Assembly of Chimeric Connexin-Aequorin Proteins into Functional Gap Junction Channels

REPORTING INTRACELLULAR AND PLASMA MEMBRANE CALCIUM ENVIRONMENTS* (Received for publication, August 27, 1997, and in revised form, November 3, 1997)

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Chimeric proteins comprising connexins 26, 32, and 43 and aequorin, a chemiluminescent calcium indicator, were made by fusing the amino terminus of aequorin to the carboxyl terminus of connexins. The retention of function by the chimeric partners was investigated. Connexin 32-aequorin and connexin 43-aequorin retained chemiluminescent activity whereas that of connexin 26-aequorin was negligible. Immunofluorescent staining of COS-7 cells expressing the chimerae showed they were targeted to the plasma membrane. Gap junction intercellular channel formation by the chimerae alone and in combination with wild-type connexins was investigated. Stable HeLa cells expressing connexin 43-aequorin were functional, as demonstrated by Lucifer yellow transfer. Pairs of Xenopus oocytes expressing connexin 43-aequorin were electrophysiologically coupled, but those expressing chimeric connexin 26 or 32 showed no detectable levels of coupling. The formation of heteromeric channels constructed of chimeric connexin 32 or connexin 43 and the respective wild-type connexins was inferred from the novel voltage gating properties of the junctional conductance. The results show that the preservation of function by each partner of the chimeric protein is dictated mainly by the nature of the connexin, especially the length of the cytoplasmic carboxyl-terminal domain. The aequorin partner of the connexin 43 chimera reported calcium levels in COS-7 cells in at least two different calcium environments.

Gap junction intercellular channels allow the direct movement between neighboring cells of ions and small molecules generally less than 1 kDa (1). These cell-cell junctions are generally believed to underpin diverse integrative events such as tissue growth and differentiation (2) and the co-ordination of signaling component allowing intercellular crosstalk may not be calcium but a more global messenger molecule such as inositol 1,4,5-trisphosphate (11). Calcium may also be a crucial environmental factor at intracellular sites such as the endoplasmic reticulum-Golgi environs, where the oligomerization of connexins into hexameric connexons is thought to occur. Many calcium-binding proteins, some with chaperone-like functions, are localized to this region of the secretory pathway and roles in the oligomerization of virally encoded proteins have been demonstrated (12, 13). The involvement of calcium in connexin oligomerization, a prelude to gap junction biogenesis, may be either direct or indirect and can involve a role for accessory proteins such as calmodulin. Calmodulin regulates junctional physiology in a variety of physiological systems (14), and calmodulin binding domains in connexins have been delineated (15). Thus, knowledge of the calcium levels in the vicinity of the gap junctions and their connexin/connexon precursors will help to elucidate its relevance in the control of cell to cell signaling through gap junctions.

The first application of aequorin as a calcium-sensitive reporter, demonstrating the importance of free ionized Ca2+ in the gating of gap junction channels, was in 1975 by Rose and Loewenstein (16). Since then, aequorin has been targeted to a number of organelles, e.g. mitochondria (17), endoplasmic reticulum (18, 19), nucleus (20), and the plasma membrane (21), where it has been used to report the subcellular calcium environment. In the present work, we have adopted a similar approach by attaching aequorin to the carboxyl tail of connexins 26, 32, and 43 targeted to gap junctions. We then have analyzed the expression and functionality of the chimeric products in mammalian cells and Xenopus oocytes (22). The results show that functional gap junction channels were constructed in

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mammalian and amphibian cells. Furthermore, measurement of chemiluminescent activity of the connexin 43-aequorin chimera in transfected COS-7 cells reported at least two different calcium environments.

EXPERIMENTAL PROCEDURES

Materials—Tag DNA polymerase, the in vitro transcription/translation system (TNT), restriction and DNA modifying enzymes, and the transcription and translation system were from Promega. ECL immunoblotting detection system and [35S]methionine were from Amersham Life Science, Inc. Tissue culture medium and reagents were supplied by Life Technologies, Inc. and monoclonal antibodies to Cx32 were supplied by Sigma unless otherwise stated. Oligonucleotides were prepared on site by J. Hoy. All other reagents were supplied by Sigma unless otherwise stated.

Construction of Connexin-Aequorin Chimeric cDNAs—Aequorin was fused in frame to the COOH terminus of Cx26, Cx32, and Cx43 by Chemicon International and Zymed Laboratories Inc.

