Phospholipase D: A Downstream Effector of ARF in Granulocytes

Shamshad Cockcroft,* Geraint M. H. Thomas, Amanda Fensome, Blandine Geny, Emer Cunningham, Ivan Gout, Ian Hiles, Nicholas F. Totty, Oanh Truong, J. Justin Hsuan

Activation of the phospholipase D (PLD) pathway is a widespread response when cells are activated by agonists that bind receptors on the cell surface. A 16-kD cytosolic component can reconstitute guanosine triphosphate (GTP)–mediated activation of phospholipase D in HL60 cells depleted of their cytosol by permeabilization. This factor was purified and identified as two small GTP-binding proteins, ARF1 and ARF3. Recombinant ARF1 substituted for purified ARF proteins in the reconstitution assay. These results indicate that phospholipase D is a downstream effector of ARF1 and ARF3. The well-established role of ARF in vesicular traffic would suggest that alterations in lipid content by PLD are an important determinant in vesicular dynamics.

Phospholipase D hydrolyses phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline. PA has effects on several cellular events including secretion, DNA synthesis, and cell proliferation and is formed in response to a number of agonists, neurotransmitters, and growth factors. Alternatively, PA can be metabolized to diacylglycerol, which can activate protein kinase C.

In cell-free assays in granulocytes the activation of PLD by guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) is dependent on the presence of both membranes and cytosol; the GTP-binding protein is a membrane-associated component (*1*). The ability of GTP-γ-S or formyl-methionyl-leucyl-phenylalanine (fMetLeuPhe) to stimulate PLD is greatly impaired in HL60 cells depleted of their cytosol by permeabilization with streptolysin O (*2*, *3*). Cytosol prepared from HL60 cells or rat brain restores GTP-γ-S–dependent PLD activity when added back to these cells (*4*). Thus, cells depleted of cytosol were used as an assay system to purify the reconstituting factor from bovine brain.

The reconstituting activity was precipitated between 40 and 70% saturation of ammonium sulfate. The precipitated proteins were dissolved, dialyzed, and chromatographed on an anion exchange column (DE-52) (Fig. 1A). Fractions were assayed for reconstitution of PLD activity either by measuring release of free choline or the formation of phosphatidylethanol (PEt). Hydrolysis of PC by PLD generates choline and PA, but in the presence of 2% ethanol, PEt is formed rather than PA (*4*, *5*). In our initial experiments both the release of free choline from cells labeled with [^{3}H]choline and production of PEt from cells labeled with [^{3}H]alkyl-lyso-PC were monitored. Both assays gave identical results, so in subsequent studies only release of [^{3}H]-choline was measured. A single peak of reconstituting activity was detected (Fig. 1A). The fractions were also analyzed in an in vitro assay with exogenous PC but no hydrolysis was detected, demonstrating that the presence of the cell membrane was essential.

The active fractions were pooled and sequentially chromatographed on heparin affinity (Fig. 1B) and gel filtration media (Fig. 1C). A single major peak of reconstituting activity was found for both chromatographic stages. The active fractions contained a 20-kD protein identified by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1E). Further chromatography of the pooled fractions on phenyl Superose consistently showed two peaks of activity (Fig. 1D). Analysis by SDS-PAGE showed an apparently homogeneous protein; the amount of this protein was proportional to the amount of reconstituting activity (Fig. 1F). Both peaks of reconstituting activity contained a protein with an apparent molecular size of 20 kD. The proteins were excised from the gel and digested with trypsin and peptide fragments were sequenced. The sequences of the peptides of the early- (peak 1) and late-eluting (peak 2) activities closely match the sequences of ARF1 and ARF3, respectively. ARF1 and ARF3 are GTP-binding proteins that belong to the Ras superfamily (Fig. 2) (*6*, *7*).

