Activin A increases Pax4 gene expression in pancreatic β cell lines

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Abstract Activin A, a member of the TGFβ superfamily, has many physiological and developmental functions. In pancreatic β cell cultures, activin promotes cell differentiation and insulin production. The author has found activin increases gene expression of the PAX4, one of the major transcription factors determining pancreatic β cell differentiation. This effect was mediated, at least in part, by the type IB activin receptor (ALK4). Moreover, the activity of human insulin promoter–reporter system was controlled by PAX4 and its isoform PAX4 Δ(G239–P251) in a unique fashion; positively by low concentrations, and negatively by high concentrations of these proteins. And the repression activities were different between these proteins. These findings confirm the importance of activin signal transduction in pancreatic β cell development and function.

Key words: Pancreas; β-Cell; PAX4; Insulin; Activin A; ALK4

1. Introduction

Gene disruption experiments have revealed that lots of transcription factors are orchestrally involved in the process of pancreatic morphogenesis and differentiation in a coordinated manner [1–3]. Initially, HB-9 [4,5], PDX-1 (also designated as IDX-1, STF-1, IPF-1) [6,7], and ISL-1 [8] have critical roles in morphogenesis. In addition, Notch signal mediating molecules including HES-1 and neurogenin-3 (ngn3) regulate cell fate as endocrine or exocrine [9–11]. In endocrine cells, HNF6 affects cell differentiation by stimulation of ngn3 expression [12]. PAX4 and PAX6 commit to determine the cell types as α cells or β/δ cells [13,14], and NKX2.2 [15] and BETA2 (also known as NeuroD1) [16] function in maturation of β cells. While in exocrine cells, p48 is required for cell lineage [17].

Activin A, a member of transforming growth factor β (TGFβ) superfamily, has many physiological and developmental functions such as stimulation of follicle stimulating hormone production in pituitary [18,19], craniofacial development [20], and promotion of erythrocyte differentiation [21]. In pancreas, activin A increases insulin content in the rat β cell line INS-1 [22] and induces the rat pancreatic exocrine acinar-derived cell line AR42J to produce insulin when treated in combination with hepatocyte growth factor (HGF) or betacellulin [23,24].

To gain insight into the mechanism of activin function in pancreatic β cells, the effect of activin on gene expression of transcription factors was investigated. Activin A was indicated to increase gene expression of the PAX4, which is one of the most crucial determinants for β cell differentiation [13], and the action of activin A was shown to be mediated, at least in part, by the type IB activin receptor, ALK4. Furthermore, the author found unique properties of PAX4 and its isoform toward human insulin promoter (HIP) as transcriptional activators and repressors depending upon their expression levels.

2. Materials and methods

2.1. Materials, cell culture, and isolation of transfectants stably expressing constitutively active type IB activin receptor ALK4 (Thr206Asp)

Human TGFβ1 was obtained from Sigma, and human activin A was purchased from Austral Biologicals or provided by Dr. Y. Eto (Ajinomoto). Murine insulin producing NIT1 [25] cells were purchased from ATCC and cultured in Ham's F12 medium containing 14% fetal calf serum (FCS). Stable NIT1 transfectants expressing constitutively active type IB activin receptor, ALK4 (T206D) [26], were isolated by transfection with human ALK4 (T206D) encoding the gene harbored in pcDNA3 vector (Invitrogen), followed by selection for G418 resistance. The vector was a gift from Dr. K. Miyazono (The Cancer Institute) with the permission of Dr. C.H. Heldin (Ludwig Institute for Cancer Research). INS-1E β cells [27] were provided by Dr. C.B. Wollheim (University of Geneva, Switzerland) and cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 5% FCS, 1 mM pyruvate, and 50 μM 2-mercaptoethanol.

