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Tissue-Specific Distribution of Differentially Phosphorylated Forms of Cx43

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Variants of the Cx43 gap junction protein have been detected on Western immunoblots by using an antipeptide antibody to the N-terminus of the protein. In heart ventricle, atrium, brain, retina, and uterus, different yet characteristic ratios of a broad 43-kDa band and a 39- to 40-kDa doublet were observed. These proteins (in lens epithelium, testes, and spleen) or their messages (in stomach, duodenum, kidney, and lung) were also detected in several nonexcitable systems but at consistently lower levels than found in electrically excitable tissues. The reproducible heterogeneity in electrophoretic mobility of Cx43 seen in different tissues does not appear to be due to proteolysis, since both the 43-kDa band and the 39- to 40-kDa doublet were recognized by an N-terminal as well as a C-terminal antibody. Furthermore, Northern (RNA) blots from different tissues show that both polypeptide profiles arise from indistinguishable transcripts. The conversion by alkaline phosphatase treatment of a predominantly 43-kDa profile (in heart) to a 39- to 40-kDa profile (characteristic of brain and protein translated in vitro from the RNA) suggests that the observed electrophoretic heterogeneity arises from tissue-wide differences in the phosphorylation state of Cx43.

Gap junctions are intercellular protein channels which provide a pathway for the exchange of ions and small molecules. This exchange of material allows electrical and metabolic coupling of cells (1, 17, 27). Rapid transmission of excitatory impulses due to electrical coupling is apparently important for cardiac myocytes to function as a syncytium (8) and for the synchronized contraction of smooth muscle cells during parturition (16). Metabolic coupling via gap junctions involving exchange of metabolites and second messengers has been implicated in embryonic development (5, 14, 25) and control of cell growth (26).

Each functional gap junction channel is formed by end-to-end pairing of hemichannels in adjoining cells. Each hemichannel, in turn, is composed of six apparently identical subunits (28). These protein subunits are members of a family, the connexins, with different but related connexins having been isolated from liver, heart, and lens (23; see reference 39 for a review). With the advent of cDNA clones, this family, called the connexins (or Cx, as used here), now includes Cx32 (24, 37), Cx43 (3), and Cx26 (46) from mammals, Cx38 (12), Cx30 (18), and Cx43 from *Xenopus laevis* (19), and Cx43 from chicken (34). All of these connexins show 40 to 60% identity in their amino acid sequences and contain conserved motifs, including two cysteine-rich extracellular domains. Different tissues and cell types have their own characteristic pattern of connexins, which can include expression of more than one connexin type in the same gap junctional plaque (35).

By using specific antibodies and cDNAs, the expression pattern of Cx32 (10, 22, 37), Cx26 (46), and Cx43 (2, 3) in several tissues has been determined. Through the use of a highly specific antipeptide antibody to the N-terminus of Cx43 in conjunction with the Cx43 cDNA, we have extended this analysis, indicating a prevalence of Cx43 in excitable tissues, although not exclusively in excitable cells. Most significantly, microheterogeneity of Cx43 was detected in different tissues. Consistent with observations made in vitro (7, 33), several lines of evidence presented here demonstrate that this microheterogeneity arises from tissue-specific differences in the posttranslational processing (specifically, phosphorylation) of the Cx43 protein, defining yet another level of diversity in the junctions of different tissues.

MATERIALS AND METHODS

Production of antibodies. A peptide corresponding to amino acids 1 to 17 of Cx43 (36), with an additional glycine and cysteine added at the carboxy terminus for cross-linking purposes, was synthesized in two batches—initially on an Applied Biosystems peptide synthesizer and subsequently on a Bioresearch peptide synthesizer. A 24-mg amount of reduced peptide was conjugated to 6 mg of keyhole limpet hemocyanin (KLH) through the bifunctional cross-linker succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce). Then, 1.25 mg of conjugated KLH in Freund's complete adjuvant was injected into a New Zealand White rabbit, approximately two-thirds intradermally and one-third intramuscularly. Three subsequent boosts at 10-week intervals were given both intradermally (near previous injection sites) and intramuscularly with 0.5 mg of the peptide-KLH conjugate. Once a titer of $>10^4$ against the peptide was detected by enzyme-linked immunosorbent assay (ELISA) with protein A conjugated to horseradish peroxidase and by a colorimetric detection system with *O*-phenyldiamine and hydrogen peroxide, the serum was collected and stored as 1-ml aliquots at −70°C for further use.

The antibody directed to the C-terminal domain of Cx43 (residues 360 to 382) was prepared and characterized in a similar manner (24a).

