Structure and Intracellular Localization of Mouse ADP-Ribosylation Factors Type 1 to Type 6 (ARF1-ARF6)[1]

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ADP-ribosylation factors (ARFs) are a family of small GTP-binding proteins that are proposed to be involved in the formation of coated transport vesicles. Although six ARF sequences have been reported in mammals to date, it has been unclear how many ARF members are present in a single organism. In this study, we provide the first direct evidence by cDNA cloning for the presence of all six ARF members in mouse. These proteins are highly conserved across mammalian species and Northern blot analysis revealed that mRNAs for all the members were expressed ubiquitously. Transfection of cells with epitope-tagged ARFs revealed that ARFs 1-3 displayed a perinuclear Golgi localization, while ARFs 4-6 appeared to be widely dispersed throughout the cytoplasm. These results suggest that although all the ARF proteins play fundamental and critical roles in cellular function, they are involved in different vesicular transport processes.

Key words: ADP-ribosylation factor, epitope tagging, Golgi complex, small GTP-binding protein, vesicular trafficking.

Multiple small GTP-binding proteins belonging to the Rab, ADP-ribosylation factor (ARF), and Sar1 subfamilies, and heterotrimeric G proteins are key regulators of vesicular trafficking through the exocytic and endocytic pathways in eukaryotic cells (for reviews, see Refs. *1-7*). Genetic and biochemical studies have revealed that Rab proteins play an essential role in various vesicular transport steps. Distinct Rab proteins are associated with almost every intracellular membrane compartment, suggesting that these small GTP-binding proteins function in vesicular targeting and/or fusion (*1, 3, 4, 6*). Sar1 is required for vesicle budding from the endoplasmic reticulum (ER) (*8-10*).

The ARF family includes six members of ARFs and several structurally related ARF-like proteins (*11-14*; for reviews, see Refs. *7* and *15*). ARF was first discovered as the cofactor required for the cholera toxin-catalyzed ADP-ribosylation of the α subunit of the heterotrimeric G protein, G_s (*16, 17*). More recently, ARF proteins have been shown to play a critical role in vesicular trafficking along the exocytic and endocytic pathways (*18-23*). Vesicle formation is triggered by the attachment of cytosolic ARF to the membrane of donor compartments. The exchange of GDP for GTP on ARF, which is inhibited by a fungal metabolite, brefeldin A (*24-26*), promotes membrane binding of ARF, the binding of which is apparently a prerequisite for the membrane binding of coatomer, a complex of seven COP proteins (*27*), and that of HA-I/AP-1 clathrin adaptor, a complex of four adaptor proteins (*28*), from the cytosol (*5, 29-31*). Subsequently, budding of COP-coated vesicles from the Golgi and that of clathrin-coated vesicles from the *trans*-Golgi network occur (*32*). Furthermore, recent evidence suggests that ARFs are capable of activating phospholipase D (*33, 34*). The stimulation of phospholipase D activity by ARFs could promote vesicle budding at distinct membrane domains, possibly by acting to direct coat protein binding in transport vesicle assembly (*35*).

Although six ARF sequences have been reported in mammals (for reviews, see Refs. *7, 15*, and *36*), there is uncertainty with respect to the number of ARF family members in a given organism. Bovine ARF1 and ARF2 (*37, 38*) and human ARFs 1 and 3-6 (*11, 12*) have been cloned to date, but there has been no report of human ARF2. In this study, however, we cloned cDNAs for all the ARF members from mouse, demonstrating that ARFs 1-6 are present in a single organism.

The six ARF proteins are highly homologous to one another and are assigned to three classes on the basis of the sequence similarity: class I, ARF1, ARF2, and ARF3; class II, ARF4 and ARF5; class III, ARF6 (*12, 36*). Owing to the

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Abbreviations: ARF, ADP-ribosylation factor; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; HA, hemagglutinin.

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high sequence similarity of ARFs, antibodies that specifically recognize each ARF member are not available, though recently D'Souza-Schorey *et al.* (*23*) have raised antiserum that reacts specifically with ARF6, and have shown that ARF6 is localized at the cell periphery and is involved in receptor-mediated endocytosis. One way to approach this problem is the epitope-tag method. Using this method, ARF1 has been shown to be localized in the Golgi region, and ARF6 in the cell periphery and endosomes (*39, 40*). However, little is known about the subcellular localization of other ARF members. In the present study, we also applied this method and determined the subcellular localization of all the ARF proteins.

A. ARF1

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GGGAGCGAAACCAACGCCTGGCTGGGAGCAGCCGCCGCGGAGGTCTTTGGCCAGTATCGATTCCACCTGTCCACAAAC   78
ATGGGGAATATCTTTGCAAACCTCTTCAAGGGCCTTTTTGGCAAAAAGAAATGCGCATTCTCATGGTGGGCCTGGATGCTGCAGGGAAG  168
M G N I F A N L F K G L F G K K E M R I L M V G L D A A G K   30

ACAACAATTCTATACAAACTGAAGCTGGGCGAAATTGTGACCACCATTCCCACCATTGGTTTCAATGTGGAGACTGTTGAATACAAGAAT  258
T T I L Y K L K L G E I V T T I P T I G F N V E T V E Y K N   60

ATCAGCTTCACCGTGTGGGATGTGGGCGGCCAGGACAAGATCCGGCCGCTGTGGCGCCACTACTTCCAGAACACCCAAGGCTTGATCTTC  348
I S F T V W D V G G Q D K I R P L W R H Y F Q N T Q G L I F   90

GTAGTGGACAGCAATGACAGAGAGCGTGTGAACGAGGCCCGTGAAGAGCTCATGAGGATGCTAGCTGAAGATGAGCTCCGAGATGCTGTT  438
V V D S N D R E R V N E A R E E L M R M L A E D E L R D A V  120

