The human epithelial sodium channel (hENaC) is a hetero-oligomeric complex composed of three subunits, α, β, and γ. Understanding the structure and function of this channel and its abnormal behavior in disease requires knowledge of the number of subunits that comprise the channel complex. We used freeze-fracture electron microscopy and electrophysiological methods to evaluate the number of subunits in the ENaC complex expressed in Xenopus laevis oocytes. In oocytes expressing wild-type hENaC (α, β, and γ subunits), clusters of particles appeared in the protoplasmic face of the plasma membrane. The total number of particles in the clusters was consistent with the whole-cell amiloride-sensitive current measured in the same cells. The size frequency histogram for the particles in the clusters suggested the presence of an integral membrane protein complex composed of 17 ± 2 transmembrane α-helices. Because each ENaC subunit has two putative transmembrane helices, these data suggest that in the oocyte plasma membrane, the ENaC complex is composed of eight or nine subunits. At high magnification, individual ENaC particles exhibited a near-square geometry. Functional studies using wild-type αβγ-hENaC coexpressed with γ-hENaC mutants, which rendered the functional channel differentially sensitive to methanethiosulfonate reagents and cadmium, suggested that the functional channel complex contains more than one γ subunit. These data suggest that functional ENaC consists of eight or nine subunits of which a minimum of two are γ subunits.

Ion channels belonging to the DEG/ENaC family have been identified in a variety of tissues from vertebrate and invertebrate species (1). The most thoroughly studied member of this large family, the epithelial Na⁺ channel (ENaC), plays a critical role in Na⁺ homeostasis, and mutations in the genes encoding ENaC cause human disease (2–5). Gain-of-function mutations that delete or disrupt a C-terminal motif increase renal Na⁺ absorption, causing Liddle’s syndrome, a genetic form of hypertension. Conversely, loss-of-function mutations cause renal Na⁺ wasting and hypotension in pseudohypoaldosteronism type I.

A large body of functional and biochemical evidence suggests that functional ENaC is composed of three homologous subunits, α, β, and γ (6–11). However, the subunit stoichiometry of the Na⁺ channel complex is uncertain. Electrophysiological studies have generated two different models of ENaC stoichiometry: one a complex of three α, three β, and three γ subunits (12); and the other a complex containing two α, one β, and one γ (13, 14). Biochemical studies using sucrose gradient sedimentation analysis also have generated two different models. Two studies found that ENaC subunits assembled into a 25 S complex, suggestive of a high-order oligomer in the range of approximately nine subunits (12, 15), whereas another reported that another DEG/ENaC protein, FaNaCh (Phe-Met-Arg-Phe-amide-activated Na⁺ channel), formed a 9 S complex, favoring a stoichiometry of four subunits (16).

To gain new insight into this problem, we used two additional approaches to examine the subunit stoichiometry of ENaC. First, we used freeze-fracture electron microscopy to examine the cross-sectional area of ENaC in the plasma membrane. In freeze-fracture microscopy, the membrane is fractured into two complementary leaflets called the protoplasmic (P) and exoplasmic (E) faces, in which integral membrane proteins appear as intramembrane particles (17). Previously, we have shown that the dimensions of the freeze-fracture particles of α-helical integral membrane proteins can be used to estimate the number of transmembrane α-helices (18). This information, along with secondary structure models, allows us to estimate the oligomeric composition of functional membrane proteins in the plasma membrane. Thus, this approach should enable us to evaluate the oligomeric structure of ENaC and distinguish between the two proposed models. In a second approach we have used a functional assay to determine whether ENaC contains one or more than one γ subunit.

