Restricted Distribution of Connexin40, a Gap Junctional Protein, in Mammalian Heart


Abstract Connexin40 (Cx40) is a member of the connexin family of gap junctional proteins. Its mRNA, abundant in lung, is also present in mammalian heart, although in lower amount. Rabbit antipeptide antibodies directed to the COOH terminus (residues 335 to 356) of rat Cx40 were characterized to investigate the distribution of Cx40 in rat and guinea pig cardiac tissues. The affinity-purified antibodies detect specifically a major protein (Mₐ, 40 000) in immunoblots of total extracts from rat lung and rat and guinea pig heart. In sections of guinea pig atrial tissue treated for immunofluorescence, a strong labeling associated with myocytes was seen with a distribution consistent with that of intercalated disks. The results of immunoelectron microscopy carried out with guinea pig atrial tissue showed that epitopes recognized by these antibodies were exclusively associated with gap junctions. These results, added to those of control experiments, demonstrate that antibodies 335-356 are specific for Cx40. Double-labeling experiments carried out with lung sections using anti-factor VIII and anti-Cx40 antibodies suggest that Cx40 is expressed in blood vessel endothelial cells. In guinea pig and rat heart sections, investigated using both immunofluorescence and immunoperoxidase techniques, a signal was also found to be associated with vascular walls. In guinea pig heart, only atrial myocytes are Cx40-positive. No labeling was detected in ventricular myocytes, including those of the His bundle and the bundle branches, which otherwise do express connexin43 (Cx43). In rat heart Cx40-expressing myocytes are localized in the conduction system, i.e., the His bundle, the bundle branches, and the Purkinje fibers. Cx43 is not detected either in the His bundle or in the proximal parts of the bundle branches, and consequently, Cx40 is the first connexin demonstrated in this region of the rat conduction system. Cx40 was not detected in the working ventricular myocytes. Double-labeling experiments carried out with hen anti-Cx43 antibodies and rabbit anti-Cx40 antibodies demonstrated that, in tissues expressing both Cx43 and Cx40, these two connexins were localized in the same immunoreactive sites. A few sites, however, appear to contain only one or the other of these two connexins. (Circ Res. 1994;74:839-851.)

Key Words • connexins • gap junctions • heart • conduction system

Adjacent cells are directly coupled, metabolically and electrically, through intercellular (or junctional) channels that aggregate to form gap junctions. Each junctional channel consists of two hemichannels (or connexons) joined together in the extracellular junctional space separating two adjacent cells.1,2 Connexons are hexamers built from proteins that belong to the connexin family.3 Twelve mammalian connexin genes have been cloned and sequenced so far.4-18 Predicted molecular masses of connexins range from 26 to 70 kD, and topological investigations carried out with connexin32 (Cx32)19-22 and connexin43 (Cx43)23-25 have demonstrated that these proteins have cytoplasmic amino and carboxyl termini and comprise four transmembrane domains bound by one cytoplasmic loop and two extracellular loops. These domains are linked by an intercellular disulfide bridge.26,27

In excitable tissues such as myocardium and smooth muscle, electrical coupling through junctional channels is responsible for action potential propagation.28 Cx43 is the major protein of mammalian heart junctional channels,5,23,29-31 but it has not been detected in all cardiac tissues. Thus, in rat heart, Cx43 cannot be detected either in the sinoatrial and atrioventricular nodes5,28 or in the His bundle and the proximal part of the bundle branches.5,23 In human and bovine heart, Cx43 cannot be detected in the sinoatrial and atrioventricular nodes,34,35 but in contrast to rat heart, it is expressed in the His bundle and the bundle branch. From electron microscopy studies, it is known that gap junctions are present in the aforementioned cardiac tissues,36,37 which cannot be stained immunohistochemically. This contradiction can only be resolved by hypothesizing that at least one connexin other than Cx43 is expressed in these tissues.

mRNAs for connexin46 (Cx46), connexin45 (Cx45), connexin40 (Cx40), and connexin37 (Cx37) have been detected in low amounts in rat, mouse, and dog heart.8,11-15,17 The following questions remain: which type(s) of cardiac cells (myocytes, fibroblasts, smooth muscle cells, or endothelial cells)38 are these connexins associated, and furthermore, if they are associated with myocytes, what is the distribution in myo-
cardium of junctional channels composed of these connexins? Cx40 from dog, rat, and mouse has recently been cloned and sequenced,13-15,17 and it has been shown that cDNAs coding for rat and mouse Cx40 express functional junctional channels.13,17,19 To find answers to some of these questions, we have raised and characterized antipeptide antibodies directed to the carboxyl terminal domain of rat Cx40. These antibodies were used to investigate the expression and spatial distribution of Cx40 in guinea pig and rat heart. The results obtained demonstrate that Cx40 is differently distributed in the heart of these species. This distribution is also different from that recently described in the dog.40,41

