PDX-1 can repress stimulus-induced activation of the INGAP promoter

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Abstract

Islet neogenesis associated protein (INGAP) promotes the generation of new islet mass in adult animal models. It is not understood what factors control the expression of INGAP. In this study, factors that regulate the expression of INGAP promoter activity are reported. To determine factors that regulate INGAP expression, we previously cloned the promoter region for INGAP. Analysis of the INGAP promoter suggested that candidate regulators of INGAP expression include the transcription factors PDX-1, NeuroD, PAN-1, STAT and AP-1. Using gene addition experiments in the 293 cell line the activity of these transcription factors on an INGAP-promoter construct linked to the β-galactosidase reporter has been determined. Induction of AP-1 activity or STAT activity using PMA or LIF stimulation respectively, or direct expression of PAN-1 specifically up-regulates INGAP promoter activity. In contrast, co-expression of PDX-1 but not NeuroD inhibits activation of the INGAP-promoter driven by PAN-1, PMA or LIF stimulation. PDX-1 binds directly to the INGAP promoter as determined in electromobility shift and antibody supershift assays. Expression of the INGAP-promoter-reporter construct in the HIT-T15 beta-cell line, a cell line that expresses endogenous PDX-1, did not reveal PMA-mediated stimulation of INGAP promoter activity. HIT-T15 cells however did efficiently transfect (>68%) and respond (2-fold) to PMA-induced signal transduction to a transfected AP-1-CAT reporter. Partial reduction of PDX-1 expression in HIT-T15 cells was associated with recovery of PMA induced INGAP promoter activity. These data suggest that expression of PDX-1 is associated with a repression of stimulus-induced INGAP promoter activity that appears to be mediated by a direct DNA interaction. These findings implicate PDX-1 in a possible feedback loop to block unbridled islet expansion.


Introduction

The pathogenesis of both type 1 and type 2 diabetes is linked to a loss of functional pancreatic beta-cell mass (Bell & Polonsky 2001). In type 1 diabetes, beta-cells are actively removed in an autoimmune attack (Mathis et al. 2001) and a reduction of approximately 50% of the beta-cell mass has occurred at the diagnosis of type 2 diabetes (UKPDS Group 1995) with a proceeding annual loss of 5–10% of the beta-cell mass. The restoration of functional beta-cell mass is an important concept in treating diabetes. Transplantation of donor islets can reverse diabetes (Ryan et al. 2002), however this approach has serious limitations rooted in an inadequate donor supply and drug toxicity related to long-term immunosuppression (Hirschberg et al. 2003). An alternative approach is to drive adult stem cells residing in the pancreas to restore the functional islet mass. Adult islet mass can be enhanced both by replication of existing islets (Bonner-Weir et al. 1993, Dor et al. 2004) and by neogenic commitment of precursor cells to new islets (Rosenberg et al. 1989, Bonner–Weir et al. 1993, Li et al. 2002, Peters et al. 2005). This latter process is termed islet neogenesis. Pathways governing islet neogenesis are poorly understood, although the process can be regulated through the administration of bio-active factors such as islet neogenesis associated protein (INGAP) (Rosenberg et al. 2004, Vinik et al. 2004), GLP-1 (exendin) (Holz & Chepurny 2003), Clusterin (Kim et al. 2001), REG (Okamoto 1999) and Gastrin/EGF (Brand et al. 2002). Several of these factors are undergoing clinical trials.

The bioactive factor, islet neogenesis associated protein (INGAP) was first identified as a differentially expressed gene in a hamster model of islet neogenesis (Rafaeloff et al. 1997). Administration of INGAP protein to hamsters elevated endogenous beta-cell mass sufficiently to decrease
hyperglycemia in drug-induced diabetes (Vinik et al. 2004). Endogenous INGAP expression is concomitant with the induction of islet neogenesis (Del Zotto et al. 2000). The INGAP protein has a molecular weight of 16-8 kDa and is related to the type 2 C-lectins (Taylor-Fishwick et al. 2003, Vinik et al. 2004). The organization of the 175 amino acids of INGAP classifies it as a member of the group 2 superfamily of reg-related proteins (Okamoto 1999). In addition to the biological efficacy of the INGAP protein, a pentadecapeptide derived from the INGAP holoprotein has been identified which retains biological activity. The exogenous administration of INGAP peptide stimulates islet neogenesis in rodents (Rosenberg et al. 2004) and dogs (G L Pittenger and D A Taylor-Fishwick, unpublished observations). Moreover, INGAP peptide can reverse diabetes in established streptozotocin-induced diabetic-C57BL6 mice (Rosenberg et al. 2004). The bioactivity of INGAP is further demonstrated in the enhancement of the beta-cell secretory response by administration of INGAP peptide (Borelli et al. 2005) and in the ability of INGAP to promote duct to islet transdifferentiation in vitro (Jamal et al. 2005).

