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## Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume

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**REGULATION of cell volume is essential for every cell and is accomplished by the regulated loss or gain of intracellular ions or other osmolytes<sup>1-4</sup>. Regulatory volume decrease often involves the parallel activation of potassium and chloride channels<sup>5-10</sup>. Overexpression of P-glycoprotein leads to volume-activated Cl<sup>-</sup> currents<sup>11,12</sup> but its physiological importance for volume regulation is unclear<sup>13</sup>. CIC-2 is a ubiquitously expressed Cl<sup>-</sup> channel<sup>14</sup> activatable by non-physiologically strong hyperpolarization. We now show that CIC-2 can be activated by extracellular hypotonicity, which suggests that it has a widespread role in volume regulation. Domains necessary for activation by both voltage and volume are localized to the amino terminus. Mutations in an 'essential' region lead to constitutively open channels unresponsive to medium tonicity, whereas deletions in a 'modulating' region produce partially opened channels responsive to both hypo- and hypertonicity. These domains can be transplanted to different regions of the protein without loss of function.**

Superfusion of *Xenopus* oocytes expressing CIC-2 with hypotonic solution produced a large increase in Cl<sup>-</sup> conductance. At isotonicity, CIC-2 Cl<sup>-</sup> current activates very slowly on strong hyperpolarization (Fig. 1a). Currents stimulated by

hypotonicity activate much faster and still display inward rectification (Fig. 1b). Although CIC-2 is normally closed at voltages less negative than -80 mV, hypotonicity induces a sizeable current in the physiological voltage range. This activation is fully reversible (Fig. 1c). Extracellular hypertonicity did not further reduce CIC-2 currents (data not shown). Roughly 10 min were needed before the effects of changes in osmolarity became apparent. This argues against a direct effect, and is compatible with slow intracellular changes in these large cells (such as cell swelling). Activation by hypotonicity was unchanged in Ca<sup>2+</sup>-free, EGTA-buffered saline, and also in oocytes additionally injected with EGTA (data not shown). Furthermore, the calcium ionophore A23187, which activates endogenous oocyte Cl<sup>-</sup> channels, did not elicit currents in oocytes injected with CIC-2 that were any larger than in control oocytes (data not shown). This argues against a role of calcium in channel activation.

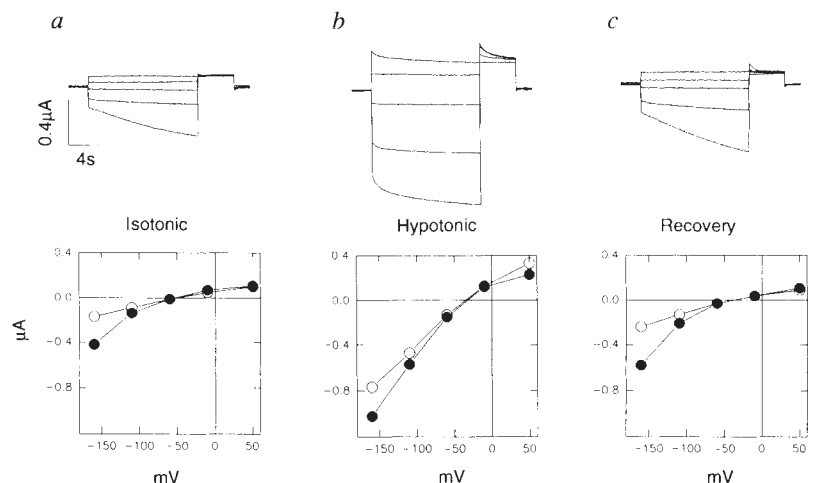
We constructed chimaeric proteins in which the region preceding the first transmembrane span D1 of CIC-2 was replaced by corresponding segments of the *Torpedo* electroplax channel CIC-0 (ref. 15) or the rat muscle channel CIC-1 (ref. 16). Expression of the CIC-(0/D1/2) construct (Fig. 2a) led to a large increase in membrane conductance, clamping the resting voltage to the Cl<sup>-</sup>-equilibrium potential. Similar but smaller currents were observed with the CIC-(1/D1/2) construct (Fig. 2b). With both chimaeras, the newly expressed conductance was almost time-independent and only slightly inward-rectifying, which contrasts with wild-type CIC-2. It fits, however, with the nearly linear instantaneous *I-V* relationship of CIC-2 observed after activation by hyperpolarization<sup>14</sup>. This suggests that a slow voltage-dependent 'gate' has been selectively removed by replacing the amino terminus.

