FEBS Letters 456 (1999) 384–388 FEBS 22213

ADP-ribosylation factor (ARF)-like 4, 6, and 7 represent a subgroup of the ARF family characterized by rapid nucleotide exchange and a nuclear localization signal

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Received 9 March 1999; received in revised form 18 May 1999

Abstract The novel ARF-like GTPase ARL7 is a close relative of ARL4 and ARL6 (71% and 59% identical amino acids). A striking characteristic of these GTPases is their basic C-terminus which, when fused to the C-terminus of green fluorescent protein (GFP), targets the constructs to the nucleus of transfected COS-7 cells. Full length ARL4 was detected in both nuclear and extranuclear compartments, whereas a construct of ARL4 lacking its C-terminus was excluded from the nucleus. Nucleotide exchange rates of recombinant ARL4, ARL6 and ARL7 were similar and appeared considerably higher than those of other members of the ARF family (ARF1, ARP). It is concluded that ARL4, ARL6 and ARL7 form a subgroup within the ARF family with similar, possibly nuclear, function.

Key words: ADP-ribosylation factor; GTPase; Ras-related; GTP binding

1. Introduction

ADP-ribosylation factors (ARFs) were initially identified, purified, and cloned because of their activity as stimulators of the ADP-ribosyltransferase activity of cholera toxin [1–3]. They constitute a subfamily of the Ras-related GTPases and regulate diverse cellular functions, e.g. vesicle traffic, endocytosis, and activity of phospholipase D (reviewed in [4–6]). The six mammalian ARFs have been divided into three classes [5]; class I (ARF1, ARF2, and ARF3), class II (ARF4 and ARF5, and class III (ARF6). In addition, seven cDNAs encoding proteins with significant similarity to ARF (ARF-like (ARL) 1–5, ARF4L[1]/ARL6, and ARF-related protein (ARP)) have been cloned, most of them by PCR-cloning with degenerate oligonucleotide primers [7–11].

Among the ARF-like proteins, ARL4 is particularly interesting because of its differential expression in the differentiating 3T3-L1 preadipocyte cell line [8], and its strong expression in testicular germ cells driven by a separate, testis-specific promoter [12]. In Northern blots with ARL4 cDNA, we detected an additional band at approximately 4 kb which appeared to represent an alternatively polyadenylated ARL4 transcript. However, the possibility could not be excluded that additional ARF-like gene with high similarity to ARL4 exists. Since the identification of this isoform was important for planning the targeted disruption of ARF-like genes, we extended our previous PCR-cloning approach to other tissues, and searched the EST databases for sequences similar to the known isoforms. In the present paper, we describe the identification of a new GTPase (ARL7) which, together with ARL4 and ARL6, forms a small subgroup within the ARF family.

2. Materials and methods

2.1. RT- and RACE-PCR

5′-RACE (rapid amplification of cDNA ends) was performed with a kit from Gibco BRL (Eggenstein, Germany) according to the instructions of the manufacturer. cDNA synthesis was performed by oligo-dT priming. Primers for RACE amplification were designed from the expressed sequence tags HSAA28756 and HS417358 (GenBank Acc. Nos. AA128756 and W68417). Samples of total RNA from human bladder epithelium or testis were used as templates. For RT-PCR amplification of a cDNA fragment comprising the full reading frame of ARL7, a forward primer corresponding with the RACE-products was used (5′-TCG CAG CCG CAG TGG CTG-3′; reverse primer, 5′-CAT TCT TTC TTG ACG CAC TC-3′). The PCR was allowed to proceed for 35 cycles of 94°C (1 min), 56°C (1 min) and 72°C (2 min). DNA fragments were isolated and subcloned into pUC18 with the Sureclone^R kit (Pharmacia, Freiburg, Germany) and sequenced in both directions.

2.2. Northern blot analysis

Northern blots of RNA from different human tissues were purchased from Invitrogen (Carlsbad, CA, USA). The membranes were hybridized under conditions of high stringency with cDNA probes comprising the full coding region labeled with [^{32}P]CTP by random oligonucleotide priming [14]. Blots were hybridized at 42°C and washed two times at 55°C with 0.12 M NaCl/0.012 M sodium citrate/0.1% SDS.

