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Selective amplification of additional members of the ADP-ribosylation factor (ARF) family: Cloning of additional human and *Drosophila* ARF-like genes

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ABSTRACT **The ADP-ribosylation factor (ARF) family is one of four subfamilies of the RAS superfamily of low molecular weight GTP-binding proteins (G proteins). Highly degenerate oligonucleotides encoding two conserved regions were used in a PCR reaction to amplify cDNAs encoding each of the known ARF proteins and eight additional cDNA fragments encoding previously unreported human members of the ARF family. Additional sequences were obtained from yeast or fly libraries by using this technique. These oligonucleotides specifically amplify members of the ARF family but not the structurally related G protein α subunits or members of the other three subfamilies of the RAS superfamily. Fragments obtained by PCR were used to obtain full-length sequences encoding highly homologous ARF-like (ARL) gene products from human and *Drosophila melanogaster* libraries, termed ARL2 and Arl84F, respectively. The encoded proteins are each 184 amino acids long and are 76% identical, with 40–45% identity to human ARF1 and *Drosophila* arf-like (arl) proteins. These genes appear to be generally expressed in human tissues and during *Drosophila* development. The purified human ARL2 protein differed in several biochemical properties from human ARF proteins, including the complete absence of ARF activity. Thus, the ARF family of low molecular weight GTP-binding proteins includes at least 15 distinct but structurally conserved members, including both the functionally conserved ARF proteins and the functionally disparate ARL proteins. The latter proteins currently comprise two distinct gene products in *Drosophila* (arl and ARL84F) and one in man (ARL2).**

ADP-ribosylation factor (ARF) was originally identified as the protein cofactor in the cholera toxin-catalyzed ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase, G_s (1, 2). It was subsequently shown to be a 21-kDa GTP-binding protein (ref. 3) with cofactor activity dependent on the binding of GTP. More recently ARF proteins have been found to localize to the Golgi complex (4) and to regulate several distinct vesicle transport steps in the exocytic and endocytic pathways, including budding from the endoplasmic reticulum (5) and fusion at the Golgi stacks (6), endosomes (7), and nuclear vesicles (8). Studies are underway to identify functions for each of the distinct gene products in the ARF family and also to define differences in the activities of the more highly conserved members—e.g., ARF1 and ARF3—which are 96% identical. To continue this work and to assess the specificity of cDNA or antibody probes useful in such studies, it is important to identify each of the members of this structurally conserved family.

As the number of ARF activities has increased in recent years, the number of structurally conserved members of the ARF family of low molecular weight GTP-binding proteins has also grown. The first ARF, bovine ARF1, was cloned by screening cDNA libraries with oligonucleotide probes based on partial sequence information (9). A variety of other techniques have now been used to clone members of the ARF family, including functional screens (10–12), low-stringency hybridizations (9, 13, 14), PCR methodologies (15), and serendipity (16, 17). At least 18 cDNA sequences of members of the ARF family from at least nine different organisms have been reported. The encoded proteins have been grouped functionally into the ARF proteins (having cholera toxin co-factor activity and/or the ability to rescue the lethal double mutant $arf1^-$ $arf2^-$ in yeast) and the ARF-like (ARL) proteins, which are structurally related but lack these activities (17, 18). Alignment of the ARF1 protein sequences with other G proteins revealed (9) that ARF proteins are the most distantly related family in the RAS superfamily and are as related to the G protein α subunits as they are to the RAS superfamily members.

All known members of the ARF family are similar in length, have glycine at position 2 (the site of *N*-myristoylation), lack the Cys-Ala-Ala-Xaa box motif (site of C-terminal carboxypeptidase cleavage, carboxymethylation, and prenylation in most members of the RAS superfamily) and have each of the consensus guanine nucleotide-binding domains. The three consensus sequences found in all guanine nucleotide-binding proteins [Gly-Xaa-Xaa-Xaa-Xaa-Gly-Lys (GXXXXGK), Asp-Xaa-Xaa-Gly (DXXG), and Asn-Lys-Xaa-Asp (NKXD), where Xaa is any amino acid (19)] are also present in the ARF family, but these regions are constrained to Gly-Leu-Asp-Xaa-Ala-Gly-Lys-Thr (GLDXAGKT in single-letter code), Asp-(Val or Leu or Ile)-Gly-Gly-Gln [D(V, L, or I)GGQ], and Asn-Lys-Gln-Asp (NKQD). In addition, the distances between these domains are remarkably conserved among the ARF proteins.

