We have studied a series of human acetyl-CoA carboxylase (ACC) 1 and ACC2 proteins with deletions and/or Ser to Ala substitutions of the known phosphorylation sites. In vitro dephosphorylation/phosphorylation experiments reveal a substantial level of phosphorylation of human ACCs produced in insect cells. Our results are consistent with AMPK phosphorylation of Ser29, Ser80, Ser1,201, and Ser1,216. Phosphorylation of the N-terminal regulatory domain decreases ACC1 activity, while phosphorylation of residues in the ACC central domain has no effect. Inhibition of the activity by phosphorylation is significantly more profound at citrate concentrations below 2 mM. Furthermore, deletion of the N-terminal domain facilitates structural changes induced by citrate, including conversion of ACC dimers to linear polymers. We have also identified ACC2 amino acid mutations affecting specific inhibition of the isozyme by compound CD-017-0191. They form two clusters separated by 60–90 Å: one located in the vicinity of the BC active site and the other one in the vicinity of the ACC1 phosphorylation sites in the central domain, suggesting a contribution of the interface of two ACC dimers in the polymer to the inhibitor binding site.

Keywords: acetyl-CoA carboxylase; binding site; human; inhibitor
Phosphorylation of Ser1,201 was reported to affect citrate affinity [32,33]. Cell cycle-dependent phosphorylation of Ser1,263 is required for ACC1 interaction with BRCA1 [34]. This Ser residue is conserved in animals. A possible regulatory mechanism involving interaction between ACC1 and Breast cancer susceptibility protein 1 (BRCA1) was suggested [2,34–39]. A new role of ACC1 in breast cancer has been proposed recently [40]. Mammalian ACCs are inhibited by malonyl-CoA and palmitoyl-CoA, while they are activated by citrate, which also induces polymerization [22,33,41–43]. Yeast ACC also has a dynamic structure and is regulated (inhibited) by phosphorylation of Ser1,157 (equivalent of human Ser1,216) by AMPK-like kinase SNF1 [44–46].

Here, we report the results of experiments on purified human ACCs expressed in insect cells, attempting to determine the relationships among phosphorylation, structure, and activity of both ACCs. Some years ago we established recombinant yeast strains in which human ACC1 or human ACC2 replaced the yeast ACC [12]. We screened possible inhibitors that could discriminate between the two human ACCs and found one, CD-017-Δ ethanol.32P radioactivity was measured by counting Cerenkov radiation. The amount of radiolabeled protein was quantitated using Image-Quant 5.0 (Molecular Dynamics, Sunnyvale, CA, USA). Two washes were applied: the first wash buffer; the NaCl concentration was then lowered to 300 mM and glycerol concentration was increased to 20% in the third wash buffer; TritonX-100 was left out of the fourth wash buffer. The elution buffer was the same as the fourth wash buffer, but contained 150 mM imidazole. The extended-wash protocol was developed to improve protein purity (Fig. S1), including elimination of insect protein (s) with kinase activity which copurified with the recombinant ACCs and led to a high level of background phosphorylation in the in vitro AMPK phosphorylation experiments (below). Such background kinase activity could also affect the ACC activity measurements. Aliquots of eluted proteins were stored at –80 °C. A BCA protein assay (Pierce, Waltham, MA, USA) was used to determine total protein concentration. Relative concentration of intact ACC was also estimated by quantitation (Image-Quant 5.0) of digital images of coomassie blue-stained gels. Mass spectrometry confirmed phosphorylation of Ser29 and Ser80 in ACC1.

