A Novel Accessory Subunit for Vacuolar H\textsuperscript{+}-ATPase from Chromaffin Granules*

(Received for publication, March 25, 1994, and in revised form, July 8, 1994)

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Three subunits, Ac115, Ac39, and the proteolipid, were positively identified in the membrane sectors of V-ATPases from different sources. We searched for organelle-specific protein in purified preparations of V-ATPase from bovine chromaffin granules. A diffused protein band at a position of about 45 kDa was identified in SDS-polyacrylamide gels of the above preparation. Following digestion with endoproteinase Glu-C (V8), a polypeptide of about 10 kDa was isolated and subjected to amino acid sequencing. Hence, the cDNA encoding the protein Ac45 was cloned from a bovine adrenal medulla library. The cDNA sequence contains an open reading frame encoding a protein of 468 amino acids with a calculated molecular mass of 51,786 daltons. A potential signal sequence comprised of the first 35 amino acids and a potential transmembrane domain at the C terminus of the protein were identified. There exist seven potential glycosylation sites between the aforementioned protein motifs. Experiments with a specific antibody against Ac45 demonstrated that it is copurifying with the V-ATPase from chromaffin granules. Immunological cross-reactivity was observed with purified V-ATPase from bovine kidney microsomes but not from plasma membranes of epithelial cells. Cell-free expression of the protein from synthetic mRNA produced a single protein band at about 50 kDa on SDS gels. Upon inclusion of dog pancreas microsomes in the reaction mixture, a slow migrating band sensitive to peptide-N-glycosidase F was observed.

V-ATPase is composed of two distinct sectors; a catalytic sector functions in the ATPase activity of the enzyme, and a membrane sector functions in proton conduction across the membrane (4). The membrane sector has several other functions including energy coupling and communicating with the lumen as well as modulating the activity of the enzyme. Mutational analysis in yeast V-ATPase showed that although the membrane sector can be assembled independently of the catalytic sector, the assembly of the catalytic sector is absolutely dependent on the previous assembly of the membrane sector (5, 6). Therefore, we expect that most of the accessory polypeptides that modify the activity of the enzyme in the different organelles will belong to the membrane sector. The proteolipid, the main functional part of the membrane sector, is highly conserved, and there are almost identical sequences in its third and fourth transmembrane helices among proteolipids from different sources (3, 7). On the other hand, the other two members of the membrane sector, Ac39 and Ac115, are not well conserved. However, significant sequence homology was observed between these subunits from yeast and bovine sources (3). It is expected that one more subunit of about 20 kDa will also be uniformly conserved in the various V-ATPases from different sources (3, 8–11). We assume that the remaining subunits that have not yet been discovered will be unique for the different organelles.

**EXPERIMENTAL PROCEDURES**

**Isolation of Peptides and Sequencing—**V-ATPase was purified from chromaffin granules as described previously (12). The purified V-ATPase was electrophoresed on 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Following brief destaining and several washes with water, the defused bands between subunits B and C were excised, briefly lyophilized, and applied on a 10-well 15% polyacrylamide gel. The slices were overlaid with 10 μl of a solution containing 0.1 M Tris-Cl (pH 6.8), 1% glycerol, Bromphenol blue, 0.1% SDS, and 1 μg of V8 protease (Boehringer Mannheim). Electrophoresis was performed at 30 V for 40 min and then 200 V for an additional 25 min. The proteins were electrotransferred onto an Immobilon-P™ filter (Millipore) according to a published procedure (13, 14). Following staining with Coomassie Brilliant Blue and destaining, a protein band of about 10 kDa was excised and sequenced directly by a Gas-phase Applied Biosystems sequenator. The following amino acid sequence was obtained: DGNLIVPDTPQ.

