Biochemical and Biophysical Research Communications **258,** 162–167 (1999)
Article ID bbrc.1999.0544, available online at http://www.idealibrary.com on IDEAL®

Cloning and Functional Activity of a Novel Truncated Form of Annexin IV in Mouse Macrophages[1]

Carol L. Sable*,†,‡,[2] and David W. H. Riches*,†,‡,[2]

*Division of Basic Sciences, Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado 80206; and *Department of Pharmacology, ‡Department of Biochemistry and Molecular Genetics, and †Department of Immunology and Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262*

Received March 21, 1999

Annexin IV was cloned and sequenced from a mouse bone marrow-derived macrophage cDNA library, and was found to exist as three different alternatively spliced transcripts. One transcript contained an additional 688 base pairs inserted within the coding region of the gene including an in-frame stop codon. Translation of this transcript *in vitro* confirmed the premature arrest of translation which resulted in a truncated annexin IV protein of approximately 22 kDa. Like other members of the annexin family, the product of the wild-type annexin IV transcript bound in a calcium-dependent manner to both phenyl-sepharose and phospholipid vesicles. In contrast, the truncated annexin IV product bound to these substrates in a Ca^{2+}-independent fashion. The existence of a novel form of annexin IV in mouse macrophages may aid in further defining the role of members of the annexin family. © 1999 Academic Press

Members of the annexin family of Ca^{2+}-dependent phospholipid binding proteins are ubiquitously distributed in eukaryotic organisms (1). Currently encompassing some thirteen members, the specific functions of many of these proteins still remain unclear although they have been implicated in a large number of biological processes including blood coagulation (2), regulation of cytoskeletal organization (3), membrane fusion events (3), and the formation of Ca^{2+}-selective ion channels (4). At the structural level, all family members possess a conserved C-terminal region which consists of a four-fold repeat of a 70 amino acid domain (with the exception of annexin VI which has an eight-fold repeat) each containing a highly conserved 17 amino acid sequence termed the endonexin-fold (5). Studies have shown this motif to be responsible for the conserved Ca^{2+}-dependent phospholipid binding properties of the annexins (5, 6). The N-terminal regions of the molecules are structurally diverse and are believed to be responsible for the more specialized functions of the annexins.

Annexin IV is functionally one of the less well-defined annexins, although recently it has been implicated in the regulation of chloride channels (7). Annexin IV is expressed in a variety of cell types (8) including mouse bone marrow-derived macrophages. Although giving rise to a single translated protein, its mRNA exists as two predominant species of 1961 bp and 1179 bp, which have arisen through the use of different polyadenylation sites in the 3′-untranslated region (Sable and Riches, unpublished observations). In this paper, we report on the cloning and sequencing of a unique annexin IV variant from a mouse bone marrow macrophage cDNA library.

MATERIALS AND METHODS

Materials. All chemical compounds, unless indicated, were purchased from Sigma Chemical Co., St. Louis, MO. Radiochemicals were from New England Nuclear, Boston, MA. Phospholipids were from Avanti Polar Lipids, Alabaster, AL. Invitrogen Corporation, San Diego, CA constructed the mouse macrophage cDNA library. cDNA for annexin IV was kindly provided by Dr. Carl Creutz, and cDNA for GAPDH was a generous gift from Dr. John Shannon. Bone marrow-derived macrophages from C3H/HeJ mice were cultured as previously described (9) for 5 days.

Construction and screening of mouse bone marrow macrophage cDNA library. A cDNA library was constructed using RNA isolated from interferon-primed C3H/HeJ mouse bone marrow macrophages and cloned into pcDNA I. The library was plated on 150 mm LB/amptet plates and screened using a random-primer labeled bovine annexin IV cDNA probe. DNA was isolated from positive clones was sequenced by the dideoxy chain termination method (10). Unknown sequences were aligned to those in the GenBank database using the BLAST search program (11).

[1] This work was supported by Public Health Service SCOR Grant HL56556 from the National Institutes of Health.

