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Immunochemical Characterization of the Gap Junction Protein Connexin45 in Mouse Kidney and Transfected Human HeLa Cells

A. Butterweck, U. Gergs, C. Elfgang, K. Willecke, O. Traub
Institut für Genetik (Abteilung Molekulargenetik) der Universität, Römerstr. 164, D-53117 Bonn, Germany

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Abstract. Antibodies to the gap junction protein connexin45 (Cx45) were obtained by immunizing rabbits with fusion protein consisting of glutathione S-transferase and 138 carboxy-terminal amino acids of mouse Cx45. As shown by immunoblotting and immunofluorescence, the affinity-purified antibodies recognized Cx45 protein in transfected human HeLa cells as well as in the kidney-derived human and hamster cell lines 293 and BHK21, respectively. In Cx45-transfected HeLa cells, this protein is phosphorylated as demonstrated by immunoprecipitation after metabolic labeling. The phosphate label could be removed by treatment with alkaline phosphatase. A weak phosphorylation of Cx45 protein was also detected in the cell lines 293 and BHK21. Treatment with dibutyryl cyclic adenosine- or guanosine monophosphate (cAMP, cGMP) did not alter the level of Cx45 phosphorylation, in either Cx45 transfectants or in 293 or BHK21 cells. The addition of the tumor-promoting agent phorbol 12-myristate 13-acetate (TPA) led to an increased ^{32}P phosphate incorporation into the Cx45 protein in transfected cells.

The Cx45 protein was found in homogenates of embryonic brain, kidney, and skin, as well as of adult lung. In kidney of four-day-old mice, Cx45 was detected in glomeruli and distal tubules, whereas connexin32 and -26 were coexpressed in proximal tubules. No connexin43 protein was detected in renal tubules and glomeruli at this stage of development. Our results suggest that cells in proximal and distal tubules are interconnected by gap junction channels made of different connexin proteins. The Cx45 antibodies characterized in this paper should be useful for investigations of Cx45 in renal gap junctional communication.

Correspondence to: K. Willecke

Key words: Antibodies to connexin45 — Connexins — Gap junctions — Kidney cell differentiation

Introduction

Gap junctions consist of aggregated membrane channels which mediate intercellular exchange of ions and metabolites thus allowing electrical and metabolic coupling of cells (*cf.* Loewenstein, 1981, Bennett et al., 1992). Intercellular communication through gap junctions has been suggested to mediate synchronization of cellular activities, growth control, embryonic differentiation, and pattern formation during development (*cf.* Loewenstein, 1988; Guthrie & Gilula, 1989). Each functional gap junction channel is formed by docking and opening of hemichannels in apposed membranes of adjacent cells. Hemichannels are thought to consist of six subunit proteins, connexins (Cx), which are coded for by a multigene family that consists of at least 12 members (*summarized in* Willecke et al., 1991; Haefliger et al., 1992; White et al., 1993).

The diversity of connexins in mouse and presumably all mammals suggests that each of these proteins may be functionally specialized. Several mammalian cell types have been shown to express more than one connexin gene. Well-documented examples are murine hepatocytes, expressing connexin32 and -26 (Nicholson et al., 1987), canine myocardiocytes, expressing Cx43, Cx40, and Cx45 (Kanter, Saffitz & Beyer, 1992) and rat keratinocytes, in which Cx43 and Cx26 were found (Brisette et al., 1991).

However, several of the newly discovered members of the connexin gene family have not been studied in detail at the protein level, since no specific antibodies were available. These reagents are needed to investigate cell type specific expression and structure *vs.* func-

tion relationships with each of the connexin molecules. For the latter purpose, we have transfected the mouse connexin genes, cloned in our laboratory (Willecke et al., 1991), into human HeLa cells, an established cell line which is deficient in dye transfer and electrical conductivity (Eckert, Dunina-Barkovskaya, Hülser, 1993). The transfected cells express the exogenous connexin transcripts at high level and are restored in dye transfer (*cf.* Hennemann et al., 1992*b*). These cells also serve as a rich and homogenous source of the corresponding connexin protein which is functionally expressed and a convenient target for characterization of the corresponding antibodies.

Here we describe characterization of new antibodies directed against a polypeptide of 138 C-terminal amino acids from mouse Cx45 protein. This peptide was expressed as a fusion protein with glutathione S-transferase in *Escherichia coli* and used for immunization after isolation. The antibodies specifically recognize their antigen in transfected human HeLa cells as well as in extracts from several mouse tissues which had previously been shown to express Cx45 mRNA (Hennemann, Schwarz & Willecke, 1992*a*). The affinity-purified Cx45 antibodies were used to locate the antigen in glomeruli and distal tubules of kidney in four-day-old mice. This finding is in contrast to the location of Cx32 and -26 in proximal tubules and suggests a distinct role for Cx45 that may be different from the one of Cx32 and -26 in renal cell physiology.

Materials and Methods

Cells and Culture Conditions

BHK21 Baby Hamster Kidney cells (ATCC CCL10), human embryonic kidney 293 cells transformed by adenovirus type 5 (ATCC CRL 1573), Hel-37 cells (passage 254) derived from C3H mouse keratinocytes (Fusenig et al., 1983; Fusenig, Dzarlieva-Petrusevka & Breitkreuz, 1985) and human cervical carcinoma HeLa cells (ATCC CCC2) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (standard medium) at 37°C in a moist atmosphere of 10% CO_2.

Animals

Embryos at 19 days of gestation, four-day-old, and adult mice, all from NMRI mice (Naval Medical Research Institute, Bethesda, MD), were used throughout the experiments.

The presence of a vaginal plug was defined as day 0 of pregnancy. After killing the mice, whole organs were quickly removed, frozen in liquid nitrogen for transcript and protein analysis or embedded in Tissuetek compound (Tissuetek, Miles Lab, Naperville, IL), and slowly frozen in isopentane/liquid nitrogen for cryostat sections.