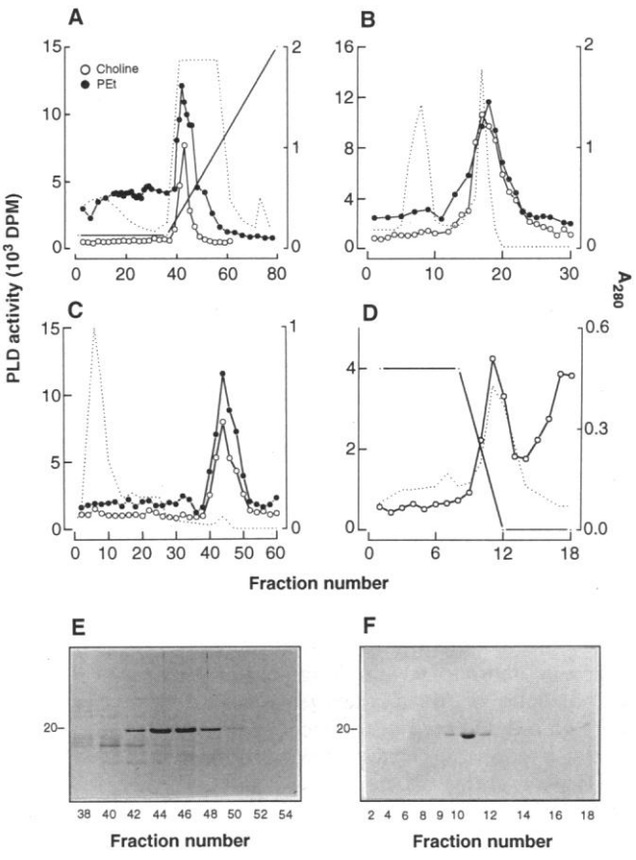

Fig. 1. Purification of the PLD activating factor from bovine brain cytosol. Cytosolic proteins precipitated between 40 and 70% saturation with ammonium sulfate were dissolved, dialyzed, and sequentially chromatographed on (**A**) DE52; (**B**) heparin Sepharose; (**C**) gel filtration (Superdex75, Pharmacia); (**D**) phenyl Superose (*27*). The fractions indicated were analyzed on SDS-PAGE after Superdex-75 (**E**) or phenyl Superose chromatography (**F**). PLD activity was monitored (*27*) either as release of [^{3}H]choline (○) or formation of [^{3}H]PEt (●) (*4*) except that 20 μl of appropriately labeled cytosol-depleted cells were incubated with 20 μl of column fractions and 5 μl of a mixture containing GTP-γ-S and Ca^{2+} (10 μM each, final concentration) for 20 min. Dotted line, optical density at 280 nm; solid line, salt gradient.

S. Cockcroft, G. M. H. Thomas, A. Fensome, B. Geny, E. Cunningham, Department of Physiology, Rockefeller Building, University College London, University Street, London WC1E 6JJ, United Kingdom.
I. Gout, I. Hiles, N. F. Totty, O. Truong, J. J. Hsuan, Ludwig Institute for Cancer Research, University College London School of Medicine, Riding House Street, London W1P 8BT, United Kingdom.

*To whom correspondence should be addressed.

To further confirm that the two ARF proteins were responsible for the GTP-dependent activation of PLD, the mixture of ARF1 and ARF3 obtained after gel filtration was analyzed by cation exchange chromatography (6). Two peaks of reconstituting activity were found and shown to match the elution of the two 20-kD proteins (8). Table 1 summarizes the purification of ARF1 and ARF3 from the bovine brain cytosol with chromatography on a cation exchange column. Yields of ARF3 were variable from preparation to preparation regardless of the final step in the purification. The purification protocol was optimized for speed rather than recovery and thus resulted in low yield.

Reconstitution of activation of PLD was dependent on ARF1 concentration; even at the highest available concentration, saturation was not observed (Fig. 3A). This indicates a stoichiometric rather than a catalytic activation of PLD by ARF1. Cytosol from HL60 cells also contains ARF proteins (8) and HL60 cells express genes for ARF1, 3, 4, 5, and 6 (*10*). We used partial purification of ARF proteins by gel filtration and assay by GTP-γ-S binding (9) to estimate that the highest concentration of ARF1 used in our reconstitution represents no more than 20% of that originally present in HL60 cells. However, it is currently not known whether ARFs 4, 5, and 6 can also activate PLD.

