

# Dominant negative effect of connexin33 on gap junctional communication is mediated by connexin43 sequestration

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## Summary

Gap junctional intercellular communication is involved in the control of cell proliferation and differentiation. Connexin33, a member of the multi-gene family of gap junction proteins, exerts an inhibitory effect on intercellular communication when injected into *Xenopus* oocytes. However, the molecular mechanisms involved remain to be elucidated. Our results show that connexin33 was only expressed within the seminiferous tubules in the testis. In contrast to the majority of connexins, connexin33 was unphosphorylated. Immunoprecipitation experiments revealed that connexin33 physically interacted with connexin43, mainly with the phosphorylated P1 isoform of connexin43 but not with connexin26 and connexin32, two other connexins expressed in the tubular compartment. In Sertoli cells and COS-7 cells, connexin43 was located at the plasma membrane, whereas in connexin33 transfected

cells, the specific association of connexin33/43 was sequestered in the intracellular compartment. High-resolution fluorescent deconvolution microscopy indicated that the connexin33/43 complex was mainly found within early endosomes. Sequestration of connexin33/43 complex was associated with a complete inhibition of the gap junctional coupling between adjacent cells. These findings provide the first evidence of a new mechanistic model by which a native connexin, exerting a dominant negative effect, can inhibit gap junctional intercellular communication. In the testis, connexin33 could exert a specific role on germ cell proliferation by suppressing the regulatory effect of connexin43.

Key words: Cx33, Cx43, Dominant negative, Cx33-Cx43 interaction, Testis

## Introduction

Gap junctional intercellular communication (GJIC) is involved in development, cell growth and cell differentiation, homeostasis and neoplastic transformation (Loewenstein, 1981; Yamasaki and Naus, 1996). Gap junctions are intercellular channels, formed by connexins (Cx), which allow direct intercytoplasmic diffusion of molecules smaller than 1 kDa (ions and signaling mediators) between two adjacent cells (Goodenough et al., 1996). Cxs oligomerize to form two juxtaposed hemichannels, or connexons, which are defined as homomeric when composed of the same Cx or heteromeric when at least two different Cxs form the connexon. Cxs are expressed in a tissue-specific manner and various cell types express more than one Cx. To date, 20 Cxs have been identified (Willecke et al., 2002) and the combination of different Cxs allows the modulation of gap junction permeability (Kumar and Gilula, 1996; Goldberg et al., 1999) and selectivity (Goldberg et al., 1999). The functional properties of heteromeric channels have been studied so far by dye coupling and electrophysiological methods in mammalian cell lines

and/or in the paired *Xenopus* oocyte system (Verselis and Veenstra, 2000).

A key observation indicates that coexpression of Cx33 with other Cxs inhibits the junctional conductance (Chang et al., 1996). However, the molecular mechanism(s) by which Cx33 impairs GJIC is unknown. We hypothesized that Cx33 might interfere with Cx function by associating with them. As the expression of Cx33 is mainly restricted to the testis (Haefliger et al., 1992; Tan et al., 1996; Chung et al., 1999) and to differentiating neuroblasts (Rozental et al., 1998), this prompted us to analyze the direct association of Cx33 with other Cxs expressed in the testis, in particular Cx43. This issue is of primordial importance because gap junctional coupling via Cx43 is essential for spermatogenesis (Juneja et al., 1999; Plum et al., 2000; Roscoe et al., 2001). In the present study, we show that Cx33 is able to associate selectively with Cx43 but not with Cx26 and Cx32. We further demonstrate that this selective complex occurred between Cx33 and two phosphorylated isoforms of Cx43 and is intracellularly sequestered within early endosomes. Most importantly, the

association formed by Cx33 and Cx43 causes a complete inhibition of the GJIC. Our data emphasize a new physiological control of gap junctional communication by intracellular sequestration of a functional Cx.

## Materials and Methods

### Cx33 antisera and expression plasmid

The anti-Cx33 antibody was obtained by immunizing rabbits with a peptide mapping C-terminal domain of rat Cx33: S<sup>272</sup>-M<sup>286</sup> (Haefliger et al., 1992) conjugated by glutaraldehyde to keyhole limpet hemocyanin (KLH). Cx33 antibody was affinity-purified from rabbit polyclonal sera. The binding specificity of the different fractions in the ELISA was confirmed by inhibition tests performed in the presence of an excess of Cx33 peptide. The rat Cx33 cDNA coding sequence was amplified by PCR from a rat testis cDNA library with forward primer 5'-CGCGGGGGATCGCCATGAGTGATTGGAGTGCCTT-3', reverse primer 5'-CGCGGGTCTAGACATATAGAAGGAAGAAAGTCCCA-C-3' and cloned in-frame into pcDNA3.1 expression plasmid with or without a myc/his tag (Invitrogen). The sequence of the rat Cx33 cDNA was confirmed by complete sequencing. The Cx43 plasmid was from ResGen (RG001053, Invitrogen) and EGFP-Rab5 (Rosenfeld et al., 2001), EGFP-Rab7 (Bucci et al., 2000) and EGFP-Lamp1 (Lebrand et al., 2002) plasmids were gifts from A. Galmiche (Inserm U 452, Nice, France).

