Exocrine specific expression of Connexin32 is dependent on the basic helix-loop-helix transcription factor Mist1

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Summary

Gap junctions are intercellular channels that provide direct passage of small molecules between adjacent cells. In pancreatic acini, the connexin26 (Cx26) and connexin32 (Cx32) proteins form functional channels that coordinate the secretion of digestive enzymes. Although the function of Cx26/Cx32 gap junctions are well characterized, the regulatory circuits that control the spatial and temporal expression patterns of these connexin genes are not known. In an effort to identify the molecular pathways that regulate connexin gene expression, we examined Cx26 and Cx32 gene activities in mice lacking the basic helix-loop-helix transcription factor Mist1 (Mist1KO). Mist1, Cx26, Cx26 and Cx32 are co-expressed in most exocrine cell types, and acinar cells from Mist1KO mice exhibit a highly disorganized cellular architecture and an altered pattern of expression for several genes involved in regulated exocytosis. Analysis of Mist1KO mice revealed a dramatic decrease in both connexin proteins, albeit through different molecular mechanisms. Cx32 gene transcription was greatly reduced in all Mist1KO exocrine cells, while Cx26 gene expression remained unaffected. However, in the absence of Cx32 protein, Cx26 did not participate in gap junction formation, leading to a complete lack of intercellular communication among Mist1KO acinar cells. Additional studies testing Mist1 gene constructs in pancreatic exocrine cells confirmed that Mist1 transcriptionally regulates expression of the Cx32 gene. We conclude that Mist1 functions as a positive regulator of Cx32 gene expression and, in its absence, acinar cell gap junctions and intercellular communication pathways become disrupted.

Key words: Pancreas, Exocytosis, bHLH, Transcription, Cell communication

Introduction

Pancreatic acini consist of exocrine cells whose main function is to produce and deliver the enzymes necessary for digestion. The appropriate release of these enzymes is accomplished through a complex and highly regulated system involving protein packaging and storage, vesicle trafficking, sensing of feeding signals and coordination of cellular secretion (Castle, 1990; Wasle and Edwardson, 2002). Enzymes are stored in granules as inactive zymogens until secretagogues, such as cholecystokinin (CCK), stimulate their release in a process referred to as regulated exocytosis. Binding of CCK to G-protein coupled receptors initiates a signaling cascade that culminates in the release of intracellular calcium, which in turn promotes movement of the zymogen granules (ZG) toward the apical cell surface and exocytosis of enzymes into pancreatic ducts (Joseph, 1996; McNiven and Marlowe, 1999). Improper signaling leads to impaired exocytosis and premature enzyme activation (Saluja et al., 1989). Owing to this specialized function, acinar cells exhibit extensive extra- and intracellular organization, with the granules aligned to the apical border adjacent to a centrally located duct while the receptors for secretagogue binding are located on the basal aspect of the cell. It is this highly organized polarity that leads to efficient enzyme release. The process of regulated exocytosis is controlled in part by the cellular junctions that exist between the individual acinar cells, with gap junctions playing a major role in modulating the exocytotic process (Meda, 1996).

Gap junctions are trans-membrane protein structures that allow passage of small solutes between adjacent cells (Kumar and Gilula, 1996). Each gap junction consists of a plaque of connexons, which are composed of hexameric complexes of connexin proteins. Nineteen different connexin genes, each with a specific tissue distribution, exist in the mouse (Willecke and Gilula, 1996). Each gap junction consists of a plaque of connexons, which are composed of hexameric complexes of connexin proteins. Nineteen different connexin genes, each with a specific tissue distribution, exist in the mouse (Willecke et al., 2002). The exocrine pancreas expresses connexin32 (Cx32; also referred to as Gjb1) and connexin26 (Cx26; also referred to as Gjb2), which is typical of most serous exocrine cells, including cells of the salivary glands, seminal vesicles and lacrimal glands (Hsieh et al., 1991; Meda et al., 1993). The existence of many different connexins with unique tissue specificities highlights the ability of gap junctions to provide a selective permeability between cells. Specific connexin expression patterns, coupled with various combinations of hexameric junctions and states of protein phosphorylation, contribute to a highly regulated process of intercellular

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signaling (Goodenough et al., 1996; Lampe and Lau, 2000). Stimulated acinar cells utilize gap junctions to communicate with adjacent cells in a common acinus to carefully regulate and coordinate the secretion of their protein products. Loss of connexin expression leads to increased levels of basal and stimulated exocytosis in acinar cells (Chanson et al., 1998), confirming the importance of gap junctions in the exocytosis pathway.

Although the expression and function of gap junctions are well characterized, the molecular regulatory circuits that control the spatial and temporal expression patterns of the connexin genes are not known. Elucidating these regulatory factors is of significant interest since much of the function associated with a specific gap junction complex is dependent on the types and amounts of connexins it contains (Hill et al., 2002; Niessen et al., 2000; Niessen and Willecke, 2000). Expression of the connexin gene family is probably regulated by key transcription factors that exhibit similar patterns of spatial expression. As previously mentioned, the Cx32 and Cx26 genes are co-expressed in cells known to exhibit regulated exocytosis (Meda et al., 1993). One transcription factor that shares a similar, but not identical, expression pattern is the basic helix-loop-helix (bHLH) protein Mist1 (Pin et al., 2000). Members of the bHLH family are instrumental in the development of numerous organ systems, and gene ablation studies in mice have revealed an essential role for bHLH proteins in the development of different cell types within the pancreas (Jenny et al., 2002; Kageyama et al., 2000; Krapp et al., 1998). Mice deficient in BETA2/NeuroD or neurogenin3 fail to develop insulin-producing β-cells, develop diabetes and die within a few days of birth (Jenny et al., 2002; Naya et al., 1997). Similarly, targeted disruption of the exocrine-specific factor PTF1-p48 results in a complete lack of exocrine cells within the pancreas, causing early postnatal death (Kawaguchi et al., 2002; Krapp et al., 1998).

