A CHO cell-derived 80-kDa recombinant polypeptide (GenBank number I15470) putatively encoding a calcium-independent phospholipase A₂ activity was expressed in S. frugiperda cells resulting in over a 15-fold increase in a calcium-independent phospholipase A₁/A₂ activity which was entirely inhabitable by (E)-6-(bromomethyl)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one. The recombinant polypeptide was purified from cytosol by sequential tandem affinity chromatographies employing ATP-agarose and calmodulin-Sepharose stationary phases. This strategy resulted in the rapid purification (36 h) of recombinant phospholipase A₂ activity in 56% overall yield to a single intense 80-kDa protein band on SDS-polyacrylamide gel electrophoresis after silver staining. The purified protein possessed phospholipase A₁, phospholipase A₂, and lysophospholipase activities. Microbore anion exchange chromatography demonstrated that the 80-kDa protein band was comprised of multiple distinct isoforms including an anionic isoform which possessed over a 5-fold higher specific activity (5 μmol/mg-min) than earlier eluting isoforms. Collectively, these results unambiguously demonstrate that: 1) the 80-kDa polypeptide catalyzes phospholipase A₁/A₂ and lysophospholipase activities with distinct kinetic parameters; 2) calmodulin and ATP both interact with the catalytic polypeptide independent of regulatory proteins; and 3) distinct isoforms of this polypeptide exist which possess markedly different specific activities.

The intracellular phospholipases A₂ represent a rapidly expanding class of enzymes which have been categorized based on their calcium dependence into calcium-dependent and calcium-independent subtypes (1). Prior work has unambiguously identified their calcium dependence into calcium-dependent and calcium-independent phospholipase A₂ activity. Of course, the assignment of catalytic function to a recombinant protein expressed in the same context from which it was originally isolated requires purification of the protein to homogeneity to unambiguously demonstrate its role as a catalytic entity and not as an activator of an endogenous activity. However, due to intractable technical obstacles, the protein catalyzing this activity has never been purified to homogeneity to unambiguously discriminate between its potential role as an activator of an endogenous phospholipase activity versus a bona fide catalytic entity.

Since at least some members in the family of calcium-independent phospholipases A₂ exhibit specific high affinity interactions with ATP and/or calmodulin (8–13), we sought to exploit potential interactions between these ligands and the expressed 80-kDa recombinant polypeptide to facilitate its purification to homogeneity to unambiguously identify its role in the catalytic process. We now report: 1) the expression of catalytically active recombinant 80-kDa calcium-independent phospholipase A₂ in a baculovirus expression system; 2) the rapid purification of the 80-kDa recombinant calcium-independent phospholipase A₂ to homogeneity through tandem sequential ATP and calmodulin affinity columns; and 3) the identification of multiple isoforms of this calcium-independent phospholipase A₂ which possess markedly different specific activities.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Sephacel and Mono-Q resins were purchased from Pharmacia. ATP-agarose and calmodulin-Sepharose resins were purchased from Sigma. The baculovirus vectors and competent DH10Bac Escherichia coli were purchased from Life Technologies, Inc. and used according to the manufacturer’s protocol. 1-a1-Palmitoyl-2-[1-14C]arachidonyl phosphatidylcholine, 1-a1-0-hexadecyl-2-[14C]arachidonyl phosphatidylcholine, and 1-1-[14C]palmitoyl lysophosphatidylcholine were purchased from DuPont NEN. (E)-6-(Bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one was obtained from Calbiochem. Competent BL21(DE3) and BL21(DE3) pLysS E. coli were purchased from Novagen. PCR reagents were purchased from Perkin-Elmer. The pCR-II vector was obtained from Invitrogen. Most other reagents were obtained from Sigma.

Cell Culture—S. frugiperda (SF) cells were cultured in 250-ml flasks equipped with a magnetic spinner containing supplemented 1 × Grace’s media (14). For expression of recombinant calcium-independent phospholipase A₂, a 250-ml flask was prepared with 80 ml of 1 × 10⁶ cells/ml and incubated at 25 °C for 24 h prior to infection with baculovirus. DUKX B1 CHO cells (ATCC #CRL 9010) were cultured in T-75 flasks containing minimal essential medium according to established methods (15).