Based on the high degree of conservation of the GTP-binding domains in ARF family members, we have developed a technique for isolating new ARF-encoding genes from any species and report the sequence of two here.§ We provide evidence that the number of ARF family members in humans alone is at least 15, rather than the 5 currently published, and probably more.

MATERIALS AND METHODS

PCR. Completely degenerate oligonucleotides were synthesized encoding most of the first two consensus regions for GTP-binding proteins, including the sense strand of D(G or

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Abbreviations: ARF, ADP-ribosylation factor; ARL, ARF-like; RT-PCR, reverse-transcribed PCR.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L13687 for human *ARL2* and L14923 for *Drosophila Arl84F*).

A)AGKT (corresponds to the consensus sequence, GXXXXGK) and the antisense strand of WD(V or I)GGQ (corresponds to DXXGQ). These oligonucleotides were approximately 1000-fold and 500-fold degenerate, respectively. The sense-strand primer encoded a protein sequence that was highly conserved among the ARF proteins but not among other regulatory GTP-binding proteins. Unique *Sal* I and *Bam*HI sites, respectively, were included outside the primer binding sites to allow directional cloning of PCR products.

Purified plasmids or phage from three different libraries were used as templates for PCR; a pCD2-based human foreskin fibroblast cDNA library (20), a *Saccharomyces cerevisiae* genomic library (21), and a genomic *D. melanogaster* library (17). Template (50–500 ng) and primers (1 μM each) were included in a standard PCR reaction for 40 cycles including 1 min at each phase; denaturation at 94°C, annealing at 50°C, and extension at 72°C. The major product in each case was a band of about 170 base pairs (bp) which was directionally cloned into the *Bam*HI and *Sal* I sites of pBluescript (Stratagene) prior to DNA sequencing. Clones containing inserts were picked at random, and both strands were sequenced by the chain-termination method of Sanger (22).

Cloning of Human *ARL2*. Approximately 5×10^5 plaques from a human brain (cortex) cDNA library in the lambda ZAP vector (Stratagene) were screened with a ^{32}P-end-labeled 49-nucleotide anti-sense oligonucleotide as described (23). This probe was derived from the central portion of the fragment of ARL2 obtained from PCR amplification of the human cDNA library. Hybridization buffer (N) was that described by Davis *et al.* (23) at 37°C, and the final washing was performed at 45°C in 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS. A single clone (no. 242) was plaque-purified and excised from the phage vector by using the EXASSIST/SOLR system (Stratagene) according to the manufacturer's protocol. The entire insert of 752 bp was sequenced and found to encode an incomplete open reading frame. The 5′ end of the open reading frame was obtained by the 5′ RACE (rapid amplification of cDNA ends) procedure as described by Frohman *et al.* (24) using nested, gene-specific, antisense primers derived from clone 242. Finally, the open reading frame was amplified from reverse-transcribed RNA (RT-PCR; ref. 25) and subcloned for sequencing. There were no differences observed between the DNA sequences obtained by amplification from the human library with redundant oligonucleotides and by the RT-PCR method. The RNA used for RT-PCR (ARL2) and Northern blots (human brain and HeLa) was obtained commercially from Clontech.

Cloning and Mapping of the *Drosophila Arl84F* Gene. A phage containing the *Drosophila Arl84F* gene was isolated by screening an iso-1 genomic phage library (17) with the PCR-amplified Drosophila *Arl84F* fragment using standard techniques. The *Arl84F* gene was mapped by Southern blotting to a 1.7-kilobase (kb) *Bam*HI fragment contained within this phage. Analysis of this sequence identified an open reading frame, interrupted by two potential introns. Oligonucleotide primers based on the predicted sequence of the 5′ and 3′ untranslated regions of the Arl84F transcript were used to amplify Arl84F cDNA fragments from *Drosophila* Oregon R embryonic RNA by using RT-PCR. The sequence of the amplified Arl84F cDNA was identical to that predicted from the sequence of the gene, with the exception of a single G → A change (resulting in a conservative Val → Ile change at residue 108 of the predicted protein). The chromosomal location of the Drosophila *Arl84F* gene was mapped by *in situ* hybridization of digoxigenin-labeled DNA fragments to salivary gland polytene chromosomes as described by Engels *et al.* (26).

