Molecular reorganization of Cx43, Zo-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen

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Summary
The gap junction protein connexin 43 (Cx43) exhibits dynamic trafficking that is altered in most tumor cells and in response to carcinogen exposure. A number of connexin (Cx)-binding proteins are known to be involved in endocytic internalization of gap junctions. Here, we analyzed the discrete molecular interactions that occur between Src, Zo-1 and Cx43 during Cx43 internalization in response to the non-genomic carcinogen γ-hexachlorocyclohexane (HCH). Internalization of the Cx43 gap junction plaque was significantly accelerated in Cx43-GFP transfected 42GPA9 Sertoli cells that were exposed to the carcinogen. HCH induced the rapid recruitment of Src to the plasma membrane, activation of Src within 3 minutes and the efficient inhibition of gap junctional coupling, but had no effect in the presence of the Src inhibitor PP2. Immunoprecipitation experiments demonstrated that HCH increased Cx43-Src interaction and concomitantly decreased Cx43–ZO-1 association. ZO-1 was detected on both sides of the gap junction plaques in untreated cells, but appeared to be mainly localized on one side during HCH-induced internalization. The dissociation of ZO-1 from Cx43 appears to occur specifically on the side of the plaque to which Src was recruited. These findings provide mechanistic evidence by which internalization of the Cx43 gap junction plaque might be initiated, suggesting that Src-mediated dissociation of ZO-1 from one side of the plaque initiates endocytic internalization of gap junctions and that this process is amplified in response to exposure to HCH.

Key words: GJA1, Cx43 gap junction plaque, Endocytosis, Src, ZO-1, Carcinogen

Introduction
Gap junction plaques, composed of associated transmembrane channels or gap junctions, allow the passage of low molecular mass (<1 kDa) signaling molecules between adjacent cells. Gap junctions are present in most tissues with the exception of erythrocytes, platelets, fully differentiated skeletal muscle cells and sperm. They are constituted by the oligomerization of transmembrane proteins called connexins (Cxs) into connexon hemichannels. So far, 21 Cxs have been identified in humans (Sohl and Willecke, 2003). Control of gap junctional intercellular communication (GJIC) occurs at the level of transcription, and is also regulated by multiple post-translational mechanisms, such as Cx assembly and disassembly, gap junction degradation, and modulation of channel properties. Phosphorylation and dephosphorylation of multiple serine, threonine and tyrosine residues in the C-terminal cytoplasmic tail of connexin 43 (GJA1, hereafter referred to as Cx43), have been reported to contribute to the orchestration of these regulatory processes (Lampe and Lau, 2004). Studies in living cells that had been transfected with Cx-GFP chimeric proteins revealed that Cx trafficking is a highly dynamic process that correlates with the short half-life of Cxs (1.5-5 hours), and that gap junction internalization might be also essential for the modulation of gap junction intercellular communication (reviewed by Segretain and Falk, 2004). However, the discrete molecular mechanism that controls endocytic internalization of gap junction plaques has not been clearly identified. Analysis of the process of gap junction internalization is of major interest because altered trafficking of Cxs, often in conjunction with an increase in degradation, has been reported in many tumor tissues and is also a typical feature of most cells exposed to carcinogens (reviewed by Pointis et al., 2007).

There is now clear evidence that Cxs can interact with a large number of signaling and scaffolding proteins that are involved in the control of GJIC (Wei et al., 2004; Herve et al., 2007). Zonula occludens-1 (ZO-1), a member of the membrane-associated guanylate kinase (MAGUK) family, is a major Cx-interacting protein. Cx43 interacts with ZO-1 through its C-terminal region that binds the second PDZ domain of ZO-1 (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). ZO-1 also interacts with other Cxs, such as Cx31.9 (Nielsen et al., 2002), Cx36 (Li et al., 2004), Cx45 (Laing et al., 2001; Kausalya et al., 2001), Cx46 and Cx50 (Nielsen et al., 2003).

Although, evidence has been provided that ZO-1 is required for localization of Cx43 into gap junction plaques (Toyofuku et al., 1998), its presence is not essential for the formation of functional channels, because Cx43 constructs that lack the C-terminal region are able to form gap junction channels and plaques (Fishman et al., 1991; Dunham et al., 1992; Unger et al., 1999). Additional studies have shown that tagged Cxs are able to form gap junction plaques...
(Jordan et al., 1999; Falk, 2000) although Cx43–ZO-1 interaction might be reduced in those fusion proteins (Gaietta et al., 2002). More recently, by using dual immunofluorescence and immunoprecipitation analyses, we and others have reported that ZO-1 can participate in the internalization of gap junction plaques in different cell types, e.g. cardiomyocytes (Barker et al., 2002), astrocytes (Duffy et al., 2004) and Sertoli cells (Segretain et al., 2004). In addition, accumulating evidence suggests that gap junction plaque endocytosis depends on the presence of additional Cx-protein partners. The nonreceptor tyrosine kinase Src is a well-known partner of Cx43, and can reduce the interaction of Cx43 with ZO-1 (Toyofuku et al., 2001). However, previously developed experimental approaches were not sufficient to elucidate the precise intermolecular interactions that occur between these proteins during internalization of gap junction plaques and formation of annular gap junctions. This is partly due to the fact that biochemical techniques based on cell membrane fractionation do not allow the examination of sequential and molecular events that drive these processes.

In this study, we have attempted to dissect the molecular interactions that occur between Cx43 and two of its binding partners, ZO-1 and Src, during the endocytic internalization of gap junctions. We also analyzed the effect of γ-hexachlorocyclohexane (HCH), a non-genomic carcinogen that is known to be a potent inducer of Cx43 internalization, on these molecular events. Our data reveal that a specific interaction between Cx43 and the activated form of the nonreceptor tyrosine kinase Src, concomitantly with a disruption of interaction of ZO-1 with Cx43 – specifically on one side of the gap junction plaque – are among the first events of endocytic internalization of gap junction plaques and formation of annular gap junctions. The current results further support the hypothesis that this mechanistic process may be abnormally accelerated in response to carcinogen exposure.