[2] To whom correspondence should be addressed at Division of Basic Sciences, National Jewish Medical and Research Center, Neustadt Rm D405, 1400 Jackson Street, Denver, CO 80206. Fax: (303) 398-1381. E-mail: richesd@njc.org.

0006-291X/99 $30.00
Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

Polymerase chain reaction of reverse transcribed RNA. One microgram portions of total RNA were reverse transcribed using oligo-dT to prime first strand synthesis. Each PCR contained 1 μM each of the upstream and downstream primers, 100 μM of each dNTP, 50 mM KCl, 10 mM Tris, pH 9.0, 0.1% (v/v) Triton X-100, 2 mM $MgCl_2$, and 2 U Taq polymerase. The amplification profile consisted of denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds, and elongation at 75°C for 1.5 minutes.

In vitro transcription and translation of annexin IV cDNA clones. cDNA clones were transcribed and translated *in vitro* using a coupled transcription/translation system. Transcription was initiated at a T7 RNA polymerase binding site and this transcript was directly translated in a rabbit reticulocyte lysate system in the presence of ^{35}S-methionine.

Phenyl Sepharose column chromatography. Macrophages were labeled with 100 μCi/ml of [^{35}S]-methionine for 4 hr before homogenization in 10 mM Hepes, pH 7.4, 150 mM NaCl (buffer A), containing 5 mM EGTA, 1 μg/ml leupeptin, and 0.1 mM PMSF. After centrifugation for 30 min at 100,000 $\times$ g the pellets were resuspended in homogenization buffer and adjusted to 1 mM $CaCl_2$. These samples were chromatographed over a 1 ml phenyl-sepharose column equilibrated in Buffer A containing 1 mM $CaCl_2$. Annexins were eluted by the substitution of 5 mM EGTA for Ca^{2+} in the buffer. A 0.1% SDS solution was used to dissociate all remaining proteins from the column and the peaks of radioactivity were pooled and analyzed by SDS–PAGE.

Phospholipid binding assay. Liposomes composed of a 1:1 ratio of either phosphatidylcholine/phosphatidylethanolamine or phosphatidylserine/phosphatidylethanolamine were prepared as described (12). Liposomes (300 μg) were incubated with 10 μl of [^{35}S]-labeled annexins in the presence of either 5.5 mM $CaCl_2$ or 5 mM EGTA for 10 min at room temperature. Proteins which bound to lipids were separated from unbound proteins by centrifugation at 150,000 $\times$ g for 30 min. The pellets and supernatants were analyzed by SDS–PAGE.

RESULTS

Cloning of a Novel Annexin IV Transcript from Mouse Macrophages

A bovine annexin IV cDNA probe was used to screen a cDNA library constructed from mouse IFNβ-primed macrophage RNA. Three different types of clones were isolated from this screen. Two of these, annexin IVA (ANX IVA) and annexin IVB (ANX IVB), were identical in their 5′ untranslated regions and in their open reading frames but contained differences in the 3′ untranslated region due to the use of alternative polyadenylation sites (C. L. Sable and D. W. H. Riches, unpublished observations). The third type of clone, annexin IVC (ANX IVC), differed from previously cloned annexin IV's in that it contained 688 bp of additional sequence inserted within the coding region proximal to the start of the third endonexin repeat. The deduced amino acid sequence of ANX IVC and its alignment with the full length protein are illustrated in Figure 1A. The first 176 amino acids of ANX IVC which include the N-terminal variable region and annexin domains I and II, were identical to the full length annexin IV. However, at a point 18 amino acids distal to the start of annexin domain III, an additional 20 amino acids, including a total of 9 hydrophobic amino acids, were found to be inserted followed by a termination codon thus yielding a protein with a predicted molecular weight of 21.9 kDa. The nucleotide sequence of the inserted region is shown in Figure 1B. This sequence is unique in that it bears no significant homology to any previously cloned genes, as determined by comparisons with known sequences using the BLAST program, indicating that the sequence is part of an unique annexin IV transcript. In addition, the inserted region contained a dinucleotide repeat, comprising a 27-fold CT repeat and a 9-fold TG repeat.

