Relationship of the Aldosterone-Induced Protein, GP70, to the Conductive Na\textsuperscript{+} Channel\textsuperscript{1}

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(J. Am. Soc. Nephrol. 1991; 2:1108-1114)

ABSTRACT

Although one of the primary effects of aldosterone is to increase apical membrane Na\textsuperscript{+} conductance, as yet none of the proteins induced by the hormone in renal epithelia have been shown to be related to the conductive Na\textsuperscript{+} channel. Because the toad urinary bladder aldosterone-induced glycoprotein, GP70, has recently been localized to the apical surface of this Na\textsuperscript{+} transporting epithelium, whether GP70 is associated with the Na\textsuperscript{+} channel was examined. The specificities of a monoclonal antibody used to characterize GP70 (mAb 20) and a polyclonal antibody raised against the purified bovine renal papillary Na channel (anti-CH) were compared. GP70 was specifically immunoprecipitated by both mAb 20 and anti-CH. Moreover, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of mAb 20 purified toad urinary bladder membrane preparations was similar to those reported for bovine and A6 cell Na\textsuperscript{+} channels. Under nonreducing conditions, a single, very large protein was evident; reduction yielded GP70, a 140-kd polypeptide, and a number of minor bands. Interestingly, only GP70 was induced by aldosterone. Thus, GP70 appears to be associated with the toad urinary bladder conductive Na\textsuperscript{+} channel; whether GP70 is an integral subunit of the channel or whether it functions as a regulatory moiety remains to be determined. Whatever the case, because GP70 is induced by aldosterone, it likely has a central role in Na\textsuperscript{+} channel modulation.

Key Words: Renal epithelial Na\textsuperscript{+} transport, sodium channel, toad urinary bladder (Bufo marinus), glycoproteins

Transcellular Na\textsuperscript{+} transport in high-resistance renal epithelia is markedly augmented by aldosterone (1). The actions of aldosterone are orchestrated in a highly coordinated manner: each of the separate steps involved in transporting Na\textsuperscript{+} across the cell appears to be enhanced. Thus, aldosterone not only increases Na\textsuperscript{+} entry (across the apical plasma membrane) (2-4) and exit (across the basolateral plasma membrane) (3,4), but it also enhances ATP production, thereby providing energy for transport (5-7).

Like all steroid hormones, aldosterone, after binding to a specific receptor, activates a gene network, which includes both primary and secondary gene products that are responsible for the cellular effects of the hormone. A number of aldosterone-induced proteins have been identified (6,8-17). The functions of some of these, such as citrate synthase and the ouabain-sensitive Na\textsuperscript{+},K\textsuperscript{-}-ATPase, are well known, and their precise roles in aldosterone-stimulated Na\textsuperscript{+} transport are the subject of active investigation.

The functions of other aldosterone-induced proteins remain to be established. One such protein is GP65,70—an electrophoretically polymorphic and microheterogeneous N-glycosylated protein that is specifically induced by natriferic corticosteroids in toad urinary bladder (TUB) and A6 cells (10-16). Recently, the higher molecular weight components of this complex (GP70) have been localized to the apical plasma membrane of TUB epithelial cells (15).

Although one of the primary effects of aldosterone is to increase apical plasma membrane Na\textsuperscript{+} conductance, as yet none of the proteins induced by the hormone have been shown to be related to the renal epithelial conductive Na\textsuperscript{+} channel. The experiments presented here were undertaken to examine whether GP70 is associated with this important transport "effector" protein.

METHODS

Female Dominican toads, Bufo marinus (National Reagents, Bridgeport, CT), were killed by double pithing. The urinary bladders were mounted on glass...
cannulae, bathed in aerated Tris-buffered amphibian Ringer’s (TBR) solution, and incubated with aldosterone [Sigma Chemical Co., St. Louis, MO; serial concentration, 1.4 x 10^{-7} M] or vehicle (methanol), as appropriate, for 10 h. [35S]-methionine (Amersham Corp., Arlington Heights, IL; SA, 1,100 Ci/mmol) was added to the serosal medium (160 μCi/mL) for the last 6 h of the incubation (10).

