ELMOD2 Is an Arl2 GTPase-activating Protein That Also Acts on Arfs*

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Regulatory GTPases in the Ras superfamily employ a cycle of alternating GTP binding and hydrolysis, controlled by guanine nucleotide exchange factors and GTPase-activating proteins (GAPs), as essential features of their actions in cells. Studies of these GAPs and guanine nucleotide exchange factors have provided important insights into our understanding of GTPase signaling and biology. Within the Ras superfamily, the Arf family is composed of 30 members in mammals, including 22 Arf-like (Arl) proteins. Much less is known about the mechanisms of cell regulation by Arls than by Arfs. We report the purification from bovine testis of an Arl2 GAP and its identity as ELMOD2, a protein with no previously described function. ELMOD2 is one of six human proteins that contain an ELMO domain, and a second member, ELMOD1, was also found to have Arl2 GAP activity. Surprisingly, ELMOD2 also exhibited GAP activity against Arf proteins even though it does not contain the canonical Arf GAP sequence signature. The broader specificity of ELMOD2, as well as the previously described role for ELMO1 and ELMO2 in linking Arf6 and Rac1 signaling, suggests that ELMO family members may play a more general role in integrating signaling pathways controlled by Arls and other GTPases.

The importance of specificity is illustrated by the Arf family, which in mammals consists of 6 Arfs, 22 Arf-like (Arl), and 2 Sar proteins (14). The Arf family is best known for the role of Arfs and Arl1 as regulators of adaptor recruitment and phospholipid metabolism, predominantly at the Golgi, although members of the Arf family are probably active at every membrane in eukaryotes (15). The remaining Arls exhibit a far greater diversity of functions than do the Arfs as they have been implicated as regulators of microtubule-dependent processes (Arl2 and Arl3 (16–20)), lysosome mobility, and microtubule binding (Arl8 (21, 22)), ciliogenesis (Arl3 and Arl6 (23–28)), and tumorogenesis (Arl11 (29, 30)). With increasing interest in these proteins, it becomes more important to identify regulators and effectors that can provide insights into the biological function of each of the Arls and to determine the extent of overlap in their actions and protein partners.

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§ The abbreviations used are: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; Arl, Arl-like; MBP, maltose-binding protein; LC-MS/MS, liquid chromatography-tandem mass spectrometry; DTT, dithiothreitol; CMC, critical micelle concentration; PH, pleckstrin homology.

2 Gaps were initially thought to simply inactivate their cognate GTPase, but more recent research suggests that GAPs can also serve as effectors of the signaling pathways regulated by the GTPase (e.g. ArfGAP1 and ARAP2 (2, 3)). The first GAPs were purified based on their biochemical activities, but sequence and mutational analyses led to the identification of GAP domains, which have proven highly predictive for identifying novel GAPs within a GTPase family. More than 170 putative GAPs have been identified and are grouped according to which GTPase they act upon and which GAP domain they possess (4, 5). Typically a protein with a given GAP domain will be active against members of one family of GTPases, or a subset within that family, but not against other families. For example, a protein with an Arf GAP domain will be active against one or more (but not necessarily all) Arfs but not against Rabs, Rhos, or Ras. Exceptions to these general rules exist (6–8), and more complete information about the determinants of GAP specificity will be required to understand the roles that GAPs play in the biology of the cell. Determining the specificity of a GAP for different GTPases is further complicated by factors that can influence the results of in vitro GAP assays, including the assay used to measure GTP hydrolysis, the need for hydrophobic surfaces on which protein interactions occur, the degree of membrane curvature (e.g. in the case of ArfGAP1 (9–11)), or the presence of co-activators (e.g. phosphatidylinositols (12, 13)).
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GAP Assays

**Charcoal GAP Assay**—Our standard GAP assay was performed by pre-loading recombinant human Arl2 with [γ-32P]GTP in a reaction that contained ~2 μM Arl2 in 25 mM HEPES, pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂, 1 mM dithiothreitol (DTT), and ~0.3 μCi/μl [γ-32P]GTP (6000 Ci/mmol; PerkinElmer Life Sciences) for 15 min at 30°C. Under these conditions, typically ~50% of the radionucleotide will be bound to Arl2 by the end of the preincubation. The amount of bound [γ-32P]GTP was determined by filtration of an aliquot of the pre-loaded Arl2 onto a nitrocellulose filter and scintillation counting, as described below. During this incubation, 25 μl of the GAP sample to be assayed was mixed with 20 μl of reaction buffer (62.5 mM HEPES, 6.25 mM MgCl₂, 2.5 mM DTT, 2.5 mM GTP, and 12.5 mM ATP) on ice. The reaction was started by adding 5 μl of the pre-loaded Arl2 to the 45-μl GAP/buffer combination, mixing, and placing at 30°C. The reaction was stopped 4 min later by the addition of 750 μl of a suspension of ice-cold activated charcoal (5% in 50 mM NaH₂PO₄). The charcoal was pelleted, and the amount of released ³²P, in 400 μl of the supernatant was determined by scintillation counting. A mock load reaction, which was identical to the pre-loading step but did not contain Arl2, was also performed and run in parallel for each sample to be assayed. This control measures the amount of GTP hydrolysis that occurs during the assay that is independent of Arl2-GTP. The control results were then subtracted from those obtained with Arl2-GTP in the reaction to obtain the Arl2-dependent GTP hydrolysis. Because Arl2 cannot hydrolyze GTP to a measurable extent by itself, this value is equal to the Arl2 GAP activity.