Materials and Methods

Preparation and Purification of Site-Directed Antibodies

The peptide corresponding to amino acids 335 to 356 (YHSDKRRLSKASKARSDDLSSV) of rat Cx4014,17 was synthesized according to Merrifield42 in 1963 and conjugated to keyhole limpet hemocyanin using bis-diazobenzidine.29 The sequence YHSDKRRLSKASKARSDDLSSV is conserved in mouse Cx40;5 histidine (H) is replaced by glutamine (Q) in the corresponding sequence of dog Cx40.19 Immunization of rabbits and the collection of serum have been previously described in detail.29 Enriched-IgG fractions were purified from sera using a Trisacryl M-DEAE column; site-directed antibodies were affinity-purified from the previous fractions using AH-Sepharose 4B coupled to the peptide by means of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.43 The peptide corresponding to amino acids 356 to 374 of connexin43 was synthesized according to previous protocols for hen egg yolk IgY.5,44 The same peptide was used to raise antibodies in hen. This peptide was conjugated to bovine serum albumin (BSA) using bis-diazobenzidine as above. The immunization protocols for hens were similar to those used for rabbits. IgY fractions were extracted from egg yolks according to Polson et al.45 Hen site-directed antibodies were affinity-purified from the previous fractions using AH-Sepharose 4B coupled to the immunogenic peptide. Preimmune sera were subjected to the same purification steps as immune sera. The collected fractions, used in control experiments, will be referred to as preimmune fractions.

Antibodies raised in rabbit to the peptide SAEOQUSRGQY (residues 314 to 322 of Cx43,6 to which a tyrosine residue was added for coupling convenience) were previously shown to be specific for Cx43 by immunoblotting, immunofluorescence, and immunoelectron microscopy.30,34 The same peptide was used to raise antibodies in hen. This peptide was conjugated to bovine serum albumin (BSA) using bis-diazobenzidine as above. The immunization protocols for hens were similar to those used for rabbits. IgY fractions were extracted from egg yolks according to Polson et al.45 Hen site-directed antibodies were affinity-purified from the previous fractions using AH-Sepharose 4B coupled to the immunogenic peptide. Preimmune sera were subjected to the same purification steps as immune sera. The collected fractions, used in control experiments, will be referred to as preimmune fractions.

In enzyme-linked immunosorbent assays,29 purified rabbit and hen antibodies reacted strongly with the synthetic peptides to which they were raised, with no evidence of cross-reactivity between antibodies and the unrelated peptide YPSSRASSRRPRPDDQLI.

Immunoblotting

Partially purified gap junction fractions were prepared from rat heart in the presence of phenylmethylsulfonyl fluoride (1 mM/L). The procedure used was similar to that previously described,28 but the last purification step on sucrose gradient was omitted. Samples were solubilized for 30 minutes in 62.5 mM/L Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10 mM/L EDTA, and 5% glycerol and fractionated by electrophoresis. Gels were stained with Coomasie brilliant blue R-250. Other samples were electrophoresed and electrotransferred onto nitrocellulose membrane.29 Immunoreplicas were incubated at 4°C for 8 hours with the saturation solution (40 mM/L Tris, pH 7.5, 0.1% Tween 20, and 4% nonfat dry milk) and then overnight with either purified hen antibodies (20 μg/mL) or rabbit anti-Cx43 antibodies (1 μg/mL).31 After several washes, the replicas were incubated for 1 hour with biotinylated-goat anti-hen antibodies (1:500, Kirkegaard and Perry Laboratories, Gaithersburg, Md) or biotinylated-goat anti-rabbit antibodies (1:1000, Jackson Immunoresearch Laboratories, West Grove, Pa), washed, and incubated for 1 hour with peroxidase-labeled streptavidin (1:1000, Jackson Immunoresearch Laboratories). Peroxidase activity was revealed as previously described.29

Lungs and whole hearts from adult rats and guinea pigs as well as atria isolated from guinea pig hearts were frozen and pulverized in liquid nitrogen before freeze drying. Freeze-dried samples were solubilized, fractionated by electrophoresis, and then electrotransferred onto nitrocellulose membranes as described above. Saturated immunoreplicas were incubated overnight at 4°C with rabbit anti-Cx40 antibodies (3 to 10 μg/mL) or rabbit anti-Cx43 antibodies (1 μg/mL),31,44 washed, and then incubated with 125I-labeled protein A. Exposure to