**EXPERIMENTAL PROCEDURES**

**Expression of ENaC in Xenopus Oocytes—α, β, and γ (or γSGAEC and γG536C) human ENaC in pMT3 were generated as described previously (9, 10, 12). Wild-type α, β, γ, or mutant γ ENaC subunits were expressed in albino Xenopus laevis oocytes by nuclear injection of cDNA for each subunit (0.2 ng each). Oocytes were maintained in Barth’s medium until used in experiments.**

**Freeze-fracture and Electron Microscopy—After measurement of the whole-cell amiloride-sensitive Na⁺ current, control oocytes and oocytes expressing wild-type αβγ-hENaC were fixed and prepared for freeze-fracture microscopy (18, 19). For particle density determinations, images of P fracture face were digitized at a final magnification of ×150,000. Particle densities were determined by counting P face particles from known areas of the membrane (NIH Image). For the measurement of the dimensions of the freeze-fracture particles, particles were sampled from 15 P fracture face regions from six replicates from six oocytes (two control and four ENaC-expressing oocytes). Particle diameter was
measured directly from the negative using a comparator (Nikon, model 6c) at a final magnification of × 1,000,000. The diameter was obtained by measuring the width of the particle edge-to-edge in a direction perpendicular to the direction of the shadow. For the frequency histograms, particle diameter measurements were placed in 0.5 nm bins, and the histograms were plotted at the center of the bin (Fig. 3A). For the examination of the particle clusters, a cluster was defined as having a particle density >1000/nm².

**Freeze-fracture Data Analysis**—Particle diameter measurements were plotted as frequency histograms which were fitted to a multiple Gaussian function (18) as shown in Equation 1.

\[
f_i = \frac{A_i}{s_i} \exp \left[ -0.5 \left( \frac{d - \mu_i}{s_i} \right)^2 \right]
\]  
(Eq. 1)

where \(f_i\) is the frequency of occurrence of a particle of size \(d\); \(d\) is the measured diameter of a given particle (nm); \(A_i\) is an approximation of the area underneath the curve of a given particle population; \(\mu_i\) is the mean diameter of a given population (nm); \(s\) is the standard deviation of the population diameter; and \(i\) is an integer from 1 to the total number of particle populations. All values are reported as means ± S.E.

**Electrophysiology of ENaC Mutants**—cDNAs encoding wild-type α and β hENaC (0.2 ng each) with 0.2 ng of γ2, γ3, or a 0.5:0.5 mixture of both (0.1 ng each) were injected as described above. 16–24 h after injection, whole-cell current was measured by two-electrode voltage clamp at −60 mV in a bathing solution of (in mM) 116 NaCl, 2 KCl, 0.4 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4. ENaC current (\(I_{\text{enac}}\)) was determined by subtracting the current with a maximal dose of amiloride (100 μM) or benzamil (100 μM) in the bathing solution from base-line current.

The magnitude of current inhibited by [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET, 1 mM) or cadmium (Cd²⁺, 10 mM) in the extracellular bathing solution (\(I_{\text{inh}}\)) was used to determine the fraction of \(I_{\text{enac}}\) inhibited by these compounds (\(I_{\text{inh}}/I_{\text{enac}}\)).

**RESULTS**

**Freeze-fracture Electron Microscopy**—We first characterized the density and size of the freeze-fracture particles of the plasma membrane of control oocytes. Consistent with our previous studies, control albino oocytes exhibited a low density of particles in the P fracture face of the plasma membrane at 275 ± 20/μm² (mean ± S.E.; \(n = 4644\) particles from 15 P face regions covering 16.9 μm² of plasma membrane) (Fig. 1A). The E fracture face of albino oocytes contained a population of particles −13 nm in diameter at a density of −900/μm² (not shown) (19). All heterologous plasma membrane proteins that we have studied thus far partition to the P face of the plasma membrane (18–22). ENaC also follows this pattern (Fig. 1, B and C; Fig. 2, A–C) and, therefore, our study focused on the P face of the plasma membrane. Analysis of the size (diameter) of the endogenous P face particles of control oocytes (Fig. 2D) revealed that the particles represented a homogenous population that could be described by a single normal distribution (Fig. 3A). The mean diameter for this particle population was 6.7 ± 0.3 nm (\(n = 502\)).

All ENaC-expressing oocytes used in freeze-fracture studies had amiloride-sensitive whole-cell currents of 5–7 μA (at −50 mV) after injection, whole-cell current was measured by two-electrode voltage clamp at −60 mV in a bathing solution of (in mM) 116 NaCl, 2 KCl, 0.4 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4. ENaC current (\(I_{\text{enac}}\)) was determined by subtracting the current with a maximal dose of amiloride (100 μM) or benzamil (100 μM) in the bathing solution from base-line current.