**Filter Trapping GAP Assay**—Each GTPase was pre-loaded with [γ-32P]GTP in a reaction that is 1 μM GTPase in 20 mM HEPES, pH 7.4, 100 mM NaCl, 50 μg/ml bovine serum albumin, 1 mM DTT, 0.5 mM MgCl₂, 1 mM EDTA, 5 mM ATP, and ~0.3 μCi/μl [γ-32P]GTP (PerkinElmer Life Sciences, 6000 Ci/mmol) for 15 min at 30°C. The pre-load reaction was then brought to a final concentration of ~20 mM MgCl₂ and placed on ice. The GAP sample (15 μl) to be assayed was mixed with reaction buffer (25 μl; 62.5 mM HEPES, 6.25 mM MgCl₂, 2.5 mM DTT, 2.5 mM GTP, and 12.5 mM ATP) on ice. A control reaction (~GAP) was run in parallel to determine the amount of [γ-32P]GTP or ³²P, released from the GTPase independent of the GAP. The reactions were started by adding 10 μl of the pre-loaded GTPase to the 40-μl GAP/buffer combination, mixing, and placing at 30°C. At each time point, 10 μl was removed from the reaction to 2 ml of ice-cold HNMD (20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT) and immediately filtered over a 0.45-μm Protran BA85 filter (Whatman), which was then washed four times with 2-ml aliquots of HNMD. The amount of GTPase-[γ-32P]GTP still bound to the filters was determined by scintillation counting. The amount of GAP activity is the difference between the counts lost from the
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GTPase in the presence of the GAP sample minus the control (−GAP) sample.

Calculation of Specific Arl2 GAP Activities

Arl2 GAP activities were determined using the charcoal GAP assay, performed in parallel with controls, as described above. To best quantify this assay we made an assumption, based upon prior ligand-binding data, that when Arl2 is exposed to high concentrations of GTP it binds very rapidly to a stoichiometry of ~20% (determined for each preparation of Arl2 but quite consistent). Thus, during the preincubation phase the Arl2 bound about 50% of the [γ-32P]GTP available to it. When the vast excess of unlabeled GTP is added at the start of the GAP assay, the free GTP-binding sites on the remaining Arl2 molecules quickly became occupied with cold GTP. Thus, we used in our calculations the 20% value to estimate the concentration of Arl2-GTP at the start of the assay, typically 1.8 pmol per assay point (2.5 nm). The GAP-dependent 32P counts, released during the 4-min incubation, were divided by the total counts available in each assay, determined by filter trapping at the end of the preincubation phase. This ratio represents the percentage of total Arl2-GTP present in the GAP reaction that was converted into Arl2-GDP during the assay. This was then multiplied by the [Arl2-GTP] to get the amount converted in the 4-min assay. Specific activity was reported in units/mg protein in the assay where 1 unit is the amount of activity required to convert 1 nmol of Arl2-GTP to Arl2-GDP/min.

To calculate the specific activity of a GAP using the filter trapping assay, we again determined the GAP-dependent loss of 32P, trapped on the filter during the assay, as described above. This number was divided by the total bound radionucleotide available at t = 0 to get the percentage hydrolyzed. Because the GTPases other than Arl2 do not bind significant amounts of unlabeled GTP during the GAP assay, because of the presence of high [Mg2+], the amount of GTPase-GTP present in the reaction was determined directly from the specific activity of the radioligand. Note that because of the differences in the ways these two assays are performed, we estimate that the concentration of Arl2-GTP was about 10-fold higher than the GTP-bound form of the other GTPases.

Purification of ELMOD2 from Bovine Testis

Numerous preparations of Arl2 GAP activity were performed, with a number of variations, and a typical example (the one that was used for mass spectroscopy analysis) is described here. 137 g (weight) of bovine testis was homogenized on ice in 500 ml of SH Buffer (25 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM DTT, 320 mM sucrose) with a PT2000 Polytron with a 20-mm TSM probe (Brinkmann Instruments, Westbury, NY) three times on setting 1 for 1 min each with a 1-min pause in between. The homogenate was transferred to a 1-liter centrifuge bottle and spun 1,000 × g for 45 min. The S1 supernatant was transferred by pipetting into 250-ml centrifuge bottles and spun 10,000 × g for 30 min. The S10 was then removed and layered in 26-ml aliquots onto 13 ml of sucrose (in 25 mM HEPES, pH 7.4, 10 mM NaCl) gradients. These gradients consisted of 8 ml of 20% sucrose layered on top of 5 ml of 60% sucrose. The gradients were spun 100,000 × g in an SW28 (Beckman, Fullerton, CA) rotor for 120 min. The interface between the 20 and 60% sucrose layers (“20/60”) was recovered by pipetting and gently vortexed to mix.

