A simple and highly sensitive radioenzymatic assay for lysophosphatidic acid quantification

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Abstract **The objective of the present work was to develop a simple and sensitive radioenzymatic assay to quantify lysophosphatidic acid (LPA). For that, a recombinant rat LPA acid acyltransferase (LPAAT) produced in *Escherichia coli* was used. In the presence of [^{14}C]oleoyl-CoA, LPAAT selectively catalyzes the transformation of LPA and alkyl-LPA into [^{14}C]phosphatidic acid. Acylation of LPA was complete and linear from 0 to 200 pmol with a minimal detection of 0.2 pmol. This method was used to quantify LPA in butanol-extracted lipids from bovine sera, as well as from human and mouse plasma. This radioenzymatic assay represents a new, simple, and highly sensitive method to quantify LPA in various biological fluids.**—Saulnier-Blache, J. S., A. Girard, M-F. Simon, M. Lafontan, and P. Valet. **A simple and highly sensitive radioenzymatic assay for lysophosphatidic acid quantification.** ***J. Lipid Res.*** **2000.** 41: **1947–1951.**

Supplementary key words radioenzymatic detection • lysophosphatidic acid acyltransferase • phosphatidic acid • serum • plasma • [^{14}C]oleoyl-CoA • butanol • recombinant protein • *Escherichia coli* • bioactive phospholipid • thin-layer chromatography

Lysophosphatidic acid (LPA; 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate) is a key intermediate in glycerolipid synthesis, and a bioactive phospholipid (1, 2). It controls a wide variety of cellular responses (mitogenesis, cytoskeletal rearrangements, cell adhesion, ion transport, and apoptosis) through the activation of specific G protein-coupled receptors (3, 4).

LPA has been detected in various biological fluids including blood (5–9), aqueous humors (10), and adipose tissue microdialysates (11). Abnormally high concentrations of LPA have been found in ascites and plasma from patients with ovarian cancer (12, 13). Several cell types have been shown to produce LPA in vitro: platelets (14, 15), adipocytes (11), and ovarian cancer cells (16). LPA has also been shown to be produced from oxidized low density lipoprotein (LDL) (17). Several possible metabolic pathways could be involved in cellular production of LPA: phospholipase A_2-dependent deacylation of phosphatidic acid (PA) (18), lysophospholipase D-dependent hydrolysis of lysophosphatidylcholine (19, 20), acyltransferase-dependent acylation of monoacylglycerol phosphate (21), or acyldihydroxyacetone phosphate reductase-dependent reduction of acyldihydroxyacetone phosphate (22).

To understand the precise role of LPA in the development of particular pathologies such as cancer or obesity, and to clarify the metabolic origin of LPA produced by cells, precise and sensitive detection of LPA is necessary. In the present work, a recombinant LPA acyltransferase (LPAAT) was used to develop a simple and highly sensitive radioenzymatic assay to quantify LPA in various biological fluids.

MATERIALS AND METHODS

Preparation of recombinant LPAAT

The rat 1-acyl-*sn*-glycerol-3-phosphate acyltransferase cDNA encoding an enzyme behaving as an LPAAT was previously cloned in bacterial expression vector pTrcHis (23). Fifty microliters of DH5α-competent cell suspension (Life Technologies, Gaithersburg, MD) was transformed with 0.5 μg of pTrcHis-AGPAT plasmid (generous gift from K. Kume, Department of Biochemistry and Molecular Biology, University of Tokyo, Japan) and grown in Luria broth (LB) medium containing ampicillin (50 μg/ml). When the A_{600} reached approximately 0.6, bacteria were cultured for 3 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. Bacteria were collected by centrifugation at 3,000 *g* for 10 min, and the pellet was disrupted by sonication in 0.2 M Tris-HCl (pH 7.4). After centrifugation at 10,000 *g* for 20 min, the supernatant was recovered and further centrifuged at 100,000 *g* (microsomes) for 90 min. The yield of a typical preparation is 6 to 7 mg of microsomal protein per liter of culture. The resulting pellet was resuspended in the same buffer to a final protein concentration of 1 μg/μl and was used for radioenzymatic detection of LPA. Kinetic analysis showed that the activity of transformation of LPA into

Abbreviations: AGPAT, acylglycerol phosphate acyltransferase; GC, gas chromatography; LB, Luria broth; LDL, low density lipoprotein; LPA, lysophosphatidic acid, 1-oleoyl-*sn*-glycero-3-phosphate; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAF, platelet-activating factor, 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol-3-phosphocholine; PBS, phosphate-buffered saline; RAS, specific radioactivity.