Enzymatic Assays—For assays of column eluents, phospholipase A₂ activity was assayed by quantifying the release of radiolabeled arachidonic acid from 5 μM 1-a1-palmitoyl-2-[1-14C]arachidonyl phosphatidylcholine in buffer containing 100 mM Tris-HCl (pH 7.0) and 4 mM EGTA.

* This research was supported by National Institutes of Health Grant 1 P01 HL57278-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Div. of Bioorganic Chemistry and Molecular Pharmacology, Departments of Medicine, Chemistry, Pharmacology and Molecular Biology, Washington University School of Medicine, St. Louis, Missouri 63110.

Matthew J. Wolf and Richard W. Gross‡

From the Division of Bioorganic Chemistry and Molecular Pharmacology, Departments of Medicine, Chemistry, Pharmacology and Molecular Biology, Washington University School of Medicine, St. Louis, Missouri 63110.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Characterization of a Recombinant 80-kDa Phospholipase A₂

Typically, assays of column eluents were conducted with 25 μL of enzyme from 210 μL. After incubation at 37°C for 30 s, reactions were quenched by addition of 1-butanol (100 μL) vortexed, and the phases were separated by centrifugation. The reaction products from column assays were resolved by thin layer chromatography (Whatman Silica Gel 60A plates) utilizing a mobile phase consisting of 50:25:5 (chloroform:methanol:ammonium hydroxide (v/v)). For kinetic assays, calcium-independent phospholipase A₁A₄ activities were assessed by quantifying the release of radiolabeled lysophosphatidylcholine or radiolabeled arachidonic acid, respectively, from 1-nal-palmityl-2-[1-14C]arachidonyl phosphatidylcholine. The release of radiolabeled fatty acid and lysophospholipid was quantified by incubating 120 ng of purified recombinant phospholipase A₁A₄ protein with 25 μM radiolabeled substrate in 100 mM Tris-HCl (pH 7.0) and 4 mM EDTA in a final volume of 100 μL. After an incubation at 37°C for 30 s, the reactions were quenched by the addition of 100 μL of 1-butanol and radiolabeled lysophospholipids and free fatty acids were quantitated by thin layer chromatography (Whatman Silica Gel 60A plates) utilizing a mobile phase consisting of 65:25:5 (chloroform:methanol:ammonium hydroxide (v/v)) or 50:20:8:1 (petroleum ether:diethyl ether:acetic acid (v/v)), respectively. Radiolabeled fatty acid and lysophospholipid were identified by staining of a standard fatty acid sample with iodine vapor, scraped, and quantitated by scintillation spectrometry. Assays of lysophospholipase activity were conducted as described above except with 1-nal-1-14C-palmityl lysophosphatidylcholine as substrate. For studies involving calmodulin-Sepharose chromatography (peaks I and II) were individually pooled, diluted 3-fold in buffer B to reduce the NaCl concentration, and resubjected to Mono-Q chromatography as described above.

The calmodulin-Sepharose column fractions which possessed recombinant calcium-independent phospholipase A₂ activity were pooled and loaded onto a PC 1.6/5 Mono-Q column previously equilibrated with buffer B. Recombinant calcium-independent phospholipase A₂ activity was eluted by the application of a linear gradient of NaCl from 0 to 1 M in buffer B. The fractions which possessed calcium-independent phospholipase A₂ activity were pooled and loaded directly onto a 1-ml ATP-agarose column previously equilibrated with buffer B. The column was sequentially washed with 10 bed volumes of buffer B, 10 mM AMP in buffer B, and buffer B, and phospholipase activity was eluted by application of 1 mM ATP in buffer B.

The ATP-agarose fractions which possessed calcium-independent phospholipase A₂ activity were pooled, adjusted to a final concentration of 5 mM CaCl₂ by addition of a 100 mM CaCl₂ buffer, and loaded onto a 0.5-ml column of calmodulin-Sepharose. Next, the column was washed with 10 column volumes of buffer containing 25 mM imidazole (pH 8.0), 500 μM CaCl₂ prior to elution of calcium-independent phospholipase A₂ activity by the application of buffer containing 25 mM imidazole (pH 8.0) and 4 mM EDTA. For studies involving calmodulin-Sepharose chromatography of Sf9 cell cytosol containing recombinant calcium-independent phospholipase A₂, freshly prepared cytosol was adjusted to 5 mM calcium and chromatographed as described above.