CHAPS (1.25 ml of 10% in 50 mM HEPES, pH 7.4) and Buffer A (148.75 ml; 25 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM DTT, 2 mM EDTA, 0.25% CHAPS) were added to 50 ml of 20/60 to bring the final protein concentration to ~2 mg/ml and the final detergent concentration to 0.25%. After a 30-min incubation on ice, the diluted 20/60 was spun at 100,000 × g for 60 min. The supernatant was recovered by pipette and loaded onto a 140-ml (2.6 × 30 cm) Macro-Prep High Q column (Bio-Rad). The column was washed with 140 ml of Buffer A, and the bound proteins were eluted with a gradient of 0–40% Buffer B (25 mM HEPES, pH 7.4, 1 mM NaCl, 1 mM DTT, 2 mM EDTA, 0.25% CHAPS) over 400 ml. Arl2 GAP activity was assayed using the charcoal assay and found to elute between 75 and 250 mM NaCl. The peak fractions were pooled, concentrated using an Amicon Ultra (Millipore, Billerica, MA) 15-ml 30,000-Da nominal molecular weight limit centrifugal concentrator, and applied to a 120-ml (1.6 × 60 cm) HiLoad Superdex 200 prep grade (GE Healthcare) gel filtration column, which was developed with Buffer C (25 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM DTT, 0.25% CHAPS). The peak fractions from the gel filtration column, corresponding to a predicted molecular mass range of 150–300 kDa, were loaded onto a 15-ml (1.6 × 20 cm) Macro-Prep ceramic hydroxyapatite type I column (20 μm; Bio-Rad). The column was washed with 10 ml of Buffer D (25 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM DTT, 5 mM K2HPO4, 5 mM KH2PO4, 0.25% CHAPS) and then 10 ml of 10% Buffer E (25 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM DTT, 0.2 mM K2HPO4, 0.25% CHAPS) before being developed with a 60 ml of 10–70% gradient of Buffer E. Fractions containing activity were pooled, concentrated, and further resolved on the 120-ml Superdex 200 column as above.

Peak fractions from the gel filtration column were pooled and brought to a final concentration of ~1% CHAPS by adding an amount of 10% CHAPS equal to one-ninth of the initial volume of the pooled fractions. After 30 min on ice, this sample was applied to a 6-ml Resource Q column (GE Healthcare). The flow-through was collected and concentrated using an Amicon Ultra 4-ml 10,000-Da nominal molecular weight limit centrifugal concentrator to a final volume of ~0.5 ml. This was then applied to a 24-ml (1.0 × 30 cm) Superdex 200 gel filtration column (GE Healthcare). Fractions (0.35 ml) from this final column were collected and assayed.

Mass Spectroscopy

Proteins in fraction 27 from the final gel filtration column (Superdex 200) were resolved on an 11% SDS-polyacrylamide gel, stained with Coomassie Blue, and the band of interest was excised and trypsinized in the gel slice. The resulting peptides were analyzed by nanoscale reverse phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described previously (39). Briefly, the digested sample was dissolved in 0.4% acetic acid, 0.005% heptfluorobutyric acid, 5% acetonitrile, ~95% water and loaded onto a 75-μm × 12-cm self-packed fused-silica C18 capillary column. The peptides were eluted during a 30-min gradient from 10 to 30% elution.
buffer (0.4% acetic acid, 0.005% heptafluorobutyric acid, 95% acetonitrile, and ~5% water) at a flow rate of ~0.3 μl/min. Eluted peptides were ionized under high voltage (1.8 kV), detected in a survey scan from 400 to 1600 atomic mass units (2 microscans) followed by three data-dependent MS/MS scans (4 microscans each, isolation width 3 atomic mass units, 35% normalized collision energy, dynamic range 1 min) in a completely automated fashion on an LCQ DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Acquired MS/MS spectra were searched against combined reference data bases (mouse, rat, and human) from the National Center for Biotechnology Information, leading to the identification of four peptides that are each present in human and bovine ELMOD2. The peptide matches were further validated by manually examining the spectra.

### Transfection, SDS-PAGE, Immunoblotting, and Northern Blotting

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and transfected with Lipofectamine Plus (Invitrogen), according to the manufacturer’s instructions. SDS-PAGE and immunoblotting were performed essentially as described previously (19). A Northern blot containing human poly(A)+ RNA from brain, colon, heart, kidney, liver, lung, muscle, placenta, small intestine, spleen, stomach, and testis (HB2010, OriGene Technologies, Rockville, MD) was probed with a random hexamer primed 32P-labeled fragment containing the entire ELMOD2 coding sequence. Hybridization was performed with ULTRAhyb (Ambion, Austin, TX) and followed standard procedures, with the highest stringency washing performed at 50°C in 0.1% SDS 0.1× SSC.

### RESULTS

#### Development of a Specific Arl2 GAP Assay

For most GTases, the dissociation of bound GTP or GDP can be slowed substantially by raising the free Mg2+ concentration (40–42). Thus, after preloading the GTase with radiolabeled GDP under optimal conditions, the addition of increased free Mg2+ “locks in” the bound GTP to maximize stability of the substrate in the GAP assay. The GAP reaction is stopped by dilution into cold buffer before trapping the remaining protein-bound [γ-32P]GTP on nitrocellulose filters. Controls run in parallel allow discrimination between intrinsic GTase activity, GAP-stimulated GTase activity, and simple GTase dissociation in the assay. This protocol was used to assess the specificity of our purified Arl2 GAP against other GTases (see below) but cannot be used with Arl2 because the rate of dissociation of guanine nucleotides from Arl2 is insensitive to [Mg2+] (43).

Instead, we developed an Arl2 GAP assay that was based upon those used earlier to assay GTase activity of the α subunits of heterotrimeric G proteins (44) and that uses the ability of activated charcoal to bind nucleotides but not free phosphate. Arl2 was pre-bound to [γ-32P]GTP and exposed to the GAP for a very limited time (typically 4 min), and the reaction was stopped by the addition of a suspension of activated charcoal. GAP-dependent GTP hydrolysis is the amount of liberated 32P, over and above that released in the absence of the GAP or in the absence of the Arl2. Because, like Arfs, Arl2 has no intrinsic GTase activity (43), the Arl2-dependent GTP hydrolysis is also a measure of the Arl2 GAP activity. It is important to clearly differentiate between GTP hydrolysis occurring as a result of GAP activity from hydrolysis resulting from contaminating nucleotidases present in every protein preparation. Thus, each assay contained two sets of parallel, control incubations as follows: one in which no GAP was added (to determine nonspecific GTase activity of the Arl2 preparation), and one for each GAP sample in which the Arl2 was absent (to determine the nonspecific GTase activity of each GAP fraction being assayed). Although contaminating GTase activities were low in our Arl2 preparations, this can be a substantial source of hydrolysis that results in low signal to noise ratios and was why we used the filter-trapping assay for several other GTases (see below).