2.3. Subcellular localization of GFP fusion constructs expressed in COS-7 cells

Fragments of the cDNA of ARL4, ARL6, and ARL7 comprising the 20 C-terminal codons were generated by PCR with primers introducing *Sac*I and *Eco*RI sites. The fragments were fused in-frame to the C-terminus of GFP by subcloning into the respective sites of the plasmid pEGFP-C1 (Clontech, Palo Alto, CA, USA). COS-7 cells were seeded on coverslips and were transiently transfected with the aid of the lipofection reagent DAC-30 (Eurogentec, Seraing, Belgium). Twenty-four hours after transfection, cells were fixed with paraformaldehyde, embedded in fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA), and visualized with a Zeiss Axiophot equipped for epifluorescence. Two–five independent transfections were performed with each fusion construct, and at least two different samples were evaluated microscopically in each experiment.

2.4. Fractionation of cells

COS-7 cells transfected with the cDNA of ARL4 subcloned into the vector pCMV were homogenized and fractionated as described pre-

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[1] The ARF-like GTPase ARL6 was also described as ARF4L (ARF4-like) [13]; the designation ARL6 is consistent with the nomenclature of the other ARF-like GTPases and is therefore preferred here.

0014-5793/99/$20.00
PII: S0014-5793(99)00759-0

viously [10] with minor modifications (legend of Fig. 4). Immunochemical detection was performed with a polyclonal antiserum against the 12 C-terminal amino acids.

2.5. Preparation of recombinant proteins

Fragments of the cDNA of ARL4 (GenBank Acc. No. X77235), ARL6 (Acc. Nos. L38490 and U25771), and ARL7 comprising the full coding regions were generated by PCR with primers introducing *Bam*HI and *Eco*RI sites, and were fused in-frame to the C-terminus of glutathione S-transferase (GST) in the vector pGEX-2TK (Pharmacia, Freiburg, Germany). Recombinant proteins were isolated from *Escherichia coli* 801-J by adsorption to GST-Sepharose (Pharmacia) and cleavage with thrombin according to the instruction of the manufacturer.

2.6. Assay of guanine nucleotide exchange

Guanine nucleotide binding to recombinant GTPases was assayed by a previously described procedure [15,16].

3. Results

3.1. Identification and cloning of ARL7

A search of the EST databases for sequences similar to ARL4 produced 26 sequence tags which corresponded to a partial sequence of an unknown ARF-like cDNA which we designated *ARL7*. Missing sequence information was obtained by 5′-RACE, and a cDNA fragment comprising the full open reading frame was amplified by RT-PCR from human bladder epithelium mRNA.

3.2. Sequence characteristics of ARL7

A database search indicated that *ARL7* represents a novel gene. Its closest relatives, ARL4 and ARL6, contain 65% and 68% identical nucleotides, respectively, with stretches of 80% identity; all other members of the ARF family exhibit considerably lower similarity. A comparison of the deduced amino acid of ARL7 sequence with that of other Ras-related GTPases indicates that the motifs presumably involved in nucleotide binding and hydrolysis [17] are detectable in ARL7 (see Fig. 2). Furthermore, several other amino acids typical for the ARF subfamily [18] were found in ARL7, i.e. glycine in position 2, and three residues which are conserved in all previously isolated members of the ARF family (N48, W79, R100, G165).