Materials and methods

Protein expression and purification

Expression of ACC1 and ACC2Δ148 (truncated ACC2 with residues 3–150 replaced with a single Glu, Table 1) in S9 insect cells was described previously [12]. For ACC1, N-terminal deletion of 103 amino acids, a single S1,201A mutation and S1,201A plus S1,263A mutations were introduced into the wild-type gene in the pFAST Bac vector by PCR and “cut and paste” of DNA restriction fragments. The 245-amino acid deletion was introduced into ACC2Δ148 by the same methods. The recombinant ACC1 and ACC2 proteins contained a His6-tag, for affinity chromatography purification, fused at the C terminus and N terminus, respectively. Recombinant baculoviruses were prepared using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Waltham, MA, USA). S9 insect cells were grown in SIF900 II SFM media, supplemented with fetal bovine serum (5%), penicillin (100 units·mL⁻¹), and streptomycin (100 µg·mL⁻¹). Two rounds of viral amplification yielded P1 and P2 viral stocks, both of which were used for large-scale (250–500 mL cultures) ACC production. Cells were harvested after 72–96 h, suspended in 50 mM Tris (pH7.5), 10 mM MgCl₂, 300 mM NaCl, and 10% glycerol, and lysed by fast freezing-thawing.

Protease-50 (G-Biosciences, St. Louis, MO, USA) was added to the lysate (20 µL for cells collected from 100 mL culture). Lysates were cleared by centrifugation at 10 000 g for 20 min, and the supernatant was collected for affinity column purification using Talon resin (Clontech, Mountain View, CA, USA). Four washes were applied: the first wash buffer contained 50 mM Tris (pH 7.5), 10 mM MgCl₂, 10 mM imidazole, 10% glycerol, 100 mM NaCl, and 0.2% TritonX-100; the NaCl concentration was increased to 500 mM in the second wash buffer; the NaCl concentration was then lowered to 300 mM and glycerol concentration was increased to 20% in the third wash buffer; TritonX-100 was left out of the fourth wash buffer. The elution buffer was the same as the fourth wash buffer, but contained 150 mM imidazole. The extended-wash protocol was developed to improve protein purity (Fig. S1), including elimination of insect protein(s) with kinase activity which copurified with the recombinant ACCs and led to a high level of background phosphorylation in the in vitro AMPK phosphorylation experiments (below). Such background kinase activity could also affect the ACC activity measurements. Aliquots of eluted proteins were stored at –80 °C. A BCA protein assay (Pierce, Waltham, MA, USA) was used to determine total protein concentration. Relative concentration of intact ACC was also estimated by quantitation (Image-Quant 5.0) of digital images of coomassie blue-stained gels. Mass spectrometry confirmed phosphorylation of Ser29 and Ser80 in ACC1.

Dephosphorylated recombinant ACC

Recombinant proteins were dephosphorylated using lambda phosphatase (New England Biolabs, Ipswich, MA, USA) following the manufacturer’s protocol. The efficiency of lambda phosphatase treatment was tested by a trichloroacetic acid (TCA) precipitation assay. A test ACC protein phosphorylated using AMPK and [γ-32P] ATP (below) and treated with lambda phosphatase was precipitated with one volume of ice-cold 10% TCA for 10 min on ice and collected on a Whatman GF-C glass-fiber filter. Filters were washed with 0.5 mL of ice-cold 5% TCA, four times with 5 mL of ice-cold 5% TCA, and once with 10 mL of 95% ethanol. 32P radioactivity was measured by counting Cerenkov radiation. The amount of radiolabeled protein decreased 86% and 96% after 10 and 60 min of the phosphatase treatment, respectively. A quantity of 30–130 µg of the affinity-purified recombinant ACCs was incubated with 2000 units of the phosphatase for 30 min at 37 °C, the proteins were repurified by affinity chromatography and their concentration was determined as described above.

AMPK phosphorylation of recombinant ACC

A quantity of 0.5–1.2 µg of protein was incubated in 20 µL of 50 mM HEPES, 150 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.15 mM AMP, 60 µM [γ-32P] ATP (10 Ci·mmol⁻¹,
2 mCi·mL⁻¹) with 30 ng of AMPK (EMD Millipore) for 90 min at 37 °C. The samples were mixed with 20 μL of 2 × SDS Tris-glycine gel-loading buffer, heated 5 min at 95 °C, and 18 μL were loaded on Tris-glycine gels. Radiolabeled protein bands were visualized by phosphoimaging. Duplicate gels were stained with Coomassie blue to determine the protein amount in the ACC full-length protein bands as described above. Radiolabeled ACC bands were excised from the gels for ³²P measurement by scintillation counting. In some experiments, such AMPK phosphorylated ACCs were used in the ACC enzymatic assay (below) without further treatment.