The molecular events regulating endogenous expression of INGAP are unclear and insight into these pathways has come from the recent cloning of the INGAP 5-prime regulatory region (Taylor-Fishwick et al. 2003). The regulatory region of INGAP has, in addition to transcriptional elements resulting from conventional signaling pathways including AP-1 and STAT, a number of predicted interaction sites for transcription factors associated with pancreas development. These include the homeodomain transcription factor PDX-1, inactivation of which results in agenesis of the pancreas (Jonsson et al. 1994, Stoffers et al. 1997), and the basic helix-loop-helix transcription factors NeuroD/Beta2 (Huang et al. 2000) and PAN-1(E47) (German & Wang 1994). NeuroD is an essential pathway in endocrine development (Naya et al. 1997) and PAN-1 is a binding partner for NeuroD (Mutoh et al. 1997) having the ability to bind to the insulin promoter to transactivate the insulin gene (Dumonteil et al. 1998).

In this report we describe the functional integration of PDX-1 to regulate INGAP-promoter mediated expression. Specifically, while PDX-1, PAN-1, AP-1 and STAT stimulate the INGAP promoter, PDX-1 exerts an inhibitory effect on factors shown to induce INGAP gene expression. This study provides the first evidence for a simple regulatory feedback loop to prevent unrestrained expansion of islet mass.

Material and Methods

Cell culture

Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The human embryonic kidney cell line, 293, was cultured in DMEM (Invitrogen) containing 10% FBS (Invitrogen). The hamster pancreatic beta-cell line, HIT-T15 (p65–69), was cultured in RPMI1640 containing 2 mM l-glutamine, 1.5 g/l sodium bicarbonate (ATCC), 10% horse serum (Invitrogen) and 2:5% FBS at 37°C. Cells were cultured at 37°C in a 5%/95% CO2/air humidified atmosphere. Cells were passaged using 0.25% trypsin-EDTA (Invitrogen).

Plasmids and antibody

Sequential deletions of the INGAP promoter were constructed as previously described (Taylor-Fishwick et al. 2003). All fragments were digested with XmaI and BglIII (New England Biolabs, Beverly, MA, USA) and subcloned into the pβgal-basic vector (Clontech), a promoterless β-galactosidase expression vector. The first start codon ATG downstream of the TATA-box in the reporter gene construct is that for β-galactosidase. The p32–1–1CAT reporter-plasmid, containing repeated AP-1 binding sites upstream of a CAT reporter, was generously provided by Dr Timothy Bos (Department of Microbiology and Molecular Cell Biology, EVMS, Norfolk, VA, USA). Expression plasmids for PDX-1, NeuroD and PAN-1; pBAT12 shPDX-1, pBAT12 mNeuroD and pBAT14 shPan-1 were generously provided by Dr Michael German (Hormone Research Institute, UCSF, CA, USA). The green fluorescent protein expression plasmid (pCMV-GFP) was obtained from Clontech. For Western blotting, the primary antibodies used were rabbit anti-PDX-1 (Chemicon International Inc., Temecula, CA, USA), anti-NeuroD (N-19) and anti-PAN-1 (N-649) (both Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-PDX-1(N-18) and anti-STAT3 (F-2) (Santa Cruz Biotechnology) were used in the EMSA as specific and non-specific PDX-1 antibodies respectively.

Transient transfection and PMA stimulation

Transient transfections of 293 and HIT-T15 cells were carried out using LipofectAMINE reagent (Invitrogen). Briefly, 4 × 10⁵ cells were cultured overnight in sixwell (35 mm) tissue culture plates (Corning Inc., Corning, NY, USA). The following day, plasmids (1 µg/4 × 10⁵ cells) and LipofectAMINE reagent (10 µl/4 × 10⁵ cells) were each diluted in 0.75 ml serum-free medium then combined, mixed and incubated at room temperature for 30 min. Following 30 min, cells in 1.5 ml serum-free medium, were added to the transfection mixture and cultured for 5 h at room temperature. Cells were then removed and placed in serum-containing media being cultured for 48–72 h. For induction studies, the transfected cells were stimulated with 50 ng/ml PMA (Sigma) or 10 ng/ml hLIF (Sigma) on the day following transfection. To normalize transfection efficiency, cells
were co-transfected with pAP4 (Flanagan & Cheng 2000), a secretory alkaline phosphatase expression plasmid kindly provided by Dr John Flanagan (Harvard, MA, USA). The substrate pNPP (Sigma) was used to determine secretory alkaline phosphatase activity in cell culture supernatant that had been heat inactivated at 70 °C for 10 min to destroy endogenous alkaline phosphatase activity.