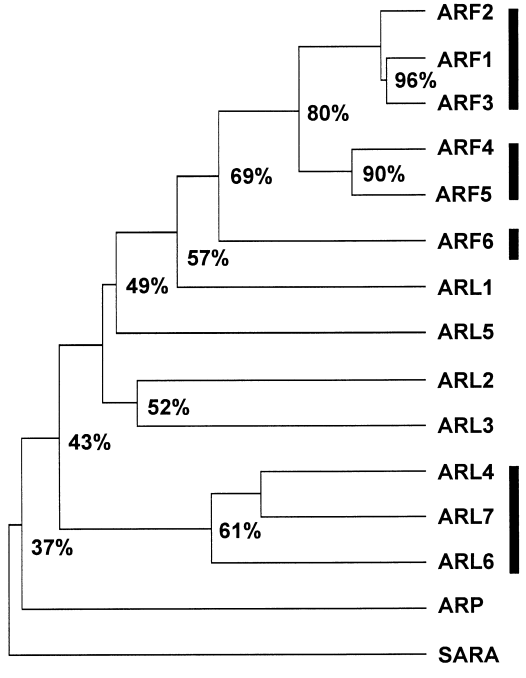

Fig. 1. Dendrogram of an alignment of human ARL7 with other members of the ARF family. The alignment was performed with the CLUSTAL program (gap penalty 5, open gap cost 10, unit gap cost 10). Bars denote the previously defined subclasses I–III and the ARL4 subgroup.

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                                    PM1      G1
          *                        *** *****
ARL7   MGN-------ISSN-ISAFQSLHIVMLGLDSAGKTTVLYRLKFNEFVNTV  42
ARL4   MGNGLSDQTSILSS-LPSFQSFHIVILGLDCAGKTTVLYRLQFNEFVNTV  49
ARL6   MGNHLTEMAPTASSFLPHFQALHVVVIGLDSAGKTSLLYRLKFKEFVQSV  50
       ***       . *.  .. **..*.*..***.****..****.*.***..*

         PM2                       PM3
         * * *                   ** ***      *  *         *
ARL7   PTIGFNTEKIKLSNGTAKGISCHFWDVGGQEKLRPLWKSYSRCTDGIIYV  92
ARL4   PTKGFNTEKIKVTLGNSKTVTFHFWDVGGQEKLRPLWKSYTRCTDGIVFV  99
ARL6   PTKGFNTEKIRVPLGGSRGITFQVWDVGGQEKLRPLWRSYTRRTDGLVFV 100
       ** *******... *..... . *************.**.* ***...*

                                      G2
          *     *                    *****
ARL7   VDSVDVDRLEEAKTELHKVTKFAENQGTPLLVIANKQDLPKSLPVAEIEK 142
ARL4   VDSVDVERMEEAKTELHKITRISENQGVPVLIVANKQDLRNSLSLSEIEK 149
ARL6   VDAAEAERLEEAKVELHRISRASDNQGVPVLVLANKQDQPGALSAAEVEK 150
       **.....*.****.***.... ..***.*.*..***** . .*. .*.**

                        G3
                         *    *
ARL7   QLALHELIPATTYHVQPACAIIGEGLTEGMDKLYEMILKRRKSLKQ-KKKR 192
ARL4   LLAMGELSSSTPWHLQPTCAIIGDGLKEGLEKLHDMIIKRRKMLRQQKKKR 200
ARL6   RLAVRELAAATLTHVQGCSAVDGLGLQQGLERLYEMILKRKKAARGGKKRR 201
        **. ** ..*  *.*  .*. * ** .*...*..**.**.*  .  **.*
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Fig. 2. Sequence alignment of ARL7 with its closest relatives in the ARF family. The alignment was performed with the CLUSTAL program (gap penalty 5, open gap cost 10, unit gap cost 10). Identical amino acids and conservative substitutions are depicted by asterisks or periods, respectively, below the sequence. Asterisks above the sequence denote residues which are highly conserved in the known members of the ARF family; residues considered characteristic for the family (G2, N48, W79, R100, and G165) are highlighted by bold italics.

A multiple alignment of ARL7 with the other members of the ARF family (dendrogram in Fig. 1) indicates that ARL4, ARL6, and ARL7 form a small subgroup within the family. Individual alignments indicate 71% identity with rat ARL4, 59% with human ARL6, 49% with human ARF1, and 43% identity with rat ARL1. The multiple alignment of ARL4, ARL6 and ARL7 as depicted in Fig. 2 illustrates the high degree of similarity between their sequences. The three GTPases are essentially identical in the GTP binding motifs and the effector loop; there are very few non-conservative substitutions in all other parts of the sequence. A striking common characteristic of the GTPases is their basic C-terminus which harbors nine arginine or lysine residues; this C-terminus exhibits a striking similarity to nuclear localization signals [19].