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mV and 100 mM extracellular Na\(^{+}\)). The density of plasma membrane particles in ENaC-expressing oocytes was 291 ± 19/\(\mu\)m\(^2\) (\(n = 4556\) particles from 27 P face regions covering 17.2 \(\mu\)m\(^2\) of plasma membrane), not significantly above background (\(p > 0.05\)). However, expression of ENaC resulted in the appearance of clusters of particles in the P face of the plasma membrane (Fig. 1, B and C; Fig. 2, A and B), which were not seen in control oocytes. The clusters were seen in ~65% of the replicas prepared from ENaC-expressing oocytes and occupied ~0.6% of the plasma membrane surface area (0.39 \(\mu\)m\(^2\) in 67.98 \(\mu\)m\(^2\) of plasma membrane examined). The average density of particles within the clusters was 2435 ± 575/\(\mu\)m\(^2\) (range, 1566–9000/\(\mu\)m\(^2\)). These clusters did not influence the overall density since each cluster occupied a small area (0.020 ± 0.006 \(\mu\)m\(^2\); range, 0.001–0.076 \(\mu\)m\(^2\)) and contained few particles (37 ± 9; range, 5–136).

Because of the unique feature of the clusters, we separately characterized the particles within the clusters and those in regions outside of the clusters. The size frequency histogram of the particles within the clusters is shown in Fig. 3A (\(n = 789\)). The fit of the data to a multiple Gaussian distribution indicated that 91% of the particles had a mean diameter of 8.0 ± 0.3 nm. 9% of the particles were larger in size (see Fig. 3A). In contrast, the size frequency histogram for the particles outside of the clusters exhibited a predominant particle population (6.6 ± 0.3 nm) (\(n = 521\); not shown) which was indistinguishable from the population of endogenous particles in un.injected oocytes (6.7 ± 0.3 nm; see Fig. 3A). There was also a very small (<4% of total) population of 8.1 ± 0.4 nm particles, similar in size to the particle in the clusters.

For proteins with a near-circular (connexin-50 hexamers) or square-shaped (aquaporin-1 tetramers) cross-sectional geometry, the area can be approximated from the diameter of their freeze-fracture particles by assuming a circular cross-sectional shape (18). Using this approach for the particles in the clusters (Fig. 2, A–C), the area calculated from the mean diameter (8.0 ± 0.3 nm) obtained from the frequency histogram was used to predict the number of membrane-spanning \(\alpha\)-helices in the integral membrane protein giving rise to these particles (Fig. 3B) (18). To correct for the thickness of the platinum-carbon film, 2.4 ± 0.4 nm was subtracted from the freeze-fracture particle diameter (18). The protein cross-sectional diameter (5.6 ± 0.5 nm) was then used to estimate its cross-sectional area (24 ± 3 nm\(^2\)). Finally, using 1.40 ± 0.03 helix/\(\pi\)nm\(^2\) (see “Discussion”) (18), the area corresponded to that of an integral membrane protein containing 17 ± 2 transmembrane \(\alpha\)-helices (Fig. 3B). Examination of the particles within the clusters at high magnification revealed that they exhibited a near-square geometry characterized by ~90° angles (\(a/b \sim 1\); where \(a\) and \(b\) are the axes) (Fig. 3C).

Electrophysiological Assay of ENaC Mutants—We used an electrophysiological assay to determine if ENaC contains more than one \(\gamma\) subunit. Our strategy was to coexpress two mutant \(\gamma\) subunits (\(\gamma_{536C}\) and/or \(\gamma_{529C}\), with wild-type \(a\) and \(\beta\) which leads to the expression of Na\(^{+}\) channels that have different responses to the cysteine-modifying reagents, MTSET and Cd\(^{2+}\). Extracellular MTSET has no significant effect on wild-type ENaC Na\(^{+}\) current (12). When Gly\(^{536}\) in the \(\gamma\) subunit was replaced with cysteine (\(\gamma_{536C}\), MTSET modification of this cysteine irreversibly decreased the Na\(^{+}\) current by 89% (Fig. 4, A and B) (12). This residue is in the second membrane-spanning segment (M2) and lies within the putative channel pore (Fig. 4C) (23). In contrast, when Ser\(^{529}\) which lies just extracellular to M2 (Fig. 4C) was replaced with cysteine (\(\gamma_{529C}\), MTSET did not inhibit the channel (Fig. 4, A and B) (12).

