# Exchange of conductance and gating properties between gap junction hemichannels

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**Abstract Gap junction channels span the membranes of two adjacent cells and allow the gated transit of molecules as large as second messengers from cell to cell. The structure of the gap junction channel pore is not resolved. For identification of pore determinants we used a chimera of two connexins, cx46 and cx32E$_1$43, that form membrane channels with distinct unit conductances and channel kinetics. Exchange of the first transmembrane segment (M1) between these connexins resulted in a chimera that exhibited most of the channel properties of the M$_1$ donor, including single channel conductance, channel kinetics, and the preference to dwell at a subconductance level. The M$_1$ segment thus appears to be an important determinant of conductance and gating properties of connexin channels.**



*Key words:* Gap junction; Connexin; Channel; Patch clamp; Chimera; Pore

## 1. Introduction

Gap junction channels span the membranes of two adjacent cells and thus provide a hydrophilic path for direct intercellular communication. Gap junction channels are made of two hemichannels each composed of six subunits (connexins). They are large conduits for molecules up to ~1 kDa. Since the first cloning of gap junction proteins it had been held that the third of four transmembrane segments (M$_1$–M$_4$) provides the lining of the pore of gap junction channels [1,2]. The conjecture was based on the presence of hydrophilic and hydrophobic amino acids in a pattern that is consistent with an amphipathic alpha helix. The discovery of connexins that form open hemichannels without apposition of two membranes [3,4] made it possible to test for pore lining amino acids by the substituted cysteine accessibility method (SCAM) [5]. Cysteines introduced in both the M$_1$ and M$_3$ segments were accessible [6]. In confirmation of the data obtained with the SCAM assay, the electron crystallographic structure of gap junctions at 7.5 Å resolution shows that the pore indeed appears to be lined by two transmembrane segments [7]. The resolution of the present crystal structure does not allow assignments of transmembrane helices to sequence. Thus, it is not clear which part of the assembly determines the pore properties, in particular the permeation filter. In the SCAM assay inhibition of conductance by thiol reagents was much more pronounced for cysteines in M$_1$ than in M$_3$ [6,8] as if M$_1$ is part of the permeation limiting part of the pore. To test this hypothesis we exchanged the M$_1$ segment between two connexins that can form open hemichannels, the lens connexin, cx46, and the chimera cx32E$_1$43, in which the first extracellular loop of cx32 is replaced by the cx43 sequence (Fig. 1). Both connexins, when expressed in Xenopus oocytes, form conductive hemichannels whose permeabilities are similar to those of complete gap junction channels. Like complete gap junction channels the hemichannels allow the passage of large tracer molecules, up to ~900 Da. They are gated by cytoplasmic acidification and voltage as are the corresponding complete gap junction channels[3,4]. Hence, the overall structures of the pore in hemichannels and complete gap junction channels likely are the same.

Cx46 and cx32E$_1$43 gap junction hemichannels can easily be distinguished from one another by their ~5-fold ratio in single channel conductances and their different kinetics. Thus, our expectation was that exchange of segments between the two open hemichannel forming connexins would result in a transfer of channel properties from the donor to the recipient connexin, provided that an exchanged segment contained determinants of channel activity. We were directed to the first transmembrane segment for this exchange by the results of the earlier SCAM study [6]. We were also steered towards M$_1$ by the observation that connexin mutations that cause connexin specific diseases cluster mainly in two areas, the extracellular loops known to be crucial for the docking of hemichannels to each other [9,10] and in the first transmembrane segment.