CTCTTGGTGTTTGCCAACAAGCAGGACCTCCCCAATGCCATGAATGCGGCCGAAATCACAGACAAGCTGGGGCTGCACTCTCTACGCCAC  528
L L V F A N K Q D L P N A M N A A E I T D K L G L H S L R H  150

AGGAACTGGTACATTCAGGCCACCTGTGCCACCAGCGGGGACGGGCTCTATGAAGGACTAGATTGGCTGTCTAATCAGCTCCGGAACCAG  618
R N W Y I Q A T C A T S G D G L Y E G L D W L S N Q L R N Q  180

AAGTGAACCAGACCCCTCCCTCCCCCTCACTTCCTCTTCTCCGCCCTCAGCTTTCCTCTCATGTGGCAAACGTGCGACTCTGTGGTCCTG  708
K                                                            181

AGTGCCAGAAGCTGTCTCCATGGGTTGGTCACAGTGTGCATCGCACCGTGCTGTACATGTGCAGACGCAGCCTGCAGCCAGGTTTTTATT  798
TAATGTAAATAGTTTCTGTTTCCACTGAGGCAGTTTCTGGTACTCCTATGCAATATTACTTAGCTTTTTTATTGTAAAAGAGAATCAAC  888
TCAACTGTCAGTACTGAGAAGGGATTTGGGTGTAGGGGCACCTGGCCTCCGGGGAGCCATTGGGCTGTAGACTGGTGTCGGTATCCATTTG  978
GTGGTTGGTTTTTAACCCAAACTCAGTGCATTTTTTAAAAATAGTTAAAAATAC  1032
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B. ARF2

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TCGGCCGAGTAGCATTTTCGTGAGTGGCTTTGTGAAGGTGTTCGCTGCGGAGACAGGTTGCAGAATTCAGCTACA   75
ATGGGGAATGTCTTTGAAAAGCTGTTTAAAAGCCTATTTGGGAAAAAGGAAATGCGGATTCTCATGGTGGGCTTAGATGCAGCTGGCAAA  165
M G N V F E K L F K S L F G K K E M R I L M V G L D A A G K   30

ACGACGATCTTGTACAAATTGAAGCTAGGAGAGATTGTGACAACCATCCCTACCATAGGTTTCAATGTGGAGACAGTAGAGTACAAAAAC  255
T T I L Y K L K L G E I V T T I P T I G F N V E T V E Y K N   60

ATCAGCTTCACAGTCTGGGATGTTGGTGGCCAGGACAAAATTAGACCTTTGTGGCGACATTACTTCCAGAACACTCAAGGTCTGATTTTC  345
I S F T V W D V G G Q D K I R P L W R H Y F Q N T Q G L I F   90

GTGGTTGACAGTAATGACCGAGAGCGGGTCAATGAGGCCCGGGAAGAATTGACCAGAATGCTAGCAGAAGATGAGCTCAGAGATGCAGTC  435
V V D S N D R E R V N E A R E E L T R M L A E D E L R D A V  120

TTGTTGGTGTTTGTAAACAAACAGGATCTTCCTAATGCTATGAATGCAGCAGAGATCACAGACAAGCTTGGCTTACACTCCCTTCGCCAG  525
L L V F V N K Q D L P N A M N A A E I T D K L G L H S L R Q  150

AGAAACTGGTACATTCAGGCTACCTGTGCGACCAGTGGAGATGGGCTTTACGAAGGCCTGGACTGGCTCTCCAACCAGCTCAAAAACCAG  615
R N W Y I Q A T C A T S G D G L Y E G L D W L S N Q L K N Q  180

AAGTGATCAGAAGCAACCCATTCCCCATGCATTGTGGCAAAGCCAGCTGGCCTTTCCCGTGTGCATGTGAGCGTGTGAGGAGCCCAGGGG  705
K                                                            181

GCTGTGTGGCTGGAGTGGGGGCAGCTTTCTCACACCGTGCCTTATACACGCTATACGAAAACCAGTATTCCATTTTAAGAAAACCAGTGT  795
TACATTTTGAATGCTACCTTCCATTTCACTAGCTTTGATGGTCATTTTTGCTGAGGCCTCCCTGGGTGTGATAGCCAGAGCATCTCTAGC  885
CTGGAAAAGAAGAAAGCAGGAGCCAGACAGGTCCTGTTGTTGCCAACCTGGGTCTGGCCTCTCCATACACCCAGAATTCTGTTGGACGTC  975
AGTGTCCTTTTATAAAAGGAAAGGAAGGATCTGTCATTCTTTCCATTGTGCCAAAGCTAGCAGCTCACTTGTAGTGCTTTAAAGCTCCCG 1065
AGAAGAAAAGATGCATTATTACCACGGGTTTGAGGAGGTTCAGAGGGGGCTACTCCCCCAAAGTTCTGTTAGCTCAGACTGACTCATTTA 1155
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C. ARF3

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TCACCGCGGTCGGGTCG   17
GCTTGCGGAGGGGAGGCAGCGGCCGGAGAGCCGGGCGGAACCTGCGGGGGCGGAGGCGGCGGTGGCAGCCGGGCGCAGGGAGCAGGAACA  107
GGGTCAAGTCCTCCATACCAAGGACCAGGCTAAAACCAGATTACACTGCCCCTCCCTGGGCCCTATTGACTTAAAGCAGCCACTGTAATC  197
ATGGGCAATATCTTTGGGAACCTTCTGAAGAGCCTAATCGGCAAGAAGGAGATGCGCATCCTGATGGTGGGCCTGGATGCTGCCGGGAAG  287
M G N I F G N L L K S L I G K K E M R I L M V G L D A A G K   30