Reconstitution of PLD activity was dependent on the presence of a GTP analog (Fig. 3B). It was most effective with GTP-γ-S but GppNHp (guanosine 5′-[βγ-imido]triphosphate), GppCH$_2$p (guanosine 5′-[βγ-methylene]triphosphate), and GTP also supported activation. GDP or aluminium fluoride was ineffective. The maximal effective concentration of GTP-γ-S was 10 μM (Fig. 3B). The activation of PLD by ARF1 was observed at 100 nM Ca^{2+} but was greatly enhanced at 10 μM Ca^{2+} (Fig. 3, A, C, and D). Activation of PLD was linear at either concentration of Ca^{2+} for at least 1 hour (Fig. 3, C and D). Because the cells were grown in the presence of [^{3}H]-choline for 48 hours, the PC was labeled to equilibrium. It is possible to calculate the amount of PC hydrolyzed because 90% of the label was incorporated into PC (*4*). Seven percent of total PC was hydrolyzed under maximal stimulation.

For comparison with purified bovine ARF1, recombinant ARF1 (rARF1) was expressed and purified (*11*) (Fig. 4, A and B). Samples obtained after gel filtration were pooled and used in the reconstitution assay. Reconstitution was dependent on GTP-γ-S and the amount of rARF1 added (Fig. 4C). Saturation with rARF1 was not observed. The amount of rARF1 required for GTP-dependent activation of PLD was greater than that of the native protein (Figs. 3A and 4C); this discrepancy is most likely attributed to the lack of myristoylation of rARF1. The characteristics of reconstitution with rARF1 were otherwise similar to those observed with purified bovine ARF1 (Fig. 4D).

ARF1 and ARF3 belong to a multi-gene family that has been found in all eukaryotic cells tested including human, bovine, rat, mouse, chicken, yeast, and slime mold (*10*). ARF was originally identified as a cofactor required for efficient adenosine diphosphate (ADP)–ribosylation of the heterotrimeric GTP-binding protein (G protein) G_s by cholera toxin (*12*). Subsequently, it was shown to be a GTP-binding protein with structural similarity to both monomeric and heterotrimeric GTP-binding proteins (*13*). ARF is a subunit of the coat of Golgi-derived coated vesicles (*14, 15*). ARF participates in the process of intracellular vesicular transport and is thought to bind to Golgi membranes, leading to the subsequent binding of the coatamer and vesicle budding (*16*). ARF may also function in endosome-endosome fusion (*17*) and in nuclear vesicle dynamics (*18*). Binding of ARF to the Golgi complex can be regulated by immunoglobulin E receptors or protein kinase C (*19*).

ARF's role in vesicular trafficking may be related to its ability to activate PLD. The hydrolysis of PC by PLD may regulate the fusion or fission process. Indeed the ratio of PI (phosphatidylinositol) to PC may be important in vesicular trafficking, and mutations in a PI transfer protein