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PA of a typical microsome preparation varied from 0.8 to 1 nmol/min per mg of protein.

Preparation of acyl- and alkyl-LPA stock solutions

Na^+ salt 1-oleoyl-*sn*-glycero-3-phosphate (LPA) (from Sigma, St. Louis, MO) was solubilized in phosphate-buffered saline (PBS) containing 1% lipid-free albumin (from ICN, Costa Mesa, CA). 1-*O*-Hexadecyl-*sn*-glycero-3-phosphate (alkyl-LPA) was obtained from 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol-3-phosphocholine (platelet-activating factor; PAF) (from Sigma). PAF was dispersed by sonication in a 50 mM Tris buffer (pH 7.4) containing 5 mM Ca^{2+}, and incubated first with pig pancreas phospholipase A_2 (from Sigma) for 2 h followed by a 30-min treatment with *Streptomyces chromofuscus* phospholipase D (from Sigma). Lipids were extracted by the Bligh and Dyer procedure (24) under acid conditions. After evaporation alkyl-LPA was solubilized in ethanol. The real concentration of the stock solutions of LPA and alkyl-LPA was determined by phosphorus measurement (25).

Lipid extraction from serum and plasma

1-Oleoyl-LPA or lipids contained in serum or plasma were extracted with 1 volume of 1-butanol. After vigorous shaking and centrifugation (5 min at 3,000 *g*), the upper butanol phase was collected and evaporated under nitrogen at 50°C. This procedure allowed 85% to 90% extraction of LPA.

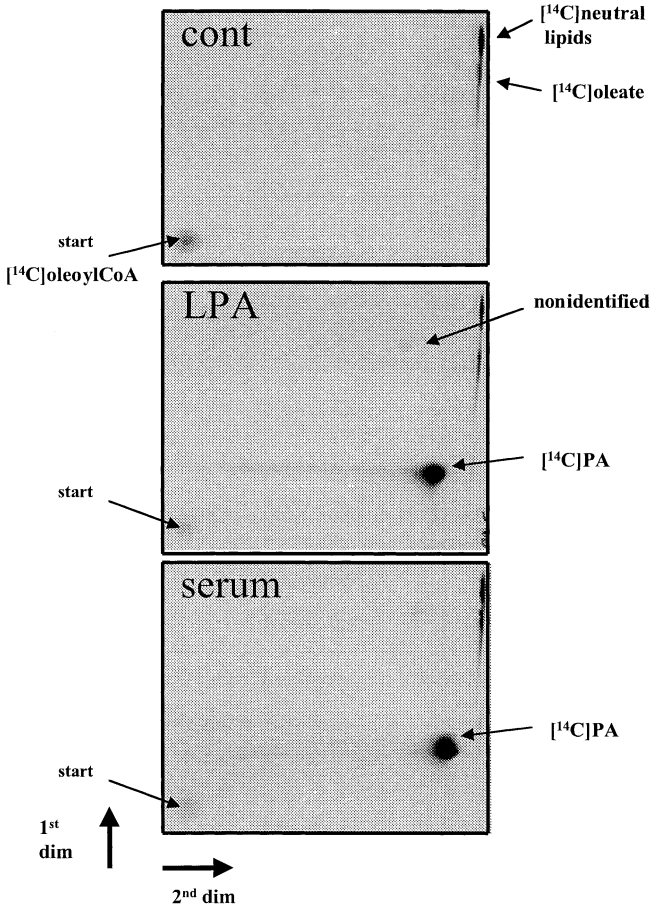

Fig. 1. LPAAT-dependent acylation of LPA to form [^{14}C]PA. Lipids were butanol extracted from 2 ml of PBS containing either 200 pmol of 1-oleoyl-LPA (LPA), 300 μl of fetal calf serum (serum), or nothing (cont). After evaporation, lipids were incubated in the presence of semipurified LPAAT and [^{14}C]oleoyl-CoA as described in Materials and Methods. The products of the reaction were separated by two-dimensional TLC and autoradiographed as described in Materials and Methods.

