Reversal of the Gating Polarity of Gap Junctions by Negative Charge Substitutions in the N-Terminus of Connexin 32

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ABSTRACT Intercellular channels formed by connexins (gap junctions) are sensitive to the application of transjunctional voltage (V_j), to which they gate by the separate actions of their serially arranged hemichannels (Harris, A. L., D. C. Spray, and M. V. L. Bennett. 1981. *J. Gen. Physiol.* 77:95–117). Single channel studies of both intercellular and conductive hemichannels have demonstrated the existence of two separate gating mechanisms, termed "V_j-gating" and "loop gating" (Trexler, E. B., M. V. L. Bennett, T. A. Bargiello, and V. K. Verselis. 1996. *Proc. Natl. Acad. Sci. U.S.A.* 93:5836–5841). In Cx32 hemichannels, V_j-gating occurs at negative V_j (Oh, S., J. B. Rubin, M. V. L. Bennett, V. K. Verselis, and T. A. Bargiello. 1999. *J. Gen. Physiol.* 114:339–364; Oh, S., C. K. Abrams, V. K. Verselis, and T. A. Bargiello. 2000. *J. Gen. Physiol*. 116:13–31). A negative charge substitution at the second amino acid position in the N-terminus reverses the polarity of V_j-gating of Cx32 hemichannels (Verselis, V. K., C. S. Ginter, and T. A. Bargiello. 1994. *Nature.* 368:348–351; Oh et al., 2000. *J. Gen. Physiol*. 116:13–31). We report that placement of a negative charge at the 5th, 8th, 9th, or 10th position can reverse the polarity of Cx32 hemichannel V_j-gating. We conclude that the 1st through 10th amino acid residues lie within the transjunctional electric field and within the channel pore, as in this position they could sense changes in V_j and be largely insensitive to changes in absolute membrane potential (V_m). Conductive hemichannels formed by Cx32*Cx43E1 containing a negatively charged residue at either the 8th or 10th position display bi-polar V_j-gating; that is, the open probability of hemichannels formed by these connexins is reduced at both positive and negative potentials and is maximal at intermediate voltages. In contrast, Cx32*Cx43E1 hemichannels with negative charges at either the 2nd or 5th positions are uni-polar, closing only at positive V_j. The simplest interpretation of these data is that the Cx32 hemichannel can adopt at least two different open conformations. The 1st–5th residues are located within the electric field in all open channel conformations, while the 8th and 10th residues lie within the electric field in one conformation and outside the electric field in the other conformation.

INTRODUCTION

Intercellular channels (gap junctions) formed by two closely related connexins, Cx32 and Cx26, are sensitive to the relative difference in the membrane potential of coupled cells, the transjunctional voltage, V_j (Barrio et al., 1991; Rubin et al., 1992). Both the initial and the steady-state conductance voltage relations of heterotypic Cx32/Cx26 junctions are asymmetric. The asymmetry in the initial conductance-voltage relation results from the rectification of open channel ionic currents, increasing when the Cx26 side of the intercellular channel is relatively positive (Bukauskas et al., 1995; Oh et al., 1999). The rectification is caused by a difference in the position of charged amino acid residues along the permeation pathway of Cx32 and Cx26 hemichannels as described by Oh et al. (1999). The asymmetry in the steady-state conductance-transjunctional voltage (G_j/V_j) relation of Cx32/Cx26 heterotypic gap junctions is due to the opposite gating polarity of the two hemichannels (Verselis et al., 1994). Cx32 hemichannels close when the potential difference across the intercellular channel (V_j) is relatively negative with respect to the Cx26 side, that is, when the potential of the Cx32 side of the intercellular channel is more negative than that of the Cx26 hemichannel. Cx26 hemichannels close when the polarity of V_j is positive. Thus, in the Cx32/Cx26 intercellular channel one or both gates present in each hemichannel are closed by adequate positivity to the Cx26 side of the junction, and neither gate is closed when the Cx26 side is relatively negative.

The difference in the gating polarity of V_j-dependence of Cx32 and Cx26 hemichannels is due to a difference in the charge of the second amino acid residue. The substitution of the neutral asparagine residue with a negatively charged amino acid (Cx32N2E or Cx32N2D) present in wild-type Cx26 reverses the gating polarity of Cx32 hemichannels, whereas the substitution of either neutral or positive charges does not change the gating polarity of Cx32 hemichannels. The substitution of a neutral or positively charged amino acid for the negatively charged aspartyl residue found in wild-type Cx26 (Cx26D2N or Cx26D2R) reverses the gating polarity of Cx26 hemichannels (Verselis et al., 1994). A negative charge substitution of the 11th amino residue (Cx32S11D) did not appear to reverse the polarity of the V_j-dependence of the Cx32 hemichannel. These results suggest that charged amino acids located in the N-terminus upstream of the 11th residue in Cx32 and Cx26 lie within the transjunctional electric field, and most likely comprise part, if not all, of the transjunctional voltage sensor. The relation between the charge of amino acids located in the

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N-terminus and the polarity of V_j-gating indicates that the gating mechanism is conserved in the two hemichannels. The movement of the N-terminus toward the cytoplasm of the cell would initiate hemichannel closure of mutant and wild-type channels.

These conclusions were originally based on macroscopic recordings of intercellular channels expressed in pairs of *Xenopus* oocytes and assumed that V_j-dependence arose from a single gating mechanism. Subsequent single channel studies demonstrated the presence of at least two separate gating mechanisms in conductive hemichannels formed by Cx46 and Cx32*Cx43E1 (Trexler et al., 1996; Oh et al., 2000) and intercellular channels formed by other several connexins including Cx32 (Bukauskas et al., 1995; Oh et al., 1997, 1999).

One gating mechanism is characterized by rapid (submillisecond) transitions between the fully open state and at least three different substates. This process was termed V_j-gating by Trexler et al. (1996) to convey the likelihood that the voltage dependence of Cx46 conductive hemichannels at positive membrane potentials and the closure of Cx46 hemichannels to a minimal conductance (G_{min}) in heterotypic pairings with Cx32 and Cx26 at positive V_j were mechanistically identical. The voltage-dependent entry of the channel into substates is likely to underlie the minimal conductance (G_{min}) that is apparent in the steady-state conductance-voltage relations of many gap junction channels (Moreno et al., 1994; Oh et al., 1997, 1999). The gating polarity of these transitions is reversed by negative charge substitutions of the second amino acid residue (Oh et al., 1999, 2000). Heteromeric conductive hemichannels containing wild-type and at least one subunit with a negative charge at the second amino acid residue display bi-polar V_j-gating (Oh et al., 2000). That is, the open probability of such heteromeric hemichannels is not described by a monotonic function of voltage, but rather the open probability is reduced substantially at both positive and negative membrane potentials and is maximal at membrane potentials around 0 mV. Recently, it has been shown that the movement of any one of the six connexin subunits is sufficient to initiate V_j-gating in Cx32 hemichannels (Oh et al., 2000).

The second gating mechanism described in intercellular channels is characterized by a sequential series of short-lived transitions to substates that together usually result in full channel closure. In this case, transitions between the fully open and fully closed state involve multiple gating transitions that when occurring sequentially can take tens of milliseconds and consequently give the appearance of "slow gating." These transitions are similar in appearance to "loop gating" described in Cx46 conductive hemichannels by Trexler et al. (1996) and the slow gating caused by uncoupling agents such as H^+, alkanols, local anesthetics, and the transitions that are observed during channel formation (Bukauskas and Peracchia, 1997; Bukauskas and Weingart, 1994). It is not known if all these slow appearing gating transitions arise by the same molecular mechanism, although this is a possibility. For simplicity, we refer to the slow transitions between fully open and fully closed states in our studies of voltage dependence of intercellular and conductive hemichannels as loop gating. Notably, loop gating transitions are infrequent and are observed at both polarities of V_j in heterotypic Cx32/Cx26 channels (Oh et al., 1999) and their polarity is not reversed by the negative charge substitution, N2E, in Cx32 hemichannels (Oh et al., 1999, 2000). Thus, the molecular determinants of V_j-gating and loop gating are fundamentally different. Loop gating appears more frequently in records of single homotypic Cx32 and Cx26 intercellular channels but appears to be only weakly sensitive to V_j over the $\pm$ 120 mV range usually examined (see Oh et al., 1997, 1999). This weak voltage dependence is consistent with the presence of a substantial G_{min} (that is attributable to V_j-gating) in macroscopic recordings of Cx32 and Cx26 junctions. If loop gating were appreciably voltage-dependent at large V_j, then G_{min} would likely approach zero at these voltages. Loop gating does not appear to contribute substantially to the steady-state conductance-voltage relation of homotypic and heterotypic junctions formed by Cx32 and Cx26 over the range of voltages usually examined ($\pm$120 mV). It may, however, contribute to the slight decrease in steady-state junctional conductance reported for Cx32N2E/Cx32 and Cx32N2D/Cx32 heterotypic junctions (see Fig. 1) as suggested by Oh et al. (1999).