If ENaC contains only a single \(\gamma\) subunit, then coexpression of a 0.5:0.5 mixture of \(\gamma_{536C}\) and \(\gamma_{529C}\) cDNA (with wild-type \(a\) and \(\beta\) will be expected to produce two populations of channels; half will be expected to contain \(\gamma_{536C}\) and be sensitive to MTSET, and half will be expected to contain \(\gamma_{529C}\) and be insensitive to MTSET. This is illustrated in Fig. 4C (top panel; \(a\) and \(\beta\) subunits are not shown). In this case, we would predict that the response to MTSET would be the average for \(\gamma_{536C}\) and \(\gamma_{529C}\) expressed individually (43% decrease in Na\(^{+}\) current; Fig. 4, B and C). However, when we coexpressed a 0.5:0.5 mixture of \(\gamma_{536C}\) and \(\gamma_{529C}\), MTSET decreased the Na\(^{+}\) current by only 21%; significantly less than the proportion of \(\gamma_{536C}\) subunits (\(p < 0.002\); Student’s unpaired \(t\) test). Thus, there was an apparent functional interaction between the two mutant \(\gamma\) subunits; \(\gamma_{529C}\) suppressed inhibition of \(\gamma_{536C}\) by MTSET.

We also tested the response to Cd\(^{2+}\). This divalent cation also interacts with cysteines, but it is much smaller than MTSET. Cadmium reversibly inhibited \(\alpha\beta\gamma_{536C}\) ENaC but had a minimal effect on \(\alpha\beta\gamma_{529C}\) ENaC (Fig. 4, A and B). However, in contrast to MTSET, Cd\(^{2+}\) inhibited the 0.5:0.5 mixture significantly more (61%; \(p < 0.002\)) than predicted (42%) for a channel containing only one \(\gamma\) subunit (Fig. 4, A and B). This result also suggests a functional interaction between \(\gamma_{536C}\) and \(\gamma_{529C}\).

DISCUSSION

ENaC Freeze-fracture Particles—A unique feature of ENaC expression in oocytes was that it led to the appearance of clusters of particles in the plasma membrane. Several observations lead to the conclusion that these particles represent ENaC: (i) the clusters only appeared in oocytes expressing ENaC as assessed by the amiloride-sensitive Na\(^{+}\) current and not in control oocytes; (ii) in ENaC-expressing oocytes, the size and shape of the particles in the clusters contrast sharply with those of the endogenous particles of the oocyte plasma membrane, whereas the vast majority (>96%) of the particles outside of the clusters are indistinguishable from the endogenous ones; (iii) in freeze-fracture studies of control oocytes, and in oocytes overexpressing a variety of other integral membrane proteins, we have not observed such clustering of particles in the plasma membrane; examples include the Na\(^{+}\)/glucose co-transporter (21), cystic fibrosis transmembrane conductance regulator (18), Shaker K\(^+\) channel (19); aquaporin-1 (19); major intrinsic protein (19); opsin (18), Na\(^{+}\)/iodide transporter (20), occludin, GABA transporter, and facilitative glucose transporter (GLUT1); and (iv) the total number of particles in the clusters was consistent with the macroscopic amiloride-sensitive currents measured in the same cells (see below).

The combination of freeze-fracture and functional data in the same oocytes provides further evidence that the particles in the clusters are responsible for the macroscopic amiloride-sensitive Na\(^{+}\) currents. The fraction of the membrane covered by the ENaC clusters (~0.006), the average particle density in the clusters (2435/\(\mu\)m\(^2\)), and the total area of the oocyte plasma membrane obtained from capacitance measurements (~3 × 10\(^7\) \(\mu\)m\(^2\)) (19, 22) can be used to estimate the total number of ENaC in the plasma membrane (\(N \sim 4 \times 10^9\)). This value, together with the macroscopic amiloride-sensitive Na\(^{+}\) current in oocytes used for freeze-fracture studies (\(I_p \sim 6 \mu A\)), and the single-channel current (\(i \sim 0.4\) pA) measured under similar conditions (2) provide an estimate for the open-state probability of functional ENaC according to \(P_o = I_p/N\). The \(P_o\) obtained (~0.04) is in reasonable agreement with that reported previously using a different technique (0.014–0.004) (24). If it is assumed that the 8.1 nm particles outside of the clusters also represent functional ENaC, \(N\) would increase to ~7 × 10\(^8\) and

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\(^{a}\) G. A. Zampighi and S. Eskandari, unpublished observations.