Expression of Human ARL2 Protein in Bacteria. The full-length coding region of *ARL2* was obtained by RT-PCR and engineered at the same time to include an *Nde* I site at the initiating methionine and a *Bam*HI site 6 bp 3′ of the stop codon to allow directional subcloning into the pET3C vector of Studier *et al.* (27, 28). Transfected BL21 (DE3) cells were grown at 30°C to a density of $A_{600} \approx 0.4$, before induction with 30 μM isopropyl thiogalactoside and continued growth for about 15 hr. At this point ARL2 represented about 10% of total bacterial protein. Purification of ARL2p was achieved by the same method used for other ARF proteins from bacteria (29). From 1 liter of bacterial culture about 7 mg of purified ARL2 was obtained.

RESULTS AND DISCUSSION

Amplification of Fragments of the Additional Members of the ARF Family. We sought a comprehensive method to identify additional members of the ARF family of low molecular weight GTP-binding proteins in a given cell type or tissue. The strategy used was PCR amplification of cDNA fragments using totally degenerate oligonucleotide primers corresponding to two highly conserved portions of ARF and ARL proteins. The distance between these two consensus GTP-binding domains (34 amino acids or 102 bp) is also well conserved in all members of the ARF family. The templates used in the original PCR reaction were plasmids derived from a human fibroblast cDNA library. After 40 rounds of amplification, the major product(s) was about 170 bp long, as predicted for a 102-bp insert, flanked by two primers ≈35 bp long (Fig. 1, lane A). This major product from the PCR reaction was purified and directionally cloned into pBluescript prior to DNA sequencing. One hundred clones were selected at random and sequenced, from which 81 inserts were read (others either lacked insert or could not be read). The >100 bp of DNA sequence generated was sufficient to unambiguously identify each of the currently published human ARF family members; including ARFs 1 (13, 30), 3 (13), 4 (10, 15), 5 (14), and 6 (14). As seen in Table 1, we also obtained eight additional cDNA fragments from the human fibroblast library. The number of times each of these cDNA fragments was detected is similar to that of the known ARF proteins (see Table 1). The encoded human protein fragments were found to be 26–97% identical to the homologous region of human ARF1 (Table 1). This is within the range of sequence conservation observed for known members of the

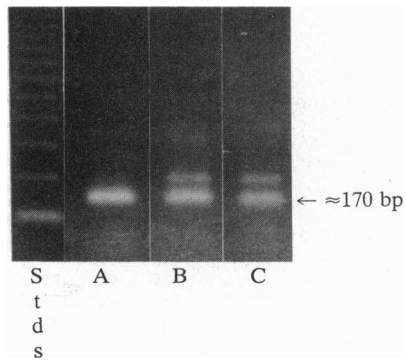

FIG. 1. Redundant oligonucleotides specifically amplify the same-size fragment from human cDNA, *S. cerevisiae*, or *Drosophila* genomic libraries. PCR reactions using the same oligonucleotide primers and human cDNA plasmids (500 ng) (lane A), yeast genomic plasmids (50 ng) (lane B), or *Drosophila* phage (100 ng) (lane C) were performed as described in text before resolving products on a 1% agarose gel. The ethidium bromide-stained products are shown along with the DNA standards (123-bp ladder) (Stds lane). A portion of the gel is shown, but no other bands were observed in the gel.

Table 1. Number of times each previously known (regular type) and additional (boldface type) ARF and ARL gene was identified by PCR from a human fibroblast cDNA library

	PCR identifications, no.	% identity to human ARF1
ARF1	3	100
ARF3	3	100
ARF4	32	97
ARF5	6	97
ARF6	1	82
ARL1	13	76
ARL2	4	84
UNK1	6	97
UNK2	3	26
UNK3	1	47
UNK4	1	63
UNK5	2	37
UNK7	2	26

UNK is the designation for a previously unknown ARF or ARL cDNA. Relatedness of the human protein fragments is shown as the percent identity compared to the same region of human ARF1, excluding primer-binding domains.

ARF family—e.g., SAR1 (12) and CIN4 (11) are 33% and 25% identical to *S. cerevisiae* ARF1, respectively. The occurrence and spacing of the consensus GTP-binding domains, the extent of sequence relatedness, and frequency of detection of the additional cDNA fragments are all consistent with the conclusion that these cDNA fragments are derived from currently uncharacterized members of the ARF family.

To confirm the human origin of the eight inserts, gene-specific oligonucleotides were used to amplify specific gene fragments (between 44 and 88 bp long) from both the original fibroblast cDNA library and a human hippocampal cDNA library (31). Specific oligonucleotides derived from each of the eight DNA sequences reported here successfully amplified fragments of the predicted sizes from each of these libraries. Thus, each of the eight fragments is present in two independently constructed human cDNA libraries, indicating that each represents a portion of a previously unreported human member of the ARF family.