In this study we further examine the range over which negative charge substitutions in the N-terminus can reverse the polarity of V_j-gating. We report that negative charge substitutions of the 5th, 8th, 9th, and 10th residues can reverse the polarity of Cx32 hemichannel V_j-gating. These results are interpreted to indicate that these residues lie within the V_j-field and most likely line a portion of the channel pore (see Purnick et al., 2000), as in this position the residues could sense changes in V_j and be insensitive to changes in the absolute membrane potential, V_m. We also demonstrate that hemichannels formed by subunits containing negative charges at the 8th and 10th residue display bi-polar V_j-gating. The open probability of hemichannels containing either T8D or L10D subunits is reduced at both positive and negative membrane potentials, and is maximal at intermediate potentials. These findings, in conjunction with those of Oh et al. (2000), strongly suggest that single T8D and L10D subunits can initiate V_j-gating at both positive and negative potentials. The simplest explanation of these findings is (assuming a single conserved V_j-gating mechanism) that the Cx32 hemichannel can adopt at least two different open states. Residues 1–5 would always be located within the pore where they sense changes in V_j, while residues 8–10 can reside either inside or outside of the channel pore and the electric field.

MATERIALS AND METHODS

Site-directed mutagenesis, RNA synthesis, and oocyte injection

Site-directed point mutations were constructed by using oligonucleotide primers and the polymerase chain reaction. Mutated fragments were inserted into wild-type Cx32 cloned into the plasmid vector pGEM7zf+ (Promega, Madison, WI), using an engineered *Sal*I site upstream of the initiation codon of Cx32 and either one of the two unique restriction sites, *Pst*I or *Eco*O109I located within the coding region. The DNA segments containing the PCR product were sequenced in their entirety. RNA was transcribed in vitro from linearized plasmid templates as described in Rubin et al. (1992). For expression of cloned connexins in *Xenopus* oocytes, ~50 nl of 1 ng/nl RNA was co-injected with 0.2 pmol/μl of the phosphorothioate antisense oligonucleotide 5′-GGT TTA GTA ATT CCC ATC CTG CCA TGT TTC–3′. This oligonucleotide is complementary to the 5′ end of endogenous *Xenopus* Cx38 and blocks all endogenous coupling between oocyte pairs that is attributable to Cx38 within 72 h (Barrio et al., 1991; Rubin et al., 1992).

Electrophysiological recording of intercellular channels in pairs of *Xenopus* oocytes

Oocytes were devitellinized and paired 12–36 h after RNA injection. Junctional currents were evident within 4–12 h of pairing and were recorded using a dual voltage clamp with glass electrodes containing 1 M KCl solutions. Coupled oocytes had similar resting potentials that ranged between −30 and −60 mV, depending on the cell pair. Cells were voltage clamped to their resting potential resulting in $V_j = 0$ mV. A family of junctional currents was generated by first applying a brief pre-pulse of ±20 mV (which was used subsequently to normalize junctional currents), then by applying a transjunctional voltage of ±5 to 120 mV in steps of 5 or 10 mV increments. Each step applied was followed by an inter-pulse interval of 90 s. Currents were digitized at two rates, at 256 Hz for 2 s, then 28 Hz for the remaining 28 s, to allow for increased accuracy in measuring initial currents. Initial and steady-state conductances were obtained from the exponential fitting of I_j.

Electrophysiological recording of conductive hemichannels

The methods for obtaining macroscopic and single channel records of conductive hemichannels formed by a Cx32 chimera, Cx32*Cx43E1, which contains the first extracellular loop of Cx43, are described by Oh et al. (2000).

RESULTS

Polarity reversal and position of negative charge substitutions

The conductance-voltage relations of homotypic Cx32 and heterotypic Cx32/Cx26 junctions are shown in Fig. 1, *A* and *B* and described below to facilitate comparisons with the other N-terminal charge substitutions examined in this study. Both the initial (▼) and the steady-state (▽) conductance-voltage relations of Cx32 homotypic junctions are symmetric about $V_j = 0$, while those of heterotypic Cx32/Cx26 junctions are asymmetric. The asymmetry in the conductance-voltage relation of initial currents in the Cx32/Cx26 junction reflects the rectification of open channel ionic currents (Bukauskas et al., 1995; Oh et al., 1999). The rectification is a consequence of a difference in the position of charged residues that lie along the pore in Cx32 and Cx26 hemichannels. The heterotypic pairing creates an asymmetric distribution of charge along the pore of the intercellular channel, which as described in Oh et al. (1999) is sufficient to account for the observed rectification with the permeation model developed by Chen and Eisenberg (1993), which numerically solves the Poisson-Nernst-Plank equation in one dimension. Homotypic pairing of hemichannels results in a symmetric distribution of charge along the channel pore, and a symmetric but not necessarily linear *I-V* relation (Oh et al., 1999). While differences in the sign of fixed charges lying within or in close proximity to the channel pore are likely to contribute to the different ionic selectivities of homotypic Cx32 and Cx26 channels (see Oh et al., 1997; Veenstra, 1996), differences in selectivity do not per se cause the rectification of ionic currents of open Cx32/Cx26 heterotypic channels, in contrast to what was recently proposed by Suchyna et al. (1999).

The asymmetry in the steady-state conductance-voltage relation of Cx32/Cx26 junctions is a consequence of the opposite gating polarity of the two hemichannels. Cx32 hemichannels enter substates in response to negative V_j, while Cx26 hemichannels enter substates in response to positive V_j (Oh et al., 1999, 2000). The rapid (submillisecond) entry of connexin hemichannels to substates is termed V_j-gating (Trexler et al., 1996; Oh et al., 2000). This gating mechanism can explain the presence of a minimal conductance (G_{min}) at large V_j in the steady-state conductance-voltage relations of homotypic and heterotypic junctions formed by Cx32 and Cx26. Homotypic and heterotypic channels formed by these two connexins also make slow transitions between a fully open and fully closed state (loop gating). The presence of a substantial G_{min} in the steady-state conductance-voltage relations of Cx32 and Cx26 junctions is consistent with the observation that loop gating in these junctions appears to be only weakly voltage-dependent over the range of transjunctional voltages examined (usually ±120 mV). Consequently, loop gating does not contribute substantially to the time-dependent changes in junctional currents at voltages in this range (see Oh et al., 1997, 1999). However, it is possible that loop gating is more sensitive to much larger transjunctional voltages and consequently, the application of a substantially larger V_j ($\gg$120 mV) might lead to a further reduction in junctional conductance such that G_{min} would approach zero. Although the two gating processes are mechanistically distinct, mutations that alter the "chemical free energy" of the open state of the channel should shift the voltage dependence of both V_j and loop gating in the same direction. Mutations that alter the chemical free energy of "closed states" may differentially affect the voltage dependence of V_j-gating and loop-gating.