### Cell culture and transfection

The 42GPA9 Sertoli cell line was maintained in Dulbecco's Modified Eagle's Medium (GIBCO BRL) containing 10% fetal calf serum at 32°C (Bourdon et al., 1998). Sertoli and COS-7 cells, seeded on glass coverslips were transfected with expression plasmids for Cx33, Cx33-myc, Cx43, EGFP-Rab5, EGFP-Rab7 and EGFP-Lamp1 alone or in combination (3 µg, at a ratio of 1:1) using Fugene reagent (Roche Molecular Biochemicals) or DEAE/Dextran (Amersham Biosciences) (Seed and Aruffo, 1987) and grown to confluency. To generate a Sertoli cell line expressing Cx33, cells were transfected with 1 µg Cx33 or Cx33-myc plasmid cDNA using Fugene reagent. Control cells were transfected with empty vector. After 48 hours, transfected cells were selected in complete medium containing 0.8 mg/ml of G418 (Geneticin, Gibco BRL). After 3 weeks, more than 100 G418-resistant clones were individually picked, expanded and assayed for Cx33 or myc expression by western blotting.

### Preparation of tissue and cell extracts

Mature rat testes, brain, heart, ovary and uterus were solubilized in NP40/Brij lysis buffer (50 mM Tris-HCl pH 7.5, 1% NP40, 1% Brij 96, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM EDTA, 1 mM aprotinin, 25 mM leupeptin, 1 mM pepstatin, 2 mM phenylmethylsulfonyl fluoride). Triton X-100 solubilization of connexons was prepared as previously described (Koval et al., 1997). Briefly, confluent cells were collected by scraping in ice-cold PBS and centrifuged at 500 g for 5 minutes. The cell pellet was resuspended in detergent-free lysis buffer and lysed with a Dounce homogenizer. The post nuclear supernatant fraction was collected by centrifugation at 500 g for 5 minutes and solubilized with Triton X-100 (final concentration 0.1%) for 30 minutes at 4°C. After centrifugation at 100,000 g for 1 hour, Triton X-100 soluble fractions were subjected to immunoprecipitation.

### Immunoprecipitation and western blotting

Lysates and Triton X-100 soluble fractions were immunoprecipitated with 2 µg of either anti-Cx43 (Transduction Laboratories), anti-Cx33 or anti-myc (gift from JF Tanti, Inserm U568, Nice, France) antibodies for 16 hours at 4°C. The immunocomplexes were

precipitated by incubation with protein A-Sepharose (Pharmacia) for 1 hour at 4°C. Immunopellets were washed four times in PBS 0.5% NP 40, once in PBS, and then eluted with 40 µl reduced Laemmli buffer at 100°C for 5 minutes. Proteins were separated on 5-15% SDS-PAGE, electroblotted onto a polyvinylidene fluoride membrane (PVDF Immobilon-P, Millipore) and analyzed by western blotting with antibodies against Cx43 (1:2000), myc (1:2000), Cx33 (1:1000), Cx26 or Cx32 (1:2000, Santa Cruz Biotechnology) as previously described (Defamie et al., 2001).

### Analysis of Cx33 phosphorylation status

The phosphorylation status of Cx33 was analyzed on testicular immunoprecipitates either by pre-incubating for 4 hours with alkaline phosphatase (Roche) or by western blotting with anti-phosphotyrosine (1:6000, Upstate Biotechnology Incorporated), anti-phosphoserine and anti-phosphothreonine antibodies (1:1000, Zymed). In other experiments, COS-7 cells were transiently transfected with Cx33-myc and Cx43. Two days after transfection, the confluent cells were labeled with [<sup>32</sup>P]orthophosphate (500 µCi) in phosphate-free DMEM for 5 hours, before being subjected to cell lysis with NP40/Bridj buffer as described above and supplemented with phosphatase and protease inhibitor cocktail. Lysates were then immunoprecipitated with myc or Cx43 antibodies. The [<sup>32</sup>P]-phosphorylated proteins were resolved by SDS-PAGE, electroblotted onto PVDF membrane and visualized by autoradiography. The presence of Cx43 and Cx33-myc in immunoprecipitates was verified by western blotting.

### Immunofluorescence staining

The localization of Cx33 and Cx43 in cells or in testis sections was assessed as previously described (Batiat et al., 2000; Defamie et al., 2001). Briefly, 5 µm testis sections or cells were fixed with cold methanol, permeabilized for 5 minutes with saponin buffer (0.5% saponin, 2.5% goat serum, 1% bovine serum albumin and 0.2% gelatin in PBS) and incubated overnight with Cx33, Cx43 and myc (1/100) antibodies at 4°C. The slides were rinsed with 0.5% saponin-PBS, incubated with FITC-conjugated anti-rabbit (DAKO) and Texas red-conjugated anti-mouse (Pharmacia) antibodies and mounted in Mowiol medium (Calbiochem). Pictures were taken with a ×63 magnification lens using a confocal microscope (Leica) fitted with a 488 or 543 nm krypton/argon laser allowing simultaneous analysis of the fluorescein and rhodamine chromophores. One representative section was chosen from the total series of 20 optical sections. Testes sections or cells incubated with the control rabbit and mouse IgG showed no staining.