Rapid dissociation of GTP from Arl2 in the GAP assay remains a problem in that the assay underestimates the GAP activity. Although there was excess unlabeled nucleotide in the assay to minimize the hydrolysis of free [γ-32P]GTP and the time of the assay was kept to a minimum, substantial release or exchange of GTP still occurs. In addition, we used sub-saturating concentrations of nucleotide in the loading reaction. The maximum concentration of the labeled substrate is predicted to be ~2 mM, well below the apparent Kₘ value of previously identified GAPs (45–49). This further lowers the activity measured and compromises the quantification that can be achieved. However, with the exception of assaying fractions during purification, the Arl2 GAP assay is performed under conditions in which activity decreases linearly with dilution of at least 10-fold, and the values reported are highly consistent between preparations and experiments. The close similarities between specific activities of the purified bovine testis and HeLa recombinant Arl2 GAP preparations (see below) is another indication that the methods adopted are valid for comparative purposes. Two other issues that had to be overcome before purification of the Arl2 GAP from tissues could be achieved were problems arising because of the quaternary structure and the low stability of the activity.

#### Arl2 GAP Activity in Testis Extracts Is Present in a Large Complex

Early attempts to measure Arl2 GAP activity in tissue homogenates were unsuccessful, presumably because of its low abundance. After Arl2 and its effector, BART, were partially localized to mitochondria (19), an enriched bovine brain mitochondria preparation was assayed and found to contain Arl2 GAP activity (36). However, the levels of activity proved to be insufficient to allow purification to homogeneity. Further searches indicated that bovine testis homogenates contained >10-fold the activity of bovine brain mitochondria but that the activity was present in a very large particle. During the initial stages of purification from testis, and prior to the addition of any detergent, Arl2 GAP activity was only found in a large complex of undetermined composition. This complex was large enough to be pelleted at 100,000 × g, but activity was inefficiently recovered from the pellet. However, the activity was preserved and could be easily resolved from smaller complexes and soluble proteins, by either gel filtration or centrifugation through discontinuous isopycnic sucrose gra...
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**FIGURE 1. Arl2 GAP activity behaves as a species of decreasing size with increasing concentrations of CHAPS.** The elution profiles of Arl2 GAP activity (squares) and total protein (A280, diamonds) are shown after resolution by Superdex 200 gel filtration chromatography. Relative migration values (Rf) of molecular mass markers were calculated by dividing the volume at which the marker eluted by the total volume of the column and are indicated by arrows. A, activity in 20/60, in the absence of detergent, migrates in the void volume with a predicted molecular mass of >600 kDa. B, peak fractions from the Macro-Prep High Q column from an Arl2 GAP purification were concentrated by ultrafiltration prior to resolution in 0.25% CHAPS. The peak of activity migrated with an apparent molecular mass of ~150 kDa. C, concentration of CHAPS was increased to 1%, and the sample was then resolved by gel filtration chromatography. This is the final step in the purification outlined in Table 1. The apparent molecular mass of the peak fraction is ~55 kDa.

The Arl2 GAP activity collected at the interface of the 20–60% sucrose layers (referred to as 20/60) eluted in the void volume of both a Sephacryl S-300 column and a Superdex 200 column (Fig. 1A), indicating an apparent molecular mass of >1.3 MDa. The activity present in this large complex proved refractory to further purification using a number of conventional chromatography resins, so a means of dissociating the complex with retention of activity was sought. The complex was not disrupted by treatment with DNase, RNase, or phospholipases A2 or C (data not shown) but was dissociated by the addition of CHAPS or sodium cholate. After more detailed studies of the effects of CHAPS on the apparent size, we discovered that with addition of the detergent to levels below the critical micelle concentration (CMC; ~0.6%) the Arl2 GAP activity eluted from gel filtration media with an apparent mass of ~150 kDa (Fig. 1B). This decrease in apparent size was even more pronounced in 1% CHAPS, in which the activity displays an apparent mass of ~55 kDa (Fig. 1C). We interpret these data as evidence that the Arl2 GAP can assemble into larger complexes via interactions that are likely hydrophobic in nature.

We also noted that the Arl2 GAP activity was relatively unstable in cholate above its CMC (Fig. 2A), and so we used CHAPS for the remainder of the purification. CHAPS was found to inhibit the Arl2 GAP assay at concentrations above ~0.1% (Fig. 2B), so during purification in CHAPS each fraction had to be diluted to a point where this inhibitory effect did not interfere with the assay.

During tests of the effects of CHAPS on the Arl2 GAP assay (Fig. 2B), we noticed that the specific activity of the crude GAP preparation (20/60) was actually increased in the presence of low amounts of detergent. To separate the effects of the detergent on our assay from effects on the large GAP complex, we treated the 20/60 with increasing concentrations of CHAPS on ice and then diluted the samples so that the detergent was less than 0.03% in the assay (Fig. 2C). The effects were relatively small, only 2-fold, and could be explained by either an effect of the detergent to increase the stability of a labile Arl2 GAP activity or to dissociate it from the effects of an inhibitory component in the complex. Tests of these two possibilities indicated that perhaps both are true. The stability of Arl2 GAP activity in 20/60 was assayed at different times at 37 °C in the presence of 0, 0.25, or 1% CHAPS (Fig. 2D). The activity became more stable with increasing concentrations of the detergent. Also, treatment of 20/60 with 0.25% CHAPS followed by centrifugation at 100,000 × g resulted in release of 85% of the activity from the complex. Only ~70% of the total protein was released, yielding a 1.2-fold increase in specific activity. More importantly, the exposure to sub-CMC levels of detergent produced activity that was smaller, as judged by gel filtration (see Fig. 1B), and could be further resolved by additional steps of chromatography (see Table 1).