Deletion of a large part (62 residues) of the amino terminus (mutant CIC-(0/10-W-63/2)) led to constitutively open channels, with currents similar to the CIC-(0/D1/2) chimaera. The extreme amino terminus of CIC-2, however, is not important for channel activation. Deletion of the first 15 amino acids, as well as several internal deletions between residues 6 and 20, resulted in wild-type activity.

Several internal deletions in a stretch of roughly 18 residues following Leu 21 led to an 'open' phenotype, defining a region that is essential for closing the channel at rest (Fig. 3a). Its amino-terminal border is remarkably sharp. Within this region, scattered deletions as small as four (or two) amino acids opened the channel. Neutralizing several charges either singly or in combination had no effect. But generating two new charges (mutant T26E/L27E) or increasing local hydrophobicity (mutant Q25L/T26L) led to the 'open' phenotype. Several 'open' mutants (listed in the legend to Fig. 3) were tested for their response to hyper- and hypotonicity: all were unresponsive (see Fig. 3b-d for mutant Δ(24-31)).

FIG. 1 Activation of CIC-2 by hypotonicity. Top, voltage-clamp traces; bottom, current-voltage relationships. ○, Currents present immediately after stepping to test voltage; ●, currents at the end of the 12-second test pulse. a, Currents under isotonic conditions (>10 min in ½ ND96 + 100 mM sucrose); b, after hypotonic activation (19 min after removing sucrose); and c, after returning to isotonicity (15 min after adding back 100 mM sucrose).

METHODS. Capped mRNA was transcribed from CIC-2 clone ptm1-b12-2 (ref. 14) by T7 RNA polymerase after linearization with *Xba*I, about 5-10 ng of which were injected into *Xenopus* oocytes prepared and handled as described<sup>27</sup>. They were stored and analysed in ND96 saline (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.4). For analysis of hypotonic stress, they were first transferred to isotonic solution containing ½ ND96 + 100 mM sucrose to eliminate potential effects of salt removal during the subsequent shift to ½ ND96. Measurements were performed at room temperature (20-24 °C) using standard two-electrode voltage-clamp technique and pCLAMP software (Axon Instruments).



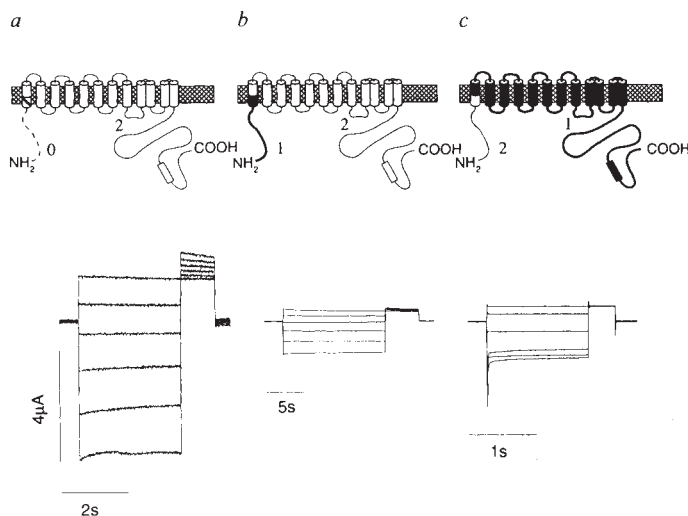
Immediately C-terminal to this 'essential' domain, we identified a region where deletions drastically changed channel activation without opening them constitutively (with the exception of  $\Delta(40-43)$ ). These 'intermediate' mutants mediated significant  $\text{Cl}^-$ -current at resting voltages, showing fast activation (compared with wild-type) with hyperpolarization and steady-state inward-rectification (Fig. 3e). They could be further stimulated by hypotonic stress (Fig. 3f). In contrast to wild-type CIC-2 and 'open' mutants, they could be closed by hypertonicity up to the point of restoring wild-type CIC-2 characteristics (Fig. 3g). These 'intermediate' mutants resemble CIC-2 channels whose set points for cell volume activation are shifted towards hypertonicity (smaller cell volume), and so are partially activated at isotonicity. This suggests that the mechanism mediating volume

sensitivity can operate in both directions, and seems to exclude second messengers (such as calcium) whose concentrations can be raised but not lowered from resting minimal levels.