For determining the subcellular localization of Cx43 and Cx33, Sertoli cells and COS-7 cells were cotransfected with EGFP-Rab5, EGFP-Rab7 or EGFP-Lamp1 and with Cx33-myc. Cells at confluency were fixed with cold methanol and then incubated with a mixture containing anti-Cx43 (1:100) and anti-myc (1:1000) antibodies. A polyclonal antibody (1:300) (Louvard et al., 1982) that was a gift from H. Zahraoui (Institut Curie, Paris France) was used to detect the endoplasmic reticulum (ER). To detect the Golgi apparatus, an anti-Golgi CTR433 monoclonal antibody (1:10, a gift from M. Bornens, Institut Curie, Paris France) was used. Antibodies were revealed with the anti-mouse IgG coupled to rhodamine, anti-rabbit IgG coupled to Cyan-5, and anti-rabbit IgG coupled to FITC. Deconvolution microscopy analysis of the immunoreactive signals was performed as described (Savino et al., 2001; Segretain et al., 2003).

### Dye transfer analysis

GJIC was assessed by the microinjection dye transfer method. Briefly, stably Cx33-myc transfected cells were microinjected with 10% Lucifer Yellow CH (Sigma) and 1% rhodamine dextran (Sigma) dissolved in 1 mM LiCl using a Narishige microinjector (IM300,

Narishige Co., Japan) and an Eppendorf micromanipulator (5171, Eppendorf, Germany). Fluorescence emission and dye transfer were examined after 5 minutes by confocal laser scanning microscopy using a Biorad MRC 1024 equipped with a 15 mW argon-krypton gas laser. The confocal unit was attached to an inverted microscope (Olympus IX70, Japan). For each condition, at least 20 microinjections were performed and the average number of communicating cells was estimated. Analysis of variance (ANOVA) with additional post hoc analysis (Newman-Keuls test) was applied to compare coupling between control cells (transfected with the empty vector) and cells stably transfected with the vector containing Cx33-myc cDNA. Differences between means were considered significant when  $P < 0.05$ .

## Results

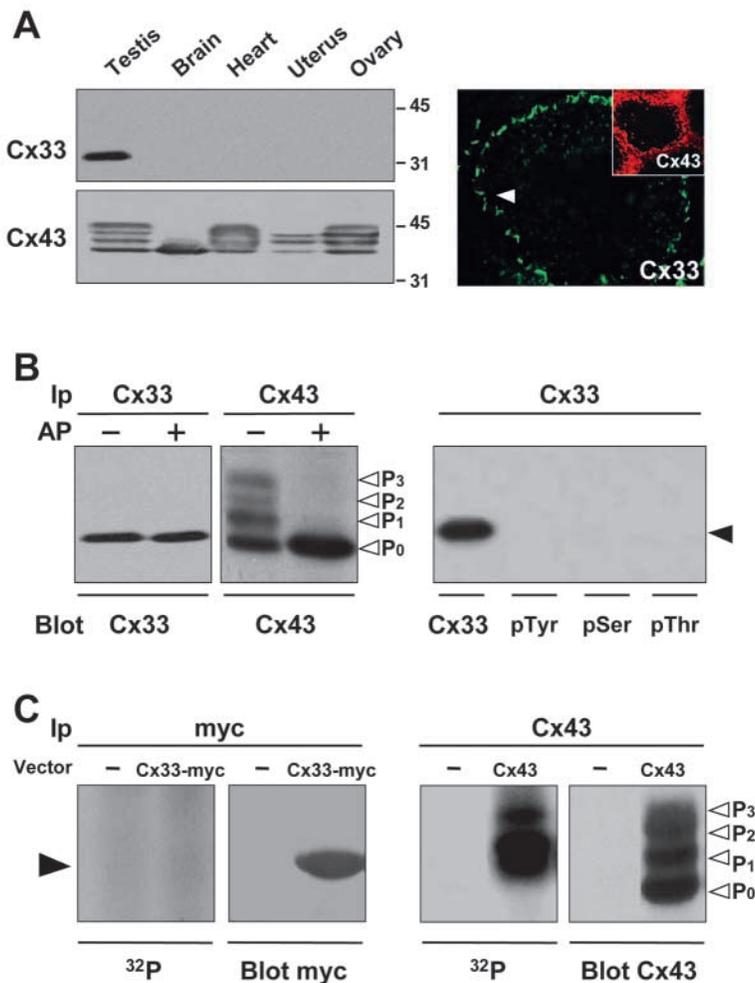
### Tissue specificity and unphosphorylated form of Cx33

To address the association of Cx33 with other Cx, we first sought to identify the tissue(s) expressing Cx33. Western blot analysis using a polyclonal anti-Cx33 reveals the presence in testis lysates of one immunoreactive band at about 33 kDa (Fig. 1A, left panel), consistent with the predicted molecular weight of Cx33 (Haefliger et al., 1992). This band was not detected in brain, heart, uterus or ovary even after overexposure of the blot. In contrast, Cx43, which was used as loading control, was found in all examined tissues as several bands between 39–45 kDa, as expected (Fig. 1A, left panel). Immunofluorescence