**Screening of Bovine Adrenal Medulla cDNA Library—**Two oligonucleotide probes were designed according to the amino acid sequence obtained as follows: 1) GAT GCC AAC CTG CTG CCT GAC ACC CAG CC; 2) GAT GG(GT) AAT CT(GT) CT(GT) CT(GT) CT(GT) CC(GT) GAT AC(GT) CAG CC. The first probe was synthesized according to LaThé’s (15) prediction, and the second included redundancies according to Béltran et al. (16). A third oligonucleotide was a combination between the above two methods as follows: GAT GCC AAC (CT(T)/CT(T) CT(T/GT) GT(T/GT) CT(GT) CC(GT) GAC ACC CAG CC. The oligonucleotides were used for polymerase chain reaction amplification of the adrenal medulla library, each with the two primers of the YPN1 plasmid in which the library was constructed (17). A polymerase chain reaction product of about 1 kilobase was obtained and used for screening the library following labeling with 32P. The screening of the bovine adrenal medulla library was per-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U10039.

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formed as described previously (17, 18). Several positive colonies were analyzed by dot blot and Southern hybridization. Among them, five showed very strong hybridization signals with the oligonucleotide probes and had a size of 2.2 kilobases. The cDNA fragments were sequenced in one direction by exonuclease III digestion and double-stranded sequencing as described previously (18, 19) and in the other direction by oligonucleotide-directed sequencing.

Preparation of Antibody Against pMAL Fusion Protein—A 0.6-kilobase DNA fragment (starting at nucleotide 839) encoding the last 197 amino acids of the protein was amplified by polymerase chain reaction and cloned into EcoRI-Hind111 restriction sites of pMAL-cRI plasmid (New England BioLabs). The correct in-frame cloning was verified by sequencing. Transformed DH5a Escherichia coli cells were grown on LB-ampicillin medium and induced for high expression of fusion protein. The overexpressed protein was purified on a maltose affinity column according to the manufacturer’s instructions. The purified fusion protein was concentrated by acetone precipitation and dissolved in a solution containing 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 0.1% SDS. Antibodies were raised in guinea pigs by a procedure similar to the one described previously for rabbits (20). In order to obtain an affinity-purified antibody (21), about 10 mg of the purified fusion protein was cross-linked onto 1 ml of Affi-Gel 10 resin (Bio-Rad) in 0.2 M Mops-solution containing 10 nM Tris-C1 (pH 7.5) and was then dialyzed overnight at 4 °C against the same solution. The solution was passed 10 times through the column with the bound fusion protein. The column was washed with 50 ml of the above solution followed by 25 ml of the same solution containing 0.5 M NaCl. The antibody was eluted by 0.1 M glycine-HCl (pH 2.8), and 0.5-m1 fractions were collected into tubes containing 0.1 ml of 1 M Tris (pH 8). The second purification step consisted of passing the affinity-purified antibody through the column of Aff-Gel with the bound maltose-binding protein, which was purified and cross-linked to the resin in the same way as the fusion protein. The affinity-purified antibody was dialyzed against a solution containing 100 mM NaCl, 100 mM sodium phosphate (pH 7.5), and 35% glycerol and was stored at −12 °C.

Determination of the Relative Amounts of Ac45 with the Other Subunits of V-ATPase—Determination of the relative amount of Ac45 in crude synaptic vesicles from various brain parts was performed by SDSPAGE followed by Western blot analysis. Fresh bovine brain was obtained from a local slaughter house and crude synaptic vesicles were prepared according to the procedure developed for synaptic vesicle preparation from rat brains (22). About 12 g of brain tissue was homogenized by Dounce homogenizer in 25 ml of solution containing 0.32 M sucrose, 10 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 0.1 mM MgCl2, 5 mM a-glycerophosphate, 0.1 mM EGTA, 10 mM Hepes-KOH (pH 7.5), and 0.3 mM phenylmethylsulfonyl fluoride. After centrifugation, the pellet was obtained in 2 ml of the same buffer and was lysed by dilution into 10 volumes of H2O. After incubation for 30 min on ice, large membrane fragments were removed by centrifugation at 40,000 × g for 15 min. Synaptic vesicles and small membrane fragments were recovered by centrifugation of the supernatant at 140,000 × g for 4 h.

Miscellaneous—Western blots were performed according to the protocol of the ECL antibody detection system from Amersham Corp. Sample loading was denatured by SDS sample buffer and were electrophoresed on 12% polyacrylamide Mini-1Gels (Amersham). The gel was stained with Coomassie Blue as described previously (16, 18). The blot was air-dried, and the specific protein bands were cut out and subjected to a quantitative amino acid analysis. A background relative to the width of the band was subtracted from the measured amounts of amino acids. After the electrophoresis was completed, the gel was stained by Coomassie Blue, and the efficiency of the transfer was estimated by the method of each subunit.