FIG. 1. (A) Deduced amino acid sequence and compartive alignment of full length mouse annexin IV and the truncated ANX IVC protein. The unique additional 20°C-terminal amino acids are overlined. (B) Nucleotide sequence of the inserted region in ANX IVC. The stop codon is indicated in bold. The long repeat sequence is underlined.

Northern analysis and PCR cloning and sequencing were used to confirm that mouse macrophages contain a transcript for ANX IVC. Northern blots of total macrophage RNA were probed with a radiolabeled cDNA probe derived from the unique sequence in ANX IVC (Figure 2) and resulted in the detection of a single band (Figure 3). cDNA corresponding to ANX IVC was also cloned from mouse macrophages by reverse transcrip-

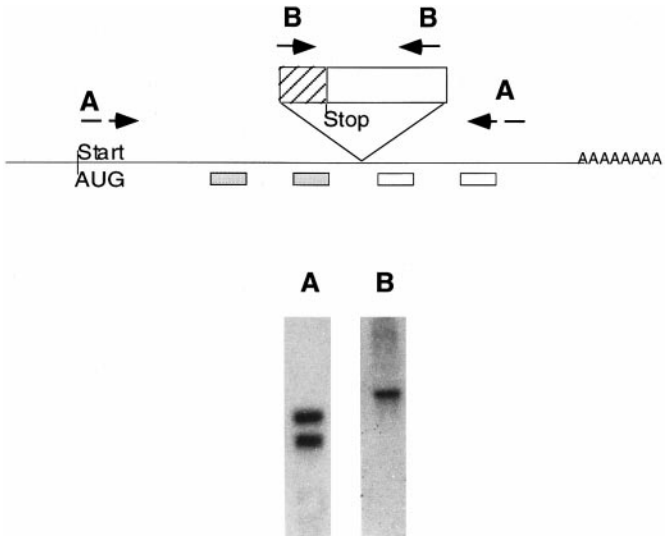

FIG. 2. Northern analysis of mouse macrophage RNA using cDNA probes specific for ANX IVC sequences. Radiolabeled cDNA probes were generated which were complementary to (A) wild-type annexin IV sequences or (B) unique sequences which are only found in ANX IVC. Northern blots containing mouse macrophage RNA were hybridized with these probes and the corresponding autoradiographs are presented.

tion and PCR. The primers used in the PCR were designed such that one primer from each set hybridized to wild-type annexin IV sequences, and the other primer from the set hybridized to sequences within the 688 additional bp in ANX IVC. The PCR gave the predicted products of 980 bp and 480 bp (Figure 3) whose sequences were identical to those in ANX IVC. To more accurately investigate the characteristics of the annexin IV transcript containing the unique sequences, the cDNA encoding ANX IVC was transcribed and translated *in vitro* using a coupled transcription-translation system in a rabbit reticulocyte lysate. Both the full length and the ANX IVC sequences were efficiently transcribed and translated in the rabbit reticulocyte lysate system. The wild-type clone gave rise to the expected product of 32.5 kD and ANX IVC resulted, as predicted, in the synthesis of a truncated protein of 23 kD (Figure 4).

The Truncated Annexin IV Binds to Phospholipid in a Ca^{2+}-Independent Fashion

Because the truncated annexin IV is missing most of the third and all of the fourth annexin repeats, we investigated the functional consequences of this loss. Two biochemical approaches, using *in vitro*-translated proteins, were used to analyze the binding properties of the wild-type and truncated annexin IV molecules, namely, (i) hydrophobic column chromatography over phenyl-sepharose and (ii) liposome binding. As predicted, the wild type annexin IV bound to the column and was specifically eluted with EGTA (Figure 5C). In contrast, the truncated annexin IV bound tightly to the column and could only be eluted with SDS (Figure 5A).