ACCACCATCCTCTACAAGCTGAAACTCGGGGAGATTGTCACCACCATCCCGACCATTGGGTTTAATGTGGAGACAGTGGAATATAAGAAT  377
T T I L Y K L K L G E I V T T I P T I G F N V E T V E Y K N   60

ATCAGCTTCACAGTCTGGGACGTAGGTGGCCAGGACAAGATTCGGCCCCTCTGGAGACACTACTTCCAGAACACCCAAGGCTTGATATTT  467
I S F T V W D V G G Q D K I R P L W R H Y F Q N T Q G L I F   90

GTGGTTGACAGCAATGATCGGGAGCGAGTGAACGAGGCCCGGGAAGAGCTGATGAGGATGCTGGCGGAGGATGAGCTCCGGGATGCTGTG  557
V V D S N D R E R V N E A R E E L M R M L A E D E L R D A V  120

CTCCTTGTGTTTGCAAACAAACAGGATTTGCCGAATGCTATGAATGCTGCTGAGATCACAGACAAGCTGGGGCTGCACTCCCTGCGTCAC  647
L L V F A N K Q D L P N A M N A A E I T D K L G L H S L R H  150

CGCAATTGGTACATTCAGGCCACCTGTGCTACCAGCGGGGACGGGCTGTATGAAGGCTTGGACTGGCTGGCCAATCAGCTCAAAAACAAG  737
R N W Y I Q A T C A T S G D G L Y E G L D W L A N Q L K N K  180

AAGTGAGAGCCAGGCAGCCCTCTCTGACTACCCCACCC  775
K                                       181
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Fig. 1. Nucleotide and deduced amino acid sequences of mouse ARF1 (A), ARF2 (B), ARF3 (C), ARF4 (D), ARF5 (E), and ARF6 (F).

D. ARF4

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CCAGACCCCCTCCTTTTGACCGTCAAGTGCCACCACCGCT   40
ATGGGCCTCACCATCTCCTCTCTCTTCTCGCGCCTGTTCGGCAAGAAGCAGATGCGCATTTTGATGGTTGGATTGGATGCTGCTGGCAAG  130
M G L T I S S L F S R L F G K K Q M R I L M V G L D A A G K   30

ACGACAATTCTGTATAAACTGAAATTAGGGGAAATAGTCACCACCATTCCTACCATTGGTTTTAATGTGGAAACAGTAGAATATAAGAAC  220
T T I L Y K L K L G E I V T T I P T I G F N V E T V E Y K N   60

ATTTGTTTCACAGTATGGGATGTTGGTGGTCAAGATAAAATTAGGCCTCTCTGGAGGCATTACTTCCAGAATACCCAGGGTCTCATTTTT  310
I C F T V W D V G G Q D K I R P L W R H Y F Q N T Q G L I F   90

GTGGTAGATAGCAATGATCGTGAAAGAATCCAGGAAGGAGCAGCTGTGCTGCAGAAAATGCTTCTGGAAGATGAGCTGCAGGATGCAGTG  400
V V D S N D R E R I Q E G A A V L Q K M L L E D E L Q D A V  120

CTGCTCCTTTTTGCAAACAAACAGGATCTGCCAAACGCTATGGCCATCAGTGAGATGACAGACAAGCTAGGTCTGCAGTCTCTCCGAAAC  490
L L L F A N K Q D L P N A M A I S E M T D K L G L Q S L R N  150

AGAACATGGTATGTCCAAGCCACTTGTGCTACACAAGGAACTGGTCTGTATGAGGGACTGGATTGGCTGTCAAATGAACTTTCAAAACGT  580
R T W Y V Q A T C A T Q G T G L Y E G L D W L S N E L S K R  180

TAAATGAAGCTGGATATCTAACCAAGGACATGTTTGATAGAATTGGTCTAGGCTTGTTACAACAAAATTAGTTTGCATCTTGGTTATTAA  670
AGGATATCTGAGACAGGTTTGGGCAGAAATTACAGCGTTTAAAACTTGTTTTGTTGCCAATTATTGTTTACCAAGAACAATGTTTGCTATT  760
TAGCAATATGCTTGGGTTTAAGAGAAATTCTCCTTGGGAAAGAAAAGTATCCATTATTATGCTTCCCTTGAACCTAAATGCCTGGATACA  850
GAGCTATCCTGACACCTTTAGACAGATCTGAGTGGTTTTTGAGCCCAAAACAATAATGTTTTAAAGTTATTCCCTTGATACTTTACTGAG  940
ACCTTTATCATTCCTCAGACAGTCTGCTGATTTAAAAATGTAGCATTCCATTTGTATTTATTTCTACCCTTTGCCAAAAAGATTTTTCTA 1030
ATAATGCTTGTACAGGCCAAGGCCGTGGTCCAAAACACTATTCAGTTTTCTTGTACTGAGGATCCCCCCACCCCACC 1107
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E. ARF5

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CAGCCCTCGCCCCGCGTCCCCTGCCCGCGCCCCCGCCCCGGGCCCCGCC   49
ATGGGCCTCACGGTGTCCGCGCTCTTTTCGCGGATCTTCGGGAAGAAGCAGATGCGGATCCTTATGGTTGGCTTGGATGCGGCTGGCAAG  139
M G L T V S A L F S R I F G K K Q M R I L M V G L D A A G K   30

ACTACCATCCTGTACAAACTGAAGTTGGGGGAGATTGTCACCACCATCCCCACTATAGGCTTCAATGTGGAAACAGTGGAATATAAGAAC  229
T T I L Y K L K L G E I V T T I P T I G F N V E T V E Y K N   60