RESULTS

To investigate the biochemical characteristics of a recently described putative calcium-independent phospholipase A₂ (Ref. 7, GenBank accession I15470), RT-PCR was performed to amplify the cDNA encoding this protein from CHO cell total RNA utilizing oligonucleotide primers corresponding to its 5’ and 3’ coding sequence. The 2.2-kb DNA product from the RT-PCR amplification was subcloned into a pCR-II vector and sequenced to authenticate the fidelity of the amplified product. The 2.2-kb DNA product was subcloned into the E. coli expression vector, pET-21a, and competent E. coli cells were transformed. Although robust amounts of recombinant 80-kDa polypeptide were produced, induced E. coli did not demonstrate additional calcium-independent phospholipase A₂ activity in comparison to control cells. We hypothesized that expression of this polypeptide in the context of a mammalian system was necessary for proper post-translational modifications and/or protein folding for expression of catalytic activity. Accordingly, the 2.2-kb product was subcloned into the baculoviral vector, pFast-Bac, and transformed into competent DH10Bac E. coli cells for helper plasmid-mediated transposition of the recombinant sequence into a bMon14272 bacmid (19). The bMon14272 bacmid was purified from the DH10Bac E. coli and used to infect Sf9 cells for subsequent formation of recombinant baculovirus and expression of recombinant protein as described in detail under “Experimental Procedures.”
phospholipase A2 activity (Fig. 1A) with greater than 80% of the recombinant activity partitioning into the cytosolic compartment (Fig. 1B). Furthermore, treatment of Sf9 cell cytosol which contained recombinant calcium-independent phospholipase A2 with the mechanism-based inhibitor (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL) (21–23) completely ablated the expressed recombinant calcium-independent phospholipase A2 activity (Fig. 1C).

To demonstrate that the 80-kDa recombinant polypeptide was responsible for catalyzing calcium-independent phospholipase A2 activity (i.e., the polypeptide was not an activator or cofactor of an endogenous catalytic entity), a strategy was developed to chromatographically purify the recombinant polypeptide to homogeneity. Since at least some calcium-independent phospholipases A2 interact in a highly selective fashion with ATP (8–12) and calmodulin (13), we attempted to exploit the power inherent in double sequential affinity chromatographies to effect the rapid and facile purification of this recombinant phospholipase A2 activity to homogeneity. First, fractions containing calcium-independent phospholipase A2 activity from the DEAE eluent were pooled and immediately loaded onto an ATP-agarose affinity column. After loading, the column was sequentially washed with buffer containing only 1 mM ATP (Fig. 3A). Over 95% of the calcium-independent phospholipase A2 activity bound to the ATP-agarose column and was quantitatively eluted by the application of 1 mM ATP. Resolution of the proteins from the ATP-agarose column fractions by SDS-PAGE and visualization after silver-staining demonstrated the specificity of the interaction between the 80-kDa recombinant phospholipase A2 and the ATP-agarose (Fig. 3B).
Since at least one calcium-independent phospholipase A2 interacts with calmodulin in a calcium sensitive fashion, we attempted to exploit the power of ternary complex affinity chromatography employing a calmodulin-Sepharose stationary phase (13). The fractions containing recombinant calcium-independent phospholipase A2 activity from the ATP-agarose column were pooled, adjusted to 5 mM calcium, and directly loaded onto a calmodulin-Sepharose column as described under “Experimental Procedures.” Recombinant calcium-independent phospholipase A2 activity quantitatively bound to calmodulin-Sepharose in the presence of calcium ion and was quantitatively eluted by dispersal of the ternary complex with application of buffer containing EGTA (Fig. 4A). SDS-PAGE of fractions from calmodulin-Sepharose chromatography displayed only a single intense band after silver staining demonstrating that recombinant calcium-independent phospholipase A2 activity copurified with the 80-kDa protein (Fig. 4B). Moreover, the resolving power of ternary complex affinity chromatography employing the calmodulin-Sepharose stationary phase was demonstrated by additional experiments in which crude Sf9 cytosol was loaded directly onto a calmodulin-Sepharose affinity column in the presence of calcium. Phospholipase A2 activity was quantitatively bound to calmodulin-Sepharose in the presence of calcium ion and was quantitatively eluted by washing with EGTA resulting in a 10-fold purification step (Fig. 4, panels C and D).

