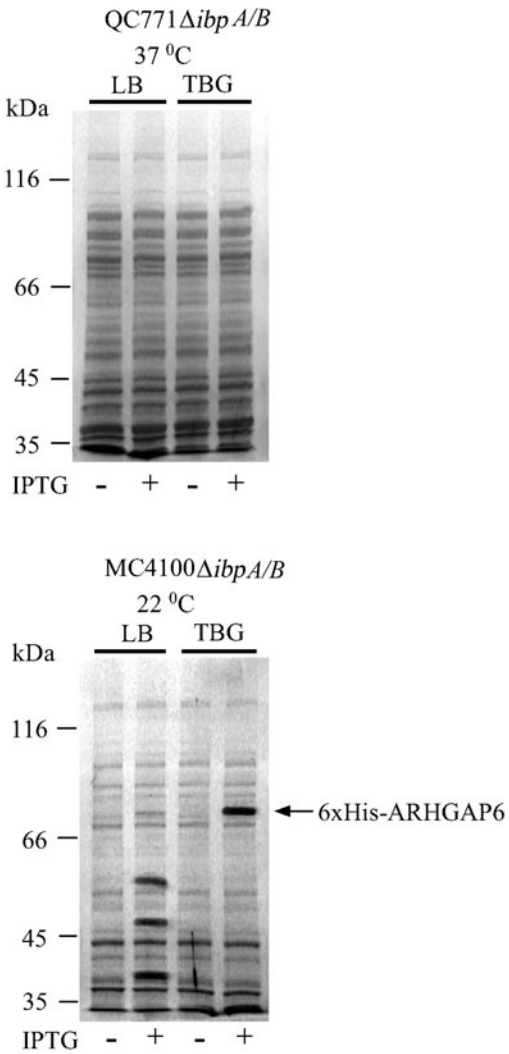

Figure 2. Expression of 6xHis-ARHGAP6 protein in QC771Δ*ibpA/B* and MC4100-Δ*ibpA/B* cells.

E. coli cells transformed with pPROEX-*ARHGAP6* were grown in media and at temperatures indicated until A_{600} reached 0.5 and the expression of 6xHis-ARHGAP6 protein was induced by adding IPTG. Expression at 37°C and at 22°C was performed for 3 and 12 h, respectively. Crude cell extracts were prepared and analyzed by 8% SDS/PAGE. The positions of molecular mass standards are indicated on the left. LB, Luria-Bertani medium; TBG, Terrific Broth supplemented with 1% glucose.

losses in the yield of 6xHis-ARHGAP6 expression in transformed cells stored on TBG agar plates for up to 6 days at 4°C. Cells stored on agar plates longer than 6 days produced cultures with a lower yield of the protein.

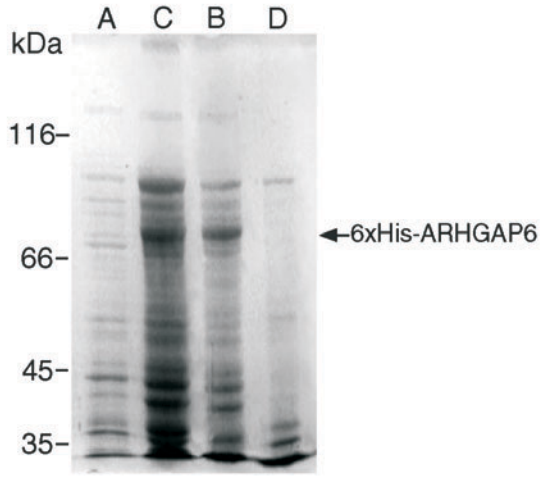

Figure 3. Evaluation of solubility of 6xHis-ARHGAP6 protein expressed in MC4100Δ*ibp*A/B cells.

MC4100Δ*ibpA/B* cells transformed with pPROEX-*ARHGAP6* were cultured in TBG medium at 22°C until A_{600} reached 0.5 (lane A). At this point IPTG was added and cells were cultured for 16 h. Crude cell extracts (lane B) and high-speed supernatant (lane C) were prepared and analyzed on 8% SDS/PAGE. The pellet (lane D) from high-speed centrifugation (30 min, 20 000 × **g**) was dissolved in loading buffer (equal volume to initial cell extract volume). On lanes B–D 25 μl of sample was loaded. The positions of molecular mass standards are indicated on the left.