Preparation of Crude Synaptic Vesicles from Various Brain Parts—Fresh bovine brain was obtained from a local slaughter house and crude synaptic vesicles were prepared according to a procedure developed for synaptic vesicle preparation from rat brains (22). About 12 g of brain tissue was homogenized by Dounce homogenizer in 25 ml of solution containing 0.32 M sucrose, 10 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 0.1 mM MgCl2, 5 mM a-glycerophosphate, 0.1 mM EGTA, 10 mM Hepes-KOH (pH 7.5), and 0.3 mM phenylmethylsulfonyl fluoride. Cell debris and nuclei were sedimented at 1000 × g for 10 min, and the synaptosomal pellet was obtained by centrifugation at 25,000 × g for 13 min. The pellet was washed by resuspension in Heps-buffered saline (142 mM NaCl, 2.4 mM KCl, 1.1 mM MgCl2, 5.0 mM a-glycerophosphate, 0.1 mM EGTA, 10 mM Hepes-KOH (pH 7.5), and 0.3 mM phenylmethylsulfonyl fluoride). After centrifugation, the pellet was suspended in 2 ml of the same buffer and was lysed by dilution into 10 volumes of H2O. After incubation for 30 min on ice, large membrane fragments were removed by centrifugation at 40,000 × g for 15 min. Synaptic vesicles and small membrane fragments were recovered by centrifugation of the supernatant at 140,000 × g for 4 h.

Results

When purified V-ATPase from chromaffin granule membranes was analyzed on polyacrylamide SDS gel, we always observed a diffused band between subunits B and C at the position of about 45 kDa (see Fig. 4 in Ref. 9). Because it is diffused, the protein gave the impression of being at substoichiometric amounts in the preparation. However, when we performed a V8 partial cleavage of this diffused band, we obtained a sharp protein band of about 800 kDa that appeared to be present in an equivalent amount with the other subunits of the vacuolar ATPase. The N-terminal sequence of this polypeptide encoded a protein of 468 amino acids with a molecular mass of 80 kDa. A search for open reading frames that potentially encoded amino acid sequence of the cDNA encoding this polypeptide indicated that the open reading frame of about 45 kDa (see Fig. 4 in Ref. 9). Because it is present in an equivalent amount with the other subunits of the vacuolar ATPase, the N-terminal sequence of this polypeptide was determined, and it had no homology to any known protein.

An open reading frame that potentially encoded a protein of 468 amino acids was deduced from the cDNA encoding this polypeptide denoted as Ac45. An open reading frame that potentially encodes a protein of 468 amino acids with a molecular mass of 51,786 daltons is apparent in the cDNA sequence. The amino acid sequence of the polypeptide reveals a potential signal sequence of 35 amino acids. The absence of this signal sequence will leave a mature protein with a molecular mass of about 48 kDa. All amino acids obtained from the N-terminal sequence of the 10 kDa V8-cleaved protein appear in the open reading frame. They follow a typical V8 cleavage site after glutamic acid. The hydropathy plot, shown in Fig. 2, indicates the presence of a potential transmembrane helix at the C-terminal end of the protein. Between the potential signal sequence and the transmembrane helix in the C terminus of the protein, there are
eight potential glycosylation sites. The V8 cleavage polypeptide contains only one potential glycosylation site that is very close to the transmembrane helix at the end of the protein. These features suggest that the protein is synthesized with a signal sequence that directs the protein into the ER and, following cleavage and glycosylation, the protein is hooked up in the membrane with the C-terminal helix, leaving the glycosylation site in the luminal side of the membrane.

Expression of the cDNA encoding Ac45 in reticulocyte lysate supports the scenario described above. As shown in Fig. 3, the expressed protein migrates on SDS-polyacrylamide gel at a position of about 50 kDa. Inclusion of dog pancreas microsomes during the cell-free synthesis of the protein significantly decreased its mobility on the gel, suggesting that the protein was glycosylated. This notion is supported by the observation that upon treatment with peptide:N-glycosidase F, the migration of the protein returned to its original position. Furthermore, the glycosylated protein was protected from digestion with proteinase K, suggesting that it is present in the luminal side of the microsomes.