Structural analysis<sup>17</sup> of the amino terminus predicts a helix-sheet-helix domain followed by a turn at about position 50-55, beyond which several mutations did not alter channel properties. The 'essential' region (where deletions produce the 'open' phenotype) is predicted to be a  $\beta$ -sheet whereas the 'modulating' region (where deletions result in 'intermediate' kinetics) lies within the second predicted helix. Both regions are highly conserved in the human CIC-2 protein (S.G., A.T. and T.J.J., unpublished results), the only difference being one conservative replacement (Glu 35 to Asp). As even the E35Q mutant had no effect, this is unlikely to have functional consequences.

FIG. 2 Electrophysiological properties of CIC chimaeric channels. Standard voltage-clamp experiments were done on chimaeric channels in which all amino acids preceding the (highly conserved) first transmembrane span D1 were exchanged between different channels. Schematic representations of the constructs are shown above the voltage-clamp traces. Putative transmembrane domains D1 through to D12 are shown as cylinders, and the conserved domain D13 is shown as a box. Recent transfer experiments described in the text suggest a cytoplasmic location of D13, according to model b of ref. 15. *a*, Construct CIC-(0/D1/2), in which the N terminus of CIC-0 precedes CIC-2; in contrast to CIC-2, the channel is constitutively open. *b*, CIC-(1/D1/2), with CIC-1 N terminus followed by CIC-2 backbone; this construct was expressed poorly and only in some experiments. *c*, CIC-(2/D1/1), N terminus of CIC-2 in front of CIC-1; the resulting current is similar to CIC-1 currents<sup>16</sup>, showing the typical deactivation at hyperpolarization and inward rectification in the positive voltage range. Voltage-clamp program: holding potential  $-30$  mV; test pulses from  $+40$  to  $-160$  mV in  $40$ -mV steps, followed by a constant  $+40$  mV pulse.

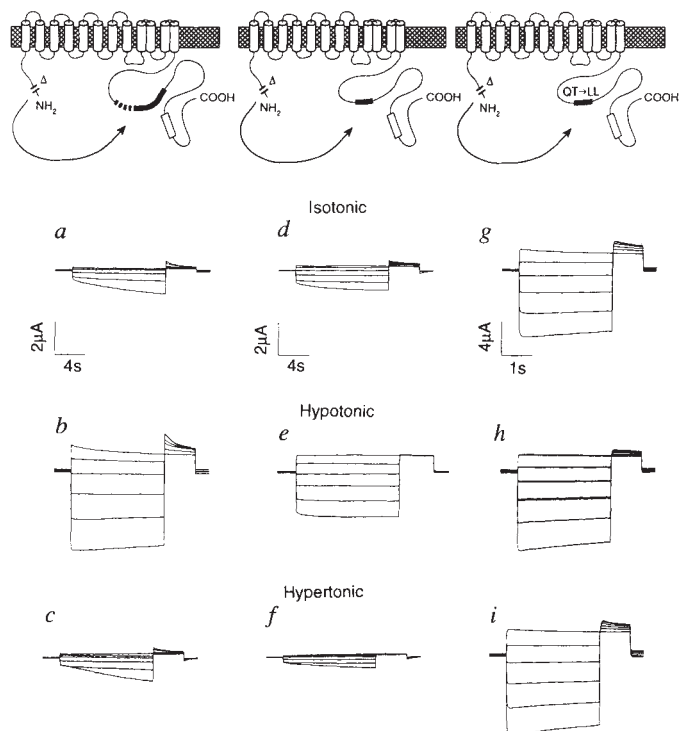
METHODS. Chimaeras were constructed by first introducing (using recombinant polymerase chain reaction<sup>28</sup> (PCR)) a silent *SpeI* restriction enzyme digestion site into all three channel cDNAs at a position coding for the conserved peptide sequence MALVS in D1. For CIC-0, Ala 60 was mutated in the same step to Leu to conform to D1 consensus sequence; Leu 56 in D1, which also differs from CIC-1 and CIC-2, was left unchanged. CIC-1 and CIC-2 N-terminal constructs always had CIC-0 5' non-coding sequence immediately preceding the initiator ATG which allows oocyte expression<sup>14,16</sup>.