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Construction of Connexin-Aequorin Chimeric cDNAs—Aequorin was fused in frame to the COOH terminus of Cx26, Cx32, and Cx43 (23, 24, and 25) using a two-step PCR procedure (26) (Fig. 1). The first step generated cDNA products containing overlapping regions of 3'-connexin minus the stop codon and 5'-aequorin minus the start codon. The chimeric cDNAs were produced by a second PCR reaction, using equimolar amounts of the initial PCR product and the connexin primer and the 3'-aequorin primer. The primers used were as follows: Cx26P1, T7 promoter and first 15 bp of Cx26 (5'-TAC ATG TGG TGT ACT GGA GT); Cx26P2, first 15 bp of Aeq (5'-ATG) and last 15 bp of Cx26 (5'-TAA) (5'-TGA TGT AAG CTT GAC AAT CTC GTC GT); Aeq32, reverse of Cx32P2; Aeq5, T7 promoter and first 15 bp of Aeq (5'-TAA TAC GAC TCA TTA TAG GGA GAA ATG GTC AAG); Aeq10, last 15 bp of aequorin including stop codon (5'-TCTG CTT GAG CTT GCC TGG GTA CAG CTA ACA TGA GAC AAT CTG GAC GG AGC AGC ACC TCC AC). The external primers contain BamHI/PstI for Cx26-Aeq and Cx32-Aeq and BglII for Cx43-Aeq.

1 The abbreviations used are: TNT, coupled in vitro transcription and translation; Cx, connexin; Aeq, aequorin; Cx26-Aeq, connexin 26-aequorin chimera; Cx32-Aeq, connexin 32-aequorin chimera; Cx43-Aeq, connexin 43-aequorin chimera; Aeq-WT, wild-type aequorin; V, voltage; I, current; Vm, holding potential; Vj, transjunctional voltage; gj, junctional conductance; PCR, polymerase chain reaction; [Ca2+], intracellular calcium concentration; bp, base pair(s).
erslips in 12-well dishes and transfected with 5 μg of the relevant cDNA. Cells were fixed 48 h after transfection in 4% formaldehyde and permeabilized in 0.1% Triton X-100, 0.1 M lysine in phosphate-buffered saline. The cellular localization of the chimeric proteins was determined using a monoclonal Cx32 antibody (generated to a sequence on the intracellular loop) or a monoclonal Cx43 antibody (generated to a sequence on the carboxyl tail). A rabbit polyonal antibody to the intracellular loop of Cx26 was also used (36). Aequorin was localized using a rabbit anti-aequorin antibody (31).

Functional Analysis of Chimeric Cx-Aeq in Stable Cell Lines—HeLa cells were transfected with 20 μg of relevant Cx-Aeq DNA in the pCR3 plasmid as described above. Forty-eight hours after transformation, the cells were split into selective media (Dulbecco's modified Eagle's medium containing 400 μg/ml G418 sulfate) and clonally propagated (37). Clones were selected on the basis of their aequorin activity. The ability to form functional gap junction channels was assessed by microinjection of confluent cell monolayers with 5% w/v Lucifer yellow CH in 0.3 M LiCl, followed by examination by fluorescence microscopy using a 440-nm filter. The cells were then fixed in 4% formaldehyde for 30 min, mounted under 16-mm coverslips, and the number of injected cells transferring dye counted. In control experiments, dye transfer was assessed in cells incubated with 180-glycyrhetinic acid (1 μM) for 30 min, a potent blocker of gap junction-mediated intercellular communication (38).

Preparation of cRNAs—Wild-type and chimeric Cx-Aeq cRNAs were linearized with ClaI and sense transcribed with T7 polymerase in the presence of the cap analogue m7G(5'ppp)5'G (Boehringer Mannheim). After DNase digestion and purification, cRNAs were quantified by absorbance (260 nm) and the proportion of full-length transcripts (>95%) was checked in 1% agarose gels stained with ethidium bromide. Expression in Pairs of Xenopus Oocytes—Oocytes were prepared for injection as described previously (40) and co-injected with an antisense oligonucleotide (10 ng/oocyte) directed against Xenopus Cx38 mRNA to block endogenous expression (40) and the cRNAs (0.1–1.0 μg/ml; 50 nl/oocyte) encoding the chimeric and wild-type connexins. Vitelline membranes were removed in hypertonic solution 24 h after injection, and pairs of oocytes were placed in contact (22).