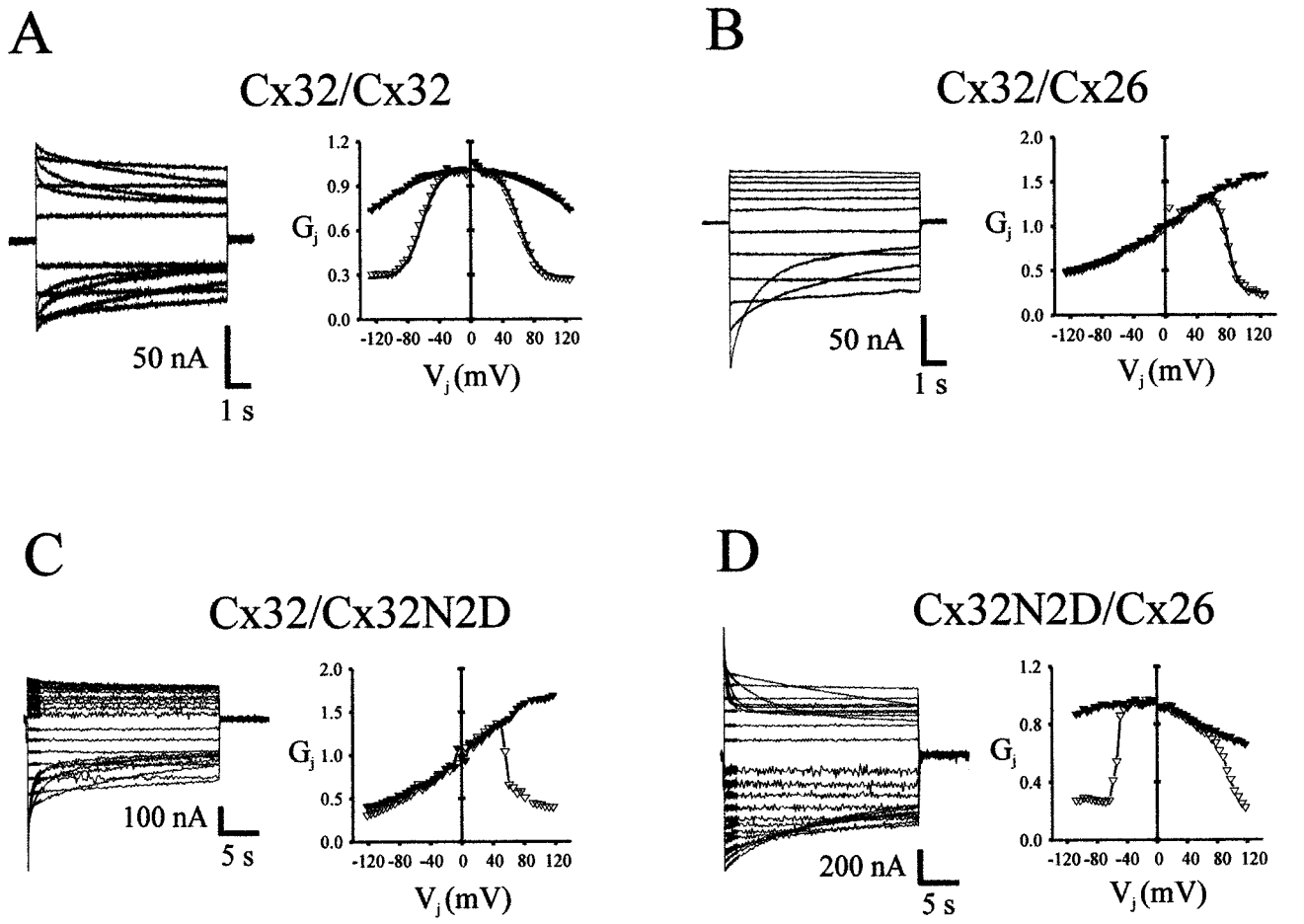

FIGURE 1 Conductance-voltage relations and representative traces of initial and steady-state junctional currents obtained in pairs of *Xenopus* oocytes. (*A*) Cx32/Cx32 homotypic junctions. (*B*) Cx32/Cx26 heterotypic junctions. (*C*) Cx32/Cx32N2D heterotypic junctions. (*D*) Cx32N2D/Cx26 heterotypic junctions. V_j corresponds to the voltage applied to the cell expressing the hemichannel appearing on the right side of the channel designation. Filled symbols represent initial conductances and open symbols represent steady-state conductances. In all cases, junctional conductance is normalized to $V_j = 0$.

As originally reported by Verselis et al. (1994), negative charge substitutions of the neutral asparagine residue located at the second position of Cx32 (Cx32N2E and Cx32N2D) reverse the gating polarity of Cx32 hemichannels. The reversal of gating polarity is made evident by comparisons of the steady-state conductance-voltage relations of wild-type junctions shown in Fig. 1, *A* and *B* with those of the Cx32N2D heterotypic channels shown in Fig. 1, *C* and *D*. Both the initial and steady-state conductance-voltage relations of the Cx32/Cx32N2D and Cx32/Cx32N2E heterotypic junctions are qualitatively identical to that of Cx32/Cx26 junctions (see also Oh et al., 1999). Junctional currents decline to steady-state values when the Cx32N2E or N2D side of the junction is made positive with respect to the Cx32 side of junction. The presence of a substantial G_{min} in these conductance-voltage relations reflects the entry of the channel into substates. Single channel studies of membrane hemichannels formed by a chimera, Cx32N2E*Cx43E1, have shown that the polarity of V_j-gating, but not loop gating, is reversed by the N2E substitution (Oh et al., 2000). The slight relaxation of junctional currents evident in Fig. 1 *C* when the Cx32N2D or Cx32N2E hemichannel is relatively negative can be ascribed to the weak voltage dependence of loop gating in these hemichannels (see Oh et al., 1999).

In the case of the Cx32N2D/Cx26 heterotypic junction (Fig. 1 *D*) the steady-state junctional conductance decreases at either polarity of V_j, but does so asymmetrically. The relaxation of junctional currents to steady-state values resemble those of Cx26 when the Cx26 side of the junction is made relatively positive and resemble those of Cx32N2D when the Cx32N2D side of the junction is made relatively positive (see Verselis et al., 1994). The simplest interpretation of these results is that the negative charge substitutions, Cx32N2E, and Cx32N2D reverse the gating polarity of V_j-gating of homomeric Cx32 hemichannels, from closure at negative V_j to closure at positive V_j.

The asymmetry in the initial conductance-voltage relation of the Cx32/Cx32N2D junction shown in Fig. 1 *C* is similar to that of Cx32/Cx26 junction. This result indicates that

charges at this position play a major role in shaping the *I-V* relation of open channels and are consistent with structural models that place the second amino acid residue within the channel pore (see Oh et al., 1999). The asymmetry in the initial conductance-voltage relation of Cx32N2D/Cx26 (Fig. 1 *D*) results from a difference in the position of other charged residues in Cx26 and Cx32N2D hemichannels (see Oh et al., 1999).

The reversal of the V_j-gating polarity by a negative charge substitution at the 5th amino acid residue (Cx32G5D) can be inferred from the conductance-voltage relations of the heterotypic pairings shown in Fig. 2, *A* and *B*. In heterotypic Cx32/Cx32G5D intercellular channels, junctional currents decline in a time-dependent manner to a non-zero G_{min} only when the cell expressing Cx32G5D is relatively positive. In the heterotypic channel, Cx32G5D/Cx26, junctional currents decline for both polarities of applied V_j, with both Cx26 and Cx32G5D closing at relatively positive V_j. The behavior of heterotypic junctions formed by pairing Cx32G5D with Cx32 and Cx26 is similar to that of heterotypic junctions formed with Cx32N2D (compare Fig. 2, *A* and *B* with Fig. 1, *C* and *D*). The presence of an appreciable G_{min} in the conductance-voltage relations of these intercellular channels is consistent with the view that V_j-gating and not loop gating contributes predominately to the decline of junctional currents to steady-state values. The simplest interpretation of these data is that the G5D substitution has reversed the polarity of V_j-gating of the Cx32 hemichannel.

Cx32 subunits with negative charge substitutions at the 3rd, 6th, and 7th positions (Cx32W3D, L6D, and Y7D) do not form homomeric hemichannels that express junctional currents in homotypic or heterotypic pairings with either Cx32 or Cx26 over the ±120 mV range of transjunctional voltage examined. The polarity of V_j-dependence of channels formed by Cx32T4D was not determined.