Chimaeras were constructed using this unique *SpeI* site. mRNA synthesis and analysis were as described in Fig. 1 legend. Experiments were done one day (*a* and *c*) or five days (*b*) after injection.

FIG. 4 Effects of transplantation of inactivating domains. Voltage-clamp experiments were done on *Xenopus* oocytes expressing mutant channels in which different parts of the N terminus were transferred into the cytoplasmic tail of an N-terminal deletion mutant:  $\Delta(16-61)$ -XTB-CT (panels *a-c*),  $\Delta(16-61)$ -SMB-CT (*d-f*), and  $\Delta(16-61)$ -SMB-(QT  $\rightarrow$  LL)-CT (*g-i*). Top, schematic representation of these mutants, which are described below. In the XTB mutant (left), an extended 'ball' (shown in black) containing a 'stuffer' (broken black region) has been inserted into the C-terminal Tail (CT); in the SMB mutant (centre), a Small 'Ball' has been transferred; in the SMB (QT  $\rightarrow$  LL) mutant (right), this segment contains the Q25L/T26L mutation described in Fig. 3 legend. The open box represents the D13 domain. *a*, *d* and *g*, Isotonic conditions ( $\frac{1}{2}$  ND96 + 120 mM sucrose; identical results were obtained with ND96); *b*, *e* and *h*, hypotonic conditions ( $\frac{1}{2}$  ND96); *c*, *f* and *i*, hypertonic conditions (ND96 + 120 mM sucrose). In the XTB mutant, wild-type characteristics are fully restored; the SMB mutant has an 'intermediate' phenotype, and the SMB(QT  $\rightarrow$  LL) mutant an 'open' phenotype.

METHODS. Transplantation mutants were constructed by ligating different PCR-amplified 5' sequences from CIC-2 (with added *NcoI* and *BstBI* sites at both ends) into the unique *NcoI* site of CIC-2 deletion mutant  $\Delta(16-61)$ . In the case of  $\Delta(16-61)$ -SMB(QT  $\rightarrow$  LL)-CT, the template for PCR was the mutant Q25L/T26L. For mutant  $\Delta(16-61)$ -XTB-CT, this results in the insertion of GFEGTRPAARDRGPVALCTRPPGLRGDQCGRGLSEPGAESRPEPEKQEEARGQCTE (Met 1-Arg 74) FEGH between His 695 and Gly 696; the 'stuffer' sequence preceding Met 1 was provided to increase potentially the mobility of the inserted sequence and stems mainly from the 5' normally untranslated region of CIC-2; for the SMB mutants, the insertion is GF(Glu 17-Lys 41)FEGH, with point mutations (already described) in the case of SMB(QT  $\rightarrow$  LL). mRNA synthesis and measurements were done as for Fig. 1. The oocyte membrane potential was clamped to values between  $+40$  and  $-160$  mV. Oocytes were incubated for at least 15 min in the respective medium before starting measurements.



We asked whether the effect of deletions could be explained by a simple length effect. We inserted stretches of 33 or 66 amino acids into wild-type CIC-2 and into two deletion mutants after the 'modulating' region (Fig. 3a). There was no significant effect on voltage- or volume-dependent activation, and no consistent effects on the rate of deactivation (data not shown).

Next we took a constitutively 'open' deletion mutant and inserted different parts of the CIC-2 amino terminus between domains D12 and D13 (Fig. 4). We first inserted a large stretch fully encompassing the 'essential' and 'modulating' regions. This

fully restored both the voltage- and volume-dependence of channel activation (Fig. 4a-c). We then inserted a shorter stretch including the 'essential' region, but which lacked part of the 'modulating' region. This resulted in an 'intermediate' phenotype (Fig. 4d-f). Finally, an otherwise identical stretch from the 'open' mutant Q25L/T26L (Fig. 3) was unable to restore sensitivity to voltage and volume (Fig. 4g-i).

