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Recently, reversible light-induced conformational changes were observed with the use of a spin label in the C-D interhelical loop in rhodopsin (16). It is significant that the changes in bR structure are also detected by a label in the C-D interhelical loop. Furthermore, the changes in both proteins evidently coincide with protonation changes at the Schiff base linkage of the retinal chromophore.

As for the functional significance of the structural changes in bR, Asp⁹⁶, five residues from the spin label at 101, is protonated in the ground state, transfers a proton to the Schiff base during M decay, and is reprotonated from solution during the decay of N (Fig. 1) (17). Evidently, the EPR signal changes are coincident with this process and thus may reflect structural changes near 101 as a result of changes in the protonation of Asp⁹⁶. An interesting possibility is that the transient change reflects the opening and closing of a pathway from the aqueous solution to Asp^{96} (3, 7). Recently, a change in local electrostatic potential around residue 101 was reported to have a similar relative time course (18).

In conclusion, a conformational change occurs during the decay of the M intermediate and reverses during the return to the ground state. The change is localized to the cytoplasmic side of the protein, in the vicinity of the E-F and C-D interhelical loops, and may reflect protonation changes in Asp⁹⁶. In principle, all of the conformational changes during the photocycle can be mapped by SDSL with a sufficiently large set of spin-labeled mutants.

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- To designate mutants, we give the single-letter code for the original amino acid, then the position of the amino acid, and finally the single-letter code for the replacement. Single-letter codes: C, Cys; G, Gly; Q, Gln; and V, Val.
- 10. Mutant plasmids were constructed by replacement of the Asp 718–Bsp HI fragment of the native bop gene with a synthetic piece carrying the appropriate mutation. Expression and purification of the mutant proteins were carried out as described previously for

other mutants [M. P. Krebs, R. Mollaaghababa, H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1987 (1993)].

- 11. Spin labeling was carried out in the presence of a fivefold molar excess of the label for 12 hours at 22°C. Excess label was removed by centrifugal washing. The stoichiometry in each case was 0.7 to 0.8 labels per bR. Spectra were recorded at 25°C at X-band frequencies in a TM101 cavity.
- 12. To designate mutants with spin-labeled side chains R1 or R2, we use the notation CXR1 or CXR2, where C is the original cysteine and X is the position of the amino acid.
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Cystic Fibrosis Heterozygote Resistance to Cholera Toxin in the Cystic Fibrosis Mouse Model

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The effect of the number of cystic fibrosis (CF) alleles on cholera toxin (CT)-induced intestinal secretion was examined in the CF mouse model. CF mice that expressed no CF transmembrane conductance regulator (CFTR) protein did not secrete fluid in response to CT. Heterozygotes expressed 50 percent of the normal amount of CFTR protein in the intestinal epithelium and secreted 50 percent of the normal fluid and chloride ion in response to CT. This correlation between CFTR protein and CT-induced chloride ion and fluid secretion suggests that CF heterozygotes might possess a selective advantage of resistance to cholera.

Cystic fibrosis (CF) is the most common, fatal, homozygous recessive disorder of the Caucasian population. One in 20 Caucasians harbors one copy of the mutated CF gene, resulting in a frequency of CF of 1 in every 2500 live births. CF is characterized by decreased Cl⁻ permeability of the apical membrane of many epithelial tissues (1), a result of mutations in the CF gene (2), and can result in meconium ileus in CF newborns and intestinal obstructions and accumulation of mucus in older individuals with CF (3). Secretory diarrhea may be caused by colonization of the small intestine with the organism Vibrio cholerae and results in a voluminous Cl⁻ and fluid secretion that can be fatal if untreated (4). Cholera mediates its action by an irreversible elevation of the concentration of intracellular adenosine 3',5'-monophosphate (cAMP), which triggers sustained Cl⁻ and fluid secretion (4, 5). These two diseases are connected by the apically located, cAMP-regulated, Cl⁻ channel CFTR. It has been proposed that CF heterozygotes may have a selective advantage against cholera-induced secretory diarrhea (6). The hypothesis suggests that

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CFTR Cl⁻ conductance is the rate-limiting step for intestinal Cl⁻ and fluid secretion, and therefore the hypothesis predicts a direct relation between CFTR expression and resultant Cl⁻ secretion.