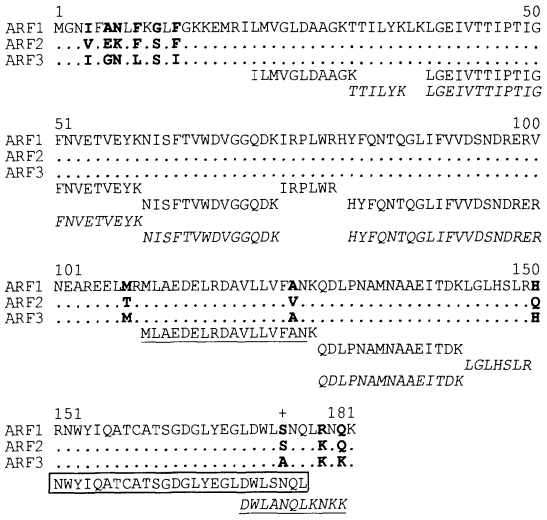

Fig. 2. Identification of the reconstitution factors as ARF1 and ARF3 by sequence analysis. Samples of activity peaks 1 (major protein peak) and 2 (minor protein peak) obtained after phenyl-Superose chromatography were sequenced as described (*28, 29*). Peak 1 peptides are shown in plain and peak 2 peptides in italic type. The single cysteine residue in peak 1 (residue 159) was the acrylamide derivative. Comparison of the initial peptide sequences from peak 1 with the Swissprot and NBRF databases with the FASTA program [Genetics Computer Group (1991), Wisconsin] revealed similarity to the sequences of bovine and human ADP ribosylation factor type I (ARF1), human type III (ARF3), and the translated bovine type II cDNA sequence (ARF2); sequence identities are represented by dots and differences are shown in bold type. The boxed peak 1 peptide sequence contained no phenylthiohydantoin-alanine at cycle 23 (+), thereby identifying peak 1 as ARF1. Seven tryptic peptide sequences derived from peak 2 failed to distinguish between ARF1 and ARF2 or ARF3. Therefore, HPLC fractions derived from an endoproteinase-Asp-NH_2 digest of peak 2 were screened for the COOH-terminal peptide (amino acid residues 171 to 181) by diode array spectroscopy, for a tryptophan residue (W), and by mass analysis. The underlined sequence was obtained showing peak 2 to contain ARF3.

Table 1. Purification of ARF1 and ARF3. Cytosol from a bovine brain (300 g) was fractionated. Proteins precipitated between 40 and 70% saturation with ammonium sulfate (AS ppt) were collected and subjected to sequential chromatography on DE52, heparin Sepharose (Hep Sep.), and gel filtration (GPC) columns. The ARF proteins were then separated by cation exchange chromatography (Mono-S, Pharmacia). The sample obtained after gel filtration was desalted and exchanged into 50 mM malonic acid buffer (pH 5.35) on Sephadex G-25 and loaded onto a Mono-S HR 5/5 column equilibrated in the same buffer. ARFs 1 and 3 were separated on a linear gradient of 0 to 300 mM NaCl. The specific activity refers to the release of labeled choline per minute per milligram of protein in the reconstitution assay.

Fraction	Total protein (mg)	Specific activity (10^3 dpm min^{-1} mg^{-1})	Yield (%)	Purification (fold)
Cytosol	4829	10	100	1
AS ppt	1439	22	65	2.2
DE52	249	28	14	2.7
Hep Sep.	16	57	1.9	5.6
GPC	0.43	1583	1.4	155
Mono-S(I)	0.3	1477	0.9	144
Mono-S(II)	0.03	1960	0.1	192

(SEC-14 mutant) in yeast can be rescued by mutations that decrease synthesis of PC (*20*). PLD can decrease the concentration of PC and therefore influence the PI:PC ratio in a membrane.

The fusion or fission process may also be influenced by PA derived from the hydrolysis of PC by PLD. In many cell types, including HL60 cells and neutrophils, the addition of short chain alcohols such as ethanol (0.5 to 2%) inhibits secretion (*3*). Alcohols at these concentrations interfere with the production of PA by PLD by competing with water and taking part in a transphosphatidylation reaction, thereby producing the corresponding phosphatidylalcohol at the expense of PA (*21*). Thus, by diverting PA to PEt, secretion is inhibited. Ethanol also inhibits the production of coated vesicles and buds in vesicular transport (*22*). The effects of ethanol on vesicular transport may be due to the interference with the production of PA by PLD.

