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Albumin Is a Major Protein Component of Transverse Tubule Vesicles Isolated from Skeletal Muscle*

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Despite multiple procedures used to isolate transverse tubule vesicles from rabbit skeletal muscle, few proteins have been identified and shown to be specific to transverse tubule vesicles. Markers for purified transverse tubules have included high affinity dihydropyridine binding, cholesterol content, Mg^{2+}-ATPase activity, (Na^+,K^+)-ATPase activity, and [^{3}H] ouabain binding. Despite these markers, few proteins from purified transverse tubules can be unequivocally identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In this report we have biochemically and immunologically identified rabbit albumin as a major component of purified transverse tubule membranes from rabbit skeletal muscle. Albumin composed between 5.1 and 9.8% ($n = 4$) of the total protein in purified transverse tubules based on scans of SDS-PAGE. Furthermore, albumin and other serum proteins are present in preparations of transverse tubules and triads but not in light sarcoplasmic reticulum. Extraction of triads with low concentrations of saponin or sodium dodecyl sulfate completely removes albumin without removing intrinsic membrane proteins. Our results suggest that albumin and other serum proteins are present in the lumen of preparations of transverse tubules and albumin may be used as a marker for the transverse tubules when analyzed on SDS gels.

In skeletal muscle fibers action potentials are conducted into the interior of the fiber via invaginations of the plasmalemma known as transverse tubules (see Refs. 1 and 2 for review). Depolarization of the transverse tubules, through an unknown mechanism, results in the release of calcium from the sarcoplasmic reticulum at the junction between transverse tubules and terminal cisternae known as the triad junction. This process is known as excitation-contraction coupling. Two of the molecular components thought to be involved in excitation-contraction coupling have recently been identified. These are the calcium release channel or ryanodine receptor of the sarcoplasmic reticulum (3, 4) and the dihydropyridine receptor of the transverse tubules (5). Although the α_1-subunit of the dihydropyridine receptor has been shown to be required for excitation-contraction coupling (6, 7), the mechanism by which it interacts with the sarcoplasmic reticulum or the calcium release channel is not known.

Multiple procedures have been developed to isolate transverse tubules in order to identify and study protein components which may be involved in excitation-contraction coupling. Procedures include loading sarcoplasmic reticulum vesicles with calcium phosphate (8) or calcium oxalate (9) prior to sucrose gradient centrifugation or French press treatment of triads (10) to separate T-system[1] from sarcoplasmic reticulum. However, even when this purified T-system is analyzed by SDS-PAGE and stained with Coomassie Blue few if any bands can be identified as specific to the transverse tubules. Even the subunits of the dihydropyridine receptor cannot be identified on SDS gels of the purified T-system. We now report that one of the major constituents of purified transverse tubule vesicles from rabbit skeletal muscle is serum albumin. The possible function of albumin in transverse tubules is discussed.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Skeletal Muscle Membranes—Transverse tubular vesicles were isolated using a modification of the method of Rosemblatt *et al.* (8). The skeletal muscle from the back and hind limbs of an adult rabbit was dissected and homogenized at high speed for 1 × 30 and 1 × 20 s in a Waring blender at 4 °C in 4 volumes of Buffer A (0.3 M sucrose, 20 mM Tris-maleate, pH 7.0) with the following protease inhibitors: 76.8 μM aprotinin, 0.83 mM benzamidine, 1 mM iodoacetamide, 1.1 μM leupeptin, 0.7 μM pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride. All other buffers contained benzamidine, iodoacetamide, and phenylmethylsulfonyl fluoride at these concentrations. The homogenate was centrifuged for 20 min at 3120 × *g*, and the supernatant was again centrifuged in a Beckman JA 10 rotor for 20 min at 9950 × *g*. The supernatant was filtered through six layers of cheesecloth prior to the addition of solid KCl to a final concentration of 0.5 M and stirred for 10 min at 4 °C. The supernatant was pelleted at 140,000 × *g* in a Beckman type 45 Ti rotor and the pellets resuspended in Buffer A. Membranes were then washed two times with Buffer A by centrifugation at 140,000 × *g* for 30 min followed by resuspension in Buffer A. Membranes (5 ml or about 50–150 mg) were then layered onto six discontinuous sucrose gradients containing 18 ml of 35% and 17 ml of 28% sucrose (w/w). Gradients were centrifuged for 16–18 h in a Beckman SW 28 rotor at 112,700 × *g*. Purified transverse tubules were collected from the interface of the sample and the 28% sucrose, diluted 4–5-fold with ice-cold 20 mM Tris-maleate, pH 7.0, and centrifuged at 140,000 × *g*. Pellets were resuspended in Buffer A, frozen in liquid nitrogen, and stored at −135 °C.