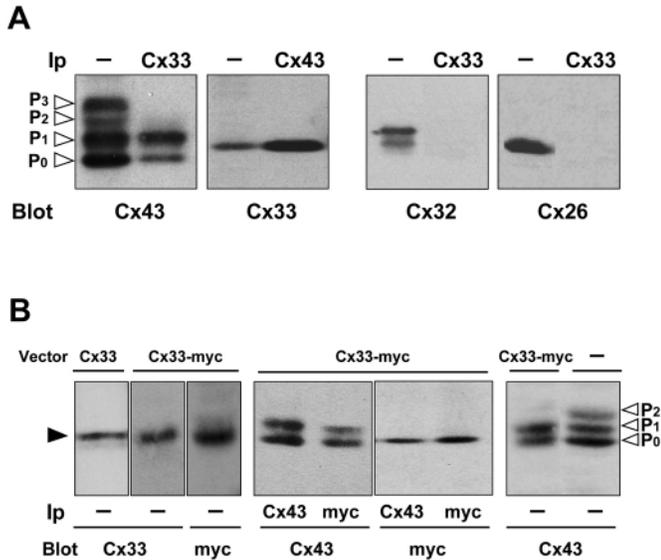
analysis demonstrates that Cx33 was specifically localized within the basal region of seminiferous tubules whereas Cx43 was expressed in both tubular and interstitial compartments (Fig. 1A, right panel). Alkaline phosphatase treatment indicates that the bands detected by western blot with the anti-Cx43 antibody corresponded to phosphorylated and unphosphorylated isoforms of the protein (Fig. 1B, left panel). However, this treatment did not modify the size of immunoprecipitated Cx33. Western blots of Cx33 immunoprecipitates with antibodies against phosphotyrosine, phosphoserine or phosphothreonine did not reveal a signal (Fig. 1B, right panel). The specificity of the three antibodies used was verified using Cx43 (data not shown). Consistently, Cx33-myc transfected COS-7 cells, prelabeled with [ $^{32}$ P] and immunoprecipitated with anti-myc antibody exhibited no [ $^{32}$ P] labeling (Fig. 1C, left panel). In contrast, [ $^{32}$ P] labeled forms migrating at about 43–45 kDa were observed in Cx43 immunoprecipitates of COS-7 cells transfected with the vector containing Cx43 cDNA (Fig. 1C, right panel). The presence of the Cx33-myc and Cx43 were detected in the same PVDF membranes by western blot analysis with Cx43 and myc antibodies.

### Specific association of Cx33 with Cx43

To determine if Cx33 and Cx43 were able to interact, Cx33



**Fig. 1.** Presence of Cx33 in the rat testis. (A) Anti-Cx33 western blot analysis detected a band migrating at 33 kDa in lysates of testis but not of brain, heart, uterus and ovary. The anti-Cx43 antibody detected the presence of different phosphorylated Cx43 isoforms in all tissues (left panel). The numbers on the right indicate molecular mass (kDa). Immunolocalization of Cx33 in seminiferous tubules (right panel). Note that the Cx33 signal is only present in the basal compartment of seminiferous tubule ( $\times 120$ ) whereas Cx43 is detected in both tubular and interstitial compartments (inset). (B,C) Characterization of the unphosphorylated status of Cx33. (B) Testis immunoprecipitates (Ip) with anti-Cx33 or anti-Cx43 antibodies were treated with (+) or without (-) alkaline phosphatase (AP) and analyzed by western blotting with Cx33 or Cx43 antibodies (left panels). Testis immunoprecipitates with anti-Cx33 were analyzed by western blot with Cx33, phosphotyrosine (Ptyr), phosphoserine (Pser) or phosphothreonine (Pthr) antibodies (right panel). (C) COS-7 cells were transfected with Cx43, Cx33-myc or empty vector (-) and labeled with [ $^{32}$ P]orthophosphate. Cell lysates were then immunoprecipitated (Ip) with myc (left panel) or Cx43 (right panel) antibodies and separated by SDS-PAGE. The [ $^{32}$ P] radiolabeled proteins were visualized by autoradiography and the presence of Cx33-myc and Cx43 was verified by western blotting with myc or Cx43 antibodies. The positions of Cx33 and Cx43 isoforms are shown by arrowheads. Figures are representative of three separate experiments. Open arrowheads indicate positions of unphosphorylated and phosphorylated forms of Cx43.



**Fig. 2.** Selective association of Cx33 with Cx43. (A) Testis lysates were untreated (–) or immunoprecipitated (Ip) with anti-Cx33 or anti-Cx43 antibodies. Lysates and immunoprecipitates were analyzed by western blotting with Cx43, Cx33, Cx32 and Cx26 antibodies; all are detected in testis lysates at the predicted sizes of 39–45, 33, 32 and 26 kDa respectively. (B) Sertoli cells were stably transfected with vectors containing Cx33 and Cx33-myc cDNAs or empty vector as a control (–). Triton X-100 soluble fractions from Sertoli cells were immunoprecipitated with either Cx43 or myc antibodies and analyzed by western blotting with Cx33, Cx43 and myc antibodies. Open arrowheads indicate positions of unphosphorylated and phosphorylated forms of Cx43. Results are representative examples of three separate experiments.