Addition of GTP-γ-S to permeabilized cells activates both heterotrimeric and monomeric GTP-binding proteins. On the other hand, fluoride in the presence of aluminium only activates heterotrimeric G proteins (*23*). Fluoride activates PLD when added to intact HL60 cells and neutrophils (*24*) but is only effective in permeabilized HL60 cells if GTP is also present (*8*). Furthermore, occupancy of the fMetLeuPhe receptor by the agonist activates two pertussis toxin–sensitive G proteins, G_{i2} and G_{i3}, and activation of PLD by fMetLeuPhe is inhibited by pertussis toxin (*25*). These results support the view that ARF activation occurs after the activation of a G protein. A role for G proteins in vesicular transport has recently been suggested (*26*).

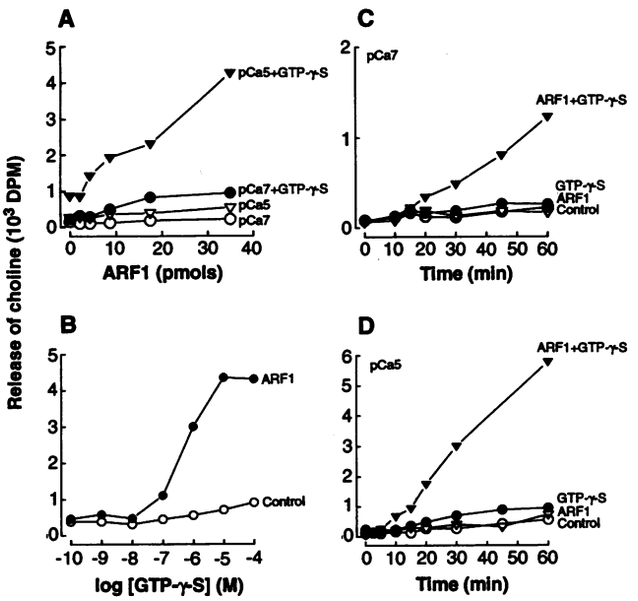

Fig. 3. Reconstitution of GTP-dependent activation of PLD. (**A**) Concentration dependence on purified bovine ARF1 at two Ca^{2+} concentrations. Cytosol-depleted HL60 cells (20 μl) were incubated with the indicated concentrations of ARF1, Ca^{2+} [buffered with 3 mM EGTA at pCa 7 (100 nM) or pCa 5 (10 μM) as indicated] and GTP-γ-S (10 μM) in a final incubation volume of 50 μl for 20 min at 37°C. (**B**) Requirement for GTP-γ-S in activation of PLD with purified bovine ARF1. Conditions were as detailed above except the amount of ARF1 added in this experiment was 17.5 pmol and the Ca^{2+} concentration was 10 μM. The assay was run for 30 min at 37°C. (**C** and **D**) Time course of PLD activation at pCa 7 (C) and pCa 5 (D). ARF1 added was 17.5 pmol and GTP-γ-S concentration was 10 μM.