Deletion of the Mist1 gene (Mist1KO), while not lethal, causes disorganization in acinus formation and numerous defects in pathways controlling regulated exocytosis (Pin et al., 2001). These include altered expression of genes involved in calcium mobilization (IP3 Receptor Type 3) and secretagogue signaling (CCK A receptor). In an effort to establish if there is a relationship between gap junction communication and the Mist1KO phenotype, we examined the expression and function of connexin molecules in Mist1KO acinar cells. In this report, we show that Mist1KO acini are deficient in gap junction-mediated intercellular communication, mainly due to the loss of Cx32 expression. At all time points examined, Cx32 mRNA and protein levels are greatly reduced in the Mist1KO exocrine pancreas, as well as in all exocrine organs that normally express the Mist1 gene. Although Mist1KO acinar cells continue to express the Cx26 gene, Cx26 protein does not accumulate in gap junction plaques, suggesting that Cx32 is required for stable incorporation of Cx26 into the membrane. Additional studies using pancreatic acinar cell lines, transgenic mice and Mist1 expression plasmids have confirmed that Mist1 transcriptionally regulates expression of the Cx32 gene. We conclude that Mist1 functions as a positive regulator of Cx32 gene expression and, in the absence of Mist1, acinar cell gap junctions and intercellular communication pathways become disrupted.

Materials and Methods

Mist1KO mice

The generation of Mist1lacZ (Mist1Het) and Mist1KO mice has been described previously (Pin et al., 2001). These mice contain a lacZ gene encoding a nuclear localized β-galactosidase (β-gal) protein driven from the Mist1 gene locus. Mist1Het mice were maintained on a C57BL/6 background. The elastase-mist1mut basic mice were generated through standard pronuclear injection protocols using a –500 +8 fragment of the rat elastase 1 promoter (Heller et al., 2001; Kruse et al., 1995). Mist1mut basic mice express the Mist1mut basic protein exclusively in pancreatic acinar cells. The complete characterization of these mice will be reported elsewhere.

Acini isolation

Pancreatic acini were obtained following standard collagenase protocols (Ohnishi et al., 1997). Briefly, animals were euthanized and the pancreas was removed and placed into KRB (0.1 M NaCl, 1 mM MgCl2, 5 mM KCl, 0.5 mM Na2HPO4, 33 mM NaHCO3, 0.5 mM CaCl2, 4 mg/ml glucose, 15 mg/ml glutamine and MEM amino acids) buffer containing 0.1 mg/ml trypsin inhibitor. Pancreatic tissue was injected with a collagenase solution (100 U/ml in KRB plus 2.5 mg/ml BSA), gassed with O2 and incubated at 37°C for 10 minutes. Tissue fragments were transferred to a fresh collagenase solution, gassed and incubated for an additional 40 minutes at 37°C with shaking. Acini were separated by manual pipetting through decreasing pipette orifices, and passed through a 150 μm nylon net filter. The filtrate was centrifuged at 500 rpm for 3 minutes and incubated at 37°C until ready to use. Cells were plated on glass coverslips in Waymouth’s medium containing 1% penicillin and streptomycin (Pen/Strep) and 0.5% fetal bovine serum (FBS).

Dye coupling and electrophysiology

Glass microelectrodes were prepared from filamented micropipette tubing (World Precision Instruments, Kwik-Fill 1B100F-3) using a Narishige vertical microelectrode puller. The glass microelectrodes were backfilled with 10 mM 6-carboxyfluorescein and individual acinar cells were injected using hyperpolarizing pulses of current (50 nA; 100 ms pulse per second) for 1 minute. Transfer of dye was monitored for at least three minutes and images were recorded using a digital camera.

Experiments to detect electrical coupling between acinar cells was performed as previously described (Mao et al., 2000). Briefly, glass microelectrodes were backfilled with 1 M KCl, single cells were injected using hyperpolarizing pulses of current (50 nA; 100 ms per second) for 1 minute. Transfer of dye was inserted into either an adjacent cell or into a cell that was positioned ~200 μm away from the current-injecting electrode. Cell membrane potentials were recorded and plotted as current (mV) vs. time.

Gene constructs

The mouse Cx32 P1 promoter spanning –680 to +20 (Hennemann et al., 1992; Neuhaus et al., 1995) was cloned from genomic DNA using PFU DNA polymerase and promoter-specific primers containing flanking restriction sites. The 700 bp PCR product was digested with BamHI and BglII and cloned into the pGL2-Basic luciferase reporter plasmid from Clontech. The entire promoter region was verified by DNA sequencing. All other DNA constructs used in this report have been described previously (Krapp et al., 1998; Lemercier et al., 1998).

Cell culture and DNA transfections

Cells from the pancreatic exocrine cell line AR42J were propagated in growth medium consisting of 40% F12K, 25% hDMEM, 25%
F12, 10% FBS, 1% Pen/Strep. Cells (1.6x10^6) were transfected by electroporation (350 mV, 960 μF; 0.4 cm gap) using a BioRad Gene-Pulser and 10 μg reporter (C32p-Luc), 5 μg of the appropriate transcription factor expression plasmid (pcDNA3, pcDNA3-Mist1, pcDNA3-Mist1mut basic), pcDNA3-Mist1mut helix 1, pcDNA3-E12, pcDNA3-E47, pcDNA3-HEB, pcDNA3-PTF1-p48, pcDNA3-NeuroD, pcDNA3-MyoD, pcDNA3-Mash1) and 5 μg pRL-Null Renilla luciferase control vector per experimental group. Cells were harvested 48 hours later by scraping in Promega Passive Lysis Buffer and luminescence values were determined using the Promega Dual Luciferase Reporter Assay System. A minimum of three independent transfections were performed for all gene constructs.