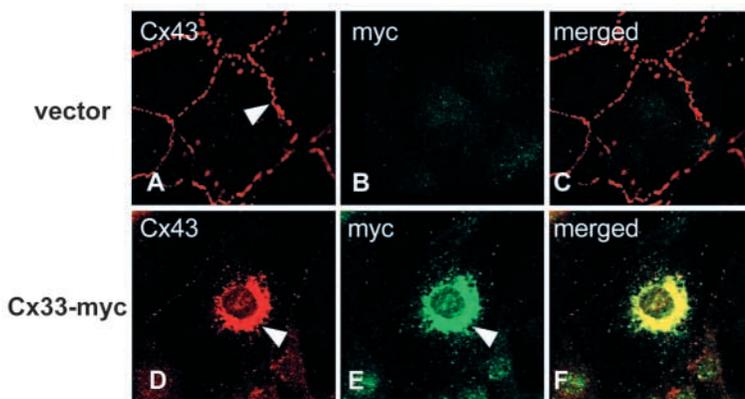
was immunoprecipitated from testis lysates and Cx33-containing complexes were analyzed by western blotting with antibodies against Cx43, Cx26 and Cx32. As shown in Fig. 2A, Cx33 co-immunoprecipitated with Cx43. The association of Cx33 and Cx43 was further supported by the presence of Cx33 in Cx43 immunoprecipitates (left panels). The specificity of this physical interaction was evidenced by the observations that Cx26 and Cx32 did not co-immunoprecipitate with Cx33 (Fig. 2A, right panels). This was also supported by the observations that Cx26 and Cx32 were unable to co-immunoprecipitate

Cx33 (data not shown). Interestingly, two bands instead of four were detected by the anti-Cx43 antibody in Cx33 immunoprecipitates (Fig. 2A, left panel). Alkaline phosphatase treatment indicates that the upper band corresponded to the P1 Cx43 isoform (data not shown). Cx33 also coprecipitated, at a lesser level with a lower molecular weight band corresponding to the unphosphorylated (P0) Cx43 isoform.

To verify the existence of direct physical interaction between Cx33 and Cx43, immunoprecipitation experiments were performed on Triton X-100 soluble Cx oligomers from Sertoli cells. As low levels of Cx33 were found in whole testis homogenates and the Sertoli cell line expressed Cx33 at a barely level detectable only by RT-PCR (data not shown), Sertoli cells were transfected with the vector containing Cx33. As indicated in Fig. 2B (left panels) western blot analysis of Cx33 transfected Sertoli cells reveals the presence of a band at about 33 kDa. Sertoli cells transfected with Cx33-myc shows the presence of a band with the same molecular weight revealed with both anti-Cx33 and anti-myc antibodies consistent with the finding that the myc epitope does not alter Cx33 molecular conformation. The 33 kDa protein was not detected with anti-Cx33 and anti-myc antibodies when cells were transfected with the empty vector (data not shown). The detergent-solubilized fractions from Cx33-myc transfected Sertoli cells were then immunoprecipitated with either anti-Cx43 or anti-myc antibodies. As indicated in Fig. 2B (middle panels), the anti-myc antibody co-immunoprecipitated Cx43. The interaction between Cx33 and Cx43 was further supported by the presence of a band of about 33 kDa revealed with the anti-myc antibody in Cx43 immunoprecipitates. Only two isoforms of Cx43 corresponding to P0 and P1 were detected in Cx33-myc transfected cells whereas three isoforms of Cx43 were observed in both the wild type and Sertoli cells transfected with the vector without Cx33-myc (Fig. 2B, right panel).

### Sequestration of Cx43 by Cx33 in early endosomes

To determine the subcellular localization of the Cx33/Cx43 complex, Sertoli cells were transfected with Cx33-myc cDNA. Punctuate linear staining for Cx43 was observed at appositional plasma membranes between adjacent Sertoli cells transfected with empty vector (Fig. 3A) or in wild-type Sertoli cells (data not shown). No immunoreactive signal was found with the antibody directed against myc (Fig. 3B). In contrast,

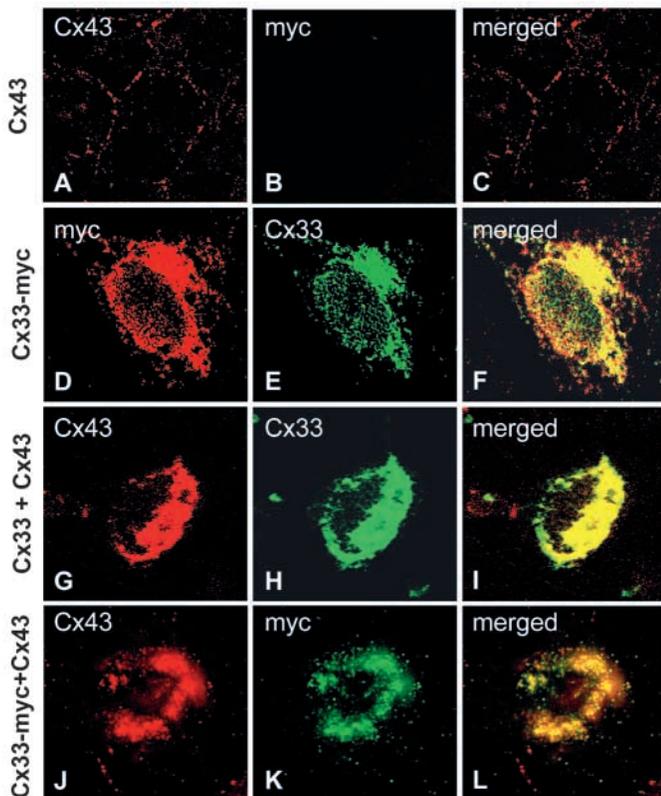


**Fig. 3.** Intracellular localization of Cx33/Cx43 complex in Sertoli cells. (A–F) Cells were transfected with the vector containing Cx33-myc cDNA (D,E,F) or vector alone (A,B,C). In the absence of Cx33-myc, Cx43 was localized as linear punctuate staining at appositional plasma membranes between adjacent Sertoli cells (A, arrowhead). In the presence of Cx33-myc, Cx43 was solely detected in the perinuclear region (D, arrowhead), the myc signal was localized in the same region (E, arrowhead). Merging Cx33 and Cx43 micrographs revealed colocalization of two signals in Cx33-myc transfected Sertoli cells (F). Images are representative of three separate experiments. Magnification,  $\times 600$ .

