Cloning of a Gap Junctional Protein from Vascular Smooth Muscle and Expression in Two-cell Mouse Embryos*

Joseph A. Lash‡, Elizabeth S. Critser‡, and Milton L. Pressler†

From the Krannert Institute of Cardiology, Departments of Medicine and Physiology and Biophysics, Indiana University School of Medicine, the Roudebush Veterans Administration Medical Center, and the Methodist Center for Reproduction and Transplantation Immunology, Indianapolis, Indiana 46202

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England Biolabs and Bethesda Research Laboratories; exonuclease III.

Gap junctional proteins (connexins) form aqueous channels that enable direct cell-cell transfer of ions and small molecules. The distribution and conductance of gap junction channels in cardiac muscle determine the pattern and synchrony of cellular activation. However, the capacity for smooth muscle to restrict contractile events temporally and spatially suggests that cell-cell coupling or its regulation may be decidedly different in this tissue. We isolated a cDNA from vascular smooth muscle which encodes a connexin (M, 43,187) structurally homologous to cardiac connexin43. Vascular smooth muscle connexin43 mRNA was expressed prominently in smooth muscle tissues, cultured vascular myocytes, and arterial endothelial cells. A model for functional expression of connexins was developed in two-cell B6D2 mouse embryos. Microinjection of in vitro transcribed vascular smooth muscle connexin43 mRNA was shown to be sufficient to induce intercellular coupling in previously uncoupled blastomeres. Through the construction of two deletion mutants of connexin43, we also show that the formation of cell-cell to-cell connections does not depend upon a predicted cytoplasmic region within 98 residues of the carboxyl terminus. Finally, the identification of connexin43 in smooth muscle and endothelial cells provides evidence for the existence of heterocellular coupling between cells of the vascular intima.

* This study was supported in part by National Institutes of Health Grants HL01451 (to J. A. L.) and HL01303 (to M. L. P.), Grants-in-Aid from the American Heart Association, the Krannert Charitable Trust, and Methodist Hospital of Indiana, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Krannert Institute of Cardiology, 1001 W. 10th St., Indianapolis, IN 46202. Tel.: 317-609-7712.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05535.

Various tissues, both functional and immunological evidence has suggested that there is structural diversity of connexins from different tissues (5–8). Recently this reasoning has been confirmed by direct comparison of the sequences of cDNAs isolated from dissimilar tissues. The deduced amino acid sequences of connexins from heart (connexin43), liver (connexin32 and connexin26), and embryonic cells (connexin38) have demonstrated clearly the structural heterogeneity of these protein subunits (9–14). However, the functional correlates of this structural heterogeneity of the various connexins is only starting to be explored (15). The significance imparted by the expression of a particular connexin to the function of both an individual cell and the entire organ remains unresolved.

In cardiac and smooth muscle, gap junctions coordinate contraction by providing low-resistance pathways necessary for cell-to-cell propagation of action potentials. Nevertheless, the capacity for smooth muscle to restrict both temporally and spatially contractile events, such as vasospasm or peristalsis, suggests that cell-cell coupling or its regulation may be decidedly different in this tissue than that which occurs in the heart. In this regard, Beyer et al. (9) have shown by Northern hybridization that connexin43, identified and isolated from heart tissue, is also expressed in the uterus, but that connexin32 is expressed in the stomach. Although gap junctions have been detected ultrastructurally and functionally in smooth muscle (16), there have been no reports of the identity or structural characteristics of connexins in this tissue. The importance of gap junctions to smooth muscle function extends not only to the contractile activity of the blood vessel, but also to areas such as direct endothelial-smooth muscle signaling, angiogenesis, and modulation of smooth muscle cell phenotype (17–20).

In this study, we constructed an oligonucleotide probe for screening a vascular smooth muscle cDNA library based on the observation that deduced protein sequences of connexins share highly conserved membrane-spanning domains. We report the nucleotide and deduced amino acid sequence of a vascular smooth muscle connexin virtually identical to cardiac connexin43 and provide evidence for mRNA expression in vascular smooth muscle, gastric smooth muscle, cultured vascular myocytes, and endothelial cells. We also demonstrate that microinjection of in vitro transcribed mRNA is sufficient to induce intercellular coupling in previously uncoupled two-cell mouse embryos. Finally, through the construction of two mutant forms of connexin43, we show that the formation of cell-cell connections does not depend upon a predicted cytoplasmic domain of the protein within 98 residues of the carboxyl terminus.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England Biolabs and Bethesda Research Laboratories; exonuclease III.
Connexin43 from Vascular Smooth Muscle

S1 nuclease, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Bethesda Research Laboratories; T5 and T7 RNA polymerase were purchased from Stratagene. Chemicals were purchased from Bethesda Research Laboratories and Sigma. Pregnant mare serum gonadotropin and human choriionic gonadotropin were from Organon Pharmaceuticals. [α-32P]dCTP, [γ-32P]ATP, [α-35S]dATP, and [35S]methionine were purchased from Du Pont-New England Nuclear.

