Rat platelets secrete two types of phospholipases upon stimulation; one is type II phospholipase A₂ and the other is serine-phospholipid-selective phospholipase A. In the current study we purified serine-phospholipid-selective phospholipase A and cloned its cDNA. The final preparation, purified from extracellular medium of activated rat platelets, gave a 55-kDa protein band on SDS-polyacrylamide gel electrophoresis. To identify the enzyme, labeled the 55-kDa protein, suggesting that this polypeptide possesses active serine residues. The cDNA for the enzyme was cloned from a rat megakaryocyte cDNA library. The predicted 456-amino acid sequence contains a putative short N-terminal signal sequence and aspartic acid, which may form a "lipase triad," were highly conserved among these enzymes. The recombinant protein, which we expressed in Sf9 insect cells using the baculovirus system, hydrolyzed a fatty acyl residue at the sn-1 position of lysophosphatidylserine and phosphatidylserine, but not appreciably hydrolyze phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, and triglyceride. The present enzyme, named phosphatidylserine-phospholipase A₁, is the first phospholipase that exclusively hydrolyses the sn-1 position and has a strict head group specificity for the substrate.

Membrane phospholipids of activated platelets serve as precursors for blood coagulation. Platelets contain at least three types of phospholipase A; cytosolic phospholipase A₁ (cPLA₁) (1, 2), secretory 14-kDa type II phospholipase A₂ (sPLA₂) (3), and another serine-phospholipid-selective phospholipase which has not yet been fully characterized (4–6). cPLA₂ is involved in the production of eicosanoids by cleaving arachidonic acid at the sn-2 position of phospholipids in various types of cells. sPLA₂ is found in inflammatory sites (7, 8) and is considered to be involved in inflammatory response progression and may participate in eicosanoid formation in certain cells, such as vascular endothelial cells, mast cells, neutrophils, hepatocytes, and others (9–13). Thus sPLA₁ and cPLA₂ are well characterized, and their cDNA and genomic structures are also known (2, 14, 15), whereas no information about the structure of the last enzyme is available.

Like sPLA₂, this new member of the phospholipase A family is secreted from activated rat platelets (4); partially purified preparations specifically act on phosphatidylserine (PS) (6) and lysophosphatidylserine (lyso-PS) (4, 5). This activity is inhibited both by diisopropyl fluorophosphate (DFP) and dithiothreitol to release a fatty acid of PS and lyso-PS, respectively (5, 6). On several chromatography columns, PS-hydrolyzing activity co-migrates with lysophospholipase activity detected using lyso-PS as the substrate, suggesting that a single polypeptide may possess both PS-phospholipase A₁ and lyso-PS-lysophospholipase activities. Therefore, throughout the present study we call this enzyme "PS-PLA₁.”

In this study, we report the purification and cDNA cloning of PS-PLA₁. The predicted amino acid sequence did not show any homology to those of phospholipases that have been reported previously, but surprisingly it showed some homology to mammalian lipases such as hepatic lipase (HL), lipoprotein lipase (LPL), and pancreatic lipase (PL). Unlike these "conventional" lipases, the present platelet-derived enzyme did not exhibit appreciable activity to triacylglyceride (TG). Platelets release a novel phospholipase A₁ upon cell stimulation, that specifically acts on serine-phospholipids and not on any other lipids.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-[¹³C] arachidonoyl-sn-glycero-3-phospho-ethanolamine, 1-palmitoyl-2-[¹³C] arachidonoyl-sn-glycero-3-phospho-ethanolaminositol, 1-palmitoyl-2-[¹³C] arachidonoyl-sn-glycero-3-phosphocholine, 1,2-di-[¹³C] oleoyl-sn-glycerol-3-phosphocholine, tri[¹³C] oleoyl-glycerol, and 1,3-[³²P] Hdiisopropyl fluorophosphate were from Dupont NEN. 1,2-Di[¹³C] oleoyl-sn-glycero-3-phosphoserine and 1-[¹³C] oleoyl-sn-glycerol-3-phosphoserine were prepared as described previously (5, 6). (Superior) was the product of Amersham International (Amersham, UK). PS, phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid, phosphatidylinositol, 1-acyl-sn-glycerol-3-phosphocholine, 1-acyl-sn-glycerol-3-phospho-inositol, and 1,2-diacylglycerol were obtained from Serdary Research Laboratories (London, UK). 1-Acyl-sn-glycerol-3-phosphoserine, 1-acyl-sn-glycerol-3-phosphoethanolamine, and lysophosphatidic acid were from Avanti Polar Lipids (Alabaster, Alabama).
AL). Triolein was the product of Sigma. DEAE-Sepharose CL-6B, heparin-Sepharose CL-6B, blue Sepharose CL-6B, and Sepharose 4B were bought from Pharmacia Fine Chemicals (Uppsala, Sweden). Other chemicals were purchased from Wako (Osaka, Japan).


