Connexin45 Interacts with Zonula Occludens-1 and Connexin43 in Osteoblastic Cells*

The relative expression of connexin43 and connexin45 modulates gap junctional communication and production of bone matrix proteins in osteoblastic cells. It is likely that changes in gap junction permeability are determined by the interaction between these two proteins. Cx43 interacts with ZO-1, which may be involved in trafficking of Cx43 or facilitating interactions between Cx43 and other proteins. In this study we sought to identify proteins that associate with Cx45 by coprecipitation in non-denaturing conditions. Cx45 was isolated with a 220-kDa protein that we identified as ZO-1. Under the same conditions, Cx43 was isolated with anti-Cx45 antiserum from Cx45-transfected ROS cells (ROS/Cx45 cells). Cx43 antiserum could also coprecipitate ZO-1 in the transfected and untransfected ROS cells. Double label immunofluorescence studies showed that ZO-1, Cx43, and Cx45 colocalized at appositional membranes in ROS/Cx45 cells suggesting that all three proteins are normally associated in the cells. Additionally, we found that in vitro translated ZO-1 binds to the carboxyl-terminal of Cx45 indicating that there is a direct interaction between the carboxyl-terminal of Cx45 and ZO-1. These studies demonstrate that ZO-1 interacts with Cx45 as well as with Cx43, and suggest that the interaction of connexins with ZO-1 may play a role in regulating the composition of the gap junction and may modulate connexin-connexin interactions.

Gap junction channels permit the transport of low molecular weight substances between the cytoplasm of neighboring cells. Each gap junction channel is made of two hemichannels, which are composed of six subunits called connexins (1). At least 14 different connexin isoforms have been identified in the mouse. Different tissues express different combinations of connexins, which might allow the formation of heteromeric or heterotypic channels and facilitate the modulation of gap junction permeability in these cells (1). Alterations in gap junction permeability affect many biological phenomena including cellular differentiation and development, metabolic homeostasis, and electrotonic coupling of excitable tissue (2).

Osteoblastic cells express connexin43 (Cx43)1. Cx45 and Cx46 (3). Although Cx46 is retained within the exocytic pathway in these cells (4), Cx43 and Cx45 are expressed on the plasma membrane and form gap junction channels. Cx43 and Cx45 have different molecular permeabilities, and changes in the relative expression of Cx43 and Cx45 in bone cells can alter the permeability of gap junctions and the expression of bone matrix proteins (2, 5). These results suggest that interactions among different gap junction proteins might alter cell-cell communication in cell networks thereby modulating the expression of bone matrix proteins and cell activities. However little is known about the processes through which different gap junction proteins interact.

One protein that may be involved in the trafficking or organization of gap junction proteins is the tight junction-associated protein ZO-1. Cx43 associates with ZO-1 (6, 7), a member of the membrane-associated guanylate kinase family of proteins, all of which contain three distinct amino acid motifs that mediate protein-protein interactions: up to three PSD95/Dlg/ZO-1 (PDZ) domains, an Src homology 3 domain, and a guanylate kinase domain (8). Membrane-associated guanylate kinases bind to the carboxyl termini of membrane proteins and to internal domains of other membrane-associated guanylate kinases, thereby organizing these proteins into two-dimensional multi-protein complexes at cell-cell boundaries (9). Mutational analysis of the interaction between Cx43 and ZO-1 suggests that the second PDZ domain of ZO-1 interacts with the carboxyl-terminal region of Cx43 (6, 7, 10).

To understand the interactions between Cx43 and Cx45 in osteoblastic cells, in the current work we sought to identify proteins that interact with Cx45 and in particular wished to determine whether ZO-1 interacts with Cx45 as well as with Cx43. We found that ZO-1 could be isolated with Cx45 from ROS/Cx45 cells using a coimmunoprecipitation assay. Immunofluorescence studies showed that Cx45 colocalized with ZO-1 and Cx43 in the transfected cells. We found that in vitro translated ZO-1 bound to an oligopeptide corresponding to the final 12 amino acids in Cx45, suggesting that ZO-1 recognizes the carboxyl-terminal of Cx45.