Thus the effects of amino-terminal determinants are largely position-independent. These experiments also support the postulated<sup>15</sup> transmembrane topology of CIC Cl<sup>-</sup> channels by

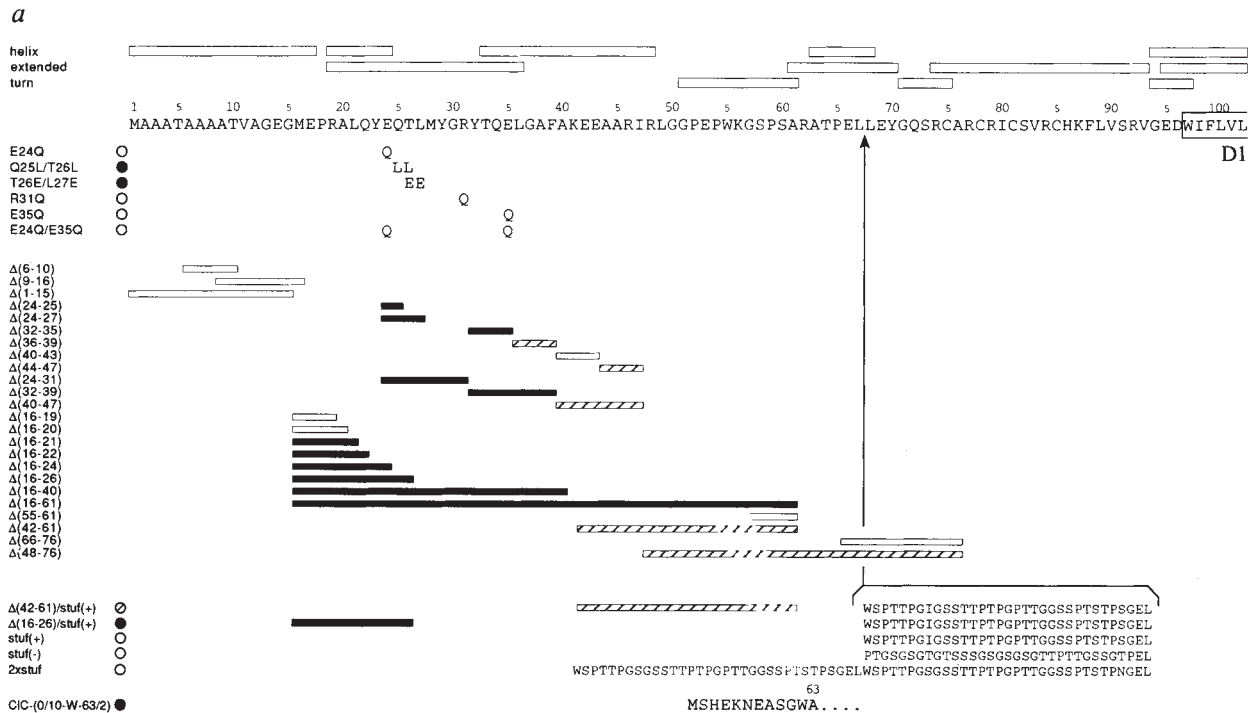
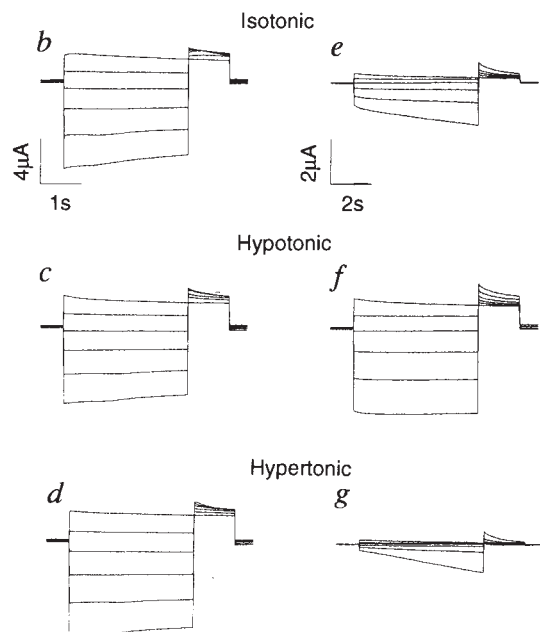