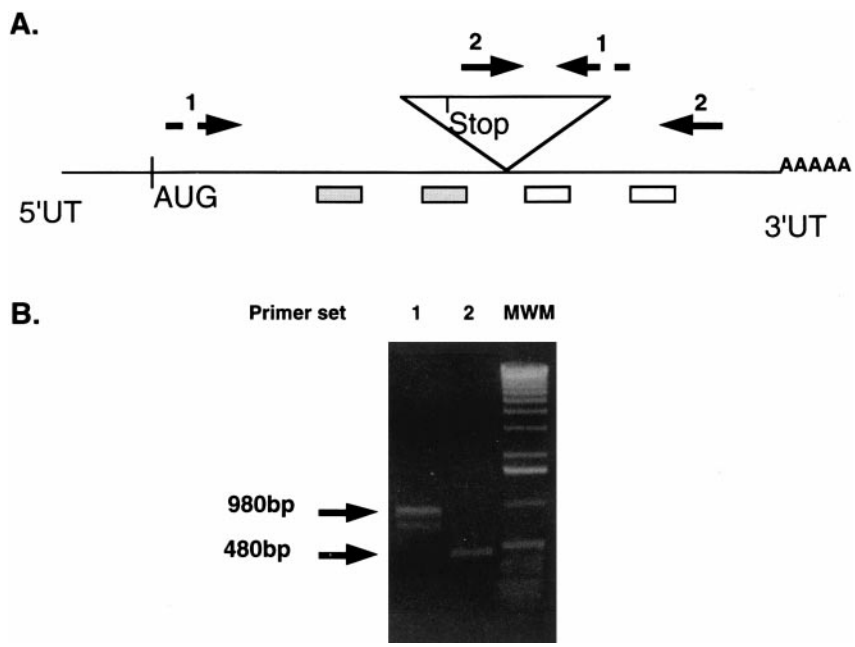

FIG. 3. Amplification of sequences corresponding to ANX IVC from reverse-transcribed mouse macrophage RNA. (A) Two different primer sets were designed to separately amplify the 5′ (1) and the 3′ (2) portions of the transcript. Within each primer set one primer bound to wild-type annexin IV sequences, and one to bind to ANX IVC specific sequences. (B) Ethidium-bromide stained agarose gel of the PCR amplification products. Bands of the predicted sizes obtained from the two PCRs are indicated.

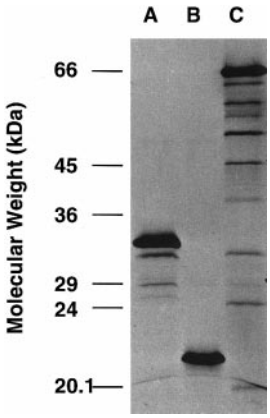

FIG. 4. *In vitro* transcription and translation of a novel annexin IV transcript. cDNA clones of murine annexin IV were transcribed and translated *in vitro* in a rabbit reticulocyte lysate system in the presence of [^{35}S]-methionine. Labeled products were analyzed by SDS–PAGE and autoradiography. Lane: (A) wild-type clone, (B) ANX IVC, (C) control (luciferase).

Liposomes composed of either phosphatidylserine: phosphatidylethanolamine or phosphatidylcholine: phosphatidyl-ethanolamine were incubated with [^{35}S]-methionine-labeled translated annexin IV proteins in either the presence or absence of Ca^{2+}. Protein which bound phospholipid was separated from unbound protein by high-speed centifugation. The pellets and supernatants were analyzed by SDS–PAGE. In the presence of 5.5 mM Ca^{2+}, more than 90% of the wild-type annexin IV protein bound to phosphatidylserine-containing vesicles, and greater than 60% of the protein bound to phosphatidylcholine-containing vesicles (Figure 6) and when protein was incubated with vesicles in the presence of EGTA, greater than 85% of the annexin IV protein was found in the supernatant of phosphatidylserine-containing vesicles, and over 65% of the protein was in the supernatant of phosphatidylcholine-containing vesicles. In contrast, Ca^{2+} had no effect on the ability of the truncated annexin IV protein to bind vesicles containing phosphatidylcholine, as approximately equal amounts of protein were bound in either the presence of Ca^{2+} (45%) or EGTA (55%). Ca^{2+} did moderately enhance the binding of the truncated protein to phosphatidylserine-containing vesicles (65% bound with Ca^{2+}), but the effect was markedly diminished compared to the effect of Ca^{2+} on the binding of the wild-type protein.