Sequence Analysis—Inserts identified in a bovine aortic smooth muscle λ gt10 library (21) were subcloned into the EcoRI restriction site of Bluescript plasmid (Stratagene). A full-length cDNA insert uncut at an internal EcoRI site was obtained by limited EcoRI digestion and also subcloned in Bluescript. Exonuclease III deletions were constructed and both strands sequenced by the dideoxy chain termination method (22) using Sequenase (United States Biochemical Corp., Cleveland, OH). Internal oligonucleotide primers were used to complete any gaps.

Northern Hybridization—Total RNA isolated from fresh tissues or cultured vascular myocytes and endothelial cells (21, 22) was separated by formaldehyde gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell). Duplicate lanes of each sample were stained with ethidium bromide for localization of the ribosomal subunits. A probe consisting of the 1.0-kb EcoRI restriction fragment of G3 (33) containing ~90% of the coding region for VSM connexin43 was gel purified and labeled (>106 cpm/μg) by random priming (Promega Biotec, Madison, WI). Hybridization and washing of nitrocellulose filters was performed as described (22) with a final wash stringency of 0.25 × SSC at room temp. Autoradiograms were exposed at ~70 °C using an intensifying screen.

In Vitro Transcription and Translation—mRNA was produced utilizing T7 RNA polymerase and capped with [35S]methionine with rabbit reticulocyte lysate (Promega) was performed according to the manufacturer's recommendations. Protein products were subjected to denaturing discontinuous polyacrylamide gel electrophoresis (23) and visualized by autoradiography with the addition of Entensify (Du Pont-New England Nuclear) utilizing T7 RNA polymerase and capped with 5'7meGppp5'G followed by autoradiography with the addition of Entensify (Du Pont-New England Nuclear). The mRNA was synthesized in vitro and pressure-injected into the uninjected cell was observed for ~45 min before scoring the results.

RESULTS

A λ gt10 cDNA library prepared from bovine aorta smooth muscle (21) was screened with an oligonucleotide probe (5'-CACGCCACACCTCTCCCTCAGCGTGAGTA-3') complementary to a portion of the first predicted transmembrane region of the published cDNA sequence of rat cardiac connexin43 (9). Three clones were identified from screening ~2.5 × 106 plaques. Each clone yielded insert fragments of ~1.9 and ~1.0 kb when excised from λ phage DNA by EcoRI, likely the result of library amplification. Southern hybridization of the oligonucleotide probe with the two restriction fragments suggested that the 1.0-kb fragment contained the coding region for the protein. The full-length, uncut insert obtained by limited EcoRI digestion of the λ phage DNA was utilized for subsequent sequencing and expression studies.

Fig. 1 shows the nucleotide and deduced amino acid sequence of clone G33. The insert consisted of a total of 2932 bases, with 10 bases upstream of the ATG codon and a poly(A) tail. An EcoRI restriction site was present at position 1039. Two polyadenylation signals (AATAAA) were identified in the 3'-untranslated region at positions 2162 and 2878. An open reading frame of 1149 base pairs encoded a protein containing 383 amino acids with M, of 43,187. The protein sequence exhibited extensive homology with connexin43 from rat cardiac muscle (9), differing by only nine amino acids (Fig. 2). Most of the amino acid substitutions in VSM connexin43 were conservative with three exceptions: insertion of an additional valine at residue 116; substitution of histidine for asparagine at residue 342; and substitution of aspartic acid for asparagine at residue 342.

FIG. 1. Nucleotide and deduced amino acid sequence of bovine vascular smooth muscle connexin43 cDNA. Polyadenylation signals (AATAAA) are boxed and located at positions 2162 and 2978.

FIG. 2. Comparison of the deduced amino acid sequences of bovine vascular smooth muscle (VSM) and rat cardiac connexin43. Predicted transmembrane domains are underlined.
for alanine at residue 349. Four of nine amino acid substitutions were found in relative proximity to the EcoRI restriction site which is notably absent in rat cardiac connexin43.

The deduced amino acid sequence of VSM connexin43 has four predicted membrane-spanning regions (27) which were identical with those of rat cardiac connexin43 (9). The amino acid charge distribution within the first signal-anchor sequence predicts that the amino terminus of the protein faces the cytoplasmic surface of the membrane (28). Consequently, this orientation constrains the carboxyl terminal also to project into the cytosol, in agreement with models proposed for connexins from liver and heart (8, 29-31).