**Purification of Arl2 GAP Activity**—The Arl2 GAP was ultimately purified ~1600-fold from bovine testis homogenates to an estimated 20% purity with an overall yield of 0.08% (Table 1), as described under “Experimental Procedures.” After production of 20/60 and treatment with 0.25% CHAPS to give a 10-fold increase in specific activity over the starting material, clarification by centrifugation at 100,000 × g for 1 h yielded a preparation that was 12-fold enriched over homogenates with close to 100% recovery. Sequential chromatography using ion exchange (Macro-Prep High Q column), hydroxyapatite, and gel filtration media (Superdex 200) was performed in 0.25% CHAPS and resulted in a net 500-fold purification with 4% yield. The yield was low as a result of both taking only peak fractions forward at each step and denaturation of the protein. Elution of Arl2 GAP activity from gel filtration chromatography in the presence of 0.25% CHAPS was consistent with an apparent molecular mass of ~150 kDa (Fig. 1B). At this point in the purification the concentration of the CHAPS was increased to 1%, and the activity no longer bound to anion exchange resins, e.g., it eluted in the flow-through of the Resource Q column. Substantial loss of activity resulted from the increase in [CHAPS] but allowed for...
resolution of the GAP from many proteins that remained able to bind to the ion exchange column. In addition, the increase in CHAPS concentration also resulted in an activity that eluted from the gel filtration column with an apparent molecular mass of 55 kDa (Fig. 1C). Fractions from this final column were analyzed by SDS-PAGE (Fig. 3A). Four major bands (one a doublet) were seen by silver staining, but only one, migrating to a predicted mass of 32 kDa by comparison to standards, peaked in fraction 27 and showed staining intensity that correlated completely with GAP activity across the fractions (Fig. 3A). This band was estimated to be one-fifth of the total protein present in the peak fraction indicating that the specific activity of fully purified bovine Arl2 GAP is 23 units/mg (Table 1) and would require 8,000-fold enrichment to achieve purity.

The region surrounding the 32-kDa band of the gel shown in Fig. 3A was excised and subjected to tryptic digestion and tandem MS/MS analysis for identification, as described under “Experimental Procedures.” Seventeen peptides were identified in this sample, which mapped to four different proteins. Four of the 17 peptides were 100% identical to regions of bovine ELMOD2 Is an Arl2 GAP activity. Each data point in the figure is the average of triplicate measurements, and each panel is a single experiment that is representative of at least two repeats. A, 20/60 preparation was exposed to detergents at different concentrations and for the indicated lengths of time, all at 4 °C. The samples were diluted 20-fold at the indicated times and assayed as described under “Experimental Procedures.” Activities shown are relative to the 20/60 starting material that had not been exposed to detergent. Note the loss of activity in 1% cholate (triangles). B, effect of CHAPS on the Arl2 GAP assay was determined by inclusion of detergent at the indicated concentrations in the assay. Activities of 20/60 shown are relative to that found in the absence of detergent. C, exposure to high concentrations of CHAPS increases Arl2 GAP activity. To determine the effect of CHAPS on the specific activity of the large GAP complex, 20/60 was treated with the indicated concentration of CHAPS for 30 min on ice. The detergent/GAP mixture was then diluted 20-fold before assaying to reduce the level of CHAPS to a concentration that does not interfere with the assay. D, Arl2 GAP activity in 20/60 becomes more thermostable with increasing concentration of CHAPS. Samples of 20/60 were brought to a final CHAPS concentration of either 0 (triangles), 0.25% (squares), or 1.0% (diamonds) and immediately moved to 37 °C for the length of time indicated. The samples were then cooled briefly on ice, diluted 20-fold, and assayed. Results are reported relative to identical samples that had not been placed at 37 °C.

Table 1

Summary of the steps used to purify Arl2 GAP activity from bovine testis

As detailed under “Experimental Procedures,” 137 g of bovine testis tissue was homogenized and clarified by centrifugation at 1,000 × g for 45 min. The supernatant was recovered and spun at 10,000 × g for 30 min to yield the material in the 1st line of the table. All table entries represent pools of peak fractions except for the final one that is a single fraction. 1 unit is the amount of Arl2 GAP activity required to convert 1 nmol of Arl2-GTP into 1 nmol of Arl2-GDP in 1 min.

| Purification step | Total activity | Total protein | Specific activity | Yield | Purification |
|-------------------|----------------|---------------|------------------|-------|--------------|
| 10,000 × g supernatant | 9.74 | 3340 | 0.00292 | 100 | 1 |
| Isopycnic sucrose gradient (20/60 interface) | 4.16 | 401 | 0.0104 | 42.7 | 3.56 |
| 20/60 in 0.25% CHAPS 100,000 × g supernatant | 9.47 | 274 | 0.0345 | 97.2 | 11.8 |
| Macro-Prep High Q peak | 4.22 | 53.9 | 0.0782 | 43.3 | 26.8 |
| Hydroxyapatite peak | 1.38 | 2.98 | 0.464 | 14.2 | 159 |
| Superdex 200 peak | 0.805 | 0.264 | 1.54 | 4.16 | 527 |
| Resource Q flow-through | 0.0852 | 0.0589 | 1.45 | 0.87 | 496 |
| Superdex 200 fraction 27 | 0.00819 | 0.00175 | 4.68 | 4.68 | 1600 |

