A novel ADP-ribosylation like factor (ARL-6), interacts with the protein-conducting channel SEC61β subunit

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Received 22 July 1999; received in revised form 25 August 1999

Abstract **We report here the isolation of a new member of the ADP-ribosylation factor (ARF)-like family (ARL-6) present in the J2E erythroleukemic cell line, but not its myeloid variants. Consistent with this lineage-restricted expression, ARL-6 mRNA increased with erythropoietin-induced maturation of J2E cells, and decreased with interleukin 6-induced differentiation of M1 monoblastoid cells. In tissues, ARL-6 mRNA was most abundant in brain and kidney. While ARL-6 protein was predominantly cytosolic, its membrane association increased following exposure to GTP-γS, like many members of the ARF/ARL family. Using the yeast two-hybrid system, six molecules which interact with ARL-6 were identified including SEC61β, a subunit of the heterotrimeric protein conducting channel SEC61p. Co-immunoprecipitation of ARL-6 confirmed a stable association between ARL-6 and SEC61β in COS cells. These results demonstrate that ARL-6, a novel member of the ADP-ribosylation factor-like family, interacts with the SEC61β subunit.**

Key words: ADP-ribosylation like factor; Protein conducting channel; Erythroid/myeloid lineage switch

1. Introduction

Leukemic cells displaying the phenotype of one hemopoietic lineage can suddenly express the features of another lineage in a process termed hemopoietic lineage switching [1]. J2E erythroleukemic cells are committed to the erythroid pathway, respond to erythropoietin and do not express any markers of other hemopoietic lineages [2]. However, when cultured under adverse conditions (i.e. extreme overgrowth and cell death), occasionally some cells emerged which displayed features of myeloid cells [3]. These cells were larger, pleiomorphic and had the appearance of monocytic cells. Ultrastructural alterations were also evident and these cells ceased expressing erythroid-specific genes and cell surface markers [3]. In addition, they displayed numerous cell surface proteins typically associated with monocytes and macrophages. These observations showed that cells committed to the erythroid lineage and capable of responding to erythropoietin were able to change hemopoietic lineages and enter the myeloid pathway. This phenomenon was remarkably similar to the conversion of mature lymphoid B-cells to macrophages that we had observed previously [1]. Interestingly, several human leukemias have been described which suddenly switch lineage, demonstrating that this is not simply a laboratory curiosity and is of clinical significance [1].

The Ras superfamily of low molecular weight GTP-binding proteins can be divided into several subgroups with diverse functions, including cellular proliferation and differentiation (Ras), intracellular trafficking (Rab), cytoskeletal remodeling (Rho) and nuclear transportation (Ran). A common feature of this superfamily is regulation by GTP hydrolysis, which cycles between an active GTP-bound, and an inactive GDP-bound state [4].

The ADP-ribosylation factor (ARF) group is part of the Ras superfamily and is subdivided into the ARF and ARF-like (ARL) molecules. There are six members of the ARF family (ARF1–6) and five ARL members (ARL1–5). The best characterized ARF is ARF-1, identified as a co-factor required for the cholera-toxin dependent ADP-ribosylation of the heterotrimeric G protein Gs, which leads to the activation of adenylate cyclase [5]. While ARF-1 is predominantly cytosolic in cell fractionation experiments, immunofluorescent microscopy has localized ARF-1 specifically to the trans-Golgi network [6]. It has also been proposed that ARF-1 is a subunit of the coat of Golgi-derived vesicles [7]. Purified ARF-1 binds to vesicles in its active GTP-bound form, via exposure of the amino terminal myristoyl group, which promotes membrane attachment [5]. The N-terminal hydrophobic α-helix also plays an important role in regulating the membrane localization of ARF-1 [5].

ARFs are also associated with cell signaling, as they are effective activators of phospholipase D [8]. An ARF-1 guanine nucleotide exchange factor (ARNO) has been identified [9,10] which is dependent upon association with phosphatidylinositol for activity. This regulation probably occurs through phosphatidylinositol binding to the pleckstrin homology domain of ARNO. The structure of ARF-1 complexed with GDP has been resolved [11] and it provides the structural basis for GTP-dependent modulation of membrane binding, the lack of intrinsic GTPase activity, and the nature of effector binding surfaces on ARFs. ARF-6, the least conserved ARF member, has been localized to the cell periphery and, depending on the nucleotide bound, cycles between the plasma membrane and intracellular endosomal vesicles [12]. Furthermore, ARF-6 regulates the actin cytoskeletal organization by binding the Rac1-interacting protein POR1 in a GTP-dependent manner [13].