ATCTGTTTCACAGTGTGGGATGTTGGAGGCCAGGATAAGATTCGGCCTCTGTGGCGGCACTACTTCCAGAACACTCAGGGCCTCATCTTT  319
I C F T V W D V G G Q D K I R P L W R H Y F Q N T Q G L I F   90

GTGGTAGACAGCAACGACCGGGAGCGGGTCCAGGAGTCTGCTGATGAACTCCAGAAGATGCTGCAGGAGGATGAGCTCCGGGATGCGGTG  409
V V D S N D R E R V Q E S A D E L Q K M L Q E D E L R D A V  120

CTGCTGGTGTTTGCCAACAAGCAGGACATGCCCAATGCCATGCCCGTGAGCGAGCTGACTGACAAGCTGGGCCTTCAGCACCTGCGTAGC  499
L L V F A N K Q D M P N A M P V S E L T D K L G L Q H L R S  150

CGCACGTGGTACGTCCAGGCCACCTGTGCCACCCAAGGCACAGGCCTGTATGATGGGCTGGACTGGCTGTCCCACGAGCTGTCAAAGCGC  589
R T W Y V Q A T C A T Q G T G L Y D G L D W L S H E L S K R  180

TAGCCAGCCAGGGGCAGGCCCCTGCTGCCCGGAAGCTCCCGCGTGCATCCCGGGATGACCAGACTCCCGGACTCCTCAGGCAGTGCCCTT  679
CCTCCCCACTCTTCCTCCCCACAGACAGGCCTCTGCTCCTGCGCCTGCCTGCATGCTCTCTCTTGTCGTTGGAGCCTGGAGCCTTGCTCT  769
CTGGGCACAGAGGGCTCTGCTCTCCTGCCTGCTGGGACCTGTGGATGGGCTTCCTGGCCAAGGCCCCCTCTTCCAGGGGAGGAGCAGGGA  859
TCTGGATTTAATTTGGTTTTGGTTTTGGTTTTTTGATTTTTTTTTCTTTTTTTTCTTTCTTTTTTTTTGTTTTG  933
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F. ARF6

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CGGGGCGGCGGCTGGAGGCGAGCACCCGCGAGTTCC   36
CGCGAGGATCCATGACCTGACCGGGCGCCGAGCCGCGCTCGTCGTCGGGGTGGTGACCGGGGTCGGTGCCGAGTCCGGTGCTCGCAGGCC  126
GGCAGGCGGCCGCAGTTGGAGCCCCGCGCGTGCCGAGGCGGCGGAGGAGCTGTCGGCGGCGCTTGCTCGCTCGCTCGTTCGCGCGGCGCCT  216
GCGGGGGGGAAGGGCAGTTCCGGGCCGGCCGCGCCTCGCCTCGCCTCGCTTGAGCAGGGCGGCGGCTCTCCCGGCGCAGACGCAGGGCCC  306
CGGCGGCGGGCGATGCCCGAGTGAGCGCAGGCCGGGCCCCTGCTCCCCTAGCCGGCAGCTTCGCGCAGGCCGCAGGCGTCCTCGCAGCG  396
GCGGCGGGACGTTTCGGGCTCGCGGCGGCGTTGTAGGCTTGAGGGGACCCGGGACACCTGAATGCCCCCCGGCCCCGGCTCTTCCGACGCG  486
ATGGGGAAGGTGCTATCCAAGATCTTCGGGAACAAGGAAATGCGGATCCTCATGCTGGGCCTGGACGCAGCCGGCAAGACAACGATCCTG  576
M G K V L S K I F G N K E M R I L M L G L D A A G K T T I L   30

TACAAGTTGAAGCTGGGCCAATCGGTGACCACCATCCCCACGGTGGGCTTCAACGTGGAGACGGTGACTTACAAAAACGTCAAGTTCAAC  666
Y K L K L G Q S V T T I P T V G F N V E T V T Y K N V K F N   60

GTGTGGGATGTGGGCGGCCAGGACAAGATCCGGCCGCTCTGGCGGCATTACTACACCGGGACCCAGGGTCTGATCTTCGTGGTAGACTGC  756
V W D V G G Q D K I R P L W R H Y Y T G T Q G L I F V V D C   90

GCCGACCGCGACCGCATCGACGAGGCCCGCCAGGAGCTGCACCGCATTATCAATGACCGGGAGATGAGGGACGCCATCATCCTCATCTTC  846
A D R D R I D E A R Q E L H R I I N D R E M R D A I I L I F  120

GCCAACAAGCAGGACCTGCCCGATGCCATGAAACCCCATGAGATCCAGGAGAAACTGGGCCTGACCCGGATTCGGGACAGGAACTGGTAT  936
A N K Q D L P D A M K P H E I Q E K L G L T R I R D R N W Y  150

GTGCAGCCCTCCTGTGCCACCTCCGGGGACGGACTCTATGAGGGGCTCACATGGTTAACCTCTAACTACAAATCCTAATGAGCGTCCTCC 1026
V Q P S C A T S G D G L Y E G L T W L T S N Y K S           175