Hybridization of total RNA from bovine aortic smooth muscle with a probe derived from the 1.0-kb EcoRI restriction fragment of clone GJ3 showed a major mRNA species hybridizing at 3.3 kb and a second band at 1.8 kb (Fig. 3). The 3.3-kb mRNA was ∼10-fold more abundant than the 1.8-kb species as determined by densitometric scanning. A similar pattern was observed in total RNA isolated from heart and gastric smooth muscle (Fig. 3), as well as in coronary artery, pulmonary artery, and carotid artery smooth muscle (not shown). Total RNA from cultured vascular myocytes and pulmonary arterial endothelial cells demonstrated the same prominent 3.3- and 1.8-kb signals. In agreement with previous studies (9), there was no hybridization signal from connexin43 detected in liver (Fig. 3). It is of note that similar levels of connexin43 mRNA were found in smooth muscle tissues, heart, and cultured endothelial cells, whereas they were ∼6-fold higher in cultured vascular myocytes.

Although the 3.3-kb mRNA species identified on Northern blots would be predicted to represent connexin43, the identity of the 1.8-kb signal is unknown. Localization of the 28 S and 18 S ribosomal subunits on ethidium bromide-stained gels demonstrated the 18 S subunit to be reproducibly smaller than the identified 1.8-kb signal. In addition, there was no hybridization signal from ribosomal subunits detected in total RNA from liver (see Fig. 3). Finally, selection for poly(A+) RNA did not change appreciably the relative ratio of the 3.3-kb to the 1.8-kb signals (not shown).

We determined whether mRNA coding for connexin43 is sufficient to induce intercellular coupling by exploiting the finding that mouse embryonic cells (B6D2F1 strain) normally are not coupled at the two-cell stage (see Table 1 below). VSM connexin43 mRNA (0.3-1.0 pg) was injected into fertilized eggs (80% viable postinjection) and Lucifer Yellow dye transferred 20-24 h later when the embryos had reached a two-cell stage (85% of injected survivors). Fig. 4 illustrates the results: connexin43 mRNA-injected embryos developed cell-cell coupling whereas no dye transfer occurred in uninjected embryos. Overall, positive dye transfer occurred in 58% of connexin43 mRNA-injected embryos versus none of 24 un.injected controls and only 3 of 20 buffer-injected controls. Statistical analysis of these groups showed that the incidence of coupling was significantly higher in the mRNA-injected embryos versus either of the control groups (p < 0.005 by Fisher’s exact test); there was no statistical significance to the small difference between buffer-injected versus uninjected controls. The intensity of dye transfer varied widely (relative fluorescence of the two blastomeres was typically ∼1:10) due partly to variation in the amount of injectate and partly to differences in the extent of coupling. Dye transfer was not immediate; Lucifer Yellow filled the injected cell within seconds, whereas minutes were required for fluorescence to be visible in the adjacent blastomeres. Cell division was not essential for development of cell-cell coupling. In a few experiments, VSM connexin43 mRNA was microinjected into each cell of two-cell embryos and the presence or absence of dye transfer determined later at the same stage. The results were similar to microinjection of mRNA in one-cell embryos; 33% showed intercellular transfer of dye versus none of the controls. Tolerance of the embryos for double injections was poor (only 6 of 20 pairs survived 24 h), and continued development limited the number of embryos that could be studied at the two-cell stage.

We also investigated the functional significance of the large predicted carboxy-terminal cytoplasmic region of VSM connexin43 with respect to induction of cell-cell coupling. Fig. 5A shows the deduced amino acid sequence of the mutant mRNA species that were produced from a Bluescript plasmid containing the 1.0-kb EcoRI restriction fragment of VSM connexin43 cDNA. The plasmid was linearized with SmaI and transcribed utilizing the T7 promoter, producing a mRNA in which the coding region was deleted for the terminal 98 amino acids of connexin43. A second mRNA was produced by linearizing the plasmid at the XbaI site within the polylinker region of the plasmid. The mRNA transcript contained the coding region for amino acids 1-346 from connexin43 followed by 8 amino acids coded by the vector. In vitro translation of the mRNAs with reticulocyte lysate yielded the protein products shown in Fig. 5B despite the absence of a termination codon and all 3'-untranslated sequence in the truncated mRNAs. The apparent molecular weights by SDS-polyacrylamide gel electrophoresis of the protein products of the full-length and truncated connexin43 mRNAs were somewhat smaller than would be predicted to be encoded by the mRNAs.