RNA expression analysis
For RT-PCR, 2 μg of total RNA was reverse transcribed using the Superscript II reverse transcription system (Gibco BRL). cDNA reactions were then amplified using gene-specific primers for Mist1 (5'-CGCCGTACCGCCTCGAATTCGCG-3', 5'-CAAGCCCTAGAGAAGATCTAG), Cx32 (5'-GCAAACAGTTGGCAGTGCTG3', 5'-GGAGGCTGCGAGCATTAAGAGC3'), and β-actin (5'-ATGTTCACAACTGGGAGC-3', 5'-TCTCTGCTGCAAAGTCCTAG). Target sequences were amplified for 35 cycles with 95°C/40 seconds, 55°C/40 seconds and 72°C/55 seconds conditions. All primer pairs amplified regions crossing intron borders.

RNA isolation and hybridization blots
Total organ RNA was isolated using the QIAGEN RNeasy isolation system following the manufacturer’s recommended protocol. Mice were euthanized and perfused with 12 ml of cold PBS and then tissues were removed and disrupted using a Tissue-Tearor mechanical homogenizer. Samples were resuspended in RNase-free water and stored at −80°C. For RNA blot analysis, 20 μg of total RNA was precipitated in ethanol, resuspended in 20 μl of RNA loading buffer and heated at 65°C for 15 minutes until completely dissolved. RNA was loaded on a 1.2% formaldehyde agarose gel and electrophoresed in 1x MOPS buffer (0.5 M Mops, 0.01 M EDTA). After separation, RNA was transferred by capillary action to Hybond® membranes in 10x SSC. Hybridizations were performed using Clontech’s ExpressHyb buffer according to the manufacturer’s recommendations. Isotopically labeled probes were synthesized by standard procedures using the Ambion Decaprime kit.

Immunohistochemistry
Frozen and paraffin-embedded pancreatic sections were processed for immunohistochemistry by standard procedures. Briefly, tissues were removed and frozen without fixation in OCT and 5 μm sections were cut at −20°C using a Zeiss cryostat. Tissue sections were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS and then blocked for 1 hour with the Mouse on Mouse (MOM) reagent (Vector). For paraffin immunohistochemistry, pancreatic tissue was fixed in 10% formalin, embedded in paraffin and sectioned at 5 μm. Sections were rehydrated, treated with 100 μg/ml protease K and post-fixed in 10% formalin for 10 minutes. Samples were washed with PBS, permeabilized with 0.1% Triton X-100 and blocked using 5% BSA and 0.1% Triton X-100. Primary antibodies were then added to frozen or paraffin-embedded sections for 1 hour at room temperature. Primary antibodies included mouse mye 9E10 (1:200, Developmental Studies Hybridoma Bank), mouse connexin32 (1:50, Developmental Studies Hybridoma Bank or 1:100, Chemicon), rabbit connexin32 (1:200, Zymed Laboratories), rabbit connexin26 (1:200, Zymed Laboratories), mouse β-galactosidase (1:100, Developmental Studies Hybridoma Bank), rabbit amylase (1:250, Sigma), rabbit occludin (1:100, Zymed) and rabbit β-catenin (1:1000, Sigma). Following primary antibody addition, sections were incubated with biotinylated or Texas Red-conjugated secondary antibodies (1:200 Vector) for 10 minutes at room temperature followed by incubation with Oregon Green-conjugated tertiary antibodies (Molecular probes) for 5 minutes. Coverslips were mounted with Fluorosave reagent and examined using an Olympus fluorescence microscope. Images were captured using a QImager MicroImager II digital camera and Empix Northern Eclipse software.

Protein immunoblot assays
Tissue protein extraction, protein electrophoresis and immunoblotting were performed as described previously (Pin et al., 2000). For immunoblot analysis, 50 μg of whole cell protein extracts were electrophoresed on acrylamide gels, transferred to PVDF membranes and incubated with primary antibodies against Mist1, Cx32, β-gal and β-actin. Following secondary antibody incubation the immunoblots were developed using an ECL kit (Pierce) as per manufacturer’s instructions.