Northern Blot Analysis

Total RNA was prepared from cells according to Chomczynski and Sacchi (1987). Aliquots (20 μg) of total RNA were electrophoresed in 1.2% agarose in the presence of 2.2 M formaldehyde (Maniatis et al., 1982) and transferred to Hybond N membranes. The Northern blots were hybridized as previously described (Willecke et al., 1991), using a ^{32}P-labeled F9-Cx45 cDNA fragment (XbaI/XhoI, 950 bp, corresponding to a region from nucleotide position 668 to the 3′ end). Autoradiographs were obtained by exposing the hybridized blot to Kodak XAR film.

DNA Transfection

For DNA transfection, a 2.1 kb KpnI/SacI fragment of the mouse Cx45 gene, derived from F9-Cx45 cDNA (Hennemann et al., 1992*a*), was cloned in the plasmid pBEHpac18 (Horst, Harth & Hasilik, 1991) and designated as pBEHpac18-Cx45. Transfection was performed by a modification of the calcium phosphate precipitation method of Graham and Van der Eb (1973). Exponentially growing HeLa cells (0.6×10^6 cells/25 cm^2) were cotransfected with 2.5 μg pBEHpac18-Cx45 DNA and 20 μg of genomic, carrier DNA from HeLa cells. After 4 hr the precipitate was removed and the cells were washed extensively with phosphate-buffered saline (PBS). Selection for transfectants was carried out with 1 μg/ml puromycin, starting 24 hr later. Individual clones were picked after three weeks and grown in selective medium for further analyses.

Production and Purification of Antisera

Production and specificity of polyclonal rabbit antibodies to Cx26, Cx32 and Cx43 have been described previously (Traub et al., 1989, 1994).

A 0.8 bp TaqI/XhoI fragment of the mouse F9 Cx45 cDNA (Hennemann et al., 1992*a*), encoding the last 138 amino acids of the carboxy terminus, was subcloned into the expression vector pGEX-3X (Pharmacia, Freiburg, FRG), cut with SmaI and XhoI. *E. coli* bacteria *M15* (Zamenhof & Villarejo, 1972) were transformed with this construct and induced with 0.1 mM isopropyl β-D thiogalactopyranoside (IPTG). This treatment led to high expression of the Cx45 DNA fragment as a fusion protein with glutathione S-transferase (Smith et al., 1986) coded for by the pGEX vector (designated as GST-Cx45c).

Isolation of fusion protein (Marston, 1986; Smith & Johnson, 1988, *modified*): After 1.5 hr of incubation in the presence of IPTG at 30°C, the *E. coli* bacteria were collected by centrifugation for 10 min at 5,000 × *g* at 4°C and resuspended in PBS containing 1% aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then lysed by sonification and Triton X-100 was added to a final concentration of 1% to minimize association of the fusion protein with bacterial proteins. Sonicated extracts were centrifuged for 5 min at 10,000 × *g* at 4°C and the supernatants were incubated for 20 min with glutathione sepharose 4b (Pharmacia, Freiburg, FRG) at room temperature. After washing three times with ice-cold PBS, fusion protein was eluted by addition of 50 mM Tris-HCL (pH 8), 15 mM reduced glutathione (final concentrations), and stored at −70°C.

Rabbits were immunized as follows: on day 0, the purified fusion protein (containing 200 μg of the Cx45 C-terminal polypeptide) in complete Freund's adjuvant (1:1) was subcutaneously injected. On day 28, 200 μg in incomplete Freund's adjuvant (1:1) was again injected subcutaneously. Afterwards, the animals were boosted five times every four weeks with the fusion protein in incomplete adjuvant

and bled two weeks after injection. The resulting antiserum was purified by affinity chromatography. GST as well as GST-Cx45c were coupled to cyanobromide-activated sepharose and the antiserum was affinity-purified, first on the GST column, and second on the GST-Cx45c column. Antibodies were eluted with 3 M KSCN and dialyzed against PBS. The specificity of the antibodies was tested in protein extracts of several tissues and cell lines.

Immunoblot Analyses

Protein content of tissue homogenates and cell lysates, obtained in Laemmli sample buffer (Laemmli, 1970), was determined according to Lowry et al. (1951). One hundred micrograms of protein from every sample were electrophoretically separated on SDS-polyacrylamide gels using the procedure of Laemmli (1970). The proteins were then electrically transferred to nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Dassel, FRG), incubated with affinity-purified antibodies to Cx45 at a dilution of 1:300 and radiolabeled with ^{125}I-protein A. Exposure to Kodak XAR films was performed at −70°C using intensifying screens.

Immunofluorescence Analyses

Cultured cells grown on glass coverslips or cryostat sections (6 μm) were fixed in absolute ethanol (−20°C) for 5 min and then processed at room temperature for immunofluorescence. The cells and sections were washed in PBS with 0.1% BSA (4°C) and incubated for 1 hr with affinity-purified antibodies in PBS containing 0.5% casein. For controls, preimmune sera were used or the specimen coverslips were only incubated with secondary antibodies. After several washes in PBS containing 0.1% BSA (4°C), the samples were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma, Munich, FRG) in PBS with 0.5% casein at a dilution of 1:50. The coverslips were first washed in PBS, then in double-distilled water, and finally mounted onto glass slides with *p*-phenylendiamine-glycerol mounting medium (Sigma, Munich, FRG). Fluorescent signals were documented on Ilford HP5 400 films using a Zeiss Axiophot photomicroscope equipped with appropriate filters.