3.3. Nuclear targeting of GFP constructs by the C-termini of ARL4, ARL6 and ARL7

In order to test whether the C-termini of ARL4, ARL6 and ARL7 indeed harbor nuclear localization signals, we generated constructs of the 20 C-terminal codons of the GTPases fused to the cDNA of GFP. COS-7 cells were transfected with these constructs, and the subcellular localization of the fusion proteins was examined by fluorescence microscopy (Fig. 3). In control cells transfected with GFP (Fig. 3, upper left panel), the fluorescence appeared evenly distributed within the cell. In contrast, in cells transfected with the GFP fusion constructs (upper right and middle panels), the fluorescence was concen-

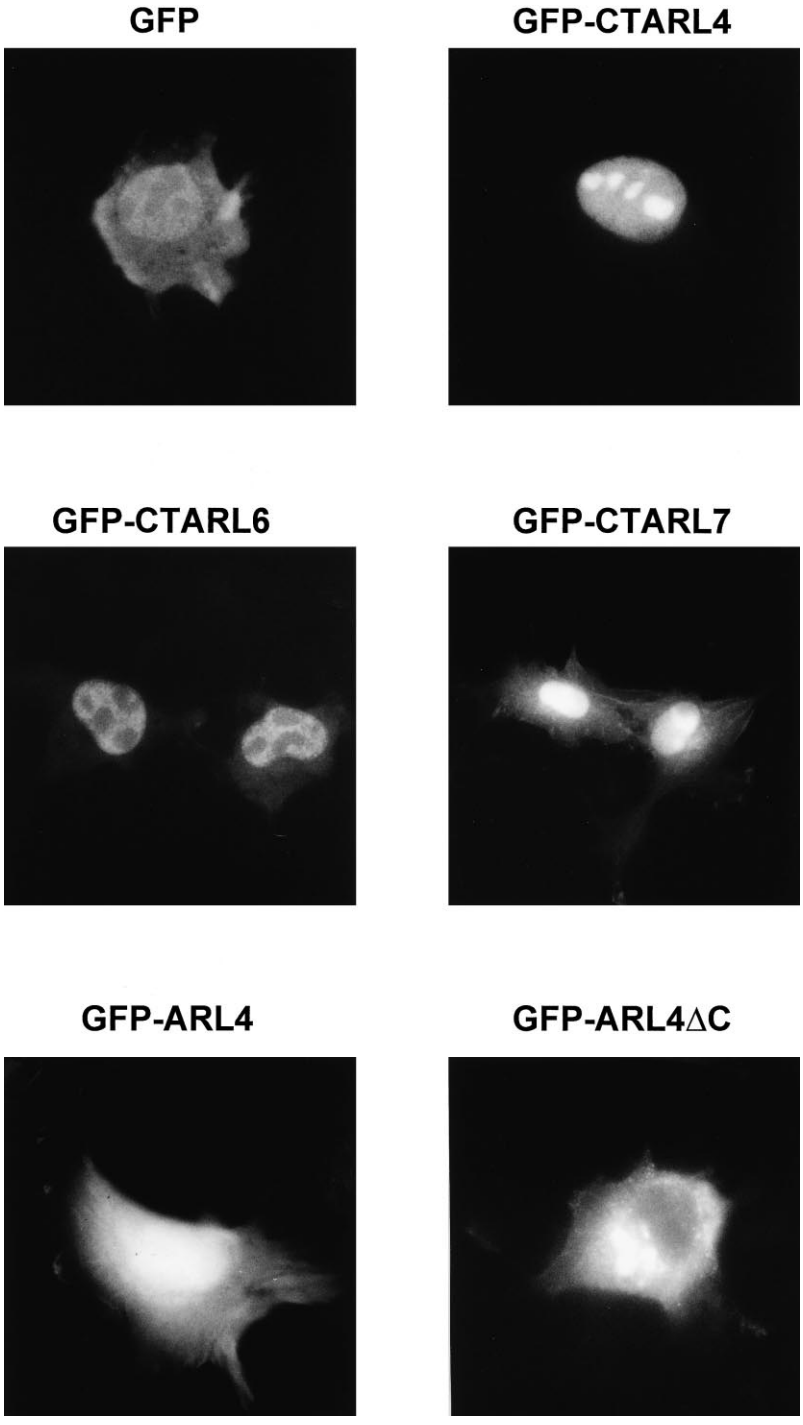

Fig. 3. Nuclear targeting of GFP constructs by the C-terminus of ARL4, ARL6 and ARL7. COS-7 cells were transfected with GFP fusion constructs of the 20 C-terminal codons of ARL4 (GFP-CTARL4), ARL6 (GFP-CTARL6), ARL7 (GFP-CTARL7), with full length ARL4 (GFP-ARL4), or with ARL4 lacking its C-terminus (GFP-ARL4ΔC). The following day, recombinant proteins were visualized by direct fluorescence microscopy.

trated within the nucleus. It should be noted that the C-termini of ARL6 and ARL7 produced a nuclear accumulation of GFP in speckles, whereas GFP fused to the C-terminus of ARL4 was concentrated in larger patches within the nucleus.

Fig. 3 also illustrates that a full length ARL4 fused to GFP was not exclusively targeted to the nucleus, but was clearly detectable in extranuclear compartments (lower left panel). Furthermore, a construct lacking the C-terminus was excluded from the nucleus (Fig. 3, lower right panel). Thus, full length ARL4 appears to be located in both a nuclear and an extranuclear compartment.

3.4. Subcellular distribution of ARL4 as assessed by fractionation of transfected COS-7 cells