Affinity purification. For purposes of affinity purification, the Cx43 (residues 1 to 20) peptide was conjugated through its amino groups, by Schiff base chemistry, to a MAC-25 membrane cartridge (MEMTEK Inc., Billerica, Mass.). Then, 0.5 ml of serum was bound to the filter, which was washed several times with 10 mM sodium phosphate (pH 7.4)–150 mM NaCl (PBS) before free aldehyde groups were blocked with 0.1% sodium borohydride in PBS. Antibody

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bound in PBS was eluted in 0.1 M glycine, pH 2.3. Four 400-μl fractions were collected and immediately passed over a 1.0-ml bed volume G-50 Sephadex spun column equilibrated with PBS. Typically we were able to recover 60% of the activity against the peptide (determined by ELISA), with most of this being collected in the first two 400-μl fractions. The affinity-purified fractions showed no reactivity against KLH or bovine serum albumin (BSA). The A_{280} of the eluted fractions indicated that the pooled, affinity-purified antibody fractions (0.4 mg/ml in immunoglobulin G [IgG]) accounted for 20% of the IgG in the original serum, as estimated from the A_{280} of the material bound to and subsequently eluted by pH 2.2 glycine from a protein A HPLC (high-pressure liquid chromatography) column (Pierce, Rockford, Ill.).

Western immunoblots. Homogenates of various tissues were prepared in 2 mM $NaHCO_3$ (pH 8.2) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 100 × *g* for 20 min. In selected cases, 1 mM vanadate or 10 mM phosphate was included in the homogenization buffer to prevent any endogenous phosphatase activity. The protein content of the resuspended pellets was determined by using the Bio-Rad protein assay kit (Coomassie based) versus a BSA standard. Then, 20 μg of protein was loaded on each lane of a 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel for electrophoresis. Electroblotting onto polyvinyl difluoride membranes (Immobilon; Millipore, Bedford, Mass.) was done by the method of Towbin et al. (41), except that the gel was not presoaked and the transfer buffer contained 10% methanol. Blots were blocked in 3% BSA in TBS (10 mM Tris hydrochloride [Tris-HCl, pH 7.4], 150 mM NaCl) and subsequently incubated with a 1:250 dilution of the affinity-purified antibody in TBS containing 0.1% BSA, 0.05% Tween 20, and, in most cases, 0.1% SDS, which we found both increased signal and reduced background. The detection system used was protein A-alkaline phosphatase conjugate in conjunction with the Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (NBT-BCIP) enhanced colorimetric reagent (Bethesda Research Laboratories, Bethesda, Md.).

Immunofluorescence. Small pieces of tissue from rat heart were frozen in liquid propane and stored in liquid nitrogen. Sections (4 μm thick) were cut on a cryostat, washed with PBS, and fixed in 1% paraformaldehyde. Unreacted aldehyde was removed by washing with PBS and subsequent incubation with 0.1 M glycine, pH 7.2. Sections were sequentially incubated with a 1:25 dilution of affinity-purified antibody to amino acids 1 to 20 of Cx43 and a 1:50 dilution of $F(ab')_2$ fragment of goat anti-rabbit IgG (Organon Biotech, Durham, N.C.). Sections were mounted in PBS-glycerol (1:9) and examined under a Zeiss Axiovert microscope.

Northern (RNA) blots. Total RNA from various rat organs was prepared by homogenization and CsCl gradient banding (15) or pelleting (6). Then, 20 μg of total RNA (as determined by the A_{280}) was glyoxylated and electrophoresed in a 1% agarose gel by the method of Maniatis et al. (29). RNA was capillary blotted onto a GeneScreen Plus membrane (DuPont Co., Wilmington, Del.) in 10× SSC (0.9 M NaCl, 0.09 M sodium citrate). The blots were deglyoxylated in 50 mM NaOH and prehybridized for 6 h at 42°C in 6× SSC containing 50% formamide, 1% SDS, 10% dextran sulfate, and 100 μg of heat-denatured salmon sperm DNA per ml. A 1.4-kb cDNA clone provided by E. Beyer was nick translated (38) and added to the prehybridization buffer at a concentration of 5×10^5 cpm/ml for hybridization at 42°C for 21 h. Blots were then washed twice with 2× SSC for 5 min at room temperature, twice with 2× SSC containing 1% SDS for 30 min at 60°C, and twice with 0.1× SSC for 30 min at room temperature before exposure to Kodak XAR film.