Results
Expression of Cx32 is significantly reduced in Mist1KO pancreatic acini
The unusual morphology of the Mist1KO pancreatic acini, coupled with the altered expression of proteins involved in regulated exocytosis, suggests that cell junction complexes may be disrupted (Pin et al., 2001). As a first step to examining cell junctions, we assessed the expression of gap junction proteins in pancreatic acini derived from wild-type (WT) or Mist1KO animals. Connexin32 (Cx32) is one of two connexin proteins expressed in serous exocrine cells and increases in gap junctions are readily detected during acinar cell development at the stage coinciding with cell organization (Bock et al., 1997; Yamamoto and Kataoka, 1985). Immunofluorescence of Cx32 in adult WT pancreatic sections revealed extensive expression, with gap junctions aggregated along the acinar cell membrane (Fig. 1A). In contrast, no Cx32-containing gap junctions were observed within Mist1KO exocrine tissue (Fig. 1B). The loss of Cx32 protein was confirmed by immunoblot assays in which Cx32 protein was not detected in Mist1KO extracts whereas it was readily observed in samples from WT pancreas (Fig. 1C). Previous work from our group described the gradual loss of β-catenin expression in older Mist1KO animals (Pin et al., 2001) and it has been suggested that the loss of one type of junctional complex (e.g. adherens) can lead to the loss of other complexes (Fujimoto et al., 1997; Kojima et al., 1999). In order to determine if the loss of Cx32 expression was a result of disruption in other cell junctions, Mist1KO and WT pancreas samples were co-stained for Cx32 and markers for zymogen granules (amylase), adherens junctions (β-catenin) and tight junctions (occludin). As previously reported (Pin et al., 2001), amylase staining is not restricted to the apical border of Mist1KO acinar cells, indicating that ZG targeting is disrupted (Fig. 1D,E). In contrast, the accumulation of β-catenin and occludin was normal in Mist1KO acinar cells, despite the absence of Cx32 protein (Fig. 1F-I). These findings indicate that the loss of Cx32-containing gap junctions is an independent event in response to the absence of Mist1, and is not linked to the observed loss of other junctional proteins (specifically β-catenin) that occurs in older Mist1KO mice (Pin et al., 2001).

The progressive deterioration of tissue architecture that is
observed in Mist1KO mice resembles chronic pancreatic injury and is associated with changes in a number of gene products (Pin et al., 2001). In many cases, the timing of the observed molecular changes suggests that they are secondary events occurring as a consequence of cell morphology, and not as a consequence of direct regulation by Mist1. In order to investigate whether the loss of Cx32 protein represents an early or late event associated with deletion of the Mist1 gene, we performed a time course analysis of Cx32 expression in WT and Mist1KO pancreatic tissue (Fig. 2). Tissue samples taken between postnatal day 1 (PN1) and adulthood revealed strong Cx32 immunofluorescence in WT pancreas, whereas virtually no Cx32-containing gap junctions were detected in Mist1KO sections, regardless of age (Fig. 2A-F).

Several mechanisms regulate protein accumulation, including changes in protein stability or mRNA transcription. To establish if the loss of Cx32 protein in Mist1KO acini was due to a corresponding loss of Cx32 transcripts, RNA blot analyses were performed on total pancreas RNA isolated from WT and Mist1KO mice. As shown in Fig. 2G, Cx32 mRNA levels in all Mist1KO samples were reduced by 75-85% compared to WT controls. This was particularly evident in the samples taken from 3-week-old mice, at which time the expression of Cx32 peaks in WT pancreas possibly because of the maturation of the regulated exocytosis process (Jamieson et al., 1988). The difference in Cx32 mRNA levels was consistent across all time points examined, with the first indication of a significant decrease detected at PN1 (Fig. 2G). These results indicate that in Mist1KO acini, Cx32 gene expression is never activated to levels in WT acinar cells, and that the decrease in Cx32 protein most probably reflects changes in mRNA transcription or mRNA stability.

**Mist1KO** pancreatic acinar cells are defective in intercellular communication

Given the significant reduction in Cx32 gene expression, we set out to examine whether Mist1KO cells remain functionally coupled. For these initial experiments, individual acini were isolated from WT and Mist1KO mice and stained for Cx32 protein. As observed previously in the pancreatic sections, Cx32 was detected in the membranes of WT acini while
3319

**Mist1null mice lack gap junctions**

Mist1KO acini were devoid of Cx32 staining (Fig. 3A,B). WT and Mist1KO acini then were tested for the ability to transfer 6-carboxyfluorescein, a small molecular mass dye, into adjacent cells. As expected, dye transfer rapidly occurred in acinar cells isolated from control mice (Fig. 3C,D). In all instances \( n=15 \) acini from 4 WT mice) three or more adjacent cells exhibited dye transfer. In contrast, acini isolated from Mist1KO mice were highly defective in dye transfer. No transfer of 6-carboxyfluorescein was detected in any of the neighboring acinar cells of Mist1KO mice \( n=12 \) acini from 4 mice), even after extended incubation times (Fig. 3E-H).

We next characterized WT and Mist1KO cells with respect to electrical coupling. Hyperpolarizing pulses of current (50 nA; 100 msec) were injected into one cell within an isolated acinus and the spread of current to neighboring cells was monitored. As shown in Fig. 3I, electrical coupling between adjacent cells from WT acini was readily detected. Electrical coupling was also evident at interelectrode distances of ~200 \( \mu \)m, suggesting that widespread coupling of WT acinar cells occurred within a single acinus (data not shown). In contrast, Mist1KO acini showed a complete absence of electrical coupling. In all cases, no transfer of the hyperpolarizing current was ever detected, even when multiple adjacent cells were tested (Fig. 3J). The complete loss of cell-cell communication in Mist1KO acinar cells is surprising since acini from Cx32KO mice maintain some coupling properties (Chanson et al., 1998). These findings suggest that other factors that are instrumental in establishing communication networks may also be affected in Mist1KO mice.