The analysis of PCR reactions using redundant oligonucleotide primers was also performed, though to a more limited extent, with genomic *D. melanogaster or S. cerevisiae* libraries as templates as described. Again, the major products of the reactions were ≈170 bp in length (Fig. 1, lanes B and C). Of 19 inserts analyzed from the fly library, 11 originated from the previously identified *Arl* gene (17). Moreover, three additional cDNA fragments encoding Arl protein sequences were identified from the fly library. The yeast library yielded four different inserts out of 20 clones sequenced, including both ARF1 (9) and ARF2 (32) and two additional sequences. However, two members of the ARF family in *S. cerevisiae*, CIN4 and SAR1, were not detected in this search. Although overall these are the two least conserved members of the ARF family, visual inspection confirms that the redundant oligonucleotides used in the PCR should anneal to each of these genes and serve as primers in the PCR. Two other ARF family members have been cloned from human cDNA libraries, and the full length of the coding regions has been sequenced (R.A.K., unpublished observation). These two ARL proteins should also have been amplified with the redundant primers used to generate the data in Table 1. These data indicate that we may not have identified all of the members of the ARF family in either organism by these analyses. Increasing the number of inserts analyzed, using other libraries, or using human genomic DNA or reverse-transcribed mRNA as template will likely lead to the identification of even more members of the ARF family. The presence of internal *Bam*HI or *Sal* I sites in this portion of a genomic or cDNA fragment would also likely prevent its isolation by this procedure. Thus, the number of members in the ARF family remains uncertain but is at least 15 distinct proteins.

To begin the work of fully characterizing the gene products identified, we have cloned and sequenced the full-length protein coding regions encoding two of these fragments, one from a human library and one from *Drosophila*. These two chosen as the fragments were found to be highly related (82% identity, 91% homology) and likely represent homologues of the same gene. This degree of structural conservation between these species also makes it likely that the protein plays an important and conserved role in cell regulation.

Cloning and Sequence Analysis of Human *ARL2* and *Drosophila Arl84F*. The cloning of a human cDNA and *Drosophila* gene, each encoding ARL proteins are described. The two predicted protein sequences are shown in Fig. 2, aligned with human ARF1 and *Drosophila* arl. Individual comparisons between proteins reveal that these human and fly proteins are 76% identical. This level of identity makes it likely that the human and fly proteins are functional homologues, but in the

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                                                  /i
Human ARL2    MG--LLTILKKMKQKERELRLLMLGLDNAGKTTILKKFNGEDIDTISPTL    48
Dm Arl84F     MG--FLTVLKKMRQKEREMRILLLGLDNAGKTTILKRFNGEPIDTISPTL    48
Human ARF1    MGNIFANLFKGLFGK-KEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTI    49
Dm arl        MGGVLS-YFRGLLGS-REMRILILGLDGAGKTTILYRLQVGEVVTTIPTI    48
              **  .   .. .  . .*.*.*..***.******* ... . . *. **.

Human ARL2    GFNIKTLEHRGFKLNIWDVGGQKSLRSYWRNYFESTDGLIWVVDSADRQR    98
Dm Arl84F     GFNIKTLEHNGYTLNMWDVGGQKSLRSYWRNYFESTDGLVWVVDSADRMR    98
Human ARF1    GFNVETVEYKNISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRER    99
Dm arl        GFNVEQVTYKNLKFQVWDLGGQTSIRPYWRCYYSNTDAIIYVVDSADRDR    98
              ***.. ..... .. .**.***...*. ** *. .*.....****.** *
                                                        /i
Human ARL2    MQDCQRELQSLLVEERLAGATLLIFANKQDLPGALSSNAIREALELDSIR   148
Dm Arl84F     LESCGQELQVLLQEERLAGATLLVLCNKQDLPGALSSNEIKEILHLEDIT   148
Human ARF1    VNEAREELMRMLAEDELRDAVLLVFANKQDLPNAMNAAEITDKLGLHSLR   149
Dm arl        IGISKDELLYMLREEELAGAILVVLANKQDMDGCMVTAEVHHALGLENLK   148
              .     **  .* *. * .*.*... ****. . . .... . * *...