in Cx33-myc transfected Sertoli cells, Cx43 was no longer detected at the cell surface but mainly in the perinuclear region (Fig. 3D). A similar localization was found for Cx33 detected with the anti-myc antibody (Fig. 3E) or with anti-Cx33 antibody (data not shown). Merging Cx43 and Cx33-myc micrographs reveals a complete colocalization of the two immunoreactive signals (Fig. 3F). Similarly, Cx33 or Cx33-myc intracellularly colocalized with Cx43 in COS-7 cells cotransfected with Cx43 and Cx33 (Fig. 4G-I) or Cx33-myc (Fig. 4J-L). Control experiments were performed in which COS-7 cells were transfected with vectors containing Cx43, Cx33-myc or Cx33 cDNAs. In COS-7 cells transfected only with Cx43, the Cx43 signal was present as expected at the plasma membrane (Fig. 4A-C). In cells transfected with Cx33-myc cDNA, the immunoreactive signal detected with Cx33 antibody was restricted to a region around the nucleus (Fig. 4E) and colocalized with the myc signal (Fig. 4D,F). A similar localization for Cx33 detected with the anti-Cx33 antibody was observed in Cx33 transfected cells (data not shown). Together, the co-immunoprecipitation and microscopy results strongly

suggested that Cx33 sequestered Cx43 intracellularly via a protein-protein interaction.

In order to localize finely the Cx33/Cx43 complex, immunofluorescence microscopy with various markers of the intracellular compartments was performed in Cx33-myc transfected Sertoli cells as previously described (Segretain et al., 2003). High-resolution deconvolution fluorescent microscopy of the signals in transfected Sertoli cells indicates that myc and Cx43 did not colocalize with markers of the Golgi apparatus, lysosomes, late endosomes (Fig. 5E-H,M-P,Q-T) or with the ubiquitin/proteasomal markers (data not shown). A faint colocalization of Cx33-myc/Cx43 was however detected with a marker of the ER (Fig. 5D). In contrast, Cx43 mainly colocalized with Cx33-myc on vesicle positively stained for the early endosomal marker Rab5 (Fig. 5L). In wild-type untransfected Sertoli cells or cells transfected with the empty vector, Cx43 staining was always present at the plasma membrane level (Fig. 3A) and was undetectable in early endosomes.



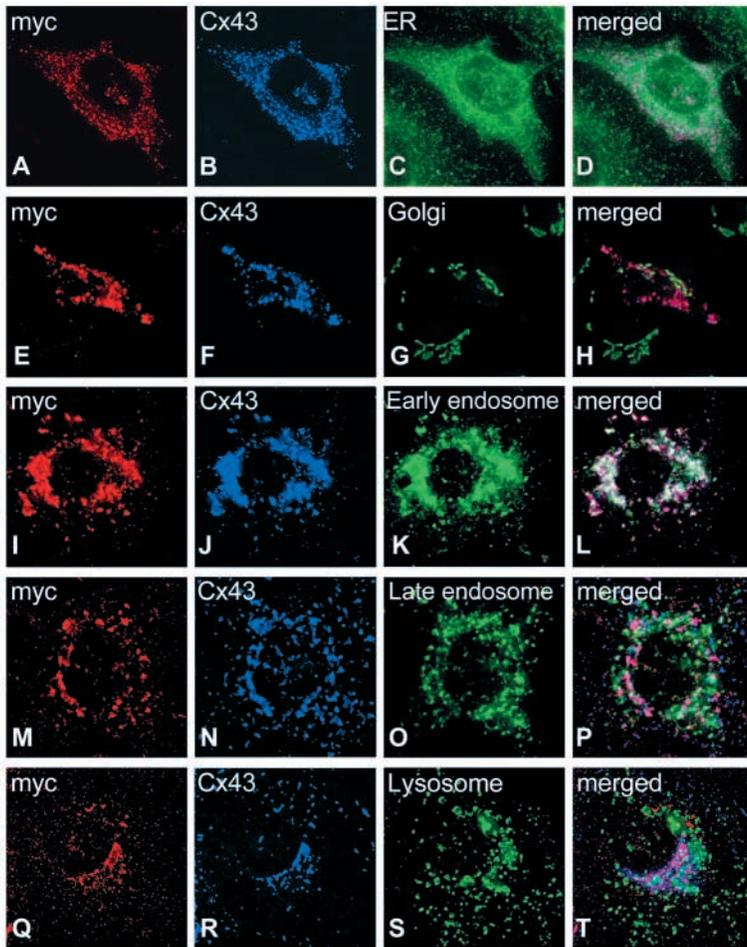
**Fig. 4.** Intracellular localization of Cx33/Cx43 complex in COS-7 cells. (A-C) In the absence of Cx33 or Cx33-myc, Cx43 staining was punctuated and linear at appositional plasma membranes between adjacent COS-7 cells and myc was undetectable. (D-F) In cells transfected with Cx33-myc cDNA the signals for myc (D) and Cx33 (E) colocalized similarly around the nucleus (F). (G-I) Cells were transfected with Cx33 or (J-L) Cx33-myc cDNAs in combination with Cx43. In the presence of Cx33 or Cx33-myc, Cx43 was solely detected in the perinuclear region (G,J). Merging Cx33 (or myc) and Cx43 micrographs revealed colocalization of the two signals in Cx33 or Cx33-myc transfected cells (I,L). Images are representative of three separate experiments. Magnification,  $\times 800$ .