c-14C]arachidonoyl-1-palmitoyl-2-acyl-sn-glycero-3-phosphoserine was used to determine PS-PLA,
activity, as described previously (5, 6). For determination of phospholipid specificity, 1-palmitoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphoserine, 1-palmitoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphoinositol, 1-palmitoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphatic acid, and 1-palmitoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphocholine (40 μM each) were incubated with recombinant PS-PLA, in 100 mM Tris- HCl pH 7.5, 4 mM CaCl₂ at 37 °C for 15 min, and the products were analyzed by thin layer chromatography using chloroform/methanol/acetic acid/H₂O (25/15/4/2; v/v/v/v).

The radioactivities corresponding to each product were detected and analyzed using a BAS 2000 imaging analyzer (Fuji Film, Kanagawa, Japan).

Purification of Phosphatidylycerine-specific Phospholipase A₁—Blood was collected from 200–300 Wistar rats by cardiac puncture, and platelet-rich plasma was prepared as described previously (5). The platelets were activated by incubating with 2 units/ml thrombin and 2 mM CaCl₂ for 20 min at 37 °C. Then, the plasma was centrifuged at 1,500 × g for 10 min at 4 °C, and the supernatant was used as the enzyme source.

Amino Acid Sequence Analysis of PS-PLA₁—The N-terminal amino acid sequence was determined by the method of Matsudaira (17). Other Analytical Methods—Protein concentrations were determined using the BCA protein assay reagent (Pierce). SDS-PAGE was performed by the method of Laemmli (18) and visualized using Coomassie blue or silver staining (19). DNA sequencing was performed using an ALFred DNA sequencer with an AutoCycle Sequencing Kit (Pharmacia Biotech Inc.).

RESULTS

Purification of PS-PLA₁ from Extracellular Medium of Thrombin-activated Rat Platelets—As rat platelets released both PS-selective phospholipase A₁ and lyso-PS-selective lysophospholipase activities upon cell activation, the extracellular medium from thrombin-activated platelet cultures was used as the enzyme source. Lyso-PS was used as a substrate for assessing enzyme activity throughout the present study. First the enzyme source was subjected to DEAE-Sepharose column chromatography. The enzyme was eluted with the linear gradient of NaCl in the presence of 300 mM Triton X-100. The lyso-PS-selective lyso-phospholipase activities were detected by reverse-phase high performance liquid chromatography using chloroform/methanol/acetic acid/H₂O (25/15/4/2; v/v/v/v) and hexane/ether/acetic acid (70/30/1; v/v/v), respectively. Radioactivity was detected using BAS 2000 imaging analyzer. In some experiments, [3H]PS (2 μM) was incubated with nonlabeled PS (2 μM) in the presence of 300 mM Triton X-100. Similarly, [3H]TG (2 μM) was mixed with nonlabeled PS (2 μM) in the presence of 300 mM Triton X-100. The lyso-PS activity was then measured by TLC using chloroform/methanol/acetic acid/H₂O (25/15/4/2; v/v/v/v), hexane/ether/acetic acid (70/30/1; v/v/v/v), respectively.