**Fig. 3.** Northern blot analysis of various tissues with a cDNA probe for VSM connexin43. Blots were probed with a 1.0-kb EcoRI restriction fragment of clone GJ3. Total RNA (∼10 μg) from bovine aortic smooth muscle (lane 1), cultured aortic smooth muscle myocytes (lane 2), porcine stomach smooth muscle (lane 3), cultured pulmonary endothelial cells (lane 4), bovine liver (lane 5), and bovine cardiac ventricular muscle (lane 6). The arrows show the positions of the 28 S and 18 S ribosomal subunits.

**Fig. 4.** Cell-cell coupling in two-cell mouse embryos injected with mRNA specific for VSM connexin43. A, light micrograph (Nomarski; × 640) of embryo prior to injection of Lucifer Yellow in the lower blastomere. B, fluorescence micrograph showing no dye transfer in control two-cell embryo (32 min after Lucifer Yellow). C, fluorescence micrograph of intercellular dye transfer in mRNA-injected embryo (19 min after Lucifer Yellow).
Connexin43 from Vascular Smooth Muscle

**DISCUSSION**

It has long been recognized that gap junctions are the membrane regions through which ionic currents pass from cell to cell during propagation of action potentials (34, 35). The distribution and conductance of gap junction channels in cardiac and smooth muscle determines the pattern and synchrony of cellular activation. Yet gap junctions are also present in other cells which are inexcitable. In these tissues, there is evidence to suggest that gap junctions provide a mechanism for transmission of small cytoplasmic molecules between cells. However, it is unknown if different functional characteristics are shared by all gap junctions or are a reflection of intrinsic structural differences in the connexin subunits comprising the gap junctions. Recent information from analysis of connexin cDNAs from heart, liver, and embryonic cells has provided a structural basis for suggesting that variations in particular domains of the protein may be the basis for different physiological functions.

In this study we have isolated and characterized a cDNA from vascular smooth muscle that encodes a gap junction protein, and we have demonstrated the ability of *in vitro* transcribed mRNA to induce cellular coupling. The deduced amino acid sequence of this cDNA yielded a protein of Mr, 43,187 which is virtually identical to the sequence of rat cardiac connexin43 (9). Eight amino acid substitutions as well as one additional residue were identified in the deduced sequence of connexin43 from vascular smooth muscle. The substitutions were all confined to predicted cytoplasmic regions of the protein. Since most of the substitutions are conservative, it seems probable that these differences are species-related and unlikely to be of functional significance. However, the extracellular and transmembrane domains of these two proteins were conserved absolutely, not surprising given the presumed role of these regions in membrane assembly and alignment with the connexin array across the gap. Yet the observation by others of single-channel activity after reconstitution of connexins in planar bilayers (36) suggests that the capacity for assembly into functional channels is not entirely dependent on alignment with an adjoining hemichannel. There must be additional factors *in vivo* that lead to localization of connexins in junctional membranes or their selective opening therein. We observed that none of the two-cell embryos in which coupling was induced by connexin43 mRNA showed leakage of dye across open channels in the surface membrane. Given the propensity of connexins to form channels in lipid bilayers, it appears improbable on the basis of chance alone that connexins would have inserted only into the junctional membrane region of the blastomere. Furthermore, our initial studies with deletion mutants of connexin43 suggest that substitutions within 98 residues of the carboxyl terminus have little significance with regard to induction of functional cell-cell channels. However, it is not yet known whether point mutations within the carboxyl-terminal region result in alterations in the probability of channel opening or closure which may be of considerable importance to cellular regulation of gap junctional function.

Northern analysis for expression of connexin43 identified a major 3.3-kb mRNA species and a minor 1.8-kb mRNA in vascular tissue, gastric smooth muscle, heart, cultured vascular myocytes, and endothelial cells. As predicted from other studies (9), connexin43 did not hybridize to RNA from liver, which has been shown to contain connexin32 and connexin26. The identity of the 1.8-kb hybridization signal to connexin43...
Connexin43 from Vascular Smooth Muscle

...likely. The two-cell mouse embryo model offers the advantage of a mammalian ...products of mRNA coding for rat liver connexin32 (47). However, the large size of Xenopus oocytes (1.8-kb mRNA) may permit the recording gap junctional channels in situ because input resistance is increased by the ~1000-fold smaller cell volume.

Acknowledgments—We thank Dr. Joe G. N. Garcia for kindly providing cultured bovine endothelial cells and Dr. David Hathaway for his advice and encouragement during this work.