**β-galactosidase reporter gene assay**

After-transfection (48–72 h), cells were harvested and washed twice with PBS (Invitrogen). Cells were lysed using a combination of lysis buffer (100 mM K2HPO4, 100 mM KH2PO4, 1 mM DTT), and three freeze-thaw cycles. The lysate was centrifuged in a microcentrifuge at 16 100 × g for 10 min to clear the lysate supernatant. Activity of β-galactosidase enzyme was determined using a chemiluminescence-based kit (Clontech). Briefly, 25 μl of the cell lysate was reacted with 200 μl of β-galactosidase chemiluminescence reaction buffer (Clontech) per well of a white opaque 96-well plate (Fisher, Pittsburgh, PA, USA) for one hour at room temperature. The luminescence value was read on a Wallac Victor2 1420 multilabel counter (PerkinElmer Life Sciences, Downers Grove, IL, USA). Proteins were normalized to protein concentration determined using the Bradford method (Bradford 1985) (Bio-Rad). Increase in luminescence for the INGAP-promoter reporter is expressed relative to transfection of the promoterless β-galactosidase reporter (pBASIC), that is, where the INGAP-promoter was omitted.

**CAT ELISA assay**

The expression of the chloramphenicol acetyltransferase (CAT) reporter gene was determined using a CAT ELISA kit (Roche). Following manufacturers directions, cells were washed three times in PBS (Invitrogen) and lysed at room temperature for 30 min. The cleared lysate (200 μl; 15 min at 16 100 × g) was added to separate wells of a 96-well microtitre plate precoated with anti-CAT. Presence of CAT was detected by anti-CAT digoxigenin and anti-digoxigenin-peroxidase before being visualized with ABTS— 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)—substrate. All incubations were for 1 h at 37 °C. Colorimetric changes were read at 405 nm on a Wallac Victor^2 1420 multilabel counter (PerkinElmer).

**Flow cytometry assay**

Both mock and pCMV-GFP (Clontech) transfected HIT-T15 cells were trypsinized, harvested by centrifugation and washed three times with cold PBS. Cell pellets were resuspended in 500 μl cold PBS and cell fluorescence determined by flow cytometry (FACSCalibur, Becton Dickinson, CA, USA). A minimum of 20 000 events per sample were acquired during analysis. Cells falling into a preset gate for fluorescent positive cells were expressed as a percentage of the total live cells acquired. Transfected cells in culture were viewed on an Olympus IX70 Confocal Microscope and scanned for brightfield and fluorescence.

**Western blotting**

Nuclear and cytoplasmic proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce, Rockford, IL, USA) containing Halt protease inhibitor cocktail (Pierce). Protein samples were mixed with an equal volume of SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 15% glycerol, 5% β-mercaptoethanol, 0.001% Bromophenol blue), and heated at 85 °C for 2 min. The denatured proteins were resolved on 12% Novex pre-cast Tris-glycine gel (Invitrogen) at 125 V for 90 min and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences) by electro elution in transfer buffer (192 mM Glycine, 25 mM Tris–base, 20% methanol) at 30 V for 90 min. Membranes were blocked with 5% (w/v) non-fat milk (Richfood Inc., Richmond, VA, USA) in TBS–T buffer (Sigma; 50 mM Tris pH 8.0, 138 mM NaCl, 2.7 mM KCl containing 0.1% Tween 20) at 4 °C overnight. Following a wash in TBS–T buffer, membranes were incubated for 1 h at room temperature in TBS–T buffer containing 1% non-fat milk and 1:5000 rabbit anti-PDX-1. After three washes in TBS–T buffer, membranes were incubated for 1 h at room temperature in TBS–T buffer containing 1:25 000 donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Biosciences). Proteins were detected using ECL plus Western blotting detection reagents as per manufactures instructions (Amersham Biosciences) and visualized by autoradiography on BioMax Light film (Sigma) for 5 min. The films were developed using Futura 3000 S automatic film processor (Diagnostic Imaging Inc. Winston-Salem, NC, USA).