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Abbreviations: ARF, ADP ribosylation factor; ARL, ARF-like; HA, hemagglutinin; ARNO, ARF-1 guanine nucleotide exchange factor

PII: S0014-5793(99)01188-6

We used differential display in an attempt to isolate lineage specific genes expressed in J2E erythroleukemic cells [14] that had undergone erythroid to myeloid lineage switching [15]. A new member of the ARL family (ARL-6) was identified and its transcript was up-regulated during erythropoietin-induced differentiation of erythroid cells and down-regulated during interleukin-6-induced macrophage differentiation. A yeast two-hybrid screen revealed that ARL-6 interacted with the protein conducting channel subunit SEC61β. This association was confirmed by hemagglutinin (HA)-tagged ARL-6 co-immunoprecipitating with SEC61β in COS cells. These results show that ARL-6, a novel ARL, interacts with the protein conducting channel SEC61β subunit.

2. Materials and methods

2.1. Molecular cloning of ARL-6

Total RNA was extracted according to the method of Chomczynski et al. [14], from which poly-A^+ RNA was isolated using the poly-A-tract mRNA isolation system (Promega, Madison, WI, USA). Differential displays were performed essentially as described by Liang and Pardee [15] using mRNA from J2E erythroid cells [2] and its myeloid derivative J2E-NR-m2 [3]. The oligonucleotide PTGC (5′-T_{11}GC-3′) was used as the reverse-transcriptase primer and primer RP2 (5′-GTG AGG CGT C-3′) was used to amplify cDNA in the presence of ^{35}S-dCTP. The differential display products were resolved on a 6% denaturing PAGE gel and exposed to X-ray film. Differentially displayed bands were excised and amplified by PCR before subcloning into the pGEM-T-Easy (Promega) vector. Both strands were sequenced using the ABI-Prisim method (PE Applied Biosystems, Branchburg, NJ, USA).

A λZAP total mouse embryo cDNA library (Clontech, Palo Alto, CA, USA) and pSPORT-J2E cDNA library made from J2E mRNA using the Superscript system (Gibco-BRL, Gaithersburg, MD, USA) were screened according to the manufacturer's instructions. Positive clones were subcloned into pBSK+ (Stratagene, La Jolla, CA, USA) and sequenced. Databases (GenBank/EMBL/DDBJ) were searched using FASTA and BLAST programs of the Genetics Computer Group software package (Madison, WI, USA).

The 5′RACE protocol was essentially as described by Edwards et al. [16], using J2E cDNA as the template with the oligonucleotide AS (5′-GGA GAC TTC CAA GGT CTT AGC TAT CAC TTA AGC AC-3′) as the 5′ extension primer. The PCR reaction was performed with oligonucleotides P2 (5′-CTG GTT CGG CCC ACC TCT GAA GGT TCC AGA ATC GAT AG-3′) and P1 (5′-GCT GAA GCC TTC CAA CG-3′) and the product that hybridized with the 0.4 kb *Eco*RI fragment of pSPORT-J2E1/2 subcloned into pGEM-T-Easy and sequenced. Northern blots were performed using random primed cDNA (Giga-Prime kit, Bresatec, Adelaide, S.A., Australia) labeled with ^{32}P-dCTP, hybridized and quantitated by a phosphoimager 445-SI (Molecular dynamics, Sunnyvale, CA, USA).

2.2. Generation of full length and HA-tagged ARL-6 constructs

Full length ARL-6 cDNA was produced by annealing the PCR product of the 5′RACE clone, generated with the oligonucleotides M13F (5′-GTA AAA CGA CGG CCA GT-3′) and P1, to the PCR product of the pSPORT-J2E1/2 clone produced with the oligonucleotides ARL-S1 (5′-CGT TGG AAG GCT TCA GC-3′) and M13R (5′-GGA AAC AGC TAT GAC CAT G-3′). ARL-6 was tagged at the carboxyl terminus with HA [17] with the oligonucleotides ARL-HA5 (5′-CTG GAT CCA TGG GCT TGC TGG ACA GAC-3′) and ARL-HA3 (5′-GGG GAA GCT TCA GGC ATA ATC TGG CAC ATC ATA AGG GTA CGT CTT CAC AGC TGG G-3′) and subcloned into pBSK+ (pB-ARL-6-HA).

