Isolation of Extracellular Recombinant Fragment of Rat Connexin-43

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Analysis of membrane topology of connexin-43 (Cx-43) made it possible to determine one of its extracellular fragments (E2): Q173-1208. The nucleotide sequence of this fragment was cloned into pCBDQ and pHPML vectors containing the sequences of calmodulin-binding domain (CBD) and HPML-domain of Ca-ATPase of human hPMCA4b cells plasma membrane. This yields two chimeric proteins with N-terminal 6-histidine motif containing the extracellular fragment Cx43 E2 and one of hPMCA4b domains (Cx43-CBD and Cx43-HPML). The latter were inserted into the recombinant polypeptide to improve solubility and enhance immunogenicity of the product. Affinity-purified on Ni-NTA agarose recombinant Cx43-CBD was used for immunization of mice and obtaining of monoclonal antibodies. Primary selection of clones was carried out by solid-phase IEA with immobilized Cx43-HPML and by immunoblotting with Cx43-HPML. The positive clones were tested immunohistochemically on rat brain sections. This two-stage testing made it possible to select two hybridomas, which produced monoclonal antibodies to Cx43 in native conformation. The resultant antibodies can be used *in vitro* and *in vivo* for immunophenotyping of various Cx43-positive cells.

Key Words: *connexin-43; gap junction; monoclonal antibodies; glioma*

Connexin-43 (Cx43, CXA1_RAT) is an integral membrane protein forming nexuses (gap junctions) between astrocytes in definitive nervous tissue and between cardiomyocytes and cells of the heart conduction system. These nexuses are involved in transmission of intercellular signals controlling proliferation, differentiation, apoptosis, and cell migration both during normal ontogeny and during invasion of tumor cells of glial origin [4,7-9,11,14]. The latter explains acute interest to Cx43 as a potential molecular target in the therapy of gliomas.

Similarly to other proteins of its family, Cx43 consists of four transmembrane domains and two short extracellular loops, while the N- and C-terminal regions of this protein are located in the cytoplasm [13]. In the membrane, Cx43 forms hexamers (connexons). The extracellular domains of Cx43 play an important role in the organization of functionally active gap junctions. Due to interaction with similar fragments in the neighboring cell, these structures promote assembly of full-value gap junctions from the connexin semichannels. The cells equipped with this communication tool can exchange certain intracellular molecules, ions, and second messengers [4,13]. Experiments showed that blocking of these fragments (specifically, with polyclonal antibodies) can disturb the assembly of gap junctions and exchange with intracellular agents [10]. In light of this, it is extremely important to produce the immunogenic preparations of recombinant extracellular Cx43 fragments.

Our aim was to construct recombinant extracellular Cx43 fragment (the second extracellular loop E2)

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integrated into highly immunogenic chimerical protein and to produce specific monoclonal antibodies interacting with E2 fragment in native conformation.

MATERIALS AND METHODS

Membrane topology of Cx43 was analyzed using HMMTOP, TMHMM, and TMPred software. This analysis determined amino acid sequences of two extracellular fragments involved in the formation of gap junctions. Further experiments were performed with Cx43 fragment Q173-I208 (QWYIYGFSLSAVYTCKRDPCPHQVDCFLSRPTEKTI of 36 AAR with molecular weight 4.28 kDa and pI=7.87), which corresponded to the second extracellular loop (E2) in Cx43, whose sequence was characterized with the smallest homology with other connexins. In order to enhance the expression of this fragment and to elevate immunogenicity of recombinant polypeptide, it was necessary to construct a hybrid consisting of the required fragment sequence and a larger "carrier". Two different domains of Ca-ATPase from the plasma membrane of human hPMCA4b cells were used as possible "carriers": C-terminal calmodulin-binding domain (CBD, 1057-1205 AAR, 17 kDa) and the first cytoplasmic loop (HPML, 166-371 AAR, 22.5 kDa). These domains demonstrated high immunogenicity for induction of antibodies and high expression level in prokaryotic cells [5,6,12,15].

To construct the hybrid proteins, the insert containing the nucleotide sequence of extracellular Cx43 fragment was cloned into pCBDQ and pHPMLQ vectors [5].

Analysis of nucleotide and protein sequences and design of the primers were performed with the help of DNAStar (Lasergene) software. Amplification of the sequence of extracellular loop of Cx43 from cDNA of rat brain library was carried out with primers CX43_173F: 5'-GATCAGATCTCAGTGGTACATCTATGGGT-3' and CX43_173B: 5'-GATCAAGCTTA GATGGTTTTCTCCGTGGGAC-3' (SibEnzim) containing the recognition sites for Bg1II and HindIII, respectively.