Phosphorylation of Cx45 in 293 and BHK21 Cells as well as in HeLa Transfectants

Cells were metabolically labeled by addition of ^{32}P sodium orthophosphate (100 μCi per ml of culture medium) to phosphate-free medium for 1 to 4 hr. To terminate the incorporation of ^{32}P, the labeling medium was removed and the cell layer washed three times with PBS without calcium and magnesium ions. The cells were then treated for 20 min with RIPA buffer [10 mM sodium phosphate buffer pH 7.2, 40 mM NaF, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1% Trasylol (Bayer AG, Leverkusen, FRG)], to facilitate solubilization of membrane proteins. The supernatants were agitated four times on a vortex mixer. After centrifugation at 13,000 × *g* (30 min, 4°C), the supernatants were aspirated, stored as aliquots at −70°C, or used directly for immunoprecipitation as described (Traub et al., 1987).

Treatment of Cx45 Expressing Cells with cAMP, cGMP or TPA

The cells were metabolically labeled with ^{32}P sodium orthophosphate for 4 hr. After three hours of labeling, cAMP or cGMP was added for 1 hr to a final concentration of 1 mM, whereas TPA was added for 15 min to a final concentration of 100 ng/ml. Afterwards, the cells were treated as described above.

Dephosphorylation of Cx45 Protein in HeLa Transfectants

Metabolically labeled Cx45 was immunoprecipitated and washed twice with buffer J (Scheidtmann et al., 1992). After washing once with phosphatase buffer, the immunoprecipitates were treated with alkaline phosphatase (Sigma-Chemie, Deissenhofen, Germany) at 37°C for 30 min, in the presence of aprotinin (1 μg/ml) and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors. Control reactions were incubated in the same buffer without added enzyme. After phosphatase treatment, the immunoprecipitates were washed again and analyzed by SDS-polyacrylamide gel electrophoresis.

Results

Analyses of Cx45 mRNA in Human HeLa Transfectants

To study expression of the cloned mouse Cx45 DNA in mammalian cells, we transfected human HeLa cells with the coding region of mouse Cx45. Human HeLa cells show a very low level of dye transfer when microinjected with Lucifer yellow, very low electrical conductivity (Eckert et al., 1993), and no Cx45 mRNA when subjected to Northern blot hybridization (Fig. 1, lane *1*). We first examined whether the transfected HeLa cells expressed Cx45 mRNA. Mouse skin from 19-day-old embryos was used as a positive control. Aliquots (20 μg) of total RNA were electrophoretically separated before transfer to a nylon membrane, and the blot was hybridized under stringent conditions to a mouse Cx45 ^{32}P-labeled cDNA fragment corresponding to the 3′ end. The Cx45 probe recognized transcripts of 2.2 kb size in independent clones of HeLa-Cx45 transfectants (Fig. 1, lanes *2* and *3*) and in embryonic skin (Fig. 1, lane *4*).

Analysis of Cx45 Protein in Lysates of HeLa/Cx45 Transfectants and Kidney-derived Cell Lines

Figure 2, lanes *B-E*, illustrates that the antibodies to the Cx45 polypeptide recognized a single protein of 45 kDa in extracts of transfected cells and of the kidney-derived cell lines 293 and BHK21. No Cx45 protein was detected in lysates of HeLa parental cells (lane *A*) and the keratinocyte cell line Hel-37 (lane *F*). In this context it should be noted that Cx45-transfected HeLa cell clones showed coupling of 50 to 70% to first-order neighboring cells when microinjected with Lucifer yellow (for details of the method used, *see* Hennemann et

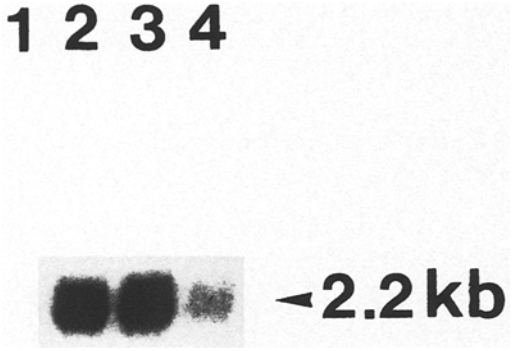

Fig. 1. Northern blot analysis of total RNA (20 μg each) isolated from HeLa parental cells (lane *1*), two independent HeLa/Cx45 transfectants (lanes *2* and *3*) and skin from 19-day-old mouse embryos (lane *4*). The Cx45 cDNA probe recognizes a single transcript of 2.2 kb size.

al., 1992*b*), whereas HeLa parental cells were dye transferred under these conditions below 5%. All Cx45-transfected HeLa cell clones described in this publication were restored for functional gap junctional communication.

Immunofluorescent Localization of Cx45 in Transfected Cells and Kidney-derived Cell Lines

Affinity-purified Cx45 antibodies were used for immunofluorescent localization in exponentially growing HeLa/Cx45 transfectants. The Cx45 immunoreactivity was detected as strong punctate spots that were located in regions of cell-to-cell contact (Fig. 3*A*). We noticed some staining in the cytoplasm with anti-Cx45, whereas HeLa parental cells (Fig. 3*I*) or controls treated with preimmune serum showed only minimal background staining (Fig. 3*E*).

The kidney-derived cell lines 293 and BHK21 were grown to confluence, incubated with anti-Cx45 or preimmune serum, and analyzed by fluorescent microscopy and phase-contrast. Cx45 protein was detected as fine, punctate immunoreaction in membrane regions of adjacent 293 cells (Fig. 3*C*). Even weaker immunolabeling with anti-Cx45 was seen in BHK21 cells (*results not documented*).