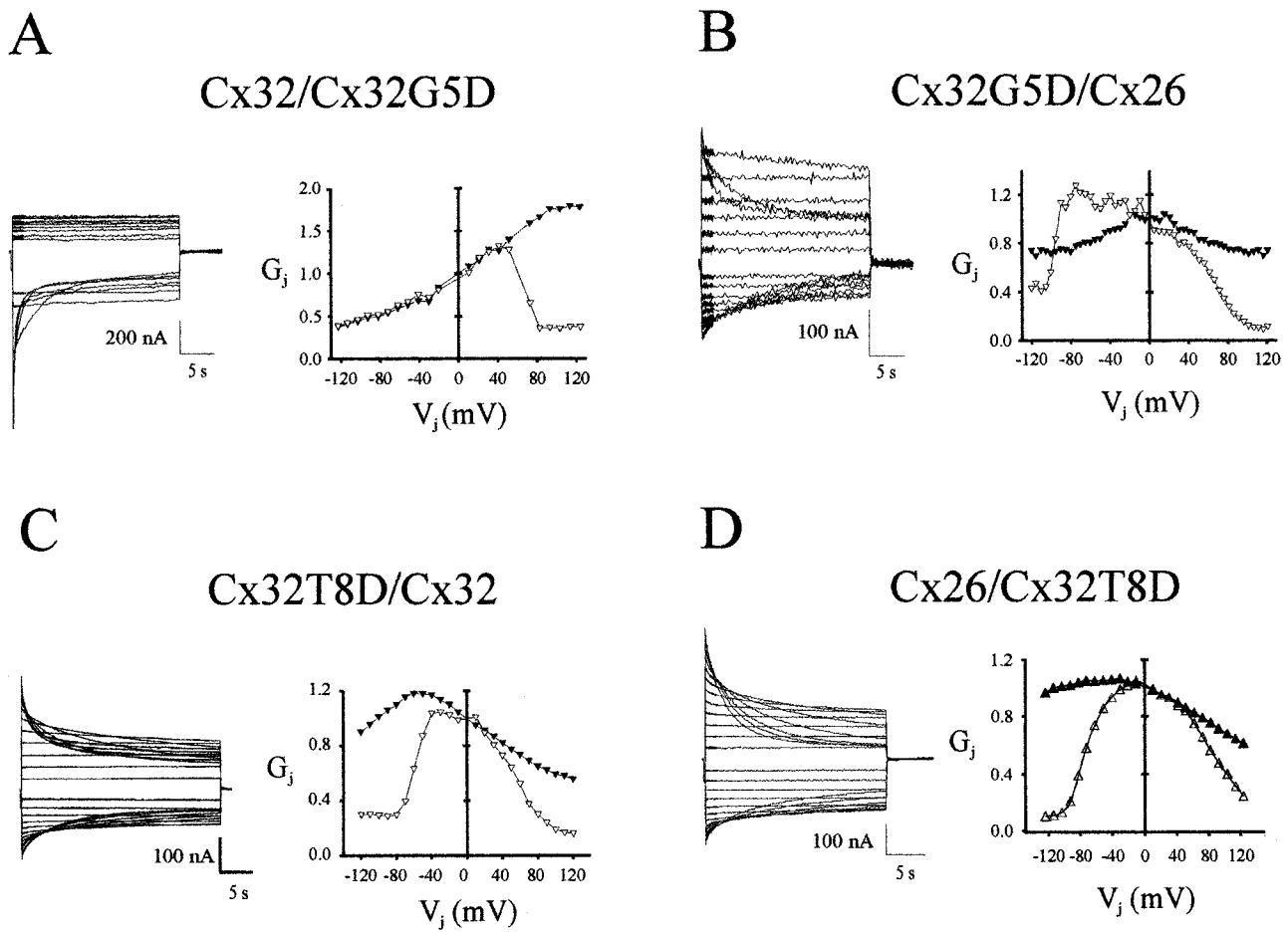

FIGURE 2 Conductance-voltage relations and representative traces of initial and steady-state junctional currents obtained in pairs of *Xenopus* oocytes. (*A*) Cx32/Cx32G5D heterotypic junctions. (*B*) Cx32G5D/Cx26 heterotypic junctions. (*C*) Cx32T8D/Cx32 heterotypic junctions. (*D*) Cx26/Cx32T8D heterotypic junctions. V_j corresponds to the voltage applied to the cell expressing the hemichannel appearing on the right side of the channel designation. Filled symbols represent initial conductances and open symbols represent steady-state conductances. In all cases, junctional conductance is normalized to $V_j = 0$. In *A*, the voltages applied to elicit the current traces shown are −40 to −120 mV and 30, 40, 80–120 mV. In *B–D* voltages are stepped in 10 mV increments beginning at ±10 mV.

The conductance-voltage relations of heterotypic intercellular channels formed by negative charge substitutions of the 8th amino acid, T8D, are surprising in that junctional currents decline for both polarities of applied V_j in heterotypic pairings with either Cx32 or Cx26 (Fig. 2, *C* and *D*). The presence of an appreciable G_{min} in both limbs of the steady-state conductance-voltage relation of Cx32T8D/Cx32 junctions (Fig. 2 *C*) suggests that V_j-gating contributes predominately to the decline of junctional currents in intercellular channels formed by Cx32T8D. The reduction in junctional conductance, when the Cx32T8D hemichannel is relatively negative (downward current deflections in Fig. 2 *C*) is solely attributable to gating of the T8D hemichannel. This result suggests that the charge substitution has not reversed the gating polarity of Cx32 hemichannel V_j-gating (i.e., the T8D hemichannel continues to close on relative negativity). Whereas, the reduction in junctional conductance that is observed when the Cx32T8D hemichannel is relatively positive (downward current deflections in the Cx26/Cx32T8D heterotypic junction, Fig. 2 *D*) can be only attributed to the T8D hemichannel. This result suggests that the T8D substitution has reversed the polarity of V_j-gating of Cx32 in the Cx26/Cx32T8D junction (i.e., the T8D hemichannel closes on relative positivity). Taken together, these observations can be interpreted as indicating either that the polarity of V_j-gating of homomeric Cx32T8D hemichannels depends upon the hemichannel with which it is paired, or alternatively that V_j-gating in Cx32T8D hemichannels is bi-polar. That is, the open probability of the Cx32T8D hemichannel decreases at both positive and negative V_j values.

To determine whether the reduction in junctional currents observed at both polarities of V_j in both heterotypic Cx32T8D/Cx32 and Cx32T8D/Cx26 junctions is due to the bi-polarity of V_j-gating of the T8D hemichannel, we examined the behavior of conductive hemichannels formed by the Cx32*Cx43E1 chimera containing the T8D substitution (Cx32T8D*Cx43E1). The Cx32*Cx43E1 chimera forms both intercellular and conductive hemichannels in *Xenopus* oocytes (Pfahnl et al., 1997; Oh et al., 2000). The mechanisms of voltage-dependent gating of Cx32*Cx43E1 conductive hemichannels are for the most part comparable to those present in Cx32 intercellular channels (Oh et al., 2000). Notably, the polarity of Cx32 V_j-gating is not changed by the Cx43E1 substitution, nor is the ability of the Cx32N2E substitution to reverse the polarity of V_j-gating altered in the chimeric hemichannel. In single channel records of conductive hemichannels formed by the parental Cx32*Cx43E1 chimera, V_j-gating corresponds to rapid (submillisecond) gating transitions to subconductance levels that occur at negative membrane potentials (Oh et al., 2000). As predicted by the behavior of intercellular channels, the polarity of these transitions is reversed by the N2E substitution (see Oh et al., 1999, 2000).

A representative macroscopic recording of conductive hemichannels formed by the homomeric aggregates of the Cx32T8D*Cx43E1 subunit is presented in Fig. 3 *A*. Depolarizations of the membrane of *Xenopus* oocytes expressing Cx32T8D*Cx43E1 from a holding potential of −90 mV result in slowly activating outward currents that begin to decline when the membrane potential is ≥+50 mV. (The resting membrane potential of oocytes expressing large numbers of conductive hemichannels is usually between −10 and 0 mV). A comparable decline in macroscopic currents is evident in recordings of membrane channels formed by Cx32N2E*Cx43E1 at positive membrane potentials (Fig. 8 *A* in Oh et al., 2000). In contrast, no relaxations of membrane currents occur in macroscopic records of Cx32*Cx43E1 membrane channels at positive membrane potentials (see Oh et al., 2000). These results suggest that T8D, like N2E, can reverse the polarity of V_j-gating of Cx32 hemichannels.

The *I-V* relation of a single Cx32T8D*Cx43E1 channel obtained with a 3-s voltage ramp over a range of ±120 mV shown in Fig. 3 *B* confirms the reversal of V_j-gating polarity. Gating transitions to subconductance levels (V_j-gating) are evident at positive membrane potentials. There is no evidence of channel closure at negative membrane potentials in these short duration ramps that are initiated from a holding potential of 0 mV.

In the segment of the record of a single Cx32T8D*Cx43E1 conductive hemichannel shown in Fig. 3 *C*, gating transitions between the fully open and subconductance levels are observed at a positive membrane potential of 50 mV. The channel remains fully open when the membrane potential is held at values between 0 and −50 mV (i.e., $P_{open} \sim 1.0$). The event seen in the rightmost portion of the record is a channel opening; the channel resided in a substate when the polarity of membrane was reversed from +50 mV to −50 mV. The behavior of the channel illustrated by this record is consistent with the *I-V* relation shown in Fig. 3 *B*. The polarity of these gating events is the same as the V_j-gating transitions observed in single channel records of homomeric Cx32N2E*Cx43E1 conductive hemichannels (Oh et al., 2000) and indicates that a T8D subunit can initiate channel closure by V_j-gating at positive membrane potentials.