Fig 1. Characterization by immunoblotting of rabbit antibodies raised to residues 335 to 356 of rat connexin40 (antibodies 335-356). Relative molecular weight (Mr) of proteins detected in the immunoreplicas was determined by comparison with molecular weight standards and cardiac connexin43 (lane a). Mobility of molecular mass standards is indicated in kilodaltons on the left of the figure (phosphorylase a, Mr 94 000; bovine serum albumin, Mr 67 000; ovalbumin, Mr 43 000; carbonic anhydrase, Mr 29 000; soybean trypsin inhibitor, Mr 21 000; and lysozyme, Mr 14 000). The primary antibodies were revealed using 125I-labeled protein A. The arrow indicates the top of immunoreplicas. Lane a shows rat heart total extracts. This replica was probed with rabbit anti-connexin43 antibodies.31 A major protein corresponding to connexin43 is detected in this immunoreplica. Lanes b, c, d, e, and f show rat lung total extracts. The wells of electrophoresis gels corresponding to these lanes were loaded with 10 times more protein than the well corresponding to lane a. Immunoreplicas a, b, and c, on one hand, and immunoreplicas d, e, and f, on the other hand, come from two separate series of experiments. Lanes b and d show replicas probed with rabbit antibodies 335-356 (5 μg/mL). A major protein of 40 kD is detected in both immunoreplicas. Lane c shows replica probed with a preimmune fraction (see "Materials and Methods" for the preparation of preimmune fractions). No protein was detected in replicas treated with preimmune fractions. Lanes e and f show replicas probed with rabbit antibodies 335-356 preincubated either with a peptide (75 μg/mL) unrelated to the immunogenic peptide (lane e) or with the immunogenic peptide (75 μg/mL) (lane f). The 40-kD band is not detected in the replica shown in lane f.
Hyperfilm (Amersham International, Buckinghamshire, UK) was carried out at 

−90°C with intensifying screens.

Specificity of the labelings due to rabbit anti-CX40 antibodies or hen antibodies was checked by (1) omission of antibodies, (2) substitution of antibodies for a preimmune fraction, and (3) substitution of antibodies for the corresponding antibodies preincubated overnight at 4°C with either the immunogenic peptide (YHSDKRRLSKASSKARSDDLSV or SAE-

GNRMQGY, 50 to 100 μg/ml) or the unrelated peptide LQLLREAEEKAGEAGTRY (100 μg/ml).

Immunofluorescence

Isolated whole hearts and heart and lung samples from adult guinea pigs (Dunkin Hartley) and rats (Wistar) were either directly frozen (in isopentane precooled at −30°C or liquid nitrogen) or fixed before freezing. Fixation was carried out with either 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, or periodate/lysine/paraformaldehyde from 1 hour to overnight. After gradual infiltration with 20% sucrose/Tissue Tek OCT compound (2:1) (Miles Laboratories, Elkhart, Ind) or 20% sucrose, the samples were frozen in liquid nitrogen. Frozen sections were collected on poly-L-lysine-coated slides and stored at −90°C until use.

After equilibration to room temperature, sections were rehydrated in PBS and saturated with 2% BSA in PBS. Only sections of fixed samples were incubated with 0.2% Triton X-100 in PBS for 1 hour and then with 0.5 mol/L NH₄Cl for 15 minutes before saturation. Sections were incubated overnight at room temperature or at 4°C with rabbit anti-CX40 antibodies (5 to 15 μg/ml), hen anti-CX43 antibodies (5 μg/ml), or rabbit anti-CX43 antibodies diluted to 1:3 μg/ml) in the saturation solution. Sections were rinsed with PBS and then incubated for 1 hour with tetraethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgGs (Jackson Immunoresearch Laboratories) or fluorescein isothiocyanate (FITC)-labeled goat anti-hen antibodies (Kirkegaard and Perry Laboratories). For the treatment of guinea pig sections, the secondary antibodies, diluted to 1:10 with the saturation solution, were mixed for 30 minutes with 10% normal guinea pig serum and centrifuged (15 minutes at 15 000 rpm). The supernatant was collected and used for labeling. The control experiments carried out to check the specificity of labelings were as follows: (1) omission of primary antibodies, (2) substitution of primary antibodies for a preimmune fraction, and (3) substitution of primary antibodies for preimmune antibodies preincubated overnight at 4°C with immunogenic peptide (15 to 50 μg/ml).