Phosphorylation of Cx45 in Transfected HeLa Cells and the Kidney-derived Cell Lines 293 and BHK21

We investigated whether mouse Cx45 is phosphorylated in transfected cells as well as in 293 and BHK21 cells expressing Cx45 protein endogenously. Thus, we used the affinity-purified Cx45 antibodies for immunoprecipitation of Cx45-transfected HeLa cells as well as 293 and BHK21 cells (*data not shown*), metabolically labeled either with ^{32}P orthophosphate (Fig. 4, lane *2*) or with ^{35}S methionine (Fig. 4, lane *1*). The results, illustrated in Fig. 4, show that the antibodies recognize

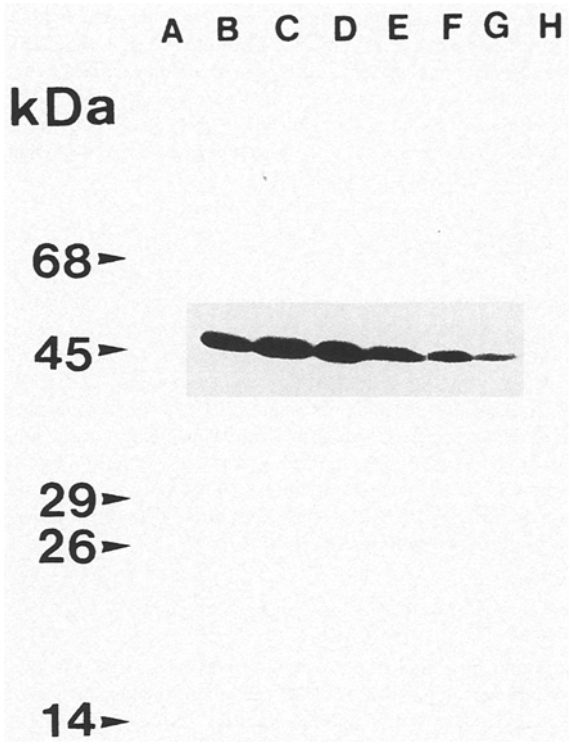

Fig. 2. Western blot analysis of Cx45 transfectants and kidney-derived cell lines. Polyclonal antibodies to Cx45 detect a 45-kDa protein in lysates of four independent HeLa/Cx45 transfectants (lanes *B-E*), 293 cells (lane *F*) and BHK21 cells (lane *G*). Lysates of untransfected HeLa cells (lane *A*) and Hel37 cells (lane *H*) were used as negative controls.

a protein of an apparent molecular mass of 45 kDa. This protein is phosphorylated (lane *2*). By comparison with the ^{35}S-methionine-labeled sample, we did not see any alteration of the electrophoretic mobility of the phosphorylated protein.

Furthermore, we investigated whether treatment with the tumor-promoting agent TPA or second messengers cAMP and cGMP influenced phosphorylation of Cx45 in transfected HeLa cells or in the cell lines 293 and BHK21, expressing the protein endogenously (*data not shown*). In comparison to untreated lysates of Cx45-transfected HeLa cells, 293, or BHK21 cells, no significant alteration of incorporated ^{32}P orthophosphate could be observed by addition of cAMP or cGMP. Treatment with TPA for 15 min resulted in a fourfold increased incorporation of ^{32}P phosphate into immunoprecipitable Cx45 protein in HeLa transfectants. In this short period of time it is likely that the effect is caused by an increase of phosphorylation and not of protein.

Dephosphorylation of Cx45 in Transfected HeLa Cells

Metabolically labeled, transfected HeLa cells were immunoprecipitated and incubated with alkaline phos-