Measurement of Macroscopic Functional Conductance—Functional conductance developed between oocyte pairs was recorded 24–72 h after pairing using the dual voltage clamp technique with two independent amplifiers (TEV-200, Dagan). Each cell of the pair was impaled with two microelectrodes (0.5–1 meq/moh) filled with 2 M KCl, 10 mM EGTA, and 10 mM Hepes pH 7.2. Functional conductance was measured as follows: V1, I1, V2, and I2 are voltages and currents, with the holding current subtracted, in oocyte 1 and 2, respectively. Initially both oocytes were clamped at the same holding potential (Vh, e.g., ~40 mV), when the transjunctional voltage (Vj) was zero since Vj = V1 – V2. A voltage step, applied in oocyte 1, defined positive V1 as greater relative positivity in oocyte 1. The current injected in oocyte 2, Ij, i.e. to hold its potential constant, was equal in magnitude and opposite in sign to the current flowing through the functional channels (Ij = –I1). Thus, the macroscopic functional conductance was directly calculated as J/NV. The stimulation and data collection were carried out with a PC-AT computer using pCLAMP software and Digidata 1200-A interface (Axon Instrument Inc.).

RESULTS

In Vivo Expression of Chimeric Connexins—The chimeric proteins were initially characterized after TNT to determine their molecular mass and whether functionality of the aequorin partner was retained. Cx-Aeq constructs were synthesized from the three chimeric constructs, namely Cx26-Aeq (47 kDa), Cx32-Aeq (53 kDa), and Cx43-Aeq (64 kDa) (data not shown). Aequorin activity of Cx43-Aeq and Cx32-Aeq was comparable to that of wild-type aequorin, but chemiluminescence of Cx26-Aeq chimeras was markedly reduced. The effect of fusion of aequorin to Cx26 on its chemiluminescence was investigated by PCR amplification of the chimera and the aequorin partner of each chimera. The PCR products were analyzed for aequorin activity in the TNT translation assay.

The aequorin partner amplified from Cx26-Aeq gave comparable aequorin activity (62 ± 1.5%) to Cx32-Aeq (78 ± 6.4%) and Aeq-WT (100%). However, chemiluminescence obtained with the full-length Cx26-Aeq construct was only 1.3 ± 0.1% of Aeq-WT activity. These results show that fusion of aequorin to the carboxyl terminus of connexin 26 inhibited aequorin activity in vitro.

In Vitro Analysis of Chimeric Connexins—Indirect immunofluorescence of transfected COS-7 cells indicated that Cx32-Aeq and Cx43-Aeq chimeras were targeted to the plasma membrane (Fig. 2a). Furthermore, double immunofluorescence with aequorin and connexin antibodies showed that aequorin was localized to the same region of the cell as its connexin partner (data not shown). Thus, the results demonstrated that the two chimeric proteins were located at the plasma membrane and at intracellular locations, especially in the endoplasmic reticulum-Golgi environs of the cell, as established by immunostaining with antibodies to TGN38, a Golgi marker (41) (data not shown). Although Cx26 was localized by immunocytochemistry using an intracellular loop domain antibody (Des 3) (36), co-localization of the connexin and aequorin components of Cx26-Aeq could not be demonstrated since no signal was detected with aequorin antibodies.

Transfected cells also expressed chimeric proteins of the predicted size for Cx26-Aeq, Cx32-Aeq, and Cx43-Aeq (Fig. 2b). Analysis of aequorin activity in cell extracts showed the activity in chimeras of Cx43 and Cx32 was comparable to Aeq-WT, but that in Cx26-Aeq transfected cells was low (Table 1). The overall levels of aequorin activity of the chimeras, compared with Aeq-WT, were Cx43-Aeq > Cx32-Aeq > Cx26-Aeq. The results suggest that the nature of the connexin partner and thus the length of the carboxyl tail may influence the functionality (i.e. chemiluminescence) of the aequorin component of the chimeric proteins.

The orientation of the chimeras in the endoplasmic reticulum was examined by determining the aequorin activity of Cx32-Aeq in living cells (18). Native recombinant aequorin reactivated in the cytosol with its prothetic group coelenterazine was used as a control (18). The results showed that Cx32-Aeq had been correctly inserted into the membrane (data not shown).