Immunoprecipitation and phosphatase treatment. Brain and ventricular homogenates (250 and 500 μg of total protein, respectively) were solubilized in 1% SDS in 10 mM Tris-HCl (pH 7.4) containing 2 mM PMSF. After dilution to a final concentration of 0.1% SDS, samples were incubated for 16 h at room temperature with the N-terminal antibody at a 1:25 dilution in TBS with 0.05% Tween 20. The Cx43-antibody complex was precipitated with protein A-Sepharose (Sigma, St. Louis, Mo.). After three washes with TBS containing 0.05% Tween 20, bound material was eluted in 10 μl of 1% SDS in 10 mM Tris-HCl (pH 7.4) and incubated with 1 to 10 U of calf intestinal alkaline phosphatase (Promega Biotec, Madison, Wis.) in a total reaction volume of 30 μl at 37°C for 4 h. For some samples from the ventricle, alkaline phosphatase was incubated with 1 mM vanadate or 10 mM phosphate for 30 min at 37°C before addition to immunoprecipitated gap junction protein. Control samples were incubated under identical conditions without alkaline phosphatase. Samples (12 μl) of both treated and control preparations were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described above. While the immunoprecipitant was demonstrated to be homogeneous with respect to Cx43 by Coomassie staining, Western blotting was used to assay material because of its higher sensitivity.

In vitro translation. About 10 μg of Cx43 cDNA in pGEM4Z was linearized with *Bam*HI and transcribed in a 100-μl reaction mixture (using a kit from Promega Biotec) in the presence of 5 U of cap analog [$m^7G(5')ppp(5')G$] per ml. RNA transcripts were purified by treatment with RQ1 DNase, phenol-chloroform and chloroform extraction, and ethanol precipitation according to the instructions provided by Promega Biotec. RNA from each reaction was dissolved in diethylpyrocarbonate-treated water containing 1 U of RNasin (Promega Biotec) per μl. Translation in 6.25 μl of rabbit reticulocyte lysate (nuclease treated, methionine depleted; Promega Biotec) was carried out at 30°C for 30 min with 1 μg of RNA and 1 μCi of [^{35}S]methionine per μl. The translation products were analyzed on a 12.5% SDS–polyacrylamide gel and processed for autoradiography.

RESULTS

Characterization of antipeptide sera to Cx43. (i) Specificity for junctional proteins. The specificity of the amino-terminal antipeptide serum for Cx43 was demonstrated in Western blots of whole ventricular homogenates. With crude serum, a prominent band at 43 kDa, a doublet at 39 to 40 kDa, and some faint bands were detected (Fig. 1a). Affinity purification of the antibody against the peptide antigen served to reduce most but not all of these bands (Fig. 1b). Affinity-purified preimmune serum showed no significant bands (Fig. 1c). The possibility that the bands of 43 to 39 kDa result from nonspecific or weak binding to more abundant cardiac proteins is minimal, since none of the bands detected on Western blots represent a major component of ventricular homogenates (Fig. 1d). The junctional nature of the bands identified was confirmed in Western blots of purified gap junction fractions prepared from rat ventricle by the method of Manjunath and Page (32) (Fig. 1e). In these fractions, demonstrated to be highly enriched for gap junctions morphologically (20, 30), the single major 43-kDa band seen with Coomassie stain (Fig. 1f) strongly binds the antibody. In many preparations, including whole homogenates, a charac-

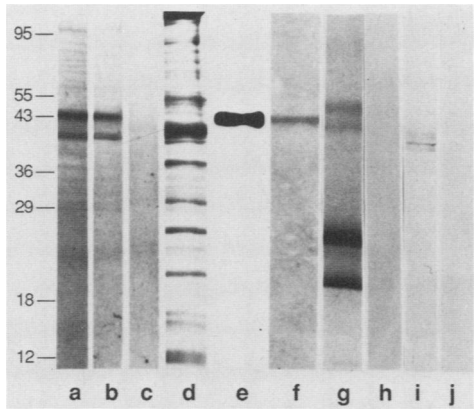

FIG. 1. Anti-Cx43(1–20) antibody is specific for gap junction proteins on Western blots. Western blot of heart ventricular homogenate with (a) crude anti-Cx43 serum, (b) affinity-purified anti-Cx43 serum, or (c) affinity-purified preimmune serum. Prominent bands of M_r 43,000 and a doublet at M_r 39,000 to 40,000 are evident in lanes a and b. The lack of correspondence between these bands and any major components in the homogenate, as visualized by Coomassie staining of unblotted material from the same gel (d), suggests that the immunoreactivity is specific. The identity of the bands was confirmed in correlated Western blots (e) and Coomassie blue-stained gels (f) of purified gap junctions from rat heart. A single band of M_r 43,000 was detected, although in many preparations traces of M_r 39,000 to 40,000 material were also visible. The Cx43 antibody does not recognize liver gap junction proteins in purified fractions from liver (h), which in Coomassie-stained gels were seen to contain Cx32 (28 kDa) and Cx26 (21 kDa) bands as well as their respective dimers at 56 and 42 kDa (g). As seen on Western blots of lens homogenates (lanes i and j), it also did not cross-react with another closely related gap junction protein of 70 kDa present in lens fibers (decapsulated lens, lane j), but did detect the Cx43 protein present in lens epithelium (intact lens, lane i)—compare Kistler et al. (23) and Beyer et al. (2, 3). Bands between 39 and 43 kDa in lanes a, b, and i represent differentially phosphorylated forms of Cx43, demonstrated in Fig. 5. Mobilities of standard marker proteins (prestained standards from Diversified Biotech) are indicated in kilodaltons in this and subsequent figures.