**Connexin26 mRNA levels are retained, but protein abundance is altered, in Mist1KO mice**

The complete loss of dye transfer and electrical coupling in Mist1KO acinar cells suggested that all gap junctional proteins were affected in these cells. Formation of normal pancreas gap junctions requires the co-expression of Cx32 and a second connexin protein, Cx26 (Meda, 1996; Zhang and Nicholson, 1994). Cx26 gene expression occurs in all acinar cells of the pancreas and the protein often cooperates with Cx32 to form heteromeric channels (Meda et al., 1993; Stauffer, 1995). In fact, Cx26 and Cx32 are often co-localized in the same cells (Zhang and Nicholson, 1994) and our immunohistochemical analysis confirmed that both proteins were localized to the same gap junction plaques in WT pancreatic acini (Fig. 4A-C). To determine if Mist1 regulates the expression of both genes in a similar fashion, we characterized the expression and localization of Cx26 in Mist1KO pancreatic tissue. As shown in Fig. 4D, RNA blot analysis revealed comparable Cx26 transcript levels in both WT and Mist1KO acinar cells. Surprisingly, no difference in Cx26 mRNA levels could be detected between normal and Mist1KO mice at any time point examined (PN1 to adult) (data not shown), suggesting that transcriptional regulation of the Cx32 and Cx26 genes remains under separate control. To confirm if this expression pattern was also observed at the protein level, Cx26 antibodies were used to detect Cx26 in Mist1KO acinar tissue. As shown in Fig. 4E, RNA blot analysis revealed equivalent levels of Cx26 protein in WT and Mist1KO acinar cells. Surprisingly, Cx26 protein was not detected in pancreatic acinar cells obtained from older (10-weeks old and adult) Mist1KO mice, despite the continued presence of Cx26 transcripts (Fig. 4E). Examination of additional young animals suggest that other factors that are instrumental in establishing communication networks may also be affected in Mist1KO mice.
3320 Journal of Cell Science 116 (16)

in WT samples (1 minute, C-D) whereas no transfer is evident in Mist1KO acinar cells (E-H), even after extended times (20 minutes). C,E,G; phase contrast; D,F,H; fluorescence. (IJ) Pancreatic acini from WT and Mist1KO mice were analyzed for electrophysiology coupling. Single cells (Cell 1) were injected with 1 M KCl (arrows) and the spread of current was assessed using a recording electrode positioned on adjacent cells (Cell 2). WT (I) acinar cells exhibit normal electrophysiological coupling whereas no coupling is detected in the Mist1KO cells (J).

Fig. 3. Mist1KO acinar cells are defective in intercellular communication. (A,B) Isolated acinar cells from wild type (WT) and Mist1KO mice were processed for Cx32 immunofluorescence (green). Similar to pancreatic sections, WT acinar cultures (A) show Cx32 plaques (arrows) on lateral cell borders whereas Mist1KO samples (B) exhibit no gap junction staining. Nuclei are stained with the DNA fluorochrome DAPI (blue). (C-H) Pancreatic acini were isolated from WT and Mist1KO mice, individual acinar cells (arrows) were injected with 10 mM 6-carboxyfluorescein and adjacent cells were monitored for the transfer of dye. Dye transfer to neighboring cells occurs rapidly in WT samples (1 minute, C-D) whereas no transfer is evident in Mist1KO acinar cells (E-H), even after extended times (20 minutes). C,E,G; phase contrast; D,F,H; fluorescence. (IJ) Pancreatic acini from WT and Mist1KO mice were analyzed for electrophysiology coupling. Single cells (Cell 1) were injected with 1 M KCl (arrows) and the spread of current was assessed using a recording electrode positioned on adjacent cells (Cell 2). WT (I) acinar cells exhibit normal electrophysiological coupling whereas no coupling is detected in the Mist1KO cells (J).

(Mist1 functions as a positive regulator of Cx32 transcriptional activity)

Our studies have shown that Cx32 gene expression, but not Cx26, is altered in the Mist1KO pancreas. The specific loss of RNA expression for only one of the connexins strongly suggests that Mist1 exerts its control directly on Cx32 gene transcription. Indeed, analysis of Mist1 and Cx32 gene expression profiles revealed a striking overlap in tissue distribution, with co-expression observed in all secretory exocrine cells, including the pancreas, submandibular gland, parotid gland and seminal vesicles (Fig. 5A). The exception to this pattern of common expression was in liver hepatocytes (Fig. 5A) and neuronal supporting cells (data not shown), both of which do not undergo regulated exocytosis and only express Cx32 but not Mist1 (Fig. 5A) (Evert et al., 2002; Meda et al., 1993; Nicholson et al., 2001; Pin et al., 2000). Analysis of various tissues in Mist1KO animals also revealed a striking correlation between Mist1 and Cx32 gene expression. Tissues that normally express Mist1 mRNA and protein, including the lacrimal and submandibular glands, exhibited greatly reduced levels of Cx32 transcripts (Fig. 5B) and an almost complete absence of Cx32-containing gap junction complexes (Fig. 5C-H). In contrast, expression of Cx32 transcripts and formation of Cx32-containing gap junction plaques remained unchanged in tissues that do not normally express Mist1, such as liver hepatocytes (Fig. 5B,I,J). These results support the hypothesis...
Mist1 null mice lack gap junctions

In order to examine whether Mist1 directly controls expression of the Cx32 gene, we cloned a portion of the mouse Cx32 P1 promoter (Neuhaus et al., 1995) spanning -680 to +20 into a luciferase reporter plasmid (Cx32p-Luc) and tested it for expression in the pancreatic exocrine cell line AR42J. Introduction of Cx32p-Luc into AR42J cells resulted in very low basal luciferase activity (Fig. 6A). Co-transfection of cells with the Cx32p-Luc reporter and an expression plasmid encoding Mist1 generated a 15- to 20-fold increase in Cx32p-Luc expression (Fig. 6A). Similar results also were obtained with the exocrine cell line ARIP (data not shown). Interestingly, no other bHLH transcription factor tested in this assay was able to activate expression of the Cx32p-Luc gene. These included both ubiquitously expressed Class A bHLH factors (E12, E47, HEB) and tissue restricted Class B bHLH factors (PTF1-p48, NeuroD, MyoD, Mash1) (Fig. 6B). The ability of Mist1 to activate expression of the Cx32p-Luc gene was dependent on its DNA binding and dimerization properties. Mutant Mist1 proteins that were defective in DNA binding (Mist1mut basic) or protein dimerization (Mist1mut helix 1) (Lemercier et al., 1998), did not activate Cx32p-Luc expression (Fig. 6C). From these studies we conclude that an active Mist1 protein is required to generate full Cx32 gene expression in secretory acinar cells.