In vitro acylation of LPA

Dry lipid extract was resuspended in 200 μl of reaction medium (1 μl of [^{14}C]oleoyl-CoA [specific radioactivity (RAS), 55 mCi/mmol; New England Nuclear, Boston, MA], 20 μl of 200 mM Tris (pH 7.5), 10 μl of recombinant LPAAT, 8 μl of 500 μM sodium orthovanadate, and 161 μl of H_2O containing Tween 20 at 1 mg/ml) and incubated for 120 min at 20°C. The mixture was vortexed every 15 min. The reaction was stopped by addition of 400 μl of $CHCl_3$–methanol–12 M HCl 40:40:0.26 followed by vigorous shaking and a 10-min centrifugation at 3,000 *g*. The lower $CHCl_3$ phase was evaporated under nitrogen, resuspended in 20 μl of $CHCl_3$–methanol 1:1, and spotted on a silica gel 60 thin-layer chromatography (TLC) glass plate (Merck, Rahway, NJ), and then separated with $CHCl_3$–methanol–NH_4OH–H_2O 65:25:0.9:3 as a solvent. Alternatively, a two-dimensional TLC using $CHCl_3$–methanol–NH_4OH–H_2O 65:25:0.9:3 as the solvent of the first migration, and $CHCl_3$–methanol–acetic acid–H_2O 40:20:5:0.5 as the solvent of the second migration, was performed. The plate was autoradiographed overnight to localize ^{14}C-labeled lipids. Identification of radioactive spots was performed by comigration with cold lipids visualized under iodine vapors. [^{14}C]PA spots were then scraped and counted with 3 ml of scintillation cocktail. The following formula was used to convert radioactivity to picomoles: dpm = 1/(2.22 × RAS) pmol, RAS being the specific radioactivity of [^{14}C]oleoyl-CoA.

Origin of bovine sera

Fetal calf sera were from BioWhittaker Europe (Verviers, Belgium; lot 6SB0011), GIBCO Life Technologies (Rockville, MD; lot 4001585K), and Boehringer Mannheim France (Meylan, France; lot 210463). Donor calf sera were from BioWhittaker Europe (lot 6M1955) and GIBCO Life Technologies (lot 3016082D).

Blood collection

Human plasma was collected in EDTA with the consent of the subjects and was approved by the Ethics Committee of the hospital. Mouse plasma was collected from FVB mice after carotid sec-

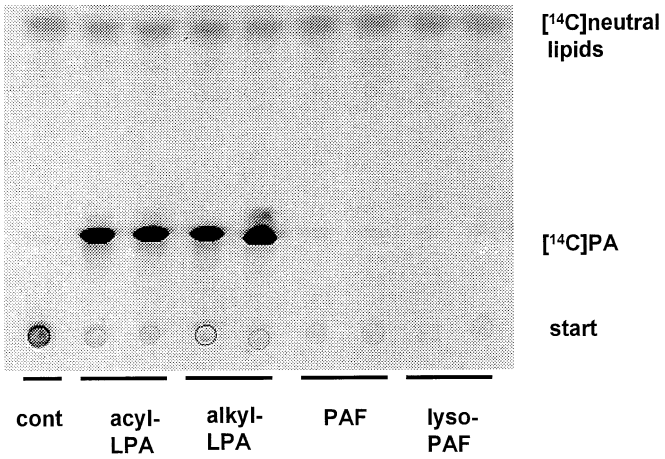

Fig. 2. LPAAT-dependent acylation of alkyl-LPA to form [^{14}C]PA. Alkyl-LPA was synthesized from PAF as described in Materials and Methods. Two hundred picomoles of acyl-LPA, alkyl-LPA, PAF, and lyso-PAF was incubated in the presence of semipurified LPAAT and [^{14}C]oleoyl-CoA as described in Materials and Methods. The products of the reaction were separated by one-dimensional TLC and autoradiographed as described in Materials and Methods.

tion. Collection was performed in tubes containing 50 μl of a 2% solution of EDTA, disodium salt. The tubes were centrifuged for 5 min at 1,500 *g* and LPA was measured in the platelet-poor plasma. Animals were handled in accordance with the principles and guidelines established by the National Institutes of Medical Research (INSERM).