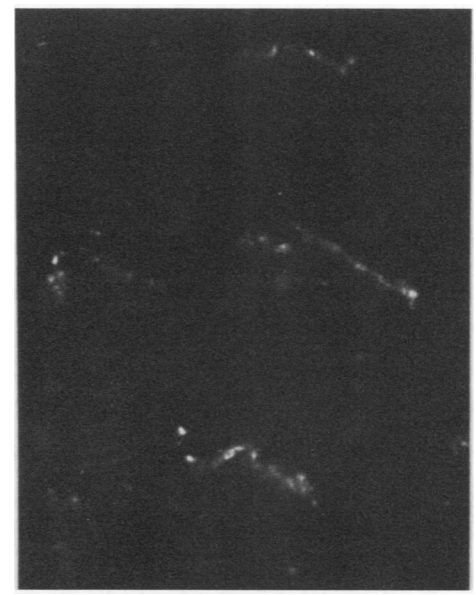

FIG. 2. Anti-Cx43(1–20) antibody also recognizes the native protein. Frozen rat heart sections incubated successively with affinity-purified anti-Cx43 and the $F(ab')_2$ fragment of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG show specific punctate labeling at the intercalated disks between myocardial cells.

teristic pattern of apparent degradation products of 29, 31, and occasionally 34 kDa was also seen (Fig. 4A; compare reference 30). Together, these results complement the earlier demonstration by Yancey et al. (45) of the specificity of this antiserum for morphologically identifiable gap junction structures.

(ii) Specificity for the Cx43 connexin. The specificity of the anti-Cx43(1–20) antibody with respect to other members of the gap junction family has not previously been analyzed extensively. This was demonstrated here by comparisons with Western blots of homogenates from rat eye lens and gap junction fractions from mouse liver (prepared as specified in reference 20). SDS-PAGE and Coomassie blue staining of mouse liver gap junction fractions showed bands for Cx32 (28 kDa) and Cx26 (21 kDa) and their respective dimers at 56 and 42 kDa (Fig. 1g). No binding of the Cx43-specific antibody could be demonstrated (Fig. 1h) even when up to 1 μg of junctional protein was loaded. Another member of the gap junctional family, even more closely related to Cx43 than the ones in liver, has recently been partially characterized in lens tissue (23). This M_r-70,000 protein is localized to lens fibers, as opposed to Cx43, which appears to be specific to the lens epithelium (2, 3). Despite the similarity in the N-termini of Cx43 and the 70-kDa lens fiber protein, our N-terminal antibody showed strict specificity and, like the C-terminal antibody of Beyer et al. (2), recognized only the epithelial junctional proteins. As shown in Fig. 1i and 1j, no bands corresponding to the lens 70-kDa protein or its degradation products were detected in whole-lens homogenates. Bands of 43 to 39 kDa were seen (Fig. 1i), but these were eliminated when the lens epithelium was removed by decapsulation (Fig. 1j).

(iii) Labeling of junctional structures. A similar degree of specificity of the anti-Cx43(1–20) antibody is also evident when intact junctional structures are labeled. In addition to recognition of the native form of the Cx43 protein by the antibody in ELISAs of purified preparations of morphologically intact gap junctions from rat ventricle (data not shown), punctate immunofluorescent labeling at intercalated disks was observed in heart sections (Fig. 2). This antibody has also been shown to specifically label, at the electron microscopic level, double membrane profiles (heptalaminar structures) in membrane fractions from heart ventricle (45). As might be expected, no labeling of mouse liver sections was found.

(iv) Antibody to the C-terminus of Cx43. An antibody to the C-terminus of Cx43 (residues 360 to 382 [24a]) was also demonstrated to bind specifically to the 43-kDa protein in heart homogenates (Fig. 3B, lane a). The specificity of this antibody for the native protein was demonstrated by immunofluorescent labeling of heart sections and myocyte cultures, which yielded patterns indistinguishable from that of the N-terminal antibody (data not shown).