* Protein concentration was estimated by Coomassie and silver staining.
ELMOD2 (Fig. 3). The bovine and human ELMOD2 proteins are each 293 residues in length with only 17 amino acid differences (94% identity), none of which were within the identified peptides. The open reading frame of the human ortholog was amplified by PCR from a cDNA library and subcloned into a pET28 vector for expression in bacterial (BL21(DE3)) cells. A large amount of recombinant protein was expressed upon induction, but it was all insoluble and pelleted during centrifugation at 100,000 g after lysis of bacteria in a French press. No Arl2 GAP activity was detected in the S100, and a variety of attempts to solubilize the protein from the pellet were unsuccessful. However, expression of the E. coli MBP as an N-terminal fusion with human ELMOD2 resulted in small amounts of soluble protein that could be purified by affinity chromatography. Both the S100 and the purified preparation of MBP-ELMOD2 were active in the Arl2 GAP assay, thus confirming ELMOD2 as an Arl2 GAP. The protein was found to precipitate progressively over a few days when stored at 4 °C or completely upon either one cycle of freeze/thaw or removal of the MBP tag by thrombin cleavage. Furthermore, the specific activity of the bacterially expressed fusion protein was ~1000-fold lower than that of the purified bovine protein, likely the result of aggregation and misfolding. Thus, we conclude that ELMOD2 possesses Arl2 GAP activity but cannot exclude (and indeed think very likely) that other proteins will be found that bind and stabilize the protein and activity in cells.

The open reading frames of the human orthologs of the three other proteins identified by MS analyses were also expressed in bacteria and HeLa cells, but none of the expressed proteins either had any detectable Arl2 GAP activity or increased the half-life or GAP activity of purified recombinant human ELMOD2.

Because another possibility for the low specific activity of the bacterially expressed ELMOD2 was the absence of post-translational modifications, we also expressed C-terminal epitope-tagged (c-Myc/His) human ELMOD2 in cultured human cells and estimated its specific activity. HeLa cells were transiently transfected with pCDNA3.1-ELMOD2, and cell lysates were collected 24 h post-transfection. Lysates were cleared by centrifugation and then assayed for Arl2 GAP activity using the purified Myc-tagged protein (Arf1) of known concentration as a standard. Control HeLa lysates prepared in this way yield Arl2 GAP activities that are close to the lower limit of detection of

![ELMOD2 Is an Arl2 GAP](image_url)
our assay. When ELMOD2-Myc/His is expressed, we found a 10–20-fold increase in activity in the lysates. Combination of results from the two assays yielded an estimate that the recombinant human ELMOD2 in HeLa lysates has a specific activity of \(~24\) nmol of Arl2-GTP hydrolyzed per min/mg protein, very close to that determined for the purified bovine testis Arl2 GAP (23 units/mg, see above), further confirming the identity of the Arl2 GAP as ELMOD2. For comparison, the specific activity of ArfGAP1 is \(~6.7\) (50) or \(10–50\) nmol (51) of Arf1-GTP hydrolyzed per min/mg protein. These results prove that ELMOD2 encodes an Arl2 GAP but leave open the question of whether any post-translational modifications or protein interactions are important modulators of activity.

**ELMOD2 Protein and Message Are Low in Abundance**—Our purification from bovine testis indicated that ELMOD2 makes up \(<0.02\)% of total testis protein. This is similar to our estimate of endogenous levels of the Arl2 GAP in HeLa cells (\(~0.04\)%), although each of these numbers is close to the lower limit of detection of the assay and each is compromised by difficulties in assaying Arl2 GAP activity in lysates. The low level of expression, apparent presence in larger complexes, and liability during purification combine to account for most of the difficulties in its isolation.

We also found extremely low levels of ELMOD2 mRNA message in our probe of a multitissue mRNA Northern blot (OriGene Technologies). This filter contained mRNA from human brain, colon, heart, kidney, liver, lung, muscle, placenta, small intestine, spleen, stomach, and testis and was probed with a hexamer primed probe generated using the entire open reading frame. ELMOD2 was barely detected, and only after \(>72\) h of exposure to a PhosphorImager screen, as a faint band of \(24\) nmol of Arl2-GTP hydrolyzed per min/mg protein. This is similar to our estimate of endogenous levels of the Arl2 GAP in HeLa cells (\(~0.04\)%), although each of these numbers is close to the lower limit of detection of the assay and each is compromised by difficulties in assaying Arl2 GAP activity in lysates. The low level of expression, apparent presence in larger complexes, and liability during purification combine to account for most of the difficulties in its isolation.

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The ELMOD1 that we amplified and subcloned from a cDNA library was based upon the sequence in GenBank™ (entry GI 34192051 dated 17 July 2006) and encoded a protein of 283 amino acids. During the course of our studies this entry was updated to one predicting a protein with an additional 51 amino acids at the N terminus. In the intervening time, we expressed the shorter open reading frame in bacteria and found that truncated human MBP-ELMOD1 possesses Arl2 GAP activity with a specific activity \(~23\)% that of MBP-ELMOD2. This fusion protein was also unstable, like MBP-ELMOD2, but it has been less thoroughly studied at this point. Thus, the protein with the highest sequence identity to ELMOD2 also possesses Arl2 GAP activity.