ACCCAGCCCCCGGAAGGAGAGAAATCCAAAACCCATTCCTAGGATTATCGCCACCTCCATCACCTCTTTGAATTGCCACTCTCTTTTTTG 1116
AATCTGAACTCTGGAGTTACTGTTCTACAGTTTAGTGGGGTTGGGGGTTTTCTTTGTTTCCTTTTTTTTTTTTTTTTTTTCCTCTTGGCTT 1206
TGCGTTAGGATGCTCTGATCTGACATTTGACACGAATACAGTGCTAGATGCCTTGTGACTTCCAGCAAACGGGGTGGGGTAATAGCAACT 1296
CTTGGTAAAGTCCTTTATAATAATGGTTTGATTTTTTTATTTCGAGAGAATCTTCCCCCCCATGTATGCTTTTTTTCCTTTTTGCCCAGG 1386
TTCTTATCACTTGCTGTAGATGGCTTATTTTGCATTCATGCAGACTATGTTCCAAGTCTGTTTCATCTAGTAAACTGAAAATTATTGCTT 1476
AATCAAACTGCCGTTTGTCTTTTATATTTAAGGCC 1511
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EXPERIMENTAL PROCEDURES

Cloning of Mouse ARF cDNAs—Approximately 5×10^5 phages of a mouse brain library in the λgt10 vector (*41*) were screened using a bovine ARF1 cDNA fragment covering the entire coding sequence (a kind gift from Dr. Richard A. Kahn, Emory University School of Medicine, Atlanta, GA; *37*) under low stringency conditions. The number of obtained cDNA clones for each ARF is shown in Table I. By this screening, cDNAs covering the entire coding sequence were obtained for ARFs 1, 3, 4, and 6, while all the cDNAs for ARF2 and ARF5 were partial. Therefore, $\sim2\times10^5$ phages of mouse liver library (*41*) were then screened using the partial ARF2 and ARF5 cDNAs, and full-length ones for both ARFs were obtained. The cDNAs were separately subcloned into the *Not*I site of pBluescript-II (Stratagene, La Jolla, CA) and sequenced using a *Bca*Best sequencing kit (Takara Shuzo, Kyoto).

Northern Blot Analysis—Total RNAs (10 μg) isolated from mouse tissues and cell lines were denatured with glyoxal, electrophoresed on a 1.5% agarose gel, and blotted

TABLE I. **ARF cDNA clones isolated from mouse brain library.** Approximately 5×10^5 clones were screened as described in "EXPERIMENTAL PROCEDURES."

ARF	Number of clones isolated
ARF1	7
ARF2	3
ARF3	6
ARF4	8
ARF5	1
ARF6	5
Total	30

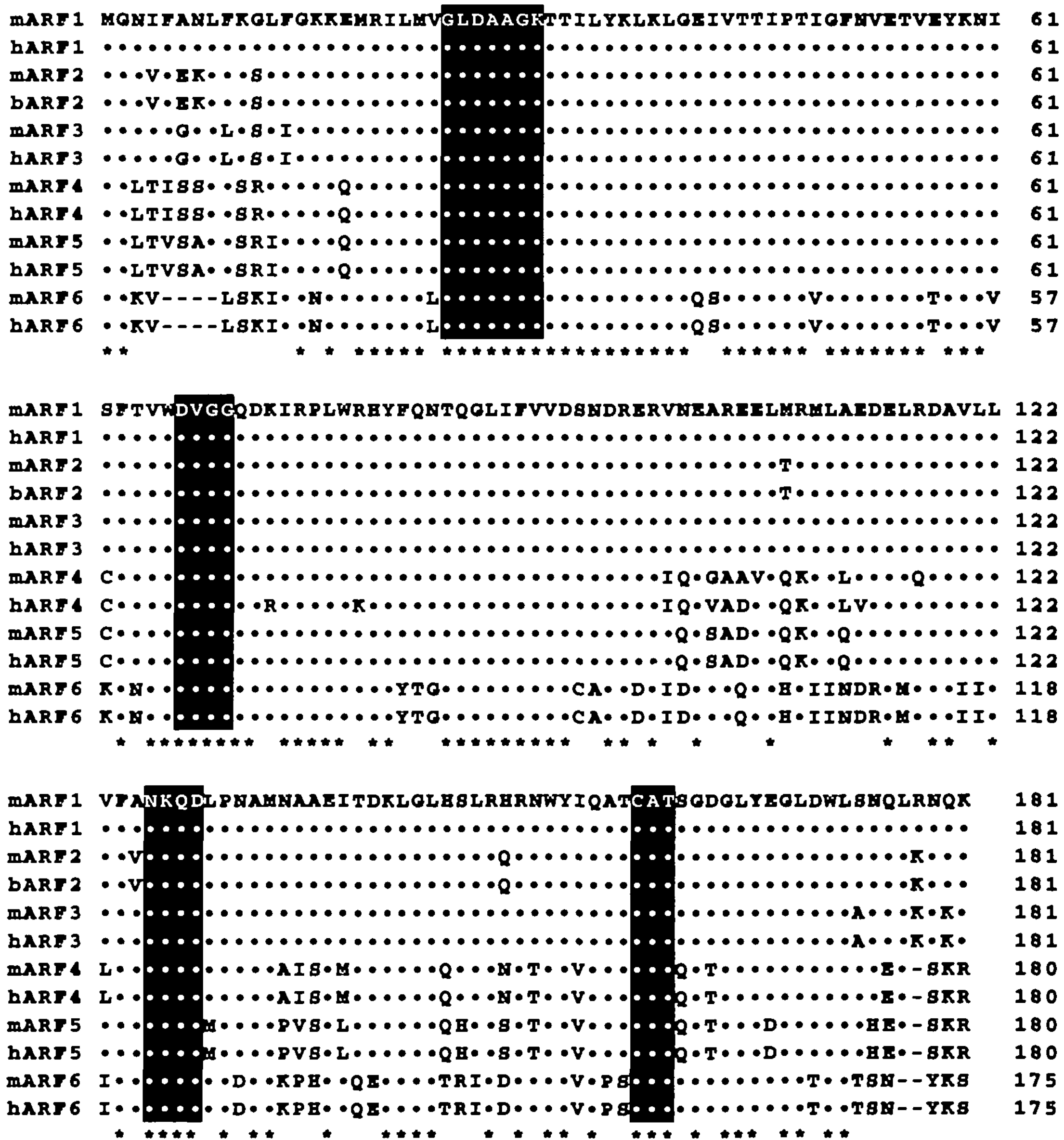

Fig. 2. **Comparison of mouse ARF amino acid sequences with those of other species.** Amino acids identical with those of mouse ARF1 are indicated by dots, and gaps introduced for optimal alignment are indicated by hyphens. Asterisks indicate the positions of amino acids identical in all the ARFs. Consensus sequences for GTP-binding and hydrolysis are shown in dark boxes.