Isolation of total RNA from rat brain and obtaining the first strands of cDNA were made with an SV Total RNA Isolation System (Promega) according to producer's guide. The resulting RNA (2 μg) was supplemented with oligo$(dT)_{18}$ primer (500 ng) and incubated at 70°C for 3 min. The mixture was placed on ice, added with iRNAsin ribonuclease inhibitor (40 U) isolated from human placenta (Promega), buffer for reverse transcriptase M-MLV (Promega), dNTP (up to 0.4 mM), and reverse transcriptase M-MLV (200 U, Promega). After incubation at 37°C for 2 h, the nucleic acids were purified successively from the proteins by extractions with a phenol-chloroform mixture (1:1) and chloroform, thereafter they were precipitated with ethanol. The resulting precipitate was resuspended in the deionized water (50 μl).

The fragments of cDNA encoding the selected region of Cx43 protein were amplified by PCR with Taq-polymerase and cloned into plasmid vector pGEM-T (Promega). The resulting plasmid DNA were used to establish structural correctness of cDNA fragments by sequencing (Genom Cooperative Research Center). The plasmid DNA without errors in the coding sequence were used to clone into the expressing vectors HPMLQ and CBDQ inserted with the help of recognition sites of Bg1II and HindIII restrictases. To express HPMLQ-Cx43 and CBDQ-Cx43 with plasmid DNA, we transformed *E. coli* cells SG13009 (Qiagen).

The transformed cells were cultured at 37°C in 100 ml LB medium containing 50 μg/ml carbenicillin and 25 μg/ml kanamycin to optical density to 0.7-0.9 at 600 nm, thereafter plasmid expression was induced with 1 mM isopropylthiogalactoside (IPTG). Incubation was performed under the same conditions for 4 h, thereafter the cells were precipitated at 3000*g* for 20 min, frozen, and stored at -70°C.

The proteins were isolated under denaturation conditions on Ni-NTA-agarose according to Qiagen recommendations. The obtained protein preparations were analyzed by electrophoresis in 12.5% PAAG with 0.1% sodium dodecyl sulfate.

The purified recombinant proteins were used to immunize the mice (20 μg per animal). Subcutaneous immunization with Freund's complete adjuvant (DiaM) was performed in 4-5 cycles with 10-12-day intervals. After two weeks, the mice were subjected to intraperitoneal buster immunization with the purified preparation; the blood was drawn after 7 days.

The mice with documented immune response were used to produce monoclonal antibodies. Isolation of splenocytes and fusion with SP/20 myeloma cells was performed as described elsewhere [1]. The hybridomas were screened by IEA with Cx43_173HPML and Cx43_173CBD immobilized on the solid phase. Then, we applied the dissolved (1:20,000) conjugate of goat antibodies raised against Fab-fragments of mouse immunoglobulins (Goat antimouse peroxidase conjugated Ab, A9917, Sigma) and TMB (00-2023, Zymed) ready-to-use solution as the substrate mixture. The positive clones selected by IEA were tested with immunoblotting with the same antigens. Subsequent visualization was performed with an ECL-advance kit (GE Healthcare) according to producer's recommendations. At the final stage, the supernatants of hybridomas were immunohistochemically tested on rat brain sections in order to select clones of hybrid cell producing monoclonal antibodies interacting with ex-

tracellular Cx43 fragment in native conformation.

Purification of monoclonal antibodies from the ascetic fluid was carried out by affinity chromatography on agarose with immobilized G-protein (Invitrogen) according to manufacturer's recommendations.

Immunochemical analysis of antibodies was performed on frozen sections of rat brain perfused with 4% neutral paraformaldehyde. The role of secondary antibodies was given to antispecies biotinylated antibodies mouse immunoglobulins BA 2000 (Vector Lab). The immunochemical reaction was visualized using ABC-Standard kit (Vector Lab) according to manufacturer's recommendations. All dilution and washing procedures were performed with phosphate-salt buffer at pH 7.4 containing 0.2% Tween-20, 0.2% Triton X-100, and 1% normal horse serum.

RESULTS

Cloning of the insert into pCBDQ and pHPML vectors followed by *E. coli* transformation yielded two strains producing chimeras of extracellular Cx43 fragment (AAR 173-208) with N-terminal polypeptides CBD and HPML (Table 1). The yield of proteins during hyperexpression under standard conditions (37°C, 4 h in the presence of 1 mM IPTG in LB medium) was 10 and 15 mg/liter culture for Cx43_173CBD and Cx43_173HPML, respectively.

The apparent electrophoretic mobility and purity of the resulting recombinant proteins were determined by disk electrophoresis in PAAG with sodium dodecyl sulfate. Electrophoretic mobility of Cx43_173CBD and Cx43_173HPML corresponded to the molecular weight of 25 and 30 kDa, respectively. According to the disk electrophoresis data, the purity of these proteins was no less than 90% (Fig. 1). An extra band near 17 kDa on the electrophoregram of Cx43_173CBD preparation No. 3 probably indicates the presence of free CBD formed as a result of partial hydrolysis of Cx43_173CBD or premature termination of translation in some bacteria under the conditions of hyperexpression. A small amount of admixtures in preparations No. 2 and No. 5 can result from aggregation of the purified proteins.