For double-labeling experiments, unfixed frozen sections from rat heart, guinea pig left atrium, and rat lung were first incubated overnight at 4°C with rabbit anti-CX40 antibodies and then either with hen anti-CX43 antibodies (8 μg/ml) (heart sections) or with goat anti-factor VIII antibodies (dilution, 1:1000; Nordic Immunological Laboratories, Tilburg, the Netherlands) (rat heart and lung sections) for 2 to 6 hours at room temperature. The sections were rinsed with PBS and incubated for 1 hour at room temperature with PBS/BSA containing either both TRITC-labeled goat anti-rabbit IgGs and FITC-labeled goat anti-hen antibodies or both TRITC-labeled donkey anti-rabbit IgGs and FITC-labeled donkey anti-goat antibodies (Jackson Immunoresearch Laboratories). The sections were examined with a Zeiss III or a Nikon Diaphot light microscope equipped for epifluorescence with the appropriate filters to distinguish FITC emission from TRITC emission.

Double-labeled heart sections were also analyzed by confocal laser scanning (CLSM) microscopy using a Leica instrument based on a Leitz Diaplan microscope interfaced with an argon ion laser (model 2020, Spectra-Physics, Mountain View, Calif) adjusted to 488 nm and with an air-cooled helium/neon laser providing a 543-nm line. Horizontal optical sections were monitored first for fluorescein by using 488-nm excitation via a 510-nm dichroic mirror, an FITC band-pass 520- to 560-nm barrier, and a green-sensitive photomultiplier. The same sections were then scanned to detect the rhodamine signal by using 543-nm excitation via a 580-nm dichroic mirror, a long-pass barrier filter >580 nm, and a red-sensitive photomultiplier. The two 8-bit-encoded 512×512 pixel images from optical sections were combined and visualized with a computergenerated green and red pseudocolor scale adapted for fluorescein and rhodamine.

Immunoperoxidase Labeling

Hearts isolated from adult guinea pigs and rats were fixed overnight with a freshly made 4% paraformaldehyde solution. After dehydration with a graded series of ethanol followed by 1-butanol or chloroform, tissue was embedded in Paraplast Plus (Monoject, Kildare, Ireland). Serial sections 7 μm thick were collected on either poly-L-lysine or 3-aminopropyltriethoxysilane-coated slides and then stored at 4°C until use. After deparaffinization of the sections, immunologic staining was carried out using the unconjugated peroxidase/antiperoxidase method. Primary antibodies used were either rabbit antipeptide antibodies to CX40 or CX43 or mouse monoclonal antibodies against human desmin (Monosan Sambio,

Fig 2. Characterization by immunoblotting of rabbit antibodies raised to residues 335 to 356 of rat connexin40 (antibodies 335-356). Relative molecular weight (Mr) of proteins detected in the immunoreplicas was determined by comparison with molecular weight standards and cardiac connexin43 (lane a). Mobility of molecular mass standards is indicated in kilodaltons on the left of the figure (see Fig 1). The primary antibodies were revealed using 125I-labeled protein A. The arrow indicates the top of immunoreplicas. Lanes a and b show rat heart total extracts; lanes c, d, guinea pig heart total extracts; and lanes e and f, total extracts of atria isolated from guinea pig heart. The wells of electrophoresis gels corresponding to lanes b to f were loaded with 10 times more protein than the well corresponding to lane a. In lane a, replica was probed with rabbit anti-connexin43 antibodies. A major protein corresponding to connexin43 is detected in this immunoreplica. In lanes b, c, and d, replicates were probed with rabbit antibodies 335-356 (5 μg/ml). A major protein of 40 kD is detected in these immunoreplicas. In lanes d and f, replicates were probed with a preimmune fraction (lane d) (see "Materials and Methods" for the preparation of preimmune fractions) or with antibodies 335-356 preincubated with the immunogenic peptide (50 μg/ml) (lane f). In both cases, no protein is detected.
Uden, The Netherlands). Sections were examined with a Nikon Diaphot light microscope.

Sections are usually incubated for 20 to 30 minutes with a 3,3′-diaminobenzidine (DAB)/H₂O₂ solution to reveal peroxidase activity. However, because more desmin is expressed in the conduction system cells compared with the ventricular cells, any given incubation time produces a more intense staining in the conduction system than in the surrounding ventricular tissue. This differential staining was used to illuminate the topography of the conduction system within the ventricles. A 10-minute reaction time with DAB/H₂O₂ solution was found to be the best time to get this differential staining.