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onto a GeneScreen*Plus* membrane (Du Pont-NEN, Boston, MA) as described previously (*42*). The blot was hybridized with the following cDNA fragments of 5'- or 3'-untranslated region specific for each mouse ARF: ARF1, nucleotides 727-954; ARF2, nucleotides 962-1155; ARF3, nucleotides 1-170; ARF4, nucleotides 871-1086; ARF5, 640-860; ARF6, nucleotides 43-510.

DNA Construction—A vector for expressing COOH-terminally influenza hemagglutinin (HA) epitope-tagged proteins in mammalian cells (pCMV-HA) was constructed by subcloning of a double-stranded oligonucleotide coding for the HA-epitope sequence (YPYDVPDYA) between the *Xho*I and *Xba*I sites of pRcCMV (Invitrogen, San Diego, CA). Each ARF cDNA having an *Xho*I recognition sequence immediately behind the codon for the last amino acid, which was generated by a PCR-based method, was subcloned into pCMV-HA.

DNA Transfection and Indirect Immunofluorescence Microscopy—The expression vector for the COOH-terminally HA-tagged ARF thus constructed was transfected into monkey kidney Vero cells by using a CellPhect transfection kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). The cells were cultured for 24 h, and then trypsinized, plated onto wells of eight-well Lab-Tek chamber slides (Nunc, Roskilde, Denmark), and cultured for a further 24 h. The cells were fixed, permeabilized, and blocked as described previously (*43*). The cells were then incubated with monoclonal mouse anti-HA antibody (12CA5, Boehringer Mannheim GmbH, Mannheim, Germany) and polyclonal rabbit anti-β-COP antibody (a kind gift from Dr. Yukio Ikehara, Fukuoka University School of Medicine, Fukuoka; *44*), followed by FITC-labeled donkey anti-mouse IgG (Chemicon, Temecula, CA) and Texas Red-labeled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA). The stained cells were observed with a laser-scanning confocal microscope (TCS4D, Leica Lasertechnik, GmbH, Heidelberg, Germany).

RESULTS AND DISCUSSION

By screening at low stringency of $\sim 5 \times 10^5$ clones from a mouse brain library (*41*) with a bovine ARF1 cDNA fragment covering the entire coding sequence, 30 positive clones were obtained. Restriction endonuclease mapping and partial nucleotide sequence analysis revealed that these included cDNA clones for all known ARFs (Table I). Since the cDNAs for ARF2 and ARF5 were partial, a mouse liver library (*41*) was then screened with the partial cDNAs, and full-length ones for both ARF2 and ARF5 were obtained. It is particularly noteworthy that cDNAs for all six ARFs were obtained from the same library. There has been uncertainty regarding the number of ARF family members in a given organism although six ARF sequences have been reported in mammals (for reviews, see Refs. *7, 15* and *36*); bovine ARF1 and ARF2 (*37, 38*) and human

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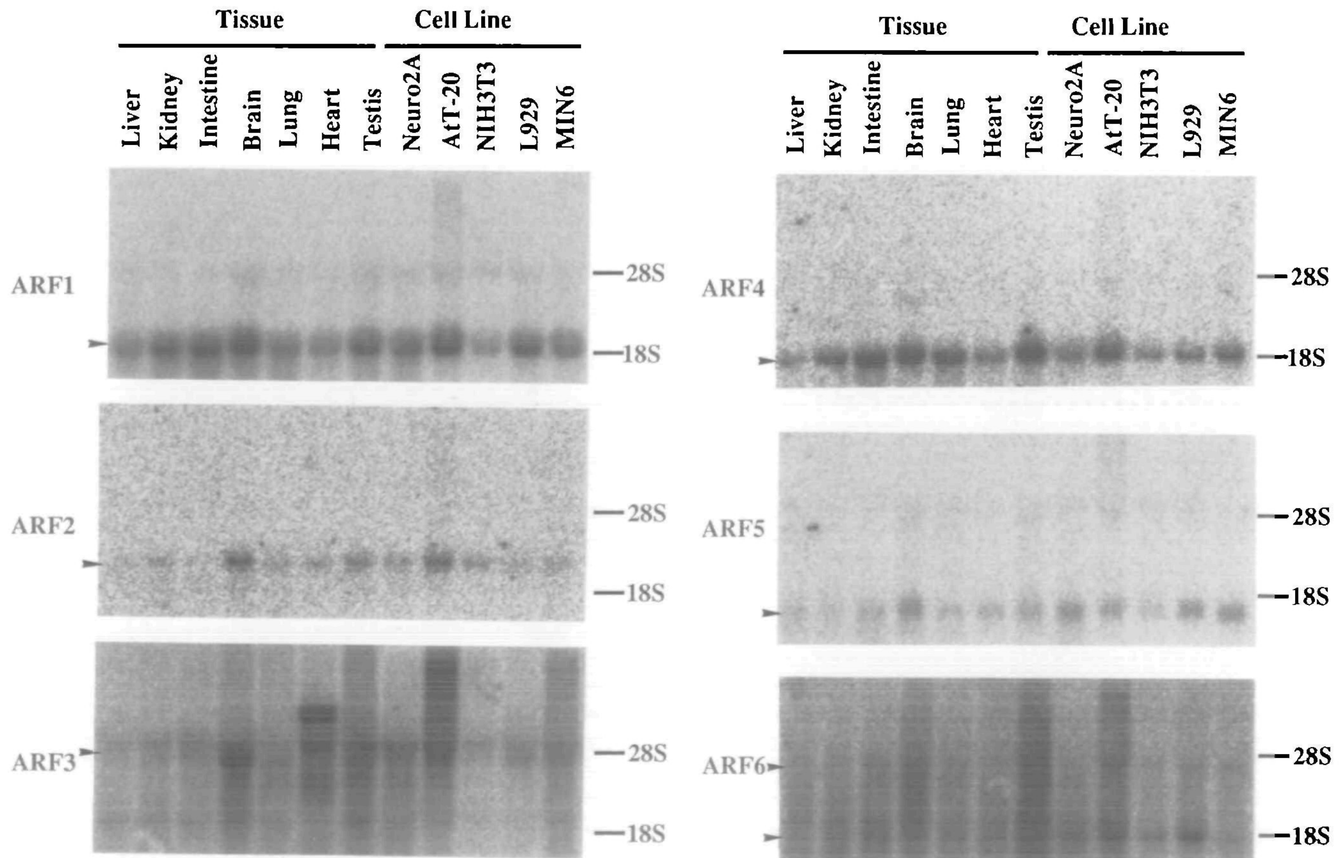