ACC activity assay

The ACC activity assay was described previously [12]. Two variants of the protocol were used, as indicated by the information shown in brackets. About 2–138 ng of ACC were first incubated for 10 (12) minutes at 37 °C in 43 (40) μL of TrisHCl (pH 7.5), MgCl₂, Na-citrate, and BSA. A quantity of 7 (10) μL of a substrate mixture was then added to a final concentration of 3.7 (4.0) mM ATP, 0.25 (0.4) μM acetyl-CoA, and 1.4 (9.3) mM NaH¹⁴CO₃ at 59 mCi·mmol⁻¹ (3.2 mCi·mmol⁻¹), and the incubation was continued for another 45 (30) minutes. The final concentrations were 50 mM for TrisHCl, 10 mM for MgCl₂, 10 mM for Na-citrate, and 0.75 mg·mL⁻¹ for BSA. Reactions were quenched with 30 μL of 6N HCl, 70 μL aliquot (two 20 μL aliquots) of the mixture were spotted on a filter paper, and dried at elevated temperature for 30–60 min. The heat-stable radiolabeled malonyl-CoA was measured by scintillation counting. Forty units of the lambda phosphatase and MnCl₂ (1.0 mM) were included in some experiments. ACC activity in the presence of 0–25 mM citrate was measured by the same method. ACC activity was also measured after AMPK treatment of proteins previously dephosphorylated using lambda phosphatase (above).

CD-017-191-resistant mutants

Human ACC2 mutations L₁₅₇0Q, A₆₉₆S, and A₁₃₀₃G were identified by sequencing the ACC2 gene cassette in plasmids isolated from yeast strain ACC2Δ148 grown in 1 mL cultures containing CD-017-191 (3–30 μM) for 1–3 weeks. A total of 24 candidate mutant yeast strains exhibiting enhanced growth in the presence of the inhibitor, relative to the wild-type strain, were analyzed. These three mutations, as well as two additional mutations, E₉₉₄S and A₈₈₅G, of residues predicted based on the crystal structure of yeast ACC to be in the immediate vicinity of A₆₉₆, were engineered by PCR cloning to produce recombinant mutant ACC2 in the baculovirus expression system as described above.

Native PAGE

Proteins were incubated for 15–30 min under conditions described above for the ACC enzymatic activity assay but without substrates and BSA, and analyzed by the Native-PAGE Novex 3–12% Bis-Tris Gel method (Invitrogen). Protein bands were revealed using the silver stain for mass spectrometry method (Pierce). NativeMark Unstained Protein Standards (Invitrogen) were used to evaluate protein sizes. The amount of protein loaded on the gel was determined experimentally to ensure best band resolution versus detection sensitivity. Citrate-containing samples were analyzed on gels prerun for 60–90 min and then run in the standard running buffers supplemented with 10 mM Sodium Citrate. Histograms were calculated using Image Lab software (Biorad) with lane background subtraction to better visualize the major protein bands.

Electron microscopy

Proteins were incubated at 37 °C for 15–30 min in ACC enzymatic activity buffer (above) without BSA and substrates, but with MnCl₂ and Na-citrate and lambda phosphatase as indicated. Unless stated otherwise, specimens were prepared for EM using a conventional negative-staining procedure [47]. Briefly, a 2.5 mL drop of sample was absorbed to a glow discharged carbon-coated copper grid for 1 min, and blotted off. The grid were stained with three drops of freshly prepared 0.75% uranyl formate drop. The grids were imaged at room temperature using a Tecnai 12 or 30 (FEI Company) electron microscope equipped with a Gatan 2K x 2K or 4K x 4K camera respectively.

Results

Phosphorylation and activity

We have studied a series of human ACC1 and ACC2 proteins with deletions and/or Ser to Ala substitutions of the known phosphorylation sites (Table 1). Stringent wash conditions were used in the affinity purification of the recombinant proteins expressed in insect cells in order to improve protein purity (Fig. S1). The resulting protein preparations were more stable and retained enzymatic activity even after prolonged storage at −80 °C. They were also more stable in terms of protein solubility in comparison with preparations made using less stringent wash conditions, which showed significant tendency to precipitate. Finally, stringent washes were also required to remove insect cell kinase(s), which otherwise copurified with ACC, thereby affecting the outcome of in vitro phosphorylation experiments and possibly also enzymatic activity assays.