2.2. Measurement of gene expression level

Total RNA was extracted from NIT1 and INS-1E cells using Isogen reagents (Nippon gene); genomic DNA contaminated was removed by DNase I digestion. The resulting RNA was converted into first-stranded cDNA using an Advantage-for-RT-PCR kit (Clontech). The gene expression levels of Pax4, insulin, Pdx-1, BETA2, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and TFIIDτ (TBP) were determined by real time quantitative PCR analysis using a PRISM™ 7700 Sequence Detection system (Perkin-Elmer Applied Biosystems) under conditions the supplier recommended [28]; briefly, the system consists of cDNA fragment amplification and fluorescence detection. The nucleotide sequences of forward and reverse primers and Taqman probes for each gene are summarized in Table 1. Taqman probes were labelled at the 5′ end with FAM (6-carboxy-fluorescein) for fluorescence and at 3′ end with TAMRA (6-carboxy-tetramethyl-rhodamine) for quenching. The expression level of each gene was normalized to that of G3PDH in Figs. 1 and 2A and to that of TFIIDτ in Fig. 2C since G3PDH expression levels seemed to vary among the clones for the latter case.

2.3. Transient transfection

Transient transfection experiments shown in Fig. 2A were carried out using lipofectamine (LF) 2000 reagent (Gibco BRL) in 6-well plates containing 2×10^6 NIT1 cells per well. DNA solution composed of 12 μl LF 2000 reagent, 300 μl OPTI-MEM and 6 μg of vector plasmids was added to each well, in which cells were already plated and grown in 1.4 ml medium. Experiments shown in Figs. 2B and 3

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Abbreviations: ARE, activin response element; FCS, fetal calf serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; HIP, human insulin promoter; TGFβ, transforming growth factor β; WT, wild type

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were performed using LF reagent (Gibco BRL) in 24-well plates containing either 4×10^5 wild type (WT) NIT1 or 2×10^5 ALK4 (T206D) expressing clone lines of NIT1 per well. The cell numbers of the clone lines are fewer than that of WT because cell size of clone lines were larger than that of WT. Transfection was performed using 380 μl FCS-free OPTI-MEM medium with 6.3 μl of LF reagent and an appropriate amount of plasmids (see figure legends) toward each well, then the medium was replaced with Ham's F12 supplemented with FCS 6 h after transfection. Luciferase activity was measured 42 h (Fig. 3) or 24 h (Fig. 2B) after medium replacement using an ML-3000B luminometer (Dynatech). The plasmid harboring *Xenopus* FAST-1 [29] was a gift from Dr. M. Whitman (Harvard University). The reporter vector pALUC5 was generated by inserting five in line repeats of activin response element (ARE) [30] upstream of the TATA box in the pGV-B2 luciferase vector (Tokyo Ink). The HIP reporter vector pIns-Luc380 was constructed by inserting the −342 to +37 region of the human insulin gene, in which +1 denotes putative capping site [31,32], directly upstream of the luciferase gene of pGV-B2 without TATA box. The expression vectors of PAX4 and its isoform were constructed by cloning PCR amplified fragments encoding each gene into pcDNA3.1 vector.

2.4. Measurement of insulin content

NIT1 cells plated in 24-well plates, containing either 4×10^5 WT or 2×10^5 clones per well, were lysed in 0.15 ml of buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF and Complete™ reagent (Boehringer). These lysates were diluted 150-fold and subjected to insulin content measurement using Phadeseph insulin RIA kit (Pharmacia-Upjohn). The data were normalized to total protein level, which was determined by a protein assay kit (Bio-Rad).

3. Results and discussion

3.1. Activin A increases Pax4 gene expression

When NIT1 cells were treated with activin, no change in homeobox transcription factor PDX-1 or basic helix-loop-helix transcription factor BETA2 transcript levels were observed. However, transcript levels of PAX4, which contains both a paired domain and a homeodomain in its molecule and whose knockout prevents β cell development [13], did increase although the direct involvement of PAX4 in insulin gene transcription has not been shown (Fig. 1A). Activin dose dependently and specifically elicited this response; both bone morphogenetic protein (BMP) 2 and TGFβ, other members of the TGFβ superfamily, had either no effect or decreased Pax4 expression (Fig. 1B,C). In addition, only activin also increased slightly the gene expression of insulin (Fig. 1C).