MATERIALS AND METHODS

Reagents and Plasmid—Anti-Cx43 (11) and anti-Cx45 antiserum (5) were previously characterized. Polyclonal and monoclonal antibodies directed against ZO-1 were obtained from Zymed Laboratories Inc. (South San Francisco, CA) whereas the monoclonal antibody directed against Cx43 was purchased from Chemicon (Temecula, CA). Unless otherwise noted, all reagents were purchased from Sigma.

1 The abbreviations used are: Cx, connexin; ZO-1, zonula occludens-1; PDZ domains, PSD95/Dlg/ZO-1 domains; IP, immunoprecipitation buffer; PBS, phosphate-buffered solution; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; RIP, radioimmune precipitation buffer.
Transfected Connexin45 Interacts with ZO-1

otherwise noted, reagents were purchased from Sigma. Myc-tagged ZO-1 (ZO-1myc) in pBluescript was a gift from Dr. Alan Fanning in the Dr. J. M. Anderson Laboratory (Yale University, New Haven, CT) (12).

Cell Culture and Transfection—ROS 17/2.8 cells are an osteosarcoma cell line that has been shown to express Cx43 and Cx46 but not Cx45 (13). Our experiments showed that these cells express ZO-1 and N-cadherin and suggest that they do not express ZO-2 or occludin (data not shown). ROS cells were cultured in minimum Eagle’s medium containing 10% heat inactivated bovine calf serum containing 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 5 units/ml penicillin, and 5 μg/ml streptomycin. The Cx45-transfected ROS/Cx45 cells had been reported previously (5, 14). Stably transfected ROS cells were selected and cultured in the same culture media containing 400 μg/ml G418.

Immunoprecipitation—The coimmunoprecipitation protocol was adapted from a procedure used to precipitate ZO-1-associated proteins (15, 16). Cells were labeled with [35S]methionine (100 μCi/ml) (Amerham Pharmacia Biotech or ICN, Costa Mesa, CA) for 2 h in methionine-depleted minimum Eagle’s medium containing 10% calf serum. The media was removed, and the cells were harvested by scraping and subsequently were solubilized in mildly dissociating conditions with an IP buffer containing PBS, 1% Triton X-100, 0.5% CHAPS, 0.1% SDS, and protease inhibitors (1:100 dilution of a protease inhibitor mixture (Sigma)). The antigen-antibody complexes were collected with protein A-Sepharose or protein A-Sepharose and anti-ZO-1 polyclonal antibody. Control peptides used in this study included the amino acids 373–382 of rat Cx43 (CSRPRPDPSKNCSTT), which we call Cx43 CT, and amino acids 367–378 on chicken Cx45 (CSKSGASAGH), which we call Cx45 CT. Antisera were transferred to Immobilon-P membranes. The membranes were blocked with PBS/1% Triton X-100/2% normal goat serum overnight and then incubated for 1 h in primary antibody. We used monoclonal anti-ZO-1 (Zymed Laboratories Inc.) at a 1:1000 dilution, monoclonal anti-Cx43 (Chemicon) at a 1:1000 dilution, or rabbit anti-Cx45 antiserum at a 1:1000 dilution (alone or in combination) as primary antibodies. The coverslips were washed and then incubated for an hour in secondary antibody. In some experiments the cells were not radioactively labeled, and the immunoprecipitated material was analyzed by immunoblotting.

Immunoblot—Proteins immunoprecipitated by anti-Cx45-antibodies were transferred to Immobilon-P membranes. The membranes were probed with 1:1000 dilution of monoclonal antibody directed against Cx43 (Chemicon) or ZO-1 (Zymed Laboratories Inc.). All blots were then probed with the appropriate peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals or Jackson Immunoresearch, West Grove, PA)) and developed with the SuperSignal West Pico chemiluminescence system (Pierce).