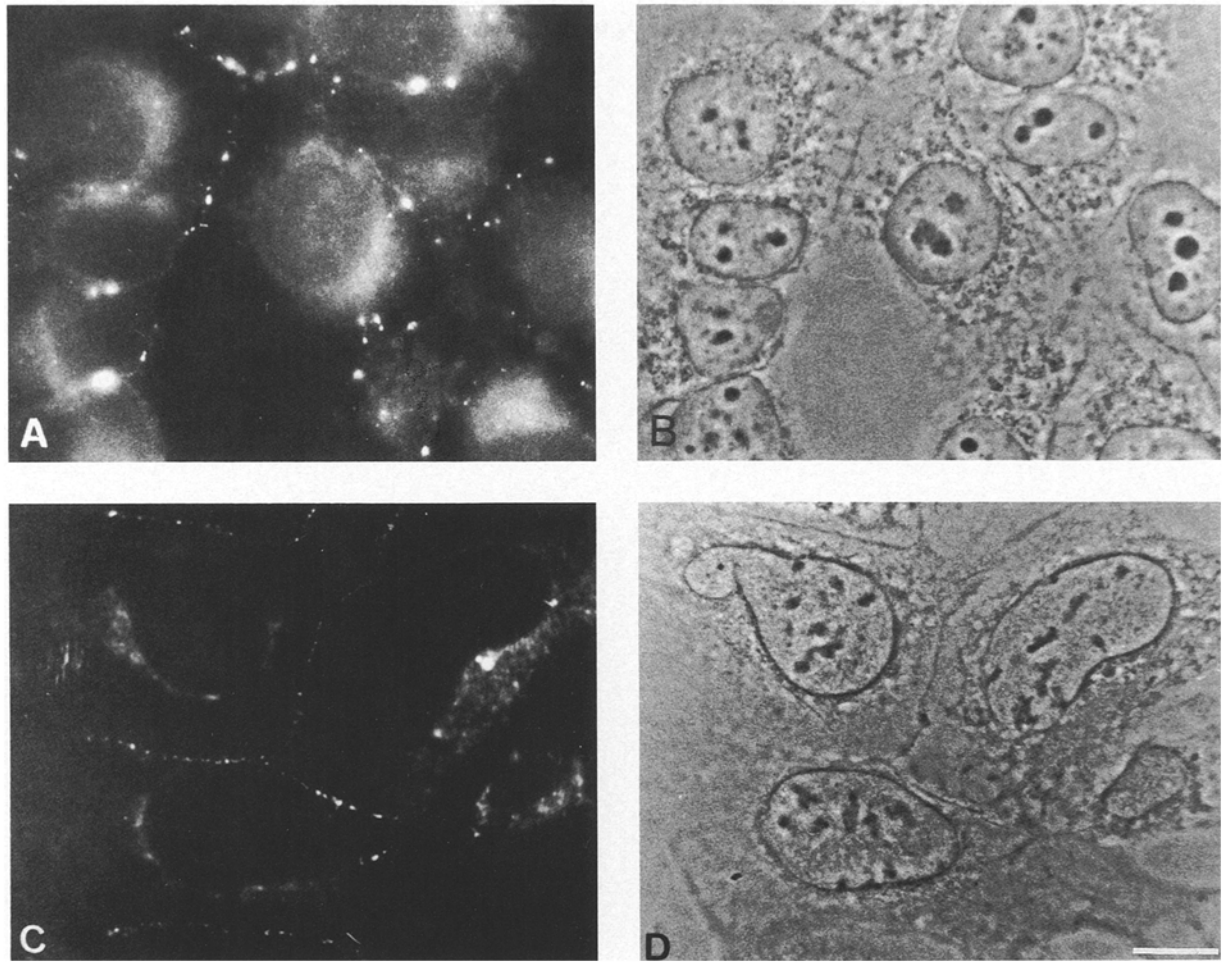

Fig. 3. Immunofluorescence analysis of Cx45 in HeLa/Cx45 transfectants and the kidney-derived cell line 293. (*A*) Cx45 immunoreactivity in HeLa/Cx45 transfectants is localized as punctate spots on areas of cell-to-cell contact. (*C*) Faint punctate Cx45 immunolabeling is observed on contacting membranes between adjacent 293 cells. (*E*) Only minimal background labeling with preimmune serum is detected in HeLa/Cx45 transfectants, No. 293 cells (*G*), or untransfected HeLa cells (*I*). (*B, D, F, H,* and *J*) Phase-contrast micrographs corresponding to fluorescence images *A, C, E,* and *G,* respectively. Scale bar: 20 μm

phatase or, for control, in phosphatase buffer without enzyme. This treatment led to removal of phosphate from the Cx45 protein in transfected HeLa cells as demonstrated in Fig. 5 (lane *2*) and did not alter mobility of the ^{35}S-methionine-labeled protein (lane *3*).

Immunoblot Analyses of Several Mouse Tissues

Figure 6 shows an immunoblot analysis with anti Cx45 in extracts of several embryonic and adult mouse tissues previously shown to express high levels of Cx45 mRNA (Hennemann et al., 1992*a*). In all cases (lung, brain, and kidney; lanes *A-E*) only a single band of 45 kDa was found. Unexpectedly, this band was relatively weak in embryonic skin (E19).

The antibodies to Cx45 detected no protein in extracts of adult skin (lane *F*) and adult liver (lane *G*) used as negative controls.

Immunolocalization of Cx45 in Kidney

Figure 7*A* and *C* illustrates that immunoreactivity with anti-Cx45 was most prominent in distal tubules and glomeruli, whereas proximal regions did not show any labeling. Polyclonal antibodies to Cx32 and Cx26 clearly labeled the epithelium of proximal tubules (*data not shown*), while immunostaining of the collecting ducts, distal tubules and glomeruli was not detected. None of these structures in kidney was labeled by Cx43 antibodies at this stage of development.

Discussion

In this study we have shown that affinity-purified antibodies, directed to a peptide of 138 C-terminal amino acids of mouse Cx45 as part of a fusion protein with glu-