Next, INS-1E cells were treated with activin A to investigate whether the effect of activin is only applicable to NIT1 cells or universally extended to other pancreatic β cell lines. Fig. 1D indicates that though no significant difference was seen for insulin expression, expression of the Pax4 was dramatically induced. This activin effect on Pax4 gene expression is also reported for exocrine acinar AR42J cells when treated with HGF together [33]. Therefore, the effect of Pax4 induction by activin A seemed to be universal in insulin-producing cell lines.

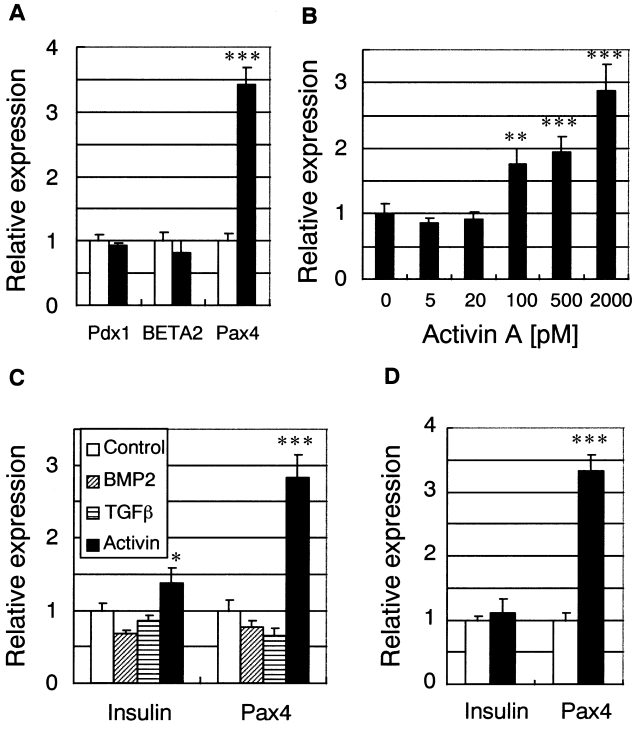

Fig. 1. Pax4 gene induction following activin treatment in NIT1 (A–C) and INS-1E (D). A: The expression levels of pancreatic β cell specific transcription factors (Pdx1, BETA2, Pax4) in NIT1 cells treated with (■) or without (□) 2 nM activin A for 12 h. B: Dose dependency of the effect of activin on Pax4 expression after 24 h treatment. C: The expression levels of insulin and Pax4 in cells treated with 2 nM of human BMP 2 (▨), TGFβ1 (▤), activin (■), or control (nothing) (□) for 24 h. D: The expression levels of insulin and Pax4 in INS-1E cells treated with 2 nM of human activin (■), or control (nothing) (□) for 24 h. The data are normalized to the expression level of G3PDH, and expressed as relative level to the no-treatment level as mean ± S.D. ($n=4$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (Student's *t*-test for A, D and Dunnett for B, C).

3.2. The effect of activin A is mediated through its receptor

In environment with high Ca^{2+} concentrations, activin A promotes insulin secretion from β cells. However, this effect seems not to be mediated by the translation via activin recep-

Table 1
Nucleotide sequences of primers and Taqman probes used for real time quantitative PCR analysis using a PRISM™ 7700 Sequence Detection system