RESULTS

The ability of LPAAT to acylate LPA to form PA was tested after semipurification of the enzyme from rat LPAAT cDNA-transformed *Escherichia coli*. For that, 1-oleoyl-LPA or butanol-extracted lipids from serum [known to contain a significant amount of LPA (6–9)] were incubated in the presence of LPAAT-transformed *E. coli* microsomes and [^{14}C]oleoyl-CoA. To identify the products of the reaction, they were separated by two-dimensional TLC (**Fig. 1**).

In the absence of LPA (Fig. 1, cont), no [^{14}C]PA was detected. A radioactive spot was found at the depot (Fig. 1, start). It corresponded to traces of [^{14}C]oleoyl-CoA extracted with 1-butanol. Two additional spots (Fig. 1, upper right corner) were also observed. The lower spot comigrating with cold oleic acid (not shown) was identified as [^{14}C]oleate. Because [^{14}C]oleate was also observed in the absence of microsomes (not shown), it resulted from a partial degradation of [^{14}C]oleoyl-CoA. The upper spot comigrated with diacylglycerol and triacylglycerol and was therefore identified as ^{14}C-labeled neutral lipids likely resulting from the presence of monoacyl- and/or diacylglycerol acyltransferases endogenously expressed in *E. coli* and copurified with LPAAT in microsomes.

In the presence of LPA (standard or from serum) a major spot comigrating with cold PA was observed. [^{14}C]PA was interpreted as the result of the transfer of [^{14}C]oleate into LPA. This confirmed the presence of LPAAT in *E. coli* microsomes. A minor but unidentified spot was also ob-