Light sarcoplasmic reticulum vesicles were isolated by the method of Campbell *et al.* (11) with the following modifications. Crude sarcoplasmic reticulum vesicles were prepared as described (11), but 0.3 M sucrose with protease inhibitors was used at all steps instead of 0.25 M. Sarcoplasmic reticulum vesicles were loaded onto six continuous sucrose gradients (28–45%) and centrifuged for 18 h at 131,500 × *g* in a Beckman SW 28 rotor. Four fractions were collected and diluted 2–4-fold with ice-cold deionized distilled H_2O as described (11). Membranes were pelleted by centrifugation at 235,000 × *g* in a

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[1] The abbreviations used are: T-system, transverse tubules; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [^{3}H]PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^{3}H]methoxycarbonyl)pyridine-3-carboxylate.

Beckman Type 45 Ti rotor. Pellets were resuspended in Buffer A, frozen in liquid nitrogen, and stored at −135 °C. Adult rabbit triads were isolated by modification of the method of Mitchell *et al.* (12) as previously described (13). Isolated triads were treated for 20 min at room temperature with 0.05% saponin (0.5 mg/ml) in 50 mM Tris (pH 7.5), 500 mM NaCl, 0.23 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 0.64 mM benzamidine at a protein concentration of 1.0 mg/ml. Following centrifugation at 146,900 × *g* in a Beckman type 70.1 Ti rotor, the membranes were washed with 20 mM Tris-maleate, pH 7.4, plus protease inhibitors, recentrifuged at 146,900 × *g*, and resuspended in Buffer A plus protease inhibitors. All membrane preparations were analyzed for high affinity [^{3}H] PN200-110 (14) and [^{3}H]ryanodine binding (15). Protein was determined by the method of Lowry *et al.* (16) as modified by Peterson (17).

SDS-PAGE and Immunoblot Analysis—Protein samples were analyzed by SDS-PAGE (5–16% gradient or 7.5% gels) using the buffer system of Laemmli (18) and either stained with Coomassie Blue or transferred to nitrocellulose according to Towbin *et al.* (19). Coomassie Blue-stained gels of nonreduced transverse tubules were scanned using a Hoefer GS-300 scanning densitometer. The peak representing albumin was identified based on the mobility of purified rabbit albumin and was integrated using the GS-360 data system software from Hoefer. Integration was performed by fitting each peak with a Gaussian distribution, and the relative area was determined.

The buffer for blocking nitrocellulose and for all incubations was either Tris-buffered saline (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0) containing 0.05% Tween or phosphate-buffered saline (154 mM NaCl, 50 mM NaH_2PO_4, pH 7.4, with NaOH) containing 5% nonfat dry milk. Nitrocellulose blots were incubated with primary antibody for approximately 1 h, washed, and incubated for 1 h with secondary anti-IgG antibodies covalently linked to horseradish peroxidase. Immunoblots were developed using 4-chloro-1-naphthol as the substrate.

Materials—Chromatographically purified rabbit albumin was purchased from Cappel (catalog no. 6012-0340). Polyclonal goat anti-rabbit albumin antibodies and polyclonal sheep anti-rabbit fibrinogen antibodies were purchased from Cappel (catalog no. 0212-0341 and 0112-0824, respectively). Normal rabbit serum was purchased from Sigma (catalog no. R-5004). Electrophoretic reagents were obtained from Bio-Rad, and prestained molecular weight standards were from Bethesda Research Laboratories. Protease inhibitors were from Sigma. Peroxidase-conjugated secondary antibodies were obtained from Boehringer Mannheim, Sigma, and Cappel. All other chemicals were of reagent grade.