2.3. Transfections and immunofluorescence

COS cells were transfected with 20 μg of plasmid DNA (pGL3E-control, pGL3-ARL-6-HA) by electroporation at 300 V, 800 μF using a Gene-Pulser II (Bio-Rad, Hercules, CA, USA) and harvested 48 h post-transfection. For cell fractionation studies, transfected cells were lysed in 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM NaCl, 10 mM $MgCl_2$, 25 mM NaF, 25 mM β-glycerol phosphate, 1 mM vanadate and 1 mM benzamidine, then centrifuged at $1000\times g$ for 5 min. The supernatant was incubated at 37°C for 30 min with, or without, 50 μM GTP-γS before centrifugation at $100\,000\times g$ for 60 min. For co-immunoprecipitations cells were lysed in 25 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% NP-40, 5 mM EDTA, 25 mM NaF, 25 mM β-glycerol phosphate, 1 mM vanadate and 1 mM benzamidine, then centrifuged at $1000\times g$ for 5 min. The supernatants were then incubated with the anti-HA.11 antibodies (BAbCo, Berkeley, CA, USA), collected with protein A beads, then immunoblotted with anti-HA.11 or anti-SEC61β antibodies (generously provided by W. Mothes, Harvard Medical School, Boston, MA, USA) before detection by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Indirect immunofluorescence was performed on transfected COS cells grown on cover slips, fixed in 50% methanol/50% acetone, using HA.11 or anti-SEC61β antibodies and an anti-mouse-FITC (Amersham) secondary antibody. DNA was counterstained with Hoechst 33258 and cells visualized using an MRC-1000/1024 UV laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA).

2.4. Yeast two-hybrid analysis

The yeast two-hybrid procedures used were essentially as described by Vojtek et al. [18], using the *Saccharomyces cerevisiae* L40 strain (MATa, his3Δ200, trp1-901, leu2-3, 112, ade2, LYS2::$(lexAop)_4$-HIS3, URA3::$(lexAop)_8$-lacZ, GAL4). Wild-type ARL-6 was subcloned into pBTM116 [18] to generate a LexA-ARL-6 fusion. The L40 strain was transformed with pBTM116-ARL-6 and used as the 'bait' to screen a pVP16 cDNA library made from mRNA derived from the lymphohemapoietic progenitor cell line EML C.1 [19]. Transformants were plated onto HIS^- plates to select for HIS3 reporter activation. Clones replated onto HIS^- plates were then assayed for β-galactosidase (β-gal) activity using the filter assay. The pBTM116-ARL-6 plasmid was then cured from the $HIS3^+/\beta\text{-}GAL^+$ clones before transformation and then assayed for HIS3 and β-gal activity. pVP16 plasmids from the cured clones were then rescued into *Escherichia coli* and sequenced. These plasmids were subsequently co-transformed with pBTM116-ARL-6 into L40 cells before HIS3 and β-gal assays.

3. Results

3.1. Isolation of ARL-6

In an attempt to identify genes involved in the hemopoietic lineage switch observed between erythroid J2E and the myeloid derivative J2E-NR-m2 cells [3], differential display was performed on cDNA prepared from these two lines (Fig. 1a). Fragments unique to each cell line were isolated, amplified and sequenced. Table 1 summarizes these data. Three of the cDNAs isolated were known (Rbtn2/LMO2, β-tropomyosin and tartrate resistant acid phosphatase) and two were novel. Significantly, the erythroid transcription factor Rbtn2/LMO2 was isolated from the parental J2E erythroid cells; deletion of the gene results in anemia and death in utero [20]. Interestingly, an alternatively spliced form of β-tropomyosin was isolated from the J2E-NR-m2 cells [21], tartrate resistant acid phosphatase [22], normally a marker of osteoclast differentiation, was isolated from J2E cells. Northern blotting confirmed the differential expression of these known

Table 1
Differential display fragments

Fragment	Size (bp)	Source	Homology	% Identity
1.11	266	J2E-NR-m2	Nil	–
3.8	177	J2E	Nil	–
4.10	350	J2E	Rbtn2/LMO2	92
5.C	215	J2E-NR-m2	β-TM	99
11.A	258	J2E	TRAP	94

Abbrevations: β-TM, β-tropomyosin; TRAP, tartrate resistant acid phosphatase.