In order to confirm the nuclear localization of ARL4 with a second method, COS-7 cells were transfected with the full length ARL4 cDNA and fractionated. Fig. 4 illustrates that the ARL4 immunoreactivity was detected in both nuclear and

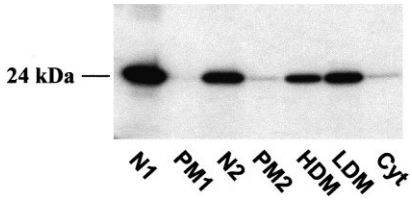

Fig. 4. Immunochemical detection of ARL4 in membrane fractions from transfected COS-7 cells. Cells were transfected with a full length ARL4 cDNA, homogenized, and fractionated. Nuclei (N) and plasma membranes (PM) were separated by sucrose gradient centrifugation of a $600\times g$ (N1, PM1) or $14\,000\times g$ (N2, PM2) pellet. High-density (HDM) and low-density microsomes (LDM) represent $50\,000\times g$ and $200\,000\times g$ pellets, respectively, of the $14\,000\times g$ supernatant.

microsomal fractions, confirming the result obtained with the GFP-tagged construct (Fig. 3, lower left panel).

3.5. Expression of ARL7 in different human tissues

Highest levels of a single 3.6 kb transcript detected with the ARL7 probe were found in brain; lower levels were found in spleen, thymus, esophagus, stomach, intestine and uterus (Fig. 5). ARL6 exhibited a different pattern of tissue distribution; highest amounts of ARL6 mRNA were found in kidney, testis, esophagus, and uterus (Fig. 5). The tissue distribution of the ARL7 mRNA is also different from that of ARL4 which is predominant in testis [12].

3.6. Guanine nucleotide exchange activity of ARL7, ARL4, and ARL6

As is illustrated in Fig. 6, the GTPases rapidly bind radiolabeled GTPγS in a saturable and magnesium dependent manner. The exchange rates were considerably higher than those of ARF or the ARF-like protein ARP [10]; they approached the exchange rate of ARF in the presence of its exchange factor mSec7-2/ARNO (Fig. 6). Furthermore, bound tracer (GTPγS) could be exchanged for GTP or GDP in a similar time course (Fig. 7).