Recombinant human ACC proteins expressed in insect cells [14,48–50] were known to be phosphorylated [26]. Mass spectrometry analysis of our recombinant human ACC1 expressed in insect cells revealed phosphorylation of Ser₂₉ and Ser₈₀. All six
ACC variants were tested using lambda phosphatase dephosphorylation/AMPK phosphorylation assay to determine their phosphorylation status at AMPK phosphorylation sites. Lambda phosphatase efficiently dephosphorylates phospho-serine, -threonine, and -tyrosine residues without protein specificity [51]. Four phosphorylation sites could be assessed by this method in ACC1 and three in ACC2Δ148 (Fig. 1). Phosphorylation levels of Ser29, Ser80, and Ser1,201 in ACC1, and Ser222 in ACC2 produced in insect cells, are as high as 90%. In ACC1, Ser1,201 is partially phosphorylated in insect cells and another residue, most likely Ser1,216, is not phosphorylated in insect cells. In ACC2, Ser222 is highly phosphorylated, one additional residue is partially phosphorylated, and one is not phosphorylated. The identity of the latter two residues, located outside of the N-terminal regulatory domain, is not known. In addition, the longest ACC2 protein lacks the first 148 residues which could carry additional AMPK phosphorylation site(s). The AMPK phosphorylation pattern of residues in ACC1 and ACC2 is very similar.

Enzymatic activity of ACC2Δ148 at 10 mM citrate is twofold lower than that of ACC1 (Fig. 2). Overall, the ACC1 variants lacking the N-terminal domain show higher level of enzymatic activity than the full-length enzyme, irrespective of their phosphorylation status. Mutations S1,201A and S1,263A do not affect the activity. Phosphorylation of Ser29 and/or Ser80 lowers the activity at 10 mM citrate twofold, while phosphorylation of Ser1,201, which occurs in insect cells, does not affect the activity.

The ACC2 phosphorylation pattern is consistent with phosphorylation of Ser222 and two other residues outside of the N-terminal regulatory domain. Enzymatic activity of the two ACC2 deletions are not affected by phosphorylation. It is possible that the 148-amino acid deletion already destroys the regulatory function of the N-terminal domain even though the key regulatory Ser222 is still present in the truncated ACC.

Our results show that no other residues in ACC1 or ACC2 affecting the enzymatic activity are phosphorylated in insect cells. AMPK phosphorylation of lambda phosphatase-treated ACC1 leads to the expected twofold decrease in the ACC enzymatic activity, but no such effect was observed for ACC1Δ103. Similarly, AMPK phosphorylation showed some effect on enzymatic activity of ACC2Δ148, but not on ACC2Δ245 (Fig. 2). These results show that AMPK

### Table 1. ACC1 and ACC2 variants.

| Variant     | Description                                      |
|-------------|--------------------------------------------------|
| ACC1*       | Full-length                                      |
| ACC1Δ103*   | Residues 2–104 deleted                          |
| ACC1Δ103M1* | ACC1Δ103 with S1,201A mutation                   |
| ACC1Δ103M2* | ACC1Δ103 with S1,201A and S1,263A mutations      |
| ACC2Δ148**  | Residues 3–150 replaced with a single Glu        |
| ACC2Δ245**  | Residues 3–246 replaced with a single Glu        |

*Contains an 8-amino acid C-terminal extension with His6 tag.
**Contains a 25-amino acid N-terminal extension with His6 tag and a TEV protease cleavage site.

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**Fig. 1.** AMPK phosphorylation. Relative level of phosphorylation of untreated/phosphorylated proteins (orange bars) and proteins dephosphorylated by λ-phosphatase and then rephosphorylated by AMPK (green bars). The difference in the phosphorylation level between dephosphorylated ACC1Δ103 and ACC1Δ103M1, differing only by the S1,201A substitution, equals 1. Two measurements were performed. The ACC enzymatic activity results for the same protein set are included in the results shown in Fig. 2. Serine residues listed next to the bars illustrate approximate contribution of their phosphorylation, ? indicates unknown ACC2 phosphorylation sites.
phosphorylation of the residues downstream of the N-terminal regulatory domain do not affect activity.