Genes	Forward primers	Taqman probes	Reverse primers
mPax4	GCCTGGGAGATCCAACACCA	AGAGCTTGCACTGGCACTGGACTCAACTCAGA	GGGAAGAACTGGAGCCA
Insulin	ATGGCCCTGTGGATGCGCTTC	TCTACACACCCATGTCCCGCC	TCAGTTGCAGTAGTTCTCCAG
mPdx-1	CCACCCAGCGCCTAA	AGTACTACGCGGCCACACAGCTCTA	GAATGCGCACGGGTC
mBETA2	AGCCCCCTAACTGACTGCA	CAGCCCTTCCTTTGACGGACCC	AGGCATAATTTTTTTCAAACTCG
mG3PDH	AAAGTGGAGATTGTTGCCAT	CATGTTCCAGTATGACTCCACTCACG	TTGACTGTGCCGTTGAATT
mTFIIDτ	TCTTTAGTCCAATGATGCCTT	CCACAGCCTATTCAGAACACCAACAG	GGTTGCTGAGATGTTGATTG
rPax4	CTTTTTGCCT GGGAGATCCA	CGCCAGCTTTGTGCTGAAGGGC	GGAGCCTTGT CCTGGGTACA
rG3PDH	AAGCAGGCGGCCGAG	TGGGCTACACTGAGGACCAGGTTGTC	ATCAAAGGTGGAAGAATGGGA

Distinctions of mouse and rat for each gene are represented by m and r, respectively, at heads of gene names. Primer sets for insulin were commonly used toward mouse and rat ones.