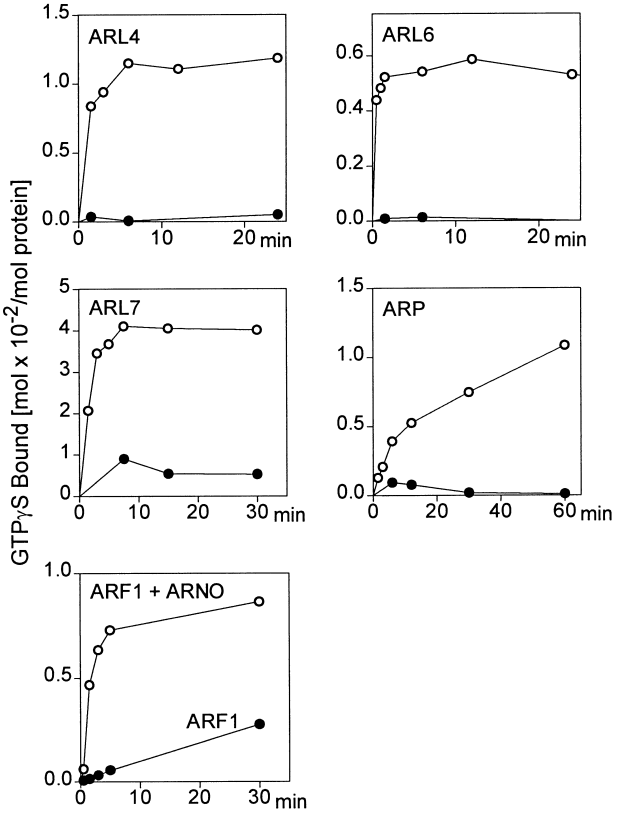

Fig. 6. Guanine nucleotide exchange (GDP/[^{35}S]GTPγS) of recombinant ARL4, ARL6, and ARL7 as compared with that of ARF1 and ARP. The recombinant GTPases ARL4, ARL6, ARL7, and ARP were incubated with radiolabeled GTPγS in the presence (open circles) or absence (filled circles) of magnesium (1 mM) for the indicated times. ARF1 was incubated in the presence of magnesium (1 mM) with (open circles) or without (filled circles) its exchange stimulator mSec7-2/ARNO.

4. Discussion

The present data indicate that the ARF-like proteins ARL4, ARL6, and ARL7 represent a subgroup of the ARF

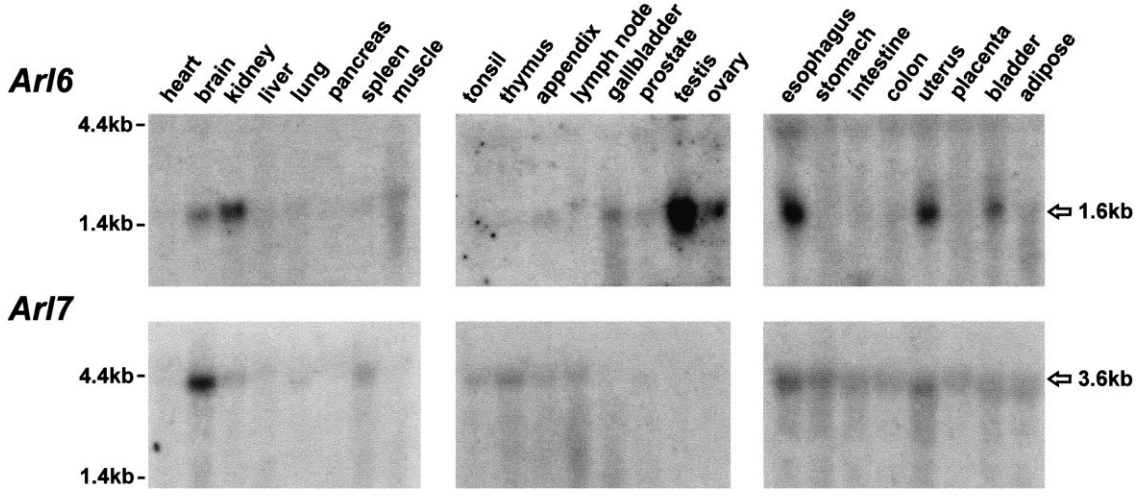

Fig. 5. Northern blot analysis of human ARL7 mRNA in different tissues. Northern blots of total RNA from the indicated tissues were hybridized with ARL6 and ARL7 cDNA fragments comprising the full coding regions. Equal amounts of RNA were present in each lane as judged from ethidium bromide staining.