Mono-Q chromatography of the calmodulin-Sepharose eluent demonstrated an elution profile identifying the presence of multiple isoforms of the 80-kDa polypeptide which each chromatographed with phospholipase A2 enzymic activity (Fig. 5). The majority of recombinant phospholipase A2 activity eluted at ~50 mM NaCl with a specific activity of 1 μmol/mg/min while a second, more anionic, peak eluted at ~120 mM NaCl with a specific activity of 5 μmol/mg/min. Each of three early eluting peaks as well as the late eluting peak contained calcium-independent phospholipase A1, phospholipase A2, and lysophospholipase activities in similar ratios. Peaks I and II were individually pooled, diluted, and subsequently rechromatographed on a re-equilibrated Mono-Q stationary phase demonstrating that each peak chromatographed according to its elution profile during the initial chromatography (i.e. chromatography of peak I resulted in a single peak eluting at 50 mM NaCl while rechromatography of peak II resulted in a single peak eluting at 120 mM NaCl) (Fig. 6). These results demonstrate that the isoforms are long-lived entities and not the result of a rapidly equilibrating mixture.

To examine the kinetic characteristics of the phospholipase A1, phospholipase A2, and lysophospholipase activities catalyzed by the recombinant 80-kDa polypeptide, substrate-activity profiles were compared. First, phospholipase A1, phospholipase A2, and lysophospholipase activities were linear with respect to time over the incubation times utilized (30 s). Second, each of the activities displayed saturation kinetics (Fig. 7, A and B). Third, phospholipase A1 activity (as assessed by the production of sn-2 labeled lysophospholipid from the substrate 1-α1-palmitoyl-2-[1-14C]arachidonylphosphatidylcholine) demonstrated an apparent maximum velocity of 1.2 μmol/mg/min.
Characterization of a Recombinant 80-kDa Phospholipase A_2

**Figure 5.** Mono-Q chromatography of recombinant calcium-independent phospholipase A_2. A, recombinant calcium-independent phospholipase A_2 activity from the calmodulin-Sepharose column eluent (10 μg of protein) was loaded onto a PC1.6/5 Mono-Q column previously equilibrated with buffer B. After washing, recombinant calcium-independent phospholipase A_2 activity was eluted by the application of a linear gradient of NaCl in buffer B. UV absorption at 280 nm (-) and NaCl concentration (- -). B, fractions from the Mono-Q column elute were dried by SDS-PAGE on 10% polyacrylamide gels and stained with silver as described under "Experimental Procedures." The calcium-independent phospholipase A_2 activity in each fraction was measured as described under "Experimental Procedures" and is expressed in dintegrations/min of [14C]arachidonic acid released from 5 μM 1-palmitoyl-2-[1-14C]arachidonoyl phosphatidylcholine and shown on the top of the gel.

**Figure 6.** Rechromatography of previously resolved recombinant calcium-independent phospholipase A_2 isoforms on a Mono-Q stationary phase. A, purified recombinant calcium-independent phospholipase A_2 from the calmodulin-Sepharose eluent (5 μg of protein) was purified on a Mono-Q column and resolved into earlier eluting (peak I) and later eluting (peak II) peaks as described under "Experimental Procedures." B, peak I from the Mono-Q chromatography in A was diluted 3-fold in buffer B to reduce the NaCl concentration and reapplied to Mono-Q resin and rechromatographed employing identical conditions as above. C, peak II from three iterative preparations similar to those represented in A was diluted 3-fold in buffer B to reduce the NaCl concentration and rechromatographed as described above.