To obtain more information on the localization of Ac45, we raised a specific antibody against a recombinant fusion protein and affinity-purified it on an Affi-Gel column to which the fusion protein was bound. Antibodies against the maltose-binding protein were removed by passing the antibody through an Affi-Gel column containing the purified maltose-binding proteins. Following solubilization of chromaffin granules by Triton X-100 and sucrose gradient centrifugation, the collected fractions were subjected to Western analysis with specific antibodies against different subunits of the enzyme. The Western blot analysis depicted in Fig. 4 clearly indicates that the Ac45 polypeptide copurified with the V-ATPase from chromaffin granules. As with all our subunit-specific antibodies, the antibody against Ac45 does not recognize the native polypeptide and therefore is not useful for immunoprecipitating the V-ATPase. Immunocytochemical staining of sections from bovine adrenal glands revealed intense staining in the medulla and almost no staining in the cortex (not shown). Further indication of the localization of Ac45 was obtained by performing Western analysis with preparations of membranes and purified enzymes from different sources (Fig. 5).}

As expected, the highest content of V-ATPase was detected in the pituitary gland, which contains high amounts of secretory granules.

To assess the relative amount of Ac45 versus the other subunits of V-ATPase, we have carried out quantitative amino acid analysis on the isolated polypeptide obtained by SDS-polyacrylamide gel electrophoresis and electrotransfer to Immobilon.
**New Subunit of the Membrane Sector of V-ATPase**

**Fig. 5.** The Ac45 protein is present in chromaffin granules and kidney microsomes. SDS-polyacrylamide gel electrophoresis, electrophoresis, electro-transfer onto nitrocellulose filters, and antibody detection were performed as described under “Experimental Procedures.” Lane 1, chromaffin granule membranes containing about 25 μg of protein. Lane 2, about 3 μg of purified V-ATPase from bovine kidney microsomes. Lane 3, about 10 μg of mouse brain synaptic vesicles. Left, antibody against subunit A of V-ATPases from bovine chromaffin granules. Right, antibody against bovine chromaffin granules Ac45.

**Fig. 6.** Distribution of Ac45 in various parts of the bovine brain. Crude synaptic vesicles were prepared from different brain parts as described under “Experimental Procedures.” Membranes containing about 20 μg of protein were dissociated in SDS buffer and electrophoresed on 12.5% polyacrylamide gels. One of the gels was stained by Coomassie Blue, and two of them were electrotransferred onto nitrocellulose filters and incubated with the indicated affinity-purified antibody as described under “Experimental Procedures.” S, Coomassie Blue-stained gel. A, antibody against subunit A of V-ATPase from chromaffin granules. Ac45, antibody against Ac45 from chromaffin granules. Lanes 1, white matter from cerebral cortex. Lanes 2, gray matter from cerebral cortex. Lanes 3, locus coeruleus. Lanes 4, cerebellum. Lanes 5, pituitary gland.

by the method of Matsudaira (13). This procedure was previously used to determine the relative stoichiometry of the subunits in coated vesicles V-ATPase complex (8). The observed amounts (in pmol) of amino acids in each protein band was normalized according to the amino acid composition of the corresponding subunit. The amino acid composition was obtained from published reading frames of cloned cDNAs as follows: Ac115 from rat brain (21); bovine subunit A (24); bovine subunit B (25); bovine subunit C (14); bovine Ac39 (17 corrected in GenBank J04204); bovine subunit D (26). The following amino acids (in single-letter code) were used for the subunit stoichiometry calculation: D, N, E, Q, S, T, A, V, I, L, Y, F, and K. The relative amount of the various subunits was normalized to subunit C. The calculated relative amounts of the various subunits without correction for the efficiency of the electrophoresis were as follows: 2.4 Ac115, 2.1 A, 2.3 B, 1.1 C, 1.6 Ac39, 0.6 D, 0.7 E, and 1.3 Ac45. After correction for the efficiency of the electrophoresis, the calculated relative amounts were as follows: 2.6 Ac115, 2.1 A, 2.8 B, 1.1 C, 1.6 Ac39, 1 D, 1.1 E, and 1.3 Ac45. The reported general organization of V-ATPase consisting of 3 A (73 kDa in Ref. 8), 3 B (58 kDa), 1 C