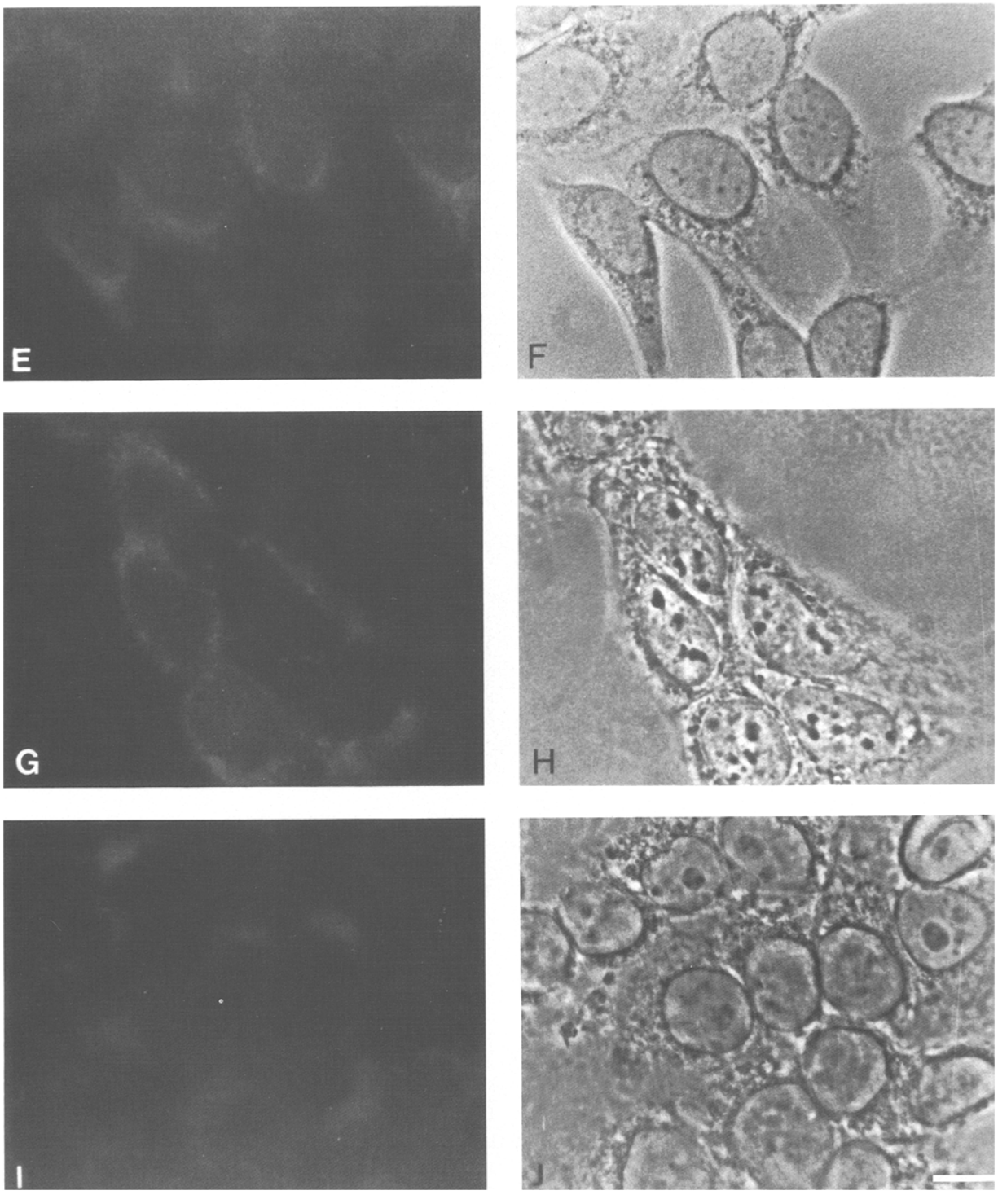

Fig. 3. *Continued.*

tathione S-transferase, detect their antigen in HeLa cells transfected with mouse Cx45 cDNA. Since the parental HeLa cells are deficient in intercellular communication (Eckert et al., 1993) and do not express Cx45 transcripts, expression of exogenous connexin genes after transfection allows the study of properties of gap junctions consisting of a single type of connexin. We found that the affinity-purified Cx45 antibodies recognized the Cx45 protein specifically after immunoblot, immunofluorescence, and immunoprecipitation. As ex-

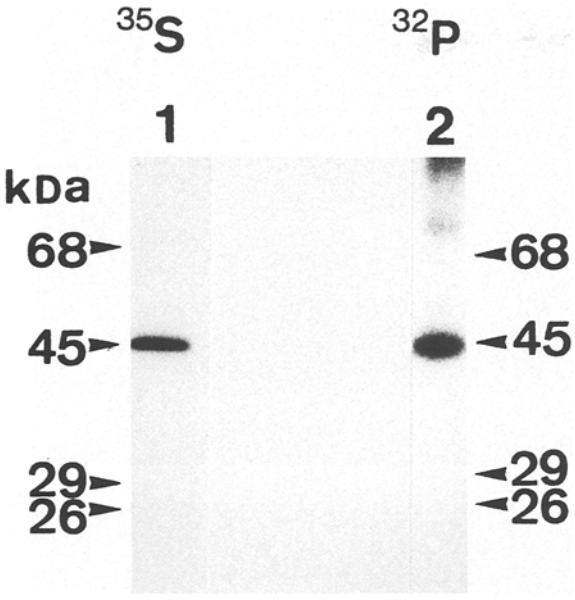

Fig. 4. Phosphorylation and methionine labeling of Cx45 protein in HeLa/Cx45 transfectants. HeLa/Cx45 transfectants were metabolically labeled with either ^{35}S methionine (lane *1*) or ^{32}P orthophosphate (lane *2*), before being lysed in RIPA buffer. Cell lysates were immunoprecipitated with affinity-purified Cx45 antibodies. After SDS-PAGE, only one band at 45 kDa was visible.

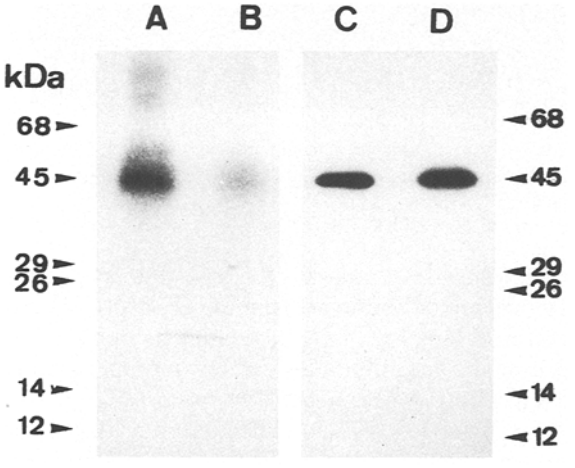

Fig. 5. Dephosphorylation of Cx45 protein in HeLa-transfected cells after treatment with alkaline phosphatase. Metabolically labeled cells (lanes *1* and *2* with ^{32}P orthophosphate; lanes *3* and *4* with ^{35}S methionine) were immunoprecipitated and incubated with alkaline phosphatase (lanes *2* and *3*) or in phosphate buffer without enzyme (lanes *1* and *4*). This treatment resulted in an almost complete removal of phosphate from the Cx45 protein (lane *2*) and did not alter mobility of the ^{35}S-methionine-labeled protein (lane *3*).

pected for a functional gap junction protein, the transfected HeLa cells expressed Cx45 on contact membranes of adjacent cells, but in addition showed some intracellular, perinuclear localization of immunoreactive