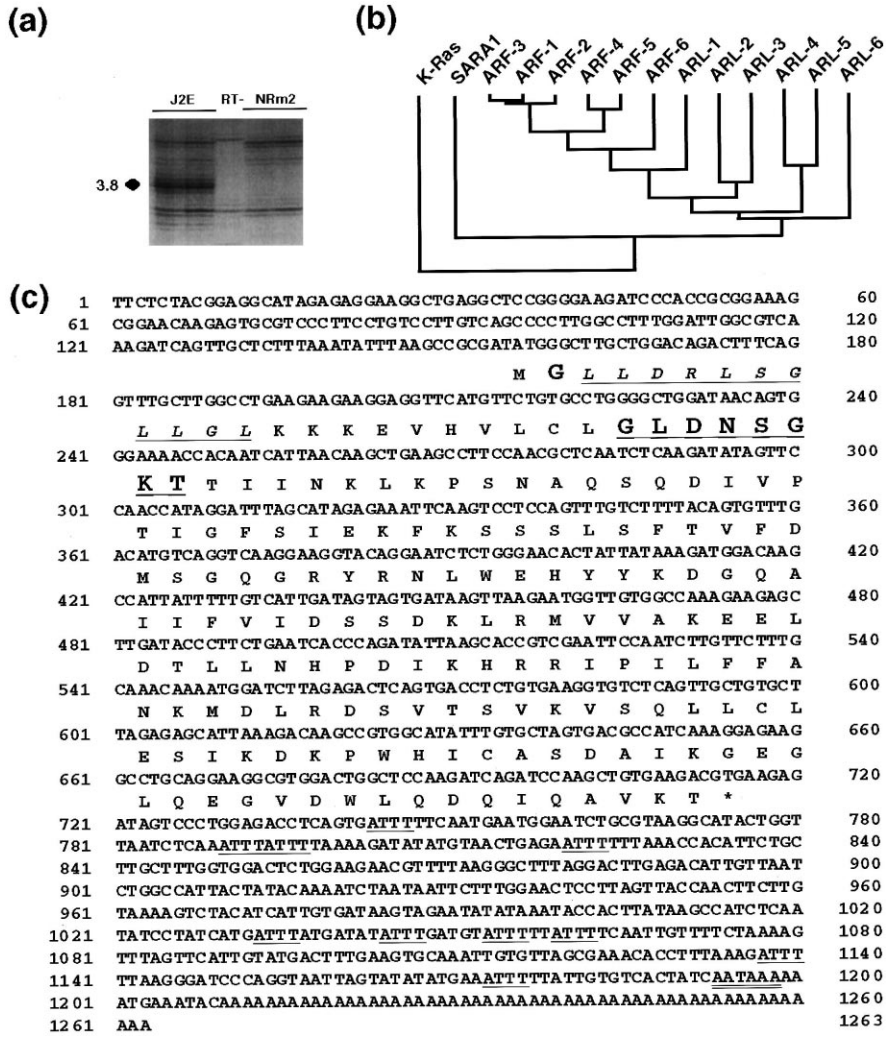

Fig. 1. Cloning of ARL-6. a: Differential display of cDNAs generated by PCR with the oligonucleotides PTGC (5′-T_{11}GC-3′) and RP2 (5′-CTG AGG CGT C-3′) from two independently prepared poly-A^+ samples of J2E and J2E-NR-m2 (NRm2) cells. A J2E reverse transcriptase negative sample (RT−) is included as a control. The fragment 3.8 that was excised and sequenced is indicated (♦). b: Schematic tree diagram of the homology between ARL-6 and members of the ARF/ARL family and two other GTPases, SARA1 and K-Ras using the Pileup program of the EGCG Wisconsin package (Genetics Computer Group, Madison, WI, USA). Sequence accession numbers are: K-ras (U09793), SARA1 (P36536), ARF-1 (P10947), ARF-2 (P16500), ARF-3 (P16587), ARF-4 (P21371), ARF-5 (P26437), ARF-6 (P26438), ARL-1 (L28997), ARL-2 (L13687), ARL-3 (U07151), ARL-4 (P40617), ARL-5 (X78604), ARL-6 (AF031903). c: Nucleotide and deduced amino acid sequence of ARL-6. Nucleotides are numbered and the deduced amino acid sequence is shown below. A polyadenylation signal found in the 3′ untranslated region is double underlined, while putative RNA destabilization motifs (AT_3) are underlined. The myristoylated glycine at position two of the amino acid sequence is in large size font, followed by the predicted hydrophobic α-helix in underlined italics and the GTP-binding site (P-loop) in underlined large size font. The sequence is available from GenBank/EMBL/DDBJ under accession number AF031903.

genes (data not shown). As fragment 1.11 was ubiquitously expressed it was discarded and fragment 3.8 was analyzed further.