**Electrophoresis mobility shift assay (EMSA)**

Nuclear extracts were prepared as described above. DNA probe (nucleotides 446–680 bp of Genbank sequence AY184211) was excised from the INGAP promoter plasmid using XmnI and Tsp509I (New England Biolabs), restriction digests being purified using the QIAquick-gel extraction kit (QIAGEN) as per manufacturer’s instructions. The probe was labeled with biotin using biotin-16-end DNA labeling kit (Pierce). Nuclear extracts were incubated with 20 fmole biotinylated probe at room temperature for 30 min in binding buffer (Pierce, 100 mM Tris pH 7.5, 500 mM KCl, 10 mM DTT, 50% glycerol, 100 mM MgCl2, 1 μg/μl poly(dI:dC), 1%NP-40, 1 M KCl). Unbiotinylated DNA probe (2 pmole) was preincubated for 10 min in competition experiments. The
samples were electrophoresed at 100 V being separated on a 4% polyacrylamide gel in 0.5 × BE buffer (4.5 mM Tris–base pH 8.0, 4.5 mM Borate, 1 mM EDTA) for 90 min. Protein complexes were transferred to Nylon membrane (Pierce), and DNA was crosslinked to the membrane under u.v. light for 8 min. Biotin-labeled DNA was detected using the LightShift Chemiluminescent EMSA kit (Pierce) following the manufacturer’s instructions and visualized by autoradiography. Alternatively, the probe was labeled with [α-32P]ATP (PerkinElmer, Boston, MA, USA) by incubating with T4 polynucleotide kinase using the DNA 5'-end labeling system (Promega). The labeled probe (40 000 c.p.m.) was added to reactions as described above. Following electrophoresis, gels were dried and bands detected by autoradiography or phosphoimager analysis using a Typhoon9410 scanner (Molecular Dynamics, Sunnyvale, CA, USA). Image Quant (Molecular Dynamics) software was used for quantification.

Construction and transfection pSilencer2-0-U6-PDX-1-siRNA: Hairpin siRNA for hamster PDX-1 mRNA (U73854) sense sequence 573–593 (GCUGAGAAG GAAUUCUUATT) and antisense sequence 571–589 (UAAGAAUUCUUUCAGCTC) was designed by Ambion Inc. Austin, Texas, USA. Oligonucleotides, 5’-GATCCGCTGGAGAAGGAATTCTTATTCAAGA GATAAGAATTCCTTCCAGCTTTTTTTGGAA AA3’ and 3’-GCCGACCTTCTTCATGTTCA TCTATTCTTAAAGGAGGTCGAGAAACCT TTTTCGAG5’ (Intergrated DNA Technologies, Inc. Coralville, IA, USA) were annealed at 94 °C for 2 mins, and cooled to room temperature. The resulting dsDNA was directionally cloned into pSilencer2-0-U6 vector (Ambion Inc.) using the incorporated restriction sites BamHI and HindIII to form pSilencer2-0-U6-PDX-1-csiRNA. For transfection of pSilencer2-0-U6-PDX-1-csiRNA, 6 × 105 HIT-T15 cells were plated into 6-well (35 mm) plates (Corning), and incubated for two days before transfections. On the day of transfections, pSilencer2-0-U6-PDX-1-csiRNA and INGAP promoter-reporter plasmid were co-transfected into HIT-T15 cells using lipofectamine2000 (Invitrogen). Cells were stimulated and assayed as described above.

Results are shown as mean ± S.E.M. Significance of the data was evaluated by non-paired Student’s t-test. P<0.05 was judged significant. Analyses were performed using Graphpad prism V4.0 software (Graphpad Software Inc., San Diego, CA, USA).

Results

Recent cloning of the genomic sequence for INGAP has identified promoter activity that is responsive to defined biochemical stimuli (Taylor-Fishwick et al. 2003). Potential binding sites for PDX-1, a homeobox transcription factor, for PAN-1 a ubiquitous class A basic helix-loop-helix transcription factor and for NeuroD a class B basic helix-loop-helix transcription factor were identified upstream to the transcriptional start site for the INGAP gene. As illustrated in Figure 1, a search on Genomatix Mat Inspector professional 6.0 (Cartharius et al. 2005; Genomatix Software GmbH, Munich, Germany) identified eleven PDX-1, one NeuroD and seven PAN-1 (E47) predicted binding sites within the 3 Kbp region upstream of the INGAP transcriptional start site. The location of these predicted sites are marked in Fig. 1 in relation to the transcriptional start site for INGAP. At the gene location 446–680, NeuroD, PDX-1 and PAN-1 co-cluster.