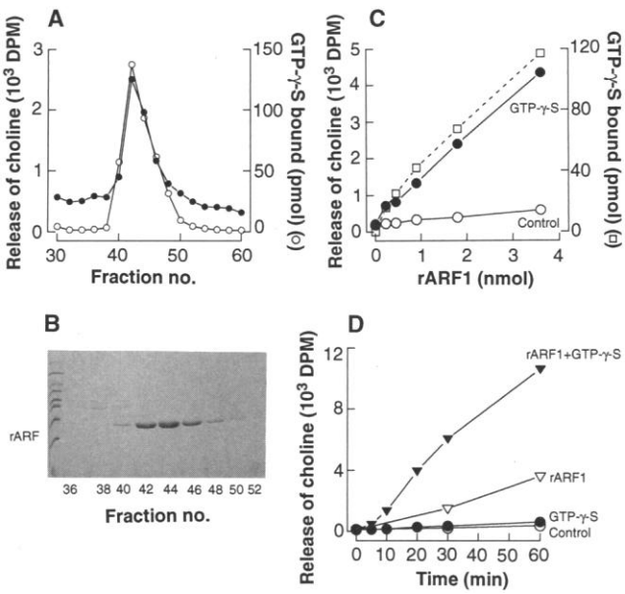

Fig. 4. Purification and assay of recombinant ARF1 (*30*). (**A**) The rARF1 protein was purified from transfected bacteria by gel filtration chromatography. Binding of GTP-γ-S in vitro (○); release of choline from cytosol-depleted HL60 cells in the presence of 10 μM each of GTP-γ-S and of Ca^{2+} (●). (**B**) Analysis of the indicated alternate gel filtration fractions by SDS-PAGE (15% gel). (**C**) GTP-γ-S–dependent reconstitution of PLD activity with rARF1 and binding of GTP-γ-S to rARF1. [^{3}H]choline release, control (○); GTP-γ-S (●); GTP-γ-S binding (□). (**D**) Time course of PLD reconstitution with rARF1. The rARF1 protein (3.6 nmol) was added and the concentrations of GTP-γ-S and Ca^{2+} were each 10 μM.

REFERENCES AND NOTES

1. J. C. Anthes *et al.*, *Biochem. Biophys. Res. Commun.* **175**, 236 (1991); S. C. Olson, E. P. Bowman, J. D. Lambeth, *J. Biol. Chem.* **266**, 17236 (1991); E. P. Bowman, D. J. Uhlinger, J. D. Lambeth, *ibid.* **268**, 21509 (1993).
2. B. Geny and S. Cockcroft, *Biochem. J.* **284**, 531 (1992).
3. J. Stutchfield and S. Cockcroft, *ibid.* **293**, 649 (1993).
4. B. Geny, A. Fensome, S. Cockcroft, *Eur. J. Biochem.* **215**, 389 (1993).
5. J. Pai, M. I. Siegel, R. W. Egan, M. M. Billah, *J. Biol. Chem.* **263**, 12472 (1988).
6. S. Tsai *et al.*, *ibid.*, p. 1768.
7. S. Tsai, R. Adamik, R. S. Haun, J. Moss, M. Vaughan, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9272 (1992).
8. G. M. H. Thomas, A. Fensome, E. Cunningham, S. Cockcroft, unpublished results.
9. R. A. Kahn, *Methods Enzymol.* **34**, 233 (1991).
10. M. Tsuchiya, S. R. Price, S. Tsai, J. Moss, M. Vaughan, *J. Biol. Chem.* **266**, 2772 (1991).
11. P. A. Randazzo, O. Weiss, R. A. Kahn, *Methods Enzymol.* **219**, 362 (1992).
12. R. A. Kahn and A. G. Gilman, *J. Biol. Chem.* **259**, 6228 (1984).
13. ———, *ibid.* **261**, 1 (1986); J. L. Sewell and R. A. Kahn, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4620 (1988).
14. T. Serafini *et al.*, *Cell* **67**, 239 (1991).
15. J. E. Rothman and L. Orci, *Nature* **355**, 409 (1992).
16. J. G. Donaldson, D. Cassel, R. A. Kahn, R. D. Klausner, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6408 (1992).
17. J. M. Lennard, R. A. Kahn, P. D. Stahl, *J. Biol. Chem.* **267**, 13047 (1992).
18. A. L. Boman, T. C. Taylor, P. Melancon, K. L. Wilson, *Nature* **358**, 512 (1992).
19. M. A. De Matteis, G. Santini, R. A. Kahn, G. Di Tullio, A. Luini, *ibid.* **364**, 818 (1993).
20. A. Cleves, T. McGee, V. Bankaitis, *Trends Cell Biol.* **1**, 30 (1991).
21. S. F. Yang, S. Freer, A. A. Benson, *J. Biol. Chem.* **242**, 477 (1967).
22. N. Pfanner *et al.*, *Cell* **59**, 95 (1989).
23. R. A. Kahn, *J. Biol. Chem.* **266**, 15595 (1991).
24. D. English, G. Taylor, J. G. N. Garcia, *Blood* **77**, 2746 (1991).
25. Y. Kanaho, H. Kanoh, Y. Nozawa, *FEBS Lett.* **279**, 249 (1991).
26. W. E. Balch, *Curr. Biol.* **2**, 157 (1992).
27. PLD reconstituting activity co-purified to a certain extent with the phosphatidylinositol transfer protein (PI-TP) up to the gel filtration step. Chromatography was done as described except that the fractions were assayed for PLD reconstitution (*28*). Final separation was achieved by hydrophobic interaction chromatography. The sample was mixed with $(NH_4)_2SO_4$ (340 mM, final concentration) and passed through a phenyl-Superose HR 5/5 column equilibrated in the same buffer at the same salt concentration; PI-TP was retained but ARF passed through. The ARF was applied again to this column after both the column and the sample were made 1 M with $(NH_4)_2SO_4$, and