To more rigorously test the importance of active Mist1 protein for Cx32 gene transcription, we took advantage of the fact that the Mist1mut basic protein functions as a dominant-negative factor to repress endogenous Mist1 activity (Lemercier et al., 1998). We reasoned that expression of Mist1mut basic in pancreatic acinar cells would inhibit the
endogenous Mist1 protein and down-regulate the expression of Mist1 target genes. Transgenic mice expressing a myc-tagged Mist1mut basic protein under the transcriptional control of the pancreas-specific elastase 1 promoter (elastasep-Mist1mut basic, myc) (Heller et al., 2001; Kruse et al., 1995), were generated and examined for Mist1mut basic expression as well as the presence of Cx32-containing gap junction plaques. As shown in Fig. 7, pancreatic acinar cells expressing the Mist1mut basic protein showed a significant decrease in Cx32 expression, whereas adjacent acinar cells that did not express the Mist1mut basic transgene exhibited normal expression and localization of Cx32. In addition, the Mist1mut basic-expressing cells no longer produced Cx26-containing gap junction complexes and instead showed the diffuse cytoplasmic Cx26 staining that was evident in Mist1KO samples (data not shown). These results demonstrate that loss of Mist1 activity, through either deletion of the Mist1 gene or through inhibition of endogenous Mist1 protein activity, leads to the loss of Cx32 expression. We conclude that Mist1 functions as a positive regulator of Cx32 gene expression in exocrine acinar cells and, in the absence of Mist1, gap junctions and intercellular communication pathways become disrupted.

**Discussion**

The intricate cell-cell communication network that is associated with most epithelial cells involves the utilization of trans-membrane gap junctions that are made up of different connexin hexamers. Gap junctions are unique among membrane channels in that they span two discrete cells and form direct intercellular conduits. Their main function is to permit rapid cellular responses to external stimuli to generate a variety of coordinated events. This is accomplished by allowing the passage of small molecules, such as Ca2+, Na+, K+, cAMP and inositol triphosphates, from one cell to another (Wasle and Edwardson, 2002). Several mechanisms govern the functionality of gap junctions, including connexin stability and post-translational modifications that maintain gap junctions in an open or closed state. However, the tissue-specific expression of the various connexin proteins underscores the true role of the junctions in cell development, differentiation and function. Loss of individual connexins characterize the pathology of several human diseases, including Charcot-Marie-Tooth disease, oculodentodigital dysplasia, cardiac ischemia and
cardiac hypertrophy (Bergoffen et al., 1993; Kelsell et al., 2001; Paznekas et al., 2003; Severs, 1994). In addition, recent mouse genetic studies have revealed unique roles for specific connexins in embryonic development, cardiac function, and growth regulation (Plum et al., 2000; Willecke et al., 1999).

Each connexin protein is distinct and the expression pattern by reduced expression of the gap junctional communication. This defect is primarily caused by reduced expression of the Cx32 gene, which leads to severe depletion of gap junction formation and gap junction-mediated cellular communication.

While it could be argued that the loss of Cx32 is a consequence of cellular disorganization and not directly of a loss of Mist1 activity, several pieces of evidence support a model of direct transcriptional regulation. First, one would predict that a simple disruption of the gap junctions by cellular disorganization would lead to decreases in both connexin genes expressed in pancreatic acini. Cx32 and Cx26 proteins are co-expressed in many of the same cell types and readily form heteromeric gap junctions (Meda et al., 1993; Stauffer, 1995). However, in Mist1KO acinar cells, Cx26 gene transcription is greatly reduced, implying that the transcriptional control of these two genes is distinct. Secondly, analysis of the Cx32 gene reveals two separate promoters, P1 and P2 (Neuhaus et al., 1995). The P1 promoter directs Cx32 gene expression in liver, salivary glands and pancreatic epithelial cells, while the downstream P2 promoter is responsible for Cx32 expression in cells of the spinal cord and brain (Neuhaus et al., 1995). Studies from the Ruch laboratory (Koffler et al., 2002; Piechocki et al., 2000) have shown that liver-specific expression of the Cx32 gene relies on the winged-helix transcription factor HNF-1, which is also present in the pancreas. However, adult pancreatic HNF-1 expression is primarily confined to cells of the endocrine compartment where Cx32 is not expressed (Edlund, 2002;
Nammo et al., 2002). Other HNF family members (HNF-6, HNF-3β) are present in the exocrine pancreas (Rausa et al., 1997), but they do not activate Cx32 gene expression in transfection assays (unpublished data). Instead, our data suggest that the P1 promoter is activated in acinar cells by Mist1. Sequence analysis of the P1 promoter reveals 5 E-box regulatory sites that potentially bind Mist1, and a mutant form of Mist1, which does not bind DNA, fails to activate Cx32 expression. The observed repression of the Cx32 gene in pancreatic acinar cells expressing a dominant-negative Mist1 protein confirms that endogenous Mist1 activity is essential for acinar cell Cx32 transcription. As predicted, the reduction in Cx32 gene expression in Mist1KO mice is observed only in cell types that normally express Mist1, such as the exocrine cells from the pancreas, salivary glands, lacrimal gland and seminal vesicle. From these studies we conclude that Mist1 functions as a direct positive regulator of Cx32 gene expression and, in its absence, acinar cell gap junctions and intercellular communication pathways become disrupted.