## 2. Materials and methods

Preparation of oocytes was as described earlier [11]. Cx46 was obtained from Dr. D. Paul [3]. Cx32E$_1$43 [4] in the vector pGEM3Z (Pomega) was cut with *Bam*HI and *Pml*I. A replacement cassette (Fig. 1) was prepared with the polymerase chain reaction (PCR) with the primers: (I) GCTGGGGGCGGCAGCCGAGTCAGCTTGGGGTGATG, (II) CCTCTTCCAGGTGAAGGGGGTC, (III) CGACTCTAGAGGATCCATGGGCGACTGGAGCTTCCTG, and (IV) CATCACCCCAAGCTGACTCGGCTGCCGCCCCCAGC, with cx32E$_1$43 and cx46 serving as templates. This yielded an intermediate construct in which the amino terminus up to the transition between the first transmembrane and the first extracellular segments had the cx46 (aa 1–41) and the rest the cx32E$_1$43 sequence. This intermediate and cx32E$_1$43 were used as templates for the production of the final insertion cassette using the primers: (V) GCATTCTACAGCCATTGGCCGAGTGTGGCTGACCGTCCTG, (II) CCTCTTCCAGGTGAAGGGGGTC, (VI) CTGCAGGTCGACTCTAGAGGATCC, and (VII) CAGGACGGTCAGCCACACTCGGCCAATGGCTGTAGAATGC. After digestion with *Bam*HI and *Pml*I the cassette was inserted into cx32E$_1$43 containing vector cut with the same enzymes. Synthetic mRNA was prepared with the mMESSAGE mMACHINE transcription kit (Ambion Inc.). Oocytes expressing the various connexins were assayed by two electrode voltage clamp to determine expression levels. Single connexin hemichannels were studied by the patch clamp technique [12] as described earlier [8]. Recordings were filtered at 5 kHz and digitized using a VR-10B digital data recorder and stored on video tape. The recordings were

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subsequently transferred to a Power Macintosh (Apple) computer using an ITC-18 Computer Interface (Instrutech Corporation) and analyzed. The acquisition and analysis software were Acquire and TAC (both from Bruxton Corporation).

## 3. Results

The chimeric connexin construct used in this study is shown in Fig. 2. We chose cx46 as the donor and cx32$E_1$43 as recipient because cx46 channels have properties distinct of those of endogenous channels in oocytes. The construction of the chimera involved an intermediate that fused cx46 (amino acids 1–41) with cx32$E_1$43 at the $M_1/E_1$ transition. This intermediate did not induce a conductance in mRNA injected oocytes. The final product cx32$M_1$46$E_1$43, however, was functional. Oocytes injected with mRNA encoding this chimera exhibited an increased membrane conductance that was voltage dependent, responsive to increased extracellular calcium concentration as well as to cytoplasmic acidification (Fig. 3). These properties are also found in both the donor and recipient connexins [3,4,8,13].

Single channel activity of cx46 gap junction hemichannels has been characterized before [8,14]. In agreement with these data cx46 channels were slothful, they opened and closed slowly and resided in the open and closed states for extended periods of time (hundreds of milliseconds). Furthermore, during both opening and closing events the channels often displayed several transitional conductance levels before reaching the full open or closed states (Figs. 4a, b, 5a, b, Table 1). Cx46 hemichannels presented a complex voltage dependence. A slow gating process closed the channel below −30 mV (not shown). As the potential was increased to positive values the channel currents monotonously became smaller, the channels rectified (Fig. 6a). At positive potentials the channels preferentially dwelled in a subconductance state, and excursions to the full open or closed states were infrequent (Figs. 4b and 6a). Single channel conductance of cx46 hemichannels were ~270 pS at −30 mV and 120 pS at +40 mV (Fig. 7), the corresponding open probabilities were 0.3 and 0.8, respectively.

Oocytes injected with cx32$E_1$43 exhibited similar levels of macroscopic membrane conductance as cx46 expressing oocytes [3,4]. The single channel activity of cx32$E_1$43 channels is shown in Figs. 4c, d, 5c, d and 6b. Typically, the transitions

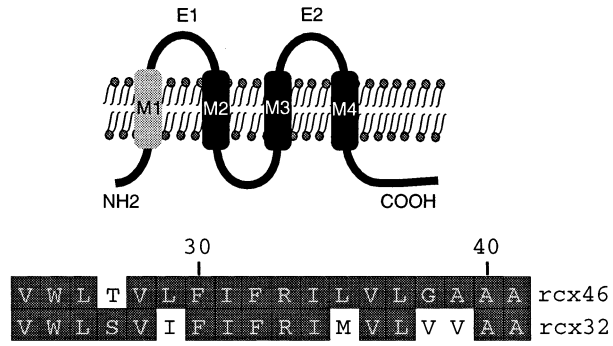


Fig. 2. Schematic of membrane topology of connexins. Based on several lines of evidence connexins have two extracellular loops, four transmembrane segments and three cytoplasmic segments. In the chimera cx32$M_1$46$E_1$43 the first transmembrane segment of cx32$E_1$43 is replaced by the corresponding sequence of cx46. Sequence alignment of the $M_1$ segments identifies the five amino acid differences.