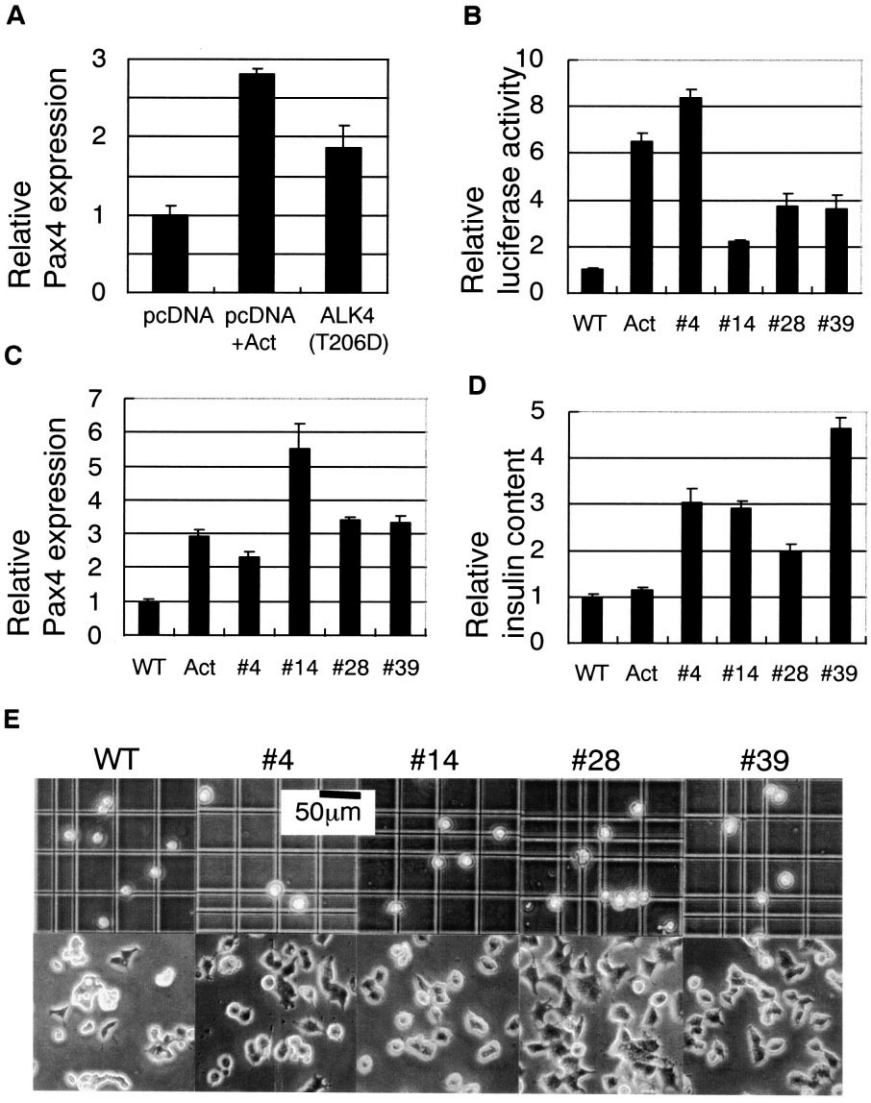

Fig. 2. The effect of transient (A) and stable (B–E) expression of ALK4 (T206D) in NIT1 cells. A: NIT1 cells were transiently transfected with control vector pcDNA3.1 (Invitrogen) for two groups or ALK4 (T206D) expression vector derived from pcDNA3. Activin A was supplemented as 2 nM to one group of control 6 h after transfection, and the Pax4 gene expression of each group was investigated 30 h after transfection. B: Intracellular activin signal intensities of WT, activin-treated WT (Act), and individual stable clone lines estimated by the ARE-luciferase system. Cells in 24-well dishes were transfected with 0.63 μg of pALUC5 and 0.063 μg of FAST-1 vector using 6.3 μl of LF reagent per well. C: The expression level of Pax4. D: Insulin content. E: Photos of WT and stable clones (#4, #14, #28, #39) just after passage (top) on hemocytometer or cultured for 18 h after passage (bottom). The data are normalized to the G3PDH gene expression level for A or to the TFIIDτ gene expression level for C, and to the total protein level for B, D, and represented as relative value to WT as the mean ± S.D. ($n = 4$).

tor since insulin secretion occurs very quickly [34]. To test whether activin-induced Pax4 expression is mediated by activin receptor, first, constitutively active form type IB activin receptor, ALK4 (T206D) [26] was transiently expressed in NIT1 cells and the responses were compared with activin treatment. Fig. 2A demonstrates ALK4 (T206D) expression induced Pax4 gene expression by 67% intensity of activin treatment. Since transfection efficiency using LF 2000 reagent was estimated to be about 50% when monitored by green fluorescent protein after transfection with its expression vector (data not shown), the effect of ALK4 (T206D) transient expression on Pax4 induction seemed to be adequate.

Next, ALK4 (T206D) was stably expressed in NIT1 cells. Four distinct clones (#4, #14, #28, and #39) were obtained. The intracellular activin signal activities of these clones were estimated taking advantage of the ARE reporter system in combination with the *Xenopus* FAST-1 transcription factor [29,30]. Clone #4 exhibited nearly the same strength as 2 nM activin stimulus, and other clones elicited moderate activities (Fig. 2B). PAX4 transcript levels of all these clones were increased as activin-treatment (Fig. 2C). The results of these transient and stable transfection experiments exhibited Pax4 expression is mediated, at least in part, by the type IB activin receptor.

Activin treatment slightly (10%) increased the insulin content of WT cells as reported for INS-1 cells [22]. Similarly but extremely, insulin contents of these stably ALK4 (T206D) expressing clones were augmented (Fig. 2D). From the morphological point of view, #4 and #39 were markedly, #28 was moderately, and #14 was slightly hypertrophic (larger cell size), respectively, compared with WT NIT1 cells (Fig. 2E). In terms of cell morphology and insulin content, it is note-

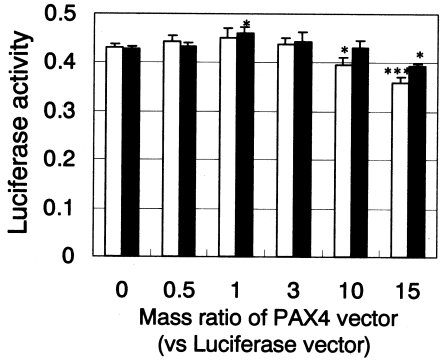

Fig. 3. The effects of PAX4 (□) and its isoform PAX4 Δ(G239-P251) (■) on HIP-controlled luciferase transcription. NIT1 cells cultured in 24-well plates were transfected with 0.05 μg of pIns-Luc380 and with PAX4 or its isoform harboring vectors at the relative mass ratio denoted, where pIns-Luc380 is 1. In each case, an empty vector pcDNA3.1 was supplemented to make the total plasmid content as the same mass. The data are expressed as the mean ± S.D. ($n = 3$). *, $P < 0.05$; ***, $P < 0.001$ (Dunnett). Luciferase activities are denoted as arbitrary units.