The original differential display fragment 3.8 was used as a probe to isolate clones from J2E and total mouse embryo cDNA libraries. A partial cDNA was isolated from the murine embryonic library, while an almost full-length cDNA clone was isolated from the J2E library. The length and sequence of the 5′ end of the cDNA was confirmed using 5′RACE. Although the nucleotide sequence showed no significant homologies in the databases, the amino acid sequence showed homology with members of the ARF family of small GTP-binding proteins (Fig. 1b), the 3.8 clone was most similar to *Caenorhabditis elegans* ARF-1 (43% identity, 65% homology). Comparison of the amino acid sequence of clone 3.8 with other members of the mammalian ARF family indicated it was the sixth member of the ARL subfamily (Fig. 1b). In accordance with the nomenclature of the family the clone was named ARL-6. It contained all the conserved features of the ARF family, including an amino-terminal myristoylation site followed by a predicted hydrophobic α-helix, and a GTP-binding site.

The 1263 bp full length cDNA (Fig. 1c) contained an open reading frame of 186 amino acids with a predicted M_r of 20.9 kDa and an isoelectric point of 8.36. A polyadenylation signal was situated between 1193–1198 bp and a poly-A tail of 53 bp began at position 1210. There were 153 bp of 5′ untranslated region and 494 bp of 3′ untranslated region. The 5′ untranslated region contained no additional in-frame or out-of-frame initiation codons, while the 3′ untranslated region contains 10 copies of the AT_3 motif implicated in mRNA destabilization [23]. Indeed, we found that ARL-6 mRNA has a relatively

short half life of 4 h in J2E cells (data not shown). The cDNA size corresponded well with a mRNA of ~1.3 kb seen in Northern blots, suggesting a full-length clone was isolated.

3.2. Expression patterns of ARL-6 mRNA

As ARL-6 was isolated from an erythroid to myeloid switch, expression during differentiation of these cell types was examined. To analyze whether ARL-6 transcripts varied during erythroid differentiation, J2E cells were induced with erythropoietin. A three-fold induction of ARL-6 transcripts was observed 12 h after hormonal stimulation and levels were maintained up to 48 h post induction (Fig. 2a). β-globin mRNA and hemoglobin production were used as markers of erythroid differentiation and both increased as expected with J2E cell differentiation [2]. ARL-6 expression was also studied during monocyte/macrophage maturation. Immature monoblastoid M1 cells were stimulated with interleukin-6 and a six-fold reduction in ARL-6 expression was observed (Fig. 2b). Lysozyme mRNA served as a marker for macrophage differentiation and rose predictably. Together these data show that ARL-6 transcript levels vary in hemopoietic lineages and alter with the differentiation status of the cells.