For determination of internal amino acid sequences, the protein was reduced by dithiothreitol, digested using trypsin, and digested with the limited protease elastase. The resulting peptide fragments were separated by reverse-phase high performance liquid chromatography using a gradient of 0–64% acetonitrile in 0.1% trifluoroacetic acid. Alternatively, the peptides were separated by SDS-PAGE using a 10–24% acrylamide gel and blotted to a polyvinylidene difluoride membrane. The isolated peptides were sequenced in an ABI model 477A protein sequencer connected on-line to an ABI model 120A phenylthiohydantoin analyzer (Perkin-Elmer).

cDNA Cloning of PS-PLA₁—A rat megakaryocyte A/11 phage cDNA library (18) was used for screening. The cDNA fragment that contained an internal amino acid sequence was amplified by the polymerase chain reaction (PCR) using the megakaryocyte A/11 phage cDNA library as the template, and the following degenerated oligonucleotide primer pairs: GTCGCCTGCGTGAGGCC and GCGGGCGTGGGAGCGAAC, based on the VPPPTQP and FHISSRV sequences of the two peptides (N-terminal and Lys-end-2) (see Table II), respectively. The resulting PCR fragments were subcloned into a pT7Blue T-vector (Novagen, Madison, WI), and the nucleotide sequences were determined. A DNA fragment which contains internal amino acid sequences was chosen and used for identifying a phage which contained same sequence as the DNA fragments by plaque hybridization. The insert DNA was then subcloned into the EcoRI site of the pBluescript II SK phagemid vector (Stratagene, La Jolla, CA). After the restriction enzyme map (Fig. 3A) was determined, the nucleotide sequence was determined by DNA sequencing. 5'-RACE was performed using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA) with rat platelet total RNA as the template for first strand cDNA synthesis according to the manufacturer's protocol. The antisense oligonucleotide TTCCGGATGTCACTGTCCTC (nucleotides 205–224 of PS-PLA₁, see Fig. 3B) was used as a primer for first strand synthesis.

Expression of PS-PLA₁ in Sf9 Cells—cDNA encoding the coding region of PS-PLA₁ (nucleotide positions 1–13729) was inserted into the SmaI/AccI sites of a baculovirus transfer vector pVL13.90, obtained from the National Institute of Allergy and Infectious Diseases, San Diego, CA. The plasmid obtained was designated as PS-PLA₁-pVL1393. A recombinant virus was prepared using the BaculoGold system (PharMingen) according to the manufacturer's protocol. Cells (6 × 10⁷ cells/ml) were infected with recombinant or wild-type Autographa californica nuclear polyhedrosis virus (AcNPV) (multiplicity of infection – 10) and incubated for 96 h at 27 °C. Phospholipase A₁ and lysophospholipase activities in the cell culture supernatant were determined as described above.

Detection of Phospholipase and Lipase Activities in a Mixed Micelle System—[14C]IPS (2 μM) or [14C]TGT (2 μM) was incubated in the presence of 100 mM NaCl, 100 mM Tris- HCl pH 7.5, 5 mCi CaCl₂ at 37 °C for 15 min in the presence of 300 mM Triton X-100. After incubation from the mixture by Bligh and Dyer's method, the release of lyso-PS and diglyceride was measured by TLC using chloroform/methanol/acetic acid/H₂O (25/15/4/2; v/v/v/v), respectively. Radioactivity was detected using BAS 2000 imaging analyzer. In some experiments, [3H]PS (2 μM) was mixed with nonlabeled T (2 μM) in the presence of 300 mM Triton X-100. Similarly, [3H]TG (2 μM) was mixed with nonlabeled PS (2 μM) in the presence of 300 mM Triton X-100. The lyso-PS activity was then measured by TLC using chloroform/methanol/acetic acid/H₂O (25/15/4/2; v/v/v/v), hexane/ether/acetic acid (70/30/1; v/v/v/v), respectively. Other Analytical Methods—Protein concentrations were determined using the BCA protein assay reagent. SDS-PAGE was performed by the method of Laemmli. DNA sequence was performed using an ALFred DNA sequencer with an AutoCycle Sequencing Kit (Pharmacia Biotech Inc.).
Absorbed proteins were subsequently eluted by 1M NaCl, 50 mM Tris-HCl, pH 4.0. Fractions (1.5 ml) were collected, examined for lysophospholipase activity (upper panel), and subjected to SDS-PAGE. The proteins were detected by Coomasie Brilliant Blue staining of the gel (lower panel). The hashed line represents absorbance at 280 nm, and the closed circle is lysophospholipase activity using lyso-PS as the substrate.