#### Inhibition of gap junctional coupling by Cx33

We therefore investigated the effect of Cx33 on gap junctional coupling. Sertoli cells transfected with the vector without Cx33-myc cDNA were able to communicate through gap junctions as demonstrated by the diffusion of Lucifer yellow from one microinjected cell to neighboring cells (Fig. 6C). Rhodamine dextran, a high molecular weight dye, was localized only inside the microinjected cells (Fig. 6B). Similar results were obtained in wild-type untransfected Sertoli cells. In this condition, the number of coupled cells was  $18.15 \pm 4.9$  (mean  $\pm$  s.d.; number of microinjections = 20). In contrast, in Sertoli cells stably overexpressing Cx33-myc no coupling occurred as indicated by the absence of Lucifer yellow transfer from the microinjected cell to adjacent cells (Fig. 6F). Quantitative analysis demonstrates that the number of communicating cells was markedly reduced ( $1.05 \pm 0.22$ ,  $n = 20$ ,  $P < 0.001$ ).

#### Discussion

There is compelling evidence that GJIC can be controlled at two major levels: (1) combination of Cxs allowing the modulation of gap junction permeability and selectivity; (2) phosphorylation of Cxs that controls the trafficking kinetics to cell surface, gap junction assembly, channel gating and turnover (Musil and Goodenough, 1991; Goodenough et al., 1996; Kumar and Gilula, 1996; Goldberg et al., 1999; Lampe and Lau, 2000). However, this control does not fit the puzzling observations that coexpression of Cx that fail to form homotypic/heterotypic channels occurs in many tissues and cell types (White and Bruzzone, 1996). Of these Cxs, much attention has been focused on Cx33 that acts as an inhibitor of channel function (Chang et al., 1996). However, the molecular basis involved is unknown. The present study provides the first evidence that Cx33, a Cx specifically expressed in the testis, formed a complex with Cx43 that was sequestered in the intracellular compartment within early endosomes and caused marked inhibition of GJIC. This was supported by the selective co-immunoprecipitation of Cx43 with Cx33 in testis lysates and in Cx33 transfected Sertoli cells and COS-7 cells.



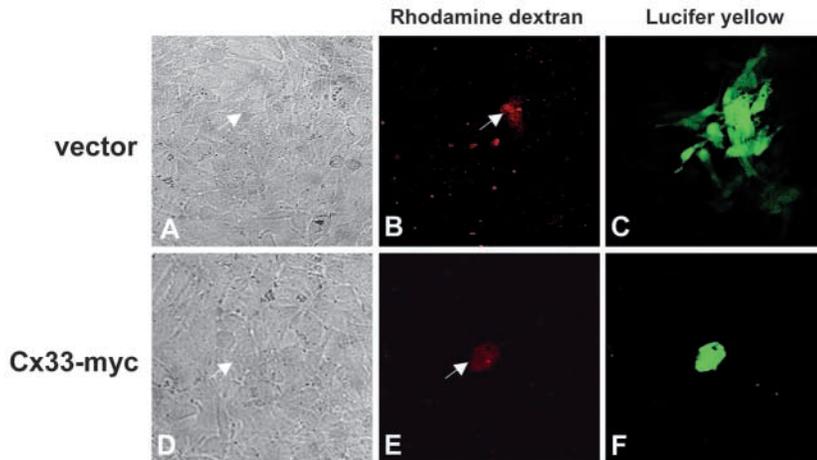
**Fig. 5.** Sequestration of Cx43 within early endosomes in Cx33 transfected Sertoli cells. (A–T) Sertoli cells expressing Cx33 were transfected with EGFP-rab5, EGFP-rab7 or EGFP-lamp vectors or stained with antibodies specific of ER or Golgi apparatus. Fluorescent deconvolution microscopy analysis allowed the simultaneous detection of Cx33 (A,E,I,M,Q), Cx43 (B,F,J,N,R) with Endoplasmic Reticulum (C), Golgi apparatus (G), early endosomes (K), late endosomes (O) or lysosomes (S). (D,H,L,P,T) Merged images of Cx43, myc and EGFP localization. White spots indicate colocalization of the three signals. Note that the Cx33/Cx43 complex colocalizes mainly with EGFP-Rab5 (L) and weakly with ER (D). Results are representative of three separate experiments. Magnification,  $\times 800$ .