Although the DNA binding and dimerization domains of Mist1 are required to achieve Cx32 gene transcription, the precise mechanism(s) by which Mist1 activates Cx32 gene expression is not known. In this regard, it will be essential to determine if Mist1 functions as a homodimer or as a heterodimer with another bHLH protein expressed in acinar cells. Preliminary data suggest that Mist1 homodimers are the preferred protein complex in the pancreas (T. Tran, unpublished data). However, Mist1 homodimers alone are not sufficient to activate the endogenous Cx32 gene in non-acinar cell types (unpublished data), suggesting that acinar cells contain cofactors that modify the Mist1 protein, or that serve as accessory transcription factors, to allow full Cx32 gene expression. Indeed, regulation of the Cx32 promoter is probably complex, since in the absence of Mist1 the Cx32p-Luc reporter gene remains active at a low basal level. This expression is not surprising given that the 680 bp 5′ flanking sequence contains protein binding sites for many general transcription factors including Sp1, NF1, Stat5 and SRF (data not shown). Future studies will focus on determining if Mist1 interacts with these other transcription factors that are bound to the Cx32 promoter to influence acinar-specific gene expression. Identification of the specific Cx32 promoter elements and additional acinar cell transcription factors will be essential to establish their role in activating Cx32 gene expression.

These studies have also provided new insight into the differential regulation of acinar-specific connexin proteins. While Cx32 appears to be regulated at the transcriptional level, this is not the case for Cx26. Despite normal levels of Cx26 mRNA, very few Cx26-containing gap junctions are detected in Mist1KO acinar cells. Instead, Cx26 protein accumulates within the cytoplasm and does not associate with the cellular membrane. This observation supports the hypothesis that Cx26 prefers to form gap junctions with Cx32 (Zhang and Nicholson, 1994). Similar events have also been reported for Cx32KO mice, where significant reductions in Cx26 gap junctions are observed, resulting in decreased dye transfer (Chanson et al., 1998). Interestingly, lacrimal glands from Cx32KO animals exhibit transient diffuse patterns of Cx26 protein accumulation that is similar to our observations with Mist1KO mice (Walcott et al., 2002). However, the complete phenotypes associated with Mist1KO and Cx32KO mice are quite distinct. Cx32KO acinar cells continue to display some electrical coupling (Chanson et al., 1998) and the disorganization observed in Mist1KO mice is not evident in Cx32KO pancreatic tissue (data not shown). The alterations in cell morphology in Mist1KO acini probably contribute to the loss of cell communication and indicate that the loss of Cx32 protein is not solely responsible for the phenotypic abnormalities observed in Mist1KO animals. It remains possible that several key defects in regulated exocytosis, including altered CCK AR and Ins(1,4,5)P3 receptor expression (Pin et al., 2001), may contribute to the inefficient dye transfer and loss of electrophysiological coupling found in this current study. The disorganization of Mist1KO acinar cells is evident early in embryogenesis, prior to significant gap junction organization (C. Johnson, personal communication). Indeed, results from our laboratory support the idea that Mist1KO pancreatic acinar cells do not reach a fully differentiated state and are developmentally blocked as immature exocrine cells. Thus, although Mist1 clearly has a role in controlling Cx32 gene expression, additional Mist1 target genes are probably important in establishing and maintaining normal acinar cell polarity and function. Identification of these genes will be crucial to fully understanding the role of Mist1 in these complex cellular processes.

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References
Bergoffen, J., Scherer, S. S., Wang, S., Scott, M. O., Bone, L. J., Paul, D. L., Chen, K., Lensch, M. W., Chance, P. F. and Fischbeck, K. H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science 262, 2039-2042.
Bock, P., Abdel-Moneim, M. and Egerbacher, M. (1997). Development of pancreas. Microsc. Res. Tech. 37, 374-383.
Castle, J. D. (1990). Sorting and secretory pathways in exocrine cells. Am. J. Respir. Cell Mol. Biol. 2, 119-126.
Chanson, M., Fanjul, M., Bosco, D., Nelles, E., Suter, S., Willecke, K. and Medina, P. (1998). Enhanced secretion of amylase from exocrine pancreas of connexin32-deficient mice. J. Cell Biol. 141, 1267-1275.
Edlund, H. (2002). Pancreatic organogenesis-developmental mechanisms and implications for therapy. Nat. Rev. Genet. 3, 524-532.
Evert, M., Ott, T., Temme, A., Willecke, K. and Dombrowski, F. (2002). Morphology and morphometric investigation of hepatocellular preneoplastic lesions and neoplasms in connexin32-deficient mice. Carcinogenesis 23, 697-703.
Fujimoto, K., Nagafuchi, A., Tsukita, S., Kuraoka, A., Okohuka, A. and Shibata, Y. (1997). Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. J. Cell Sci. 110, 311-322.
Goodenough, D. A., Golger, J. A. and Paul, D. L. (1996). Connexins, connexons, and intercellular communication. Annu. Rev. Biochem. 65, 475-502.
Heller, R. S., Stoffers, D. A., Bock, T., Svenstrup, K., Jensen, J., Horn, T., Miller, C. P., Habener, J. F., Madsen, O. D. and Serup, P. (2001). Improved glucose tolerance and acinar dysmorphogenesis by targeted expression of transcription factor PDX-1 to the exocrine pancreas. Diabetes 50, 1553-1561.
Hemmennan, H., Kozjek, G., Dahl, E., Nicholson, B. and Willecke, K. (1992). Molecular cloning of mouse connexin26 and -32: similar genomic organization but distinct promoter sequences of two gap junction genes. *Eur. J. Cell Biol.* 58, 81-89.