Immunofluorescence Microscopy—ROS cells were cultured on cover-slips to 60–80% confluence and fixed in 50% methanol/50% acetone for 2 min at room temperature and permeabilized in 1% Triton X-100/PBS. The cells were blocked with PBS/1% Triton X-100/2% normal goat serum overnight and then incubated for 1 h in primary antibody. We used monoclonal anti-connexin-32 (Chemicon) at a 1:1000 dilution, monoclonal anti-Cx43 (Chemicon) at a 1:1000 dilution, or rabbit anti-Cx45 antiserum at a 1:1000 dilution (alone or in combination) as primary antibodies. The coverslips were washed and then incubated for an hour in secondary antibody. In some experiments we used a 1:2000 dilution of fluorescein 3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA)) and developed with the SuperSignal West Pico chemiluminescence system (Pierce).

RESULTS

A 220-kDa Polyepitope Precipitates with Cx45 in ROS/Cx45 Cells—We identified proteins that associate with Cx45 in ROS/Cx45 transfectants by immunoprecipitation of Cx45-containing complexes in mild detergent conditions (Fig. 1A). Detergent lysates were made from [35S]methionine-labeled cells solubilized in IP buffer (PBS containing 1% Triton X-100, 0.5% CHAPS, and 0.1% SDS). Anti-Cx45 antibodies were added to the lysate, antigen-antibody complexes were collected with protein A-Sepharose, and the immunoprecipitated material was extensively washed with IP Buffer or the more stringent RIPA buffer (PBS containing 1% Triton X-100 and 0.6% SDS) (Fig. 1A) prior to analysis by SDS-PAGE and fluorography. As shown in Fig. 1A, the immunoprecipitated material derived from ROS/Cx45 cells revealed a 45-kDa band in both IP buffer-washed and RIPA buffer-washed samples. However, the IP buffer-washed samples also contained a 220-kDa band that was not detected in the RIPA buffer washed precipitates. As expected, nothing was detected in the IP or RIPA buffer-washed precipitates from the untransfected ROS cells (Fig. 1A). To confirm that this 220-kDa coimmunoprecipitated band occurred in cells expressing endogenous Cx45 as well as in the ROS/Cx45 transfectant, we isolated Cx45 immunoprecipitates from the osteoblastic cell line UMR 106–01, which expresses endogenous Cx45, and again detected a band that migrated at ~220 kDa (Fig. 1B). The molecular mass of this polyepitope is similar to that of ZO-1, a protein that is known to bind to Cx43.

ZO-1 and Cx43 Are Present in Cx45 Immunoprecipitates—We next asked whether the 220-kDa protein that coimmunoprecipitated with Cx45 was ZO-1. In the experiments shown in Fig. 2A, ROS cells, ROS/Cx45 cells, and UMR cells were harvested and solubilized in IP buffer, and the soluble material was immunoprecipitated with an anti-Cx45 antiserum. The antigen-antibody complexes were collected with protein A-Sepharose, and the precipitated material was washed with either IP buffer or RIPA buffer and analyzed by immunoblotting with a monoclonal antibody directed against ZO-1. ZO-1 was found in IP buffer-washed anti-Cx45 immunoprecipitates derived from ROS/Cx45 cells but not in the RIPA buffer-washed immunoprecipitates (Fig. 2A). As expected, the anti-Cx43 antibody did not precipitate detectable ZO-1 from ROS cells.

Because Cx43 is a known binding partner of ZO-1 and our previous data suggest an interaction between Cx43 and Cx45 in cells expressing both connexins (14), we asked whether Cx43...
Proteins immunoprecipitated from transfected ROS cells were analyzed by immunoblotting with a monoclonal antibody directed against Cx43, showing that Cx43 was associated with the anti-Cx45 immunoprecipitate from the ROS/Cx45 cells (Fig. 2B). The anti-Cx45 antiserum did not immunoprecipitate Cx43 from the untransfected ROS cells. Similarly there was no Cx43 in the RIPA buffer-washed immunoprecipitates derived from either cell line.