FIG. 2. a, immunolocalization of Cx32-Aeq and Cx43-Aeq chimeric proteins in transiently transfected COS-7 cells. A, chimeric Cx32-Aeq stained with a monoclonal antibody against Cx32. B, chimeric Cx43-Aeq stained with a monoclonal antibody to Cx43. Arrows indicate targeting of the chimeric proteins to the plasma membrane. b, Western blot analysis of the Cx-Aeq chimeric proteins expressed by transient COS-7 cell transfectants. After SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose the blots were probed with antibodies to aequorin. Lane 1, Cx26-Aeq; lane 2, Cx32-Aeq; lane 3, Cx43-Aeq.
Formation of Cell-Cell Channels in HeLa Cell Transfectants—To study the formation of functional gap junction channels, stable HeLa cell lines expressing the chimeric proteins were generated and their intercellular communication properties assessed by Lucifer yellow transfer (Fig. 3 and Table II). Fig. 3 (A1 and A2) shows that two transfected HeLa cell lines expressing Cx43-Aeq (D7 and E9) transferred dye to up to 25 neighboring cells, whereas negligible dye transfer occurred in nontransfected cells (Fig. 3, B1). Confirmation that direct communication had occurred between cells across gap junction channels was obtained by inhibition of dye transfer following incubation of the cells for 30 min with 18α glycyrrhetinic acid, an inhibitor of intercellular communication across gap junctions (Fig. 3, A3 and B2). Additionally, a correlation was noted between dye transfer and the level of aequorin expression (Table II). In HeLa cell line D7, which showed the higher aequorin activity, 74% of the cells injected with Lucifer yellow transferred dye to 10 or more cells. In cell line E9, which exhibited significantly lower aequorin activity, 59% of the cells transferred the dye to the same number of cells. Few (<2%) nontransfected HeLa cells transferred dye to neighboring cells. These results indicate that the expression properties of the two partners of the chimeric protein are closely linked. Thus, HeLa cells expressing Cx32-Aeq transferred the dye only when protein expression from the cytomegalovirus immediate early promoter was stimulated by 5 mM sodium butyrate. No intercellular dye transfer was observed between HeLa cells expressing Cx26-Aeq.

Formation of Cell-to-Cell Channels in Xenopus Oocytes—The voltage gating properties of the channels formed by Cx43-Aeq and Cx32-Aeq were examined. To study the assembly of the chimeras into functional gap junctions alone (i.e., as homomeric channels) or in combination with wild-type connexins (i.e., as heteromeric and heteromorphic channels), paired oocytes were injected with various combinations of connexin and Cx-Aeq cRNAs. Homomeric and heterotypic channel formation was determined by measuring the macroscopic junctional conductance. Formation of heteromeric channels was inferred by comparing the voltage gating properties of the junctions formed in pairs of oocytes expressing only wild-type connexins and the results obtained with pairs co-expressing the chimeras.

Homomeric and Heterotypic Channels Comprising Cx-Aeq Subunits—To examine the formation of homotypic channels, paired oocytes were injected with equal amounts of the same connexin-aequorin chimera cRNA (Table III, a and b). Although large junctional conductances were induced by cRNA of wild-type connexins, pairs expressing Cx26-Aeq or Cx32-Aeq showed no detectable levels of electrical coupling. In contrast, pairs injected with Cx43-Aeq were electrically coupled, although junctional conductance was 1–2% of that of wild-type Cx43, despite the fact that both pairs expressed similar levels of connexins. These experiments show that expression of Cx26-Aeq and Cx32-Aeq in oocytes does not result in the formation of intercellular channels, whereas the Cx43-Aeq was able to form channels, although this chimera was far less effective than Cx43 in inducing homomeric coupling.

The formation of heterotypic channels was examined by pairing oocytes expressing the Cx-Aeq with oocytes expressing wild-type connexin (Table III, c and f). Conduction was restricted to Cx43-Aeq paired with cells injected with Cx43 or Cx32-Aeq/Cx43. These heterotypic junctions showed higher conductance values than those shown by homotypic Cx43-Aeq pairs. However, the presence of wild-type hemichannels in the partner cell did not rescue the loss of function in oocytes expressing Cx26-Aeq or Cx32-Aeq.

Voltage-gating Properties of Cx43-Aeq Channels—Many vertebrate junctions are controlled by transjunctional voltage, i.e., the voltage difference between cell interiors (\(V_j\)), in which voltage-gating properties are specific depending on the type of connexin forming the intercellular channels (42). The voltage gating properties of intercellular channels comprising Cx43-Aeq subunits showed that junctional conductances (\(g_j\)) of homotypic Cx43-Aeq and Cx43 junctions displayed a common maximal \(g_j\) at \(V_j = 0\) and symmetrical \(g_j\) reduction in response to positive and negative \(V_j\) (Fig. 4). However, they possessed different kinetic properties since the \(g_j\) transitions of Cx43-Aeq junctions showed markedly slower time courses.