with an apparent K_m of 3.3 μM. Fatty acid production catalyzed by the recombinant 80-kDa polypeptide (which reflects both direct phospholipase A_2 catalyzed release of the sn-2 radiolabeled arachidonic acid moiety as well as fatty acid generated through sequential phospholipase A_1 and lysophospholipase activities) demonstrated an apparent maximum velocity of 0.9 μmol/mg/min and an apparent K_m of 1.6 μM. We point out that a substantial amount of fatty acid release could result from sequential phospholipase A_1 and lysophospholipase activities. Incubation of enzyme with 1-palmitoyl-O-hexadecyl-2-[3H]arachidonoyl phosphatidylcholine demonstrated the expressed 80-kDa polypeptide possessed a phospholipase A_2 activity of 0.5 μmol/mg/min. Since the sn-1 alkyl ether linkage is not susceptible to hydrolysis by esterolytic processes, these results unambiguously establish phospholipase A_2 activity as an inherent catalytic function of the expressed polypeptide. Fourth, kinetic analysis of lysophospholipase activities demonstrated that the enzyme rapidly catalyzes hydrolysis of monomeric lysophosphatidylcholine (critical micellar concentration of palmitoyl lysophosphatidylcholine = 7 μM (24)) with only modest increase of enzymic activity at supramicellar concentrations of substrate.

**DISCUSSION**

The present results demonstrate that the recombinant chromatographically pure 80-kDa polypeptide catalyzes phospholipase A_1/A_2 and lysophospholipase activities when expressed in a baculoviral expression system. Moreover, individual isoforms of the 80-kDa polypeptide can be chromatographically resolved and calcium-independent phospholipase A_1/A_2 and lysophospholipase activities congregate with each peak of 80-kDa protein mass. Collectively, these results identify the 80-kDa polypeptide as a catalytic entity mediating phospholipolysis. Furthermore, they underscore the necessity of expression of this protein in the context of a mammalian cell since E. coli expressed robust quantities of recombinant protein which did
not possess either inherent or inducible (in our hands) catalytic activity.

The recombinant protein expressed calcium-independent phospholipase A₁/A₂ and lysophospholipase activities in similar amounts. Potentially, two mechanisms can be responsible for the sn-2 fatty acid release from choline glycerophospholipids including: 1) the sequential hydrolysis of the sn-1 acyl group followed by lysophospholipase activity; or 2) direct hydrolysis of the sn-2 acyl group and the concomitant generation of sn-1 acyl-lysophospholipids. Both mechanisms likely contribute to the production of radiolabeled free fatty acid from specifically radiolabeled sn-2 1-α1-palmitoyl-2-[1-14C]arachidonoyl phosphatidylcholine. We point out the potential possibility that all of the observed release of free fatty acid could result from sequential phospholipase A₁ and lysophospholipase activities since the recently generated sn-2 labeled lysophospholipid is present at the active site of the enzyme and could serve as the preferred substrate for a second round of hydrolysis. In this paradigm, the relative fractional percentage of radiolabeled fatty acid to lysophospholipid generated reflects the relative rates of a second round of enzymatic cleavage in comparison to the rate of release of the radiolabeled lysophospholipid bound at the active site. However, the recombinant protein has substantive amounts of phospholipase A₂ activity since 1-α1-O-hexadecyl-2-[3H]arachidonoyl phosphatidylcholine (where the sn-2 acyl group is the only acyl group which can be hydrolyzed by this enzyme) is a good substrate. Finally, the rate of lysophospholipase activity was similar to that of phospholipase A₂ activity, suggesting the similar interactions of the carboxyl moiety destined for hydrolysis with critical amino acids at the active site are present (i.e. the activation energies are similar for the hydrolysis of both substrates).

There are several features of the purification strategy which merit consideration. First, the utilization of tandem sequential affinity columns facilitates the procurement of geometric increases in the high purification factors typically obtained through affinity chromatographic approaches. Second, ternary complex affinity chromatography with calmodulin-Sepharose resin facilitates the discrimination not only between entities which can recognize calmodulin but also between those proteins which possess an obligatory requirement for binding to the calcium-calmodulin complex. Third, the ATP-agarose affinity chromatography exploited the molecular recognition of ATP by the protein in the context of an anionic stationary phase. Since the proteins had been previously selected for binding to a cationic stationary phase (DEAE-Sepharose resin), the subsequent forced affinity binding to a negatively charged affinity column further amplified the power and selectivity of ATP affinity chromatography. The integration of these three key principles in the purification strategy allowed the complete purification of this polypeptide in 36 h in >50% overall yield (Table I) while prior attempts employing conventional chromatographic strategies have resulted in technically demanding approaches accompanied by poor yields of inhomogenous preparations.