Distribution of Cx43 and its variants in different tissues. (i) Protein. Given the specificity of our antibody for Cx43, we screened various tissue homogenates to determine the distribution of this protein. Of the tissues screened (Fig. 3A), the 43-kDa and/or related bands were readily detected in ventricle (Fig. 3A, lane a), brain (b), atrium (c), term uterus (d), retina (e), and intact lens (f). Minimal levels of these

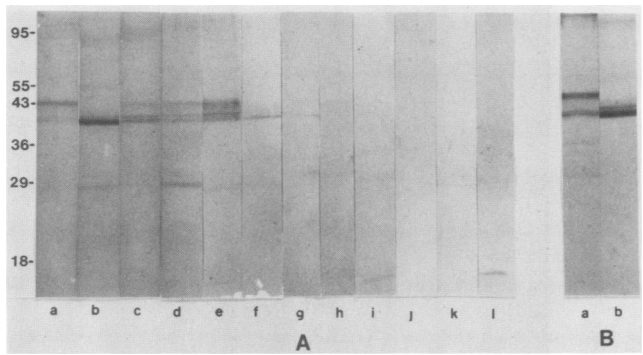

FIG. 3. Distribution of Cx43 and a related protein in homogenates of different tissues. (A) 20 μg of total protein from heart ventricle (a), brain (b), heart atrium (c), uterus (d), retina (e), lens (f), testes (g), spleen (h), kidney (i), stomach (j), liver (k), and lung (l) were Western blotted and probed with the anti-Cx43(1–20) antibody. In tissues where Cx43 was detected (a through g), different ratios of the 43-kDa and the 39- to 40-kDa doublet characteristic of each tissue are seen. Some tissue preparations showed evidence of degradation products of 29 and 31 kDa (e.g., lanes b and d). Minor bands at 16 kDa in lanes e, i, and l were also seen in equivalent lanes stained with preimmune sera. (B) Both the 43-kDa band and the 39- to 40-kDa doublet were also detected by an antibody to the C-terminus (amino acids 360 to 382) of Cx43, as seen on Western blots of ventricle (a) and brain (b) homogenates.

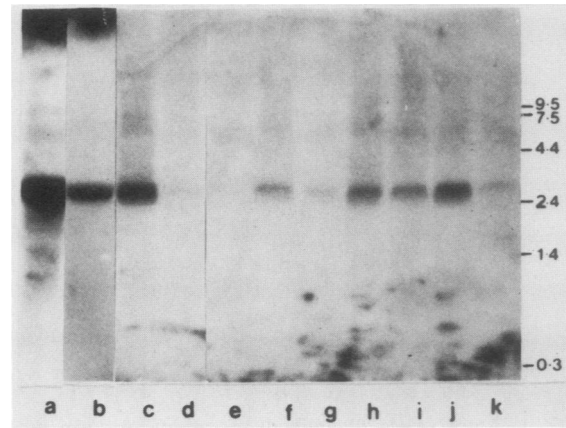

FIG. 4. Cx43 transcripts are indistinguishable between tissues. Northern blots with 20 μg of total RNA were hybridized under high stringency (as described in the text) to a 1.4-kb cDNA probe (containing the entire coding region plus 5′ untranslated material of Cx43 [3]). Lanes a and b are from one blot, and lanes c through k are from another. Lanes contained RNA from heart (a), brain (b), heart (c), duodenum (d), liver (e), lung (f), kidney (g), spleen (h), stomach (i), testes (j), and skeletal muscle (k). A 3.3-kb message was found, albeit in different abundances, in all tissues except liver, in which even longer exposures failed to detect a signal.

bands were also seen in testes (g), spleen (h), and kidney (i). No signal above preimmune levels was detected in stomach (j), liver (k), lungs (l), duodenum, pancreas, or trachea (data not shown). A 16-kDa band evident in some tissues (e.g., lanes e, i, and l) appears to represent nonspecific activity of the serum, as it was also recognized by preimmune sera. A surprising but reproducible result from these screens was the marked heterogeneity in mobility of the bands detected by our antibody. A somewhat diffuse band of 43 kDa and a doublet at 39 to 40 kDa were both detected, with different ratios of the two being characteristic of each tissue. In heart tissue, ventricular homogenates contained a prominent 43-kDa band and a minor 39- to 40-kDa doublet (Fig. 3A, lane a), while the converse was true for the atrium (Fig. 3A, lane c). In term uterus (Fig. 3A, lane d) and retina (Fig. 3A, lane e), the 43-kDa band and the 39- to 40-kDa doublet were present in comparable amounts. In brain (Fig. 3A, lane b), the 43-kDa band was absent. A single prominent band at 39 kDa was found, with only minor amounts of the upper 40-kDa part of the doublet. This is similar to the pattern seen in lens epithelium (Fig. 3A, lane f). Surprisingly, from the intensity of the bands in Western blots, there appears to be more of the Cx43 protein in brain per gram of total protein than in any other tissue, including the heart.