Effect of 20-Amino Acid Truncation on Phenyl Sepharose Binding

The ability of the truncated annexin to bind to the column could be explained by (1) the loss of annexin repeats 3 and 4, or (2) the unique C-terminus of the truncated product which has a high abundance of hydrophobic amino acids. Sequence analysis revealed that the unique C-terminal 20 amino acids of the truncated protein contains 9 hydrophobic amino acids within a 14 amino acid stretch (VFQHSWIPDLFLTL). To test whether the unique 20 residues imparted enough hydrophobic properties to the truncated annexin IVC to explain the altered binding to the columns, an ANX-IVC protein lacking these residues was constructed (ANX IVC-u). When the protein from this clone was produced *in vitro* using the transcription/translation system in the presence of [^{35}S]-methionine, and chromatographed over phenyl-sepharose, its binding properties were identical to those of the truncated protein from ANX IVC (Figure 5B). In addition, the ANX IVC-u lacking the unique C-terminal amino acids bound liposomes in a manner indistinguishable from that of ANX IVC (data not shown) suggesting that the unusual phenyl-sepharose- and phospholipid-binding properties of the truncated annexin IV are not likely to be due to the addition of hydrophobic residues to the C-terminus of this protein, but rather may reflect the absence of annexin repeats 3 and 4.

DISCUSSION

In this report, we have shown that murine macrophages contain a transcript which codes for a novel, truncated form of annexin IV. The existence of this transcript was confirmed by: (1) cloning from a mouse macrophage cDNA library, (2) detection of a transcript in mouse total RNA using Northern analysis, (3) cloning from macrophage RNA using reverse transcription and PCR amplification, and subsequent sequencing of the amplified product. The novel annexin IV transcript differs from the mRNA encoding the wild-type protein by the presence of 688 bp within the coding region of

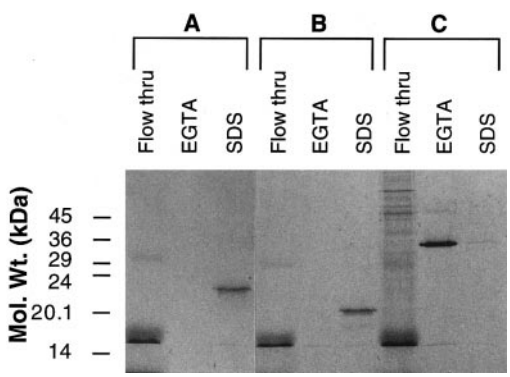

FIG. 5. Phenyl-sepharose column chromatography of murine annexin IV proteins produced *in vitro.* Annexin IV clones were transcribed and translated *in vitro* as described in Figure 6. Proteins were subjected to phenyl-sepharose chromatography as described in Methods. Fractions from flow-through, EGTA eluates, or SDS eluates were pooled, and proteins were precipitated using acetone and analyzed by SDS–PAGE. (A) ANX IVC; (B) ANX IVC-u; (C) Wild-type.

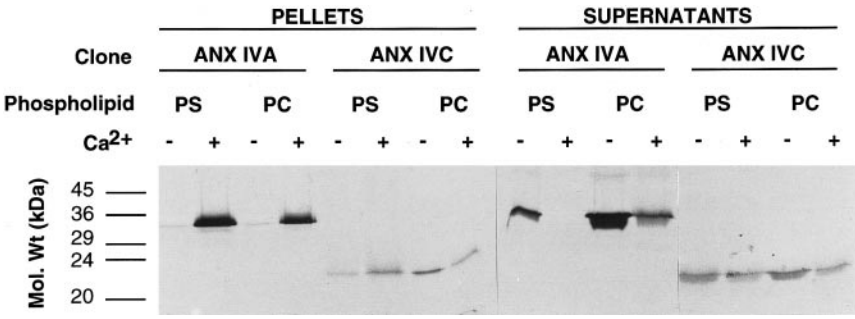

FIG. 6. Binding of annexin IV proteins to phospholipids. Wild-type (Clone IA) or truncated (ANX IVC) murine annexin IV proteins were transcribed and translated *in vitro* as described in Figure 5. Proteins were incubated in the presence of Ca^{2+} (+) or EGTA (−), with 1:1 mixtures of either phosphatidylethanolamine:phosphatidylserine (PS) or phosphatidylethanolamine-phosphatidylcholine (PC). Phospholipids were separated by centrifugation, and both the pellets and the supernatants were analyzed by SDS–PAGE for the presence of radiolabeled protein.