**Immunoelectron Microscopy**

Guinea pig hearts were fixed by perfusion with 4% paraformaldehyde in sodium cacodylate (0.1 mol/L, pH 7.4). Small tissue samples (≤0.5 mm³) from atria were immersed for 1.5 to 2 hours at 4°C in the same fixative. Samples from guinea pig right atrium were also fixed directly by immersion with periodate/lysine/paraformaldehyde. After quenching with 0.5 mol/L NH₄Cl or 50 mmol/L glycine for 1 hour, the samples were dehydrated and embedded in Lowicryl K4M at 4°C or at −35°C. Ultrathin sections were saturated with 5% normal goat serum and 1% BSA in PBS for 10 minutes and then incubated overnight with antibodies 335-356 (8 μg/mL) diluted in the saturation solution. After rinsing with PBS/BSA, sections were incubated for 4 hours with 5-nm gold-labeled goat anti-rabbit IgG (Amersham) diluted to 1:10 with PBS/BSA, extensively rinsed, and stained with uranyl acetate and lead. Sections were examined with a Philips 420 electron microscope. Control experiments were similar to those carried out for immunofluorescence experiments.

**Results**

**Characterization of Site-Directed Rabbit Anti-CX40 Antibodies**

**Immunoblotting**

Affinity-purified antibodies raised to residues 335 to 356 of rat CX40 (antibodies 335-356) were first used to probe replicas of total extracts of rat lung. [125I]–protein A reveals in these replicas a single protein with Mr of 40 000 that migrates just below cardiac Cx43 (Fig 1, lanes a, b, and d). This protein is not detected in replicas incubated either with preimmune fractions (Fig 1, lane c) or with antibodies 335-356 preincubated with the immunogenic peptide YHSDKRRLSKASSKARS-DLSSV (Fig 1, lane c). In contrast, it is detected in replicas probed with antibodies preincubated with the peptide LOLLREAKA AKEAGGTRY, unrelated to the immunogenic peptide (Fig 1, lane e). Identical results were obtained with guinea pig lung (not shown). In replicas of total extracts of rat and guinea pig heart, probed with antibodies 335-356, [125I]–protein A reveals a major band that migrates just below that of cardiac Cx43, as shown above (Fig 2, lanes a, b, and c). This band, which is heavily labeled in guinea pig atria replicas (Fig 2, lane e) and is usually rather weak in rat heart, is not detected in replicas probed either with preimmune fractions (Fig 2, lane d) or with antibodies preincubated with the immunogenic peptide (Fig 2, lane f).

**Immunofluorescence**

The results were identical whatever the method of fixation used. In lung sections treated with antibodies 335-356, the main immunoreactive sites are associated with blood vessel walls (Fig 3a and 3b). Double-labeling experiments with antibodies 335-356 and anti–factor VIII antibodies suggest that these immunoreactive sites are localized between factor VIII–positive cells (Fig 3c and 3d). Immunoreactive sites are also seen at high magnification between alveolar cells (not shown), but no attempt was made to identify these cells. In sections of guinea pig atrial tissue treated with antibodies 335-356, a strong labeling associated with myocytes was observed (Fig 4a). Distribution of this labeling is consistent with

![Fig 3](http://circres.ahajournals.org)
that of intercalated disks, which otherwise also contain Cx43 (Fig 4b). Immunoreactivity to antibodies 335-356 was also seen in blood vessel walls (Fig 4a). No labeling was seen in the lung and cardiac tissue section subjected to the various control experiments (see “Materials and Methods”), and in particular, labeling was abolished by preincubation of antibodies 335-356 with the immunogenic peptide (15 μg/mL).

**Immunoelectron Microscopy**

Examination of ultrathin sections from guinea pig atrial tissue treated for immunoelectron microscopy shows that colloidal gold particles are exclusively associated with gap junctions (Fig 5). Identical results were obtained whatever the method of fixation used. No labeling was seen in the sections subjected to the control experiments.

**Distribution of Myocyte-Associated Cx40 in Guinea Pig and Rat Heart**

To detect Cx40 and Cx43 in the same sections by double-labeling experiments, antibodies to residues 314 to 322 of rat Cx43 were raised in the hen. Characterization of these antibodies is presented in Figs 6 and 7. The hen antibodies detect a major protein of 45 to 43 kD in replicas of partially purified rat heart gap junctions (Fig 6, lane d). This protein has the same electrophoretic mobility as Cx43 (Fig 6, lane c) as judged from comparison with the labeling of twin replicas incubated with previously characterized rabbit anti-Cx43 antibodies. In frozen sections of rat ventricular tissue incubated with the hen antibodies and examined by fluorescence microscopy, the pattern of labeling is consistent with the distribution of intercalated disks and indistin-
guishable from that obtained with rabbit anti-Cx43 antibodies (**Fig** 7). In sections subjected to control experiments, no fluorescence was detected.