As stated above, closures are not observed in homomeric T8D hemichannels at moderate negative potentials (Fig. 3 *C*). However, at more negative membrane potentials (−100 mV in Fig. 3 *D*), the same homomeric T8D hemichannel also closes by transitions ascribable to V_j-gating. This negative polarity of V_j-gating is the same as that of the parental Cx32*Cx43E1 conductive hemichannel. Taken together, the data shown in Fig. 3 illustrate that the open probability of Cx32T8D*Cx43E1 hemichannels is reduced at both positive and negative membrane potentials and is maximal at intermediate membrane potentials. We conclude that V_j-gating is bi-polar in Cx32T8D hemichannels. The simplest

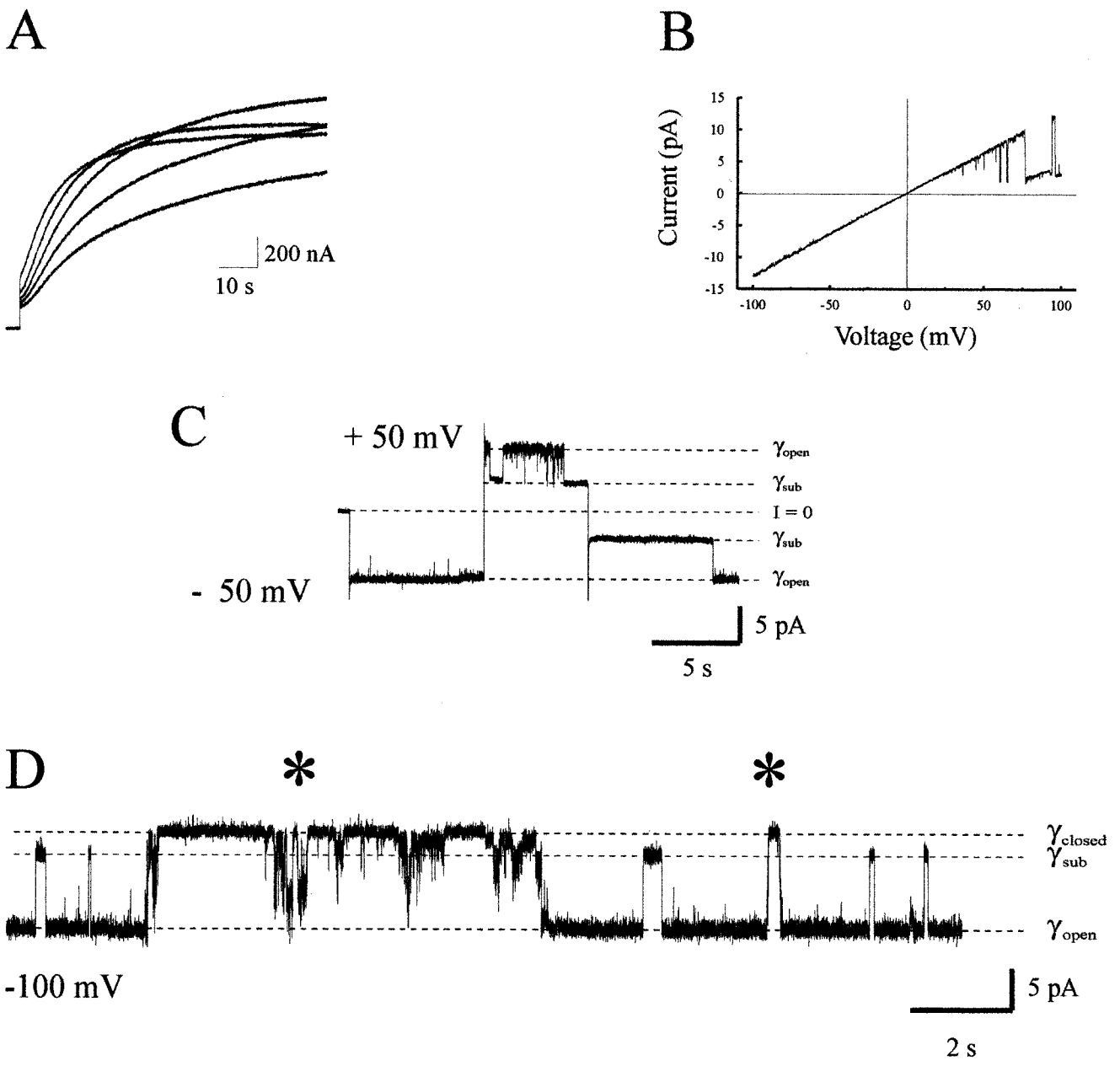

FIGURE 3 Representative macroscopic and single channel records of conductive hemichannels formed by Cx32T8D*Cx43E1. (*A*) A representative macroscopic recording of currents elicited from a single *Xenopus* oocyte injected with Cx32T8D*Cx43E1 mRNA. Voltage steps, from +20 to +60 mV, were applied to this cell in +10 mV increments from a holding potential of −90 mV. (*B*) The *I-V* relation of a single Cx32T8D*Cx43E1 channel resulting from a voltage ramp with a 3 s duration over a range of ±100 mV. The initial holding potential was 0 mV. V_j-gating can be seen at positive membrane potentials >50 mV. (*C*) A single channel record of a Cx32T8D*Cx43E1 membrane channel. From an initial holding potential of 0 mV ($I = 0$ in *C*) the membrane potential was stepped to −50 mV. No gating events were observed at this potential. When the membrane potential was "flipped" to +50 mV, gating transitions to a subconductance level are observed. The reversal of the polarity of membrane potential (to −50 mV) when the channel resided at a subconductance level resulted in the opening of the channel after a delay of ~5 s. (*D*) A single channel record of a Cx32T8D*Cx43E1 membrane channel at holding potential of −100 mV. Multiple transitions to subconductance levels, which correspond to V_j-gating, are observed. Loop gating transitions are denoted by the asterisks. In all cases, single channel records were obtained in a cell-attached configuration.

interpretation is that a T8D subunit can initiate V_j-gating at both positive and negative polarities of membrane potential.

The Cx32T8D hemichannel also displays loop gating. The complex series of gating transitions between fully open and fully closed conductance levels, which occur at negative membrane potentials and are marked by the asterisks in Fig. 3 *D*, are loop gating events. The polarity of loop gating is unaffected by the T8D substitution, occurring only at neg-

ative membrane potentials in both the parental and the mutant Cx32 chimera. This result further supports the assertion that loop gating results from a molecular mechanism that is fundamentally unrelated to V_j-gating (Trexler et al., 1996; Oh et al., 2000). Although both V_j-gating and loop gating are likely to contribute to the decline of junctional currents observed in macroscopic recordings of heterotypic Cx32T8D/Cx32 and Cx32T8D/Cx26 intercellular channels (Fig. 2, *C* and *D*), the single channel analyses indicate that V_j-gating of the T8D subunit is bi-polar.

Thus, the conductance-voltage relation of the Cx32T8D/Cx32 heterotypic junction (Fig. 2 *C*) can be explained in the following way, with respect to the operation of the V_j-gates. When the Cx32 hemichannel is relatively positive (Cx32T8D is negative), the observed reduction in junctional currents reflects the closure of the V_j-gate in the apposed Cx32T8D hemichannel. Recall that Cx32 hemichannels close only for negative V_j. When the Cx32 side of the junction is relatively negative (Cx32T8D is positive), the reduction in junctional conductance can be ascribed to the closure of both Cx32 and Cx32T8D hemichannels.

The conductance-voltage relation of the Cx26/Cx32T8D heterotypic junction (Fig. 2 *D*) can be explained as follows. When the Cx32T8D side of the junction is relatively positive (Cx26 is negative), the reduction in junctional currents result from the closure of the V_j-gate in the Cx32T8D hemichannel. Recall that Cx26 hemichannels only close when V_j is positive. When the Cx32T8D side of the junction is relatively negative, the reduction in junctional currents can be ascribed to the closure of V_j-gates in both Cx26 and Cx32T8D hemichannels. Note that the loop gating attributable to the Cx32T8D hemichannel would also contribute to the reduction of junctional currents when the T8D hemichannel is relatively negative.

Homomeric hemichannels containing negative charge substitutions at either the 9th or 10th amino acid residues, Cx32L9D and Cx32L10D, do not express junctional currents in either homotypic pairings or heterotypic pairings with Cx32 or Cx26 hemichannels. Similarly, no currents attributable to connexin membrane hemichannels are observed in *Xenopus* oocytes that have been only injected with either Cx32L9D*Cx43E1 or Cx32L10D*Cx43E1 RNA. However, membrane currents attributable to connexin hemichannels are observed in single oocytes injected with 1:1 mixtures of Cx32*Cx43E1 and either Cx32L9D*Cx43E1 or Cx32L10D*Cx43E1 RNA (Fig. 4).

The single channel record shown in Fig. 4 *A* is from an oocyte co-expressing both Cx32*Cx43E1 and Cx32L9D*Cx43E1 subunits. The behavior of this channel is markedly different from that of homomeric Cx32*Cx43E1 membrane channels (see Oh et al., 2000). As Cx32L9D*Cx43E1 does not express membrane currents as a homomeric hexamer, the single channel record shown in Fig. 4 *A* must contain both Cx32*Cx43E1 and Cx32L9D*Cx43E1 subunits, although it is not possible to precisely define the subunit composition of the heteromeric channel shown in this record.

The observed rapid closures to subconductance levels that occur at negative membrane potentials can be ascribed to the presence of at least one Cx32*Cx43E1 subunit, as Oh et al. (2000) have shown that comparable V_j-gating transitions in channels formed by this chimera occur only at negative membrane potentials and that a single subunit is sufficient to initiate V_j-gating. The frequency and duration of closures ascribable to V_j-gating increases as the membrane potential becomes more negative. Loop gating transitions comparable to those shown in Fig. 3 *D* are observed infrequently at substantial negative potentials (not shown). The closures to subconductance levels that are observed at positive membrane potentials in the heteromeric Cx32L9D*Cx43E1/Cx32*Cx43E1 hemichannel must therefore reflect the action of at least one Cx32L9D*Cx43E1 subunit. The open probability of the heteromeric channel is reduced at both positive and negative membrane potentials. This is illustrated by the *I-V* relation shown in Fig. 4 *B*. Thus, heteromeric channels comprised of WT and L9D subunits display bi-polar V_j-gating. The simplest conclusion is that V_j-gating at negative potentials is initiated by the presence of at least one Cx32*Cx43E1 subunit, while V_j-gating at positive potentials is initiated by at least one Cx32L9D*Cx43E1 subunit. Thus, the L9D substitution can reverse the polarity of V_j-gating but does not reverse the polarity of loop gating.