RESULTS

T-system membranes were analyzed by SDS-PAGE followed by Coomassie Blue staining to determine the major protein components of these membranes. Fig. 1*A* shows triads and T-system run in the absence and presence of reduction and stained with Coomassie Blue. The predominant band in the T-system has a molecular mass of approximately 100 kDa which reacts on immunoblots with antibodies against the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum (not shown). The Mg^{2+}-ATPase of the T-system also has an apparent molecular mass of approximately 100 kDa (8) and is not separated from the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase by SDS-PAGE in this gel system (8). This figure also shows the presence of another large band around 65–70 kDa in triads and the T-system which changes mobility on SDS-PAGE upon reduction (*double arrow*). The molecular weight and the large shift in mobility of this protein are characteristic of pure rabbit serum albumin (Fig. 1*A*). In isolated triads, albumin and calsequestrin (*CS*) have a similar mobility under reducing conditions. The identity of this band as albumin was confirmed using immunoblots of identical samples stained with antibodies against rabbit albumin (Fig. 1*B*). Furthermore, scans of four distinct preparations of the T-system run under nonreducing conditions showed that albumin is a major component of this preparation composing 6.7 ± 1.1% (mean ± S.E.) of the total protein (range, 5.1–9.8%).

Fig. 2*A* shows an immunoblot of light sarcoplasmic reticulum, triads, T-system, and rabbit albumin stained with anti-

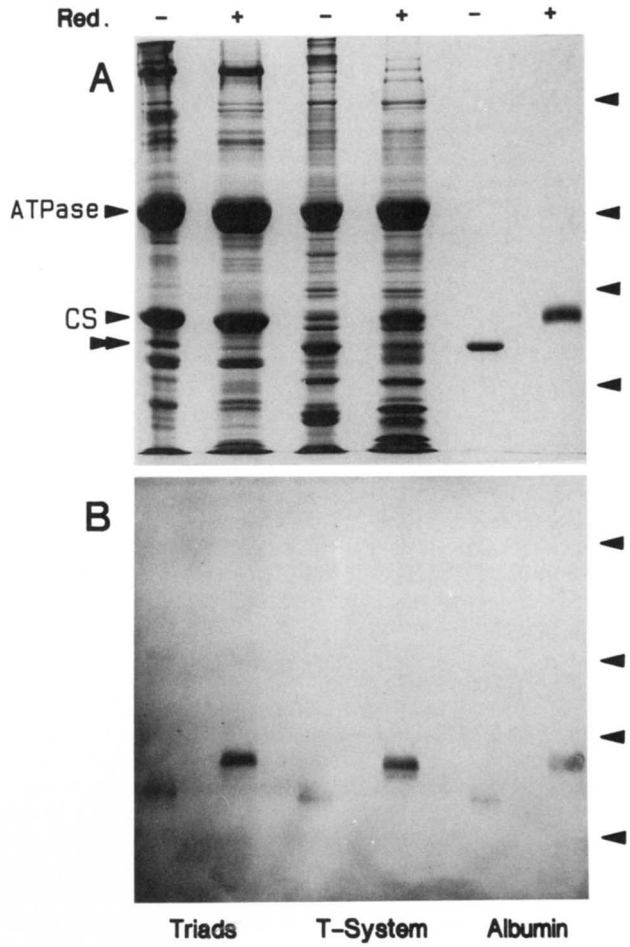

FIG. 1. **SDS-PAGE and immunoblot analysis of the T-system, triads, and rabbit albumin.** Isolated triads (*left two lanes*), T-system (*middle two lanes*), or purified rabbit albumin (*right two lanes*) were treated in the absence (−) or presence (+) of reduction (*Red.*) and subjected to SDS-PAGE on a 7.5% gel and stained with Coomassie Blue (*panel A*) or transferred electrophoretically to nitrocellulose (*panel B*). Nonreduced samples contained 20 mM *N*-ethylmaleimide, and reduced samples were boiled for 5 min in the presence of 2% 2-mercaptoethanol. Molecular mass standards are indicated on the *right* (prestained molecular mass standards were from Bethesda Research Laboratories and are from *top* to *bottom*; myosin, 200,000 Da; phosphorylase *b*, 97,400 Da; bovine serum albumin, 68,000 Da; and ovalbumin, 43,000 Da). *Panel A* contains 100 μg each of triads and T-system and 2 μg of chromatographically purified rabbit albumin. *Panel B* contains 25 μg each of triads and T-system and 0.2 μg of chromatographically purified rabbit albumin stained with polyclonal goat anti-rabbit albumin antibodies. The (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum (*ATPase*) and calsequestrin (*CS*) are indicated on the *left*. The *double arrowhead* indicates nonreduced albumin.

bodies directed against rabbit albumin. Staining clearly shows that albumin is enriched in the T-system and triads and nearly absent in light sarcoplasmic reticulum. The distribution of albumin in the different membrane preparations is similar to the distribution of [^{3}H]PN200-110 binding and dihydropyridine receptor based on immunoblots using anti-α_1 antibodies (14). These results show that albumin is a major component of isolated transverse tubule vesicles and is not present in light sarcoplasmic reticulum. Staining of similar blots with either anti-fibrinogen antibodies (Fig. 2*B*) or anti-whole rabbit serum antibodies (data not shown) revealed the presence of multiple serum proteins in the T-system but not in light sarcoplasmic reticulum.