After incubation, bladders were rinsed in cold (4°C) TBR and the epithelial cells were scraped from the mucosal surface, pooled, washed three times with TBR, and collected by centrifugation (1,200 x g; 5 min). The pellet was homogenized (Dounce homogenizer, 40 strokes) in 2 mM EDTA–220 mM sucrose–50 mM Tris (pH 7.5), centrifuged (120,000 x g; 1 h), and solubilized in 0.1% Triton X-100–5 mM Tris (pH 8.0) (10).

Monoclonal antibody no. 20 (mAb 20) was obtained from a hybridoma line as previously described (15). Antibody-rich ascites was prepared in standard fashion and was purified by ammonium sulfate fractionation and fast protein liquid chromatography with a mono-Q column (Pharmacia Inc., Piscataway, NJ) (18). The purified fraction was coupled to cyanogen bromide-activated Sepharose-4B (Pharmacia) (19). Rabbit polyclonal directed against the bovine renal papillary Na+ channel (anti-CH) (20) was kindly provided by Dr. D. Benos.

Solubilized membranes were immunoprecipitated with mAb 20 hybridoma supernatant (500 μL; 100 μL of anti-rat immunoglobulin G-Sepharose) or anti-channel antisera (anti-CH) (20 μL; 100 μL of protein A) as previously described (15). In other experiments, membranes were first purified by mAb 20 affinity chromatography and then were immunoprecipitated with anti-CH. For the affinity chromatography, solubilized membranes were loaded on the antibody column and allowed to bind for 10 to 15 min. The column was then washed with 50 mM Tris (pH 8.0), 1 mM EDTA, and 0.1% Triton X-100 until 35S counts in the collected fractions were back to baseline. The wash solution was then changed to 50 mM Tris (pH 8.0), 1 mM EDTA, and 0.5% sodium deoxycholate until the counts again returned to baseline. The final wash consisted of 50 mM Tris (pH 8.0), 0.5 M NaCl, and 0.1% Triton X-100. The bound proteins were then reverse eluted with 100 mM sodium citrate (pH 2.6), 3.0 M KCl, and 0.1% Triton X-100. Fractions (1 mL) were collected into 0.5 mL of 2.0 M Tris (pH 8.0).

Immunoprecipitates were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) under reducing conditions, nitrocellulose blotting, and autoradiography (10, 14). mAb 20 affinity-purified material was also separated on 3% and 5 to 15% gradient sodium dodecyl sulfate (SDS)-PAGE gels run under both reducing and nonreducing conditions as described by Benos et al. (21), except that 128 mM 2-mercaptoethanol was substituted for dithiothreitol in the reducing buffer and the sample was heated to 100°C for 5 min.

RESULTS

 Autoradiographs of 2D-PAGE gels of biosynthetically labeled TUB proteins before and after immunoprecipitation with mAb 20, anti-CH, or preimmune rabbit serum are illustrated in Figure 1. As we have previously shown (15), GP70, a microheterogeneous protein (isoelectric point, 5.8 to 6.2) with an apparent molecular mass after reduction with 2-mercaptoethanol of 70 kD is the predominant specific component of mAb 20 immunoprecipitates analyzed by 2D-PAGE. In contrast, anti-CH immunoprecipitates the entire GP65,70 complex. GP65,70 is not present in control immunoprecipitates performed with preimmune rabbit serum. Thus, mAb 20 and anti-CH specifically immunoprecipitate very similar 70-kd proteins.

 Autoradiographs of biosynthetically labeled TUB proteins purified by mAb 20 affinity chromatography alone or first purified by mAb 20 affinity chromatography and then immunoprecipitated with anti-CH are shown in Figure 2. Identical 70-kd proteins (GP70) are evident in the autoradiographs of both 2D gels. It is apparent that anti-CH recognizes a protein eluted from the mAb 20 affinity column. Assuming that anti-CH is specific for the epithelial Na channel (20), mAb 20 must recognize a component of the channel or a protein that copurifies with the channel. This could be GP70 itself or another protein (that is excluded from 2D-PAGE gels). We therefore further examined the specificity of mAb 20 by SDS-PAGE.