Spermatogenesis is a complex process of germ cell multiplication and differentiation that takes place under the control of Sertoli cells. Within the seminiferous tubules, germ cells are closely associated with supporting Sertoli cells and cross talk between these cells is mediated through paracrine regulations, direct cell-cell interactions and communication via gap junctions. In the testis, ten Cx mRNAs are presumably translated within the seminiferous epithelium (Risley, 2000). We and others reported the presence of Cx43 in the basal compartment of seminiferous tubules in both Sertoli and germ cells (Risley et al., 1992; Pelletier, 1995; Batias et al., 2000) and its physiological implication in male fertility (Juneja et al., 1999; Plum et al., 2000; Defamie et al., 2001). More recently, we have characterized the presence of Cx43-based gap junctions between neighboring Sertoli cells, between Sertoli cells and spermatogonia and between Sertoli cells and spermatocytes (Decrouy et al., 2004). The current study demonstrates that Cx33 traps Cx43 intracellularly and inhibits Cx43 gap junctional coupling. Thus, it is tempting to suggest that Cx33, through this process, could alter the formation of Cx43 gap junctions within the seminiferous epithelium. Our results also show that Cx33 was unable to immunoprecipitate and colocalize with Cx26 and Cx32, two other Cxs expressed in the testis but differently localized within the seminiferous epithelium (Risley et al., 1992). Together these data suggest that the selective association of

Cx33 with Cx43 plays an essential role in the control of spermatogenesis.

There is growing evidence that phosphorylation may play a role in Cx43 trafficking and/or assembly (Lampe and Lau, 2000). Interestingly, our data indicate that Cx33 was mainly associated with the P1 phosphorylated isoform of Cx43. The current results also indicate that Cx43, which was normally localized at the plasma membrane, was intracellularly colocalized with Cx33 in Sertoli cells expressing Cx33. This raises the possibility that the interaction between Cx33 and Cx43 contributed to the regulation of Cx43 phosphorylation and subsequent Cx43 trafficking.

Another interesting feature is that Cx33, in contrast to other identified Cxs except Cx26, appeared as an unphosphorylated Cx (Traub et al., 1989; Saez et al., 1990). The reason(s) for such a characteristic are presently unknown. It is well recognized that the length of the cytoplasmic C-terminal tail is essential in the regulation of the protein function since several recognition sites for different protein kinases are present in this domain of the protein (Lampe and Lau, 2000). Among the Cxs identified, Cx26 is the smallest. It has almost no C-terminal tail and is unphosphorylated (Traub et al., 1989; Saez et al., 1990). After Cx26, Cx33 has the shortest C-terminal tail (Haefliger et al., 1992). Thus, it is possible that lack of the phosphorylated isoform of Cx33 may be due to the structural characteristics of this Cx.



	Number of communicating cells
Sertoli cell (vector)	18.15 (± 4.90)
Sertoli cell (Cx33-myc)	1.05 (± 0.22) *

**Fig. 6.** Inhibition of gap junctional coupling by Cx33. (A-F) Phase-contrast and immunofluorescence photomicrographs ( $\times 300$ ) of Sertoli cells transfected with vector containing Cx33-myc cDNA (D,E,F) or vector alone (A,B,C) microinjected with a mixture of Lucifer yellow and rhodamine dextran. No transfer of dye was observed in Sertoli cells overexpressing Cx33 (D-F). By contrast, Lucifer yellow was transferred to adjacent cells in the control (C), whereas rhodamine dextran was localized only within the microinjected cell (B). Microinjected cells are identified with arrows. The average number (mean $\pm$ s.d.,  $n=20$ ) of communicating cells in control (vector alone) and Cx33-myc transfected Sertoli cells is given in the table. \* $P<0.001$ .

Previous studies have shown that in normal or pathological conditions Cx may accumulate in intracellular stores that correspond to the ER-Golgi intermediate compartment (ERGIC) or Golgi apparatus (George et al., 1999). The reasons for the current observation that the Cx33/Cx43 complex colocalized in the early endosomes are presently unknown. Recently, it has been suggested that early endosomes are the first sorting platform from which endocytosed materials are targeted to various intracellular destinations (Deneka and van der Sluijs, 2002). Thus, the possibility that Cx33 acts as a chaperone that alters the stability of the gap junction plaques by maintaining Cx43 in the early endosome before recycling to the plasma membrane or to other cytoplasmic components can be hypothesized. Altered trafficking of the Cx33/Cx43 complex to the plasma membrane could be also considered since it has been demonstrated that early/recycling endosomes can also be part of the exocytic pathway (Laird and Spiess, 2000). In addition, a role of Cx in processes independent of the formation of gap junctional channels has been suggested (Huang et al., 1998; Krutovskikh et al., 2000; Plotkin et al., 2002; Olbina and Eckhart, 2003). Whether the Cx43/Cx33 trapped in the early endosomes could play such roles remains to be elucidated.

Our data also demonstrate that the presence of Cx33 caused a dramatic inhibition of GJIC in Sertoli cells. These results are consistent with previous observations that coexpression of Cx33 with Cx43 dramatically reduces or inhibits the junctional conductance in the paired *Xenopus* oocyte system (Chang et al., 1996). From our data, one can infer that the inhibitory effect of Cx33 on GJIC stems from intracellular sequestration of the Cx33/Cx43 complex. Whether this model is applicable to other Cxs remains to be determined. In this regard, it is noteworthy that the transfection of dominant negative Cx43 constructs leads to aberrant cytoplasmic localization of Cx46 or P0/P1 Cx43 isoforms (Krutovskikh et al., 2000; Lampe and Lau, 2000). So far, such behavior has only been described for

mutated Cx constructs. The current findings provide a new mechanism for the control of gap junctions in which a native Cx (like Cx33) can have dominant negative effects by sequestering Cxs that associate with it. Thus, further studies on the function(s) of Cx33 within the seminiferous epithelium will provide new insights into the role of this Cx in the control of the spermatogenic process.

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