the gene. Sixty bp downstream of the start of this inserted sequence is an in-frame stop codon. Using *in vitro* translation, we have confirmed that this stop codon is utilized, and therefore the annexin IV protein from this transcript is truncated after the addition of 20 amino acids to the C-terminus that are not present in the wild-type protein.

We proposed that the inclusion of this 688 bp sequence within the unique annexin IV transcript is the result of an alternative splicing event. The genes of annexins I, II, VI, and VII (13–16) have been cloned, and, with the exception of annexin VII, exhibit a high degree of conservation of gene structure, especially in regards to intron/exon gene boundaries. Although the annexin IV gene has yet to be cloned, work done by Tait (17) has shown that the intron/exon boundaries in this gene conform to the same pattern as annexins I, II and VI. Alignments of the annexin I, II, and VI genes with the cDNA sequence of the novel murine annexin IV suggest that the position of insertion of the additional sequences corresponds exactly to an intron/exon boundary in the other genes. In addition, this site also corresponds to the site of an alternative splicing event in annexin VI (18). Sequences at the boundaries between introns and exons are highly conserved (19), and the sequences surrounding the inserted region in annexin IVC conform to recognized splice donor and acceptor sequences.

The ANX IVC transcript was transcribed and translated *in vitro* into a truncated 22 kD protein that contained only repeats 1 and 2. Phenyl-sepharose column chromatography and liposome binding, indicated that the truncated molecule had lost the ability to bind phospholipid in a Ca^{2+}-dependent manner. Elucidation of the crystal structures of annexins I (20) and V (6, 21–23) have demonstrated that there are multiple Ca^{2+}-binding sequences within the molecules. They are composed of a consensus sequence, GXGT(X38)D/E (6), which is found in annexin IV in repeats 1, 2, and 4. Mutational analysis of the Ca^{2+}-binding sites in annexin IV have revealed that the repeat 4 is most prominant in Ca^{2+}-dependent phospholipid binding (24). In addition, mutation of a highly conserved arginine at the C-terminus of the endonexin-fold in the repeat 4 also had profound effects on the ability of annexin IV to interact with membranes (24). These findings are consistent with our data demonstrating that the truncated annexin IV, lacking repeat 4, is defective in phospholipid binding.

Residues in repeat 3 have also been demonstrated to be important in regulating phospholipid binding of the annexins (25). In annexin V, trp187 is exposed upon binding of Ca^{2+} to the annexin, and is demonstrated to directly make contact with the membrane (21, 25). It is proposed that the conformational changes induced by binding of Ca^{2+} in domains 1, 2 and 4, which result in the exposure of trp187, are important in establishing membrane contact and anchoring the annexin to the membrane (21, 23). Therefore, the third annexin repeat is essential to membrane binding, which is also consistent with the reduced phospholipid-binding properties in the truncated annexin IV.

Since the full range of functions of annexin IV are still unknown, it is currently not possible to assign a role to the truncated version of the protein. However, some speculations can be made. Firstly, the truncated protein may playing a regulatory role by removing the functional activities of the full-length annexin IV, i.e., by acting as a dominant negative inhibitor. The lack of Ca^{2+}-dependent phospholipid-binding activities in the truncated annexin is consistent with this function. Secondly, the truncated protein may have a distinct cellular function. To elucidate this function we have attempted to make cell lines which overproduce the truncated annexin IV, however, to date none of the transfections have yielded viable cells.