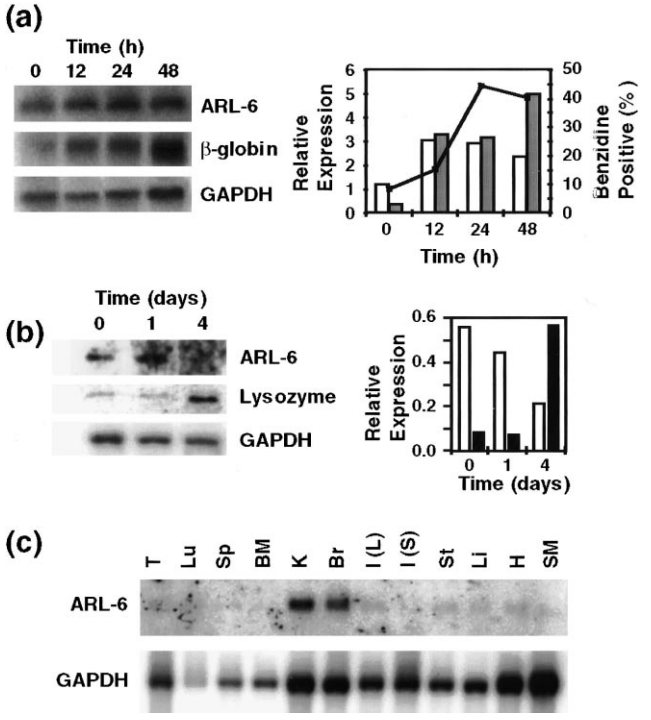

Fig. 2. Analysis of ARL-6 mRNA. a: J2E cells stimulated with erythropoietin (5 U/ml) were harvested and analyzed for hemoglobin production by benzidine staining [2]. Total RNA was then extracted and analyzed for ARL-6, β-globin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels. Levels of ARL-6 (clear histograms) and β-globin (stippled histograms) relative to GAPDH and the percentage of benzidine positive cells (■-■) are shown to the right. b: M1 monoblastoid cells were stimulated with interleukin-6 (6 ng/ml) then harvested and analyzed for ARL-6 and GAPDH transcript levels. Levels of ARL-6 (clear histograms) and lysozyme (black histograms) relative to GAPDH are shown to the right. c: Analysis of various mouse tissues for ARL-6 mRNA expression. Poly-A^+ RNA was isolated from mouse tissues and hybridized to an ARL-6 cDNA probe. The same blot was then stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Lanes are: T, thymus; Lu, lung; Sp, spleen; BM, bone marrow; K, kidney; Br, brain; I (L), intestine (large); I (S), intestine (small); St, stomach; Li, liver; H, heart and SM, smooth muscle

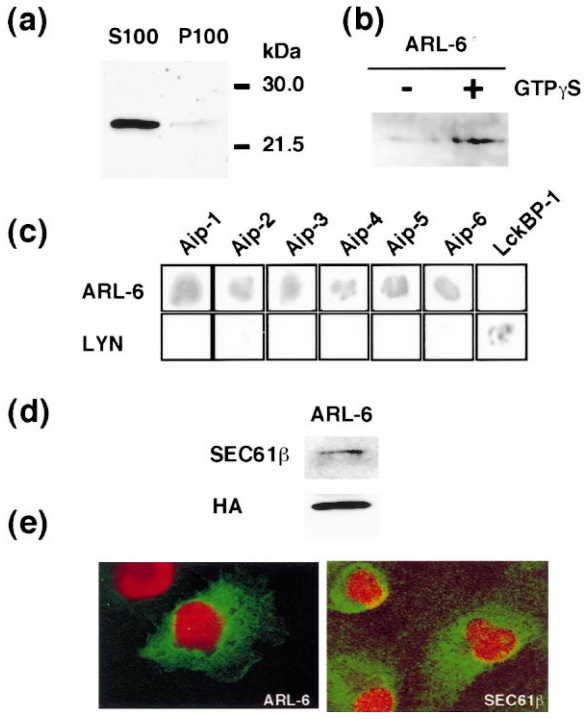

Fig. 3. Analysis of ARL-6 protein and interacting partners. a: Subcellular localization of ARL-6. COS cells transiently expressing HA-tagged ARL-6 were fractionated into cytosolic (S100) and particulate/membrane (P100) components, then immunoblotted with anti-HA antibodies. Size markers are shown to the right. b: HA-tagged ARL-6 shows an increased particulate/membrane (P100) localization upon GTP-γS stimulation. Lysates from transfected COS cells expressing the HA-tagged ARL-6 were incubated with (+) or without (−) 50 μM GTP-γS for 30 min prior to fractionation and then immunoblotted with anti-HA antibodies. c: The yeast strain L40 [18,19] was co-transfected with the plasmids pBTM116-ARL-6 (ARL-6) or pBTM116-Lyn (Lyn) and pVP16-Aip1-6 (Aip-1–6) or pVP16-LckBP1 (LckBP-1). The resultant colonies were replated onto Leu^-/Trp^- plates and assayed for β-gal activity by the filter assay. The known interaction between Lyn and LckBP-1 was used as a positive control. d: Lysates from COS cells transfected with HA-tagged ARL-6 were immunoprecipitated with HA-specific antibodies, then probed with anti-SEC61β or anti-HA antibodies. (e) COS cells transiently expressing HA-tagged ARL-6 were fixed and subjected to indirect immunofluorescence, stained for ARL-6 with anti-HA antibodies or for SEC61β with anti-SEC61β antibodies and counterstained with Hoechst 33258 for DNA. Cells were visualized using an MRC-1000/1024 UV laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA).

Intriguingly, when mouse tissues were examined, expression of ARL-6 was very low in hemopoietic organs such as spleen and bone marrow (Fig. 2c). Several other tissues also had low expression levels and the highest levels of ARL-6 mRNA were found in brain and kidney.