The transcription factors PDX-1, NeuroD and PAN-1 were expressed in 293 cells, either alone or in combination, to determine if they had an active role in regulating the reporter gene expression driven by the INGAP promoter. Expression plasmids for each defined transcription factor and an INGAP-promoter-reporter plasmid were co-transfected into 293 cells. Expression of the factor was confirmed by Western blot analysis (data not shown). The human embryonic kidney epithelial cell line, 293, has no detectable expression of endogenous INGAP and can support INGAP-promoter reporter gene expression in response to defined exogenous stimuli (Taylor-Fishwick et al. 2003). The ability of each transcription factor to activate the INGAP promoter was measured by the production of the reporter gene, β-galactosidase using a luminescence-based assay. Specificity for INGAP promoter activation was confirmed by substituting the INGAP-promoter reporter gene with a promoterless β-galactosidase reporter–gene. The data shown is expressed as the fold induction over the promoterless reporter gene activity. Introduction of the PDX-1 expression plasmid into cells resulted in a two-fold increase in INGAP-promoter regulated β-galactosidase activity.
Introduction of the NeuroD expression plasmid into cells resulted in a two-fold increase in INGAP-promoter regulated β-galactosidase activity. In contrast, introduction into 293 cells of the expression plasmid for the ubiquitous transcription factor PAN-1(E47) resulted in a five-fold increase in INGAP-promoter regulated β-galactosidase activity. Additionally, the consequence of combining transcription factors was explored. Expression plasmids for multiple transcription factors along with the INGAP-promoter mediated β-galactosidase activity expressed as a fold induction over the β-galactosidase activity of the promoterless reporter construct. Significant reduction in PAN-1-induced reporter activation in marked (* P<0.05).

In order to determine if the PDX-1 inhibition was restricted to PAN-1 stimulation or provided a general block of stimulation, the effect of PDX-1 on PMA and LIF stimulation of the INGAP promoter was tested. We have previously shown that both the phorbol ester PMA (Taylor-Fishwick et al. 2003) and the cytokine LIF (Taylor-Fishwick et al. 2002) stimulate INGAP-promoter activity in transfected 293 cells. The 293 cell line was co-transfected with the INGAP-promoter-reporter construct and with either the expression plasmid pBAT12 PDX-1 encoding for PDX-1, or the expression plasmid pBAT12 NeuroD encoding for NeuroD to provide an expression plasmid control. Following transfection, cells were stimulated with 50 ng/ml PMA for 24 h and the activity of the reporter gene assayed. The PMA-induced stimulation of the INGAP-promoter-reporter in control 293 cells was compared with the PMA-induced stimulation of the INGAP-promoter-reporter in 293 cells expressing PDX-1. In control 293 cells PMA stimulated a greater than three-fold induction of β-galactosidase activity (Fig. 3A). Whereas the presence of PDX-1 inhibited the PMA-induced stimulation of β-galactosidase driven by the INGAP promoter (P<0.05, t-test). This inhibition was not observed in cells transfected with NeuroD (Fig. 3A). The NeuroD expressing cells showed the equivalent three-fold induction in promoter activity in response to PMA. The expression of PDX-1 protein in 293 cells transfected with pBAT12 PDX-1 was confirmed by Western Blot analysis (Fig. 3A, inset). Control 293 lysate (lane a) did not have a PDX-1 immuno-reactive band whereas lysate from cells transfected with PDX-1 (lane b) did have a PDX-1 immuno-reactive band. Similarly, there was a 2.5 fold induction of INGAP-promoter activity stimulated by LIF in control 293 cells. This stimulation was inhibited by 50% (P<0.05, t-test) in 293 cells expressing PDX-1 (Fig. 3B). Thus the negative action of PDX-1 on INGAP gene regulation extends to both PMA-mediated and LIF-mediated stimuli and is not restricted to stimulation of the INGAP-promoter mediated by the transcription factor PAN-1 in the 293-transfection model.