 When biosynthetically labeled TUB proteins are purified by mAb 20 affinity chromatography and then separated on 3% SDS-PAGE gels under nonreducing conditions, a single, very large protein is evident (Figure 3A). Under reducing conditions, there appears to be a slight shift in the apparent molecular mass of this protein and the appearance of a new band running just behind the dye front. Because the protein(s) comprising the new band migrated close to the bottom of the gel, a molecular mass cannot be assigned with accuracy. However, because ferritin (220 kD) also runs in this region of the gel, their molecular masses must be less than or equal to 220 kD.

 Figure 3B shows the same mAb 20 affinity-purified material separated on 5 to 15% gradient SDS-PAGE gels before and after reduction with 2-mercaptoethanol. Reduction is associated with the appearance of several polypeptides, including two major (68- to 75- and 135- to 140-kd, respectively) and a number of minor bands. Because proteins below 220 kD should
Figure 1. Comparison of antibody specificities. Parallel protocol. Solubilized membranes from six toad urinary hemi-bladders incubated and prepared as described in Methods were divided into three aliquots of $7 \times 10^4$ cpm and immunoprecipitated with mAb 20 hybridoma supernatant (15) (500 µL; 100 µL of anti-rat immunoglobulin G-Sepharose), rabbit polyclonal directed against the bovine renal papillary Na$^+$ channel (20) (20 µL; 100 µL of protein A) and preimmune rabbit serum as previously described (15). All of the material immunoprecipitated by each of the antibody preparations was analyzed by 2D-PAGE under reducing conditions, nitrocellulose blotting, and autoradiography (4,9,10). In each panel, the location of GP65,70 is indicated by brackets. The open arrows indicate the position of albumin (68 kd). The typical GP65,70 complex is evident in the starting material (membrane-rich fraction (MRF)) (2.0 x $10^6$ cpm; 8-day exposure) and the anti-CH immunoprecipitate (5.6 x $10^5$ cpm; 21-day exposure) but is completely absent in the preimmune control (2.9 x $10^6$ cpm; 42-day exposure); GP70 is the predominant specific component of the mAb 20 immunoprecipitate (6.6 x $10^5$ cpm; 6-day exposure). Thus, mAb 20 and anti-CH specifically immunoprecipitate very similar 70-kd proteins.

Figure 2. Comparison of antibody specificities. Sequential protocol. Solubilized membranes were purified by mAb 20 affinity chromatography as described in Methods and then immunoprecipitated with anti-CH. The affinity-purified material (upper panel; mAb 20, 1.5 x $10^5$ cpm; 10-day exposure) and the material subjected sequentially to mAb 20 affinity purification and immunoprecipitation with anti-CH (lower panel; anti-CH, 1.0 x $10^5$ cpm; 20-day exposure) were analyzed by 2D-PAGE under reducing conditions, nitrocellulose blotting, and autoradiography (4,9,10). Identical microheterogeneous 70-kd proteins (GP70) are evident in both panels (arrows). Control immunoprecipitates, performed with preimmune rabbit serum, were completely negative (data not shown). Thus, anti-CH recognizes a protein (or proteins) eluted from the mAb 20 affinity column (either GP70 itself or a protein that copurifies with GP70 and that is excluded from 2D gels).
Figure 3. Autoradiographs of SDS-PAGE profiles of mAb 20 affinity-purified membrane proteins. Solubilized membranes, purified by mAb 20 affinity chromatography as described in Methods, were separated on 3% (panel A, 3.9 \times 10^3 cpm each lane) and 5 to 15% (panel B, 6.0 \times 10^3 cpm each lane) SDS-PAGE gels under both reducing (R) and nonreducing (NR) conditions. The solid arrows indicate the predominant proteins identified under the different conditions; no bands were present in the 5 to 15% gels run under nonreducing conditions. The open arrows indicate the positions of thyroglobulin and ferritin subunits (330 and 220 kd, respectively). mAb 20 recognizes a very large, mercaptoethanol-sensitive glycoprotein complex that consists of several discrete polypeptides, the major one of which is GP70.