Fig. 3. **Northern blot analysis.** Total RNAs (10 μg) of the mouse tissues and cell lines indicated were analyzed as described under "EXPERIMENTAL PROCEDURES." Positions of ARF transcripts are indicated by arrowheads. Neuro 2A, a neuroblastoma cell line; AtT-20, a corticotrophic tumor cell line; NIH 3T3, an embryo cell line; L929, a connective tissue cell line; MIN6, an insulinoma cell line.

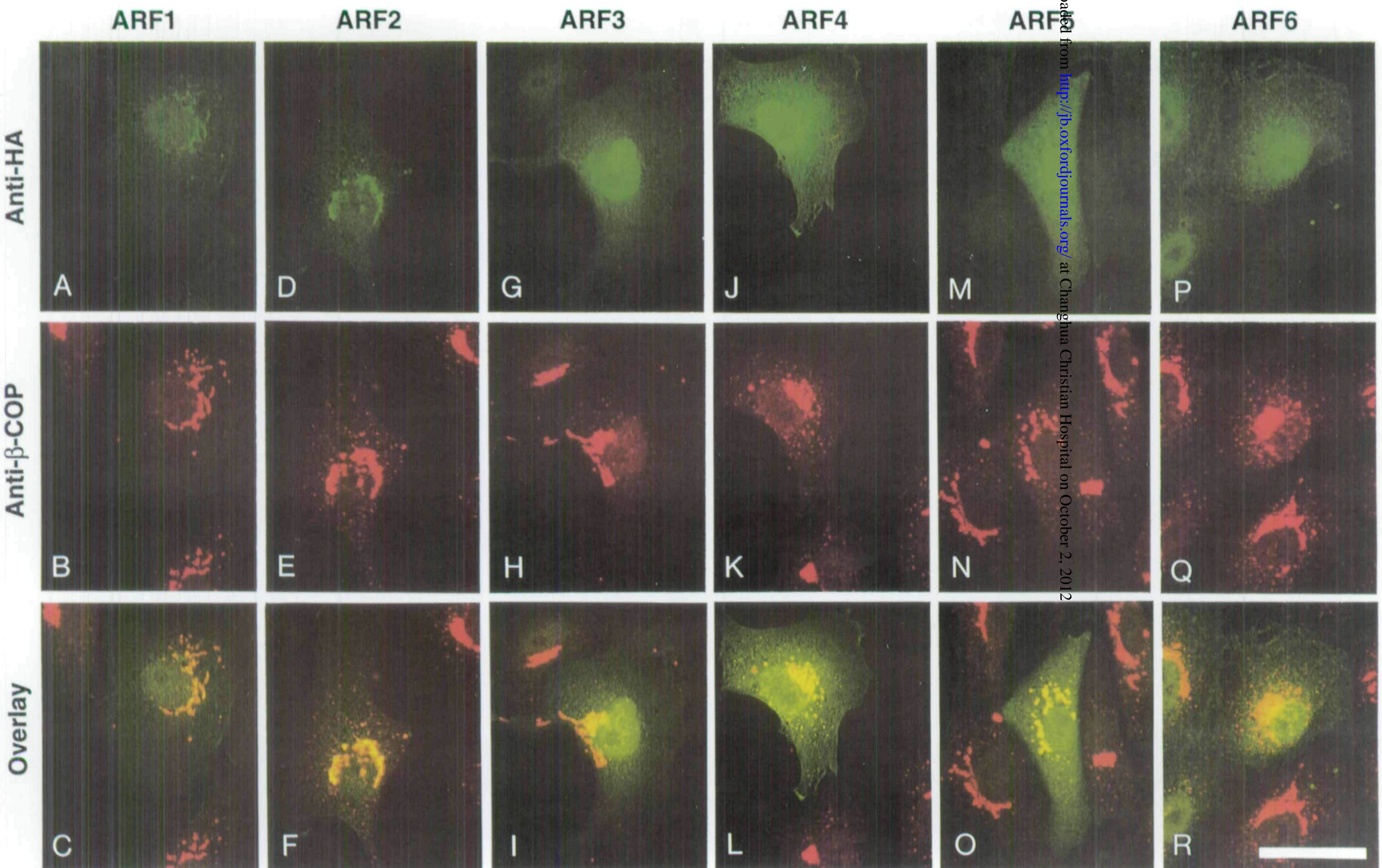

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Fig. 4. **Immunofluorescence analysis of cells expressing epitope-tagged ARFs.** Vero cells expressing HA-tagged ARF1 (A–C), ARF2 (D–F), ARF3 (G–I), ARF4 (J–L), ARF5 (M–O), and ARF6 (P–R) were stained with monoclonal anti-HA antibody (upper panels) and polyclonal anti-β-COP antibody (middle panels) followed by FITC-labeled anti-mouse IgG and Texas Red-labeled anti-rabbit IgG as described under "EXPERIMENTAL PROCEDURES" and observed with a confocal microscope. Overlays are shown in the lower panels.

ARFs 1 and 3-6 (*11, 12*) have been cloned to date, but there has been no report on human ARF2. In this study, however, we cloned cDNAs for all the ARF members from mouse, demonstrating that ARFs 1-6 are present in a single organism.

Figure 1 shows the nucleotide and deduced amino acid sequences of the six mouse ARFs. Alignment of the mouse ARF sequences with those of other mammals (Fig. 2) revealed that the ARFs are highly conserved proteins. The deduced amino acid sequences of mouse ARFs 1-3, 5, and 6, were in complete agreement with those of the human and bovine counterparts so far reported. By contrast, seven out of 180 amino acids of mouse ARF4 were different from those of human ARF4. The very high sequence conservation suggests that the whole region of each ARF protein plays a critical role in its function.

It is curious that only the mouse ARF4 sequence is not identical with that of other species. This could imply that we cloned one isoform of ARF4 and there may exist another ARF4 isoform that we failed to isolate. Nonetheless, we think that the cDNAs we cloned encode the sole mouse counterpart of ARF4, since the partial nucleotide sequences containing both the translated and untranslated regions of eight cDNA clones for ARF4 isolated from the brain library and five clones from the liver library were identical with one another (data not shown).

We then performed Northern blot analysis to examine the tissue and cellular distribution of the ARF members. In this analysis, we used a fragment covering an untranslated region of each ARF cDNA to detect specifically mRNA transcripts of each member, since the nucleotide sequences in the coding region of ARFs 1-6 are highly homologous to one another. The analysis revealed that all ARF transcripts were expressed in all examined mouse tissues (liver, kidney, intestine brain, lung, heart, testis) and cell lines (NIH 3T3, L929, AtT-20, MIN6, Neuro2A) (Fig. 3), suggesting all the ARF proteins play a fundamental role in cellular function.