Expression of PS-PLA₁—To confirm that the cDNA obtained encoded PS-PLA₁, a recombinant baculovirus was prepared and used to infect Sf9 insect cells. As shown in Fig. 4A, the culture medium of Sf9 cells infected with the recombinant baculovirus exhibited appreciable lysophospholipase activity, whereas the culture medium obtained with wild-type virus did not. The medium of cells infected with the recombinant virus also showed PS-hydrolyzing activity (Fig. 4B), confirming that both lysophospholipase and PS-hydrolyzing activities were mediated by the same protein. The activity was mostly recovered from the medium, suggesting that the enzyme was spontaneously secreted from Sf9 cells. In platelets the enzyme might be stored in granules and secreted upon cell activation.

Sequence Analysis—We searched the protein data base for similar sequences using the BLASTN program (19). LPL, HL, and PL of various species such as human, rat, and mouse showed significant homology with the enzyme (Fig. 5A). The enzyme showed 30.8, 31.1, and 29.1% identity to rat LPL (20), HL (21), and PL (22), respectively. Each of these lipases (LPL, HL, and PL) is composed of two domains (the N- and C-terminal regions (amino acids 1–280 in PS-PLA₁) are in the first half of the molecule, which corresponds to the N-terminal domain of the parent molecule, which corresponds to the N-terminal domain of the enzyme). The amino acid sequences corresponding to the active site Ser-142 in PS-PLA₁, Asp-166, and His-236 in PS-PLA₁ are well conserved in these three lipases and may form a “catalytic triad” in PS-PLA₁.

New Member of the Lipase Family
New Member of the Lipase Family

TABLE I
Partial purification of lyso-phospholipase active on lyso-PS from supernatant of activated platelets
Lyso-phospholipase activities were measured by the modified Dole's method using 1-[14C]oleoyl-sn-glycero-3-phosphoserine as a substrate (see "Experimental Procedures").

| Procedure applied        | Total protein | Total activity | Specific activity | Recovery |
|--------------------------|---------------|----------------|------------------|----------|
| Supernatant of activated platelets | 571           | 4.46           | 0.008            | 1        | 100    |
| DEAE-Sepharose            | 345           | 4.00           | 0.012            | 1.5      | 90     |
| Heparin-Sepharose         | 5.9           | 3.62           | 0.614            | 77       | 81     |
| Blue Sepharose            | 0.1           | 1.46           | 14.0             | 1800     | 31     |

LPL and HL is essential for the synthesis of fully active enzymes (26, 27). The latter half of the PS-PLA1 molecule (amino acids 281–426) did not show significant homology with LPL, HL, or PL (Fig. 5A). Lipase lids exist in LPL and HL, and are surrounded by two cysteine residues (216 and 239 in LPL, and 239 and 262 in HL) (24). These lids are reported to be involved in determining the substrate specificity of the two enzymes (28). In PS-PLA1 the region corresponding to the lipase lids (amino acids 222–233, the putative lid of PS-PLA1) is composed of 12 amino acid residues, whereas the lids of both LPL and HL are composed of 22 amino acid residues. Thus the putative lid of PS-PLA1 is very short. The phylogenetic relationship between the lipase family and PS-PLA1 is shown in Fig. 5B. This shows that PS-PLA1 is more closely related to LPL and HL than PL.

Substrate Specificity of PS-PLA1—From 500 ml of culture medium of S99 cells infected with recombinant virus we purified about 160 μg of recombinant enzyme by the same method as that used for rat platelet enzyme. This recombinant enzyme exhibited a smaller molecular mass (50 kDa) on SDS-PAGE analysis compared with enzyme derived from rat platelets (data not shown). The specific activity of recombinant enzyme toward PS was 2.6 μmol/min/mg, whereas those toward phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and phosphatidylinositol were less than 0.1 μmol/min/mg (Table III). The substrate specificity of the platelet enzyme was the same as that of the recombinant enzyme (data not shown).

Next we examined whether the enzyme hydrolyzed TG, because the structure of the enzyme resembles the lipases (Fig. 5A). No appreciable hydrolysis was observed against triolein in the Triton X-100 micelle system, whereas PS was hydrolyzed under the same conditions (Fig. 6, lanes 1 and 2). Hydrolysis of triolein was not detected even under the conditions in which the same amount of lipids was introduced in the assay (Fig. 6, lanes 3 and 4). Thus PS-PLA1 cannot digest TG in spite of its similarity in structure to the mammalian lipases.