worthy to refer that not only transgenic mice expressing dominant negative type II activin receptor but also those expressing constitutively active ALK4 (T206D) exhibit hypoplasia of pancreatic islets (small area) and a lower insulin content [35]. Fig. 2D,E seems to be consistent with the results of the former case of transgenic mice, and not with the latter case. These results seem to imply that appropriate strength of activin signal is required for the maintenance of β cell function and signal intensity might affect the size of β cells.

3.3. PAX4 and its isoform affect HIP by dual modes

To investigate the role of PAX4 in insulin gene expression, a HIP reporter system was utilized in NIT1 cells. Previous reports indicate that there are three and four splicing isoforms of PAX4 in mouse [36] and rat [37], respectively. The intact PAX4 isoform is most abundant and the levels of two deleted isoforms, PAX4 Δ(Gly-239–Pro-251) and PAX4 Δ(Phe-278–Ser-314) are only one-tenth that of the intact form in MIN6 murine β cells [36]. In NIT1 cells intact PAX4 was also the major isoform, and PAX4 Δ(G239–P251) was expressed at one-third the intact isoform amount as estimated by RT-PCR, but the level of Pax4 Δ(F278–S314) was faint or invisible. And activin treatment to NIT1 seemed to increase proportionally both intact PAX4 and PAX4 Δ(G239–P251) isoform (data not shown).

The cDNAs of these two proteins were cloned to expression vectors and their effects on the HIP were analyzed. At low levels, both proteins exhibited slight (5–7%) transactivation activities. However, at higher doses both proteins suppressed transcription (Fig. 3), implying their dual roles as activators and as repressors for insulin gene expression depending upon their intracellular levels, though the effect might not be so critical. These dual modes functions of PAX4 are similar to the case of PDX-1 for insulin expression found in the rat RIN38 β cell line [38].

Recently, PAX4 is regarded to work in β cells as a repressor, especially competing against PAX6, which is associated with pancreatic α cell function [14], but does have transactivation function when expressed by a C-terminal domain form [39–42]. The present results matched these ones. It is intriguing that repression activity was attenuated in the PAX4 Δ(G239–P251) isoform compared with the intact one, suggesting G239–P251 domain, which is just adjacent to the homeobox domain and not found in PAX6 as an activator molecule when the amino acid sequence of PAX6 is aligned with that of PAX4 [35], might be crucial for repression activity by dint of unknown mechanisms such as affecting whole conformation of the PAX4 molecule and/or interacting with unknown factors so as to reduce activator activity.

PAX4 is shown to bind directly on rat insulin-1 promoter [40], however, putative paired domain binding motifs not always seem to be the same (G(A/C)ANTCANGCGTG or GNN(G/T)T(C/G)A(T/A)GCGTG or ANNTTCACGC) [39–41], therefore, it remains to be elucidated whether PAX4 directly binds to its cognate region on the HIP.

3.4. Conclusions

The results of the present study indicated that (i) Pax4 gene expression is induced by activin A and the signal is mediated through its receptor, ALK4, (ii) PAX4 and its isoform might positively and negatively regulate the HIP activity depending on their expression levels and they showed difference in repression activities. Consequently, these findings confirm the significance of activin signal transduction in pancreatic β cell function. Activin signal is known to be mediated by transcription factors termed Smad2/3 and Smad4 [43–47], and Smad2 is involved in the transdifferentiation of AR42J cells into insulin producing ones induced by activin A plus HGF [48]. Taken together, modulators of activin signal transduction might be promising therapeutic targets toward pancreatic β cell dysfunction caused by diabetes mellitus.

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