ARFs 1 and 3 were separated on a gradient of decreasing $(NH_4)_2SO_4$ concentration.

28. G. M. H. Thomas *et al.*, *Cell* **74**, 919 (1993).
29. The nonconserved NH_2-termini of ARF proteins are chemically blocked by acylation. The solid underlined peak 1 peptide sequence was found in two discrete high-performance liquid chromatography (HPLC) fractions. The absence of phenylhydantoin (PTH)-valine at position 16 of this peptide (*) in Fig. 2 identified peak 1 as either the type I or III isoform. Time of flight mass analysis of the two HPLC fractions performed on a Finnigan Lasermat with an α-cyano-4-hydroxycynammic acid matrix gave values of 2047 and 2064 D, corresponding to the type I or III peptide containing unoxidized and oxidized methionine residues, respectively.
30. Sequence corresponding to full-length ARF1 was amplified from a rat brain cDNA library with specific oligonucleotides. Polymerase chain reaction (PCR) primers were designed with Nde I and Bgl II restriction sites to facilitate cloning of the amplified cDNA fragments into the pET3C expression vector. Several of the cloned PCR fragments were sequenced with the T7 Sequensing system (Pharmacia) to prove the identity of the amplified products. BL21 (DE3) cells were transformed with pET3C-ARF construct and expression of recombinant ARF1 was induced with isopropylthio-β-galactoside at a final concentration of 0.4 mM. Two hours after induction, bacterial cells were collected by centrifugation. Purification of the recombinant protein was done as described (*11*) except that gel filtration (Superdex 75) was done in PIPES buffer. Purity was assessed by SDS-PAGE. Samples obtained after gel filtration were concentrated and used directly. GTP-γ-S binding to ARF was assessed as described (*11*).
31. We thank the Wellcome Trust and the Royal Society for their support. A.F. and E.C. were supported by studentships from Yamanouchi and SERC, respectively.

22 November 1993; accepted 14 December 1993

Maspin, a Serpin with Tumor-Suppressing Activity in Human Mammary Epithelial Cells

Zhiqiang Zou, Anthony Anisowicz, Mary J. C. Hendrix,* Ann Thor, Mark Neveu,† Shijie Sheng, Kristina Rafidi, Elisabeth Seftor,* Ruth Sager‡

A gene encoding a protein related to the serpin family of protease inhibitors was identified as a candidate tumor suppressor gene that may play a role in human breast cancer. The gene product, called maspin, is expressed in normal mammary epithelial cells but not in most mammary carcinoma cell lines. Transfection of MDA-MB-435 mammary carcinoma cells with the maspin gene did not alter the cells' growth properties in vitro, but reduced the cells' ability to induce tumors and metastasize in nude mice and to invade through a basement membrane matrix in vitro. Analysis of human breast cancer specimens revealed that loss of maspin expression occurred most frequently in advanced cancers. These results support the hypothesis that maspin functions as a tumor suppressor.

We have used subtractive hybridization (*1–5*) and the "differential display" method (*6, 7*) to identify candidate tumor suppressor genes that are defective in human breast carcinoma cells. These genes, now totaling more than 30, were identified initially by searching for mRNAs whose expression is reduced or absent in tumor cells compared with normal cells grown under similar conditions.