(ii) mRNA. To determine whether the different polypeptide profiles shown in Fig. 3A were the products of identical or similar transcripts, total RNA from most of the tissues screened by Western blots was analyzed by Northern blots. Each tissue was found to express a 3.3-kb message indistinguishable from that of Cx43. The disparity in the size of the Cx43 message reported here and by Beyer et al. (2) probably arises from our use of specific RNA markers (Bethesda Research Labratories). As shown in Fig. 4, the highest levels of the 3.3-kb message were observed in heart (lanes a and c) and brain (lane b). While term uterus and retina, in which we had detected significant Cx43 on Western blots, were not tested, spleen (Fig. 4, lane h) and testes (j), which showed similar or lower levels of Cx43, showed readily detectable messages. Weaker signals were seen in lung (f) and stomach (i). Minimal but detectable amounts were found in duodenum (d), kidney (g), and skeletal muscle (k), but none was found in liver (e). In all cases the message was identical in size and was recognized by the Cx43 probe under conditions of high stringency, as described in the Materials and Methods section. The detection of an RNA transcript for Cx43 in tissues where no protein product had been detected probably reflects the differential sensitivity of Northern and Western blots, although differential translational efficiency cannot be ruled out.

Nature of Cx43 variants. The above analyses demonstrated that all tissues showing either the 43- or 39- to 40-kDa profile or a mixture of the two expressed indistinguishable messages, suggesting that the proteins derive from a common gene and probably a common transcript. Although proteolytic degradation is evident in several of the tissue samples (bands of 29 and 31 kDa in several lanes in Fig. 3A), proteolysis does not seem to explain the variation in the range of 39 to 43 kDa. This is indicated by the fact that minimizing exogenous proteolysis during isolation (e.g., by inclusion of protease inhibitors) reduces these lower-molecular-weight products yet does not affect the pattern of bands at 43 and 39 kDa. More conclusive evidence that proteolysis is not the cause of the electrophoretic heterogeneity of Cx43 is provided by the recognition of both the 43- and 39- to 40-kDa bands not only by the antibody to the N-terminal 20 amino acids of Cx43 (Fig. 3A), but also by an antibody raised against the C-terminal 22 amino acids of the Cx43 protein (Fig. 3B).

However, consistent with all our results and with parallel observations in culture (7, 34) is the possibility that the variation in electrophoretic mobility reflects different phosphorylation states of the protein. To test this, immunoprecipitated material from heart ventricle and brain was treated with alkaline phosphatase. This caused the appearance of additional bands in the Western blots corresponding to

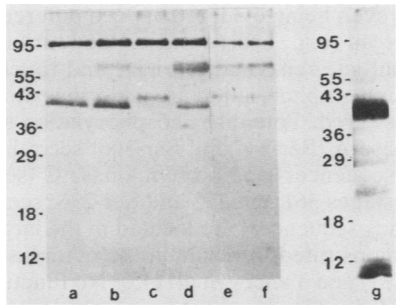

FIG. 5. Dephosphorylation of Cx43 from heart ventricle renders it indistinguishable from that in brain or the in vitro-translated product. Homogenates from heart ventricle (c through f) and brain (a and b) were immunoprecipitated with anti-Cx43(1–20) and subsequently incubated in the presence (b, d, e, f) or absence (a and c) of alkaline phosphatase prior to SDS-PAGE and Western blotting. In lanes e and f, alkaline phosphatase was pretreated with 1 mM vanadate (e) or 10 mM phosphate (f). Blots were probed with anti-Cx43(360–382) and protein A-alkaline phosphatase prior to colorimetric detection. Alkaline phosphatase treatment of heart ventricular immunoprecipitates reduces the prominent 43-kDa band seen in control samples (c) to a 39-kDa band (d) indistinguishable from that seen in both control (a) and phosphatase-treated (b) brain samples. In vitro translation of Cx43 RNA in the rabbit reticutolysate system also yields a major band of 39 kDa (g). Preincubation with inhibitors of alkaline phosphatase prevented this change in mobility of Cx43 from heart ventricle (e and f). Additional bands seen at 95 and 75 kDa were demonstrated to arise from residual intact IgG from the immunoprecipitation (which binds the protein A-alkaline phosphatase conjugate) and alkaline phosphatase (which apparently renatures sufficiently on the blot to give a positive colorimetric reaction), respectively.