Efforts to test this hypothesis have been limited by the absence of an animal model for CF (7). The CFTR(-/-) mouse was created by disruption of the CF gene at exon 10 by insertion of an in-frame stop codon (designated X) to replace Ser489 (S489X). This stop codon results in an unstable, truncated CFTR message and no full-length CFTR protein (8). Heterozygotes carrying one copy of the normal CFTR gene and one copy of the disrupted gene were crossed to give offspring that express zero, one, or two normal CFTR alleles and were designated CFTR(-/-) (homozygote CF), CFTR(+/-) (heterozygote), or CFTR(+/+) (homozygote normal), respectively. Heterozygote CFTR(+/-) mice were bred to C57BL/6 mice for at least six generations, with selection at each generation for the presence of the S489X mutation. After six generations, when over 98% of the loci were expected to be identical to C57BL/6, these heterozygous animals were mated with one another, and CFTR(+/+), CFTR(+/-), and CFTR(-/-) mice were obtained. These breeding strategies provided an opportunity to

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test CF heterozygote resistance to cholera in isogenic animals such that CT response was directly related to the amount of CFTR.

We assessed the relation between CFTR genotype and CT-induced fluid secretion in these mice by use of the sealed adult mouse model for secretory diarrhea (9). Normal homozygotes were given 10 μ g of CT through an oro-gastric feeding tube and then were incubated for 6 hours, which caused an accumulation of clear fluid in the small intestine and cecum. Cystic fibrosis homozygotes treated identically had no response. Intestinal fluid secretion was highest in the CFTR(+/+) mice, intermediate in the CFTR(+/-) mice, and lowest in the CFTR(-/-) mice (Fig. 1).

The physiological mechanism that results in fluid secretion in the mammalian small intestine is Cl⁻ secretion (10). The short-circuit current response (I_{sc}) of the murine jejunum is dominated by a Cl⁻ current that is mediated by apical CFTR Cl⁻ conductance (11). We studied the I_{sc} of isolated murine jejunal segments exposed to CT for a total of 6 hours (12). Consistent with the in vivo fluid secretion data, murine intestinal segments mounted in Ussing chambers differed in their I_{sc} response to



Fig. 1. Fluid accumulation (FA) ratios in mouse small intestine. Fluid accumulation ratios were measured by comparing fluid mass to gut mass after administration of 10 μ g of CT through orogastric feeding tubes to mice of the indicated genotypes. Values represent means + SEs of 12 animals for CFTR(+/+), 17 animals for CFTR(+/-), and 8 animals for CFTR(-/-). Mice (regardless of genotype) treated with saline alone responded with a fluid accumulation value that was not different from that of the CFTR(-/-) mice (27). Groups were significantly different from each other with a *P* < 0.001, as determined by a single-factor analysis of variance.

CT, correlating with the number of normal CFTR alleles (Fig. 2). The response to CT (10 μ g/ml) in the CFTR(+/+) jejunum was a sustained $I_{\rm sc}$ that had a peak $\Delta I_{\rm sc}$ of $46.3 \pm 7.8 \ \mu \text{A/cm}^2$. The CFTR(+/-) jejunum treated with CT showed both a smaller peak ΔI_{sc} of 23.0 ± 2.6 μ A/cm² [49.6% of the CFTR(+/+) peak I_{sc}] and an overall decrease in the net Cl⁻ secretion (net Cl⁻ secretion was measured as the area under the secretion rate versus time curve over the 6-hour period) to approximately half of the value for the CFTR(+/+) jejunum. The ratio of the cumulative 6-hour CFTR(+/-) value to that of the cumulative 6-hour CFTR(+/+) value was 0.41. The sustained CT effect in CFTR(+/+)tissues could reflect several possible mechanisms, including differential recruitment of endosomal CFTR channels or a regulatory effect of CT on phosphodiesterases (13). The CFTR(-/-) jejunum was routinely unresponsive to stimulation by CT (14).