PS-PLA1 releases fatty acid from 1-acyl-lyso-PS, so it can cleave the fatty acid of lyso-PS at the sn-1 position. To test whether PS-PLA1 has phospholipase B activity toward PS, the ability of PS-PLA1 to cleave a fatty acid at the sn-2 position was investigated using 1-palmitoyl-2-[14C]arachidonoyl-PS as the substrate. As shown in Fig. 7 radioactivity was not detected at the TLC migration position of free fatty acid, but was detected at the position of lyso-PS. Thus the acyl chain at the sn-2 position in PS is not hydrolyzed by PS-PLA1. When 1,2-di[14C]oleoyl-PS was used as the substrate for a PS-PLA1 assay, radioactivity was detected in spots corresponding to both free fatty acid and lyso-PS (data not shown). These results suggest that PS-PLA1 releases fatty acid from the sn-1 position in PS and lyso-PS.

DISCUSSION
In this study we purified PS-PLA1 that was secreted from activated platelets and established its primary structure by cDNA cloning. The primary structure of the enzyme did not exhibit homology to any phospholipase A2 or lyso-phospholipase that has been reported previously (type I PLA2 (29), type II PLA2 (14), cPLA2 (2), hepatic lysophospholipase (30), Escherichia coli lysophospholipase (31), human eosinophil lyso-phospholipase (32), and rat pancreas lysophospholipase (33)). Unexpectedly PS-PLA1 resembles some mammalian lipases (LPL, HL, and PL) although it did not show any detectable lipase activity.

Substrate Specificity of PS-PLA1—The recombinant form of PS-PLA1 hydrolyzed PS and lyso-PS at the sn-1 position of the glycerol backbone to release a fatty acid (Fig. 7), but did not hydrolyze other phospholipids (Table III). A previous study using partially purified enzyme showed that the enzyme hydrolyzed lyso-phosphatidylserine but did not hydrolyze other lyso-phospholipids such as lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidic acid (5). The enzyme did not hydrolyze TG, even though it did hydrolyze PS under the same conditions (Fig. 6, and Fig. 7, lane 1). Thus PS-PLA1 cannot hydrolyze TG, even though it shares some sequence homology with mammalian lipases. HL is known to possess both lipase and phosphatidylase activities. HL is known to possess both lipase and phosphatidylase activities. HL is known to possess both lipase and phosphatidylase activities.
The substrate specificity of PS-PLA₁ is different from that of lipases because it cannot react with TG, but does recognize the glycerophosphoserine structure and hydrolyzes a fatty acyl residue bound at the sn-1 position of the glycerol backbone.

Which part of the molecular structure of PS-PLA₁ determines its substrate specificity? Under aqueous conditions, lipases are inactive, as the "catalytic triad" is buried beneath a short amphipathic α-helix (the "lid") (23). Compared with the lid amino acid sequences of other lipases, the putative lid of PS-PLA₁ is extremely short (Fig. 5A). Rat LPL and HL have
Phospholipase A1 (PS-PLA1) is a member of the lipase family, known for its role in hydrolyzing phospholipids. The enzyme is composed of 22 amino acids between two conserved cysteine residues, whereas the corresponding region in PS-PLA1 is composed of only 12 amino acid residues. It has been reported that the substrate specificities of LPL and HL are determined by the lids of the molecules (28). Guinea pig PL has a short lid and exhibits both phospholipase A1 and lipase activities (35), and hornet phospholipase A1, which has only 7 amino acids in the corresponding region, has weak lipase activity (34). Thus, the "phospholipase A1 activity" of lipase family molecules may depend on the length and amino acid sequence of the lid.

The mechanism by which PS-PLA1 specifically recognizes serine-phospholipids is not known at present. A cluster of positively charged amino acids (Lys and Arg) is present in the sequence of PS-PLA1 (348–353). It is interesting that the amino acid sequences around this cluster (VEFNLKEKRD) resemble the PS-binding motif found in protein kinase C and PS deacylase (FXFLKXXXXXK) (36). Amino acid sequences of lipases around these regions are full of variety. This region may be responsible for determining the substrate specificity of PS-PLA1. Mutation studies will test this hypothesis.