Treatment of triads with 0.05% saponin in the presence of

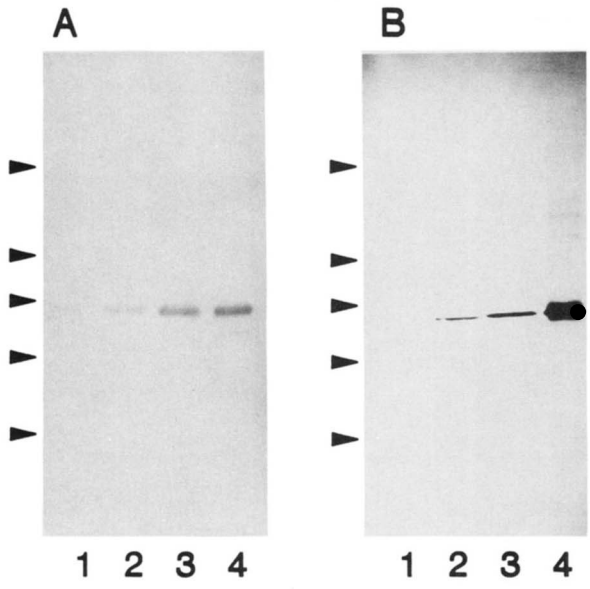

FIG. 2. **Immunoblots of light sarcoplasmic reticulum, triads, and the T-system stained with antiserum antibodies.** Samples of light sarcoplasmic reticulum (*lane 1*), triads (*lane 2*), T-system (*lane 3*), and albumin (*panel A, lane 4*) or rabbit serum (*panel B, lane 4*) were subjected to SDS-PAGE on a 5–16% gradient gel and transferred electrophoretically to nitrocellulose. *Panel A* contains 25 μg each of light sarcoplasmic reticulum, triads, and the T-system and 0.2 μg of albumin run in the presence of 2% 2-mercaptoethanol. The immunoblot was stained with goat anti-rabbit albumin antibodies followed by a rabbit anti-goat IgG secondary antibody conjugated to horseradish peroxidase. *Panel B* contains 100 μg each of light sarcoplasmic reticulum, triads, and the T-system and 3.3 μl of rabbit serum (diluted 1:10) run in the absence of reduction. The immunoblot was stained with sheep anti-rabbit fibrinogen antibodies followed by a rabbit anti-sheep IgG secondary antibody conjugated to horseradish peroxidase. Molecular mass standards are indicated on the *left* (prestained molecular mass standards were from Bethesda Research Laboratories and are from *top* to *bottom*: myosin, 200,000 Da; phosphorylase *b*, 97,400 Da; bovine serum albumin, 68,000 Da; ovalbumin, 43,000 Da; and α-chymotrypsin, 25,700 Da).