To confirm that PDX-1 interacts directly with the INGAP promoter, EMSA was performed. Region 466–680 (Genbank AY184211) of the INGAP promoter (marked in Fig. 1) was biotinylated and used as a binding probe on nuclear extracts from 293 cells (control) and 293 cells that had been transfected with pBAT12 PDX-1, the PDX-1 expression plasmid. A shifted band was detected that was specific for the PDX-1 expressing 293 nuclear extract (Fig. 4A, lane a). Furthermore, the band-shift was blocked in conditions where the nuclear extracts were incubated with both biotinylated probe and a 50-fold molar excess of unbiotinylated probe (Fig. 4A, lane b). Additionally, to demonstrate that the shifted complex contained PDX-1, an antibody mediated supershift experiment was performed. In the presence of the PDX-1 antibody the specific complex migrated at a...
higher molecular mass confirming the presence of PDX-1 in the INGAP-promoter complex (Fig. 4A, lane c). This supershift was not observed in extracts incubated with a non-PDX-1 recognizing antibody (Fig. 4B). Therefore PDX-1 directly interacts with the INGAP promoter.

Endogenous PDX-1 is selectively expressed in beta-cells of the mature endocrine pancreas. HIT-T15 cells are a transformed hamster beta-cell line. Western blot analysis for PDX-1 protein expression showed that HIT-T15 cells constitutively express PDX-1 which could be detected in both the cytoplasmic and the nuclear compartments of the cell (Fig. 5A, inset). Unlike the activation of the INGAP promoter seen in 293 cells, which have no detectable PDX-1 expression, transient transfection of the INGAP-reporter-promoter construct into the PDX-1-expressing HIT-T15 cells did not result in a basal expression of the β-galactosidase reporter gene. Further, stimulation with PMA of HIT-T15 cells transfected with the INGAP-reporter-promoter resulted in no detectable expression of the β-galactosidase reporter gene, i.e. no detectable activation of the INGAP promoter. For both conditions, reporter expression was compared with that detected with a promoterless β-galactosidase reporter-construct (pBASIC, Fig. 5A). Transfection efficiency and PMA responsiveness were confirmed in the HIT-T15 cells. Transfection of HIT-T15 cells with pCMV-GFP, an expression plasmid for green fluorescent protein, under the identical protocol used for the INGAP-promoter showed greater than 68% of the cells expressed green fluorescent protein (GFP) when analyzed by flow cytometry (Fig. 5B). Cells were clearly positive for GFP as analyzed by immunofluorescence of pCMV-GFP transfected cells.

Figure 3 PDX-1 inhibits stimuli of the INGAP promoter. Mock transfected 293 cells were compared with 293 cells transfected with PDX-1 or NeuroD. The cells were stimulated with 50 ng/ml PMA (A) or 10 ng/ml LIF (B). Inset (A) shows Western blot analysis for PDX-1 expression in 293 cells (a) or 293 cells transfected with PDX-1 (b). Data shows β-galactosidase reporter gene activity (A) and fold induction (B) relative to the promoterless β-galactosidase reporter.

Figure 4 PDX-1 binds the INGAP promoter. EMSA of nuclear extracts from PDX-1 transfected 293 cells incubated with 20 fM labeled probe alone (a) or together with either 2 pM unlabeled probe (b) or antibody specific for PDX-1 (c). Positions of shifted (*) and antibody-supershifted (**) bands are marked on the gel (A) and densitometry analysis (B) of nuclear extracts incubated with either 20 fM probe alone (solid black line), or with 2 pM unlabeled probe (solid grey line), PDX-1 antibody (broken black line) or non-specific antibody (broken grey line).
HIT-T15 cells were also transfected with the reporter plasmid pAP-1-CAT. PMA stimulation of pAP-1-CAT-HIT-T15 cells resulted in a two-fold increase in CAT reporter gene activity (Fig. 5C). The plasmid pAP-1-CAT is a reporter construct in which tandem AP-1 consensus binding sites are upstream of a chloramphenicol acetyl transferase reporter gene. These data show that HIT-T15 cells both transfect efficiently and are responsive to PMA induced signaling. Introduction of the pAP1-CAT promoter-reporter plasmid into 293 cells resulted in an equivalent two-fold induction in CAT activity following stimulation with PMA (Fig. 5C). Thus, in contrast to 293 cells, the intracellular environment of HIT-T15 cells does not permit PMA-mediated stimulation of the INGAP promoter. HIT-T15 cells can however, be transfected and are responsive to PMA as a stimulus of gene expression. To reduce the expression of endogenous PDX-1 in HIT-T15 cells, siRNA for hamster PDX-1 sequence 573–593 (Genbank sequence U73854) was introduced. PDX-1 expression was analyzed by Western blot analysis. The siRNA-PDX-1, but not a control siRNA probe (data not shown), reduced the protein expression level of PDX-1 by 60 ± 15% (Fig. 6 A). The expression of the housekeeping gene tubulin was not affected by siRNA-PDX-1. Stimulation of the INGAP-promoter by PMA was tested in siRNA-PDX-1 treated HIT-T15 cells. Reporter-gene activity was increased 0·62 ± 0·1 fold in HIT cells treated with siRNA-PDX-1 that had a reduced expression of PDX-1. The increase seen was only in response to PMA stimulation, no increase in basal reporter gene activity was detected. To determine if the partial reduction in PDX-1 expression could explain the partial recovery of reporter gene activity to PMA in HIT-siRNA-PDX cells, PDX inhibition of PMA-mediated activation of the INGAP-promoter-reporter gene was established in 293 cells (Fig. 6B). Decreasing amounts of pBat12 PDX-1 were transfected into 293 cells and the plasmid backbone (pBAT12) was used to standardize the DNA transfection ratios. PMA stimulation of the INGAP-reporter gene was dose-dependently ...
inhibited by PDX-1. A partial recovery of the PMA response correlated with reduced amounts of PDX-1 transfected \((P<0.05, t\)-test). Collectively, these data imply the presence of a feedback inhibition of INGAP promoter activity mediated by PDX-1 in HIT-T15 cells.