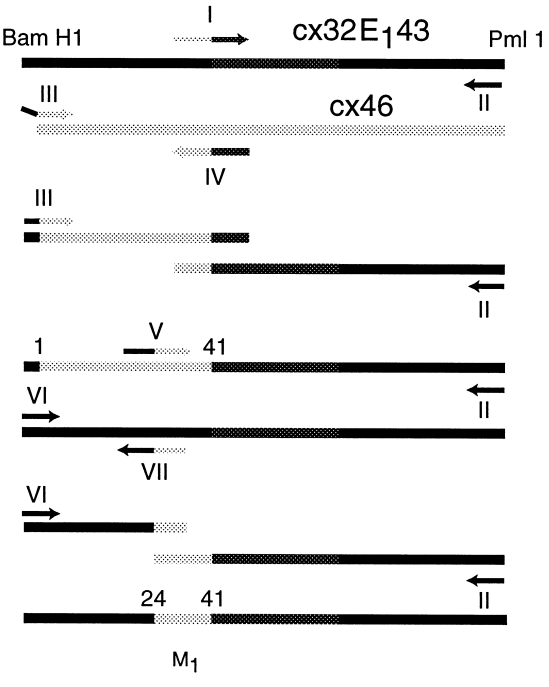


Fig. 1. Construction of cx32$M_1$46$E_1$43. A replacement cassette flanked by the restriction enzyme sites *Bam*HI and *Pml*I was generated by PCR. The sequences of connexins are coded as follows: cx32, black; cx43, dark gray; cx46, light gray. The positions of the primers I–VII are indicated by arrows. Arabic numbers denote amino acid positions in cx46.

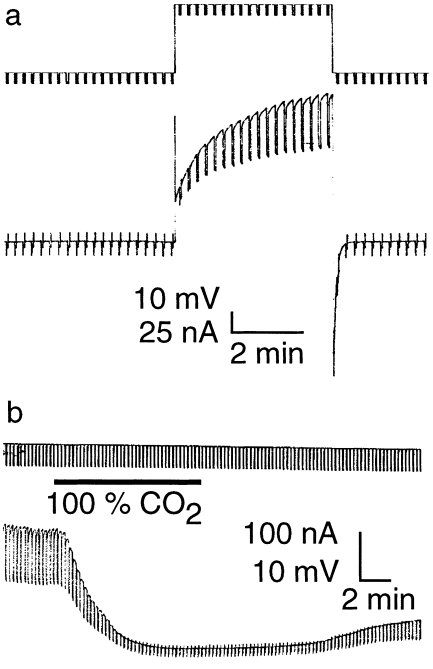


Fig. 3. Effect of membrane potential (a) and cytoplasmic acidification (b) on whole cell membrane currents induced by 5 mV voltage steps in oocytes expressing cx32$M_1$46$E_1$43. a: The membrane potential (upper trace) was held at −30 mV and stepped to 0 mV resulting in a slow activation of currents. b: Superfusion of oocytes held at 0 mV with OR2 solution saturated with 100% $CO_2$ resulted in a reversible inactivation of currents (lower trace).