**Results**

**Accelerated internalization of Cx43-GFP gap junction plaques in response to HCH exposure**

Fluorescence deconvolution microscopy performed on Cx43-GFP-transfected 42GPA9 Sertoli cells allowed to observe many steps of Cx trafficking: cytoplasmic accumulation within the Golgi region (Fig. 1A, upper left panel) labeled using antibody CTR433, gap junction plaque between two adjacent cells at the plasma membrane visualized by the occludin signal (Fig. 1A, upper right panel), cytoplasmic spots of various sizes and shapes representing accumulation of early endosomes as depicted with Rab5 (Fig. 1A, left lower panel), and degradative elements revealed by using the lysosomal marker Lamp2 (Fig. 1A, right lower panel). Colocalization of Cx43-GFP in these different cellular compartments was evidenced by yellow fluorescence (Fig. 1A, insets). After 1-hour exposure to HCH that is known to induce Cx43-based gap junction internalization (Defame et al., 2001), gap junction plaques had disappeared and only large green fluorescent vesicles corresponding to the endocytic internalization of gap junction plaques remained within the cytoplasm of treated cells (Fig. 1B). Kinetics of Cx43-GFP gap junction-plaque internalization and of annular gap junction formation, which we identified as described previously (Segretain and Falk, 2004; Piehl et al., 2007), were then analyzed using time-lapse video-microscopy in untreated control cells and cells that had been treated with HCH (Fig. 1C). The two images at the top show the edge of gap junction plaques identified by the green fluorescent line that was located between two adjacent 42GPA9 Sertoli cells at time point 0. As expected, addition of HCH to the culture medium of Cx43-GFP-transfected cells markedly decreased the time-course of Cx43-GFP internalization. The mean time for the internalization of gap junction plaques was 1164.5±88.4 minutes (n=8) 24 hours after transfection in untreated cells. This time was significantly reduced (to 21.9±7.4 min, n=8, P<0.01) following exposure of the cells to the HCH. Similar effects of HCH were also obtained 48 hours after transfection of Cx43-GFP (data not shown). HCH exposure reduced the number of gap junction plaques in Cx43-GFP-transfected cells in a time-dependent manner, with a maximal significant (P<0.01) effect after a 30-minute exposure to the carcinogen (Fig. 1D).

**Activation of Src is associated with Cx43 gap junction internalization and impaired coupling**

To investigate whether Src activation promotes Cx43 gap junction internalization, 42GPA9 Sertoli cells – which endogenously express Cx43 – were incubated for different time periods in the presence of 50 μM HCH and activation of Src was assessed by western blotting using a specific antibody against phosphorylated Src (anti-Src [pY418]). Since we have previously reported that HCH activates MAP kinases (Mograbi et al., 2003), we also analyzed whether the MAP kinases ERK1/2, p38 and JNK are activated within 60 minutes of HCH exposure, using antibodies directed against their phosphorylated (activated) forms. HCH induced the rapid activation of Src in Sertoli cells (Fig. 2A,B). Significant phosphorylation (P<0.05) of Src occurred already after 3.5 minutes and was maintained throughout the experiment (duration of up to 60 minutes). Similarly, during the same time course, HCH stimulated the phosphorylation of both p42 (ERK2) and p44 (ERK1) (Fig. 2C), in agreement with our previous observations (Mograbi et al., 2003). However, phosphorylation of JNK or p38 was not stimulated by HCH (Fig. 2D).

To better define the role of Src in the early steps of endocytic internalization of gap junction plaques, the numbers of fluorescent gap junction plaques in Cx43-GFP-transfected cells as well as the coupling between cells were evaluated after HCH exposure for increasing periods of time (5-30 minutes), in the presence or absence of the selective Src inhibitor PP2 or the ERK1/2 inhibitor PD98059 (Fig. 3). Whereas PP2 prevented both, the reduction in gap junction plaque number (Fig. 3A) and in cell-cell coupling (Fig. 3C) 5 minutes after exposure to HCH (analyzed by gap FRAP, Fig. 3B), PD98059 did not inhibit HCH-induced effects during those time periods (Fig. 3A,C, right panels). Indeed, PD98059 was unable to block the significant decrease (P<0.05) of these two parameters in response to HCH. Altogether these results suggest that the early steps of gap junction internalization are Src-dependent and MAP kinase-independent.

Endogenous Cx43 was then investigated by immunofluorescence analyses in HCH-exposed Sertoli cells with or without PP2 or the inactive Src family protein tyrosine kinase inhibitor analog PP3 (Fig. 4). As expected, punctate Cx43-specific staining that is characteristic of gap junctions was detected along cell borders, outlining cell boundaries of untreated cells (Fig. 4a). Intensities of plasma membrane-localized Cx43 staining were significantly reduced after 10 minutes of HCH treatment and, instead, appeared as cytoplasmic puncta 2 hours later (Fig. 4b). Addition of HCH and PP2 together mainly prevented the delocalization of the Cx43 (Fig. 4c), whereas addition of PP3 did not inhibit the effect that HCH had on the cells (Fig. 4d). The rapid activation of Src by HCH was associated with the relocation of Src to the plasma membrane (Fig. 4, lower panels).
Indeed, in control cells, Src was detected as numerous diffuse spots that were randomly distributed within the cytoplasm (Fig. 4e). In the presence of HCH, however, Src immunofluorescence changed from an intracellular location to one at the plasma membrane, as evidenced by the strong linear immunoreactive signal between two adjacent cells (Fig. 4f). Sometimes this redistribution was incomplete in all cells observed, and remaining cytoplasmic signals were detected (data not shown). Treatment of Sertoli cells with HCH and PP2 together prevented the carcinogen-induced relocation of Src to the plasma membrane (Fig. 4g), whereas PP3 did not (Fig. 4h).