**ZO-1 Immunoprecipitates with Cx43 in ROS and ROS/Cx45 Cells**—To confirm the interactions identified above, we performed coimmunoprecipitations from radioactively labeled ROS and ROS/Cx45 cells using anti-Cx43 serum. The immunoprecipitated proteins were then analyzed by SDS-PAGE and fluorography (Fig. 3A). Anti-Cx43 precipitated both 45-kDa and 220-kDa radioactively labeled polypeptide proteins from both cell lines. There were more of both proteins in the ROS/Cx45 cells than in the ROS cells. Another striking feature of these immunoprecipitates is that other than the 45-kDa and 220-kDa proteins there are no other [35S]methionine-labeled proteins that copurify with Cx43 on these gels.

To confirm that the anti-Cx43 antiserum also immunoprecipitated ZO-1, anti-Cx45 immunoprecipitates that were derived from unlabeled ROS and ROS/Cx45 were subject to immunoblotting with a monoclonal antibody directed against ZO-1. ZO-1 was found in anti-Cx43 immunoprecipitates derived from each cell type (Fig. 3B). The immunoprecipitates derived from ROS/Cx45 cells had more ZO-1 than the immunoprecipitates derived from ROS cells. These data confirm that anti-Cx43 antiserum can isolate ZO-1 in the ROS cells.

**Cx45 Colocalizes with ZO-1 and Cx43 in Cx45-transfected ROS Cells**—Subsequently we examined the localization of Cx45, ZO-1, and Cx43 in the transfected ROS cells by confocal microscopy of immunofluorescence microscopy. The merged image shows that there is substantial colocalization of Cx45 and ZO-1 in both cell lines. Merging the Cx45 (Alexa-488) and ZO-1 (CY3) micrographs revealed areas where the two signals colocalized. While plasma membrane Cx45 was associated with ZO-1, some of the ZO-1 at the plasma membrane was not associated with Cx45. In contrast there was very little Cx45 staining seen in the ROS cells. Thus Cx45 at the cell surface colocalized with ZO-1, consistent with the data above suggesting that these proteins could associate.

We next confirmed that Cx43 also colocalized with Cx45 in these cells as was seen previously (14). The Cx45-transfected cells were simultaneously stained with an antibody directed against Cx43 and Cx45 as seen in Fig. 5. In ROS cells not expressing Cx45, Cx45 was present at cell-cell boundaries and some staining in cytoplasmic vesicles, while minimal staining was seen with the Cx45 antibody. The anti-Cx43 antiserum and the anti-Cx45 antibody stained the ROS/Cx45 cells in discrete spots along the plasma membrane, and a significant amount of staining was evident in a perinuclear region as well. The merged image shows that there is substantial colocalization of Cx43 and Cx45 in the ROS/Cx45 cells. These results are consistent with the hypothesis that transfected Cx45 associates with Cx45 in the ROS/Cx45 cells.

**ZO-1 Binds to the Cx45 Carboxyl-terminal**—ZO-1 binding usually occurs via interactions with the carboxyl terminus of membrane proteins. To determine whether ZO-1 binds to the carboxyl-terminal of Cx45 oligopeptides corresponding to the final 12 amino acids in Cx45 (Cx45CT), the carboxyl-terminal...
panels, the Cx45 and Cx43 images were merged to show revealed with a CY3-conjugated donkey anti-mouse IgG. In the Alexa-488-conjugated donkey anti-rabbit IgG, and mouse antibody was monoclonal antibody against Cx43. Rabbit antibodies were seen with lized, and stained with rabbit antibodies against Cx45 and a mouse
ROS cells. Furthermore Cx45 and ZO-1 colocalize in the same immunoprecipitates suggests that it is unlikely that Cx43 and Cx45 bind to the same PDZ domain.