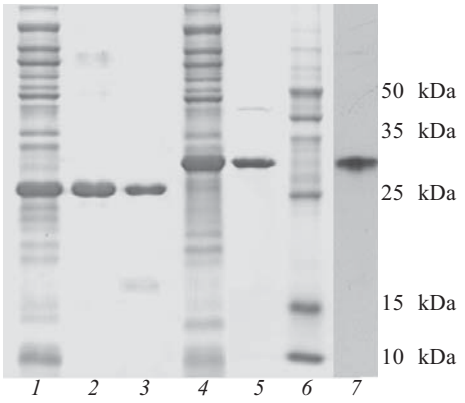

Fig. 1. Electrophoretic analysis of recombinant polypeptides Cx43_173CBD and Cx43_173HPML in 12.5% PAAG with sodium dodecyl sulfate. *1*) cell lysate of Cx43_173CBD hyperproducer strain; *2*, *3*) purified Cx43_173CBD preparations; *4*) cell lysate of Cx43_173HPML producer strain; *5*) purified Cx43_173HPML preparation; *6*) molecular weight standard (Promega) V8491; *7*) immunoblotting of Cx43_173HPML performed with purified monoclonal antibodies against Cx43_173CBD, antispecies antibodies (Goat antimouse HRP-conjugated Ab; A9917, Sigma), and chemoluminescent reagent ECL-advance (GE Healthcare).

The obtained preparations of Cx43 extracellular fragment were used for immunization of mice in order to obtain the monoclonal antibodies. Immunochemical specificity of affinity-purified monoclonal antibodies was characterized by immunoblot analysis. To protect the analytical procedure from the effect of anti-

TABLE 1. Physicochemical Parameters of Chimeric Recombinant Proteins

Name	Molecular weight, kDa	Molecular weight by electrophoresis in PAAG, kDa	pI	Primary structure
Cx43_173CBD	22.5	25	6.02	MRGSHHHHHHLKEAGHGTTKEEITKDAEGLDEIDHAEMELRRGQILWF RGLNRIQTQIKVVKAFHSSLHESIQKPYNQKSIHSFMTHPEFAIEEELP RTPLLDEEEEENPDKASKFGTRVLLLDGEVTPYANTNNNAVDCNQV QLPQSDSSLQSLETSVRQWYIYGFSLSAVYTCKRDPCPHQVDCFLS RPTEKTI
Cx43_173HPML	27.9	30	8.36	MRGSHHHHHHTAFNDWSKEKQFRGLQCRIEQEQKFSIIRNGQLIQL PVAEIVVGDIAQVKYGDLLPADGILIQGNDLKIDESSLTGESDHVKKSL DKDPMLLSGTHVMEGSGRMVVTAVGVNSQTGIILTLLGVNEDDEGEK KKKGKKQGVPENRNKAKTQDGVALEIQPLNSQEGIDNEEKDKKAVKV PKKEKSVLQGKLTRLAVQIGKAGLLMSQWYIYGFSLSAVYTCKRDPCP HQVDCFLSRPTEKTI