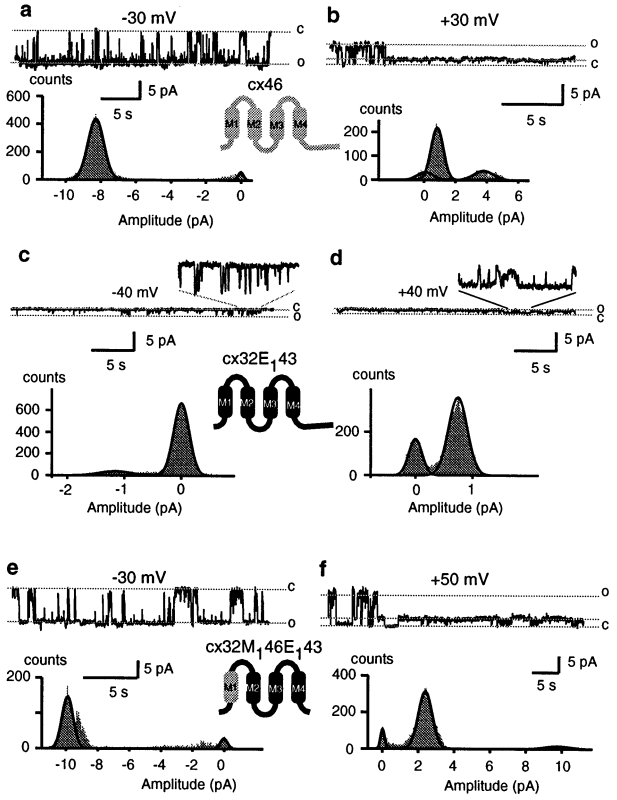


Fig. 4. Single channel currents recorded from inside-out patches that were excised from oocytes expressing cx46 (a, b), cx32$E_1$43 (c, d), and the chimera cx32$M_1$46$E_1$43 (e, f). Channels were held at negative (a, c, e) or positive (b, d, f) potentials as indicated. Representative records are shown and all-point amplitude histograms are based on analysis of 15–30 s segments. The insets in c and d are at 5 times extended scales. At negative potentials the channels gate mainly between open and closed states. Cx46 and cx32$M_1$46$E_1$43 channels can be seen to remain in the open and closed states for several hundred milliseconds and the transitions are slow and can involve intermediate current levels. In contrast cx32$E_1$43 channels gate faster and the open times usually are brief. At positive potentials both cx46 and cx32$M_1$46$E_1$43 channels assume preferentially a subconductance state whereas cx32$E_1$43 channels mainly gate between full closed and open states. Open (o), closed (c), and subconductance levels are indicated by dotted lines. All single channel records in this study were obtained in symmetric solutions, both pipette and bath contained 140 mM potassium gluconate, 10 mM potassium chloride and 5 mM (*N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), pH 7.5.

between open and closed states occurred in single steps, no transitions through intermediate conductance levels were apparent. These channels opened and closed more rapidly than cx46 channels (Fig. 5, Table 1). Cx32$E_1$43 channels exhibited brief open durations at negative potentials. They displayed a similar voltage dependence as cx46, they closed slowly at potentials below −30 mV and rectified at positive potentials.

Table 1
Rise times of channel openings

| cx46 wt | cx32$E_1$43 | cx32$M_1$46$E_1$43 |
|---|---|---|
| 80 ± 7 ms | 17 ± 2 ms | 96 ± 16 ms |

For each channel type ten successive channel openings in a record were analyzed. Only transitions from closed to full open state were measured. The membrane potential was held at −30 mV. Means ± S.E.M. are given.

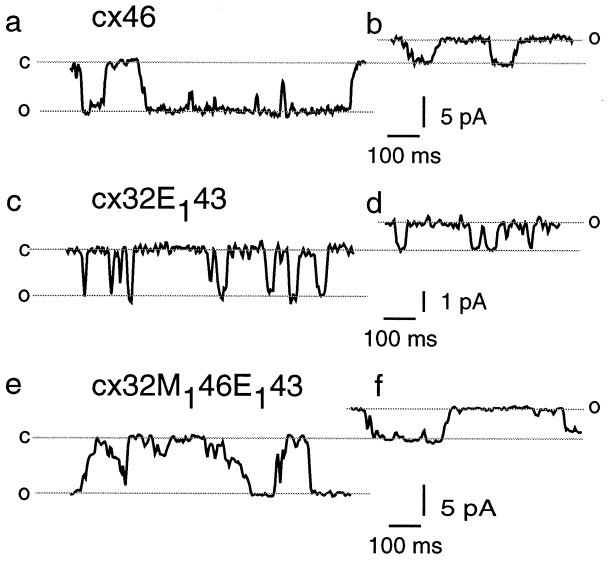


Fig. 5. Samples of single channel currents recorded from inside-out patches that were excised from oocytes expressing cx46 (a, b), cx32$E_1$43 (c, d), and the chimera cx32$M_1$46$E_1$43 (e, f). Holding potentials were −30 mV (a, c, e) or +30 mV (b, d, f). Currents are displayed at extended time scale to illustrate differences in channel kinetics. Transitions between open and closed states are fast in cx32$E_1$43 channels and slow in cx46 wt and cx32$M_1$46$E_1$43 channels.