**DISCUSSION**

Studies of yeast mutants lacking genes encoding specific subunits of V-ATPase suggest that the assembly of the enzyme starts with its membrane sector. It was shown that the catalytic sector cannot be assembled in the absence of the proteolipid that is part of the membrane sector (5, 6). On the other hand, the proteolipid can be assembled into the membrane without the presence of an assembled catalytic sector. In yeast, except for Ac115, only a single gene encodes each of the V-ATPase subunits (27-33). Therefore, there is an enigma of the V-ATPase assembles correctly in the numerous organelles present in mammalian cells and membranes. The question of what directs V-ATPase to such diverse organelles as chromaffin granules, synaptic vesicles, lysosomes, endosomes, and plasma...
membranes has no apparent answer. The membrane sector of V-ATPases consists of a very conserved polypeptide called the proteolipid that is implicated directly in proton conductions across the membrane as well as two less conserved polypeptides, Ac115 and Ac39 (17, 23, 34, 35). These polypeptides may be present in all known V-ATPases of eukaryotic cells. It is known that Ac115 is a genuine glycosylated membrane protein containing few transmembrane helices and is facing the luminal side of the organelles (23). The Ac39, on the other hand, is facing the cytoplasmic side of the membrane and contains no potential transmembrane helices (8, 17). It is most probably associated with another subunit of the membrane sector. It is likely that we are still missing subunits of the membrane sector that will be present in all membrane sectors of V-ATPases of eukaryotic cells. One may be analogous to subunit a of F-ATPases. All of these subunits together are going to participate in the fundamental function of the membrane sector, namely, proton conduction and energy coupling with the catalytic sector. Consequently, specific membrane proteins are likely to be required for the correct assembly of the different membrane sectors in different organelles.

The distribution of Ac45 in the adrenal gland shows a high concentration in the medulla and little, if any, in the cortex. Observations at higher magnification indicated that the protein is concentrated in chromaffin cells and not in blood vessels or other cells of the medulla. The experiments described in Fig. 3 shed some light on the biogenesis and orientation of the protein. The translation product of the cDNA migrates on gels at a position of about 51 kDa, which is close to the molecular mass calculated from the open reading frame. Inclusion of dog pancreas microsomes shifted the migration of the protein in SDS gels to a position of about 70 kDa, indicating that at least some potential glycosylation sites are being utilized. The slow migrating protein band, but not the band at the original position, is protected from digestion by protease. This suggests that Ac45 is translocated into the dog pancreas microsomes during its cell-free translation from the synthetic mRNA. It is worth noting that after the removal of the sugars from the protein by proteases, the glycosidase F, the protein migrated to an identical position of 51 kDa on the gel as the protein synthesized in the culture protein. This may not apply to the processing of the Ac45 proteolipid that is implicated directly in proton conductions facing the cytoplasmic side of the membrane and contains no functional homologues of Ac115 (37). It is likely that mammalian cells also contain more than one Ac115, and the presence or absence of these homologues may modulate the activity of the enzyme in the various organelles and membranes.

Acknowledgments—We thank Dr. Stephen Gluck for providing the purified ATPase from kidney microsomes and plasma membranes, and we thank Kurt Höffelder and Doreen J. Čiolk for protein analysis.