Glycoprotein Nature of PS-PLA1—Of the known mammalian phospholipases, rat platelet PS-PLA1 is the only one that is a glycoprotein. Three potential N-glycosylation sites are present in the deduced amino acid sequence of PS-PLA1. The idea that PS-PLA1 is a glycoprotein was confirmed by two additional observations: 1) in our preliminary experiments, PS-PLA1 and lyso-PS lysophospholipase activities were retained on concanavalin A-, WGA-, and RCA-Sepharose columns and were eluted by the haptens sugar of each lectin; and 2) the calculated molecular mass of PS-PLA1 is 47 kDa, whereas the purified protein had a molecular mass of 55 kDa by SDS-PAGE analysis. This difference may be due to the addition of an N-linked sugar moiety because treatment of the purified protein with N-glycosidase F, which removes N-linked sugar moieties, reduced the molecular mass of platelet PS-PLA1 to 48 kDa (data not shown). The molecular mass of the recombinant protein, produced in a baculovirus system, was 50 kDa. The difference in molecular mass observed between rat platelet and recombinant PS-PLA1 may be due to differences in the sugar structure because the calculated molecular mass of recombinant PS-PLA1 after N-glycosidase F treatment was also 48 kDa. A strict carbohydrate structure seems not to be required for PS-PLA1 activity. This fact is in contrast with LPL and HL, since it has been reported that an N-linked sugar on LPL and HL at the N-terminal (amino acid 75) is necessary for enzyme activity (26, 27). In addition, PS-PLA1 has no homology with phospholipase B of Penicillium notatum (37) or Saccharomyces cerevisiae (38), both of which are glycoproteins.
with thrombin (5). The mechanism of PS-PLA₁ sorting to secretory granules was analyzed by TLC. The positions of cold PS, lyso-PS, and free fatty acids, detected by \(^{14}C\)-vapor, are indicated at the right.

Secretion of PS-PLA₁ from Activated Platelets—PS-PLA₁ was released from rat platelets when the cells were stimulated with thrombin. The enzyme is also released in response to other stimuli such as ADP and A23187 (5). In unstimulated platelets, PS-PLA₁ may be stored in α-granules, since type II PLA₂, which is known to be present in α-granules, is released from rat platelets in a similar manner to PS-PLA₁ upon stimulation with thrombin (5). The mechanism of PS-PLA₁ sorting to secretory granules is not known at present. The N-terminal hydrophobic sequence of PS-PLA₁, which consisted of 24 amino acids, may be a signal sequence because Gly\(^{25}\) is an N-terminal amino acid of the purified enzyme (Table II). According to the PSORT program, which predicts protein sorting from the primary amino acid sequence (39), the 24 hydrophobic amino acid sequence of PS-PLA₁ does not show features typical of the signal sequences of "secretory" proteins. The enzyme was secreted from infected Sf9 cells, supporting the fact that the signal sequence of PS-PLA₁ is not known at present. The N-terminal hydrophobic sequence of PS-PLA₁ may play important roles in the attack on the fatty acyl residue at the sn-1 position of serine-phospholipids. PS and lyso-PS may be involved in the regulation of these processes through control of the concentration of PS or lyso-PS.