REFERENCES
1. Bennett, M. V., L., and Goodenough, D. M. (1978) Neurosci. Res. Program Bull. 16, 372-388
2. Loewenstein, W. R. (1981) Physiol. Rev. 61, 829-913
3. Urwin, P. N. R., and Zarnik, G. (1989) Nature 333, 545-549
4. Manjunath, C. K., and Page, E. (1985) Am. J. Physiol. 249, H781-H791
5. Nicholsson, B. J., Gross, D. B., Kent, S. B. H., Hood, L. E., and Revel, J.-P. (1985) J. Biol. Chem. 260, 6514-6517
6. Nicholsson, B., Dormitche, R., Teplow, D., Treub, O., Willecke, K., and Revel, J.-P. (1987) Nature 329, 732-734
7. Kistler, J., Christie, D., and Bullivant, S. (1988) Nature 331, 721-723
8. Beyer, E. C., Kistler, J., Paul, D. L., and Goodenough, D. A. (1989) J. Cell Biol. 108, 695-700
9. Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1987) J. Cell Biol. 105, 2621-2629
10. Paul, D. L. (1986) J. Cell Biol. 103, 123-124
11. Kumar, N. M., and Gihla, N. B. (1986) J. Cell Biol. 103, 767-776
12. Gimlich, R. L., Kumar, N. M., and Gihla, N. B. (1988) J. Cell Biol. 107, 1023-1072
13. Ribbo, L., Beyer, E. C., Swenson, K. I., Paul, D. L., and Goodenough, D. A. (1989) Science 243, 1194-1195
14. Zhang, J.-L., and Nicholsson, B. J. (1990) J. Cell Biol. 109, 3391-3401
15. Loewenstein, W. R. (1987) Cell 48, 725-726
16. Bienenstock, M., d'. M., Kinnair, N. M., and Garfield, R. E. (1987) Am. J. Physiol. 253, C580-C589
17. Larson, D. M., Carson, M. P., and Haudenschild, C. C. (1987) Microsc. Res. Tech. 13, 184-190
18. Chamley-Campbell, J., Campbell, G. R., and Ross, R. (1979) Physiol. Rev. 59, 1-68
19. Ather, J., Nilsson, J., Palmberg, L., and Sjolund, M. (1985) Cell Tissue Res. 239, 60-74
20. Dilley, R. J., McGeachie, J. K., and Prendergast, F. J. (1987) Atherosclerosis 65, 89-207
21. Mcclelland, P., Lash, J. A., and Hathaway, D. R. (1989) J. Biol. Chem. 264, 17425-17431
22. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, A. J., and Struhl, K. (eds) (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York
23. Bertazzol, U. R. (1970) Nature 227, 78-86
24. Robb, J. M., Gilligan, B., Critser, E. S., and First, N. L. (1986) Biol. Reprod. 34, 723-739
25. Bevier, R. D., Lishfried, M. L., and Lieberman, G. (1983) Biol. Reprod. 28, 235-247
26. Whitten, W. K. (1971) Adv. Biochem. 6, 129-141
27. Racu, J. K., and Argos, P. (1986) Biochim. Biophys. Acta 869, 197-214
28. Hartmann, E., Rapt, U., and Lodish, H. F. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 5786-5790
29. Revel, J.-P. (1987) Nature 329, 732-734
30. Zirn, J., Green, C. R., Evans, W. H., and Gilula, N. B. (1987) J. Biol. Chem. 262, 7751-7763
31. Hartmann, E., Rapt, U., and Lodish, H. F. (1989) J. Cell Biol. 108, 2241-2254
32. Green, C. R., Harst, E., Gourdie, R. G., and Severs, N. J. (1988) Proc. R. Soc. Lond. B Biol. Sci. 233, 165-174
33. Swenson, K. L., Jordan, J. R., Beyer, E. C., and Paul, D. L. (1989) Cell 57, 145-166
34. Bazzar, D. M., and Berger, W. (1965) J. Gen. Physiol. 48, 797-823
35. Bazzar, L., Berger, W., and Dewey, M. M. (1968) J. Gen. Physiol. 51, 347-373
36. Young, J. D. E., Cohn, Z. A., and Gilula, N. B. (1987) Cell 50, 733-743
37. Ceveney, S. (1985) Annu. Rev. Physiol. 47, 319-335
38. Hartmann, E., Rapt, U., and Lodish, H. F. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 5786-5790
39. Reves, J.-P. (1987) Nature 329, 732-734
40. Podd, C. J., and Feil, A. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2470-2477
41. Dehbi, T., Miller, T., Page, J., Starrett, T., and Wanner, R. (1986) Science 236, 1290-1293
42. Warner, K., Levine, K., Khatib, I., and Dahl, G. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 5380-5384