Cholera toxin acts by adenosine diphosphate (ADP) ribosylation of the α subunit of the stimulatory G protein, which results in an accumulation of intracellular cAMP that is maintained for the lifetime of the intestinal epithelium and which leads to the activation of cAMPregulable apical Cl⁻ channels and to subsequent fluid secretion (4, 5). The decreased fluid secretion we observed in CF murine heterozygotes could result, in theory, from decreased cAMP concentrations or from decreased Cl⁻ conductance due to decreased CFTR expression. We examined the ability of CT stimulation to produce cAMP in the three different CFTR genotypes. We found no difference in either the basal concentration of cAMP or the cAMP concentration after 6 hours of



Fig. 2. $I_{\rm sc}$ response to CT of isolated murine jejunum from CFTR(+/-) and CFTR(+/+) mice. After 30 min of basal recording, CT (10 µg/ml or approximately 120 nM) was administered to the mucosal bathing solution (as indicated by the arrow), and the $I_{\rm sc}$ and tissue resistance were monitored for 6 hours. Values represent means and standard errors of 16 individual preparations for both CFTR(+/-) (**D**) and CFTR(+/+) (**O**) mice, comprising five mice for each genotype with either two, three, or four tissues mounted from each mouse.

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CT stimulation in the CFTR(+/+), CFTR(+/-), or CFTR(-/-) jejunum (Fig. 3), consistent with normal and CF human jejunal biopsies (15). Thus, the decreased secretory capacity in heterozy-gotes occurs at a site distal to the production of cAMP.

Chloride permeability of the apical membrane of the mammalian small intestinal tract is mediated entirely through CFTR (11, 16). Thus, a plausible explanation for our results is that fluid and Cl⁻ secretion are proportional to the amount of CFTR protein expressed in the intestinal epithelium. We measured the amount of CFTR protein present in CFTR(+/+)and CFTR(+/-) small intestine by protein immunoblot (Fig. 4). We were unable to detect murine CFTR using antiserum developed against various epitopes of the human CFTR sequence. Polyclonal antiserum (number 3634), raised to a 23amino acid COOH-terminal peptide of the murine CFTR sequence (17), detected the murine CFTR homolog (180 kD) in CFTR(+/+) enterocytes but not in a comparable human colon adenocarcinoma cell line (T84). This protein band was confirmed to be murine CFTR by the absence of a comparable reacting antigen in CFTR(-/-) cells (no signal detected in six individual samples from four different gels). The CFTR(+/-) enterocytes, however, contained the murine CFTR homolog at a lower intensity that corresponded to approximately half of the normal homozygote CFTR protein (relative densitometry of CFTR protein) as a per-



Fig. 3. Basal and CT-stimulated cAMP concentrations in mouse jejunum. The cAMP accumulation was measured (in picomoles of cAMP per milligram of protein) in jejunal segments isolated from animals of the indicated genotype (*28*). Values represent means and standard errors of 10 to 12 individual measurements for each genotype (total of four mice per genotype) for both basal and stimulated data.

centage of CFTR protein in (+/+) cells: (+/-), 54.6 ± 4.7 and (-/-), 0.00 ± 0.0 (six to eight samples from each genotype separated on four individual acrylamide gels). Thus, the amount of fluid secretion, Cl⁻ secretion, and CFTR protein correlated with CFTR genotype in the ratio of 0:1:2 for CF homozygotes, CF heterozygotes, and normal homozygotes.

A higher than expected CF heterozygote frequency (1 in 20) may be explained by genetic drift, increased fertility, or a heterozygote selective advantage. A heterozygote selective advantage is the most plausible explanation for the maintenance of the high CF heterozygote frequency in the human population because evidence does not support genetic drift (18) or increased fertility (19). Several hypotheses have been proposed to account for the CF heterozygote selective advantage, including resistance to pulmonary tuberculosis (20), resistance to influenza (21), and resistance to cholera (6, 7). A selective heterozygote advantage of 2% occurring approximately 23 generations ago could explain the high observed incidence of CF (22). Of all the hypotheses, only resistance to cholera is favored by the finding that CFTR is a cAMPregulable Cl⁻ channel (23). Other reports have provided indirect support for a relation between cholera and CF, including decreased sweat secretion in response to β-adrenergic stimulation of CF heterozygotes, as compared with that in normal individuals (24), and CT stimulation of normal, but not CF, human intestinal epithelium (25). Our data show that fluid and Cl⁻ secretion in response to CT varies directly with the number of CFTR alleles in each mouse.