REFERENCES

1. Nelson, N. (1990) Biochim. Biophys. Acta 1060, 109-124
2. Henkem, P., Gun, X.-L., Wang, Z.-Q., Zhang, K., and Gluck, S. (1992) J. Biol. Chem. 267, 9948-9957
3. Nelson, N. (1990) Bioenerg. Biomembr. 24, 407-414
4. Bowman, B. J., Docherty, W., Harris, T., and Bewnam, E. J. (1989) J. Biol. Chem. 264, 15066-15072
5. Nomiki, T., Beltrão, C., Nelson, H., and Nelson, N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1938-1942
6. Kane, P. M., Kuehn, M. C., Howald-Stevenson, I., and Stevens, T. H. (1992) J. Biol. Chem. 267, 447-454
7. Sze, H., Ward, J. M., Lai, S., and Perera, I. (1992) J. Exp. Biol. 173, 223-235
8. Ash, H., Terres, G., Pink, S., and Forgue, M. (1988) J. Biol. Chem. 263, 8796-8802
9. Moriyama, Y., and Nelson, N. (1989) J. Biol. Chem. 264, 3577-3582
10. Moriyama, Y., and Nelson, N. (1989) Biochim. Biophys. Acta 990, 241-247
11. Moriyama, Y., and Nelson, N. (1989) J. Biol. Chem. 264, 18441-18449
12. Moriyama, Y., and Nelson, N. (1987) J. Biol. Chem. 262, 9175-9180
13. Matsuoka, P. (1987) J. Biol. Chem. 262, 10035-10038
14. Nelson, H., Mandiy, S., Nashi, T., Yamanaka, Y., Miedel, M., and Nelson, N. (1990) J. Biol. Chem. 265, 20860-20863
15. Lathe, R. (1985) J. Mol. Biol. 180, 1-12
16. Bellrín, U., Kopecky, J., Pan, Y.-C., Nelson, H., and Nelson, N. (1992) J. Biol. Chem. 267, 37877-37882
17. Wrang, S.-Y., Moriyama, Y., Mano, M., Hulmes, J. E., Pan, Y.-C., Danho, W., Nelson, H., and Nelson, N. (1990) J. Biol. Chem. 265, 17638-17642
18. Sambrook, J., Prites, E., F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Henikoff, S. (1984) Gene (Amst.) 28, 361-369
20. Nelson, N. (1983) Methods Enzymol. 77, 50-513
21. Pringle, J. R., Adams, A. E. M., Dabora, D. G., and Haar, B. K. (1991) Methods Enzymol. 194, 565-602
22. Bennett, M. K., Caleske, N., Kreider, T., and Scheller, R. H. (1992) J. Biol. Chem. 267, 761-775
23. Perin, M. S., Fried, V. A., Stone, D. K., Xie, X.-S., and Sudhof, T. C. (1991) J. Biol. Chem. 266, 3877-3881
24. Puopolo, X., Kamatomo, C., Adachi, I., and Forgac, M. (1991) J. Biol. Chem. 266, 24544-24572
25. Puopolo, X., Kamatomo, C., Adachi, I., Magner, R., and Forgac, M. (1992) J. Biol. Chem. 267, 3699-3706
26. Hirsh, S., Strauss, A., Masood, K., Lee, S., Sukhatme, V., and Gluck, S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3004-3008
27. Nelson, H., Mandiy, S., and Nelson, N. (1989) J. Biol. Chem. 264, 1775-1778
28. Nelson, H., and Nelson, N. (1989) FEBS Lett. 247, 147-153
29. Nelson, H., and Nelson, N. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5033-5037
30. Hirota, R., Ohnami, Y., Nakano, A., Kawasaki, H., Suzuki, K., and Amraku, Y. (1990) J. Biol. Chem. 265, 6732-6733
31. Beltrán, C., and Nelson, N. (1992) Acta Physiol. Scand. 140, 41-47
32. Forsy, F. (1990) J. Biol. Chem. 265, 18554-18560
33. Stevens, T. H. (1990) J. Biol. Chem. 265, 47-55
34. Manolos, M. F., Proteza, D., Preston, R. A., Stenbit, A., Roberts, B. M., Preuss, D., Mandiy, S., Kubota, D., and Jones, E. W. (1992) J. Biol. Chem. 267, 14294-14300.
35. Mandel, M., Moriyama, Y., Hulmes, J. E., Pan, Y.-C. E., Nelson, H., and Nelson, N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5521-5524
36. Freeze, H. H. (1993) Curr. Prot. Mol. Biol. 2, 1-18
37. Mandian, M. F., Wu, R., Tubbs, D. L., Talton, R. E., Roberts, B. T. M., and Jones, E. W. (1994) J. Biol. Chem. 269, 14094-14074
38. Eyto, J., and Doolittle, R. F. (1982) J. Biol. Chem. 257, 101-103