3.3. Subcellular localization of ARL-6

To determine the subcellular localization of ARL-6, a carboxyl-terminal HA tagged ARL-6 was transiently expressed in COS cells. Fractionation studies revealed that most of the ARL-6 protein was cytosolic, and only a small fraction was present in the particulate/membrane fraction (Fig. 3a). In support of the cell fractionation data, when ARL-6 subcellular localization was analyzed by confocal microscopy (Fig. 3e),

Table 2
Amino acid sequence of ARL-6 interacting proteins (Aip1–6)

Name	ARL-6 interacting protein (Aip1-6) sequence						Homology	% Identity	Function
Aip-1	1	WCWGDSVCSL	VDNRESGTMA	EGDNRSSNLL	AVETASLEEQ	LQGWGEVMLM	KIAA0069	91	Proposed transmembrane
	51	ADKVLRWERA	WFPPAIMGVV	SLLFLIIYYL	DPSVLSGVSC	FVMFLCLADY			
	101	LVPILAPRIF	GSNKWTTEQQ	QRFHEICSNL	VKTPPKLWAG	GNASFPRKKT			
	151	NVLHEHDHFS	CCGGLG						
Aip-2	1	SDLERKHLDL	KEVALKQFRS	VKKMGGDEFC	RRYQDQLEAE	IEETYANFIK	cDNA vc27f05	100	Unknown
	51	HNDGKNIFYA	ARTPATLFAV	MFAMYIISGL	TGFIGLNSIA	VLCNLVMGLA			
	101	LTSLCTWAYV	KYSGEFREIG	TMIDQIAETL	WEQVLKPLGF	DGGKHKAVCN			
	151	KLYQSRPDPV	SHQAGR						
Aip-3	1	PGPTPSGTNV	GSSGRSPSKA	VAARAAGSTV	RQRKNASCGT	RSAGRTTSAG	SEC61β	98	Translocon subunit
	51	TGGMWRFYTE	DSPGLKVGPV	PVLVMSLLFI	AAVFMLHIWG	K			
Aip-4	1	KRVKEKAVAV	HQAEALPGPS	LDQWHRSAGE	DNDGPVLTDE	QKSRIQAMKP	cDNA EST02690	100	Unknown
	51	MTKEEWDARQ	SVIRLLLPFL	LLLLPLLLLV	LRSLLP				
Aip-5	1	AKQRNMAREP	RPAPCLGRFL	PGLSFRPAGL	QGHFQMEQPC	SEQSALLPDQ	JWA	90	Proposed cytoskeletal
	51	LPGGGCHDDF	GCWVSEPLQH	DPRRSHCGAG	VHGVRVGSAQ	RHPPPDEEQY			
	101	PTAFVMVVML	PATSSYPCFG	G					
Aip-6	1	ARRRRQVTPG	PATRPGYSDY	TQGDSWGEGE	GDENEGCDQV	ARDLRAEFSA	cDNA mo20b06	93	Unknown
	51	RASSETKRAP	LLPRVGDGSP	VLPDKRNGIF	PATAAKRTQA	RRWPIQALSI			
	101	LCSLLFAVLL	AFLL						

The deduced amino acid sequence of Aip1-6 is shown with potential hydrophobic α-helices underlined. The closest homologue (% identity indicated) of each Aip and its function, if known, is also listed. The nucleotide sequences for Aip-1 to Aip-6 have been deposited in the GenBank database, accession numbers are: Aip-1; AF133669, Aip-2; AF133670, Aip-3; AF133910, Aip-4; AF133911, Aip-5; AF133912, Aip-6; AF133913.

most of the staining was cytoplasmic. As members of the ARF and ARL families are small GTP-binding proteins, the effect of the non-hydrolyzable GTP analogue GTP-γS on subcellular localization was investigated. Significantly, the amount of particulate/membrane-associated ARL-6 was increased 3–5 fold by GTP-γS (Fig. 3b).

3.4. ARL-6 interacting molecules

In an attempt to identify proteins that interact with ARL-6, a yeast two-hybrid screen was performed. Of 10^7 clones screened 70 were HIS3 positive, 13 of which were also β-gal positive. After curing, only nine clones reactivated the reporters upon reintroduction of the pBTM116-ARL-6 plasmid. Plasmids from these nine clones were rescued and sequenced, identifying six ARL-6 interacting proteins, termed Aip-1 to Aip-6, (Table 2, Fig. 3c). Of the six interacting molecules only Aip-3 was a previously well characterized protein, with 98% amino acid identity to the human SEC61β subunit of the protein conducting channel (Table 2). Aip-1 has 91% amino acid identity with KIA0069, which contains four potential transmembrane domains, while Aip-6 has 93% amino acid identity with JWA, a proposed cytoskeletal protein. Curiously, each of the interacting molecules contained potential hydrophobic α-helices, which may indicate a means of associating with ARL-6 via its hydrophobic α-helix. The specificity of the Aip interactions with ARL-6 was shown by their inability to associate with the unrelated protein tyrosine kinase Lyn (Fig. 3c).