**Discussion**

The studies described in this report show that PDX-1 can directly modulate INGAP promoter activity. Expression experiments in 293 cells reveal that PDX-1 binds to the INGAP promoter and inhibits gene expression when stimulated by the agents PAN-1, PMA and LIF. The stimulus response of the INGAP promoter is absent in HIT-T15 cells that have a constitutive expression of PDX-1. This response can be partially recovered by knocking down PDX-1 expression. Transcriptional control of INGAP by PDX-1 may clarify a mechanism to regulate induced islet mass.

In the studied transfection model, NeuroD, PDX-1 and PAN-1 increased INGAP promoter-mediated gene expression. The greatest stimulation of INGAP promoter activity was observed with PAN-1 which resulted in a five-fold induction in INGAP promoter mediated gene expression. The enhanced efficacy of PAN-1 compared with PDX-1 is unlikely to reflect greater occupancy on the INGAP promoter since more PDX-1 sites are predicted in the INGAP promoter region assayed. The homodimeric/heterodimeric properties and non-specific nature of PAN-1 in its DNA interaction (Dumonteil et al. 1998, Poulin et al. 2000, Qiu et al. 2002) suggest that PAN-1 could exert its action by interaction with a binding partner present in 293 cells. The identification of a protein partner is being investigated. Previous interactions of PAN-1 with PDX-1 and NeuroD have been reported (Ohneda et al. 2000). For the insulin gene, these interactions involve co-operative DNA-binding and synergy for gene induction. Whether these interactions are relevant to the regulation of INGAP were explored in a multi-factor transfection model. It was a surprise to discover that PDX-1 inhibited PAN-1-mediated activation of the INGAP-promoter rather than enhancing promoter stimulation. There are two possible explanations of this result: (1) either PDX-1 is interacting with PAN-1 or a PAN-1-binding partner to sequester a PAN-1 and/or PAN-1-binding partner away from a functional DNA-binding complex or (2) PDX-1 directly interacts with the INGAP promoter and induces a conformational change that is inhibitory to additional stimulus-induced transcription. Using the electromobility gel shift assay, PDX-1 was shown to interact with the INGAP promoter, suggesting the mechanism of PDX-1-mediated inhibition is by direct DNA interference. To strengthen this concept, the consequence of PDX-1 expression in distinct models for stimulating the INGAP promoter were explored. The phorbol ester, PMA and the cytokine, LIF induce signaling pathways and transcription cascades that are distinct to activation mediated by PAN-1. While PMA and LIF induce INGAP-promoter activity in 293 cells, the introduction of PDX-1 into 293 cells inhibited INGAP-promoter activity stimulated by PMA or LIF. Furthermore, INGAP promoter activity could not be stimulated by PMA in cells that endogenously express PDX-1, even though these cells transfected efficiently and are responsive to PMA-mediated stimuli, both in the studies shown in this report and those of others (Goodison et al. 1992,
Yaney et al. 2002). Whereas, a reduction in the expression of endogenous PDX-1, using a siRNA knockdown strategy, partially restored the ability of PMA to activate the INGAP promoter. The absence of a complete knockdown of PDX-1 is likely to explain the partial recovery of the PMA response and the sustained absence of the basal down of PDX-1 is likely to explain the partial recovery of the INGAP promoter. The absence of a complete knockdown strategy, partially restored the ability of PMA to activate the INGAP promoter may be unresponsive to additional stimuli. PDX-1 exerts a direct negative influence on the INGAP expression in 293 cells. Thus, the data indicate that the partial expression of PDX-1 could correlate with a partial inhibition at the INGAP promoter was reproduced in 293 cells. Thus, the data indicate that PDX-1 exerts a direct negative influence on the INGAP promoter such that in the presence of PDX-1 the INGAP promoter may be unresponsive to additional stimuli.