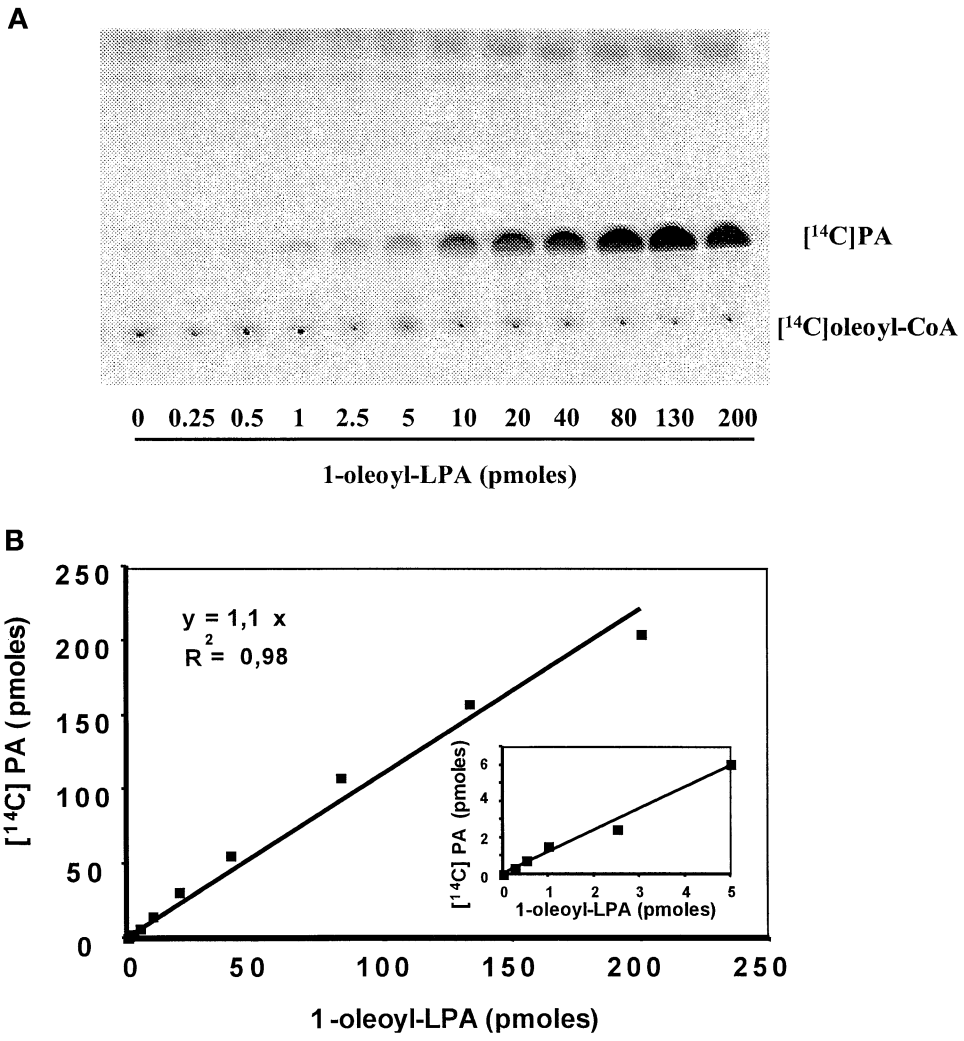

Fig. 3. Sensitivity and linearity of LPAAT-dependent acylation of LPA to form [^{14}C]PA. Butanol-extracted 1-oleoyl-LPA (0 to 200 pmol) was evaporated and incubated in the presence of semipurified LPAAT and [^{14}C]oleoyl-CoA as described in Materials and Methods. The products of the reaction were separated by one-dimensional TLC and autoradiographed (A) as described in Materials and Methods. Each spot of [^{14}C]PA was scraped and counted, and the radioactivity was converted into picomoles (B). The inset in (B) represents the lowest amounts of LPA.

TABLE 1. Quantification of LPA in different bovine sera

Calf Serum	Company	Concentration of LPA
		μM
Fetal	BioWhittaker	0.65 ± 0.21
	GIBCO	0.28 ± 0.04
	Boehringer Mannheim	0.71 ± 0.07
Donor	BioWhittaker	2.56 ± 0.43
	GIBCO	1.31 ± 0.11

Lipids present in 25 μl of fetal or donor calf serum were extracted with butanol, evaporated, and incubated in the presence of semipurified LPAAT and [14C]oleoyl-CoA as described in Materials and Methods. The products of the reaction were separated by one-dimensional TLC and autoradiographed as described in Materials and Methods. Each spot of [14C]PA was scraped and counted. The radioactivity was converted into picomoles and the concentration of LPA was calculated. Values correspond to means ± SEM of three separate determinations.

served. Because it represented less than 2% of [^{14}C]PA this minor spot was further neglected.

Whereas the vast majority (about 90%) of LPA species found in biological fluids and tissues are acyl-LPA, a small proportion (about 10%) are alkyl-LPA (26). LPAAT-dependent acylation of alkyl-LPA was tested. Alkyl-LPA was synthesized from PAF (see Materials and Methods) and exposed to semipurified LPAAT and [^{14}C]oleoyl-CoA. As shown in **Fig. 2**, both acyl-LPA or alkyl-LPA led to the formation of [^{14}C]PA. No [^{14}C]PA was formed from PAF or lyso-PAF.

The sensitivity of the radioenzymatic assay was then tested. For that, increased quantities of 1-oleoyl-LPA were incubated in the presence of semipurified LPAAT and [^{14}C]oleoyl-CoA. This led to the formation of an increased amount of [^{14}C]PA (**Fig. 3A**). Conversion of the radioactivity into picomoles revealed that LPA acylation into PA was complete and linear (slope close to 1) from 0 to 200 pmol, with a detection set point of 0.2 pmol (Fig. 3B).

The radioenzymatic assay was finally used to quantify LPA in bovine sera of various origins. As shown in **Table 1**, the concentration of LPA measured in 20 μl of serum varied from 0.28 to 2.56 μM. The concentration of LPA in donor calf sera was significantly higher (2.4- to 4.7-fold) than the mean concentration (0.54 μM) of LPA found in fetal calf sera. Although present at much lower concentrations than in serum, significant concentrations of LPA (from 51 to 283 nM) were also found in human and mouse platelet-poor plasma (**Table 2**).

TABLE 2. Quantification of LPA in human and mouse plasma

	Concentration of LPA	n
	nM	
Human	83 ± 8 (51–136)	14
Mouse	170 ± 50 (80–283)	12

Lipids present in 200 μl of human or mouse platelet-poor plasma were extracted with butanol, evaporated, and incubated in the presence of semipurified LPAAT and [14C]oleoyl-CoA as described in Materials and Methods. The products of the reaction were separated by one-dimensional TLC and autoradiographed as described in Materials and Methods. Each spot of [14C]PA was scraped and counted. The radioactivity was converted into picomoles and the concentration of LPA was calculated. Values correspond to means ± SEM of n separate determinations. Extreme values are reported in parentheses.