The predominant mutation causing CF in the Caucasian population is a deletion of phenylalanine residue 508 (Δ F508), which occurs in approximately 70% of individuals with CF (2), and results in no functional CFTR reaching the apical membrane (26). Thus, like our mouse model, Δ F508 heterozygotes are expected to have 50% of the normal amount of

Fig. 4. CFTR protein expression in CFTR(+/+), CFTR(+/-), and CFTR (-/-) intestinal tissue. Murine CFTR protein was isolated from jejunal enterocytes of the appropriate genotype and analyzed



by conventional protein immunoblot techniques (29). Lane 1, 50 μ g of protein extracted from T84 cells. Lanes 2 through 4, 50 µg of jejunal enterocyte protein from animals with the following genotypes: lane 2, CFTR(-/-); lane 3, CFTR(+/-); and lane 4, CFTR(+/+). Arrowheads indicate molecular size markers.

CFTR protein. This smaller amount of expressed CFTR in human heterozygotes would translate to a decreased fluid secretory response to CT and might be effective as a protective mechanism in avoiding the dehydration that is often lifethreatening in cholera. The data presented here support the hypothesis that human CF heterozygotes have a selective advantage in surviving the potentially fatal effects of secretory diarrhea and possibly provide an explanation for the high incidence of CF carriers.

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- 12. Murine jejunal segments were isolated from 36- to 40-day-old mice [five mice from both CFTR(+/-) and CFTR(+/+) groups were used]. Jejunal segments were mounted full thickness (unstripped) in Ussing chambers with an aperture of 0.28 cm² and bathed in bilateral oxygenated Krebs' bicarbonate Ringer buffer [N. J. Willumsen, C. W. Davis, R. C. Boucher, Am. J. Physiol. 256, C1033 (1989)] containing 5 mM mannitol in the luminal solution Measurements were made under voltage clamp conditions. Under these conditions, the I_{sc} is predominantly a measure of electrogenic Cl secretion (11). After an initial 30-min period of basal recording, CT (10 µg/ml) was added to the luminal solution, and bioelectric measurements were made for 6 hours. Then 5 mM glucose was added to the luminal bath to induce Na⁺/glucose absorptive co-transport
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- 28. Basal measurements were made from jejunal segments isolated 6 hours after animals were administered saline by an oro-gastric tube. Stimulated measurements involved administration of 10 µg of CT through the oro-gastric feeding tube and incubation for 6 hours, followed by removal of jejunal segments. Jejunal segments (3 to 4 cm in length) were isolated, rapidly frozen on dry ice, and homogenized in icecold 6% trichloroacetic acid. Precipitated protein and supernatant were collected and centrifuged (1000g for 5 min). Pellets were neutralized with 0.1 M NaOH, and the protein concentration was determined. Ether extracts of the supernatants were reconstituted and assayed for cAMP content by an acetylated radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).
- 29. Jejunal segments were removed from mice of all three genotypes, and enterocytes were isolated by a procedure of divalent cation precipitation and centrifugation [L. D. Lawson and D. W. Powell, Am. J. Physiol. 252, G783 (1987)]. Jejunal enterocytes or dissociated T84 cells were immediately solubilized in a modified Laemmli buffer [B Sarkadi et al., J. Biol. Chem. 267, 2087 (1992)], protein concentration was determined, and 50 µg of protein was loaded per lane on a 6% polyacryl amide resolving gel with a 5% stacking gel. Electrophoresis was at 100 mV for 4 hours in standard solutions. After protein transfer to polyvinylidine difluoride membrane, the membrane was incubated with a 1:20 dilution of affinity-purified antibody number 3634 in TTBS [200 mM NaCl, 50 mM tris (pH 7.4) 0.1% Tween, and 5% w/v milk powder (Carnation)] for 1 hour. After we washed the membrane, a 1:100 dilution of the secondary antibody, peroxidase-conjugated goat antibody to rabbit immunoglobulin G (Jackson Laboratory, Bar Harbor, ME), was incubated with the membrane for 1 hour. The membrane was then treated with chemiluminescence reagents (Amersham, Arlington Heights, IL) for detection of CFTR bands. A comparable blot was incubated with polyclonal antibody number 858 (directed against the COOH-terminus of human CFTR), and only the T84 sample in lane 1 reacted with a positive signal at the expected location for CFTR (27). A total of eight individual jejunal samples were collected from both CFTR (+/+) and CFTR(+/-) mice. Direct densitometry comparisons were carried out only on samples that had been exposed to identical electrophoretic separation, protein transfer, and immunostaining techniques. Murine CFTR migrates with an apparent molecular size of 180 kD, as indicated in Fig. 4.
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