Effect of HCH on the Cx43–ZO-1 and Cx43-Src complexes during endocytic internalization of Cx43 gap junction plaques

To investigate whether the interaction of Src with Cx43 promotes the internalization of gap junction plaques in cells incubated with or without HCH, we further examined interactions between Cx43 and Src, and between Cx43 and ZO-1, the latter of which is known to participate in the control of gap junction function. Cx43, ZO-1 and Src were immunoprecipitated from Sertoli cells, and the presence of Cx43-containing complexes was assessed by western blotting (Fig. 5). As shown in Fig. 5A, HCH treatment reduced the intensity of the Cx43 signal in the ZO-1 immunoprecipitation (IP), and of the ZO-1 signal in the Cx43 IP. Densitometric analyses confirmed that the intensity of the ZO-1 band in Cx43 IP was reduced by about 36±12% (P<0.05) (Fig. 5A, left panel). By contrast, exposure of cells to HCH enhanced the levels of Cx43 in the Src IP. A weak but clearly detectable band corresponding to Src, verified in the Src IP, was detected in the Cx43 IP in HCH-treated cells but not in untreated control cells (Fig. 5A). As expected, ZO-1 and Src did not immunoprecipitate with each other. Interestingly, the phosphorylated isoforms of Cx43 were upregulated in the Src IP of HCH-exposed Sertoli cells. The significantly enhanced association of Cx43 and Src in response to HCH exposure was confirmed by quantitative analysis of the relative optical densities of Src immunoreactive signals in Cx43 IP (Fig. 5A, right panel, P<0.01).

To test whether the increased interaction of Cx43 and Src in response to HCH exposure requires the activation of Src, cells were...
cultured for 30 minutes in the presence or absence of HCH with or without PP2 (Fig. 5B). Cx43 and ZO-1 IP were subjected to western blot analyses using antibodies that were directed specifically against the phosphorylated form of Src (P-Src). Results presented in Fig. 5B revealed that HCH treatment increased the interaction between Cx43 and P-Src, as evidenced by the stronger P-Src-band intensity. Quantitative analysis revealed that the relative P-Src-signal intensity was approximately seven times higher in Cx43 IP of HCH-treated cells than in untreated cells or in cells exposed to HCH and PP2 together (P<0.01) (Fig. 5B, right panel). The markedly reduced Cx43 IP signal in HCH- and PP2-treated cells suggests that Cx43 is preferentially associated with P-Src (Fig. 5B). In contrast to Cx43, ZO-1 did neither associate with unphosphorylated nor with phosphorylated Src, as evidenced by the inability of ZO-1 to communoprecipitate with P-Src (Fig. 5B).

Analysis of molecular interactions between Cx43, ZO-1 and Src during gap junction endocytic internalization induced by HCH

To reveal the close molecular interactions that might occur between Cx43, ZO-1 and Src during endocytic internalization of gap junction plaques, 42GPA9 Sertoli cells were transiently transfected with Cx43-GFP. Since the 42GPA9 Sertoli cell line constitutively expresses endogenous Cx43 (Lablack et al., 1998; Fiorini et al., 2004) (and our work; Fig. 4), the cell model we used here is a mixed population of cells that express either endogenous Cx43 only or both endogenous Cx43 and Cx43-GFP. We first evaluated the number of adjacent cells that were capable of forming Cx43-GFP plaques and that expressed the tagged Cx43 in one or in both adjacent cells. Approximately 75% of the fluorescent plaques were found between Cx43-GFP expressing cells (identified by the presence of green fluorescence within the cytoplasm) and wild-type cells expressing only endogenous Cx43 (Fig. 6A). ZO-1 was detected near gap junction plaques that had formed between Cx43-GFP expressing cells (Fig. 6B) or between Cx43-GFP transfected and wild-type cells (Fig. 6C). However, in the latter, the intensity of the immunoreactive ZO-1 signal was stronger. This difference confirms that Cx43-GFP could be mixed with endogenous Cx43 within the same gap junction plaques, and that a larger number of ZO-1 polypeptides could be associated with the gap junction plaque that contained endogenous untagged Cx43. Although ZO-1 was expressed at higher levels in
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cells expressing endogenous Cx43, we were unable to determine a role for ZO-1 in the limitation of the size of the gap junction plaques as it has been reported previously (Hunter et al., 2005). When a Cx43-GFP-expressing cell was adjacent to a wild-type cell, high-resolution deconvolution microscopy revealed that immunoreactive ZO-1 appeared to be localized on both sides of the Cx43-GFP-containing gap junction plaques, with a slightly higher signal intensity on one side of the plaque corresponding to the cell expressing only endogenous Cx43 (Fig. 6D) – as described above. In response to HCH exposure, ZO-1 appeared to be preferentially located on the inner side of the invaginating gap junction plaques (Fig. 6E,F) and inside annular gap junction vesicles (Fig. 6G). This observation was confirmed by immunogold electron microscopy (data not shown). In addition, an intense signal for ZO-1 was found inside annular gap junctions near the plasma membrane, whereas a few punctae were seen when these structures were located around the nuclear region (data not shown). This relative reduction in the levels of ZO-1 signal from the membrane to the cytoplasmic nuclear compartment might reflect a degradation process of the annular gap junction, in accordance with a recent published study (Leithe et al., 2006). Previous publications have suggested that the nonreceptor tyrosine kinase Src is actively involved in the dissociation of the Cx43–ZO-1 complex (Toyofuku et al., 2001). We hypothesize that Src association with Cx43 is a prerequisite for gap junction plaque internalization. In agreement with our data presented in Fig. 4, immunofluorescence analyses of endogenous Src revealed, that Src is not closely associated with Cx43-GFP gap junction plaques in control cells (Fig. 6H). In response to HCH exposure, Src was mainly detected as small dots along the internalizing plaque (Fig. 6I,J) and, in contrast to ZO-1, was preferentially confined to the outer side of annular gap