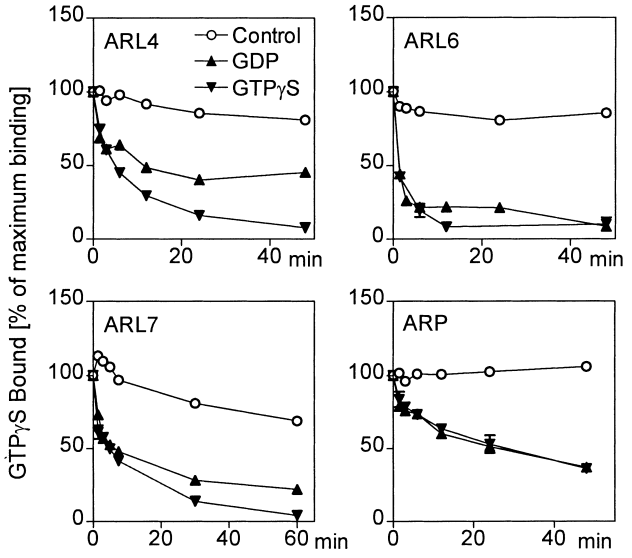

Fig. 7. Guanine nucleotide exchange ([^{35}S]GTPγS/GTPγS or [^{35}S]GTPγS/GDP) of recombinant ARL4, ARL6, and ARL7 as compared with that of ARP. The recombinant GTPases were pre-incubated with radiolabeled GTPγS, and 200 μM (final concentration) of unlabeled GTPγS or GDP were indicated at time zero. After the indicated times, bound nucleotides were isolated by filtration through nitrocellulose membranes.

family with similar structural and functional characteristics. ARL4 has previously been cloned from 3T3-L1 cells as a differentially expressed gene [8], and has later been shown to be expressed in testicular germ cells under the control of a separate promoter [12]. ARL6 has been identified as an open reading frame in a genomic region comprising the BRCA1 gene [13,20]. ARL7 is a novel gene identified in this study, and is the closest relative of ARL4 and ARL6 on the basis of a sequence comparison. In addition to their structural similarity, the three GTPases share a nuclear localization signal and the unusually high guanine nucleotide exchange rate.

The C-termini of ARL4, ARL6, and ARL7 are capable of targeting GFP to the nucleus of COS-7 cells. Therefore, it appears safe to conclude that the stretches of basic amino acids within the C-termini represent a nuclear localization signal. Furthermore, ARL4 expressed in COS-7 cells is indeed abundant in a nuclear fraction, and a construct lacking the nuclear localization signal is excluded from the nucleus. It is unclear whether the discrete differences in the patterns of subnuclear distribution observed here between ARL4 and ARL6/ARL7 reflect a functional difference. However, it appears reasonable to assume that the three GTPases are indeed targeted to the nucleus and exert a specific, presumably related, nuclear function.

We can only speculate on the specific function of ARL4, ARL6 and ARL7. It might be assumed that the GTPases are involved in protein transport between organelles, since members of the ARF family have been shown to regulate cellular trafficking (reviewed in [4,5,21]), and since ARF-like proteins appear to be similar to the ARF isoforms in their functional characteristics [22]. Transport through the nuclear pore complex is GTP dependent and is regulated by the Ras-related GTPase Ran (reviewed in [23,24]). Cargo proteins bind to importins α and β by their nuclear localization signal; the translocation and the subsequent dissociation of this complex appears to be regulated by Ran. Because of their nuclear localization signal, ARL4, ARL6 and ARL7 appear to be cargo proteins rather than Ran-like regulators of the interaction of cargo proteins with the translocators importin α and β. It is conceivable, however, that the GTPases represent adaptors for specific cargo proteins that lack a nuclear localization signal. Furthermore, the possibility has to be considered that the ARL isoforms exert a regulatory function within the nucleus.