Heterotypic junctions constructed of homomorphic Cx43 and Cx43-Aeq hemichannels responded asymmetrically to the

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**TABLE I**

Aequorin activity of chimeric proteins expressed in COS-7 cells

The level of chemiluminescence was measured using 100 ng of total cellular protein prepared from COS-7 cells transfected with Cx26-Aeq, Cx32-Aeq, Cx43-Aeq, or Aeq-WT. The results are the mean of triplicate experiments ± S.E. and are expressed as the % aequorin activity compared to Aeq-WT (100%).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Aequorin activity %</th>
</tr>
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<tbody>
<tr>
<td>Cx26-Aeq</td>
<td>6.02 ± 0.85</td>
</tr>
<tr>
<td>Cx32-Aeq</td>
<td>7.25 ± 7.25</td>
</tr>
<tr>
<td>Cx43-Aeq</td>
<td>88.5 ± 8.09</td>
</tr>
<tr>
<td>Aeq-WT</td>
<td>100</td>
</tr>
</tbody>
</table>

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**TABLE II**

Dye transfer and aequorin activity in HeLa cells

Cell populations were assessed for their ability to transfer Lucifer yellow dye and aequorin activity as described in methods. For dye transfer, the total number of cells injected (\(n\)) is indicated and the results are expressed as the % of cells transferring dye to 10 or more cells. Aequorin activity is given as relative photon counts obtained from 4 × 10⁶ cells. The results are the mean ± S.E.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cells coupled</th>
<th>Aequorin activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type ((n = 55))</td>
<td>1.8</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>Cx43-Aeq (D7) ((n = 69))</td>
<td>74</td>
<td>69,803 ± 2,782</td>
</tr>
<tr>
<td>Cx43-Aeq (E9) ((n = 68))</td>
<td>59</td>
<td>12,792 ± 2,072</td>
</tr>
</tbody>
</table>

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**FIG. 3.** Lucifer yellow transfer in HeLa cells. Stable cell lines D7 and E9 expressing Cx43-Aeq were analyzed for their ability to transfer Lucifer yellow (A1 and A2). Lucifer yellow transfer in nontransfected cells is represented in B1. Communication via gap junctions was inhibited by incubation of the cells with 1 μM 18α glycyrrhetinic acid for 30 min (A3 and B2). B3 shows a typical monolayer of cells viewed under phase. Original magnification, × 20.
transjunctional voltage (Fig. 4, Cx43:Cx43-Aeq), suggesting that (i) two independent $V_j$ gates exist, with one in the Cx43 hemichannel and another in the Cx43-Aeq hemichannel; (ii) each channel closes in response to $V_j$ negative on its cytoplasmic side; and (iii) the characteristics of $V_j$ displayed by each hemichannel were only partially affected by association with the other hemichannel. In summary, the $V_j$ dependence of the heterotypic Cx43 and Cx43-Aeq junctions reflected the gating properties of each hemichannel in their homomeric junctions.

**Formation of Heteromeric Cx/Cx-Aeq Channels**—The formation of heteromeric channels comprising chimeric Cx-Aeq and wild-type connexin and the effect of chimeric Cx26, Cx32, and Cx43-Aeq on the coupling induced by cells expressing wild-type connexins were studied (Table III, d, e, and f). Pairs co-expressing Cx32 and Cx32-Aeq in both oocytes developed significantly lower levels of coupling than those expressing solely the wild-type connexin and the effect of chimeric Cx26, Cx32, and Cx43-Aeq on the coupling induced by cells expressing wild-type connexins were studied (Table III, d, e, and f). Pairs co-expressing Cx32 and Cx32-Aeq in both oocytes developed significantly lower levels of coupling than those expressing solely the wild-type connexin. This reduction of junctional conductance was less in pairs in which one cell was co-injected with cRNA to Cx32-Aeq and the other with cRNA to Cx32. This dominant-negative inhibition may be explained by oligomerization of chimeric and Cx32 subunits. Support for this conclusion was obtained by comparing the voltage gating properties of junctional conductance developed between pairs expressing only wild-type Cx32 with those formed by co-injecting one cell with the cRNAs to Cx32 and Cx32-Aeq with the counterpart oocyte being injected solely with cRNA to Cx32 (Fig. 5). In contrast to wild-type Cx32 junctions, where conductance responded symmetrically to positive and negative pulses of $V_j$ conductance (40), the junctions in Cx32/Cx32-Aeq:Cx32 pairs reacted asymmetrically to $V_j$ steps of opposite polarity. This asymmetry strongly suggests heterogeneity in the connexin composition of the connexon hemichannels contributed by each cell. Since Cx32-Aeq was unable to form functional channels alone or in heterotypic combination with the wild-type hemichannels described above, this result also points to the co-oligomerization of chimeric and wild-type Cx32 subunits into heteromeric connexon hemichannels. These heterotypic junctions showed complex voltage regulation because the incorporation of chimeric Cx32-Aeq subunits into junctional channels only slightly modified the voltage gating properties of heteromeric Cx32-Aeq/Cx32 hemichannels. There was also a more dramatic reduction in voltage sensitivity and a slowing of the junctional conductance inactivation of the apposed Cx32 homomeric hemichannels. It was found that $V_j$ positive in the cytoplasmic side of the oocyte expressing both Cx32 and Cx32-Aeq induced junctional currents that were less voltage-sensitive and that displayed slower kinetics than those induced by negative $V_j$, as was