To determine if the ARL-6/SEC61β interaction occurred in vivo, HA-tagged ARL-6 was immunoprecipitated from transfected COS cells and probed with antibodies for SEC61β. Fig. 3d shows the interaction of SEC61β with ARL-6, which confirms the interaction of these two molecules in the yeast two-hybrid system. In addition, confocal microscopy revealed that SEC61β and ARL-6 had similar staining patterns (Fig. 3e), suggesting that the two molecules may co-localize within the cell. Therefore, in addition to associating in the yeast two-hybrid system and co-immunoprecipitating (Fig. 3c,d), ARL-6 and SEC61β show similar intracellular localization patterns (Fig. 3e).

4. Discussion

In an attempt to identify erythroid/myeloid lineage switch genes using differential display, we have isolated a novel gene with sequence homology to the Ras superfamily of small molecular weight GTP-binding proteins, particularly the ARF/ARL subfamily. The high degree of homology between this novel gene and members of the ARF/ARL family (35–40% amino acid identity) suggests that it is the sixth ARL. Of the 15 residues in ARF that interact directly with the bound Mg-GDP [11], 12 are identical in ARL-6. The notable differences are a substitution of the alanine-alanine doublet (ARF-1, 24-GLDAAGK-30) in the Walker sequence [11] with asparagine-serine (ARL-6, 24-GLDNSGK-30). These residues interact with the β-phosphate of the Mg-GDP. While the A27 to N27 substitution in ARL-6 is seen in some ARLs, the A28 to S28 substitution has not been described previously. The other significant difference is the substitution of A52 (ARF-1), which would interact with the α-phosphate of the Mg-GDP, with S54 (ARL-6). While this substitution is not seen in other ARF/ARL members, it is present at the homologous site in Ras. Taken together, these results suggest ARL-6 may function mechanistically like ARF-1, but is likely to have a distinct intracellular role.

ARL-6 has a unique expression pattern among the ARF/ARL family with the highest mRNA levels present in brain and kidney. This may indicate that ARL-6 has an important

function in these tissues, as they both have important secretory vesicle function e.g. neurotransmitter release in the brain and erythropoietin secretion from the kidney. Although some members of the ARF/ARL family are ubiquitous, others have tissue-specific expression patterns. ARF-4 is expressed widely, while ARF-1, 3 and 5 are found predominantly in kidney [24]. ARF-6, on the other hand, is present at high levels in ovaries [24]. Of the ARLs, ARL-5 has the highest expression in brain, intestine and thymus [25]. Interestingly, ARL-6 mRNA levels increased during erythroid differentiation, but fell as macrophage maturation proceeded, suggesting it may play a role in hemopoietic development. This observation may be significant as ARL-6 was isolated following an erythroid to myeloid lineage switch.

As with other ARF/ARL proteins [24], ARL-6 was found mainly in the cytoplasm. ARF/ARL molecules bind to phospholipid micelles and membranes in a GTP-dependent manner [26] and our fractionation data demonstrate an increase in ARL-6 membrane association after stimulation with GTPγS. These data suggest that ARL-6 may cycle between the cytoplasm and membranes in a GTP-dependent manner.

A yeast two hybrid screen and co-immunoprecipitation revealed that ARL-6 could interact with the SEC61β subunit of the translocon, while confocal microscopy indicated a possible intracellular co-localization of these two molecules. There are several points at which ARL-6 could influence the SEC61p complex, including, assembly of the heterotrimeric SEC61p complex, formation of the SEC61p membrane channel, regulation of the translocation or the lateral channel exit [27]. The SEC61p complex containing SEC61β is capable of GTP exchange activity for the GTP binding signal recognition particle receptor-α in the translocon [28]. Perhaps, SEC61β within the SEC61p complex acts as a GTP exchange factor for the novel molecule ARL-6. The precise biological function of the interaction between ARL-6 and SEC61β awaits elucidation.

Acknowledgements: The authors thank W. Mothes (Harvard Medical School, Boston, MA, USA) for the SEC61β rabbit antiserum. Assistance with confocal microscopy was kindly provided by P. Rigby and S. Codey, UWA. The authors are also grateful to Prof. D. James (Center for Molecular and Cellular Biology, University of Queensland, Qld., Australia) for critically evaluating this manuscript. The NHMRC (Grant # 98-0610), MEDWA, AMRAD and the Cancer Foundation of WA supported this work.

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