A similar conclusion can be drawn for the L10D substitution based on the single channel records shown in Fig. 4 *C*. The closures to substates observed at positive membrane potentials in this heteromeric channel can be ascribed to the actions of an L10D subunit, while those that occur at negative potentials can be ascribed to the actions of a Cx32*Cx43E1 subunit. The open probability of the heteromeric L10D/WT channel is maximal at membrane potentials around 0 mV and decreases at both positive and negative potentials (not shown). Thus, both L9D and L10D can initiate V_j-gating at positive membrane potentials and form heteromeric channels with wild-type subunits that display bi-polar V_j-gating.

A segment of an on-cell patch recording of an oocyte injected with a 1:1 mixture of Cx32N2E*Cx43E1 and Cx32L10D*Cx43E1 RNA is shown in Fig. 4 *D*. This heteromeric channel enters substates at positive and negative membrane potentials and also displays bi-polar V_j-gating (not shown). However, in this case, the closures to subconductance levels that become more prevalent at larger negative membrane potentials must be due to the presence of an L10D subunit, as the N2E subunit only initiates V_j-gating at positive membrane potentials (Oh et al., 2000). The simplest interpretation is that L10D, like T8D, can initiate closures by V_j-gating at both positive and negative membrane potentials. Loop gating transitions are observed infrequently and only at large negative membrane potentials in

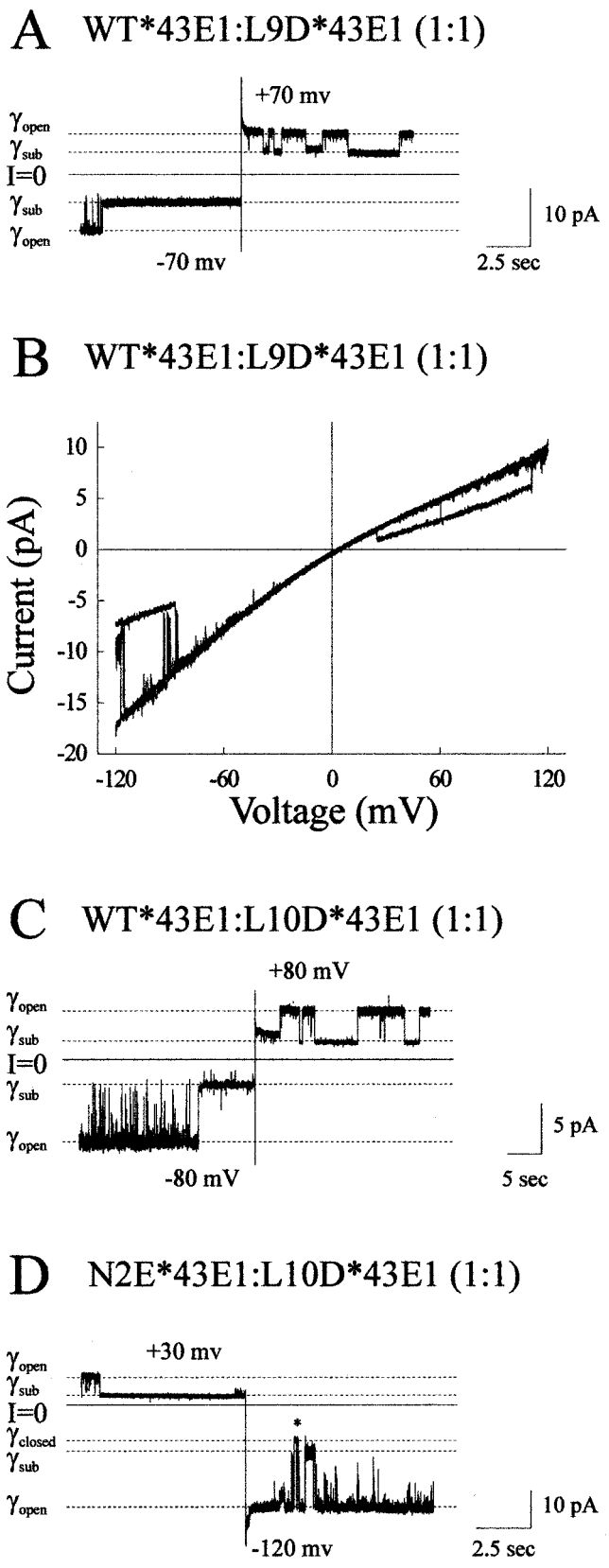

FIGURE 4 Cell-attached patch clamp recording of the designated heteromeric membrane channels illustrating bi-polar V_j-gating. (*A*) A single channel record obtained from an oocyte injected with a 1:1 mixture of Cx32*Cx43E1/Cx32L9D*Cx43E1 RNA. Gating transitions to subconductance levels are observed at holding potentials of ±70 mV. (*B*) The current-voltage relation of a Cx32*Cx43E1/Cx32L9D*Cx43E1 RNA conductive hemichannel shown was determined by applying a 3 s voltage ramp over a ±120 mV range. The time axis of the voltage ramp runs from +120 mV to −120 mV. Three sequential current traces are superimposed and illustrate that the open probability of the heteromeric hemichannel is maximal at membrane potentials near 0 mV and decreases at both positive and negative membrane potentials. (*C*) A single channel record obtained from an oocyte injected with a 1:1 mixture of Cx32*Cx43E1/Cx32L10D*Cx43E1 RNA. Gating transitions to subconductance levels are observed at holding potentials of ± 80 mV. (*D*) A single channel record obtained from an oocyte injected with a 1:1 mixture of Cx32N2E*Cx43E1/Cx32L10D*Cx43E1 RNA. V_j-gating transitions to are observed at holding potentials of +30 mV and −120 mV. The asterisk denotes a loop gating transition. The adjacent transition to a subconductance level corresponds to a V_j-gating event.

heteromeric channels containing at least one L10D subunit. An example of a loop gating event is marked by the asterisk in Fig. 4 *D*. The subsequent, adjacent event of longer duration (600−800 ms), exemplifies a V_j-gating transition that could only have been initiated by an L10D subunit.

In contrast, a negative charge substitution at the 11th amino acid residue (Cx32S11D) does not appear to change the gating polarity of Cx32 hemichannels (Fig. 5, *A* and *B*). In Cx32S11D/Cx26 heterotypic junctions, junctional currents decline to steady-state values with an appreciable time constant only when the Cx26 side of the junction is relatively positive. The failure to observe any reduction in junctional currents when the Cx32S11D side of the junction is relatively positive is consistent with the assignment of negative polarity to V_j-gating of the Cx32S11D hemichannel. If S11D had reversed the polarity of Cx32 V_j-gating, a decline in the junctional currents would have been observed in the current traces of the heterotypic Cx32S11D/Cx26 junction when the Cx32S11D side of the channel was relatively positive (upward current deflections in Fig. 5 *B*). Thus, the S11D hemichannel is likely to have the same V_j-gating polarity as the wild-type Cx32 hemichannel when it is expressed as a heterotypic junction with Cx26. The observed reduction in junctional currents in the heterotypic Cx32S11D/Cx32 junction at both polarities of V_j is also consistent with the assignment of a negative V_j-gating polarity to the Cx32S11D hemichannel. However, the reduction in G_{min} to a value close to zero that is observed when the Cx32S11D hemichannel is relatively negative in both heterotypic junctions suggests that the voltage dependence of loop gating has been shifted such that closure by this mechanism is more likely to occur at potentials closer to zero. It is possible that the reduction in junctional currents that is observed when the S11D hemichannel is relatively negative reflects the operation of both V_j and loop gating mechanisms. To explore this possibility we examined the behavior of Cx32S11D*Cx43E1 conductive hemichannels. The segment of the single channel record of a