Although there have been many reports on the subcellular localization and function of ARFs, most studies have been restricted to ARF1; the functions of other ARFs have not been defined. One of the reasons for this is the lack of antibodies that specifically recognize individual ARF members, since the ARF proteins are highly homologous to one another (see Fig. 2). For example, a widely used monoclonal antibody to ARF, 1D9 (*45*), recognizes all the ARF members (data not shown). To circumvent this problem, we first constructed expression vectors for ARFs with a COOH-terminal extension of the influenza HA epitope, since the HA epitope tag has been shown not to alter the intracellular localization of ARF1 and ARF6 (*39, 40*), although we could not completely exclude the possibility that such a tag affects the localization of other ARFs.

Monkey kidney Vero cells were transiently transfected with these ARF-HA constructs and were examined to determine the intracellular localization of each ARF using monoclonal anti-HA antibody (12CA5) by indirect immunofluorescence microscopy (Fig. 4). In cells expressing class I ARFs, ARF1 (panel A), ARF2 (panel D), and ARF3 (panel G), the staining was mainly restricted to a perinuclear region. For ARF1, a similar staining pattern was observed when it was tagged COOH-terminally with the human myc epitope in place of HA (data not shown). The staining of class I ARFs was superimposed on that of β-COP, a component of the coatomer protein complex, suggesting that these class I ARFs are localized in the Golgi region (panels A-I). Teal *et al.* (*39*) showed, by expressing HA-tagged ARF1 in COS-1 cells, that ARF1 colocalizes with β-COP in the perinuclear region. Our results thus showed that this is also the case with other class I ARFs, ARF2 and ARF3. Our results are also compatible with the previous report showing that ARF1 binding is a prerequisite for binding of coatomer to the Golgi membranes (*29*), and suggest that ARF2 and ARF3 play similar roles.

The staining patterns of ARFs 4-6 were different from those of ARFs 1-3. The class II and class III ARFs were localized in punctate structures throughout the cytoplasm and at the cell periphery (panels J, M, and P). Although the punctate staining was relatively intense in the perinuclear region, it was not completely superimposed on the β-COP staining (panels J-R). While this study was in progress, D'Souza-Schorey *et al.* (*23*) and Peters *et al.* (*40*) showed using an antibody specific for ARF6 and using the epitope tag method, respectively, that ARF6 is localized in endosome-like structures and in the plasma membrane, in accordance with our finding for ARF6. For ARFs 3-6, relatively intense nuclear staining was also observed, but its significance is currently unknown. We are now addressing this question.

In this study, we showed that ARFs constitute a protein family that is highly conserved across mammalian species and expressed ubiquitously. These findings indicate that ARF proteins play fundamental and critical roles in cellular function. However, ARF proteins belonging to classes I, II, and III appear to show different subcellular localizations, suggesting that they are involved in different vesicular transport processes. Experiments are under way in our laboratory to address this issue.

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