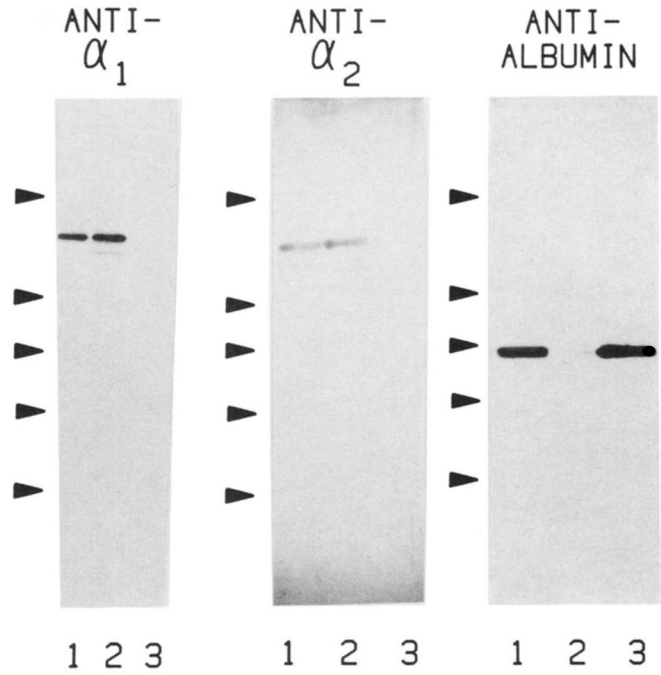

FIG. 3. **SDS-PAGE and immunoblot analysis of triads treated with 0.05% saponin.** Isolated triads were treated with low concentrations of saponin as described under "Experimental Procedures." Triads (*lane 1*), extracted triads (*lane 2*), and the supernatant from the first centrifugation (*lane 3*) were subjected to SDS-PAGE under reducing conditions (2% 2-mercaptoethanol) on a 5–16% gradient gel and transferred electrophoretically to nitrocellulose for indirect immunoperoxidase staining. The *left panel* contains 40 μg of membranes/lane and 40 μl of supernatant stained with monoclonal antibody IIF7 against the α_1-subunit of the dihydropyridine receptor (14). The *center panel* contains 100 μg of membranes/lane and 100 μl of supernatant stained with polyclonal antibodies against the α_2-subunit of the dihydropyridine receptor (7). The *right panel* contains 50 μg of membranes/lane and 50 μl of supernatant stained with polyclonal goat anti-rabbit albumin antibody. Molecular mass standards are as indicated in Fig. 2.

0.5 M NaCl (Fig. 3, *right panel*) removes albumin from triads without removing either of the high molecular weight subunits of the dihydropyridine receptor (Fig. 3). Staining of similar immunoblots with antibodies against the 52- and the 32-kDa subunits of the dihydropyridine receptor showed that these subunits remain in the membrane fraction after this treatment (data not shown). Analysis of SDS-PAGE gels of saponin-treated triads and supernatants shows that the (Ca^{2+} + Mg^{2+})-ATPase and the calcium release channel are not removed by this treatment (not shown). These results demonstrate that albumin is removed from triads when the vesicles are rendered permeable by concentrations of detergent which do not completely solubilize the membranes. This suggests that albumin and other serum proteins are located in the lumen of transverse tubule vesicles and not lightly attached to the outside of the vesicles. Since both saponin and SDS were used at low concentrations to remove albumin, it is unlikely that albumin is bound to a membrane protein found in triads. Since it is unlikely that these serum proteins are produced endogenously in muscle (21), these data suggest that albumin reaches its location in the T-system via passive diffusion from plasma.

DISCUSSION

Horgan and Kuypers (20) have recently purified the T-system using calcium phosphate loading and identified three major protein components of 104, 70, and 30 kDa based on scans of their preparation. The 70-kDa band was removed by low concentrations of SDS, while the 104- and 30-kDa bands were not. The authors suggested that the 104-kDa band was the Na^+,K^+-ATPase but could not identify the other bands in their preparation. The results presented here suggest that the 70-kDa band may well have been albumin since it is removed by low concentrations of detergent and is present in the T-system. Recently, another component of the transverse tubules has been identified and purified (22) from rabbit transverse tubules. This protein has been termed the transverse tubule coupling protein and has been proposed to be involved in coupling transverse tubules to the terminal cisternae of the sarcoplasmic reticulum via an interaction with the calcium release channel. The transverse tubule coupling protein has a similar molecular weight, and the shift in molecular weight on SDS-PAGE gels upon reduction is strikingly similar to that of rabbit albumin. If the transverse tubule coupling protein is shown to be rabbit albumin, then at least two explanations may explain these previous results. One possibility is that the band initially identified as interacting with the calcium release channel is distinct from the band which was purified. This would explain the highly nonspecific labeling of the calcium release channel with purified and radioactivity labeled transverse tubule coupling protein. Alternatively, albumin may have some nonspecific interaction with the radiolabeled calcium release channel.

Albumin is a globular protein found in plasma (40–50 mg/

ml) which functions to transport small molecules through the plasma (including bile acids, long chain fatty acids, thyroid hormone, aspirin, digitalis, and other poorly soluble drugs) and to provide oncotic pressure in the capillary (23). Albumin is the major protein component of plasma making up greater than 50% of the total plasma protein (24). Albumin is produced predominantly in liver (23) but may also be produced in small quantities in other tissues. In skeletal muscle albumin has been localized to the interstitium (21) as well as the T-system (25). Heileg and Pette (21) were unable to show endogenous expression of albumin in rabbit skeletal muscle based on *in vivo* labeling with [^{35}S]methionine and could not identify albumin as a product of *in vitro* translation of RNA isolated from skeletal muscle. It should be noted that conflicting results concerning the localization of albumin and its endogenous expression have been reported (26).