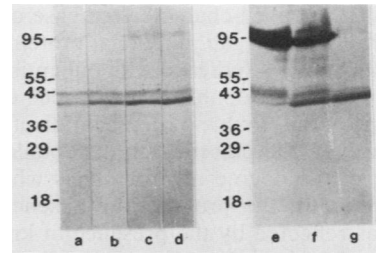

FIG. 6. Cx43 heterogeneity is not due to release of endogenous phosphatases. Whole heart and telencephalon from rat were rapidly isolated and homogenized in 2 mM sodium bicarbonate containing 1 mM PMSF. Homogenates were immediately mixed in different ratios and centrifuged in a microfuge. The pellet was then subjected to SDS-PAGE and Western blotting. Heart and brain homogenates mixed at a ratio of 1:0 (lane a), 1:0.25 (lane b), 1:1 (lane c), and 0.5:1 (lane d) show that the intensity of the 43-kDa band of the heart homogenate (a) is decreased with the amount of homogenate (d) but is not affected by addition of increasing amounts of brain homogenate (b and c). In a separate experiment, Cx43 was immunoprecipitated from heart ventricle (e) and then added to brain homogenate (f). Brain homogenate alone is shown in lane g. Each sample was then processed for Western blotting. Lane f reflects a simple addition of the elements in lanes e and g with no relative change in the expected levels of the 43- and 39-kDa bands. A slight reduction in all bands (including IgG at 95 kDa) is evident because of apparent overloading of protein on the gel.

residual IgG (95 and 74 kDa) and phosphatase (75 kDa). However, these did not obscure the effects on Cx43. In the case of heart ventricle, this treatment caused disappearance of the 43-kDa band and a concomitant increase in the 39-kDa band (Fig. 5, lane d). No such change occurred when equivalent incubations were carried out in the absence of alkaline phosphatase (Fig. 5, lane c) or jointly with the enzyme and its specific inhibitors, i.e., 1 mM vanadate (Fig. 5, lane e) or 10 mM phosphate (Fig. 5, lane f). Similar alkaline phosphatase treatments of immunoprecipitates from brain caused no shift in the polypeptide profile (Fig. 5, lanes a and b). Consistent with these results is the observation that unmodified protein translated directly from an in vitro-synthesized Cx43 transcript in rabbit reticulocyte lysate had the same electrophoretic mobility (Fig. 5, lane g) as the profile in brain homogenates or in heart homogenates following phosphatase treatment.

Finally, we also considered the possibility that the electrophoretic pattern of Cx43 in different tissues represents an artifact of isolation or preparation (e.g., activation of a phosphatase or unequal transfer of blots). Western blots of freshly prepared heart and brain homogenates, mixed in various ratios, were carried out to determine whether the brain homogenate (in which the 43-kDa band was not observed) contained factors which might affect the banding pattern in heart homogenate. As shown in Fig. 6, the intensity of the 43-kDa band in heart homogenate (lane a) did not decrease when mixed with increasing amounts of brain homogenate (lanes b and c). However, a slight decrease in the 43-kDa band was observed when the amount of the heart homogenate was reduced (lane d). A similar result was observed when immunoprecipitated Cx43 from heart ventricle was mixed with fresh brain homogenate. While the intensity of the 43-kDa band in the immunoprecipitated material in the presence of the brain homogenate (lane f) is apparently less than that of the immunoprecipitated material alone (lane e), this decrease (72.9%) is similar to that observed for the IgG band at 95 kDa (74%), suggesting reduced solubility of all immunoprecipitated material in the presence of additional protein (from brain homogenate). The solubility of the 39-kDa band from the brain homogenate (lane g) appears to be similarly reduced when mixed with the immunoprecipitated material (lane f). In addition, the presence of a phosphatase inhibitor (1 mM vanadate or 10 mM phosphate) during both initial homogenization and isolation failed to change the electrophoretic profiles of Cx43 in either heart or brain homogenates (data not shown). These results strongly suggest that the different phosphorylation states of Cx43 are a property of the tissues themselves rather than an artifact of sample preparation.

DISCUSSION

In this article, we further characterize the specificity of an antibody raised against the N-terminal 20 amino acids of the heart gap junction protein. Earlier studies of Yancey et al. (45) have demonstrated the specificity of this antibody for the native form of Cx43 and its ability to block dye transfer in cultured myocytes. In this study, we demonstrate its absolute specificity for Cx43 with respect to other proteins and other members of the connexin family (Fig. 1). Using this antibody in conjunction with the Cx43 cDNA, we observed that the protein (Fig. 3) and its transcript (Fig. 4) were present in a variety of tissues and were particularly predominant in those with an electrically excitable character (e.g., heart, uterus, brain, and retina). However, earlier studies of Beyer et al. (3) and our own observations indicate that expression is not confined to excitable cells per se (e.g., in brain the principal expressors of Cx43 are astroglia [11; unpublished data]).