\(^{b}\) S. Eskandari and E. M. Wright, unpublished observations.
the $P_o$ would be $-0.02$. Therefore, the total number of ENaC particles can reasonably account for the macroscopic amiloride-sensitive currents.

An association with cytoskeletal proteins might explain the unique clustering of ENaC. The C terminus of ENaC subunits contains a conserved PXXXYXX sequence (2) that fits the consensus of a PY (PXY) protein-protein interaction motif (25). The PY motif binds to proteins containing a WW domain, a 35–40-amino acid sequence containing two conserved tryptophan residues (25). Moreover, Nedd4, a protein containing three WW domains, binds and alters the function of ENaC (26, 27). We speculate that binding of endogenous oocyte Nedd4 or a related protein may be responsible for the characteristic clustering of ENaC in the membrane. It is important to note that any putative association of ENaC with factors in the cytoplasm will not alter the freeze-fracture particle diameter measurements as ENaC partitions to the P face of the plasma membrane, and such interactions are not seen in this fracture face.

**ENaC Oligomeric Assembly**—The subunit stoichiometry of ENaC has attracted considerable interest. Although it is well established that all three subunits ($\alpha$, $\beta$, and $\gamma$) are required for the assembly of a functional channel with maximal activity, the oligomeric composition has been proposed to be either a complex composed of four subunits, two $\alpha$, one $\beta$, and one $\gamma$ (13, 14), or nine subunits, three $\alpha$, three $\beta$, and three $\gamma$ (12, 15). Since biochemical and functional studies have led to two different oligomeric structure models (four versus nine subunits), we reasoned that examination of ENaC freeze-fracture particles would provide an independent method to examine its oligomeric assembly.

Our freeze-fracture method is based on the finding that the cross-sectional area of the freeze-fracture particles reflects the cross-sectional area of their membrane-spanning region (18). In addition, for proteins with $\alpha$-helical transmembrane domains, the cross-sectional area is proportional to the number of helices spanning the plasma membrane. This was based on the comparison of the cross-sectional area of ENaC or subunits belonging to other homologous members of this large family of ion channels predicts two transmembrane helices (M1 and M2) connected through a large extracellular loop, in which both the N and C termini are cytoplasmic (28–30). Therefore, a heterotetrameric structure of ENaC would be composed of a total of eight transmembrane helices, whereas a structure composed of nine subunits would contain 18 transmembrane helices. By using the relationship described above, the corresponding cross-sectional area for the two proposed ENaC models would be 12.2 and 25.2 nm$^2$.

The cross-sectional area of $>90\%$ of the particles in the clusters of ENaC-expressing oocytes was $24 \pm 3$ nm$^2$, corresponding to a complex composed of 17 $\pm$ 2 transmembrane helices.
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α-helices. For ENaC this implies an oligomer of eight or nine subunits, which would predict a molecular mass of ~700–800 kDa for the channel complex. This observation in the oocyte plasma membrane is consistent with results from sucrose sedimentation data obtained from ENaC expressed in COS-7 cells and in vitro, and with previous functional data (12, 15). Interestingly, analysis of the overall shape of individual ENaC particles revealed a near-square geometry with ~90° angles and \( a/b \approx 1 \). From structural considerations, such an overall shape constrains the number of subunits forming the channel to four or multiples of four. Therefore, based on the shape and size of the ENaC particles, we are biased toward an oligomeric structure in which the ENaC complex is composed of eight subunits (Fig. 3C). However, the freeze-fracture data do not allow us to determine the relative stoichiometries of \( a, \beta \), and \( γ \) subunits.