![Fig. 4. Subcellular localization of Cx43 and Src in response to HCH exposure. Sertoli cells were treated with 50 μM HCH for 10 minutes in the presence or absence of 30 μM PP2 or 30 μM PP3. Cx43 and Src were then detected by immunofluorescence. Notice that HCH treatment resulted in both aberrant cytoplasmic localization of Cx43 and recruitment of Src to the plasma membrane (arrows), is totally prevented by the presence of PP2 but not of PP3. In both situations, proteins relocation are easily noticeable by membranous lines (arrows) or cytoplasmic dots (arrowheads). Images are a typical example of three separate experiments.](image-url)

![Fig. 5. Changes in Cx43–ZO-1 and Cx43-Src associations after exposure to HCH. (A) Lysates from 42GPA9 Sertoli cells that had been incubated with or without 50 μM HCH were immunoprecipitated (Ip) with antibodies against ZO-1, Src or Cx43. Immunoprecipitates were then analyzed by western blotting using antibodies against ZO-1, Src and Cx43. Bands of ZO-1, Src and Cx43 were detected at the predicted molecular masses of 120, 55 and 43 kDa, respectively. In the presence of HCH, levels of ZO-1 associated with Cx43 decreased when lysates were immunoprecipitated with either ZO-1 or Cx43. After Cx43 Ip, low but detectable levels of Src were associated with Cx43 in HCH-treated cells, but were totally absent in control cells. (B) Lysates from 42GPA9 Sertoli cells subjected for 1 hour to 50 μM HCH with or without 30 μM PP2 were immunoprecipitated with antibodies against Cx43 or ZO-1. Cx43 and ZO-1 immunoprecipitates were then analyzed by western blotting for P-Src (p-Src) using anti-Src [pY418] antibody. For each experiment in A and B, a representative blot of three different experiments is shown. Relative optical densities of the bands in arbitrary units are presented in the right panels. Results are the average of three different experiments. *P<0.05, **P<0.01 significantly different from untreated cells.](image-url)
Fig. 6. Close molecular interactions between Cx43, ZO-1 and Src during HCH-induced endocytic internalization of Cx43-GFP gap junction plaques. (A) Percentage of two adjacent cells in which one Cx43-GFP transfected (T) cell is in contact with a wild-type cell expressing endogenous Cx43 (Wt), or in which the two cells express Cx43-GFP (T/T). The transfected cells were identified by the presence of GFP within cells. (B,C) Localization of immunoreactive ZO-1 between two adjacent cells that (B) express Cx43-GFP, or between (C) one cell that expresses Cx43-GFP (T) and a wild-type cell (Wt) that expresses only endogenous Cx43. Dashed lines correspond to the zone of contact between the two adjacent cells. (D-K) High-resolution deconvolution fluorescence microscopy analysis of the interaction of Cx43-GFP with ZO-1 and Src during the internalization of gap junction plaques in response to 50 μM HCH. (D) Side view of a gap junction plaque (green) decorated on both sides by ZO-1 (red). Colocalization is shown in yellow. (E) Presence of ZO-1 only on one side of the plaque after HCH treatment, indicating the beginning of the plaque endocytosis. (F) Localization of ZO-1 on the internal side of the gap junction plaque during internalization. (G) View of an early Cx43-GFP annular gap junction associated with ZO-1 in its center. (H) Cx43-GFP gap junction plaque (green) and distribution of Src (red) in control cells. (I) Side view of a gap junction plaque associated at one side with Src after HCH exposure. Colocalization is shown in yellow. (J) At the beginning of plaque internalization the presence of Src (yellow) was mainly detected on the external side of the gap junction plaque. (K) View of an early Cx43-GFP annular gap junction associated in its periphery with Src (yellow). Images are representative of five different experiments.

Discussion

The internalization of gap junction plaques in order to form cytoplasmic annular gap junctions is a dynamic process that involves the clathrin-mediated endocytic machinery (Segretain and Falk, 2004; Piehl et al., 2007). In a wide variety of cancers the dynamics of gap-junction-plaque maintenance in the plasma membrane and of gap junction internalization is altered (reviewed by Mesnil et al., 2005; Pointis et al., 2007). There is now compelling evidence that internalization of gap junction plaques requires a precisely tuned interaction of Cx with a large number of protein partners (Wei et al., 2004; Kirkham and Parton, 2005; Herve et al., 2007). However, the strict molecular interactions that occur between Cx43 and these regulatory partners during gap junction internalization are not well characterized yet. Identification of such proteins and of their roles is required for the understanding of gap junction maintenance and internalization under physiological and pathological conditions. By combining biochemical approaches with wide-field fluorescence deconvolution microscopy in cells that express Cx43-GFP, we showed for the first time that: (1) the partial dissociation on one side of the gap junction plaque, between Cx43 and its preferential partner ZO-1, is associated with the endocytic internalization of gap junction plaques; (2) the internalization of gap junction plaques is dependent upon the activation of Src, the recruitment of activated Src to the plasma membrane and its association with Cx43; (3) these dynamic and rapid processes are amplified in response to the non-genomic carcinogen HCH.