DISCUSSION

One of the major functions of aldosterone in renal epithelia is to increase the transport of Na\(^+\) across the cell. Although aldosterone-stimulated Na\(^+\) transport is dependent on new protein synthesis, the proteins induced by the hormone have not been completely characterized (1). One such protein, GP65,70, is an electrophoretically polymorphic (molecular masses, 65 and 70 kd) and microheterogeneous (isoelectric point, 5.8 to 6.2) N-glycosylated protein that is specifically induced by natriferic corticosteroids in TUB and A6 cells (10–16). The molecular mass and extensive glycosylation of GP65,70 exclude the possibility that it represents either citrate synthase (22) or the \(\alpha\)-subunit of amphibian renal Na\(^+\),K\(^+\)-ATPase (23). In addition, GP65,70 is not recognized by polyclonal antibodies raised against the \(\beta\)-subunit of amphibian renal Na\(^+\),K\(^+\)-ATPase (14). Thus, GP65,70 represents neither citrate synthase nor the Na\(^+\),K\(^+\)-ATPase, both of which are well-known aldosterone-induced proteins (6,9).

To further elucidate the role of GP65,70 in aldosterone-stimulated Na\(^+\) transport, we raised a panel of mAb to partially purified TUB epithelial cell membranes containing this glycoprotein (15). One of these antibodies (mAb 20) was of particular interest. mAb 20 immunoprecipitates a group of microheterogeneous polypeptides that correspond (on 2D-PAGE) to the higher molecular mass component (GP70) of the GP65,70 complex (15). Moreover, immunoelectron microscopy studies revealed that mAb 20 is directed against an epitope in the apical plasma membrane and subapical granules of TUB granular cells, the
Na\textsuperscript+} transporting cells of this epithelium (15). These findings raised the possibility that mAb 20 could be directed against the conductive Na\textsuperscript+} channel and that GP70 may be associated with the channel (either as an integral subunit or as a regulator of channel function).

Recently, several investigators have purified putative Na\textsuperscript+} channels from renal epithelia (20,21,24,25). However, there is still considerable controversy with regard to the overall size and subunit structure of the channels. At the present time, the best-characterized of these "channels" is the one described by Benos and collaborators in bovine renal papillae and A6 cells (20,21,24). This approximately 700-kd multimeric glycoprotein has an amiloride-binding site (associated with the 149-kd subunit), a protein kinase A-sensitive phosphoprotein (molecular mass, 315 kd), and at least three other polypeptides (molecular masses, 95, 71, and 55 kd).

To test the hypothesis that GP70 is associated with the apical Na\textsuperscript+} channel, we first compared the specificities of mAb 20 (15) and a polyclonal antibody raised against the purified bovine papillary Na\textsuperscript+} channel (anti-CH) (20). As shown in Figures 1 and 2, GP70 (or a complex containing GP70) is specifically immunoprecipitated by both of these antibodies. These cross-reacting specificities are compatible with the hypothesis that GP70 is associated with the renal epithelial Na\textsuperscript+} channel and that mAb 20 may be directed against the channel. Interestingly, whereas the anti-CH antibody immunoprecipitated both GP65 and GP70 in approximately equal proportions, the mAb 20 immunoprecipitate contained an abundance of GP70. Because we have previously demonstrated the GP65 and GP70 are the same proteins, differing only in their degree of glycosylation (16), mAb 20 may have a higher affinity for channels containing or associated with GP70. On the other hand, the anti-CH antibody appears to have similar affinities for channels containing or associated with either GP65 or GP70. Thus, the native channel complex may exist in two discrete forms.

We next examined the immunoprecipitation profiles of the two antibodies. When biosynthetically labeled TUB, purified by mAb 20 affinity chromatography, are subjected to SDS-PAGE (rather than 2D-PAGE), proteins other than GP70 are present: Two major bands (68 to 75 and 135 to 140 kd), and a number of minor bands, are evident (Figures 3B and 4). In addition, when such affinity-purified material is subjected to SDS-PAGE on 3% gels under nonreducing conditions, a single, very large protein is evident; reduction of this complex yields GP70 and at least one additional 140-kd polypeptide (Figure 3). Thus, the SDS-PAGE profiles obtained with mAb 20 are similar (though not identical) to those reported for the putative bovine renal papillary and A6 cell Na\textsuperscript+} channels (21,24). These findings provide additional support for the hypothesis that GP70 is associated with the renal epithelial Na\textsuperscript+} channel.