One of the genes we have identified by this approach encodes a member of the serpin family of protease inhibitors, which we have termed "maspin." A single 3.0-kb maspin mRNA is expressed in normal mammary epithelial cell strains (*8*), but not in most mammary tumor cell lines examined (those shown in Fig. 1, as well as MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-468, BT-549, and Hs 578T cells) nor in foreskin- or breast-derived fibroblasts (*9*). Southern (DNA) blot analysis (*9*) of Xba I–restricted DNA from normal and tumor cells with a maspin complementary DNA (cDNA) probe revealed no gross structural alterations of the maspin gene in the tumor cells. This result suggests that the maspin gene is down-regulated but not mutated in cancer cells.

Maspin cDNA was isolated from a library prepared from normal human mammary epithelial (76N) cells. The cDNA sequence contains 2584 nucleotides, including 75 nucleotides of 5′ and 1381 nucleotides of 3′ untranslated sequence. It encodes a protein of 375 amino acids, with an NH_2-terminal methionine and COOH-terminal valine, and eight internal cysteine residues that may form two or more disulfide bonds to stabilize the protein's tertiary structure (Fig. 2A). The initiation codon and surrounding nucleotides fit the Kozak consensus (*10*).

Maspin displays significant sequence similarity to the serpin superfamily of serine protease inhibitors (Fig. 2B), with highest amino acid identity to the equine and human neutrophil-monocyte elastase inhibitors (43% and 39%, respectively), human

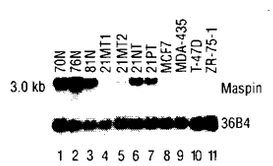

Fig. 1. Northern (RNA) blot analysis of maspin mRNA in normal and tumor cells. Total cellular RNA was isolated from exponentially growing cells cultured in DFCI-1 medium (*8*). Total RNA (20 μg of RNA per lane) was subjected to electrophoresis on a 1% formaldehyde-agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-labeled maspin probe. Lanes labeled 70N, 76N, and 81N contain RNA from normal breast epithelial cells; all other lanes contain RNA from breast tumor cells. 21NT and 21PT are primary tumor lines; 36B4 is a loading control (*5*).

Table 1. Tumorigenicity of maspin-transfected MDA-MB-435 cells. Cells (5×10^5) were resuspended in phosphate-buffered saline and injected into the mammary fat pads of nude mice. Each mouse was injected at two sites. In one experiment, the mice were 8 to 10 weeks old when injected; in the second, they were 4 to 6 weeks old. Tumor development was monitored weekly. Numbers in parentheses are the numbers of tumors at 10 weeks after injection. The number of tumors is smaller at 10 weeks than at 6 weeks because some animals died. CA, carcinoma; ND, not done; NT, no tumor. $P = 0.034$ for mice T1 through T6; $P = 0.00057$ for mice T4 through T6 (Student's one-sided t test).

Cells	Tumors/sites injected at 6 weeks	Mean tumor weight at 10 weeks (g)	Metastases	
			Lung	Lymph nodes
pCMVneo N1	8/10	0.74 (7)	CA	CA
pCMVneo N2	10/10	1.77 (6)	CA	CA
pCMVmaspin T1	8/10	1.67 (4)	ND	ND
pCMVmaspin T4	6/10	0.31 (7)	NT	NT
pCMVmaspin T5	5/10	0.35 (7)	NT	NT
pCMVmaspin T6	8/10	0.43 (9)	NT	NT

Z. Zou, A. Anisowicz, M. Neveu, S. Sheng, K. Rafidi, R. Sager, Division of Cancer Genetics, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.
M. J. C. Hendrix and E. Seftor, University of Arizona Cancer Center, Tucson, AZ 85724.
A. Thor, Department of Pathology, Massachusetts General Hospital, 100 Blossom Street, Boston, MA 02114.

*Present address: Pediatric Research Institute, 3662 Park Avenue, St. Louis, MO 63110.
†Present address: Pfizer Central Research Building, Groton, CT 06340.
‡To whom correspondence should be addressed.