DISCUSSION

Several procedures have previously been used to detect LPA. Gas chromatography (GC) allows quantification of methylated fatty acids derived from TLC-purified LPA (15). This is an indirect assay allowing determination of the relative fatty acid composition of LPA. However, this method is not sensitive and requires relatively high amounts of purified LPA. In addition, classic GC does not allow determination of the presence of alkyl-LPA. For that it is necessary to employ both mild alkaline hydrolysis and GC coupled to mass spectrometry (26).

The presence of LPA in a biological fluid can also be demonstrated by using bioassays. This type of assay consists of measuring the influence of an LPA-containing fluid on a cellular response known to be controlled by LPA, such as platelet aggregation (17), calcium mobilization in *Xenopus* oocytes (10), or cell spreading (11). Because bioactive phospholipids other than LPA are also able to generate those biological responses, bioassays cannot be considered specific enough for LPA quantification. LPA can also be detected after labeling cellular phospholipid with [^{32}P]orthophosphate, and separation of [^{32}P]LPA by TLC (7, 11, 18). Although sensitive, this method is applicable only to cultured cells, and is not quantitative.

LPAAT, also known as 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (EC 2.3.1.51), catalyzes LPA conversion into PA, a key step in glycerolipid synthesis. LPAAT has been demonstrated to be highly specific for LPA and not influenced by LPA fatty acid composition (27). Therefore the high specificity of LPAAT made this enzyme an interesting partner in the development of a new assay for LPA quantification.

By using a rat-recombinant LPAAT produced in *E. coli* (23) we have developed a simple, rapid, and highly sensitive radioenzymatic assay to quantify LPA in various biological fluids. By using this assay it is possible to quantify LPA in crude butanol-extracted lipids without prior time-consuming and poorly efficient TLC purification of LPA. It is also important that this assay allows quantification of both acyl- and alkyl-LPA. Although present at a much lower concentration than acyl-LPA, alkyl-LPA has been reported to be much more active than acyl-LPA in platelet aggregation (28). Although our assay does not discriminate between acyl-LPA and alkyl-LPA, the amount of alkyl-LPA present in a biological fluid can nevertheless be determined. For that the biological fluid can be treated with a lysophospholipase, such as phospholipase B, known to specifically hydrolyze acyl-LPA without altering alkyl-LPA. Phospholipase B-resistant LPA will correspond to alkyl-LPA.

Our radioenzymatic assay is also highly sensitive. It is possible to detect less than 0.5 pmol of LPA. This high sensitivity allows determination of LPA concentration from

low volumes of biological samples. It was, for example, possible to quantify LPA in 200 μl of plasma or 25 μl of serum. Those volumes are entirely compatible with current blood sampling in both human and animals. This high sensitivity allowed us to easily quantify as low a concentration as 51 nM LPA in human and mouse plasma. It is important to notice that, by using GC, Xu et al. considered that the lowest detectable concentration of LPA in human plasma was 100 nM (5).

Finally, the method is reliable because the concentration values determined in bovine sera are in agreement with those previously published (6–9). However, it is noticeable that donor calf sera contain a higher concentration of LPA than fetal calf sera. Although the precise reasons of this difference remain to be determined, this information needs to be taken into consideration when choosing cell culture serum.

In conclusion, this radioenzymatic assay represents a new and highly sensitive method for LPA quantification. Because of its simplicity and rapidity of execution, this method could easily be introduced in any research or hospital laboratory interested in fundamental or pathological issues concerning LPA.

We thank Dr. Kazuhiko Kume (University of Tokyo) for having generously provided us with pTrcHis-AGPAT vector. We also thank David Sibrac for skillful technical assistance and Stéphane Gesta for fruitful discussion. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (APEX #4X405D), the Association pour la Recherche sur le Cancer (#5381), the Institut de Recherche Servier, and the Laboratoires Clarins.

Manuscript received 12 May 2000 and in revised form 19 July 2000.

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