Human ARL2    SHHWCIQGCSAVTGENLLPGIDWLLDDISSRIFTAD   184
Dm Arl84F     THHWLVAGVSAVTGEKLLSSMDWLIADIAKRIFTLD   184
Human ARF1    HRNWYIQATCATSGDGLYEGLDWLSNQLRNQ----K   181
Dm arl        NRTFQIFKTSATKGEGLDQAMDWLSNTLQSR----K   180
               . . .   .*..*. *  ..*** . . ..    .
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FIG. 2. Alignment of human ARL2 and *Drosophila* Arl84F proteins with human ARF1 and *Drosophila* arl. Identities in all four proteins are indicated by an asterisk and homologies by a period. Overall identity is 31% and similarity 70%. The locations of the two introns that interrupt the coding region of *Arl84F* are indicated by "/i" at positions 22 and 140 of the protein. The locations of the regions used to design redundant oligonucleotide primers are shown in boldface type.

absence of any functional data, they are being given different names. The name ARL2 was given for the human ARL protein and Arl84F is assigned to the *Drosophila* protein, as a result of its cytological location (see below). These two proteins are 45% (ARL2) and 42% (Arl84F) identical to human ARF1, and each is about 43% identical to the previously described (17) *Drosophila* arl protein. A search of the SwissProt data base using the FASTA alignment program revealed that each of the top 13 scores were ARF proteins, and this search failed to find any protein with a higher homology to ARL2 than human ARF1. In addition, the predicted ARL2 and ARL84F protein sequences contain all of the structural hallmarks of a member of the ARF family, including (*i*) a glycine at position 2, the site of N-myristoylation; (*ii*) the highly conserved consensus GTP-binding domains GLD(G or A)AGKTT (indicative of an ARF protein), WDXGGQ (found in all ARF and heterotrimer GTP-binding proteins (G proteins) in contrast to the RAS and related proteins in which DTAGQ is typical), and NKQD (found in all members of the ARF family); and (*iii*) the absence of a cysteine near the C terminus, which is found in most G protein α subunits and members of the RAS superfamily.

Expression of the Fly Arl84F and Human ARL2 Messages. Northern blot analysis of total *Drosophila* RNA with an Arl84F cDNA probe revealed a single hybridizing species at about 0.8 kb (Fig. 3*A*). The expression of Arl84F is not significantly different at the embryonic, larval, pupal, or adult stages (Fig. 3*A*). In addition, this RNA is expressed uniformly throughout embryogenesis, as revealed by *in situ* hybridization of Arl84F cDNA probes to whole-mount preparations of *Drosophila* embryos (data not shown). A more limited number of human poly(A)$^+$ RNA samples, including human brain, HeLa cell, and NCI-H209 (a small-cell lung cancer line) were probed with the human ARL2 probe, and a single band at ≈880 bp was observed with uniform levels of expression in the different cell types (Fig. 3*B*). Thus, both human ARL2 and *Drosophila* Arl84F messages appear to be ubiquitously expressed in human and fly tissues, and expression of Arl84F is not limited to a particular stage of development.

Cytological Mapping of the *Drosophila* Gene. The *Arl84F* gene maps to the right arm of the third chromosome in salivary gland region 84F. Interestingly, one mutation in the 84F region, neurally altered carbohydrate (*nac*), causes the loss (or alteration) of a glycoconjugate specific to neural glycoproteins. Given the role of ARF proteins in the secretory pathway, it is tempting to speculate that *nac* is a mutation in the *Arl84F* gene.

Biochemical Characteristics of ARL2. The human ARL2 protein was produced in and purified from bacteria to allow biochemical characterization. High-affinity binding of guanosine-5′-[γ-(^{35}S)thio]triphosphate to purified recombinant ARL2 was rapid, reaching steady state within 10–15 min at 30°C (data not shown). Guanine nucleotide exchange on ARL2 lacked the requirement for phospholipids previously described (3) for ARF proteins. Optimal conditions for binding included millimolar levels of magnesium and 0.1% Lubrol PX. Under these conditions a stoichiometry of 0.72 mol of guanine nucleotide-binding site per mol of ARL2 protein was achieved. Specific binding was detected in the absence of detergents or phospholipids but was highest and stabilized in 0.1% Lubrol PX. Intermediate levels of binding were observed in 1% sodium cholate or 3 mM dimyristoyl phosphatidylcholine and 0.1% sodium cholate [optimal conditions for nucleotide exchange on ARF proteins (3)]. Binding in low levels of magnesium reached a maximum at very early time points but then rapidly declined. This phenomenon was also observed when binding was performed in the absence of any detergent and likely represents denaturation of the protein under these conditions. Addition of higher levels of magnesium was thought to stabilize the binding of nucleotides and