Cx32$E_1$43 channels, however, did not exhibit the preference for a subconductance as cx46 channels at positive potentials, instead they mainly flickered between closed and full open states (Figs. 4d and 6b). Their unit conductance was 40 pS at −30 mV and 20 pS at +40 mV, five times smaller than the conductance of cx46 channels (Fig. 7). The open probabilities were 0.2 at negative potential and 0.75 at positive potential.

Oocytes injected with mRNA for the chimera cx32$M_1$46$E_1$43 exhibited channel activity similar to the one of cx46 channels (Fig. 4e and f). The gating pattern of the chimera resembled that of cx46 channels, the transitions to open and closed states were slothful and involved transitional conductance levels instead of the rapid gating of the cx32$E_1$43 channel (Fig. 5e, f, Table 1). Unlike either parent channel at negative potentials the chimera wobbled at the open state giving rise to a double peak for the open conductance (Figs. 4e and 6c). Whether this wobble is caused by two juxtaposed open states remains to be determined. The chimeric channel rectified, but rectification was weaker than in the parent channels (Figs. 6 and 7). Like the $M_1$ donor, cx46, the chimera conducted at a subconductance level at positive potentials (Fig. 4f). The unit conductance of the channel was considerably larger than that of the parent channel, cx32$E_1$43, and was similar to that of cx46, the donor of the first transmembrane segment (Fig. 7). Both the kinetic and conductance properties of the chimera cx32$M_1$46$E_1$43 were similar to cx46. Thus, as the result of the transfer of the $M_1$ segment, the recipient connexin acquired channel properties from the donor connexin.

## 4. Discussion

The present results are at variance with the preponderant view of pore forming moieties in gap junction channels ac-

cording to which the third transmembrane exclusively provides the gated aqueous pathway through the membrane. Instead the present results strongly suggest that the first transmembrane segment of connexins (or a part of it) provide a critical portion of the lining of the channel and thus complement data obtained with the SCAM procedure [6]. The almost identical unit conductances of the $M_1$ donor and the chimeric connexin channels suggest that $M_1$ contains the flux limiting, narrow part of the channel.

The $M_1$ segment alone may not be sufficient to furnish the complete channel lining [6]. Only two amino acid positions were found to be reactive in the SCAM procedure. Thus it is possible that other transmembrane segments, including $M_3$, which gave marginal results in the SCAM assay, supply the remainder of the channel lining. Even a contribution by a segment not predicted to be transmembranous and thus equivalent to the P segment of voltage gated ion channels cannot be excluded. As a potential candidate for such a contribution the transition segment between $M_3$ and the second extracellular loop has been considered earlier [9]. Based on the present study an additional contributor, regardless of its identity, would be expected to provide the wider intracellular and/or extracellular vestibuli to the channel.

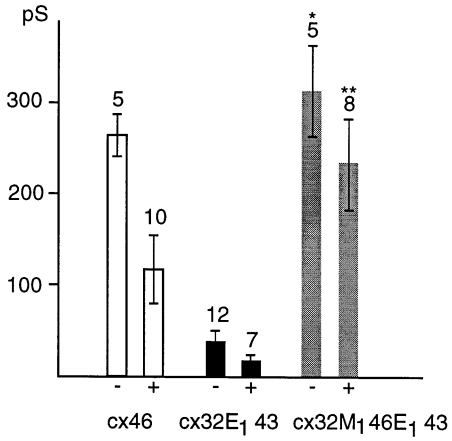


Fig. 7. Statistical analysis of single channel conductances of cx46 (white bars), cx32$E_1$43 (black bars), and cx32$M_1$46$E_1$43 (gray bars) channels held at negative (range: −40 to −30 mV) and positive potentials (range: +30 to +40 mV) as indicated by + and − signs. Means of maximal conductance levels with error bars indicating standard deviation; the number of measurements is indicated above each bar. Analyses are based on 96 s segments of activity. *: not significantly different from wt cx46 at negative potential. **: different from wt cx46 at positive potential, $P < 0.05$.