**TABLE III**

Conductance values in microsiemens are mean ± S.D. of the number of pairs indicated (n). NC, no coupling was detected.

<table>
<thead>
<tr>
<th></th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cx43-Cx43</td>
<td>Cx43A-Cx43A</td>
<td>Cx43-Cx32</td>
<td>Cx43/Cx43A-Cx43/Cx43A</td>
<td>Cx43/Cx32A-Cx32A</td>
<td>Cx43/Cx32A-Cx32A</td>
</tr>
<tr>
<td></td>
<td>19.9 ± 13.7 (6)</td>
<td>0.34 ± 0.3 (9)</td>
<td>1.0 ± 0.6 (6)</td>
<td>22.6 ± 11.6 (9)</td>
<td>32.3 ± 6.1 (8)</td>
<td>0.72 ± 0.4 (9)</td>
</tr>
<tr>
<td></td>
<td>35.0 ± 8.4 (6)</td>
<td>0.3 ± 0.1 (12)</td>
<td>1.3 ± 0.5 (8)</td>
<td>41.3 ± 12.6 (6)</td>
<td>36.8 ± 16.3 (7)</td>
<td>1.4 ± 0.8 (8)</td>
</tr>
<tr>
<td></td>
<td>28.3 ± 11.6 (6)</td>
<td>0.18 ± 0.1 (6)</td>
<td>1.1 ± 0.7 (7)</td>
<td>25.9 ± 15.3 (6)</td>
<td>21.1 ± 9.7 (6)</td>
<td>0.57 ± 0.46 (7)</td>
</tr>
<tr>
<td>Cx32-Cx32</td>
<td>33.0 ± 10.7 (6)</td>
<td>NC (7)</td>
<td>NC (6)</td>
<td>8.4 ± 9.4 (8)</td>
<td>30.5 ± 12.3 (9)</td>
<td>NC (8)</td>
</tr>
<tr>
<td>Cx32A-Cx32A</td>
<td>58.3 ± 12.2 (8)</td>
<td>NC (6)</td>
<td>NC (9)</td>
<td>10.6 ± 3.5 (8)</td>
<td>36.2 ± 16.6 (9)</td>
<td>NC (6)</td>
</tr>
<tr>
<td>Cx32-Cx32A</td>
<td>35.5 ± 17.6 (8)</td>
<td>NC (7)</td>
<td>NC (7)</td>
<td>2.3 ± 1.4 (9)</td>
<td>8.4 ± 10.7 (9)</td>
<td>NC (8)</td>
</tr>
<tr>
<td>Cx32A-Cx32A</td>
<td>21.1 ± 9.4 (6)</td>
<td>NC (10)</td>
<td>NC (8)</td>
<td>25.3 ± 10.8 (6)</td>
<td>20.5 ± 8.1 (7)</td>
<td>NC (9)</td>
</tr>
<tr>
<td>Cx26-Cx26</td>
<td>39.9 ± 13.2 (7)</td>
<td>NC (8)</td>
<td>NC (6)</td>
<td>45.0 ± 15.0 (8)</td>
<td>33.5 ± 16.4 (8)</td>
<td>NC (8)</td>
</tr>
<tr>
<td>Cx26A-Cx26A</td>
<td>43.2 ± 15.7 (6)</td>
<td>NC (8)</td>
<td>NC (6)</td>
<td>41.9 ± 18.3 (8)</td>
<td>38.5 ± 14.9 (6)</td>
<td>NC (7)</td>
</tr>
</tbody>
</table>
observed in the homotypic Cx32 junctions.