Rosemblatt *et al.* (8) have proposed that serum proteins are present in the T-system purified by calcium phosphate loading. In these studies, however, they were unable to identify any of the potential serum proteins. Here we have shown that both albumin and fibrinogen are present in purified preparations of transverse tubules. The function, if any, of albumin in the T-system is not clear. It is possible that it passively diffuses into the T-system and serves no essential function. This is supported by the presence of other serum proteins in purified transverse tubules. Alternatively, the presence of albumin in the T-system may prevent the osmotic collapse of the tubules similar to the role albumin plays in maintaining osmotic equilibrium in capillaries. Regardless of the function of albumin in transverse tubules we have clearly shown that albumin is a major component of purified transverse tubules and is likely present in the lumen of the vesicles based on the treatment with low concentrations of detergent. Attempts to confirm the lumenal localization of albumin using proteases were inconclusive due to the resistance of albumin to digestion. Based on our results albumin may serve as a marker for the T-system on SDS-PAGE gels. Proteins of similar molecular weight to albumin have been reported in the T-system by multiple investigators (8, 20, 22). This clear demonstration of albumin in the T-system and triads may allow for the identification of some of these proteins as albumin.

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REFERENCES

1. Schneider, M. F. (1981). *Annu. Rev. Physiol.* **43,** 507–517
2. Ebashi, S. (1976). *Rev. Physiol. Biochem. Exp. Pharmacol.* **38,** 292–313
3. Imagawa, T., Smith, J. S., Coronado, R., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 16636–16643
4. Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q., and Meissner, G. (1988) *Nature* **331,** 315–319
5. Campbell, K. P., Leung, A. T., and Sharp, A. H. (1988) *Trends Neurosci.* **11,** 425–430
6. Tanabe, T., Beam, K. G., Powell, J. A., and Numa, S. (1988) *Nature* **336,** 134–139
7. Knudson, C. M., Chaudhari, N., Sharp, A. H., Powell, J. A., Beam, K. G., and Campbell, K. P. (1989) *J. Biol. Chem.* **264,** 1345–1348
8. Rosemblatt, M., Hidalgo, C., Vergara, C., and Ikemoto, N. (1981) *J. Biol. Chem.* **256,** 8140–8148
9. Kirley, T. L. (1988) *J. Biol. Chem.* **263,** 12682–12689
10. Caswell, A. H., Lau, Y. H., and Brunschwig, J-P. (1976) *Arch. Biochem. Biophys.* **176,** 417–430
11. Campbell, K. P., Franzini-Armstrong, C., and Shamoo, A. E. (1980) *Biochim. Biophys. Acta* **602,** 97–116
12. Mitchell, R. D., Palade, P., and Fleischer, S. (1983) *J. Cell Biol.* **96,** 1008–1016
13. Sharp, A. H., Imagawa, T., Leung, A. T., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 12309–12315
14. Leung, A. T., Imagawa, T., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 7943–7946
15. Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. T., Sutko, J. L., Kahl, S. D., Raab, C. R., and Madson, L. (1987) *J. Biol. Chem.* **262,** 6460–6463
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
17. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685
19. Towbin, H., Staehlin, T., and Bordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
20. Horgan, D. J., and Kuypers, R. (1988) *Arch. Biochem. Biophys.* **260,** 1–9
21. Heileg, A., and Pette, D. (1988) *Eur. J. Biochem.* **171,** 503–508
22. Chadwick, C. C., Inui, M., and Fleischer, S. (1988) *J. Biol. Chem.* **263,** 10872–10877
23. Peters, T., Jr. (1975) in *The Plasma Proteins* (Putman, F. W., Ed) Vol. 1, pp. 133–181, Academic Press, New York
24. Blomback, B., and Hanson, L. A. (1979) in *Plasma Proteins* (Putman, F. W., ed) John Wiley & Sons, New York
25. Yokota, S. (1982) *Histochemistry* **74,** 379–386
26. Muller, G., and Heizmann, C. W. (1982) *Eur. J. Biochem.* **123,** 577–582