Although mAb 20 appears to be directed against a component of the Na\textsuperscript+} channel complex, its precise specificity is uncertain. In previous studies that used 2D-PAGE analysis of mAb 20-purified material (15), GP70 appeared to be the only protein specifically immunoprecipitated by mAb 20. Consequently, it was assumed that mAb 20 was directed against GP70. The studies presented here that used SDS-PAGE indicate that GP70 is the only induced protein immunoprecipitated by the antibody but raise the possibility that mAb 20 may be directed against one of the other putative Na\textsuperscript+} channel components copurified with GP70 (or against a multisubunit epitope). Whatever its precise specificity, however, it is clear that mAb 20 can be used to purify and characterize the large GP70-containing glycoprotein complex recognized by this antibody, a complex that presumably represents the TUB conductive Na\textsuperscript+} channel. Whether GP70 is an integral subunit of the channel or whether it functions as an associated regulatory moiety remains to be determined.

Aldosterone selectively increases the incorporation of [35S]methionine into the 70-kd subunit of the putative TUB Na\textsuperscript+} channel (Figure 4). Whether this reflects increased synthesis, decreased degradation, or both, aldosterone appears to regulate at least one of the components of the Na\textsuperscript+} channel complex. This conclusion contrasts with the prevailing view that the Na\textsuperscript+} channel is not induced by aldosterone (26–29). However, the approaches used in earlier studies (26,27) to reach the conclusion that aldosterone activates preformed channels in the apical plasma membrane were, of necessity, indirect, having been performed well before the recent biochemical characterization of the channel. Moreover, a recent study (28), with amiloride photoaffinity probes to identify the cellular pool of Na\textsuperscript+} channels, demonstrated only that the amiloride-binding subunit of the channel was not induced by aldosterone. Because the 70-kd component of the bovine and A6 cell Na\textsuperscript+} channels does not appear to be the amiloride-binding subunit (21,25), these studies are compatible with the hypothesis that GP70 is both a component of the channel and specifically induced by aldosterone.

A recent report from the laboratory of Benos et al. (29) with a polyclonal antibody raised against purified bovine renal papillary Na\textsuperscript+} channels also concluded that aldosterone does not alter the number of channels in the apical membrane. However, the polyclonal antiserum used in these studies did not recognize the 70- to 85-kd subunits of these putative channels. These data, therefore, also do not exclude a role for GP70 in aldosterone-related channel modulation.

The subunits of a multimeric protein can be synthesized at unequal rates. In fact, differential rates
of synthesis would allow for one of the subunits to serve a regulatory role. In this regard, GP70 bears intriguing similarities to the β-subunit of the Na+ pump. Both are highly N-glycosylated, exhibit similar electrophoretic microheterogeneity, and have very similar molecular masses in both their glycosylated and nonglycosylated forms (14,16,23). Increasing evidence suggests that the β-subunit may serve a regulatory or targeting function for the Na+ pump (30,31). It is possible that the 70-kd component of the renal epithelial conductive Na+ channel serves a similar function.

The biochemical mechanisms that underlie the hormonal modulation of the conductive Na+ channel remain to be delineated. Only when the renal epithelial apical membrane Na+ channel has been isolated, all of its components characterized, and the question of what distinguishes a functional from a nonfunctional Na+ channel clearly understood, will it be possible to determine the precise mechanisms involved in hormone-mediated channel modulation. However, the work presented here has shown that the aldosterone-induced glycoprotein (GP70), appears to be associated with the TUB conductive Na+ channel and that it also appears to be the only component of the channel induced by aldosterone. It is likely, therefore, that GP70 has a central role in aldosterone-mediated channel modulation. We speculate that GP70 is an aldosterone-induced channel regulator that converts preformed, nonfunctional protochannels into functional holochannels, either by activating protochannels already present in the apical plasma membrane or by inserting holochannels into the membrane from a preformed protocchannel pool.

ACKNOWLEDGMENTS

This work was supported by grants from the Department of Veterans Affairs to Malcolm Cox, M.D. (Merit Review 104:14M) and Paul Palevsky, M.D. (Associate Investigator) and Biomedical Research Support and Research Foundation Grants from the University of Pennsylvania to Bonnie Blazer-Yost, Ph.D. We express our gratitude to Dr. Dale Benos, University of Alabama, Birmingham (for providing the antichannel antiserum), Ms. Joanne Deithorn, the Medical Media Service, Philadelphia VA Medical Center (for preparing the Figures), and Mrs. Lorrettie Grove (for secretarial assistance).

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