The levels of junctional conductance in pairs co-injected with wild-type Cx43 and Cx26 and the corresponding chimeric connexins were comparable to those induced by wild-type connexin alone. However, whereas the voltage gating properties of Cx26Aeq/Cx26:Cx26Aeq/Cx26 pairs were indistinguishable from Cx26:Cx26 pairs, they were different in pairs co-expressing Cx43-Aeq and Cx43 subunits (Fig. 4). Oocytes co-expressing Cx43 and Cx43-Aeq evoked modified currents in which the properties were intermediate to those exhibited by respective homomeric Cx43 and homomeric Cx43-Aeq hemichannels. The formation of heteromeric Cx43-Aeq/Cx43 hemichannels is evident when the time courses of the two components of current inactivation in the co-injected site were compared with the kinetics of the homomeric junctions. The faster component had a slower time course than that of the rapid transitions found in the homomeric Cx43 hemichannels (Fig. 4). Thus, the assembly of Cx43-Aeq and Cx43 into separate gap junctions did not occur, and the gating properties suggest the formation of heteromeric hemichannels comprising both Cx43-Aeq and Cx43 subunits; these display intermediate voltage gating properties.

Utility of Cx-Aeq Chimerae for Monitoring Calcium Levels—COS-7 cells expressing Cx43-Aeq were used to measure by chemiluminescence the intracellular calcium concentration ([Ca^{2+}]_{i}) in the cell, namely in the endoplasmic reticulum-Golgi environs and at the plasma membrane (Fig. 2a), and data are means ± S.E. (n = 12). B, the effect of addition of Ca^{2+} to cells maintained in Ca^{2+}-free medium. Recombinant aequorin in cells expressing luciferase-aequorin or Cx43-Aeq was activated with 5 μM coelenterazine in Ca^{2+}-free medium (+1 mM EGTA). Cells were initially perfused in KRH + 1 mM EGTA, KRH was supplemented with 1.3 mM CaCl_{2}, and data are means ± S.E. (n = 3). The arrow indicates the time point at which Ca^{2+} was added back to the cells.

**DISCUSSION**

The Assembly of Chimeric Proteins into Functional Gap Junctions—The chimerae examined in this work comprise a targeting component (connexin) and a reporter group (aequorin). During the assembly of functional gap junction channels from connexins with attached reporter groups, each partner should retain biological activity. The reporter group is required to function irrespective of its position in the cell, but the functionality of the targeting component can only be assessed following its assembly into connexon hemichannels that align and interact at the plasma membrane with connexons in neighboring cells to generate a gap junction. The chimerae should oligomerize and traffic along the secretory pathway with fidelity. At the plasma membrane, the hemichannels should dock subject to the connexin compatibility rules determined in studies on gap junction formation in HeLa cells (43) and in Xenopus oocytes (44). A further objective is that the gap junctions should operate as near to normal as possible, although the main task of the chimera is to report the calcium environment en route to and at its functional residence. The present work shows that Cx43-Aeq best satisfied these demanding criteria; Cx43-Aeq was targeted to the gap junctions as shown by Lucifer yellow transfer in HeLa cells and maintained the ability to form homotypic channels in oocytes. The chimeric Cx43-Aeq subunits induced low levels of macroscopic junctional conductance relative to wild-type connexin. Finally, the utility of Cx43-Aeq was evident for it reported the calcium environment in at least two different Ca^{2+} environments in live cells.

The Cx32-Aeq chimera showed characteristics similar to
other chimeric connexins that have been studied (45) as well as to mutants of human Cx32 associated with the X-linked form of Charcot-Marie-Tooth disease (46). These modified connexons combine the loss of ability to form homomeric channels with the effect of dominant-negative inhibition over the development of coupling induced by wild-type connexins. However, in the present work, Cx32-Aeq also incorporated together with wild-type subunits into electrophysiologically distinguishable heteromeric channels, indicating that oligomerization with a functionally competent connexin partner rescued the complete loss of function of Cx32-Aeq. The ability to form a functional heterotypic channel may be critically determined by the stoichiometry of Cx32-Aeq and wild-type subunits in the connexon hemichannels and a number of different hemichannels could result from different combinations of wild-type and Cx32-Aeq subunits. The assembly of two types of subunits into heteromeric hemichannels may inhibit the correct alignment of the two participating hemichannels, the docking, or the gating of the complete channel into the open configuration. The results also suggest that only a few classes of the multiple possible combinations, (probably those with lowest Cx32-Aeq:Cx32 ratio), resulted in the formation of functional heteromeric channels. In contrast to results obtained with Cx32-Aeq and Cx43-Aeq, the Cx26-Aeq chimera was unable to incorporate into functional gap junction channels.