In contrast to the well-characterized case of heart ventricle, in which the major gap junctional protein has an M_r of 43,000, in many tissues screened with out antibody the major reactive product was a doublet at M_r 39,000 to 40,000. One possible origin of this doublet is degradation of the M_r-43,000 protein. However, the presence of this doublet seems to be independent of the degree of proteolysis which can occur during isolation (in the presence or absence of protease inhibitors), as reflected by the presence of known degradation products (bands at 34, 31, and 29 kDa [32]). Furthermore, both the 43-kDa band and the 39- to 40-kDa doublet are recognized by antibodies raised against the last 20 residues from both the N-terminus (Fig. 3A) and C-terminus (Fig. 3B) of the deduced Cx43 protein sequence, eliminating the possibility of terminal cleavages of greater than 15 residues.

It is also unlikely that the 39- to 40-kDa doublet and the 43-kDa polypeptide arise from different transcripts. The messages detected in tissues having either polypeptide pattern (brain and heart) have identical sizes and hybridize equivalently at high stringency to probes including either the 5′ untranslated and coding regions (nucleotides 1 to 1400 in reference 3) shown in Fig. 4 or the 3′ untranslated region alone (nucleotides 1352 to 1785 in reference 3) (data not shown). It is possible that internal splicing of the Cx43 RNA could produce the different products shown in Fig. 3 yet escape detection in the above analyses. However, this is inconsistent with the results of Willecke et al. (43), showing that both brain and heart Cx43 messages can protect the 5′ 1.4 kb of the Cx43 cDNA from heart (which contains the entire coding region) from S1 nuclease digestion. Moreover, it should be noted that the genes encoding other connexins, i.e., Cx32 (33) and Cx26 (46), seem to lack introns within the coding region.

Direct evidence, however, does support differential phosphorylation as the cause of the electrophoretic heterogeneity of Cx43. A similar heterogeneity in the electrophoretic mobility of differentially phosphorylated forms of Cx43 has been demonstrated in vole fibroblasts (7) and chicken lens (34). Alkaline phosphatase treatment of immunoprecipitates can reduce the 43-kDa protein found in heart (Fig. 5, lane c) to a 39-kDa band indistinguishable from that in brain (Fig. 5, lanes a and d). This reduction is not due to the presence of contaminating proteases, since it was prevented by the presence of a specific phosphatase inhibitors, such as vanadate or excess phosphate (Fig. 5, lanes e and f). The conclusion from this analysis, that Cx43 in the brain is present in a largely unphosphorylated state, is supported by the observation that Cx43 translated in a cell-free system (and thus presumably not covalently modified; Fig. 5, lane g) has mobility identical to that of immunoreactive Cx43 in the brain (Fig. 5, lanes a and b).

While these analyses are limited by the sensitivity of electrophoretic mobility to changes in phosphorylation, it clearly does serve to demonstrate different relative levels of phosphorylation in different tissues. These differences seem to be an inherent and reproducible property of the tissues rather than an artifact of isolation. Inclusion of phosphatase inhibitors during isolation failed to change the polypeptide profiles, and in a variety of experiments in which heart and brain homogenates were mixed, all results are consistent with a combination of the profiles of the separate homogenates (Fig. 6). While heart ventricle and brain represent the extremes, each tissue that we screened had its own characteristic fingerprint of Cx43 phosphorylation, with consistent differences even being evident between different portions of the same organ (e.g., heart ventricle and atrium).

The actual site, enzymatic origin, and functional significance of this phosphorylation are major questions which are still not answered. Potential phosphorylation sites for both protein kinase A (Ser-244 and Ser-306; see reference 13 for consensus sequences) and protein kinase C (several serines between residues 361 and 372 and Ser-296; see reference 44 for consensus sequences) are located in the large C-terminal domain demonstrated to reside in the cytoplasm by Manjunath et al. (31) and Yancey et al. (45). No functional changes in junctional coupling have yet been correlated with protein kinase C action. However, cyclic AMP has been demonstrated to increase junctional conductance in heart cells, which express only Cx43 (4, 9). While the directness of this effect is unclear, the cyclic AMP-dependent phosphorylation of another connexin, Cx32, has been demonstrated and was shown to correlate with an increase in junctional conductance (40, 42).

The surprising result of the current work is not that Cx43 can be phosphorylated, a possibility that has been raised several times in the past, but rather that this should occur differentially between whole organs (e.g., heart and brain) and also within a single organ (atrium and ventricle of heart). This confers a further dimension of diversity on this complex gene family. Its significance for junctional function in these tissues remains obscure, emphasizing the critical need for identifying specific sites of phosphorylation and their influence on the biosynthesis and channel function of Cx43. Such analyses, correlated with those presented here, should help elucidate the differential role of gap junctional communication in various tissues, particularly those involved in the conduction of electrical signals.

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