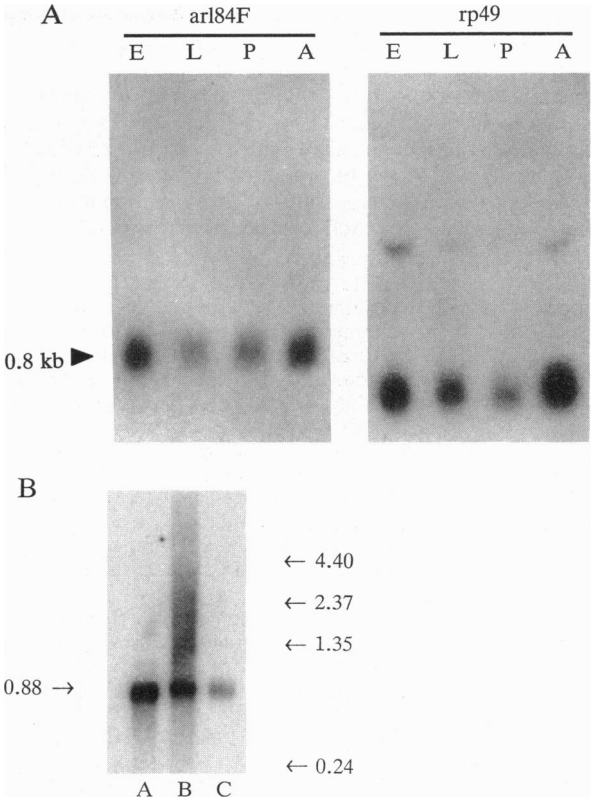

FIG. 3. Both *Arl84F* and *ARL2* are ubiquitously and uniformly expressed in fly and human cells. (*A*) Temporal expression of the *Arl84F* gene during *Drosophila* development. A Northern blot containing equal amounts of total RNA isolated from embryos (lane E), larvae (lane L), pupae (lane P), or adult (lane A) flies was hybridized to a radiolabeled Arl84F cDNA probe. As a control for equivalent loading, the same blot was subsequently hybridized to a radiolabeled *Drosophila* ribosomal protein 49 (rp49; ref. 33) probe. (*B*) Poly(A)$^+$ RNA from human brain (10 μg) (lane A), HeLa cell (6.5 μg) (lane B), or NCI-H209 (2.5 μg) (lane C) was hybridized with a radiolabeled ARL2 coding region probe. The location of DNA size standards in kilobases is shown on the right, and the estimated size of the ARL2 message (0.88 kb) is shown on the left.

protect against denaturation. The intrinsic rate of GTP hydrolysis was also determined under different conditions and found to be quite low. The maximal rate of GTP hydrolysis observed was 0.0074 min^{-1}. This rate is slower than the rate of nucleotide exchange and likely represents the limiting rate of hydrolysis itself. Thus, the guanine nucleotide-binding characteristics of ARL2 are quite distinct from those of ARF proteins, including the rapid attainment of high stoichiometries of nucleotide binding in the absence of phospholipids and presence of millimolar free magnesium.

The ability of ARF proteins to serve as cofactor in the ADP-ribosylation of G_s by cholera toxin is not currently understood within the context of its physiological role in the regulation of vesicle transport. However, this ARF assay remains an important functional discriminator of ARF proteins (10, 17, 34). Recombinant ARL2 was found to be completely lacking in this activity. Indeed, recombinant ARF1 has at least a 10^4-fold higher specific activity in the ARF assay. This finding, more than any other, explains the naming of this new protein and gene as an ARL rather than an ARF.

Techniques such as 5′ and 3′ rapid amplification of cDNA ends and RT-PCR should allow the rapid cloning of the full-length coding regions of the other human ARF family members. The regulated expression of dominant alleles of ARF proteins has recently been shown to cause dramatic

changes in phenotype, which can be used to help define the physiological role(s) for these proteins in cell regulation (R.A.K., unpublished observation). In addition, expression in bacteria of members of the ARF family provides large amounts of purified material for biochemical tests of nucleotide binding, ARF activity, and other protein functions. Thus, the identification of new members of the family may now be followed relatively quickly with functional studies to define unique roles for each member of this expanding family of regulatory proteins.

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