The role of ZO-1 in the internalization of gap junction plaques has been investigated previously (Barker et al., 2002; Duffy et al., 2004; Segretain et al., 2004). Disruption of the Cx43–ZO-1 interaction by stably transfecting rat osteosarcoma cells with a connexin-binding N-terminal fragment of ZO-1 decreases gap junctional communication and punctuate appositional membrane staining for Cx43 (Laing et al., 2005). For other authors, gap junction plaques formed by Cx43-GFP, a connexin fusion construct unable to interact with ZO-1, do not affect internalization of tagged gap junction plaques in HeLa cells, a communication-deficient cell line (Hunter et al., 2005). Our data, obtained by using 42GPA9 Sertoli cells, revealed that ZO-1 is usually localized on both sides of the gap junction and its presence is restricted to one side of the gap junction plaque during the endocytic internalization of gap junction plaques and the formation of annular gap junctions. This suggests that the dissociation of ZO-1 from Cx43 – preferentially on one side of the gap junction plaque – has a prominent role in gap junction endocytosis. In testis, Sertoli cells form multiple cell-cell junctions in which adherens-, communicating- and tight-junction proteins are co-expressed at the basal compartment of the seminiferous epithelium to form the blood-
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The altered colocalization of Cx43 and ZO-1 during gap junction internalization that we observed here – together with our previous data (Segretain et al., 2004) – strongly supports the hypothesis that, in Sertoli cells (which constitutively express endogenous Cx43), Cx43 gap junction internalization is a ZO-1 dependent process. This conclusion is consistent with previous results, which suggest that the role of ZO-1, in the control of gap junction plaque internalization, varies in different types of cells and depends on whether cells express endogenous Cx43 or not (Laing et al., 2005).

The molecular mechanism(s) and/or the potential factors that lead to the dissociation of ZO-1 from Cx43 are still unknown, although it probably results from altered interaction of the extreme C-terminus of Cx43 with the second PDZ domain of ZO-1, as previously reported (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). However, a modified interaction with other Cx partners could also induce dissociation of Cx43 from ZO-1. Indeed, recent studies based on immunoprecipitation, immunolocalization or yeast two-hybrid approaches revealed that Cxs can interact with a large number of additional partners, including α- and β-catenin (Fujimoto et al.,...
Fig. 8. Illustration of the molecular dynamics of the interactions of Cx43, ZO-1 and Src during endocytic internalization of gap junction plaques in response to treatment with HCH. If a sufficient amount of activated Src is present in pathological tissues or in cells that were treated with carcinogen, dissociation of the Cx43–ZO-1 complex occurs after the kinase Src associates with Cx43, rapidly resulting in both internalization of the gap junction plaque and formation of the annular gap junction. The reduced level of ZO-1 on one side of the Cx43 gap junction plaque at the beginning of internalization could be indicative of the cell that endocytizes the gap junction plaque.

Well established is the fact that interactions between ZO-1 and Cx43 are needed for both the stability of Cx43 at the plasma membrane and the endocytotic process of Cx43 (Barker et al., 2002; Duffy et al., 2004; Segretain et al., 2004). Thus, it is possible that in our study the accelerated internalization of gap junction plaques in response to HCH occurs preferentially into contacting cells that express Cx43-GFP, and in which Src could more easily decrease the interaction between Cx43 and the reduced levels of ZO-1 that are present in these cells. This does not exclude the possibility that, under different conditions, the Cx43-GFP plaque can also be internalized in adjacent cells that do not express Cx43-GFP, as reported by others (Jordan et al., 2001).

It has previously been reported that Src can control Cx43 channel closure by directly phosphorylating of tyrosine residues and by activating other signaling pathways (reviewed by Pahuja et al., 2007). Our observations suggest that activation of Src is also a crucial step in the endocytotic internalization of gap junction plaques. This hypothesis is supported by our in vitro experiments, which indicate that exposure of cells to the selective Src inhibitor PP2 completely inhibited the negative effect that HCH had on gap junction plaque numbers and coupling, whereas the inactive analog PP3 was without effect. However, regarding the structural association of Src, Cx43 and ZO-1 that we describe here, the mechanism(s) by which activation of Src leads to internalization of gap junction plaque has not been identified. Whether internalization of gap junctions in response to HCH is dependent (Toyofuku et al., 2001) or independent of tyrosine phosphorylation (Duffy et al., 2004), and whether it is mediated through displacement of the Cx43CT–PDZ-2-domain complex by the SH3 domain of Src (Sorgen et al., 2004) has not yet been investigated. The current data suggest that Src activation does not only lead to a rapid closure of the gap junction channel, but also accelerates the endocytotic internalization of Cx43. This hypothesis is in agreement with recent observations in astrocytes, which suggest that the loss of interaction of Cx43 with its scaffolding protein, consecutive to a specific association with Src, alters Cx43 internalization and degradation rather than trafficking of Cx43 to the plasma membrane (Duffy et al., 2004).

A recent study provides evidence that, under pathological conditions (chemical ischemia/hypoxia), Src is located in a membrane fraction insoluble in Triton-X-100, and which predominantly contains

1997; Ai et al., 2000), α- and β-tubulin (Giepmans et al., 2001), caveolin (Schubert et al., 2002) and, more recently, drebrin (Butkevitch et al., 2004). Previous evidence suggested that Src is involved in both, dissociation of the Cx43–ZO-1 complex (Toyofuku et al., 2001) and downregulation of gap junctional communication between cells (Postma et al., 1998; Sorgen et al., 2004; Duffy et al., 2004). Our present study clearly demonstrates that dissociation of ZO-1 and Cx43 occurred in one side of the gap junction plaque, preferentially on the side where Src is associated with Cx43. This association to a specific side was maintained during internalization of gap junction plaques and was also seen on annular gap junctions. Whether additional protein partners of Cx43 are implicated in this process remains to be investigated.