The separation of the 80-kDa phospholipase A₂ into distinct chromatographically resolvable isofoms with differing specific activities was unanticipated. Rechromatography of constituent isofoms eluting with their original chromatographic profiles demonstrated that these isofoms are isolatable entities (on a laboratory time scale) and are not the result of a dynamic interchange of an equilibrium mixture. It is intriguing to speculate that the higher specific activity isoform of this polypeptide has a higher phosphorylation state giving rise to an increased retention time on an anionic exchange resin and a higher specific activity. Whatever covalent modifications are eventually determined to be responsible for these effects, the results suggest a potential biochemical mechanism for agonist-induced increases in calcium-independent phospholipase A₂ activity whereby a high specific activity isoform can be generated from a lower specific activity pool during cellular stimulation.

Recently, studies of the crystal structure of phospholipase Cδ identified the unanticipated finding of an EF-hand calcium binding motif (residues 133–281) which shares substantial homology with calmodulin (25). The present results demonstrate a direct interaction between calmodulin and the recombinant 80-kDa phospholipase A₂. Indeed, the recombinant phospholipase Cδ could show regions of homology and provide insights into the structure of an evolutionarily distant ancestral polypeptide from which both phospholipases are derived.

Acknowledgment—We gratefully acknowledge the assistance of Rose Gubitosi-Klug with Sf9 cells in experiments utilizing the baculovirus expression system.

REFERENCES

1. Dennis, E. A. (1994) J. Biol. Chem. 269, 13057–13060
2. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesh, C. S., Stultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) Cell 65, 1043–1051
3. Sharp, J. D., White, D. L., Chou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportman, J. E., Becker, G. W., Kang, L. H., Roberts, E. F., and Kramer, R. M. (1991) J. Biol. Chem. 14550–14553
4. Clark, J. D., Milona, N., and Knopf, J. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7708–7712
5. Channon, J. Y., and Leslie, C. C. (1990) J. Biol. Chem. 265, 13057–13060
6. Glover, S., Bahr, B., Jonas, M., and Gelb, M. H. (1995) J. Biol. Chem. 270, 15359–15367
7. Jones, S. S., Tang, J., Kriz, R., Shaffer, M., Knopf, J., and Seehra, J. (1996) PNAS 10, 5156–5160
8. Hazen, S. L., Stuppy, R. J., and Gross, R. W. (1990) J. Biol. Chem. 265, 10022–10030
9. Hazen, S. L., and Gross, R. W. (1991) J. Biol. Chem. 266, 14526–14534
10. Hazen, S. L., and Gross, R. W. (1991) Biochem. J. 280, 581–587
Characterization of a Recombinant 80-kDa Phospholipase A₂

11. Ackermann, E. J., Kempner, E. S., and Dennis, E. A. (1994) J. Biol. Chem. 269, 9227–9233
12. Ramanadham, S., Wolf, M. J., Jett, P. A., Gross, R. W., and Turk, T. (1994) Biochemistry 33, 7442–7452
13. Wolf, M. J., and Gross, R. W. (1996) J. Biol. Chem. 271, 20989–20994
14. O’Reilly, D. R., Miller, L. R., and Luckow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W. H. Freeman and Co., New York
15. Jakoby, W. B., and Pastan, I. H. (1979) Methods Enzymol. 58, 132–140
16. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
17. Sturdier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130
18. Sturdier, F. W. (1991) J. Mol. Biol. 219, 37–44
19. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) J. Virol. 67, 4566–4579
20. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) J. Biol. Chem. 266, 7227–7232
22. Zupan, L. A., Weiss, R. H., Hazen, S. L., Parnas, B. L., Asten, K. W., Lennon, P. J., Getman, D. P., and Gross, R. W. (1991) J. Med. Chem. 34, 95–100
23. Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) J. Biol. Chem. 270, 445–450
24. Stafford, R. E., Fanni, T., and Dennis, E. A. (1989) Biochemistry 28, 5113–5120
25. Essen, L. O., Perisic, O., Cheung, R., Katan, M., and Williams, R. L. (1996) Nature 380, 595–602