A plasmid purported to encode full-length human ELMOD3 was purchased from OriGene but was later found to encode a variant (GenBank™ entry GI 85662659). This variant (referred to here as ELMOD3_C) is identical to the current GenBank™ entry for ELMOD3 (GI 34147693) through amino acid 314 but differs at the C terminus and presumably is an alternatively spliced version. The C terminus of this variant exhibits similarity to the ELMOD3 proteins from chimpanzee, macaque, cow, dog, and mouse, whereas the C terminus of the official ELMOD3 GenBank™ entry only exhibits sequence similarity to a chimpanzee ortholog. Therefore, we expressed ELMOD3_C in HeLa cells as a Myc/His-tagged protein and failed to detect any Arl2 GAP activity in cell lysates. These tests for Arl2 GAP activity among the human ELMO domain-containing proteins reveal that only ELMOD1 and ELMOD2, which share 53% identity (Fig. 4C), are active. It is possible that other family members have activity that was not detected as a result of the need for orienting the protein on a membrane surface, because of the presence of the PH domain (57), or possibly interference of the tags used. It is also possible that the...
other family members are active as GAPs for GTPases other than Arl2.

**ELMOD2 Is an Arl2 GAP**

![Diagram](image.png)

**FIGURE 4.** ELMOD2 is one of six human family members containing the ELMO domain. A, all six human ELMO domain-containing proteins are depicted with ELMO domains (block box) aligned. ELM01–3 also contain a PH domain (hatched box) located C-terminal to the ELMO domain. B, amino acid sequence identities between human ELMO domain family members are shown. The percent identity of pairwise combinations is shown above the diagonal line. The number of identical residues and the total number of residues compared between each pair is indicated below the diagonal line. The ELMOD2 has 53% identity to ELMOD1 over 294 amino acids but 20% or lower identity with the remaining family members. C, amino acid sequences of the Arl2 GAP activity containing ELMOD1 and ELMOD2 proteins are shown with conserved residues identified. The ELMO domain region is boxed.

ELMOD2 Also Possesses GAP Activity Against Arf6 but Not Ran, Rac1, or RhoA—To determine the specificity of the GAP activity of ELMOD2 toward GTPases other than Arl2, we examined the activity of recombinant human MBP-ELMOD2 against Arl3, Arf1, Arf6, RhoA, Rac1, and Ran. Because of the nucleotide handling properties of these GTPases and the varying presence of contaminating nucleotidase activity, we used the filter-binding assay described above and under “Experimental Procedures.” Because each GTPase has a different rate of release of bound nucleotide, in each case the relevant parameter is not the absolute rate of loss of radioactivity but the difference in the rate of loss between the sample with and without (buffer alone) the GAP. ArfGAP1 and 20/60 were included as positive controls, as the latter is a crude sample predicted to contain a number of GAP activities. Note that the rate of dissociation of GTP
from Arl2 under these conditions is too rapid to allow it to be assayed in this way.

Arl2 is more closely related to Arl3 than to any other GTPase (20, 58), so it was not surprising to see that ELMOD2 was also active against Arl3 (Fig. 5A). As expected, both MBP-ELMOD2 and 20/60 were active against Arl3, but ArfGAP1 was not. None of the GTPase activities of proteins outside the Arf family (RhoA (Fig. 5D), Rac1 (Fig. 5E), and Ran (Fig. 5F)) were increased upon exposure to MBP-ELMOD2 or ArfGAP1, although each was increased by 20/60. Thus, MBP-ELMOD2 is active as a GAP for Arl2 and Arl3 but not for these GTPases outside the Arf family.

Surprisingly, MBP-ELMOD2 exhibited substantial GAP activity for Arf1 (Fig. 5B) and Arf6 (Fig. 5C), although less for the latter. This is despite the fact that ELMOD2 lacks the consensus Arf GAP domain (32, 59) and clearly provides opportunities for cross-signaling between Arl and Arf proteins, which are thought to have quite distinct functions.

To further test the ability of MBP-ELMOD2 to act as a GAP for Arfs, we assayed Arf1 and Arf6 in the charcoal assay. Despite the varying signal to noise ratios in these assays, the measured specific ELMOD2-dependent GAP activity for the Arfs was very similar in each assay (Fig. 6).

Relative GAP activities of ELMOD2 against all GTPases tested are shown in Fig. 6. These relative activities were obtained using the same concentration of each GTPase and [γ-32P]GTP, the same time points, and the same buffer conditions and were repeated at least three times with similar results. However, because the conditions of the assay were developed and therefore optimized for Arl2, differences in the binding stoichiometries of each GTPase yield different substrate (GTPase-GTP) concentrations at t = 0. Specifically, because of differences in the nucleotide binding properties of Arl2, it is predicted that the concentration of GTP-bound Arl2 in these assays was ~10-fold higher than the GTP-bound form of the other GTPases, making it likely that activity toward these other GTPases is underestimated.

**DISCUSSION**

We report the purification, identification, expression, and initial characterization of ELMOD2 as the first mammalian Arl GAP. ELMOD2 was found to exist in cells as part of a large protein complex, the components of which are likely to con-
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tribute to the stability, activities, and biological function of ELMOD2. Surprisingly, ELMOD2 was also found to be active as an Arf GAP. The presence of an ELMO domain in this and five other human proteins, one of which we showed also possesses Arl2 GAP activity, leads us to speculate that the ELMO domain is a GAP domain for Arl2 and other members of the Arf family, with specificities yet to be determined. The biological functions of the ELMO proteins, their locations, and their ability to act as terminators or effectors of Arf family GTPases are currently unknown but will likely provide new insights into signaling by these GTPases and the potential for cross-talk between Arfs and Arls.