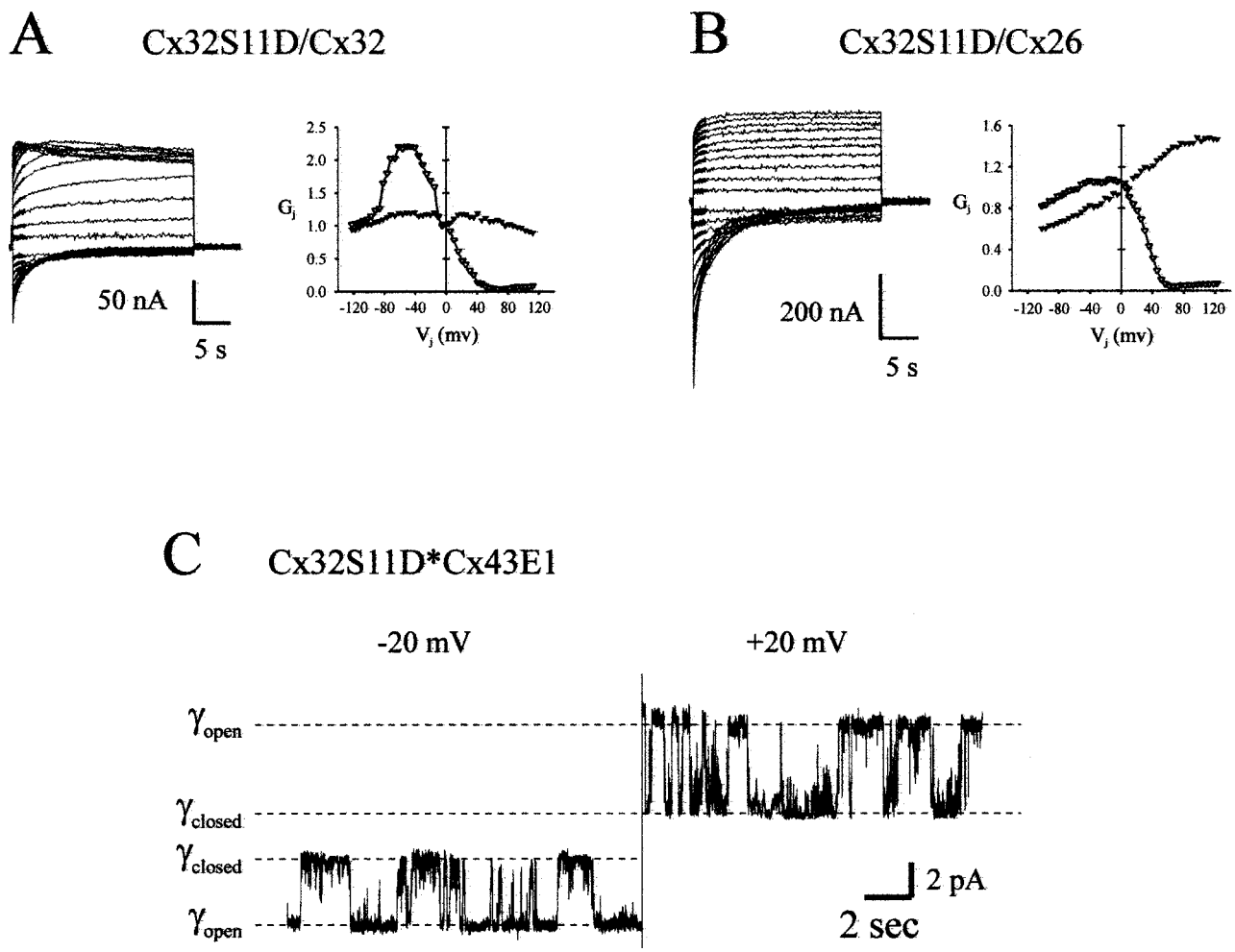

FIGURE 5 Macroscopic and single channel records of the S11D mutation. Conductance-voltage relations and representative traces of initial and steady-state junctional currents obtained in pairs of *Xenopus* oocytes. (*A*) Cx32S11D/32 heterotypic junctions. (*B*) Cx32S11D/Cx26 heterotypic junctions. V_j corresponds to the voltage applied to the cell expressing the hemichannel appearing on the right side of the channel designation. Filled symbols represent initial conductances and open symbols represent steady-state conductances. In all cases, junctional conductance is normalized to $V_j = 0$. (*C*) A segment of a record of a single Cx32S11D*Cx43E1 conductive hemichannel at a holding potential of ± 20 mV in a cell-attached patch recording configuration. All gating transitions can be ascribed to loop gating (see text).

Cx32S11D*Cx43E1 hemichannel shown in Fig. 5 *C* demonstrates the high frequency and prevalence of loop gating transitions at small depolarizing and hyperpolarizing membrane potentials (20 and −20 mV). This behavior is consistent with the properties of the Cx32S11D hemichannel that can be inferred from the conductance-voltage relation of the Cx32S11D/Cx32 heterotypic junction. Note that when $V_j = 0$, junctional conductance is approximately half-maximal. That is, the open probability of the S11D hemichannel is ~0.5 at $V_j = 0$ (Fig. 5 *A*). The high frequency of loop gating transitions and voltage dependence of these transitions at negative membrane potentials (not shown) prohibits the identification of V_j-gating transitions at larger negative membrane potentials. Therefore, we cannot unequivocally assign a negative V_j-gating polarity to the S11D hemichannel, although the macroscopic data are consistent with this view.

DISCUSSION

The description of the equilibrium and kinetic properties of voltage gating of amphibian gap junctions by Spray et al. (1981) and Harris et al. (1981) provides the framework that underlies much of our current understanding of transjunctional voltage dependence (V_j-dependence) of intercellular channels formed by the connexin gene family. The voltage dependence of most vertebrate gap junction channels is remarkable in that it depends solely on the difference in the resting membrane potential of coupled cells (V_j) and not on the absolute membrane potential of either cell (V_m). The symmetry in the steady-state conductance-transjunctional voltage relation of most homotypic gap junctions is explained by a model in which each of the two oppositely oriented hemichannels functions separately, but not necessarily independently (see Fig. 2 *A* in Verselis et al., 1994).

All available evidence indicates that each hemichannel contains separate voltage sensors and gates. The application of a transjunctional voltage of given polarity would favor the closure of the voltage gate in one hemichannel and the opening of the voltage gate in the oppositely oriented hemichannel. An open gate would be more likely to close if the second gate with which it is in series would be fully open. Thus, voltage gating is viewed as being essentially a hemichannel property, but contingent in that changes in open probability also depended upon the state of the gates in both hemichannels (see Harris et al., 1981). The sensitivity of intercellular gap junctions to V_j and not V_m suggests that the transjunctional voltage sensor is located within and near the cytoplasmic surface of the large aqueous pore.

Asymmetries in the conductance-voltage relations of gap junction channels can arise if the intercellular channel displays sensitivity to V_m (see Obaid et al., 1983; Verselis et al., 1991; Bukauskas and Weingart, 1994), or if different hemichannels paired heterotypically have different sensitivities to V_j (Swenson et al., 1989; Barrio et al., 1991). For example, the asymmetry in the steady-state conductance-voltage relation of heterotypic Cx32/Cx26 junctions is due to the difference in the polarity of the transjunctional voltage dependence of the two hemichannels (Verselis et al., 1994; Oh et al., 1999). Closure of Cx32 hemichannels is favored when the cytoplasm of the Cx32 expressing cell is negative with respect to that of the cell with which it is paired (negative V_j), while closure of Cx26 hemichannels is favored at positive V_j. The difference in the gating polarity is due to the electrostatic effect of a charge difference at the second amino acid residue in the two connexins. The substitution of the neutral asparagine residue present in wild-type Cx32 with the negatively charged aspartate residue present in Cx26 reverses the gating polarity of Cx32 hemichannel V_j-dependence. The reciprocal substitution, Cx26D2N, reverses the gating polarity of the Cx26 hemichannel. Consequently, the opposite gating polarities of Cx32 and Cx26 hemichannels reflect the opposite valence of the voltage sensors formed by the two connexins.

The opposite polarity of V_j-dependence of Cx32 and Cx26 hemichannels corresponds to rapid (submillisecond) gating transitions between fully open and subconductance levels (Bukauskas et al., 1995; Oh et al., 1999). This process has been termed V_j-gating (see Trexler et al., 1996) and can explain the presence of a minimal conductance (G_{min}) in the conductance-voltage relation of homotypic and heterotypic gap junctions formed with Cx32 and Cx26. A second gating mechanism termed loop gating by Trexler et al. (1996) cannot account for the difference in the polarity of V_j-dependence of Cx32 and Cx26 hemichannels. Negative charge substitutions of the second amino acid residue of Cx32 reverse the polarity of V_j-gating transitions, but do not change the negative gating polarity of loop gating transitions (Oh et al., 1999, 2000). In Cx32 and Cx26 channels, loop gating appears to be weakly voltage-dependent (see Oh et al., 1997, 1999), although in some channels it may contribute to the decline of junctional currents to steady-state levels (Oh et al., 1999).