The position where the reporter is attached to the connexin is important. The options are the amino terminus, the intracellular loop and the carboxyl tail, all cytoplasmically located (4). The amino terminus (~22 amino acids) shows high sequence homology in all connexins, suggesting a crucial function, especially in membrane insertion and possibly targeting (47). The intracellular loop, highly variable in sequence, is implicated in channel regulation (48). The carboxyl-terminal tail was chosen in the present work for it may position the aequorin reporter group further away from the membrane, thereby optimizing retention of functions of both partners. Furthermore, functional analysis of a truncated Cx43, in which most of the carboxyl-terminal domain was removed, has suggested that the cytoplasmic tail does not play an important role in transjunctional voltage gating (49). However, in the absence of a detailed three-dimensional structure, the proximity and interactive dynamics of these domains remain to be investigated. Connexins 26, 32, and 43 have cytoplasmic carboxyl domains of 18, 78, and 156 amino acids respectively (3). The present work shows that Cx26-Aeq was not functional, with even the aequorin activity impaired. Cx32-Aeq alone was also defective in its ability to form channels in oocytes although the chimera’s topography in the membrane was correct and dye transfer occurred in transfected HeLa cells upon induction of protein expression with sodium butyrate. In oocytes, electrical communication was absent but was rescued if the chimera was incorporated into heteromeric channels with Cx32. These observations, combined with the fact that Cx43-Aeq formed functional homomeric channels both in oocytes and in HeLa cells, lead to the view that the length of the carboxyl tail emerges as one of the most important criteria in maintaining optimal aequorin expression. We conclude that the ratio of the molecular weight of connexin to its partner protein is likely to be important in maintaining optimal functionality of both partners of the chimeric protein. For example, fusion of the large reporter molecule, β-galactosidase (mass of 116 kDa), with the carboxyl terminus of Cx43 was nonfunctional and prevented gap junction communication mediated by wild-type Cx43 (50).

Chimeric Connexins Modify Gap Junction Channel Operation—The basis by which gap junction channels are gated by voltage, intracellular Ca2+, or pH acidification remains unresolved. Calcium has been suggested to trigger a twisting action of connexins leading to pore closure at the cytoplasmic side of the junction (8). Gating by pH may involve a “particle-receptor” interaction between the intracellular loop and the carboxyl terminus of Cx43 leading to closure of the gap junction (51). Several connexin domains have been implicated in the gating involved in transjunctional voltage dependence. Point mutations in Cx32 and Cx26, which alter the charge of the NH2-terminal second amino acid and at the M1/E1 boundary, reversed gating polarity (52). The substitution of Pro by Leu in Cx26 suggests that the second membrane traverse (M2) is active in the transduction event in voltage gating (53), and evidence for participation of the cytoplasmic loop in voltage gating has also been reported (46). Clearly, attachment of a large molecule of similar overall size such as aequorin to the carboxyl tail of a connexin can modify any of the gating mechanisms in junctional channels. Indeed, as was shown by the novel gating properties displayed by Cx43-Aeq homotypic and Cx32-Aeq heterotypic channels, at least the voltage gating mechanism was altered in oocytes. However, it should be noted that, despite these differences, gap junctions constructed of Cx43-Aeq in transfected HeLa cell lines actively transferred Lucifer yellow. Thus, mammalian cells and oocytes behave differently with respect to their handling and expression of connexin chimeras and their assembly into connexon channels. These observations support the view that eukaryotic cells and oocytes can display different gap junction communication characteristics (54).

Cx43-Aeq Reports Intracellular Calcium Levels in COS Cells—Finally, we show that the chimeric connexin probes can be used to report [Ca2+]i, in live cells. Basal [Ca2+]i, reported by Cx43-Aeq was approximately 415 nM. However, after the aequorin moiety of the chimera had been reactivated in medium containing EGTA, a transient level reaching 2 µM was reported. These results show that two different components of intracellular [Ca2+]i, can be reported by the same probe. The transient (2 µM) calcium signal may reflect store-operated calcium influx (55). However, this value (2 µM) is similar to that obtained using a SNAP25-Aeq chimera targeted exclusively to the plasma membrane (21). The basal Ca2+ level reported by Cx43-Aeq was higher than that reported by the cytosolically located luciferase-aequorin probe (approximately 100 nM). This elevated [Ca2+]i, may reflect a mean of the [Ca2+]i, in the endoplasmic reticulum-Golgi areas where the intracellular stores of Cx43-Aeq reside, and the high level reported below the plasma membrane. However, since the results obtained with oocytes and HeLa cells show that Cx43-Aeq was targeted to the plasma membrane and that gap junction mediated intercellular communication occurred, it is also possible that the chimera is reporting the calcium level near to the gap junction pore entrance. The utility of these functional connexin-aequorin probes will allow many areas of work imposing on gap junction biogenesis and gating to be explored.

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REFERENCES
Connexin-Aequorin Chimerae Monitor the Calcium Environment

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