In summary, we have described a novel form of annexin IV transcript in mouse macrophages. Although the protein product corresponding to this transcript has not been detected, several unique features of the RNA warrant further investigation. In addition, if a truncated protein is produced, its lack of Ca^{2+}-

dependent phospholipid-binding properties make it a novel member of the annexin family.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical support provided by Linda Remigio. We also wish to thank Dr Carl Creutz for generously providing the bovine cDNA probed that was used to screen the mouse macrophage cDNA library. Lastly, we thank Nadia de Stackleberg, Barry Silverstein and Leigh Landskroner for help in the preparation of the illustrations and photography.

REFERENCES

1. Raynal, P., Hullin, F., Ragab-Thomas, J. M. F., Fauvel, J., and Chap, H. (1993) *Biochem. J.* **292,** 759–765.
2. Funakoshi, T., Heimark, R. L., Hendrickson, L. E., McMullen, B. A., and Fujikawa, K. (1987) *Biochemistry* **26,** 5572–5578.
3. Ikebuchi, N. W., and Waismann, D. M. (1990) *J. Biol. Chem.* **265,** 3392–3400.
4. Creutz, C. E., Pazoles, C. J., and Pollard, H. B. (1978) *J. Biol. Chem.* **253,** 2858–2866.
5. Geisow, M. J. (1986) *FEBS Lett.* **203,** 99–103.
6. Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Romisch, J., and Paques, E. (1992) *J. Mol. Biol.* **223**(3), 683–704.
7. Kaetzel, M. A., Chan, H. C., Dubinsky, W. P., Dedman, J. R., and Nelson, D. J. (1994) *J. Biol. Chem.* **269,** 5297–5302.
8. Kaetzel, M. A., Pula, G., Campos, B., Uhrin, P., Horseman, N., and Dedman, J. R. (1994) *Biochem. Biophys. Acta* **1223,** 368–374.
9. Riches, D. W. H., Henson, P. M., Remigio, L. K., Catterall, J. F., and Strunk, R. C. (1988) *J. Immunol.* **141,** 180–188.
10. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**(12), 5463–5467.
11. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**(3), 403–410.
12. Johnston, P. A., Perin, M. S., Reynolds, G. A., Wasserman, S. A., and Sudhof, T. C. (1990) *J. Biol. Chem.* **265,** 11382–11388.
13. Horlick, K. R., Cheng, I. C., Wong, W. T., Wakeland, E. K., and Nick, H. S. (1991) *Genomics* **10,** 365–374.
14. Shirvan, A., Srivastava, M., Wang, M. G., Cultraro, C., Magdenzo, K., McBride, O. W., Pollard, H. B., and Burns, A. L. (1994) *Biochemistry* **33,** 6888–6901.
15. Smith, P. D., Davies, A., Crumpton, M. J., and Moss, S. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2713–2717.
16. Spano, M., Raugei, G., Palla, E., Colella, C., and Melli, M. (1990) *Gene* **95,** 243–251.
17. Tait, J. F., Smith, C., Frankenberry, D. A., Miao, C. H., Adler, D. A., and Disteche, C. M. (1992) *Genomics* **12,** 313–318.
18. Moss, S. E., and Crumpton, M. J. (1990) *FEBS Lett.* **261,** 299–302.
19. Breitbart, R. E., Andreadis, A., and Nadal-Ginard, B. (1987) *Annu. Rev. Biochem.* **56,** 467–495.
20. Weng, X., Luecke, H., Song, I. S., Kang, D. S., Kim, S. H., and Huber, R. (1993) *Protein Sci.* **2**(3), 448–458.
21. Sopkova, J., Renouard, M., and Lewit-Bentley, A. (1993) *J. Mol. Biol.* **234**(3), 816–825.
22. Huber, R., Romisch, J., and Paques, E. P. (1990) *EMBO J.* **9**(12), 3867–3874.
23. Concha, N. O., Head, J. F., Kaetzel, M. A., Dedman, J. R., and Seaton, B. A. (1993) *Science* **261,** 1321–1324.
24. Nelson, M. R., and Creutz, C. E. (1995) *Biochemistry* **34**(9), 3121–3132.
25. Meers, P. (1990) *Biochemistry* **29**(13), 3325–3330.