Subsequent chromatography on a cation exchange (Uno S) column gave a significant improvement in the purity of the 6xHis-ARHGAP6 protein. The expression system employed and the described purification pro-

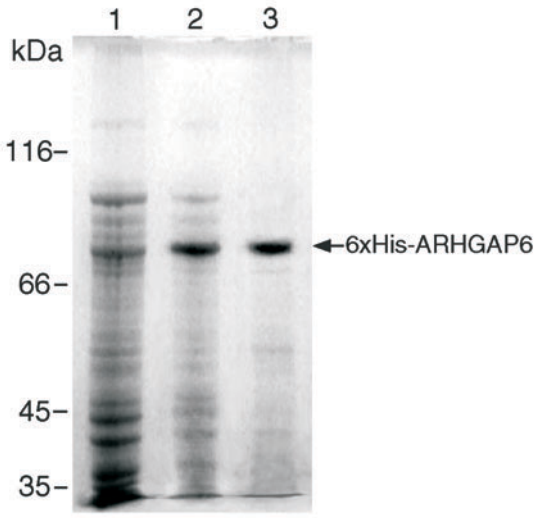

Figure 4. Purification of 6xHis-ARHGAP6 protein expressed in *E. coli*.

Proteins from MC4100Δ*ibpA/B* cells transformed with pPROEX-*ARHGAP6* were electrophoresed on 8% SDS/PAGE. Expression of 6xHis-ARHGAP6 protein was performed at 22°C for 16 h. Individual lanes of the SDS/PAGE contained the following: high-speed supernatant of crude extract of cells expressing 6xHis-ARHGAP6 protein (lane 1), proteins eluted from the Ni-NTA column by 100 mM imidazole (lane 2), proteins eluted from the cation exchange column (UnoS) by 120 mM KCl. The positions of molecular mass standards are indicated on the left.

Purification of 6xHis-ARHGAP6 protein

Usually the process of protein purification started with about 1.5 g of *E. coli* cells obtained from 300 ml culture. Soluble bacterial lysate was first subjected to chromatography on Ni-NTA. The purity and apparent molecular mass of the eluted proteins were checked by SDS/PAGE. The advantage of using His-tagged protein is a relative ease of purification on a metal-affinity column. However, despite extensive washes the fractions eluted by 100 mM imidazole contained also other proteins besides 6xHis-ARHGAP6 (Fig. 4). cedure allowed us to obtain 1–2 mg of pure 6xHis-ARHGAP6 protein from 1.5 g of *E. coli* cells. The purified 6xHis-ARHGAP6 protein migrated on 8% SDS/PAGE as a protein with a molecular mass of 85 kDa (Fig. 4). This is in good agreement with 87.8 kDa calculated from the predicted amino-acid sequence of the recombinant protein.

6xHis-ARHGAP6 antibodies

The 6xHis-ARHGAP6 protein purified as described above was electrophoresed on 8%

PAGE/SDS. The protein bands were visualized with Ponceau S and the 6xHis-ARHGAP6 band was cut out and electroeluted from the gel. The 6xHis-ARHGAP6 protein was then used to generate polyclonal antibodies in rabbits. The obtained anti-ARHGAP6 polyclonal antibodies recognized with high specificity the 6xHis- ARHGAP6 protein. As can be seen in Fig. 5 the antibodies did not recognize any rat protein. Immunoblot analysis performed on extracts of human blood cells showed the presence of one major (about 85 kDa) and two minor protein bands (about 100 kDa and about 60 kDa) reactive with anti-ARHGAP6 (Fig. 5). The protein bands reactive with anti-

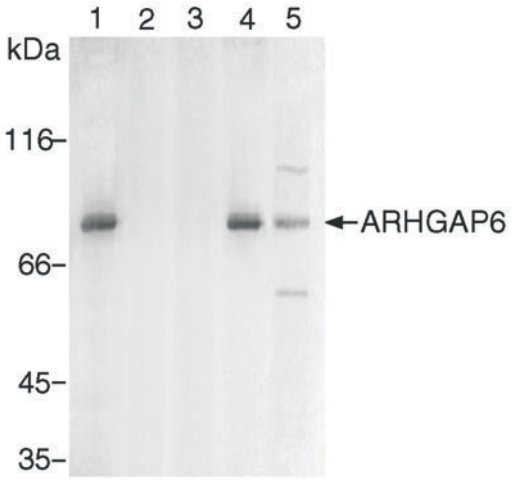

Figure 5. Specificity of anti-ARHGAP6 polyclonal antibodies.

Tissue extracts (50 μg of protein) were subjected to SDS/PAGE (8%) and transferred to an Immobilon transfer membrane. The membrane was immunoblotted with anti-ARHGAP6 polyclonal antibodies. On lane 1, recombinant ARHGAP6 protein was loaded (30 ng). On lanes 2 and 3, extracts of rat kidney and heart were loaded, respectively. On lane 4, rat kidney extract supplemented with 30 ng of recombinant ARHGAP6 was loaded. On lane 5, extract of human blood cells was loaded. The positions of molecular mass standards are indicated on the left.