Hill, C. E., Rummery, N., Hickey, H. and Sandow, S. L. (2002). Heterogeneity in the distribution of vascular gap junctions and connexins: implications for function. *Clin. Exp. Pharmacol. Physiol.* 29, 620-625.

Hsieh, C. L., Kumar, N. M., Gilula, N. B. and Francke, U. (1991). Distribution of genes for gap junction membrane channel proteins on human and mouse chromosomes. *Somat. Cell Mol. Genet.* 17, 191-200.

Jamieson, J. D., Gorelick, F. S. and Chang, A. (1988). Development of secretagogue responsiveness in the pancreas. *Scand. J. Gastroenterol.* Supplement 151, 98-103.

Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Joseph, S. K. (2000). Regulation of gap junctions by Mist1 expression. *J. Neurosci.* 26, 23-36.

Kaminski, F., Rose, S. D., Swift, G. H., Hammer, R. E. and MacDonald, R. J. (2002). Structural and functional diversity of connexin genes in the mouse and human genome. *FEBS Lett.* 466, 112-114.

Ohnishi, H., Samuelson, L. C., Yule, D. L., Ernst, S. A. and Williams, J. A. (1997). Overexpression of Rab3D enhances regulated amylase secretion from pancreatic acini of transgenic mice. *J. Clin. Invest.* 100, 3044-3052.

Paznekas, W. A., Boyadjiev, S. A., Shapiro, R. E., Daniels, O., Wollnik, B., Keegan, C. E., Innis, J. W., Dinulos, M. B., Christian, C., Hannibal, M. C. et al. (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am. J. Hum. Genet.* 72, 408-418.

Petrich, B. G., Gong, X., Lerner, D. L., Wang, X., Brown, J. H., Saftiz, J. E. and Wang, Y. (2002). c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. *Circ. Res.* 91, 640-647.

Piekocki, M. P., Toti, R. M., Strom, J. M., Burk, R. D. and Ruch, R. J. (2000). Liver cell-specific transcriptional regulation of connexin32. *Biochim. Biophys. Acta* 1491, 107-122.

Pin, C., Bonvisutto, A. C. and Konieczny, S. F. (2000). Mist1 expression is a common link among serous exocrine cells exhibiting regulated exocytosis. *Anat. Rec.* 259, 157-167.

Pin, C. L., Ruckstuhl, J. M., Johnson, C. and Konieczny, S. F. (2001). The bHLH transcription factor Mist1 is required to maintain exocrine pancreas cell organization and acinar cell identity. *J. Cell Biol.* 155, 519-530.

Plum, A., Hallas, G., Magin, T., Dombrowski, F., Hagendorff, A., Schumacher, B., Wolkert, C., Kim, J., Lamers, W. H., Evert, M. et al. (2000). Unique and shared functions of different connexins in mice. *Curr. Biol.* 10, 1083-1091.

Rausa, F., Samadani, U., Ye, H., Lim, L., Fletcher, C. F., Jenkins, N. A., Copeland, N. G. and Costa, R. H. (1997). The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 beta in the developing murine liver and pancreas. *Dev. Biol.* 192, 226-246.

Saluja, A. K., Saluja, M., Printz, H., Zavertnik, A., Sengupta, A. and Steer, M. L. (1989). Experimental pancreatitis is mediated by low-affinity cholecystokinin receptors that inhibit digestive enzyme secretion. *Proc. Natl. Acad. Sci. USA* 86, 8968-8971.

Severs, N. J. (1994). Pathophysiology of gap junctions in heart disease. *J. Cardiovasc. Electrophysiol.* 5, 462-475.

Stauffer, K. A. (1995). The gap junction protein beta1-connexin (connexin-32) and beta2-connexin (connexin-26) can form heteromeric hemichannels. *J. Biol. Chem.* 270, 6768-6772.

Walcott, B., Moore, L. C., Birzgalis, A., Claros, N., Valiusas, V., Ott, T., Willecke, K. and Brink, P. R. (2002). Role of gap junctions in fluid secretion of lacrimal glands. *Am. J. Physiol. Cell Physiol.* 282, C501-C507.

Wasse, B. and Edwardson, J. M. (2002). The regulation of exocytosis in the pancreatic acinar cell. *Cell Signal.* 14, 191-197.

Willecke, K., Eiberger, J., Degen, J., Eckardt, D., Romualdi, A., Guldenagel, M., Deutsch, U. and Soll, G. (2002). Structural and functional diversity of connexin genes in the mouse and human genome. *Biochim. Biophys. Acta* 1579, 725-737.

Willecke, K., Parkinson, S., Plum, A., Temple, A., Thonissen, E. and Ott, T. (1999). Biological functions of connexin genes revealed by human genetic defects, dominant negative approaches and targeted deletions in the mouse. *Novartis Found. Symp.* 219, 76-88; discussion 88-96.

Yamamoto, M. and Kataoka, K. (1985). Large particles associated with gap junctions of pancreatic exocrine cells during embryonic and neonatal development. *Am. J. Anat.* 171, 305-310.

Zhang, J. T. and Nicholson, B. J. (1994). The topological structure of connexin 26 and its distribution compared to connexin 32 in hepatic gap junctions. *J. Membr. Biol.* 139, 15-29.