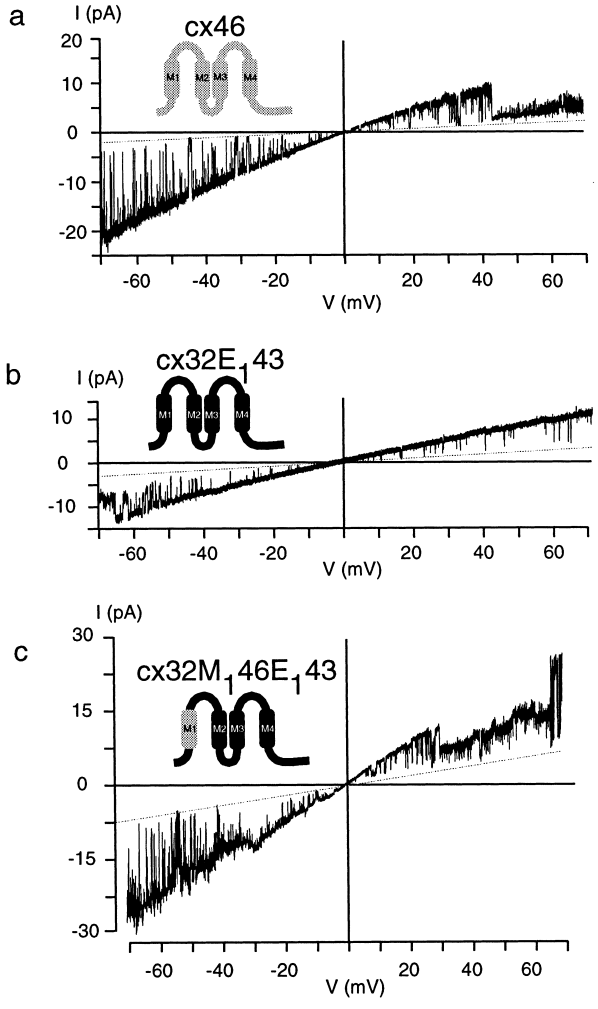


Fig. 6. Single channel currents in membrane patches excised from oocytes expressing cx46 (a), cx32$E_1$43 (b), or cx32$M_1$46$E_1$43 (c) during voltage ramps applied over 80 s from +70 to −70 mV. The dotted lines indicate leak conductance (assumed to be linear).

The observation that gating properties, the channel kinetics and the preference for a subconductance level at positive potentials, are acquired by the chimera from the $M_1$ donor, cx46, suggests that the $M_1$ segment also contains structural elements for gating. Thus connexins appear to follow a modular building principle and the first transmembrane segment apparently contains both the conductance module and the gating module that determines the gating to a subconductance level at positive potentials. The mechanism for rectification, on the other hand, does not appear to be fully represented in the $M_1$ segment.

Despite the large difference in single channel conductance the permeability of cx46 and cx32$E_1$43 channels for large tracer molecules is similar. The cut-off limit for both types of channels is close to 900 Da. Oocytes expressing these channels take up from the bath medium effectively carboxyfluorescein (molecular weight (MW) 376), at barely detectable rate Lucifer yellow cadaverine biotin-X (MW 873) and exclude Stachiose fluorescein (MW 1146) ([4], Pfahnl, Hu and Dahl, unpublished). Thus, these tracer molecules are not useful to distinguish the channels. However, support that channel permeability is determined by the $M_1$ segment comes from site directed mutagenesis data [15]. In the SCAM assay, position 35 in cx46 yielded the highest inhibition of conductance [6,8]. Replacing leucine 35 by glycine not only increases the single channel conductance, but also yields channels with larger permeability. Oocytes expressing L35G mutants take up Stachiose fluorescein and allow permeation of spermine in contrast to wt cx46 channels that are impermeable for both compounds [15].

The structure of several membrane channels has now been determined by crystallography and each follows a different blueprint for the pore architecture [16–18]. It appears that the gap junction channel does not follow any of these building principles but has its own unique pore structure which allows

the gated transit of molecules as large as second messengers from cell to cell.

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