The mechanism(s) that maintains the association of ZO-1 with Cx43 within the internal side of the gap junction plaque during the course of internalization of this structure is also unknown. The specific localization of ZO-1 in this side is in agreement with the current observation that the ZO-1 signal is only detected within the annular gap junction and not around the structure. Since the internalization of gap junction plaques might occur in one cell or in the contiguous cell, the precise detection of ZO-1 in a given side of the gap junction plaque prior to internalization, might provide information on the cell into which the gap junction plaque is going to be internalized. In the experimental model developed here, it is obvious that the Cx43-GFP gap junction plaque originated from one cell by Cx43-GFP mixed with endogenous Cx43, and from the contacting cell by endogenous Cx43 alone – just as it has previously been demonstrated (Thomas et al., 2005). The recent observation that ZO-1 associates specifically with endogenous Cx43 but not with tagged Cx (Hunter et al., 2005; Girao and Pereira, 2007), is supported by our data, which show that higher ZO-1 signals are found in the contacting cell that does not express Cx43-GFP. For this effect, several reasons can be postulated. First, GFP could alter Cx43 cis-oligmerization, which in turn might affect the accessibility or binding affinity of ZO-1 to endogenous and Cx43-GFP in mixed connexons. Second, GFP might impair the binding affinity of ZO-1 to endogenous Cx43 by steric hindrance. Third, it is possible that the GFP tag in the mixed connexon interferes with the association of other proteins within the complex of ZO-1 and endogenous Cx43.
gap junctions (Li et al., 2005). Under physiological conditions, however, endogenous Src might be predominantly located within the membrane fraction that is soluble in Triton-X-100 (Naruse et al., 1998; Wu et al., 2000; Li et al., 2005). Our present data, together with our previous observations (Defamie et al., 2001), indicate that Src activation by the carcinogenic HCH is associated with an increased internalization of Cx43 gap junction plaques. Consistent with this observation, an aberrant cytoplasmic localization of Cx43 has been described as a common feature of many tumor cells (reviewed by Mesnil et al., 2005; Pointis et al., 2007). In addition, Src is known to be overexpressed and functionally upregulated in many types of human cancer (Brown and Cooper, 1996), and it has been suggested that the Src family kinases are important for several aspects of tumor progression, including destabilization and disassembly of cadherin-dependent cell-cell contacts (Owens et al., 2000). Increased Src activity, associated with reduced gap-junctional communication, has been also reported in the pathophysiology of heart failure (Toyofuku et al., 1999).

Taken together, our data reveal that a non-genomic carcinogen such as HCH might lead to severe disruption of cell-cell communication by interfering with gap junction plaques following the rapid internalization of these structures (see Fig. 8 for a summary of results). It is possible that this step is associated with the movement of cytoskeletal components that are implicated in the endocytic process and whose nature remains to be identified. The current observations suggest that exaggerated dissociation of the Cx43–ZO-1 complex results from interaction with other proteins – such as Src (which is known to be overexpressed in many cancers or, here, in response to carcinogenic exposure) – can be one of the first events to allow the internalization of gap junction plaques and the disappearance of cell-cell communication, a specific feature of many tumor cells. In support of this model, accelerated Cx43 internalization has been suggested as an early process that is associated in situ with uncontrolled cell proliferation before the onset of testicular tumor cell invasion (Segretain et al., 2003). Although our experimental data indicate that the reduced levels of ZO-1 on one side of the gap junction plaque might dictate into which cell the plaque internalizes, the reason why the dissociation of the Cx43–ZO-1 complex preferentially occurs on one side of the plaque is presently unknown. It is reasonable to speculate that this phenotypic heterogeneity results from cell-cell variability (Chabot et al., 2007).

**Materials and Methods**

**Cell culture and transfection**

The 42GPA9 Sertoli cell line was cultured as previously described (Bourdon et al., 1998). To study the dynamics of gap junctions, Sertoli cells (70% confluent) were transfected with a previously described Cx43-GFP construct (Falk, 2000). When indicated, cells were also transfected with an expression plasmid for Rab5a-wt-mRFP1C (gift from M. Zerial, Max Planck Institut, Dresden, Germany). Transfections were performed using Lipofectamin (Invitrogen) according to the manufacturer’s instructions, as recently described (Gillieron et al., 2006). To induce internalization of gap junction plaques, Cx43-GFP transfected Sertoli cells were incubated in the presence of 50 μM γ-hexachlorocyclohexane (HCH), an organochlorine compound that has previously been reported to efficiently stimulate Cx43 internalization (Guan and Ruch, 1996; Defamie et al., 2001). The effect of HCH was reversible and at the dose used here not cytotoxic (Defamie et al., 2001). When indicated, 30 μM PP2 (inhibitor of the Src tyrosine kinase family; Calbiochem) or 10 μM PD98059 (MEK1-specific inhibitor, Calbiochem) were added to the medium for 90 minutes, prior to the addition of HCH. In control conditions, cells were incubated with the inactive Src inhibitor PP3, vehicle alone, or were left untreated.

**Immunofluorescence and cell imaging**

Transfected cells were transferred on slides, fixed and permeabilized as previously described (Defamie et al., 2001; Segretain et al., 2004). ZO-1 was immunodetected with a mouse monoclonal anti-ZO-1 antibody (1:100), purchased from CliniSciences (Montrouge, France). Afterwards, the slides were incubated with goat TRITC-conjugated anti-mouse IgG (1:100), obtained from Interchim (Montluçon, France). High-resolution deconvolution microscopy analysis was performed with a wide-field fluorescence deconvolution microscope (Nikon TE2000E, Service Commun de Microscope, IFR Biomédicales des Saint-Pères, Paris) and acquired image stacks were deconvoluted using Autoquant (MediaCybernetics) image package algorithms. Surface rendered three-dimensional volume reconstructions were generated using Amira software 3.1.1. Immunofluorescence staining and emission filter (chroma) in fast prior filter well, and forced air-cooled CCD camera (CoolSnap HQ2, Roper-Princeton Instrument). Six positions were randomly chosen in each well and for each position a Z series of video images was captured every 3 minutes. Individual image sequences were deconvoluted and rendered into movie sequences using the software package NIS elements (Nikon software). For cellular localization of Cx43-GFP, indirect immunofluorescence was performed using specific markers of Golgi complex (CTR433 antibody, 1:10, a gift from M. Bornens, Curie Institute, Paris, France), of plasma membrane (occludin antibody, 1:100, from Zymed Laboratories, CA) and of lysosomes (CD1107b/Lamp-2, 1:100, from Pharmingen, Becton Dickinson, Le Pont de Claix, France) as previously described (Defamie et al., 2001; Fiorini et al., 2004). Early endosomal localization of Cx43-GFP was analyzed in cells co-transfected with the expression plasmid for Rab5a-wt-mRFP1C.