PDX-1 expression is implicated in the development of the pancreas (Jonsson et al. 1994, Stoffers et al. 1997), in lineage commitment (Edlund 2001) and in homeostasis of the mature beta-cells of the endocrine pancreas (Ahlgren et al. 1998). Thus, the functional implication of the observations made in this report may impact the regulation of INGAP expression both in development and/or in preserving the integrity of functional islet mass. INGAP is present in the pancreatic anlage during embryogenesis (Rafaeloff et al. 1998, N S Hamblet, A M Bowman, D A Taylor-Fishwick, unpublished observations), suggesting INGAP may have a role in the regulation of cell commitment, differentiation and/or expansion of the developing pancreas. During pancreas organogenesis it is possible that PDX-1 expression may serve to restrict INGAP expression. Additionally, the recent identification of INGAP expressing cells within the mature endocrine pancreas (Flores et al. 2003, Taylor-Fishwick et al. 2004) raises the possibility that the actions of INGAP may not be restricted to the expansion of islet mass. INGAP expression in the endocrine region of the pancreas is mutually exclusive to the PDX-1 expressing beta-cell. Immuno-reactivity for INGAP and the alpha cell marker glucagon has been shown to co-localize in islets (Flores et al. 2003, Taylor-Fishwick et al. 2004). It is therefore feasible that an interaction between PDX-1 and INGAP expression is important in endocrine lineage commitment, such that expression of PDX-1 is associated with repression of INGAP expression. Moreover, evidence for a cross-talk signal originating from the beta-cell and regulating the expression of INGAP can be inferred in the studies of Takatori et al. (2003) which showed an increase in INGAP mRNA following destruction of beta-cells with the toxin streptozotocin.

Could PDX-1 have a role in regulating INGAP-mediated islet neogenesis in the mature pancreas? In models of islet neogenesis, surgical occlusion of the pancreatic duct or administration of exogenous INGAP, unbridled expansion of islet mass has not been observed (Gold et al. 1998, Rosenberg 1998, Rosenberg et al. 2004). Clearly negative feedback mechanism(s) exist to avoid unregulated generation of new islet mass. The attraction in our findings of PDX-1 exerting a negative role on INGAP expression lies in the simplicity of this direct feedback model. Precedent for a simple feedback loop involving PDX-1 does exist. PDX-1 binds to its own promoter and is implicated in a direct negative loop controlling its own expression (Marshak et al. 2000). Other regulating mechanism(s) must also exist to downregulate INGAP-driven islet neogenic signals. These are likely to be at the level of the INGAP receptor since administration of exogenous INGAP bypasses a negative feedback based upon repression of endogenous INGAP expression. Administration of exogenous INGAP upregulates PDX-1 expression in pancreatic ductal cells (Rosenberg et al. 2004) and ductal PDX-1 expression is a purported marker of precursor islet stem cells (Gagliardino et al. 2003), the cells most likely to be expressing the INGAP receptor.

Diabetes results from a loss of the functional beta-cell mass. Significant restrictions in organ supply and toxicity associated with long-term immunosuppression currently present major limitations to exogenous islet repopulation strategies (Hirshberg et al. 2003). Islet regeneration achieved by reprogramming endogenous pathways within the pancreas present an intriguing paradigm to repopulating the functional islet mass in the diabetic pancreas. As INGAP appears to function as part of a neogenic cascade, additional and equally attractive molecular targets are the regulators of INGAP expression, both for their theoretical potential as novel therapies for diabetes and for providing a more complete understanding of the processes governing neogenesis and pancreas organogenesis. Knowledge of the events governing INGAP expression, as reported herein, opens opportunities towards the realization of rational treatments for diabetes.

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**References**


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