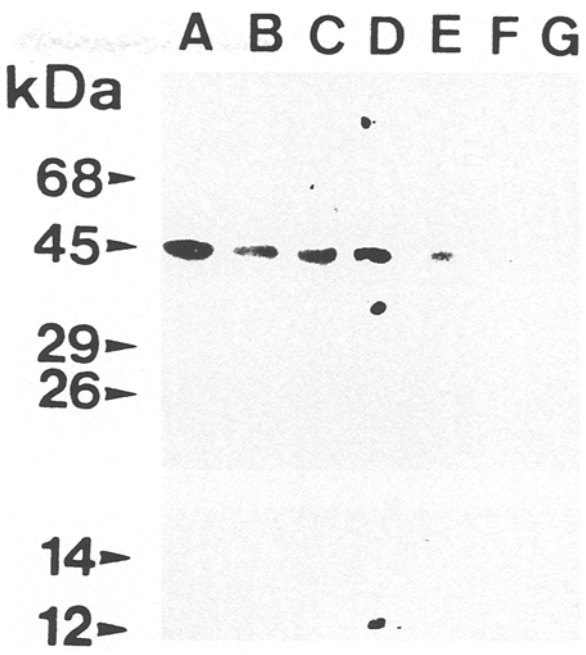

Fig. 6. Western blot analysis of several tissues prepared from 19-day-old embryos or adult mice. Affinity-purified antibodies to Cx45 recognize a single band of 45 kDa in homogenates of adult lung (lane *A*), embryonic brain (lane *B*), embryonic kidney (lane *C*), kidney from four-day-old mice (lane *D*), and embryonic skin (lane *E*). No protein was detected in homogenates of adult skin (lane *F*) and adult liver (lane *G*).

Cx45 protein. This is also seen with HeLa cells expressing other exogenous connexins and is likely to be caused by overexpression of connexins in these cells. Kidney-derived human 293 cells and hamster BHK21 cells show only faint, but specific immunofluorescence with anti-Cx45 on contact membranes. The low reactivity in these cells could be due to low levels of Cx45 protein or to differences in the C-terminal sequence of Cx45 in mouse *vs.* human or hamster.

The Cx45 protein, functionally expressed in HeLa cells, is phosphorylated as shown by metabolic labeling with ^{32}P and immunoprecipitation. Phosphorylation does not alter the electrophoretic mobility of Cx45 in SDS-polyacrylamide gels. This is confirmed by removal of phosphate after treatment with alkaline phosphatase that did not influence the electrophoretic position of the 45 kDa band.

Treatment of Cx45-transfected HeLa cells with the tumor promotor TPA induced a fourfold increased phosphorylation of Cx45 protein, since it is unlikely that an increase of protein after 15 min of incubation caused the intensified incorporation of ^{32}P phosphate. Treatment with cAMP or cGMP resulted in no significant alteration of Cx45 phosphorylation in transfected HeLa cells or the kidney-derived cell lines 293 and BHK21. In contrast, identical treatment of HeLa/Cx40 transfectants led to an increased incorporation of ^{32}P phosphate into the Cx40