Distribution of Cx40 in rat and guinea pig heart was investigated in serial sections by immunofluorescence and immunoperoxidase labeling. The results obtained were similar whatever the method of fixation and investigation (immunofluorescence versus immunoperoxidase labeling) used. In guinea pig heart, only atrial myocytes were found to express Cx40 (**Fig** 4a). Cx40 was not detected either in the ventricular lateral walls (**Fig** 4a) or in any regions of the interventricular septum, although this connexin is expressed in blood vessel walls of these tissues. In contrast, Cx43 was detected both in atrial and ventricular myocytes (**Fig** 4b) and also in the His bundle and the bundle branches (**Fig** 8). Distribution of both Cx40 and Cx43 was investigated by **CLS** microscopy in the guinea pig atrial tissue (**Fig** 9). Analysis of the results shows that both connexins are colocalized in most of the immunoreactive sites. However, in some sites, only Cx43 or Cx40 is detected (**Fig** 9).

Cx40 was found to be expressed in rat heart ventricular tissue, but its expression is restricted to a few well-defined regions: (1) the upper apical region of the interventricular septum (**Fig** 10), topographically corresponding to the His bundle as shown by desmin staining, (2) two subendocardial regions extending along the septum and localized on both sides of the septum (these regions correspond to the localization of the His bundle branches (**Fig** 11), (3) the false tendons (chordae tendineae spuriae),**6,6** joining the interventricular septum to the lateral ventricular walls and containing Purkinje fibers**6,6** (**Fig** 11), and (4) some subendocardial regions of the lateral walls, mainly in their distal parts. In these latter regions, Cx40 is associated with some groups of cells, forming short cablelike arrangements. Cx40 was not detected in the myocytes of any other parts of the ventricular tissue, although this connexin is expressed in the wall of

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**Fig** 5. Characterization by immunoelectron microscopy of rabbit antibodies raised to residues 335 to 356 of rat connexin40 (antibodies 335-356) in paraformaldehyde-fixed guinea pig heart. Ultrathin sections from guinea pig heart atrial tissue were treated as described in "Materials and Methods." Note that gap junctions (arrows), easily identifiable as double-membrane profiles, are strongly and exclusively labeled with colloidal gold particles. Arrowhead indicates a desmosome; m, mitochondria; and my, myofilaments. Bar=400 nm.

**Fig** 6. Characterization by immunoblotting of hen antibodies raised to residues 314 to 322 of rat connexin43 (CX43). Lanes a and b show Coomassie blue-stained gels of standard proteins (molecular masses in kilodaltons are indicated on the left) and partially purified rat heart gap junctions, respectively. Note in lane b a major band of M, of 45 000 to 43 000 corresponding to CX43. Lanes c and d show immunoreplicas of partially purified gap junctions probed with rabbit anti-CX43 antibodies (**Fig** 10, 11, 12) (1 μg/mL) and hen antibodies (20 μg/mL) raised to residues 314 to 322 of rat CX43, respectively. These primary antibodies were revealed using biotinylated secondary antibodies and peroxidase-labeled streptavidin. CX43 detected in lanes c and d is indicated by arrowhead. No protein was detected in replicas of control experiments. Arrow indicates the top of separating gels and immunoreplicas.
certain blood vessels irrigating these regions (Fig 12). Subendocardial groups of cells localized in the lateral walls contain both Cx40 and Cx43. The distribution of both connexins was investigated in these cells by CLS microscopy. Examination of sections shows that, as in guinea pig atrial myocytes, most of the immunoreactive sites contain both Cx40 and Cx43, whereas some of them contain only one or the other of the connexins (not shown). In the rat atrial tissue, besides the labeling of vascular walls, immunoreactivity to rabbit anti-Cx40 antibodies was seen as a very few scattered fluorescent dots.

Distribution of Cx40 and Cx43 in guinea pig and rat heart is summarized in the Table.