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REFERENCES
1. Kim, D. K., Kudo, I., Fujimori, Y., Mizushima, H., Masuda, M., Kikuchi, R., Izawa, K., and Inoue, K. (1990) Biochim. (Tokyo) 108, 903–906
2. Clark, J. D., Lin, L. L., Krik, R. W., Ramesha, C. S., Su, R., Milona, N., and Knope, J. L. (1991) Cell 65, 1043–1051
3. Horigome, K., Hayakawa, M., Inoue, K., and Nojima, S. (1987) Biochim. (Tokyo) 101, 625–631
4. Horigome, K., Hayakawa, M., Inoue, K., and Nojima, S. (1987) Biochim. (Tokyo) 101, 53–61
5. Higashi, S., Kobayashi, T., Kudo, I., and Inoue, K. (1988) Biochim. (Tokyo) 103, 442–447
6. Yokoyama, K., Kudo, I., and Inoue, K. (1995) Biochim. (Tokyo) 117, 1280–1287
7. Chang, H. W., Kudo, I., Haru, S., Karasawa, K., and Inoue, K. (1988) Biochim. (Tokyo) 105, 385–389
8. Murakami, M., Kudo, I., and Inoue, K. (1993) J. Biol. Chem. 268, 839–844
9. Murakami, M., Kudo, I., and Inoue, K. (1993) J. Biol. Chem. 268, 1099–1101
10. Hara, S., Kudo, I., Chang, H. W., Matsuda, K., Miyamoto, T., and Inoue, K. (1989) Biochim. (Tokyo) 105, 385–389
11. Hara, S., Kudo, I., and Inoue, K. (1995) Eur. J. Biochem. 209, 257–261
12. Suga, H., Murakami, M., Kudo, I., and Inoue, K. (1995) Eur. J. Biochem. 218, 807–813
13. Polakowski, J., Schalkwijk, C., Briner, V. A., and van den Bosch, H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2532–2536
14. Komada, M., Kudo, I., Mizushima, H., Kitamura, N., and Inoue, K. (1989) Biochim. (Tokyo) 106, 545–547
15. Komada, M., Kudo, I., and Inoue, K. (1990) Biochem. Biophys. Res. Commun. 188, 1059–1065
16. Hattori, M., Ariai, H., and Inoue, K. (1995) J. Biol. Chem. 268, 18748–18753
17. Matsuzawa, P. (1987) J. Biol. Chem. 262, 10037–10038
18. Choi, D., Greenberg, S. M., and Rosenberg, R. D. (1987) J. Clin. Invest. 80, 1024–1026
19. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
20. Brault, D., Toy, T., and Schott, M. C. (1987) J. Biol. Chem. 262, 4031–4037
21. Komarnysh, C. M., and Schott, M. C. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 1526–1530
22. Payne, R. M., Sims, H. F., Jennens, M. L., and Lowe, M. E. (1994) Am. J. Physiol. 266, G914–G921
23. Winkler, P. K., Arccc, A., and Huisken, W. (1990) Nature 344, 711–774
24. van T., Roussel, A., Lalouel, J. M., and Cambilau, C. (1994) J. Biol. Chem. 269, 4626–4633
25. Emmerich, J., Beg, O. U., Peter, J., Prevost, L., Bronzelli, J. D., Brewer, H. J., and Santamarina, F. S. (1992) J. Biol. Chem. 267, 4161–4165
26. Ben, Z. O., Stahlke, G., Liu, G., Davis, R. C., and Doolittle, M. H. (1994) J. Lipid Res. 35, 1511–1523
27. Buesa, R., Pujana, M. A., Ponce, P., Auerwey, J., Deeb, S. W., Reina, M., and Vilaro, S. (1995) J. Lipid Res. 36, 939–951
28. Dugue, R. A., Debi, H. L., and Santamarina, F. S. (1995) J. Biol. Chem. 270, 25396–25401
29. Verheij, H. M., and de H. G. (1991) Methods Enzymol. 197, 214–223
30. Sugimoto, H., Hayashi, H., and Yamashita, S. (1996) J. Biol. Chem. 271, 7705–7711
31. Kobayashi, T., Kudo, I., Karasawa, K., Mizushima, H., Inoue, K., and Nojima, S. (1985) Biochim. (Tokyo) 86, 1017–1025
32. Ackerman, S. J., Correute, S. E., Rosenberg, H. B., Bennett, J. C., Mastroianni, D. M., Nicholson, W. A., Weller, P. F., Chin, D. T., and Tenen, D. G. (1993) J. Immunol. 150, 456–466
33. Han, J. H., Stratowa, C., and Rutte, W. J. (1987) Biochemistry 26, 1617–1625
34. Soldatova, L., Kochoumian, L., and King, T. P. (1993) FEBS Lett. 329, 145–149
35. Hjorth, A., Carriere, F., Woldike, H., Mittra, E., Lawson, D. M., Nicholson, W. A., Weller, P. F., Chin, D. T., and Tenen, D. G. (1993) Biochemistry 32, 4702–4707
36. Igashira, K., Kaneda, M., Yamaji, A., Saito, T. C., Kikukawa, U., Ono, Y., Inoue, K., and Umeda, M. (1995) J. Biol. Chem. 270, 29075–29078
37. Masuda, N., Kitamura, S., and Saito, K. (1991) J. Biol. Chem. 266, 783–787
38. Lee, K. S., Patton, J. L., Fido, M., Hines, L. K., Kohlwein, S. D., Paltauf, F., Henry, S. A., and Levin, D. E. (1994) J. Biol. Chem. 269, 19725–19730
39. Nakai, K., and Kanehisa, M. (1992) Genomics 14, 897–911