FIG. 3 Amino-terminal CIC-2 mutants and associated phenotypes. **a**, Schematic representation of mutants. At the top, structural predictions<sup>17</sup> are shown for the N terminus of CIC-2, whose amino-acid sequence is given below. The start of the first transmembrane span D1 is boxed. Point mutations are indicated by the substituted amino acid, and deletion mutants by bars below the corresponding amino acids. These bars also indicate gross functional phenotype: blank, wild-type; black, 'open'; hatched, 'intermediate'. For other mutants, the phenotype is indicated by similar symbols (circles) after the construct name. The sequences for the stuffer fragments inserted after Leu 67 into wild-type CIC-2 and into two deletion mutants are shown with corresponding phenotype indicated by bars and/or circles. **b-g**, Representative voltage-clamp traces of N-terminal mutations. Typical measurements of oocytes injected with an 'open' mutant ( $\Delta(24-31)$ ) (**b-d**) and an 'intermediate' mutant ( $\Delta(44-47)$ ) (**e-g**) are shown. Experiments were done under isotonic conditions (**b, e**) ( $\frac{1}{2}$ ND96 + 120 mM sucrose for >10 min); (**c, f**) hypotonic conditions ( $\frac{1}{2}$ ND96 for 30 min (**c**) or 21 min (**f**)); (**d, g**) and hypertonic conditions (ND96 + 120 mM sucrose for 27 min (**d**) or 15 min (**g**)). Voltage was varied between +40 and -160 mV in 40 mV steps, followed by a constant step to +40 mV. Holding potential was equilibrium potential. The following 'open' mutants were tested for response to volume changes and were found to be unresponsive:  $\Delta(16-21)$ ,  $\Delta(16-22)$ ,  $\Delta(16-61)$ ,  $\Delta(24-31)$ ,  $\Delta(32-35)$ , Q25L/T26L, T26E/L27E.

**METHODS.** Internal deletion mutants and changes of single amino acids were constructed by recombinant PCR<sup>28</sup> using *Pfu* DNA polymerase (Stratagene). In  $\Delta(1-15)$  the CIC-0 5' noncoding region was put (by PCR) immediately in front of the second ATG in frame (coding for Met 16). The chimaeric deletion mutant CIC-(0/10-W-63/2), in which the first ten amino acids of CIC-0 are fused to CIC-2 after amino acid 62, was constructed by ligation of the 5' *EcoRV* fragment of CIC-0 to the 3' *SmaI* fragment of CIC-2, which leads to an additional Trp residue at the ligation site. For 'stuffer' mutants, partially overlapping synthetic oligonucleotides (64 bases each) were annealed, extended by T7 DNA polymerase, self-ligated, cut with *SstI* (its recognition sequence was provided at both ends), and ligated into the 5' *SstI* site of CIC-2. Insertions of both orientations (stuf(+), stuf(-)) and a tandem head-to-tail insertion (2Xstuf) were obtained. Oligonucleotides



were designed to code for  $\alpha$ -helix and  $\beta$ -sheet breaking, mostly polar amino acids on both strands. In the stuf(+) orientation, a *BstXI* site (with *SstI*-compatible overhang) is generated at the 5' end of the insertion, which was used to ligate its 3' fragment to the 5' *SstI* fragments of the corresponding deletion mutants for constructs  $\Delta(16-26)$  stuf(+) and  $\Delta(42-61)$  stuf(+). All constructs were confirmed by sequencing. Structural predictions<sup>17</sup> were calculated using DNASTAR software (Madison, WI). Measurements were made as described for Fig. 1.

suggesting that the amino terminus and the region following D12 are on the same (cytoplasmic) side of the plasma membrane. The inactivating domain also functions when inserted after D13 (results not shown), effectively excluding D13 as a transmembrane domain.

To determine whether the CIC-2 amino terminus can impose slow activation by hyperpolarization upon another member of the CIC family, we put it in front of CIC-1 (construct CIC-(2/D1/1)). This did not significantly change its properties<sup>16</sup> (Fig. 2c). Although it is not yet known whether hyperpolarization-induced slow activation<sup>15,18</sup> of CIC-0 is caused by its amino terminus, we know from the properties of chimaera CIC-(0/D1/2) (Fig. 2a) that this domain cannot substitute for the CIC-2 amino terminus. Thus, the CIC-2 inactivating domain seems to interact specifically with a region (a putative 'receptor') present in CIC-2, but not in CIC-1.

Our findings are reminiscent of the 'ball-and-chain' model of inactivation<sup>19</sup> experimentally verified<sup>20,21</sup> for *Shaker* K<sup>+</sup> channels. In this model, an amino-terminal 'ball' can block the open pore of the channel. It is tethered to the channel by a polypeptide 'chain'. The CIC-2 'ball' should correspond mainly to the 'essential' region, with the 'modulating' region possibly acting to modify binding affinity. In contrast to K<sup>+</sup> channels, the 'ball' inactivates CIC-2 at rest and can be removed by voltage or hypotonicity (cell swelling).