Human ELMOD2 was shown to be an Arl2 GAP by purification of the activity from bovine testis and the demonstration that the purified recombinant protein is active. However, Arl2 GAP activity is most stable when part of a large cellular complex and exhibits a shortened half-life when monomeric. The findings that sub-CMC concentrations of CHAPS allowed partial dissociation of the large complex and increased the thermal stability of Arl2 GAP activity suggest that hydrophobic residues are involved in ELMOD2 interactions. Because lability of the recombinant and purified testis proteins contributed to difficulties in working with each preparation and limited the accuracy of quantification that can be achieved, development of a more stable preparation is a high priority. This will likely require the identification of ELMOD2-binding partners. In addition to providing access to a more stable preparation of Arl2 GAP activity, interaction of ELMOD2 with these binding partners in vitro may result in changes in catalytic rates or specificity among GTPases. Knowledge of the composition of ELMOD2 complexes should also provide additional insights into its cellular locations and biological functions.

The finding that ELMOD2 had GAP activity toward Arf1 was surprising because it lacks the canonical, cysteine-rich, zinc finger Arf GAP signature, CX_CX_{16–18}CX_C (where X is any amino acid) (32), that is present in every previously reported protein with Arf GAP activity (e.g. see Refs. 12, 31, and 33) and found in >20 proteins in the human proteome. ELMOD2 has five cysteines, but their arrangement does not resemble the Arf GAP signature and only one of them is completely conserved among metazoan orthologs. Thus, we predict a novel structure and mechanism of promoting GTP hydrolysis by Arf family members will be found for ELMOD2.

We tested all six human ELMO domain proteins and found that ELMOD1 also had Arl2 GAP activity, whereas the other proteins did not. We cannot exclude the possibility that other ELMO domain proteins possess GAP activity against other members of the Arf family. Indeed, we speculate that the ELMO domain is an Arf family GAP domain that may provide cross-talk between Arf and Arl proteins in cells. A more systematic screening of GAP activities of ELMO domain proteins for Arf family members is planned but must await more stable preparations of ELMOD1 and ELMOD2, as well as identification of the complexes that exist in cells. It will also be important to evaluate potential roles of other molecules (including lipids and phosphoinositides) to serve as cofactors in the GAP assay, as has been shown for some Arf GAPs (60), and the need for a membrane surface to assist in the orientation of reactants, particularly for the ELMO proteins that contain PH domains (57). The more challenging, but also more important, question is the extent to which ELMO domain proteins serve as regulators or effectors of Arl2 or other Arf family GTPases in vivo.

An intriguing hint that ELMOs may act to link Arf and other GTPase signaling pathways is found in the role of Arf6 to stimulate actin reorganization in lamellipodia formation. The Arf guanine nucleotide exchange factor ARNO activates cell migration through activated Arf6 in a Rac1-dependent manner (61). The Rac1 dependence was recently shown to require the colocalization of DOCK180/ELMO1 Rac GEF activity (62). It is not yet known how Arf6 is inactivated along the sides and trailing edge of the forming protrusions, although recruitment of Arf GAPs has been proposed as a possibility (62). But another intriguing possibility is that the Arf GAP activity is already there in the form of an ELMO domain protein. Although ELMOD1 did not have Arf GAP activity in our in vitro assays, it is certainly possible that the activity may be found under different conditions. Thus, the position of ELMO1 between Arf6 and Rac1 signaling may be a prototype for other ELMO domain proteins. Because ELMOD2 was found to be active against Arl3, it is also tempting to speculate that it functions in the regulation of cytokinesis. We recently showed (20) that knockdown of Arl3 by short interfering RNA leads to inhibition of cytokinesis in HeLa cells and ELMOD2 was one of only a few genes discovered in an RNA interference screen in flies for genes required for cytokinesis (63).

Although opportunities for cross-talk between GTPase signaling pathways are evident, it is still most likely that the biological role of ELMOD2 will result from its actions as GAP and potential effector of Arl2 signaling. Arl2 was first found to be involved in tubulin and microtubule dynamics in a yeast genetic screen (17, 64) and later biochemically through its binding to tubulin folding chaperone cofactor D (16). Although monomeric Arl2 binds GTP readily in the absence of a GEF and would therefore be expected to exist in the activated form in cells, the bulk of cellular Arl2 is bound to cofactor D and in this form cannot bind GTP (65). We recently showed that an excess of activated Arl2 causes the loss of microtubules and cell cycle arrest (20), and so cofactor D may act as a sink for Arl2, keeping it locked in the inactive form. It is likely that ELMOD2, as an Arl2 GAP, provides an additional level of control in the critical function of regulating the levels of activated Arl2. Although most cellular Arl2 is in the cytosol, a smaller pool is found in mitochondria where it can bind BART and the adenine nucleotide transporter 1 (19). The function of Arl2 in mitochondria is not clear, but it may be involved in regulating energy metabolism (19). Thus Arl2 and ELMOD2 may act together to sense and transmit information about the general health of the cell between mitochondria and the cell division apparatus. Further studies of the localization of ELMOD2 and the identification of its binding partners and regulators will contribute significantly to our understanding of these pathways.

Finally, the fact that Arl2 is an ancient protein, with orthologs in the earliest eukaryotes, whereas orthologs of ELMOD2 and ELMOD1 are not evident in early eukaryotes, suggests that other Arl2 GAPs may exist in early eukaryotes that perhaps are
also expressed in mammals. Detailed analyses of the key functional residues and structures of the ELMO domain proteins may allow the detection of more divergent proteins, from potentially any eukaryote, that share Arl GAP activities.

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