**Discussion**

**Antibodies 335-356 Specifically Recognize a Protein With M, of 40 000 Localized in Gap Junctions: Cx40**

Cx40 mRNA has been shown to be present in various rat and mouse organs and tissues. This mRNA is expressed in large quantities in the lung and in smaller quantities in the heart. For this reason, characterization of antibodies 335-356 was carried out in the lung first. In total extracts of rat lung probed with antibodies 335-356, a major protein with $M_r$ of 40 000 was specifically detected (Fig 1). A protein with the same electrophoretic mobility was also detected, specifically, in rat and guinea pig hearts (Fig 2). Immunofluorescence experiments showed that the epitopes recognized by antibodies 335-356 are present only in certain regions of the guinea pig and rat heart (eg, the guinea pig atria [Fig 4] or subendocardial regions of rat heart interventricular septum [Fig 11]). In these regions, the pattern of labeling is similar to that of intercalated disks, where Cx43 junctional channels have been also demonstrated to be localized (Figs 4 and 9 and Reference 32). Finally, immunoelectron microscopic analysis of guinea pig atrial tissue showed that antibodies 335-356 recognized myocyte-associated gap junctions (Fig 5). These data demonstrate that the site-directed antibodies we purified are specific for Cx40. For the first time, the $M_r$ of endogenous Cx40 is reported. This $M_r$ is similar to that deduced from immunoprecipitation experiments carried out either with *Xenopus* oocytes injected with rat Cx40 cRNA or with cells transfected with cDNA coding for Cx40.

Besides certain myocytes, Cx40 was found to be also associated to blood vessel walls. The regularly spaced labeling seen in cross-sectioned vessels (Figs 4 and 12) and its close association with the borders of factor VIII-positive cells (Fig 3) suggest that Cx40 might be expressed in the endothelial cells lining the lumen of vessels. Endothelial cells are connected in situ through functional gap junctions, and immunofluorescence...
FIG 9. Distribution of connexin40 (CX40) and connexin43 (CX43) in guinea pig atrial myocytes (unfixed organ). Double-labeling experiment was analyzed by confocal laser scanning microscopy. Sections were incubated successively with rabbit anti-CX40 antibodies (antibodies 335-356), then with hen anti-CX43 antibodies. These primary antibodies were revealed with tetraethyl rhodamine isothiocyanate-labeled goat anti-rabbit antibodies and fluorescein isothiocyanate-labeled goat anti-hen antibodies, respectively. Distribution of CX43 and CX40 is illustrated by micrographs a and b, respectively. Micrograph c is a merged picture of the fluorescence signals shown in a and b. Examination of the micrographs shows that CX43 and CX40 are colocalized in most of the immunoreactive sites. However, in a few sites, only one or the other of the connexins seems to be expressed. The two arrows in a and c indicate immunoreactive sites where CX43 only is detected. The two arrowheads in b and c indicate sites where CX40 only is detected. Bar=20 μm.
experiments have already led authors\textsuperscript{39} to conclude that endothelial cells of rat heart blood vessels express Cx40. The presence of Cx40 mRNA has been also demonstrated in primary cultures of bovine aortic endothelial cells.\textsuperscript{17} Thus, results from various sources converge to suggest that Cx40 might be expressed in vascular endothelial cells, at least in the heart and lung. However, conclusive confirmation of the localization of Cx40 in endothelial cell gap junctions by immunoelectron microscopy remains to be provided.

**Cx40-Expressing Myocytes Are Localized in Guinea Pig Heart Atrial Tissue and Rat Heart Conduction System**

Expression of Cx40 in myocytes is controversial: Kanter and colleagues\textsuperscript{15,40,41} have demonstrated that Cx40 is associated with myocyte gap junctions of dog heart ventricles, whereas Bruzzone et al\textsuperscript{39} have reported that rat heart ventricular myocytes do not express Cx40. This is only an apparent contradiction. We demonstrate in the present study that guinea pig heart atrial myocytes express Cx40 (Fig 4) but that, in contrast, Cx40 could not be detected in ventricular myocytes, which, like atrial myocytes, do express Cx43 (Figs 4 and 8). We also show that in guinea pig heart the His bundle and the bundle branches abundantly express Cx43 (Fig 8), as in human and bovine heart,\textsuperscript{35} but do not express Cx40 (Table). A quite different situation is apparent in rat heart (Table). In this species, myocytes expressing Cx40 are localized only in a few regions of ventricular tissue. The topography of these well-defined regions (Figs 10 and 11) matches the conduction system of the rat heart (including the Purkinje fibers), as described in detail by light and electronic microscopy.\textsuperscript{50,54,55} In particular, Purkinje fibers of type I and type II have been described in false tendons and in subendocardial regions of rat ventricular walls.\textsuperscript{50} Expression of Cx40 in these cells is in agreement with the recent results reported for dog heart.\textsuperscript{40,41} In contrast Cx40 was not detected in rat contractile ventricular myocytes, whereas it was detected in the dog.\textsuperscript{40,41} Absence of Cx40 mRNA in these myocytes, as shown by situ hybridization (M. van Kempen, D. Paul, D. Gros, T. Vermeulen, A. Moorman, W. Lamers, unpublished data), strengthens our results. As previously reported,\textsuperscript{32} Cx43 was not detected either in the His bundle or in the proximal regions of the bundle branches of rat heart, and consequently Cx40 is the first connexin whose expression has been demonstrated in this part of the rat conduction system.