**Immunoprecipitation and western blotting**

Confluent Sertoli cells, exposed to 50 μM HCH, were collected by scraping in ice-cold NP40/β-lysis buffer (50 mM Tris-HCl pH 7.5, 1% NP40, 1% Brij96, 1 mM NaVO3, 1 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM EDTA, 1 mM aprotinin, 25 mM leupeptin, 1 mM pepstatin, 2 mM phenylmethylsulfonyl fluoride). Protein lysates (800 μg) were immunoprecipitated overnight with 2 μg of anti-Cx43 (mouse; Transduction Laboratories, Lexington, KY), anti-ZO-1 (mouse; Zymed Laboratories, CA) or anti-Src (rabbit; Santa Cruz Biotechnology Inc., CA) antibodies. The immunocomplexes were precipitated by incubation with protein-A-Sepharose for Src and proteinG-Sepharose for Cx43 and ZO-1 (Pharmacia) for 1 hour at 4°C. Immunopellets were washed four times in PBS 0.5% NP-40, once in PBS, ended with 10 μl of reduced Laemmli buffer and boiled 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 using either anti-Cx43 (1:2000), anti-ZO-1 (1:2000) or anti-Src (1:1000) as previously reported (Defamie et al., 2001; Fiorini et al., 2004). For detection of the activated form of Src, cell lysates were analyzed by western blotting with a polyclonal antibody directed against the phosphorylated active forms of Src (anti-Src [pY418]; 1:2000, BIOSOURCE, Camarillo, MA). After stripping, equal loadings of proteins were verified by reprobing the same blots with anti-Src antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which detects unphosphorylated and phosphorylated Src isoforms. The presence of primary antibodies was revealed using horseshadish peroxidase-conjugated antimouse or anti-rabbit (1:10,000) IgG (Dako, Trappes, France) and visualized using an enhanced-chemiluminescence detection kit (ECL; Amersham, UK).

**Dye-coupling procedures**

Functionality of gap junction channels was evaluated by analyzing the passage of a fluorescent dye through these junctions as previously described (Gilleron et al., 2006). Briefly, cells were incubated with 5 μM of calcein-AM (Invitrogen SARL, Cergy Pontoise, France) in DMEM for 30 minutes at 32°C. Extracellular calcein was removed by three washes in DMEM. Individual cells were selected by using the region-of-interest function of the Zeiss LSM software, and photobleached by strong laser pulses (488 nm and 40 iterations) on a Zeiss confocal microscope (LSM 510, S.C.M., IFR Biomédicales des Saint-Pères, Paris, France). Confocal images were taken every 3 minutes during a 12-minute period after calcein photobleaching. Fluorescence was quantified using LSM software before bleach, just after, and following 12 minutes of photobleaching. The percentage of fluorescence recovery in bleached cell was determined by averaging all cells (n=50) for each experiment. Similar analyses were performed in four independent sets of experiments.

**Statistical analyses**

Data were expressed as the mean ± standard error of the mean (± s.e.m.). Statistical analyses were assessed using Student’s t-test or one-way analysis of variance and Duncan’s new multiple range tests. Differences were considered to be significant at p<0.05.

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References

Ak, Z., Fisher, A., Spray, D. C., Brown, A. M. and Fishman, G. I. (2000). Wnt-1 regulation of connexin43 in cardiac myocytes. *J. Clin. Invest.* 105, 161-171.

Barker, R. J., Price, R. L. and Gourdie, R. G. (2002). Increased association of ZO-1 with connexin43 during remodeling of cardiac gap junctions. *Circ. Res.* 90, 317-324.

Boardun, V., Lablack, A., Abbe, P., Segretain, D. and Pointis, G. (1998). Characterization of a clonal Sertoli cell line using adult PyLT transgenic mice. *Bio Reprod.* 58, 591-599.

Brown, M. T. and Cooper, J. A. (1996). Regulation, substrates and functions of src. *Biochim. Biophys. Acta.* 1337, 121-140.

Butkevitch, E., Häusmann, S., Wenzel, D., Shirado, T., Duden, R. and Majoul, I. (2004). Debrin is a novel Connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton. *Curr. Biol.* 14, 650-658.

Cheitlin, M. D., Cohn, J. E. and van Ouwerkerken, A. (2007). Stochastic gene expression out-of-steady-state in the cyanobacterial circadian clock. *Nature* 450, 1249-1252.

Delamé, N., Mograbi, B., Roger, C., Cronier, L., Malassine, A., Brucker-Davis, F., Fiorini, C., Segretain, D. and Pointis, G. (2001). Immunolocalization and expression of connexin43 and zonula occludens-1 in 42GPA9 Sertoli cells. *Carcinogenesis* 22, 1537-1542.

Duffy, H., Ashton, A., O'Donnell, P., Coombs, W., Taffet, S., Delmar, M. and Spray, D. (2004). Regulation of connexin43 protein complexes by intercellular acidification. *Circ. Res.* 94, 215-222.

Dunham, B., Liu, S., Taffet, S., Trabka-Janik, E., Delmar, M., Petrushy, R., Zheng, S., Moller, L. F. and Afflano, M. I. (1992). Immunolocalization and expression of functional and nonfunctional cell-to-cell channels from wild-type and mutant rat heart connexin43 cDNA. *Circ. Res.* 70, 1233-1243.

Falk, M. M. (2000). Connexin-specific distribution within gap junctions revealed in living cells. *Cell. Sci.* 113, 411-420.

Fiorini, C., Mograbi, B., Cronier, L., Bourget, I., Ducroz, X., Ferrua, B., Malassine, A., Samson, M., Fenchel, P., Segretain, D. et al. (2004). Dominant negative effect of connexin33 on gap junctional communication is mediated by connexin43 sequestration. *J. Biol. Chem.* 279, 15125-15129.