Acknowledgements: The nucleotide sequence of ARL7 has been deposited in the EMBL database (Acc. No. Y17804). The authors are indebted to Drs. S. Paris and B. Antonny for samples of recombinant ARF and ARNO, and to P. Mühl-Zürbes and S. Detro-Dassen for technical assistance. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Jo 117/10-2).

References

[1] Kahn, R.A. and Gilman, A.G. (1984) J. Biol. Chem. 259, 6228–6234.
[2] Sewell, J.L. and Kahn, R.A. (1988) Proc. Natl. Acad. Sci. USA 85, 4620–4624.
[3] Price, S.R., Nightingale, M., Tsai, S.-C., Williamson, K.C., Adamik, R., Chen, H.-C., Moss, J. and Vaughan, M. (1988) Proc. Natl. Acad. Sci. USA 85, 5488–5491.
[4] Boman, A.L. and Kahn, R.A. (1995) Trends Biochem. Sci. 20, 147–150.
[5] Moss, J. and Vaughan, M. (1995) J. Biol. Chem. 270, 12327–12330.
[6] Exton, J.H. (1997) Physiol. Rev. 77, 303–320.
[7] Clark, J., Moore, L., Krasinskas, A., Way, J., Battey, J., Tamkun, J. and Kahn, R.A. (1993) Proc. Natl. Acad. Sci. USA 90, 8952–8956.
[8] Schürmann, A., Breiner, M., Becker, W., Huppertz, C., Kainulainen, H., Kentrup, H. and Joost, H.G. (1994) J. Biol. Chem. 269, 15683–15688.
[9] Cavenagh, M.M., Breiner, M., Schürmann, A., Rosenwald, A.G., Terui, T., Zhang, C., Randazzo, P.A., Adams, M., Joost, H.G. and Kahn, R.A. (1994) J. Biol. Chem. 269, 18937–18942.
[10] Schürmann, A., Maßmann, S. and Joost, H.G. (1995) J. Biol. Chem. 270, 30657–30663.
[11] Breiner, M., Schürmann, A., Becker, W. and Joost, H.G. (1996) Biochim. Biophys. Acta 1308, 1–6.
[12] Jacobs, S., Schürmann, A., Becker, W., Böckers, T.M., Copeland, N.G., Jenkins, N.A. and Joost, H.G. (1998) Biochem. J. 335, 259–265.
[13] Smith, S.A., Holik, P.R., Stevens, J., Melis, R., White, R. and Albertsen, H. (1995) Genomics 28, 113–115.
[14] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[15] Kahn, R.A., Kern, F.G., Clark, J., Gelmann, E.P. and Rulka, C. (1991) J. Biol. Chem. 266, 2606–2614.
[16] Paris, S., Beraud-Dufour, S., Robineau, S., Bigay, J., Antonny, B., Chabre, M. and Chardin, P. (1997) J. Biol. Chem. 272, 22221–22226.
[17] Valencia, A., Chardin, P., Wittinghofer, A. and Sander, C. (1991) Biochemistry 30, 4637–4648.
[18] Kahn, R.A., Der, C.J. and Bokoch, G.M. (1992) FASEB J. 6, 2512–2513.
[19] Dingwall, C. and Laskey, R.A. (1991) Trends Biochem. Sci. 16, 478–481.
[20] Harshman, K., Bell, R., Rosenthal, J., Katcher, H., Miki, Y., Swenson, J., Gholami, Z., Frye, C., Ding, W., Dayananth, P., Eddington, K., Norris, F.H., Bristow, P.K., Phelps, R., Hattier, T., Stone, S., Shaffer, D., Bayer, S., Hussey, C., Tran, T., Lai, M., Rosteck, P.R., Skolnick, M.H., Shattuck-Eidens, D. and Kamb, A. (1995) Human Mol. Genet. 4, 1259–1266.
[21] Rothman, J.E. (1994) Nature 372, 55–63.
[22] Hong, J.-X., Lee, F.-J.S., Patton, W.A., Lin, C.Y., Moss, J. and Vaughan, M. (1998) J. Biol. Chem. 273, 15872–15876.
[23] Görlich, D. and Mattaj, I.W. (1996) Science 271, 1513–1518.
[24] Nigg, E.A. (1997) Nature 386, 779–787.