Citrate dependence

Full citrate activation requires dephosphorylation of the N-terminal regulatory domain of ACC1 (Fig. S2). In addition to the apparent repression of the enzymatic activity by the N-terminal regulatory domain in phosphorylation-independent manner (Fig. 2), the inhibitory effect of the N-terminal phosphorylation of ACC1 is more profound at citrate concentrations below 2 mM (Fig. 3), physiological range of citrate concentration in human cells [41]. For ACC2Δ148, activity is not affected by phosphorylation at any citrate concentration (Fig. 3).

The effect of citrate on molecular properties of the full-length ACC1 and ACC1Δ103 (phosphorylated and dephosphorylated) was assessed by native PAGE. Citrate induced a shift of a significant fraction of ACC1Δ103 towards higher molecular weight part of the gel, in phosphorylation-independent manner (Fig. 4). A similar behavior was observed for ACC1, but only for the dephosphorylated protein. These gel-shifts indicate significant structural changes induced by citrate in the absence of the N-terminal phosphorylation mimicked by deletion of the N-terminal domain.

These changes correlate well with the observed effect of dephosphorylation or N-terminal deletion and citrate activation on enzymatic activity.

Finally, we have evaluated association state of different proteins using negative-staining electron microscopy.
Fig. 4. Effect of the N-terminal deletion, phosphorylation, and citrate on protein migration on native gels in the absence of citrate (A) and in the presence of 10 mM citrate (B). Histograms are aligned with images of the corresponding lanes from silver-stained gels. Top of the gel is on the left. Numbers on the lane images identify lanes on the source images of silver-stained gels shown in Fig. S3. pACC is the phosphorylated (in insect cells) version of ACC1 (treated with λ-phosphatase). Single-asterisk indicates ACC dimer and double-asterisk indicates a putative ACC polymer.

Fig. 5. Negative-staining EM images of ACC1Δ103 (A) and ACC2Δ245 (B) showing elongated oligomers and other states of the protein (dimers, unfolded protein, smaller degradation products, and contaminants) under conditions used for ACC enzymatic assay in the presence of 10 mM citrate. Dephosphorylation (addition of λ-phosphatase) was not required for the oligomer formation.
in order to determine their tendency to form higher order polymers. We have found that ACC1Δ103 and ACC2Δ245 were the only ACC variants able to form such structures in the presence of 10 mM citrate (Fig. 5) (independent of λ-phosphatase treatment). The conditions used to prepare proteins for the evaluation were the same as for the ACC enzymatic assays.

Efficient formation of stable dimer polymers is apparently not required for activity under conditions used in our experiments. Formation of such stable structures at lower citrate concentration requires participation of at least one other protein MIG12 [41]. It is not known, if ACCs produced in insect cells and purified under stringent wash conditions contain MIG12 homolog or any other proteins affecting ACC activity/structure.

Mutations affecting ACC2 inhibition by specific inhibitor CD-017-191

ACC2-specific inhibitor CD-017-191, 2-[[5-methyl-2-[4-(1-methylethoxy)phenyl]-4-oxazolyl]methyl]-oxazolo[4,5-b]pyridine, which inhibits human ACC2 with an IC50 of 2.8 μM, was identified by a yeast-based screening test using a gene-replacement yeast strain depending for growth on truncated ACC2 (ACC2Δ148) and then confirmed in vitro using a recombinant protein [12]. Human ACC1 is not inhibited by the compound at concentrations up to 100 μM. The yeast gene-replacement strain allowed screening for ACC2Δ148 mutants resistant to the inhibitor [12]. Sensitivity of the mutant screen depends on the potency of the inhibitor and yeast genomic mutations (ACC2 expression cassette is located on a plasmid) occur frequently [32] leading to false positives. Plasmid sequencing and in vitro tests of recombinant mutant proteins is required to verify results of the mutant screen. Analysis of mutant ACC2Δ148 gene-replacement yeast strains partially resistant to compound CD-017-191 revealed three ACC2 mutations affecting its inhibitory strength: A696S, A1,303G, and L1,570Q. The effect of the mutations was confirmed by in vitro enzymatic assay using mutant ACC2Δ148 produced in insect cells (Fig. 6). Two additional mutations were also engineered and tested in vitro: E694S showed no effect, but A687G lowered EC50 for compound CD-017-191 (Fig. 6).