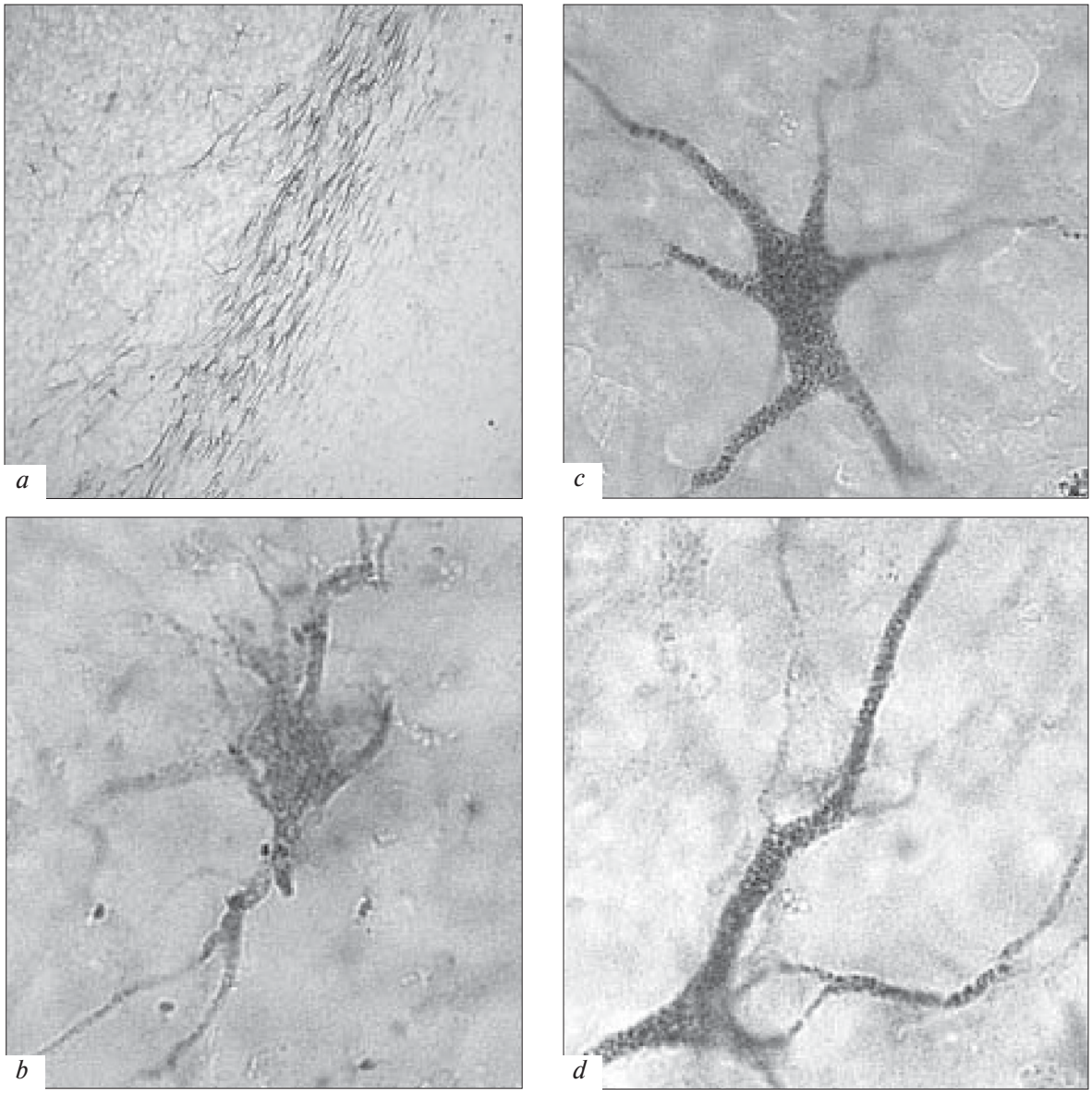

Fig. 2. Immunohistochemical visualization of Cx43-positive astrocytes with monoclonal antibodies against extracellular fragment of Cx43_173. *a*) overview of astrocytic border around glioma, ×100; *b-d*) individual Cx43-positive astrocytes at high magnification, ×1000. Characteristic placoid structures of membranous connexons are visualized. No staining was observed in the absence of specific antibodies.

bodies against the hybrid domains CBD and HPML, the sera and supernatants of hybridomas obtained by immunization with Cx43_173CBD were tested with Cx43_173HPML and vice versa (Fig. 1, *7*).

After confirming affinity interaction of the obtained antibodies with recombinant polypeptide immunogens, we demonstrated immunochemical specificity of these antibodies towards the native form of Cx43. To this end, the clones selected with ELISA and immunoblotting were tested immunochemically on rat brain sections with experimental C6 glioma. These sections were considered as Cx43-positive, because it is widely known that almost all stages of C6 glioma development are accompanied with intensive astroglial border around it [2] consisting of reactive astrocytes characterized by intensive expression of GFAP [13] and Cx43 [9].

Immunoperoxidase visualization of rat brain sections with C6 glioma with monoclonal antibodies against Cx43_173CBD revealed reactive astrocytes around the glioma at the same peritumoral area where they were visualized with the use of anti-GFAP antibodies (Fig. 2, *a*). At high magnification (×1000), reactive astrocytes and their processes demonstrated characteristic granularity corresponding to placoid arrangement of membrane connexons (Fig. 2, *b, c, d*).

Two-stage selection of hybridomas from several dozens of ELISA-confirmed positive clones yielded

two clones most actively producing monoclonal antibodies against native extracellular Cx43 fragment.

Cloning of Cx43 Q173-I208 fragment in chimeric proteins with water-soluble hPMCA4b domains yielded highly immunogenic and well-soluble recombinant proteins containing the sequence of the second extracellular E2 Cx43 loop. After immunization of mice with these proteins and subsequent fusing procedure, we obtained two clones of hybrid cells, whose monoclonal antibodies specifically interacted with Cx43-positive astrocytes on rat brain sections with experimental C6 glioma fixed with paraformaldehyde.

These monoclonal antibodies can be used *in vitro* and *in vivo* for immunophenotyping of various Cx43-positive cells.

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