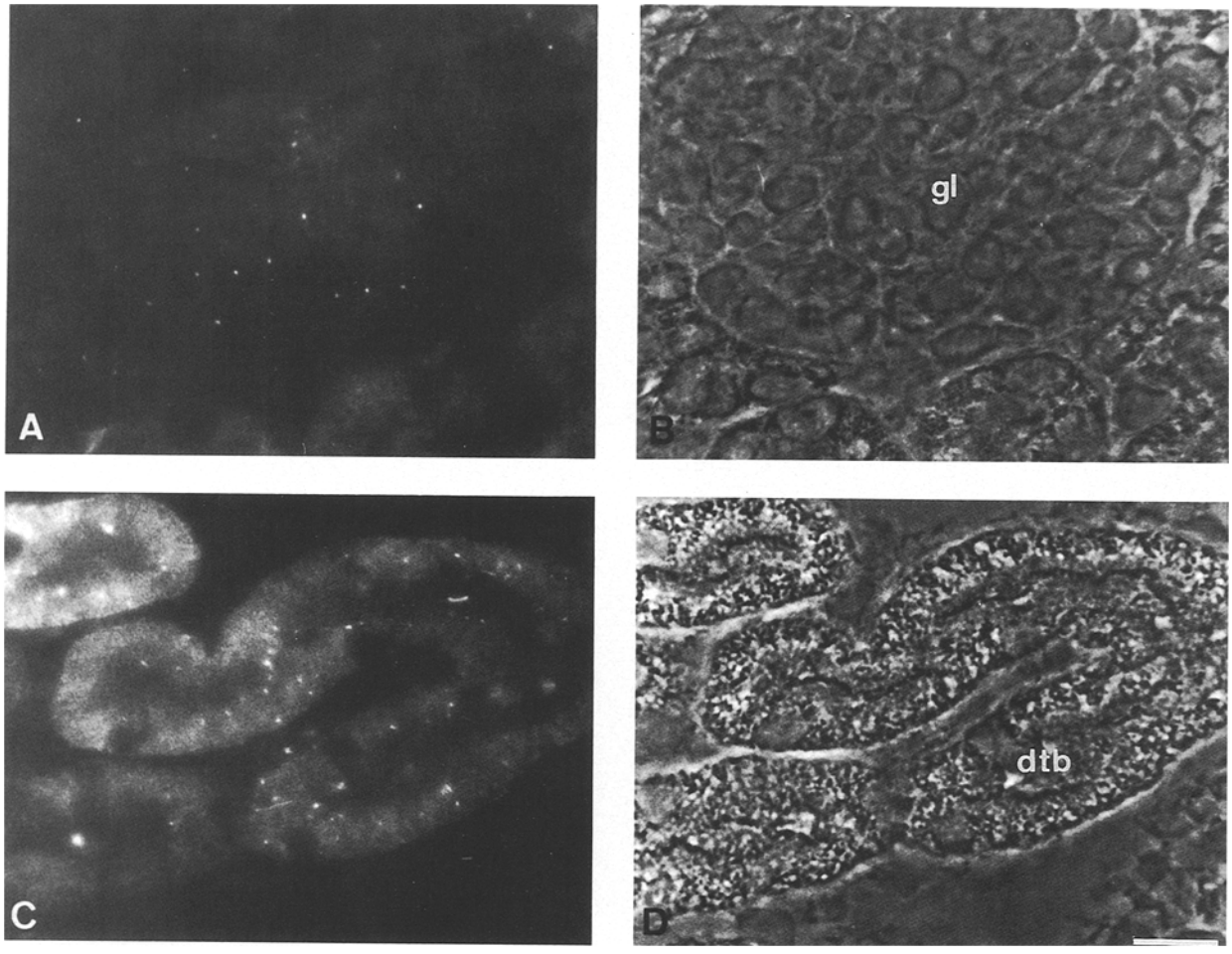

Fig. 7. Immunohistochemical localization of Cx45 gap junction antigen in cross section from kidney of four-day-old mice shown by immunofluorescence (*A, C*) and the corresponding phase-contrast micrographs (*B, D*). (*A*) Localization of Cx45 antigen in a glomerulus and (*C*) distal tubules. Letter code: *gl,* glomerulus; *dtb,* distal tubule; *ptb,* proximal tubule. Scale bar: 7.5 μm.

protein (Traub et al., 1994). Moreover, addition of 8-bromo-cAMP resulted in a 1.6-fold increase of ^{32}P phosphate into immunoprecipitable Cx32 in paired rat hepatocytes (Saez et al., 1986). Traub et al. (1987) had previously shown in mouse hepatocytes that Cx32, but not Cx26, is phosphorylated in the presence of cAMP. Since phosphorylation of Cx45 in HeLa transfectants is stimulated by TPA but not significantly by cAMP, it is likely that a cAMP-independent kinase is involved, for example, protein kinase C. At present, the function of this phosphorylation is unknown — as with all other phosphorylated connexin proteins. Musil et al. (1992) studied phosphorylation of Cx43 in detail but could not distinguish between effects on assembly or gating of Cx43 gap junction channels.

The Cx45 protein shares a similar serine-rich region in the carboxy terminus with Cx43 and some other connexins, containing predicted phosphorylation sites for protein kinase C or other protein kinases. Cx43 has previously been shown to be phosphorylated on serine residues (Musil et al., 1990). We have not yet exactly determined which amino acids in the Cx45 protein are phosphorylated, but there is evidence that serine residues in the last third of the carboxy terminus are involved (A. Butterweck, *unpublished results*).

Veenstra et al. (1992) have demonstrated that chick Cx45 forms gap junctional channels with a unitary conductance of about 30 pS and a strong voltage dependence. Very recently, Steinberg et al. (1994) reported that Cx45 gap junction channels in osteoblastic cells are much less permeable to Lucifer yellow than Cx43 channels. This could mean that Cx45 gap junctions preferentially allow diffusion of relatively small ions and metabolites, whereas Cx43 channels can also be penetrated by bigger molecules such as Lucifer yellow. Gap junctions in the HeLa/Cx45-transfected cells used in this study are clearly permeable by Lucifer yellow (C. Elfgang et al., *in preparation*). Certain metabolites may penetrate through Cx45 gap junction channels more slowly than through other connexin channels. Further

work is required to correlate the permeability properties of Cx45 channels with their specific location in distal tubules and glomeruli of kidney.

The affinity-purified antibodies to Cx45, described in this paper, react specifically with Cx45 in adult lung as well as embryonic brain, kidney, and skin, as shown by immunoblot analyses. We analyzed these tissues because work from our laboratory (Hennemann et al., 1992*a*) had shown that Cx45 mRNA was most abundant in them. Previously characterized antibodies to canine Cx45 protein (Kanter et al., 1992) have not been reported to be characterized by immunoblot of tissue extracts. Our immunoblot results show that Cx45 protein is expressed in relatively high amounts in adult lung as well as in embryonic brain and kidney. Unexpectedly, however, embryonic skin (E19) expresses much less Cx45 protein than the other embryonic tissues tested, although the amounts of Cx45 mRNA in these tissues are similar. Furthermore, expression of Cx45 protein in kidney of four-day-old mice was significantly lower than in embryonic kidney (E19), although our previous analyses had shown that the amounts of Cx45 mRNA were highest in kidney at day 4, postnatally, and decreased thereafter. Apparently, the amount of Cx45 protein decreases before that of Cx45 mRNA during late stages of kidney development.

We have detected expression of Cx45 protein in glomeruli and distal tubules of kidneys in four-day-old mice. In contrast, Cx32 and Cx26 are both expressed in cells of proximal renal tubules. Sainio et al. (1992) found that the expression of Cx32 and Cx26 transcripts in kidney increased significantly during development and after birth. Conversely, Cx43 transcript was expressed at high abundance during early development, but the expression decreased dramatically after birth. Sainio et al. suggested a cell-specific utilization of different gap junction genes during different stages of kidney organogenesis. Based on results with experimentally induced metanephric mesenchyme, they found that the Cx43 gene is induced during the early segregation of the secretory tubule, while Cx32 and Cx26 expression is not detected until advanced stages of development. Therefore, they suggested that Cx43 may contribute to compartmentalization, whereas Cx32 and Cx26 may be correlated with the physiological activity of the proximal tubules. Similar investigations with Cx45 could give information whether this protein is more likely involved in organogenesis, especially tubulogenesis, than in physiological function(s) of the kidney.

We cannot exclude yet that other connexins (with the exception of Cx43) are coexpressed with Cx45 in renal cells. For example, in isolated canine ventricular myocytes, Kanter et al. (1992, 1993) have shown that Cx45 is coexpressed together with Cx40 and Cx43. It appears that each cell type expresses a characteristic combination of connexin proteins for gap junctional communication. The antibodies characterized in this paper should help to unravel the contribution of Cx45 to this complex pattern of intercellular communication.

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