Based on the size distribution of the particles in the clusters, no particle population consistent with ENaC heterotetramers (i.e. \( αγβγ \)) was detected in the frequency histograms, and only 3% (24 out of 789) of the particles examined had a size that would suggest a complex of four subunits (~6.1 nm). Nevertheless, the low density of ENaC in the plasma membrane, and the similarity of size of a putative ENaC heterotetramer with that of the endogenous population, prevents us from ruling out the existence of a very small number of ENaC heterotetramers in the plasma membrane. Finally, other particle populations larger in size were also present in the clusters but were considerably lower in abundance (<9% of total). At present we cannot account for these larger particles, but they may represent higher oligomeric states of ENaC.

Electrophysiological Assay of ENaC Mutants—The freeze-fracture data provided evidence for an ENaC complex composed of eight or nine subunits, but it could not be ruled out that a small number of ENaC channels exist in the membrane as heterotetramers (i.e. \( αγβγ \)). Therefore, to overcome this limitation, we carried out an assay to determine if the functional form of ENaC contains more than one \( γ \) subunit. Our assay was based on the differential sensitivity of two functional Na\(^+\) channel mutants to MTSET and Cd\(^{2+}\). The results indicated that both MTSET and Cd\(^{2+}\) significantly inhibited \( αβγ_{529C} \) ENaC, but neither led to a notable inhibition of amiloride-sensitive macroscopic currents in the channel complex. Thus, functional assay of ENaC expressing two different \( γ \) mutants argues against functional ENaC heterotetramers (i.e. \( αγβ \)) and predicts the presence of a minimum of two \( γ \) subunits in the functional channel complex.

Our new observations together with consideration of previous data suggest a way to reconcile the apparently discrepant interpretations of earlier work. Studies using electrophysiologic assays measured current in oocytes expressing combinations of wild-type subunits and subunits bearing point mutations that alter sensitivity to channel blockers, including methanethiosulfonate compounds, amiloride, and Zn\(^{2+}\) (12–14). Such assays require assumptions regarding the dominant effect of the mutation, the relative expression of wild-type and mutant subunits, and random association of subunits into the channel complex. Consequently, these assays tend to provide a minimal estimate of the number of subunits in the channel complex. Thus, data suggesting that ENaC channels are heterotetramers with 2 \( α \), 1 \( β \), and 1 \( γ \) subunit (13, 14) may be explained if the channel is in fact a multiple of a tetramer containing 8 subunits, perhaps with a stoichiometry of 4 \( α \), 2 \( β \), and 2 \( γ \) subunits. Our functional data suggest that the channel complex contains more than one \( γ \) subunit. Thus, a channel composed of eight subunits would not simply be a combination of two tetrameric channels but rather both \( γ \) subunits would contribute to a common pore. Moreover, sucrose gradient centrifugation provided differing results depending on the detergent used. When ENaC was solubilized in CHAPS or Triton X-100, migration was consistent with a tetrameric size (15), but when it was solubilized in digitonin, the data suggested a higher-order complex consistent with eight or nine subunits (12, 15). Because results obtained from this technique depend on the ability of the channel complex to remain intact in the presence of detergent, such studies may also underestimate the number of subunits. The freeze-fracture technique used here provides an independent estimate of eight or nine subunits by directly examining the area of the channel complex in the natural lipid environment of the oocyte plasma membrane. Although our present data leave open the question of the relative stoichiometry of the channel complex, they do provide important new insights into the total number of subunits in the complex, which may help reconcile earlier reports, and they influence future models of the channel and its investigation.

Conclusion—When expressed in Xenopus oocytes, ENaC appeared in the plasma membrane in clusters of particles, and the total number of ENaC particles in these clusters was consistent with amiloride-sensitive macroscopic currents in the same cells. The cross-sectional area of ENaC in the plasma membrane obtained from the dimensions of the particles suggested that the functional ENaC complex contains eight or nine subunits. However, the overall shape of ENaC particles (near-square) leads us to hypothesize that ENaC is composed of eight subunits. Furthermore, our functional data suggested that each functional ENaC contains a minimum of two \( γ \) subunits. The functional and biochemical similarity of ENaC to other members of the DEG/ENaC family of channels suggests that our findings may represent a common structural and functional feature of this class of membrane proteins.

Acknowledgments—We thank Zachary E. Lloyd, Dan Bucher, and Diane Olson for technical assistance.
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