Discussion

Sequence and structure comparisons of human multidomain and bacterial (e.g. E. coli) multisubunit ACCs reveal highly conserved domains required for the basic enzymatic activity. In addition, human ACCs contain an additional N-terminal domain and a large central domain. These domains are significantly less conserved and provide structural elements for divergent regulatory functions. The N-terminal domain is not present in some other multidomain ACCs, such as yeast [45] and grass cytosolic ACCs [53], but a different sequence element is present in grass plastid ACCs [54] and in apicoplast
Figure 7 revealed a two-component CD-017-191-binding EC50 shift was observed: A687G, A696S, A1,303G, and their mutants found no effect of AMPK phosphorylation/activity assessments of the recombinant human ACCs involved in regulation (Introduction). Phosphorylation/regulatory phosphorylation site and ACC contains a regulatory phosphorylation site and inhibition is stronger (2.5 fold) at lower, physiological citrate concentrations. The central domain of yeast ACC2 cannot be inactivated by phosphorylation and the inhibitor binding site. The truncated version of the interface of two ACC dimers in the polymer, creatine kinase, suggests contribution of amino acid residues located at the active site and the other in the vicinity of the phosphorylation sites in the central domain. Such an arrangement suggests contribution of amino acid residues located at the interface of two ACC dimers in the polymer, creating the inhibitor binding site. The truncated version of ACC2 cannot be inactivated by phosphorylation and shows a tendency to form polymers in vitro and presumably in vivo in the yeast gene-replacement screening strain. In this arrangement of the dimers, the BC active site on one dimer is located opposite a part of the central domain of the other dimer where phosphorylation sites, reportedly important for ACC1 function and interaction with BRACAl, are located. However, phosphorylation and regulatory importance of this part of ACC2 central domain are not known. The existence of two independent inhibitor binding sites, both affecting ACC enzymatic activity, is less likely. The BC half-binding site is located in a highly conserved region of all ACCs. However, the central domain half-binding site is located in a region highly divergent even in human ACCs, a possible source of the inhibitor specificity [12].

Our initial library screen was designed to identify only such specific inhibitors. The CD-017-191 binding site(s) is different from the well-established binding sites of inhibitors such as Soraphen A [57] and herbicides targeting grass plastid ACC [52].

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article:

**Fig. S1.** Coomassie blue-stained protein gels each comparing two fractions with the highest protein concentration of ACC1 affinity-purified by a fast one-wash protocol (A) and two fractions purified by the extended stringent wash protocol (B). Total protein from insect cells expressing human ACCs (C): 1, nocodavirus control; 2, protein markers; 3, ACC2Δ148; 4, ACC1Δ103; 5, ACC1 (prep 1); 6, ACC1 (prep 2); 7, ACC2Δ245.
**Fig. S2.** Citrate dependence of enzymatic activity of recombinant human ACCs, ACC1 (A), ACC1Δ103 (B) and ACC2Δ148 (C). Relative activity of the dephosphorylated form (orange line) and untreated proteins (green line). The activity of the dephosphorylated form of each protein at 10 mM citrate equals 1. Standard errors of 8 to 24 measurements are shown.

**Fig. S3.** Silver-stained native protein gels without citrate (A) and with 10 mM citrate (B) citrate. Source images used to calculate histograms shown in Fig. 4. *indicates corresponding lanes with double the amount protein loaded not used in further analysis. – indicates protein lanes not used in the analysis. Deformation of the gels during staining is due to low gel concentration at the top (3%).

**Table S1.** EC50 and EC50 shifts for inhibition of wild-type and mutated ACC2Δ148 by CD-017-191.