With K<sup>+</sup> channels, 'chain' length influences the rate of inactivation<sup>20</sup> by determining the space volume in which the 'ball' can move. Inactivation of CIC-2 is nearly two orders of magnitude slower than that of *Shaker B*, although their amino termini are comparable in length. Thus 'chain' length cannot be the common principle governing inactivation kinetics for both channels. Indeed, we observed no consistent effect with amino-terminal insertions, and inactivation times seemed unchanged in the transplantation mutants. Hence a different step (like a conformational change on binding the 'ball') seems to be rate-limiting.

The CIC-2 'ball' lacks known target sequences for regulatory mechanisms such as phosphorylation. Its small size seems to preclude simultaneous binding to the cytoskeleton and to the putative 'receptor'. Thus cell-swelling and voltage may act by changing the affinity of its 'receptor'.

Mutational analysis shows that the structural requirements on the CIC-2 'ball' are relaxed. This is akin to K<sup>+</sup> channels, in which several mutations within the 'ball' have no effect<sup>20</sup>, and for which the 'balls' from different channels share no homology<sup>22</sup>. Whereas very different K<sup>+</sup> channels can be inactivated by the same 'ball'<sup>21-26</sup>, the CIC-2 'ball' interacts quite specifically with a 'receptor' present only on CIC-2. Identification of this 'receptor', analysis of its interaction with the 'ball', and an understanding of the destabilisation of this interaction by changes in cell volume are important goals. □

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## Phosphorylation of the *S. cerevisiae* Cdc25 in response to glucose results in its dissociation from Ras

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IN the yeast *Saccharomyces cerevisiae*, addition of glucose to starved cells triggers a transient rise in the intracellular level of cyclic AMP that induces a protein phosphorylation cascade<sup>1</sup>. The glucose signal is processed by the Cdc25/Ras/adenylyl cyclase pathway<sup>2</sup>, where the role of Cdc25 is to catalyse the GDP-GTP exchange on Ras<sup>3</sup>. The molecular mechanisms involved in the regulation of the activity of Cdc25 are unknown. We report here the use of highly selective anti-Cdc25 antibodies<sup>4</sup> to demonstrate that Cdc25 is a phospho protein and that in response to glucose it is hyperphosphorylated, within seconds, by the cyclic AMP-dependent protein kinase. It is also demonstrated that, concomitantly with hyperphosphorylation, Cdc25 partially relocalizes to the cytoplasm, reducing its accessibility to membrane-bound Ras. These results are of general significance because of the highly conserved sequence of Ras-guanyl nucleotide exchange factors from yeasts to mammals.

Wild-type yeast cells (SP1)<sup>5</sup> were labelled with <sup>32</sup>P-phosphoric acid before glucose starvation, and then incubated with glucose. Levels of cAMP and <sup>32</sup>P incorporation into immunoprecipitated Cdc25 were monitored in parallel. Figure 1 shows a temporal correlation between the diminished mobility of Cdc25 in the electrophoretic field, the extent of incorporation of <sup>32</sup>P into Cdc25 and the transient rise in intracellular cAMP levels. The incorporation of <sup>32</sup>P into Cdc25 peaks at 10 to 20 s (Fig. 1b, lanes 2, 3), and at the same time the cAMP level starts to rise sharply and peaks at 30 s (Fig. 1a). Thirty seconds after glucose addition, cAMP levels start to decrease and Cdc25 seems to be dephosphorylated in parallel to a lesser phosphorylated state (Fig. 1b, lane 4). In glucose-starved cells after 1 min the cAMP levels decay to an intermediate level, and, in parallel Cdc25 reaches an intermediate state of phosphorylation that is higher than the state of phosphorylation of Cdc25 (Fig. 1b, lanes 5 and 1, respectively). Exposure to glucose is also accompanied by a partial redistribution of the hyperphosphorylated Cdc25 to the cytoplasm whereas in the prestimulated state the less phosphorylated protein is exclusively localized to the membrane (Fig. 1b, lanes 1-3 and 6-8). This suggests that redistribution of Cdc25 to the cytoplasm results from its enhanced phosphorylation. The diminished electrophoretic mobility of the hyperphosphorylated Cdc25 generated in response to glucose and its redistribution to the cytoplasm is clearly observed in western blots using anti-Cdc25 antibodies (Fig. 1c). In these blots no change in the

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