Fishman, G. I., Moreno, A. P., Spray, D. C. and Leinwand, L. A. (1991). Functional analysis of human cardiac gap junction channel mutants. *Proc. Natl. Acad. Sci. USA* 88, 3525-3529.

Fujimoto, K., Nagafuchi, A., Tsukita, S., Kuraoka, A. and Ohokuma, A. (1997). Dynamics of connexin43-E-cadherin and occludin on cell membranes during gap junction formation. *J. Cell Sci.* 110, 311-322.

Gaitta, G., Deerinck, T. J., Adams, S. P., Bourer, J., Tour, O., Laird, D. W., Sosinsky, G. E., Tsien, R. Y. and Ellisman, M. H. (2002). Multicolor and electron microscopic imaging of connexin trafficking. *Science* 296, 503-507.

Giepmans, B. and Moolenaar, W. (1998). The gap junction protein gap43 interacts with the second PDZ domain of the zona occludens-1 protein. *Circ. Biol. Sci.* 8, 931-934.

Giepmans, B., Verlaan, I. and Moolenaar, W. (2001). Connexin-43 interactions with ZO-1 and E-cadherin and beta-catenin require juxtamembrane sequences. *J. Biol. Chem.* 276, 219-223.

Gilleron, J., Nebot, S., Scarabelli, L., Senegas-Balas, F., Palmero, S., Segretain, D. and Pointis, G. (2006). Involvement of the gap junction connexin43 in the control of Sertoli cell proliferation by thyroid hormones. *J. Cell Physiol.* 209, 153-161.

Girao, H., Perez, P. and Siffroi, J. P. (2007). The proteasome regulates the interaction between Cx43 and ZO-1. *J. Cell Biochem.* 102, 719-728.

Guo, X. and Ruch, R. J. (1996). Gap junction endocytosis and lysosomal degradation of connexin43. *J. Biol. Chem.* 271, 719-726.

Herve, J. C., Bournemeyer, S., Sarrouille, D. and Duffy, H. H. (2007). Gap junctional complexes: from partners to functions. *Prog. Biophys. Mol. Biol.* 94, 29-65.

Hunter, A., Barker, R., Zhu, C. and Gourdie, R. (1997). Acute loss of cell-cell communication caused by G protein-coupled receptors: a critical role for c-src. *J. Cell Biol.* 140, 1199-1209.

Schubert, A., Schubert, W., Spray, D. and Lissanti, M. (2002). Connexin family members target to lipid raft domains and interact with caveolin-1. *Biochemistry* 41, 5754-5764.

Segretain, D. and Falk, M. (2004). Regulation of connexin biosynthesis, assembly, gap junction formation and removal. *Biochim. Biophys. Acta.* 1662, 3-21.

Segretain, D., Ducroz, X., Dompierre, J., Escalier, D., Rahman, N., Fieroni, C., Mograbi, B., Siffroi, J. P., Hulthen, I., Fenchel, P. et al. (2003). Sequestration of connexin43 in the early endosomes: an early event of Leydig cell tumor progression. *Exp. Cell Res.* 313, 4083-4093.

Pflieh, M., Lechmann, C., Gumpert, A., Denizet, J. P., Segretain, D. and Falk, M. M. (2007). Internalization of large double-membrane intercellular vesicles by a clathrin-dependent endocytotic process. *Mol. Biol. Cell* 18, 337-347.

Postma, F. R., Hengeveld, T., Abbas, J., Giepmans, B. N. G., Zondag, G. C. M., Jalink, K. and Moolenaar, W. (1998). Loss of cell-cell communication caused by WOM proteins: a critical role for c-src. *J. Cell Biol.* 140, 1199-1209.

Sako, Y., Sakai, N., Yamada, S., Kuroki, T., Kato, S., Nishizawa, T., Shinoda, M., Miki, K., Terui, T., Nakamura, Y. et al. (1999). Direct association of the gap junction protein connexin-43 with rat cardiac myocytes. *Biochim. Biophys. Acta.* 1457, 27-36.

Sako, Y., Sakai, N., Yamada, S., Kuroki, T., Kato, S., Nishizawa, T., Shinoda, M., Miki, K., Terui, T., Nakamura, Y. et al. (1999). Direct association of the gap junction protein connexin-43 with rat cardiac myocytes. *Biochim. Biophys. Acta.* 1457, 27-36.

Segretain, D., Dompierre, X., Escalier, D., Rahman, N., Fieroni, C., Mograbi, B., Siffroi, J. P., Hulthen, I., Fenchel, P. et al. (2003). Sequestration of connexin43 in the early endosomes: an early event of Leydig cell tumor progression. *Exp. Cell Res.* 313, 4083-4093.

Sako, Y., Sakai, N., Yamada, S., Kuroki, T., Kato, S., Nishizawa, T., Shinoda, M., Miki, K., Terui, T., Nakamura, Y. et al. (1999). Direct association of the gap junction protein connexin-43 with rat cardiac myocytes. *Biochim. Biophys. Acta.* 1457, 27-36.

Sako, Y., Sakai, N., Yamada, S., Kuroki, T., Kato, S., Nishizawa, T., Shinoda, M., Miki, K., Terui, T., Nakamura, Y. et al. (1999). Direct association of the gap junction protein connexin-43 with rat cardiac myocytes. *Biochim. Biophys. Acta.* 1457, 27-36.

Sako, Y., Sakai, N., Yamada, S., Kuroki, T., Kato, S., Nishizawa, T., Shinoda, M., Miki, K., Terui, T., Nakamura, Y. et al. (1999). Direct association of the gap junction protein connexin-43 with rat cardiac myocytes. *Biochim. Biophys. Acta.* 1457, 27-36.

Sako, Y., Sakai, N., Yamada, S., Kuroki, T., Kato, S., Nishizawa, T., Shinoda, M., Miki, K., Terui, T., Nakamura, Y. et al. (1999). Direct association of the gap junction protein connexin-43 with rat cardiac myocytes. *Biochim. Biophys. Acta.* 1457, 27-36.