ARHGAP6 are likely to be isoforms of ARHGAP6 generated by alternative splicing. Based on cDNA sequences deposited in GeneBank (accession Nos. NM001174, AF022212, AF012272, AF177663, and AF177665) five isoforms of ARHGAP6 protein with calculated molecular masses of 106, 75.9, 84 (two isoforms) and 62.9 kDa could be predicted to be present in human tissues.

DISCUSSION

According to our knowledge this is the first report describing expression in *E. coli* cells and purification of human ARHGAP6 protein. The expression and purification system described in this contribution enables obtaining reasonable quantities of soluble 6xHis-ARHGAP6 protein. The successful expression of this protein was made possible by the use of the MC4100Δ*ibpA/B* mutant strain.

Numerous data indicate that foreign proteins often do not express well in *E. coli* cells. Some proteins when expressed in *E. coli* induce cell lysis. The lysis of non-induced BL21(DE3) cells transformed with pPROEX-*ARHGAP6* observed in our experiments could be due to background expression of the toxic protein (Studier, 1991). Partial improvement of cells stability noted when the transformed cells were cultured in the presence of 1% glucose supports such an assumption. Decreased background expression of the T7 polymerase gene and improved plasmid stability in BL21(DE3) cells cultured in the presence of 1% glucose have been reported (Pan & Malcolm, 2000). The reduction of background expression in BL21 (DE3) cells in the presence of 1% glucose can be explained by catabolite repression of the *lacUV5* promoter, which is a mutated version of the original *lac* promoter. The mutation (G → A at –9) reduces *lacUV5* dependence on the cAMP/catabolite gene activator protein (CAP) complex to initiate transcription (Hirschel *et al.*, 1980). Previously it was demonstrated that the affinity of *lacUV5* for cAMP/CAP is significantly reduced but not fully abolished (Fried & Crothers, 1984). Therefore, the cAMP/CAP complexes present in cells growing in media with poor carbon sources (LB, TB) may acti-

vate to some extent the *lacUV5* promoter. Glucose, by decreasing the level of cAMP, suppresses this so-called "promoter leaking".

The lysis of BL21(DE3) cells when expression of the 6xHis-ARHGAP6 protein was induced indicates that this protein is toxic to *E. coli* cells. Such a toxicity could be caused by native 6xHis-ARHGAP6 protein or/and by accumulation of denatured aggregates. The results obtained with the use of MC4100Δ*ibA/B* cells point out rather to the second possibility. The IbpA and IbpB chaperones are small heat-shock proteins (sHSP) whose physiological role in *E. coli* cells is still enigmatic. IbpA and IbpB are the major components of denatured and aggregated proteins (the S fraction) accumulating in *E. coli* cells during heat-shock (Kucharczyk *et al.*, 1991; Laskowska *et al.*, 1996) and are found associated with non-native recombinant proteins expressed in bacterial cells (Allen *et al.*, 1992). It was reported that *in vitro* IbpB oligomers bind and stabilize heat-denatured proteins preventing the irreversible aggregation (Veinger *et al.*, 199ir8). Kitagawa *et al.* (2002) reported that IbpA and IbpB protected several enzymes against inactivation by heat, oxidants or guanidine hydrochloride. The recently reported data from *in vivo* experiments on the refolding of heat-denatured fructose-1-6-bisphosphate aldolase indicate that the IbpA/B proteins may inhibit DnaK/DnaJ-dependent refolding of some proteins by blocking DnaK/DnaJ-binding sites in denatured proteins (Kuczynska-Wisnik *et al.*, 2002). These authors also found that mild heat stress produced less aggregated proteins (fraction S) in mutant cells not expressing the IbpA/B proteins compared to WT bacteria cells. It may be assumed that the 6xHis-ARHGAP6 protein expressed in MC4100Δ*ibpA/B* cells lacking the IbpA and IbpB proteins instead of being held in an aggregated form by the IbpA/B proteins is effectively refolded by the DnaK/DnaJ chaperone system. Furthermore, it may be speculated that the lack of accumulated insoluble 6xHis-ARHGAP6 protein together with the suppression of promoter leaking by glucose are the factors responsible for cell viability during ARHGAP6 expression in *E. coli* cells. On the other hand, the reasons for the lack of expression of the 6xHis-ARHGAP6 protein in QC771Δ*ibpA/B* cells remain unknown. It may be assumed that in QC771Δ*ibpA/B* cells besides the IbpA/B proteins other genome- determined factors influence the expression of foreign proteins.

In summary, the methodology described here allowed the production of soluble 6xHis-ARHGAP6 protein in *E. coli* cells, which could be effectively purified by a simple two step procedure.

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