Recently, we reported that heteromeric Cx32 (Cx32*Cx43E1) conductive hemichannels comprised of wild-type and Cx32N2E subunits were bi-polar and that a single connexin subunit was sufficient to initiate V_j-gating (Oh et al., 2000). Taken together, these results suggest that the connexin V_j-sensor is contained within the N-terminus. While we have not established the electrostatic mechanism underlying the reversal of polarity, it likely involves the orientation of a electrical dipole created by the positively charged N-terminal methionine residue and a negative charge located within the first 10 amino acid residues. The incorporation of a negative charge at any of the first 10 amino acid residues might result in the formation of a dipole such that the negative pole is oriented toward the intracellular side of the hemichannel. The application of a positive potential would result in the movement of the negative pole toward the cytoplasm. The positive charge of the unmodified N-terminal methionine residue (see Hertzberg et al., 1988) and/or the partial charge created by the helical dipole in the amino-terminus could account for the positive valence of the Cx32 voltage sensor. The movement of the positive charge toward the cytoplasmic surface would initiate Cx32 hemichannel closure when V_j is relatively negative.

The data presented in this paper indicate that a negative charge substitution of N-terminal amino acid residues up to and including the 10th residue (Cx32L10D) can reverse the polarity of V_j-gating of a Cx32 hemichannel. Therefore, Cx32 subunits must be able to adopt a conformation in which the first 10 residues are located within the electric field, and these residues most likely reside within the channel pore. It is likely that this conformation results by the formation of turn in the N-terminus, which we propose is made possible by the inherent flexibility of the glycine residue located at the 12th position in all members of Group I or β connexins. A turn in this position could position the N-terminus of Group I connexins within the channel pore, thus satisfying the requirement that the V_j-sensor lie within the transjunctional electric field near the channel surface, where it would be largely insensitive to changes in absolute membrane potential.

The ability of negative charge substitutions at the 8th and 10th residues to initiate V_j-gating at both positive and negative membrane potentials can be explained by postulating that the Cx32 hemichannel can adopt at least two open channel conformations. In one conformation, the 8th and 10th residues would be located outside the electric field created by V_j, while in the second conformation, these residues would be located within the electric field. When all six subunits adopt a conformation in which residues 8 and 10 lie outside the field, the polarity of V_j-gating would be determined by the positive charge of the N-terminal Met

residue and not by the negative charge substitution at the 8th or 10th position. Consequently, the polarity of V_j-gating would be the same as that of wild-type Cx32. However, when all six subunits containing a negative charge at either the 8th or 10th position adopt a conformation that places all six respective negative charges into the electric field, the polarity of Cx32 V_j-gating would be reversed and closure would occur only at positive membrane potentials. When the conformation of subunits is mixed, with both positive and negative charges located in the field, V_j-gating would be bi-polar. The relative frequency and duration of closures at positive and negative potentials would depend upon the number of subunits in each orientation and on the relative sensitivity of the voltage sensor contained in each subunit to V_j (see Oh et al., 2000).

The linearity of the *I-V* relation and the observation of gating transitions only at positive potentials in the T8D channel shown in Fig. 3, *B* and *C* can be explained if all six connexin subunits adopt the same conformation with the 8th amino acid residue located within the electric field at membrane potentials close to 0 mV (the resting potential of the oocyte). This statement is based on our previous results, where we have shown that the *I-V* relation of Cx32N2E*Cx43E1 conductive hemichannels is linear and noted that the *I-V* relation rectifies inwardly as Cx32*Cx43E1 subunits are substituted to form heteromeric channels (see Oh et al., 2000). The bi-polarity of V_j-gating that becomes apparent in Fig. 3 *D* (-100 mV) can be explained if at least one subunit adopts a conformation in which the 8th amino acid residue lies outside the electric field when the membrane is substantially hyperpolarized. Note that no V_j-gating transitions are observed at -50 mV. These observations suggest that the open states may be voltage-dependent. At membrane potentials more positive than -50 mV it is possible that most if not all six subunits adopt a conformation in which the 8th residue lies within the transjunctional electric field, while at more negative holding potentials (-100 mV) at least one subunit may adopt a conformation in which the 8th residue lies outside the electric field in the open state of the channel.

The preceding interpretation of bi-polarity is based on the assumption that V_j-gating arises by a single mechanism that is initiated by the movement of the N-terminus toward the cytoplasmic side of the channel (see Verselis et al., 1994). Bi-polarity might also arise if the N-terminus could move in the opposite direction (toward the channel interior) at larger membrane potentials and if this movement of the N-terminus also initiates V_j-gating. However, there is little or no evidence that supports the existence of such a mechanism at the present time. Notably, V_j-gating is not bi-polar in wild-type and Cx32N2E*Cx43E1 homomeric hemichannels (over ± 100 mV), and Cx32G5D homomeric hemichannels appear to close only at positive V_j.

Other evidence supporting the existence of multiple open Cx32 intercellular channel conformations has been presented by Oh et al. (1997). In the determination of the radius of Cx32 intercellular channels using the non-electrolyte procedure described by Vodyanoy and Bezrukov (1992), Bezrukov and Vodyanoy (1993), and Krasilnikov et al. (1995), Oh et al. reported a bi-modality in the single channel conductance of intercellular Cx32 channels when PEG 300 (hydrodynamic radius of 6.0 Å) is incorporated into the recording solution. This result can be explained if Cx32 channels adopt two different open conformations. In one conformation the pore radius would be <6.0 Å and PEG 300 would be excluded from the channel pore. In the other conformation the channel radius would be >6.0 Å and PEG 300 would occupy the channel pore and result in a decrease in single channel conductance. It has also been suggested that multiple open states exist in Cx26 intercellular channels (Oh et al., 1999).

At the present time we cannot unequivocally define the position of the 11th amino acid residue with respect to the transjunctional electric field. The shift in voltage dependence of the Cx32S11D hemichannel that is inferred from the behavior of the Cx32S11D/Cx32 and Cx32S11D/Cx26 heterotypic junctions most likely reflects the action of the loop gate. Loop gating events predominate in the Cx32S11D*Cx43E1 conductive hemichannel and obscure the identification of V_j-gating events. However, the failure to observe any decline in junctional currents at positive V_j (relative to the Cx26 hemichannel) in heterotypic Cx32S11D/Cx26 junctions is consistent with the assignment of a negative polarity to V_j-gating of the S11D hemichannel. This is the same polarity as the wild-type Cx32 hemichannel. If this inferred polarity of V_j-gating is correct, the S11D residue would lie outside the electric field in all channel conformations at the voltages examined in this study. The placement of the 10th residue within the field (at least in some conformations, see above) suggests that the electric field changes rather sharply between the 10th and 11th residues. The determination of the precise boundary of the drop in V_j will require further studies of other mutations in the N-terminus.

The ability of connexin subunits containing L9D and L10D subunits to form functional channels only when co-expressed with wild-type subunits is somewhat surprising, as several Cx32 mutations have been reported to behave as dominant negatives (negative complementation, see Bruzzone et al., 1994; Omori et al., 1996). In fact, the L9D and L10D mutations have the opposite effect; they are "complemented" by wild-type Cx32 subunits in that the formation of heteromeric hemichannels "rescues" the expression of ionic currents not observed in L9D and L10D homomeric channels. The "complementation" of wild-type and mutant subunits can be explained if the presence of one or more wild-type subunit compensates for a large shift in the voltage dependence of V_j-gating and/or loop gating of the homomeric hemichannel containing six mutant subunits. This possibility arises from the reported ability of a single

connexin subunit to initiate V_j-gating and the possibility that electrostatic interactions among subunits containing oppositely charged N-termini would stabilize the open state of the heteromeric channels (see Oh et al., 2000). Thus, while the open probability of a homomeric hemichannel comprised of L9D or L10D subunits may be very small over a range of given voltages, the presence of one or more wild-type subunits may shift the voltage dependence such that the open probability is substantially increased at comparable voltages. We cannot exclude the possibility that the presence of one or more wild-type Cx32 subunits might compensate for a defect in "protein trafficking" or in the mechanism that targets hemichannels containing only L9D or L10D subunits to the membrane, but there does not appear to be a need to invoke such a mechanism at the present time.

In summary, the structural picture that emerges from this study is that the N- terminus of Cx32 forms a vestibule to the channel pore and that charged amino acid residues located at the first 10 positions of this domain can contribute to a voltage sensor that initiates V_j-gating. It appears that the Cx32 hemichannel can adopt at least two different open conformations. The 11th and upstream residues most likely lie outside the channel pore in both conformations, as the placement of a negatively charged residue at the 11th position does not appear to alter the V_j-gating polarity of Cx32 hemichannels, although this aspect must be explored further. As Cx32 subunits containing either the N2D or the G5D substitution reverse the gating polarity of Cx32 hemichannels and are not bi-polar, the first five residues must always be located within the channel pore. The bi-polarity of V_j-gating of negative charge substitutions at the 8th and 10th residues can be explained if these residues are located in the pore in some, but not all, open channel conformations.

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