Cx42 is the bird analogue of mammalian Cx40, and there is 70% amino acid homology between these two connexins.\textsuperscript{56} Recent studies\textsuperscript{57} have suggested that the distribution of Cx42 in avian heart is similar to that of Cx40 in rat heart. Despite this similarity, one should not forget that Cx40 is differently distributed in rat and guinea pig hearts. Thus, the distribution of this connexin in the myocardium of one species is not necessarily a basis for drawing conclusions about its distribution in the myocardium of another species. This also applies to the expression of Cx43 in the proximal regions of the mammalian heart conduction system (His bundle and its branches), as shown in the present study and others.\textsuperscript{32,35}
Colocalization of Cx40 and Cx43 in Immunoreactive Sites

Hen antibodies to residues 314 to 322 of rat Cx43 were affinity-purified and characterized by immunoblotting and immunofluorescence. These antibodies specifically recognize a single protein of the same electrophoretic mobility as Cx43 (Fig 6) localized in intercalated disks (Figs 7, 9, and 12). These results provide reasonable evidence for the specificity of these antibodies for Cx43.

Hen anti-Cx43 antibodies and rabbit anti-Cx40 antibodies were used to detect simultaneously Cx43 and Cx40 (double-labeling experiments) in guinea pig and rat heart sections. Analyses by CLS microscopy were focused on guinea pig atrial tissue and subendocardial regions of rat heart lateral ventricular walls. These latter regions contain cell groups expressing both Cx40 and Cx43 that likely correspond to subendocardial Purkinje fibers described in the rat.50 Only results obtained with guinea pig atrial tissue are illustrated (Fig 9), but in both cases the results are similar: Cx40 and Cx43 are colocalized in most of the immunoreactive sites, a finding in agreement with the colocalization of Cx40 and Cx43 in gap junctions of dog Purkinje fibers.41 Some sites remain monoreactive (Fig 9), suggesting that they only contain Cx40 or Cx43. However, the major connexin type could be "contaminated" by a very few molecules of the other type, undetectable by CLS microscopy in spite of the sensitivity of this technique. Colocalization of two connexins as described above is not unknown (both liver connexitin32 and connexin26 were identified in the same gap junctions50), but it raises the question of their interactions. There is no junctional communication between paired Xenopus oocytes when one of them expresses Cx40 and the other expresses Cx43.39 This inability of Cx43 to interact with Cx40, if it is confirmed in other biological systems, reduces the number of possible arrangements between the two connexins.

CX40 and the Conduction of Action Potentials

The presence of both Cx43 and Cx40 in certain cardiac tissues (which does not preclude the expression of other types of connexins in these same tissues) should offer additional possibilities for the regulation of conduction velocity. The unitary conductance of Cx43 junctional channels of myocytes has been estimated to be 40 to 60 picoSiemens.50,60 A recent investigation carried out with HeLa cells transfected with Cx40 cDNA reported the unitary conductance of Cx40 channels to be \( \approx \) 150 picoSiemens.61 If it is assumed that the kinetics of both Cx43 and Cx40 channels are approxi-
account other factors, such as cell geometry, longitudinal resistance, and the number of channels per junction. Clearly, further investigations are needed to analyze the conduction process in molecular terms before a firm statement can be made about the physiological significance of the pattern of connexin distribution in cardiac gap junctions.

Note added in proof: While this article was in proof, two articles appeared dealing with the distribution of Cx40 in rat heart. Gourdie et al. used antibodies raised to residues 360 to 379 of chick Cx42 to demonstrate that Cx40 is present in all main components of the rat conduction system, whereas it is not detected in the working ventricular tissue. In contrast, Bastide et al. using antibodies raised to residues 337 to 358 of mouse Cx40 (residues that are conserved in rat Cx40), reported the coexpression of Cx40 and Cx43 in working ventricular myocytes, although they noted a preferential expression of Cx40 in the ventricular conductive myocardium. The discrepancy between these results and ours is unknown.

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Restricted distribution of connexin40, a gap junctional protein, in mammalian heart.

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