It is still unclear what role ZO-1 plays in the life cycle of a connexin. One hypothesis is that ZO-1 makes a scaffold that temporarily secures the different connexins in gap junction plaques at the cell-cell boundary. The amino acid sequences of the carboxyl-terminal of all of connexins, with the exception of Cx32, end in a hydrophobic residue suggesting that they might bind to PDZ domains in ZO-1 (18). This might indicate that sets of connexins that bind to ZO-1, like Cx45 and Cx43, could be found in the same gap junctions along with ZO-1. In contrast Cx32 and Cx43 sort to different plasma membrane locales in thyroid epithelial cells, and only Cx43 colocalized with ZO-1 (19). It is also possible that interaction with a ZO-1 scaffold stabilizes the connexin at the gap junction. Recent data from Toyofuku et al. are consistent with this notion as Cx43 that cannot interact with ZO-1 (due to mutation or phosphorylation by c-Src) turns over much more rapidly than Cx43 that can interact with ZO-1 (10). In a scaffolding model, the different domains of ZO-1 serve as docking modules for kinases and phosphatases that interact with the different connexin polypeptides. ZO-1 could then serve a common function in the life cycle of a number of different connexins by providing a docking platform for these signaling molecules.

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REFERENCES
1. Goodenough, D. A., Goliger, J. A., and Paul, D. L. (1996) Ann. Rev. Biochem. 65, 569
2. Lecanda, F., Warlow, P. M., Sheikh, S., Furlan, F., Steinberg, T. H., and Civitelli, R. (2000) J. Cell Biol. 151, 931–943
3. Steinberg, T. H., Civitelli, R., Geist, S. T., Robertson, A. J., Hick, E., Veenstra, R. D., Wang, H. Z., Warlow, P. M., Westphale, E. M., Laing, J. G., and Beyer, E. C. (1994) EMBO J. 13, 744–750
4. Koval, M., Harley, J. E., Hick, E., and Steinberg, T. H. (1997) J. Cell Biol. 137, 847–857
5. Lecanda, F., Towler, D. A., Ziambaras, K., Cheng, S. L., Koval, M., Steinberg, T. H., and Civitelli, R. (1998) Mol. Cell. Biol. 9, 2258
6. Toyofuku, T., Yabuki, M., Oyos, K., Kusuya, T., Hori, M., and Tada, M. (1998) J. Biol. Chem. 273, 12725–12731
7. Giepmans, B. N., and Moolenaar, W. H. (1998) Curt. Biol. 8, 931
8. Fanning, A. S., and Anderson, J. M. (1999) J. Clin. Invest. 103, 767–772
9. Gonzalez-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000) Semin. Cell Biol. 11, 315–324
10. Toyofuku, T., Zhang, H., Akamatsu, Y., Kusuya, T., Tada, M., and Hori, M. (2001) J. Biol. Chem. 276, 1780–1788
11. Laing, J. G., Tadros, P. N., Westphale, E. M., and Beyer, E. C. (1997) Exp. Cell Res. 236, 482–492
12. Fanning, A. S., Jameson, B., Jessaitis, L., and Anderson, J. M. (1998) J. Biol.
13. Steinberg, T. H., Civitelli, R., Geist, S. T., Veenstra, R. D., Wang, H. Z., Westphale, E. M., and Beyer, E. C. (1993) Mol. Biol. Cell 4, 329 (abstr.)
14. Koval, M., Geist, S. T., Kemendy, A. E., Westphale, E. M., Civitelli, R., Beyer, E. C., and Steinberg, T. H. (1995) J. Cell Biol. 130, 987–995
15. Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M., and Tsukita, S. (1999) J. Cell Biol. 147, 1351–1363
16. Haskins, J., Gu, L., Wittchen, E. S., Hibbard, J., and Stevenson, B. R. (1998) J. Cell Biol. 141, 199–208
17. Deleted in proof
18. Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 275, 73–77
19. Guerrier, A., Fonlupt, P., Morand, I., Rabilloud, R., Audebet, C., Krutovskikh, V., Gros, D., Rousset, B., and Munari-Silem, Y. (1995) J. Cell Sci. 108, 2609–2617
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